



Nongenomic and genomic effects of estradiol and xenoestrogens and their mechanisms of action in zebrafish and rat testicular cells

Hemily Batista da Silva

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THÈSE

Pour obtenir le diplôme de doctorat

Spécialité ASPECTS MOLECULAIRES ET CELLULAIRES DE LA BIOLOGIE

Préparée au sein de l'Université de Caen Normandie

En cotutelle internationale avec Universidade Federal de Santa Catarina , BRESIL

Nongenomic and genomic effects of estradiol and xenoestrogens and their mechanisms of action in zebrafish and rat testicular cells

**Présentée et soutenue par
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Hemily Batista da Silva

**NONGENOMIC AND GENOMIC EFFECTS OF ESTRADIOL AND
XENOESTROGENS AND THEIR MECHANISMS OF ACTION IN ZEBRAFISH AND
RAT TESTICULAR CELLS**

Ph.D. thesis in cotutelle submitted to the *Université de Caen Normandie* (UNICAEN) and to the *Universidade Federal de Santa Catarina* (UFSC) as a partial requirement for obtaining the Ph.D. in *Aspects Moléculaires et Cellulaires de la Biologie* and in Biochemistry, respectively.

Advisors: Dr Christelle Delalande Lecapitaine (UNICAEN) and Dr Fátima Regina Mena Barreto Silva (UFSC).

Caen / Florianópolis
2022

I dedicate this thesis to my husband and my parents, who always encouraged and supported me, and never let me give up on my dreams.

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ABSTRACT

The spermatogenesis is characterized by the development of germ cells into spermatozoa and its correct performance is related to the physical and energy support provided by Sertoli cells. However, aquatic and terrestrial animals are susceptible to exposure to the bisphenol A (BPA), which negatively affect the endocrine and male reproductive systems. Therefore, the general objective of this study was to investigate the effects of BPA and/or 17 β -estradiol (E2) on calcium (Ca²⁺) signaling, testicular energy metabolism and spermatogenesis in adult zebrafish and/or immature rats. Chapter I, part I: Results showed that L-type voltage-dependent Ca²⁺ channels, Ca²⁺-dependent chloride channels, protein kinase C, inositol triphosphate receptor (IP3R), MEK 1/2, PI3K and nuclear estrogen receptors (ESR) are involved in the mechanism of action of 10 pM BPA in stimulating ⁴⁵Ca²⁺ influx in zebrafish testis. Moreover, 10 μ M BPA caused an increase in lactate dehydrogenase (LDH) release and triacylglycerol content dependent on IP3R activation. Hence, BPA triggers cytosolic Ca²⁺ overload involving crosstalk between nongenomic and genomic signaling pathways in zebrafish. Chapter I, part II: Results showed that lactate and glycogen contents were reduced in zebrafish testes after *in vitro* (1 hour) and *in vivo* (12 hours) exposures to BPA. LDH activity was reduced only after *in vitro* exposure. In contrast, aspartate aminotransferase activity was increased, but decreased after *in vivo* exposure to 10 pM and 10 μ M BPA. However, alanine aminotransferase activity was reduced in the testes incubated with BPA, whereas its activity was increased after *in vivo* exposure. Additionally, a decrease in the proportion of the surface of spermatids and spermatozoa, as well as an increase in the percentage of apoptotic spermatocytes were observed after *in vivo* exposure to 10 pM and 10 μ M BPA. Furthermore, the relative expression of LDHBa, alanine aminotransferase 2 (GPT2), pyruvate carboxylase, ESR2b and P-element induced wimpy-like 1 was increased in testes incubated with 10 pM BPA for 6 hours, while pyruvate kinase M1/2 (PKMA) relative expression was reduced after 72 hours. In addition, the reduction in the relative expression of outer dense fiber protein 3b by 10 pM BPA was dependent on ESR α / β activation. Moreover, the reduction in the proportion of spermatids and spermatozoa by BPA was also via ESR α / β . The relative expression of monocarboxylate transporter 4, ESR2a, ESR2b and synaptonemal complex protein 3 was increased, while the relative expression of GPT2 and estrogen-related receptor α was reduced in testes incubated with 10 μ M BPA for 72 hours. Additionally, the reduced relative expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2a and LDHBa, as well as the increased glycogen phosphorylase relative expression by 10 μ M BPA were dependent on ESR α / β activation. Furthermore, the increased number of testicular cells expressing LDH by BPA was also via ESR α / β . Moreover, BPA resulted in reduced PKM and proliferating cell nuclear antigen immunocontents, as well as increased ERK 1/2 phosphorylation. Overall, BPA impairs testicular energy metabolism and causes defective spermatogenesis in zebrafish. Chapter II: Results showed that the relative expression of glycogen phosphorylase, PKM and LDHc was increased in immature rat testis explants incubated with E2 and BPA (1 nM or μ M) for 6 hours. Furthermore, the relative expression of GPT2 and glycerol kinase 2 was increased by BPA. Therefore, incubations of immature rat testis with E2 and BPA affect testicular energy metabolism. Overall, our data evidence that short-term exposures to BPA impair Ca²⁺ homeostasis, energy metabolism and spermatogenesis.

Keywords: Bisphenol A, spermatogenesis, calcium, energy metabolism, testis.

LIST OF FIGURES

Figure 1: Schematic representation of different stages of spermatogenesis in which mature and motile spermatozoa are produced.....	20
Figure 2: Formation and depolymerization of synaptonemal complexes.....	23
Figure 3: Comparison of morphological differences between (A) non-cystic and (B) cystic spermatogenesis.....	26
Figure 4: Stages of postnatal testicular development and maturation.....	28
Figure 5: Timeline and histology of gonad differentiation in zebrafish.....	30
Figure 6: Schematic representation of the main metabolic pathways involved in energy production in the testis with emphasis on the glycolytic pathways in Sertoli cells.....	36
Figure 7: Schematic representation of the participation of calcium (Ca^{2+}) as a key second messenger in cellular processes as a life and death signal.....	40
Figure 8: Schematic representation of the crosstalk between nongenomic and genomic estrogen receptor signaling pathways.....	47
Figure 9: Chemical structures of BPA, diethylstilbestrol and E2.....	51
Figure 10: Schematic representation of the BPA metabolism in zebrafish.....	52
Figure 11: Schematic overview of organotypic culture of zebrafish testis.....	59
Figure 12: Schematic overview of organotypic culture of rat testis explants.....	69
Figure 13: Concentration-response curve and time-course of $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish incubated with BPA.....	74
Figure 14: Membrane ion channels involvement in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish.....	75
Figure 15: Involvement of Ca^{2+} release from intracellular stores in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish.....	76
Figure 16: Involvement of protein kinases in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish.....	77
Figure 17: Involvement of $\text{ESR}\alpha/\beta$ in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish.	78

Figure 18: IP3R activation induces plasma membrane damage in the testis of zebrafish.....	79
Figure 19: Schematic representation of the effects and mechanism of action of BPA on Ca^{2+} influx in the testis of zebrafish.....	84
Figure 20: Effects of BPA on lactate content, glycogen content and ^{14}C -deoxy-D-glucose uptake in the testis of zebrafish.....	86
Figure 21: Effects of BPA on LDH, ALT and AST activities in the testis of zebrafish...	87
Figure 22: Effects of BPA on the proportion of spermatocytes, spermatids and spermatozoa in the zebrafish testis.....	89
Figure 23: Effects of BPA on apoptosis in spermatocytes.....	90
Figure 24: Effects of 10 pM BPA on the relative expression of energy metabolism-associated genes in zebrafish.....	91
Figure 25: Effects of 10 μM BPA on the relative expression of energy metabolism-associated genes in zebrafish.....	92
Figure 26: Analysis of the involvement of $\text{ESR}\alpha/\beta$ in the mechanism of action of 10 pM BPA on PKMA relative expression in the zebrafish testis.....	93
Figure 27: Involvement of $\text{ESR}\alpha/\beta$ in the mechanism of action of 10 μM BPA on the relative expression of testicular energy metabolism-associated genes in zebrafish...	94
Figure 28: Effects of BPA on the immunocontent of pyruvate kinase M1/M2 (PKM)....	95
Figure 29: Immunolocalization of LDH and effects of BPA on density of LDH-expressing cells in testicular histological sections of zebrafish.....	96
Figure 30: Involvement of $\text{ESR}\alpha/\beta$ in the increase in density of LDH-expressing cells mediated by BPA in testicular histological sections of zebrafish.....	97
Figure 31: Effects of 10 pM BPA on the gene expression of nuclear estrogen receptors and estrogen-related receptors in the zebrafish testis.....	98
Figure 32: Effects of 10 μM BPA on the gene expression of nuclear estrogen receptors and estrogen-related receptors in the zebrafish testis.....	98
Figure 33: Effects of 10 pM BPA on the expression of specific marker genes of testicular cells in zebrafish.....	99

Figure 34: Effects of 10 μ M BPA on the expression of specific marker genes of testicular cells in zebrafish.....	100
Figure 35: Involvement of ESR α/β in the mechanism of action of BPA in modulating the expression of ODF3b and SYCP-3 in the zebrafish testis.....	101
Figure 36: Effects of BPA on the immunocontent of proliferating cell nuclear antigen (PCNA).....	101
Figure 37: Effects of BPA on the ERK 1/2 phosphorylation (p-ERK 1/2 / t-ERK 1/2)..	102
Figure 38: Effects of BPA and involvement of the ESR α/β on the proportion of spermatocytes, spermatids and spermatozoa in the zebrafish testis.....	103
Figure 39: Schematic representation of the proposed mechanism of the <i>in vivo</i> effects of BPA on testicular energy metabolism and spermatogenesis in zebrafish.....	113
Figure 40: Schematic representation of the proposed mechanism of the 1-hour and 6-hour <i>in vitro</i> effects of BPA on testicular energy metabolism.....	114
Figure 41: Schematic representation of an overview of the involvement of ESR α/β in 72-hour genomic effects of BPA (<i>in vitro</i>) in the zebrafish testis.....	115
Figure 42: Effects of 10 pM, 1 nM and 1 μ M E2 on the relative expression of energy metabolism-associated genes in immature rats.....	117
Figure 43: Involvement of ESR α/β in the mechanism of action of E2 on the relative expression of testicular energy metabolism-associated genes in immature rats.....	119
Figure 44: Effects of 10 pM, 1 nM and 1 μ M E2 on the relative expression of spermatogonia and spermatocytes cell marker genes in immature rats.....	120
Figure 45: Effects of 10 pM, 1 nM and 1 μ M BPA on the relative expression of energy metabolism-associated genes in immature rats.....	121
Figure 46: Involvement of ESR α/β in the mechanism of action of BPA on the relative expression of testicular energy metabolism-associated genes in immature rats.....	122
Figure 47: Effects of 10 pM, 1 nM and 1 μ M BPA on the relative expression of spermatogonia and spermatocytes cell marker genes in immature rats.....	124

LIST OF TABLES

Table 1: Morphological and neuroendocrine features of postnatal testicular development.....	27
Table 2: Age comparison of developmental stages between rat and human.....	29
Table 3: Primary and secondary antibodies used in Western Blot.....	64
Table 4: Zebrafish-specific primer sequences for gene expression analysis by real-time RT-PCR.....	72
Table 5: Rat-specific primer sequences for gene expression analysis by real-time RT-PCR.....	73

LIST OF ABBREVIATIONS

2-APB	2-Aminoethoxydiphenyl Borate
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
AP-1	Activator Protein-1
AST	Aspartate aminotransferase
BPA-S	Sulfate Bisphenol A
Ca ²⁺	Calcium
CaCC	Calcium-dependent Chloride Channels
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
Cl ⁻	Chloride
CRE	cAMP Response Element
CREB	cAMP Response Element Binding Protein
Ct	Cycle Threshold
CURB	<i>Centre Universitaire des Ressources Biologiques</i>
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
DMC1	DNA meiotic recombinase 1
DMEM/F-12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
dNTP	Deoxyribose Nucleotide Triphosphate
DPBS	Dulbecco's Phosphate Buffered Saline
dpf	Days post-fertilization
E2	17 β -estradiol
ECL	Enhanced Chemiluminescent Substrate
EDC	Endocrine Disrupting Chemicals
EFSA	European Food Safety Authority
EGFR	Epidermal Growth Factor Receptor
ERE	Estrogen Response Element
ERK	p44/42 MAPK/Extracellular Signal-Regulated Kinases
ERR	Estrogen-Related Receptors

ESR1	Nuclear Estrogen Receptor α gene
ESR2	Nuclear Estrogen Receptor β gene
ESRR	Estrogen-Related Receptor gene
ESR α	Nuclear Estrogen Receptor α
ESR β	Nuclear Estrogen Receptor β
ETC	Electron Transport Chain
F	Forward
FSH	Follicle-Stimulating Hormone
GA-BPA	Glucuronic Acid Bisfenol A
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GDE	Glycogen Debranching Enzyme
GK	Glycerol-Kinase
GLUT	Glucose Transporter
GnRH	Gonadotropin-Releasing Hormone
GPER	G-Protein coupled Estrogen Receptors
GPR	G-Protein coupled Receptors
GPT2	Alanine aminotransferase 2 gene
GTH	Gonadotropins
HDAC	Histone Deacetylase
HPG	Hypothalamic-Pituitary-Gonadal axis
HRP	Horseradish Peroxidase
HSP	Heat Shock Proteins
HVA	High Voltage-Activated
IGF	Insulin-like Growth Factor
IGFR	Insulin-like Growth Factor Receptor
IHC	Immunohistochemistry
IP3	Inositol triphosphate
IP3R	Inositol triphosphate Receptor
ITGa6	Integrin alpha-6
kDa	Kilodalton
KOH	Potassium Hydroxide
L-VDCC	L-type Voltage-Dependent Calcium Channels
LaCl ₃	Lanthanum Chloride
LBD	Ligand-Binding Domain

LDH	Lactate Dehydrogenase
LDHBa	Lactate Dehydrogenase Ba gene
LH	Luteinizing Hormone
LVA	Low Voltage-Activated
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
MAPK	Mitogen-Activated Protein Kinase
MCT	Monocarboxylate Transporter
MNAR/PELP-1	Nongenomic estrogen receptor activity modulator protein
NADH	Reduced Nicotinamide-Adenine Dinucleotide
NCoR	Nuclear receptor Corepressor
NF- κ B	Nuclear Factor kappa B
OAA	Oxaloacetate
ODF3b	Outer Dense Fiber Protein 3b
p-ERK 1/2	Phospho-p44/p42 MAPK
p53	Tumor-suppressor Protein p53
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PCXA	Pyruvate Carboxylase A gene
PDH	Pyruvate Dehydrogenase complex
PDP1	Pyruvate Dehydrogenase Phosphatase 1
PEP	Phosphoenolpyruvate
PFK-1	6-PhosphoFructo-1-Kinase
PFK-2/FBPase-2	6-PhosphoFructo-2-Kinase/Fructose-2,6-Bisphosphatase
PFKFb	6- PhosphoFructo-2-Kinase/Fructose-2,6-Bisphosphatase gene
PI3K	Phosphatidylinositol-3-Kinase
PIWIL-1	P-element-Induced Wimpy-Like protein 1
PKA	Protein Kinase A
PKC	Protein Kinase C
PKM	Pyruvate Kinase M1/M2 isoform
PKMA	Pyruvate Kinase M1/2a gene
PND	Postnatal Day
PYGL	Glycogen Phosphorylase Liver form gene
PYGM	Glycogen Phosphorylase Muscle form gene

R	Reverse
RIP-140	Receptor-Interacting Protein 140
RPL8	Ribosomal Protein L8
RT	Reverse Transcription
S.E.M.	Standard Error of the Mean
SC	Synaptonemal Complex
SCP	Synaptonemal Complex Protein
SDS	Sodium Dodecyl Sulfate
SERCA	Sarco/Endoplasmic Reticulum Calcium ATPase
SOC	Store-Operated Calcium Channels
SRC/p160	Steroid Receptor Coactivators/p160 family
SRE	Serum Response Element
SRF	Serum Response Factor
STAT	Signal Transducer and Activator of Transcription
SYCP3	Synaptonemal Complex Protein 3 gene
t-ERK 1/2	Total-p44/p42 MAPK
TAG	Triacylglycerol
TBS	Tris-Buffered Saline
TIFF	Tagged image File Format
T _m	Melting Temperature
TRP	Transient Receptors Potential
TRPV1	Transient Receptor Potential Vanilloid subfamily 1
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UFSC	<i>Universidade Federal de Santa Catarina</i>
UNICAEN	<i>Université de Caen Normandie</i>
USA	United States of America

TABLE OF CONTENTS

1. INTRODUCTION	19
1.1 MORPHOPHYSIOLOGICAL ASPECTS OF THE TESTES	19
1.1.1 Structural and morphological aspects of the testes	19
1.1.2 Germ cells and the spermatogenesis	19
1.1.2.1 Marker genes and proteins for germ cells and spermatogenesis specific stages: A molecular approach to analyzing germ cell population and spermatogenesis	21
1.1.3 Differences in morphophysiological characteristics of testis, sexual maturation and spermatogenesis of zebrafish and rats	24
1.1.3.1 Testis development and sexual maturation in male rats	26
1.1.3.2 Testis development and sexual maturation in male zebrafish.....	29
1.1.4 Regulation of spermatogenesis.....	30
1.1.5 Role of Sertoli cells and testicular energy metabolism in the regulation of spermatogenesis	31
1.1.5.1 Influence of testicular glucose metabolism.....	33
1.1.5.2 Influence of testicular glycogen metabolism.....	34
1.1.5.3 Influence of amino acid metabolism	35
1.1.5.4 Influence of lipid metabolism	35
1.2 CALCIUM SIGNALING	37
1.2.1 Calcium influx from the extracellular medium through ion channels located at the plasma membrane	37
1.2.2 Nongenomic signaling pathways and calcium release from intracellular calcium stores triggered by second messengers.....	38
1.2.3 Role of Ca²⁺ in the regulation of cellular processes: a life and death signal.....	39
1.3 ESTRADIOL (E2) IN THE MALE REPRODUCTIVE SYSTEM.....	41
1.3.1 Estrogen receptors expression in the testes of mammals and fish ..	41
1.3.2 Effects of estrogens on male reproductive system, spermatogenesis and energy metabolism in mammals and fish	43
1.4 ESTROGENS RECEPTOR SIGNALING MECHANISMS	44
1.4.1 Direct genomic signaling: Classical pathway	44
1.4.2 Nongenomic signaling.....	45
1.4.3 Crosstalk between nongenomic and genomic signaling pathways...	46
1.5 ESTROGEN-LIKE ENDOCRINE DISRUPTING CHEMICALS: BISPHENOL A	47
1.5.1 Effects of BPA on male reproductive system, spermatogenesis and energy metabolism in mammals and fish.....	50
1.5.2 BPA metabolism in rats and fish	50
JUSTIFICATION.....	53
2 OBJECTIVES.....	54

2.1 GENERAL OBJECTIVE.....	54
2.2 SPECIFIC OBJECTIVES.....	54
2.2.1 Specific objectives in zebrafish.....	54
2.2.2 Specific objectives in rats.....	54
3 MATERIALS AND METHODS.....	55
3.1 CHEMICALS.....	55
3.2 ANIMALS.....	56
3.2.1 Fish.....	56
3.2.2 Rats.....	57
3.3 TREATMENTS AND EXPERIMENTAL PROCEDURES IN ZEBRAFISH.....	57
3.3.1 Acute <i>in vitro</i> treatments of zebrafish testes.....	57
3.3.2 Organotypic culture of zebrafish testis.....	57
3.3.3 Acute <i>in vivo</i> treatments with BPA.....	59
3.4 METHODOLOGICAL APPROACHES USED IN ZEBRAFISH EXPERIMENTS.....	59
3.4.1 Calcium (⁴⁵Ca²⁺) influx.....	59
3.4.2 ¹⁴C-D-deoxy-D-glucose uptake.....	61
3.3.3 Measurement of lactate content.....	61
3.3.4 Extraction and measurement of glycogen content.....	61
3.3.5 Activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST).....	62
3.3.6 Measurement of triacylglycerol (TAG) content.....	62
3.3.7 Western Blot.....	62
3.3.7.1 Extraction and denaturation of proteins.....	62
3.3.7.2 Electrophoresis, transfer of proteins and antibody incubations.....	63
3.3.8 Processing of zebrafish testes and acquisition of histological images.....	64
3.3.9 Immunohistochemistry (IHC).....	65
3.3.9.1 Stereology: Quantitative analysis of the positive (brown-stained) LDH cells.....	66
3.3.10 Processing and digital analysis of histological images of zebrafish testis.....	66
3.3.11 TUNEL assay.....	67
3.3.11.1 Stereology: Quantitative stained cells analysis.....	67
3.4 TREATMENTS AND EXPERIMENTAL PROCEDURES IN IMMATURE RATS.....	68
3.4.1 Organotypic culture of testis explants from immature rats.....	68
3.5 METHODOLOGICAL APPROACHES USED IN ZEBRAFISH AND IMMATURE RATS EXPERIMENTS.....	69
3.5.1. Extraction and quantification of total RNA.....	69
3.5.2 Reverse Transcription (RT).....	70

3.5.3 Relative gene expression analysis by real-time Polymerase Chain Reaction	70
3.6 STATISTICAL ANALYSIS	73
4 RESULTS, DISCUSSION AND CONCLUSIONS.....	74
4.1 CHAPTER I: STUDY OF THE NONGENOMIC AND GENOMIC EFFECTS OF BPA ON TESTICULAR ENERGY METABOLISM AND SPERMATOGENESIS IN ADULT ZEBRAFISH TESTIS.....	76
4.1.1 Results of Part I of Chapter I: Study of the <i>in vitro</i> effects of BPA on Ca ²⁺ signaling in the testis of zebrafish.....	74
4.1.1.1 <i>In vitro</i> acute incubation of zebrafish testis with low-concentration BPA increases calcium influx.....	74
4.1.1.3 <i>In vitro</i> BPA increases ⁴⁵ Ca ²⁺ influx via IP3R activation	76
4.1.1.5 BPA-induced plasma membrane damage is dependent on activation of IP3R.....	78
4.1.2 Discussion of Part I of Chapter I.....	79
4.1.3 Conclusions of Part I of Chapter I	85
4.1.4 Results of Part II of Chapter I: Study of the <i>in vitro</i> and <i>in vivo</i> effects of BPA on testicular energy metabolism and spermatogenesis in zebrafish	86
4.1.4.1 <i>In vivo</i> and <i>in vitro</i> BPA disturbs testicular energy metabolism by modulating enzymatic activities and reducing energy substrates.....	86
4.1.4.2 <i>In vivo</i> BPA impairs zebrafish spermatogenesis by decreasing the proportion of the haploid cells.....	88
4.1.4.3 <i>In vivo</i> BPA impairs spermatogenesis by increasing the spermatocytes apoptosis	89
4.1.4.4 <i>In vitro</i> BPA disturbs testicular energy metabolism by modulating the expression of genes involved in carbohydrate and amino acid metabolism...	90
4.1.4.5 Nuclear estrogen receptors (ESR α/β) are involved in the mechanism of action of 10 μ M BPA in modulating the relative expression of PYGL, PFKFb2a and LDHBa	92
4.1.4.7 Immunolocalization and positive LDH cells density: <i>In vitro</i> exposure to 10 μ M BPA results in increased number of testicular cells expressing LDH in the zebrafish	95
4.1.4.8 The increase in the number of testicular cells expressing LDH after incubation with 10 μ M BPA is dependent on nuclear estrogen receptors (ESR α/β).....	96
4.1.4.9 <i>In vitro</i> BPA modulates nuclear gene expression of estrogen receptors and estrogen-related receptors in zebrafish testis.....	
4.1.4.10 <i>In vitro</i> BPA modulates the expression of PIWIL-1, SYCP-3 and ODF3b respective marker genes of spermatogonia, spermatocytes and spermatids	99
4.1.4.11 The decreased expression of ODF3b (spermatid marker gene) mediated by the lowest concentration of BPA is dependent on nuclear estrogen receptors activation	100

4.1.4.13 Impaired spermatogenesis by decreasing the proportion of haploid cells in testes mediated by the lowest concentration of BPA (<i>in vitro</i>) is dependent on nuclear estrogen receptors activation.....	102
4.1.5 Discussion Part II of Chapter I	104
4.1.6 Conclusions of Part II of Chapter I	116
4.2 CHAPTER II: STUDY OF THE <i>IN VITRO</i> GENOMIC EFFECTS OF E2 AND BPA ON TESTICULAR ENERGY METABOLISM AND SPERMATOGENESIS IN IMMATURE RAT TESTIS	117
4.2.1 Results E2 of Chapter II.....	117
4.2.1.1 Acute <i>in vitro</i> exposure to E2 disturbs testicular energy metabolism of immature rats by modulating the expression of genes involved in carbohydrate and amino acid metabolism.....	117
4.2.1.2 Changes in the expression of PYGM, PKM and LDHc in immature rat testis after 6 hours of incubations with E2 are not dependent on nuclear estrogen receptors.....	118
4.2.1.3 Acute <i>in vitro</i> exposure to E2 does not modulate the expression of spermatogonia and spermatocytes cell marker genes in immature rats	119
4.2.2 Results BPA of Chapter II.....	120
4.2.2.1 Acute <i>in vitro</i> exposure to BPA disturbs testicular energy metabolism of immature rats by modulating the expression of genes involved in carbohydrate and amino acid metabolism.....	120
4.2.2.2 Changes in the expression of PYGM, PKM, LDHc, GPT2 and GK2 in immature rat testis after 6 hours of incubations with BPA are not dependent on nuclear estrogen receptors.....	122
4.2.2.3 Acute <i>in vitro</i> exposure to BPA does not modulate the expression of spermatogonia and spermatocytes marker genes in immature rat testis	123
4.2.3 Discussion of Chapter II	124
4.2.4 Conclusions of Chapter II.....	129
5 CONCLUSIONS	130
6 FUTURE PERSPECTIVES	131
RÉSUMÉ SUBSTANTIEL.....	132
REFERENCES.....	156
APPENDIX.....	189

1. INTRODUCTION

1.1 MORPHOPHYSIOLOGICAL ASPECTS OF THE TESTES

1.1.1 Structural and morphological aspects of the testes

The testis is a paired organ that has two main functions: spermatozoa production (spermatogenesis) and steroid hormones production (steroidogenesis) (MATTA, 2002). The testes are surrounded by a fibrous capsule of connective tissue called the tunica albuginea. The mammalian testes have an ellipsoid (ovoid) shape and are located outside the body, in the scrotum (SHARMA, 2007). The testes of teleost fish, on the other hand, have an elongated shape and are attached to the dorsal wall within the abdominal cavity by a mesorchium (URIBE; GRIER; MEJÍA- ROA, 2015). However, in all vertebrates, from fish to mammals, the testis is composed of two main compartments, the interstitial and the tubular compartment (KOULISH; KRAMER; GRIER, 2002). The interstitial compartment is composed of steroidogenic Leydig cells, blood and lymphatic vessels, as well as macrophages, mast cells and connective tissue cells, which are continuous with the tunica albuginea (SCHULZ et al., 2010). The tubular compartment houses the germinal epithelium containing the somatic Sertoli cells and germ cells, and it is delineated by a basement membrane and peritubular myoid cells (SCHULZ et al., 2010; URIBE; GRIER; MEJÍA-ROA, 2015).

1.1.2 Germ cells and the spermatogenesis

Spermatogenesis is a complex biological event highly organized in which spermatogonial stem cells become spermatozoa through a series of events involving cell proliferation, division and differentiation (Figure 1). The different stages of germ cells development are identified and differentiated based on morphological and functional aspects. Therefore, spermatogenesis is composed of three main phases that are very similar between different classes of vertebrates, such as fish and mammals: I) Mitotic/spermatogonial phase; II) Meiotic/spermatocytary phase; and III) Spermiogenic/differentiation phase (NÓBREGA; BATLOUNI; FRANÇA, 2009).

The mitotic phase is characterized when a spermatogonial stem cell (diploid cell) proliferates through mitotic divisions giving rise to undifferentiated spermatogonia,

type A differentiated spermatogonia, intermediate spermatogonia and type B spermatogonia. The cells resulting from the mitotic divisions of a single spermatogonia remain connected by cytoplasmic bridges due to incomplete cytokinesis (NÓBREGA; BATLOUNI; FRANÇA, 2009; SCHULZ et al., 2010). The meiotic phase begins when type B spermatogonia enter in the first meiotic prophase giving rise to preleptotene spermatocytes (HERMO et al., 2010). Meiosis is a complex process unique to germ cells that requires many genes and involves one round of DNA duplication followed by two consecutive cell divisions for diploid cells to form haploid cells. In meiosis I, the first prophase is subdivided into different stages giving rise to leptotene, zygotene, pachytene and diplotene spermatocytes (COBB; HANDEL, 1998; HERMO et al., 2010; SCHULZ et al., 2010). Prophase is followed by metaphase, where paired chromatids attach to the equator of the spindle. Then, in anaphase, the chromatids are paired to opposite poles of the spindle, and telophase gives rise to daughter cells called secondary spermatocytes. In meiosis II, they divide to form haploid cells called spermatids. Subsequently, spermiogenesis occurs, where round spermatids are transformed into highly elongated spermatids, which in turn undergo drastic morphological and functional changes such as nuclear condensation and flagella formation, giving rise to mature spermatozoa (HESS; DE FRANCA, 2008; NÓBREGA; BATLOUNI; FRANÇA, 2009; SCHULZ et al., 2010).

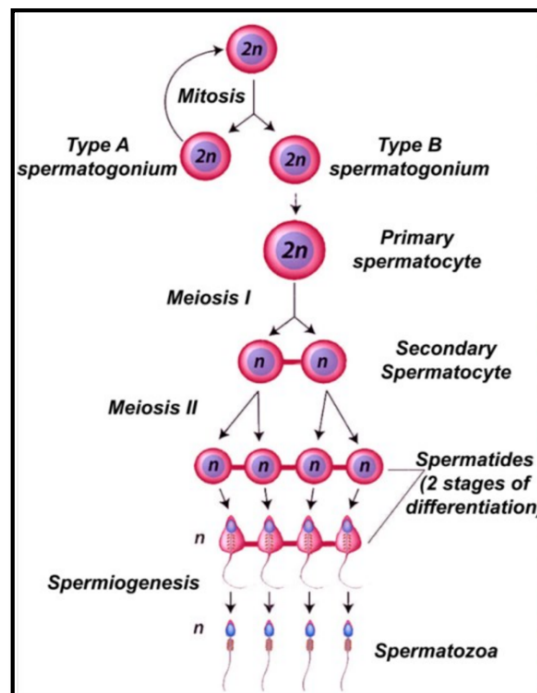


Figure 1: Schematic representation of different stages of spermatogenesis in which mature and motile spermatozoa are produced. In adult males, the mitotic division of the spermatogonial stem cells results

in two types of spermatogonia; type A spermatogonia are reserved for the renewal of spermatogonial stem cells, while type B spermatogonia further differentiate into (diploid) primary spermatocytes. The primary spermatocytes undergo meiosis I resulting in two (haploid) secondary spermatocytes and these cells, through meiosis II, produce four (haploid) spermatids. In the final stage called spermiogenesis, spermatids undergo numerous morphological changes, such as the formation of the acrosome, nuclear condensation, development of the flagellum and reorganization of the cytoplasm, to give rise to mature spermatozoa. Source: Adapted from KHAWAR; MEHMOOD; ROOHI, 2018.

1.1.2.1 Marker genes and proteins for germ cells and spermatogenesis specific stages: A molecular approach to analyzing germ cell population and spermatogenesis

The expression of many specific genes and proteins during different phases of spermatogenesis has been revealed through molecular biology techniques (ANWAY et al., 2003). Determination of transcripts specifically expressed by germ cells provides an extensive list of probable critical proteins. Total transcript profiles in the testes of developing mice identified 1652 genes whose transcript abundance markedly increased coincidentally with the onset of meiosis (SCHULTZ et al., 2003). In addition, further analysis of the UniGene EST database coupled with real-time polymerase chain reaction (PCR) has already indicated that approximately 4% of the mouse genome is dedicated to expression in post-meiotic male germ cells. Targeted disruption of 19 of these genes has indicated that a majority have roles critical in male fertility (SCHULTZ et al., 2003).

The first phase of spermatogenesis is characterized by spermatogonial stem cell proliferation through mitotic divisions, which give rise to different spermatogonia populations. Thus, spermatogonial proliferation serves to ensure a constant supply of germ cell precursors for spermatogenesis. The detection of several antigens which are selectively expressed during defined stages of the cell cycle has become widely used approach for the study of cell proliferation (CELIS; CELIS, 1985; SCHLATT; WEINBAUER, 1994). One of the most used antigens is the proliferating cell nuclear antigen (PCNA) (SCHLATT; WEINBAUER, 1994) which constitutes an anchor point for DNA polymerases δ during DNA replication (BRAVO et al. 1987). PCNA is a highly conserved 36 kDa (kilodalton) eukaryotic nuclear protein which is differentially regulated during the cell cycle, reaching maximum expression at the G1/S phase transition and lowest levels during M phase (CELIS; CELIS, 1985). Thus, studies use PCNA as an important marker of proliferating spermatogonia (MIURA; MIURA; YAMASHITA, 2002; LEAL et al., 2009a; HERMO et al., 2010). The detection of PCNA immunolocalization and its expression in the germinal epithelium are widely used

approaches to investigate cell proliferation. Studies obtained in Japanese eel fish (MIURA; MIURA; YAMASHITA, 2002) and zebrafish (LEAL et al., 2009a) have been reported that PCNA is predominantly expressed in spermatogonia. Furthermore, the immunolocalization of PCNA has been detected exclusively in nuclei of spermatogonia and early spermatocytes from mice (LIU; WANG, 2021), rats and monkeys (SCHLATT; WEINBAUER, 1994), as well as from Pacific oysters (FRANCO et al., 2010). In addition to DNA synthesis, PCNA participates also of other important cellular processes, including cell cycle control, nucleotide excision repair, post-replication mismatch repair, base excision repair and apoptosis (JÓNSSON; HÜBSCHER, 1997). Low and moderate cellular quantities of p53 tumor-suppressor protein (p53) positively stimulate PCNA expression, while high levels of p53 inhibit its expression (MERCER et al., 1991; YAMAGUCHI et al. 1994; PAUNESKU et al. 2001). It has already been reported that if PCNA protein is present in abundance in the cell in the absence of p53, DNA replication occurs. On the other hand, if PCNA becomes non-functional, present in low quantities or absent in the cells, they cell die by apoptosis (PAUNESKU et al. 2001).

In addition to PCNA, integrins are also expressed during defined stages of the cell cycle which plays an important role for cell proliferation, differentiation and cell survival. Adult rodent spermatogonial stem cells express integrin alpha-6 (ITGa6), a subunit of a receptor for laminin-332 (laminin-5) (SHINOHARA; AVARBOCK; BRINSTER, 1999; EBATA; ZHANG; NAGANO, 2005), which has also been used as one of the spermatogonia marker genes (HERMO et al., 2010). Furthermore, Piwi-element-induced wimpy-like protein 1 (PIWIL-1) is also expressed during defined stages of the cell cycle, which is more commonly used as a spermatogonia marker gene in zebrafish studies (NÓBREGA et al., 2010; WONG; COLLODI, 2013). PIWIL are endoribonucleases that associate with small non-coding RNAs, piRNA, forming a functional complex that plays a central role in maintaining the integrity of the germline by repressing transposable elements and preventing their mobilization (FERNANDES et al., 2018).

Among the several spermatocytes markers, DNA meiotic recombinase 1 (DMC1) gene is considered one of the main and most used spermatocyte and prophase meiotic markers (HERMO et al., 2010). In most organisms, homologous recombination occurs during meiosis and is essential to ensure accurate segregation of homologous chromosomes at the first meiotic division. In the first meiotic prophase, DMC1 is required to generate a cross-over between homologous chromosomes which

ensures the segregation of the chromosomes at meiotic division I (SHINOHARA; SHINOHARA, 2004). In the first meiotic prophase, homologous chromosomes undergo homology recognition, align and synapse, at which point the crossover events are referred to as chiasmata, which are points of physical contact between homologous chromatids (PITTMAN et al., 1998). DMC1 knockout mice result leads to infertility due a defect in cross-over and in synaptonemal complex (SC) formation, which leads to the arrest in late prophase of meiosis I (PITTMAN et al., 1998). The SC is a scaffold composed of proteins that connect homologous chromosomes along their entire length to form synapses of homologous chromosomes during prophase of meiosis I (HERMO et al., 2010; ZHANG; ZHANG; GAO, 2021). The axial/lateral elements of the SC consist of scaffold proteins, SC protein 2 (SCP2) and SC protein 3 (SCP3) (Figure 2), which are encoded by SYCP-2 and SYCP-3, respectively (SCHALK et al., 1998). SCP3 knockout mice has been revealed an unformed synaptonemal complex with arrest of meiosis in zygote spermatocytes, rendering them infertile (YUAN et al., 2000). Therefore, SYCP-3 is one of the main and most used spermatocyte and prophase meiotic markers, it is widely used as a spermatocyte marker gene in zebrafish studies (LEAL et al., 2009b; OZAKI et al., 2011; CRESPO et al., 2019; GONZÁLEZ-ROJO et al., 2019; TOVO-NETO et al., 2020).

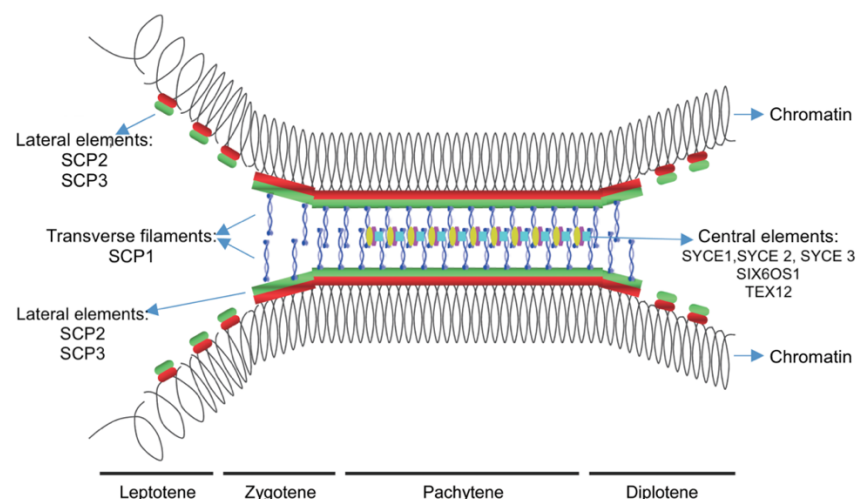


Figure 2: Formation and depolymerization of synaptonemal complexes. In leptotene spermatocytes, the assembly of the lateral elements, composed of SCP2 and SCP3, begins. In the zygotene phase, homologous chromosomes begin to pair, while central elements and transverse filaments (composed of SCP1) are formed between the paired axes. In pachytene spermatocytes, homologous chromosomes synapse completely with elements formed along the entire length of the chromosomal axes. In diplotene spermatocytes, the synaptonemal complex begins to disassemble. SYCE – Synaptonemal complex central element protein; SIX6OS1 – SIX6OS1 protein; TEX12 – Testis-expressed protein 12. Source: Adapted from JIANG et al., 2020.

Thus, meiotic cell divisions ensure that diploid germ cells, spermatocytes, produce haploid cells, spermatids and later mature spermatozoa. In the final process of haploid cells development (spermiogenesis), nuclei of round spermatids condense and the cells elongate to form the characteristic morphology of spermatozoa (HESS; DE FRANCA, 2008). Thus, proteins or protein-coding genes specific of the flagellum of spermatids and elongated spermatozoa can be used as markers of haploid cells. Among them, outer dense fiber protein 3b (ODF3b) is a testis-specific protein which is distributed in the intermediate and main parts of the flagella of elongated spermatids and spermatozoa (DE CARVALHO et al., 2002). As ODF3b gene is transcribed more specifically in spermatids (PETERSEN et al., 2002), it is widely used as a spermatid marker gene in zebrafish studies (LEAL et al., 2009b; CRESPO et al., 2019; TOVO-NETO et al., 2020).

1.1.3 Differences in morphophysiological characteristics of testis, sexual maturation and spermatogenesis of zebrafish and rats

The testes of fish and rats have the same function regarding to spermatozoa production, but there are some relevant morphophysiological differences. Among them, the main one is that the tubular compartment and spermatogenesis are the cystic type in fish and non-cystic type in mammals (Figure 3) (SCHULZ et al., 2010; URIBE; GRIER; MEJÍA-ROA, 2015). In addition, another important difference is that fish Sertoli cells maintain their ability to proliferate even in the adult phase (SCHULZ et al., 2005; LEAL et al., 2009a), unlike rat Sertoli cells, which proliferate only until infantile or juvenile phases, before reaching puberty (ORTH et al., 1982). Hence, adult mammalian testis contains a fixed number of Sertoli cells defined before puberty that determines the size of the testis and the ability to support successive spermatogenic waves (SHARPE et al., 2003; JOHNSON; THOMPSON; VARNER, 2008). Therefore, a ratio of spermatids supported by a Sertoli cell is reported to be about 100/1 in fish (SCHULZ et al., 2005; LEAL et al., 2009a), which is approximately 10 times higher than in mammals (RUSSEL; PETERSON, 1984). Thus, it has been suggested that the spermatogenesis cystic type is more efficient than the way Sertoli cells are distributed in the seminiferous tubules of mammals (MATTA et al., 2002).

Spermatogenesis (cystic type) in fish occurs within cysts which are formed inside the seminiferous tubules when an undifferentiated spermatogonia is completely

enveloped by the cytoplasmic extension of one or two Sertoli cells. Therefore, fish spermatogenesis allows the synchronous development of a single group of germ cells in clonal development derived from a single spermatogonia (MATTA, 2002; SCHULZ et al., 2005, 2010; LEAL et al., 2009a). From cross-sections of the testis of an adult zebrafish it is possible to identify spermatogonia, spermatocytes and spermatids cysts, as well as spermatozoa dispersed in the lumen of the tubule after the opening of the cysts (SCHULZ et al., 2010). The combined duration of the meiotic and spermiogenic phases of spermatogenesis is approximately 6 days, one of the shortest periods among teleost fish ever investigated (LEAL et al., 2009a).

Unlike fish, spermatogenesis is the non-cystic type in mammals. From cross-sections of the testis of an adult rat, it is possible to identify four or five layers of germ cells. The cells of each layer comprise a generation, a cohort of cells that develop as a synchronous group (SHARMA, 2007). The synchronized development of germ cells results from the repetitive appearance of specific cellular associations of germ cells, named stages of the seminiferous epithelium (LEBLOND; CLERMONT, 1952a). Leblond and Clermont (1952a) defined such cellular associations based on the nineteen stages of spermatid development identified in rat spermiogenesis (LEBLOND; CLERMONT, 1952b). Therefore, 14 specific cellular associations were defined as I–XIV stages of the seminiferous epithelium. During the first 8 stages of young spermatid development, the seminiferous epithelium also contains an older generation of spermatids that reach the last stage of development and are released when the younger generation completes stage 8. Therefore, the release of immature spermatozoa from the epithelium occurs at the end of stage VIII of the seminiferous epithelium (LEBLOND; CLERMONT, 1952a). The series of changes that occur between two successive appearances of the same cellular association in a given area of the rat seminiferous tubule was defined as a cycle of the seminiferous epithelium (LEBLOND; CLERMONT, 1952a). Its duration has been estimated to be around 13 days in Wistar and Sprague-Dawley rats (CLERMONT; HARVEY, 1965; CLERMONT, 1972) and 16 days in humans (HELLER; CLERMONT, 1963). The process of spermatogenesis extends over a period equal to four complete cycles of the seminiferous epithelium. Therefore, the estimated duration of spermatogenesis is approximately 52 days in rats (CLERMONT; HARVEY, 1965; CLERMONT, 1972) and approximately 64 days in humans (HELLER; CLERMONT, 1963).

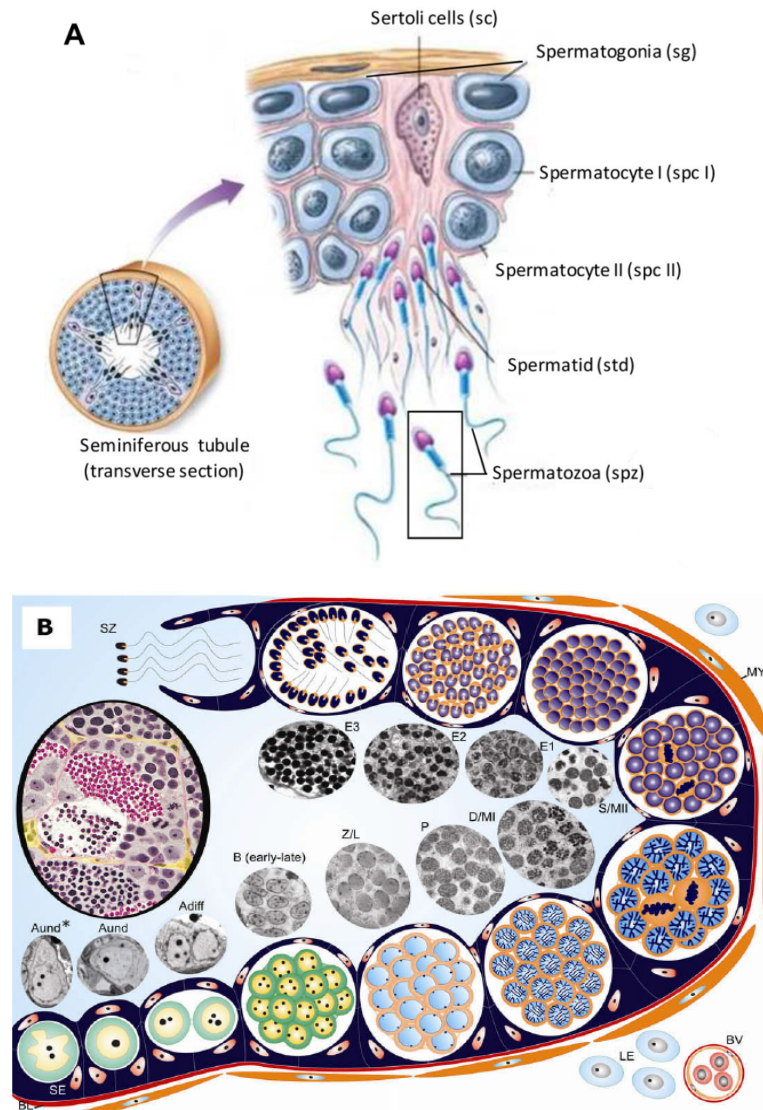


Figure 3: Comparison of morphological differences between (A) non-cystic and (B) cystic spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). Interstitial Leydig cells (LE) and blood vessels (BV) are shown in the interstitial compartment. (B) Aund* – undifferentiated spermatogonia*; Aund – type A undifferentiated spermatogonia; Adiff – type A differentiated spermatogonia; B early-late – type B spermatogonia; L/Z – Leptotenic/Zygotenic primary spermatocytes; P – Pachytenic primary spermatocytes; D/MI – Diplotenic spermatocytes/Metaphase I; S/MII – Secondary spermatocytes/Metaphase II; E1 – early spermatids, E2 – intermediate spermatids; E3 – late spermatids; SZ – Spermatozoa. Source: Adapted from SCHULZ et al., 2010.

1.1.3.1 Testis development and sexual maturation in male rats

Puberty is the period in the vertebrate life cycle that marks the transition from sexual immaturity to maturity, enabling an animal to acquire reproductive functions. Male sexual maturation involves postnatal development of the testes, which occurs through a range of endocrine and morphological changes (PICUT et al., 2015). Thus, testicular development and sexual maturation of male rats can be divided into five

stages. These stages include the neonatal which corresponds to the first 7 postnatal day (PND); infantile (8–20 PND) and juvenile (21–32 PND), which correspond to the prepubertal period; peri-pubertal (33–55 PND) and late pubertal (56–70 PND) periods (OJEDA; ADVIS; ANDREWS 1980; OJEDA; SKINNER, 2006). Therefore, male rats are sexually mature at 70 PND (OJEDA; SKINNER 2006; PICUT et al., 2015). The main features of morphological (Figure 4) and neuroendocrine parameters observed during neonatal, early and late infantile, juvenile and peripubertal stages are summarized in Table 1.

Age (PND)	Stage	Morphology	Neuroendocrine parameters
0 to 7	Neonatal	<ul style="list-style-type: none"> • Tubules lined by Sertoli cells and gonocytes • Gonocytes transition to spermatogonia Type A • High mitotic rate in Sertoli cells • No tubular stages apparent 	<ul style="list-style-type: none"> • PND 0 to 3: Fetal testosterone level high • FSH level high (gonocyte transition occurs) and then diminishes due to rising inhibin level • Regression of fetal Leydig cells and development of progenitor Leydig cells that produce DHT • Androgen-independent spermatogonial mitosis
8 to 14	Early infantile	<ul style="list-style-type: none"> • ↑ proliferation/density of Sertoli cells and spermatogonia (types A and B) • High mitotic rate in all cell populations • No tubular stages apparent 	<ul style="list-style-type: none"> • Testosterone level low • FSH level low • PND 9 to 16: E2 levels rise
15 to 20	Late infantile	<ul style="list-style-type: none"> • Apoptosis of spermatogonia; mitotic activity wanes • PND 15: Sertoli cells mature and stop dividing • First wave of spermatogenesis with appearance of: <ul style="list-style-type: none"> • PND 16: leptotene/zygotene spermatocytes • PND 18: pachytene spermatocytes • PND 16: tubular stages become apparent • PND 18: tubular lumina develop • PND 18 to 22: double-layered rosettes present 	<ul style="list-style-type: none"> • Testosterone level low • PND 16: Blood–testis barrier (BTB) develops • DHT levels high and support first wave of spermatogenesis • E2 level wanes as BTB forms and Sertoli cells mature
21 to 32	Juvenile	<ul style="list-style-type: none"> • Loss of rosette architecture • Tubular diameter increases • Apoptosis of pachytene spermatocytes (due to relatively low testosterone level) • PND 26: round spermatids appear • PND 20 to 70: marked increase in tubular diameter 	<ul style="list-style-type: none"> • Testosterone level starts to increase (produced by immature Leydig cells) • PND 21: progenitor Leydig cells peak • PND 28 to 30: PRL plays role in maturation of HPG axis with parallel increasing levels of LH and T and decreased sensitivity of feedback
33 to 55	Peri-pubertal	<ul style="list-style-type: none"> • Spermiogenesis • Elongating spermatids appear • PND 46: stage VII tubules; step 19 spermatids • PND 52: spermatozoa in epididymis 	<ul style="list-style-type: none"> • PND 35: immature Leydig cells maximum • High FSH levels stimulate maturation of Leydig cells and increased testosterone production • High PRL levels increase LH receptors on Leydig cells to stimulate higher testosterone production • PND 45: FSH levels peak (important for final maturation of Leydig cells and release of spermatocytes) • Mature Leydig cells form • By PND 90: testosterone levels are at adult levels

Table 1: Morphological and neuroendocrine features of postnatal testicular development. BTB – Blood–testis barrier; DHT – dihydrotestosterone; E2 – Estradiol; FSH – Follicle-stimulating hormone; HPG – Hypothalamic–pituitary–gonadal; LH – Luteinizing hormone; PND – Postnatal day; PRL – prolactin. Source: PICUT et al., 2015.

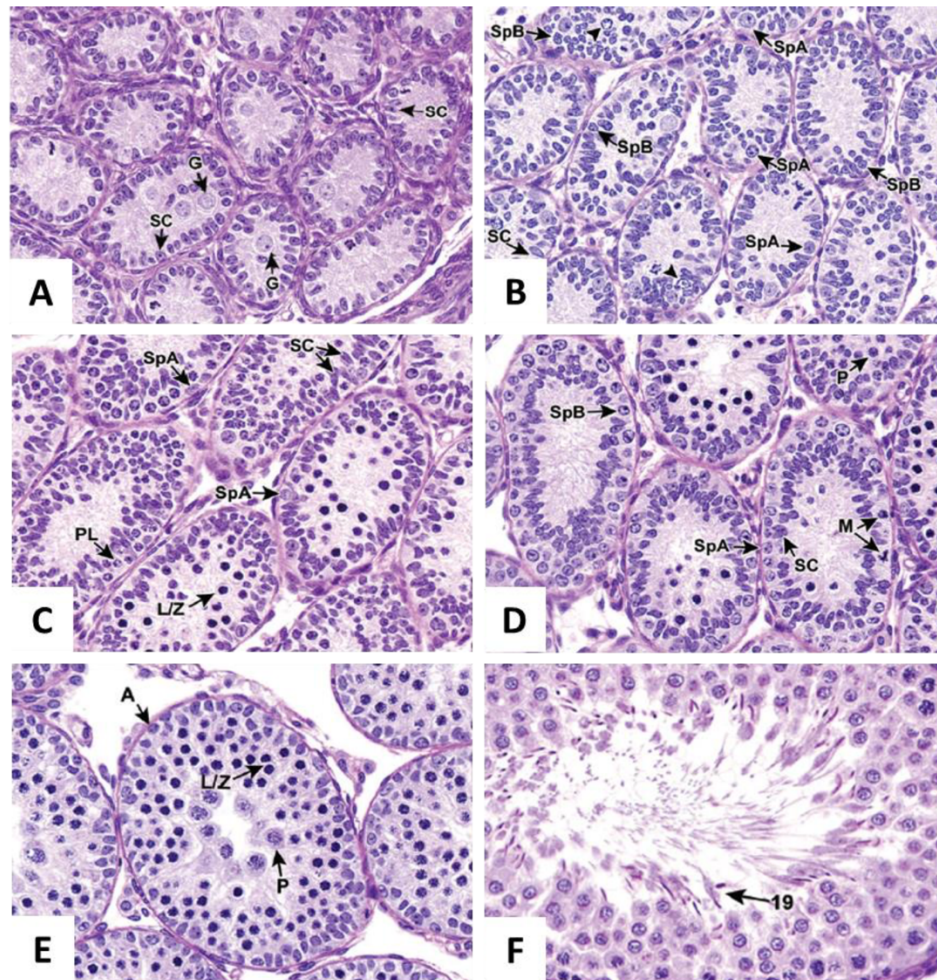


Figure 4: Stages of postnatal testicular development and maturation. (A) PND 3 (neonatal): tubules lined by numerous Sertoli cells (SC) in the basement membrane. Gonocytes (G) have a large round nucleus and are located in the center of the tubules. (B) PND 9 (infantile): tubules are lined by Sertoli cells and spermatogonia, forming a pseudostratified layer. Sertoli cells have an elongated to polygonal nucleus with scattered chromatin and a prominent nucleolus. Type A spermatogonia (SpA) have a large, oval nucleus and type B spermatogonia (SpB) have a small and round nucleus. It is possible to observe mitotic figures in spermatogonia populations and Sertoli cells, as well as many cells in prophase (arrowhead). (C) PND 16 (infant): Formation of spermatocytes in adluminal leptotene and zygotene (L/Z) in some tubules. Other tubules contain spermatocytes in preleptotene (PL). (D) DPN 18 (infantile): Spermatogonia and Sertoli cells form a double layer called rosette, with an outer row of types A and B spermatogonia and an inner row of Sertoli cell nuclei. Pachytene spermatocytes (P) appear and few mitotically active spermatogonia (M) are still present. (E) DPN 25 (juvenile): Development of spermatocytes in pachytene continues and Sertoli cells and spermatogonia recede into a single layer as the tubule diameter expands. (F) PND 46 (peri-pubertal): Spermatids in stage 19 present in tubule at stage VII (arrow). Source: Adapted from PICUT et al., 2015.

Interestingly, those stages of testicular development and sexual maturation in rats loosely correlate with similar developmental stages in humans (BARROW; BARBELLION; STADLER, 2011). An age comparison of developmental stages in rat^a (OJEDA; ADVIS; ANDREWS 1980) and human^b (BARROW; BARBELLION; STADLER, 2011) is summarized in Table 2.

Stage name in rat/human	Rat ^a	Human ^b
Neonatal/newborn	PND 0 to 7	0 to 28 days
Infantile/infant	PND 8 to 20	1 to 23 months
Juvenile/child	PND 21 to 32	2 to 12 years
Peri-pubertal/adolescent	PND 33 to 55	12 to 16 years
Late puberty/adolescent	PND 56 to 70	

Table 2: Age comparison of developmental stages between rat and human. Source: PICUT et al., 2015.

As spermatogenesis is not yet established in prepuberty stages, this is a critical period of reproductive development that is highly vulnerable to changes caused by environmental factors. Exposures to endocrine disrupting chemicals (NORIEGA et al. 2009; MOURITSEN et al., 2010; OLIVEIRA et al., 2017) and toxic metal (COUTO-SANTOS et al., 2020) during the prepubertal phase have been shown to delay the onset of puberty and lead to damage to the testes that may disrupt the fertility in adulthood. Despite the age difference, there is a close similarity and conservation of key events in the development and sexual maturation of rats and humans (OJEDA; SKINNER, 2006). In this way, many of the results obtained in studies with rats as an animal model can be extrapolated to humans.

1.1.3.2 Testis development and sexual maturation in male zebrafish

The zebrafish lifecycle is divided into four major periods: embryo, larva, juvenile and adult. The process of gonad differentiation has been described to be juvenile hermaphroditic, since juvenile zebrafish gonads develop first as ovary-like ones followed by differentiation into the ovary or testis (MAACK; SEGNER, 2003). The timeline of gonad differentiation in zebrafish is shown in Figure 3. Gonad differentiation in zebrafish begins at around 25 days post-fertilization (dpf) (Figure 5B). At 25 dpf, the gonads contain oocyte-like germ cells that differentiate into oocytes or undergo apoptosis to give rise to testicular tissues (CHEN; GE, 2013). Ovarian differentiation is finished at 35 dpf (Figure 5C), while testis differentiation is finished around 45 dpf (Figure 5D) (CHEN; GE, 2013). The timing of testis differentiation takes longer than ovarian differentiation, probably because it involves additional steps of removal of oocyte-like germ cells by apoptosis (UCHIDA et al., 2002). The gonads at 20 dpf are full of proliferating germ cells. At 25 dpf, the gonads contained oocyte-like germ cells

that differentiate into oocytes or undergo apoptosis to give rise to testicular tissues. Zebrafish reach sexual maturity and is considered adult at around 60 to 90 dpf, which is considered fast in relation to its average lifespan of three to five years (PARICHY et al., 2009). Thus, zebrafish is widely used as a model organism in the biology of reproduction field (RIBAS; PIFERRER, 2013).

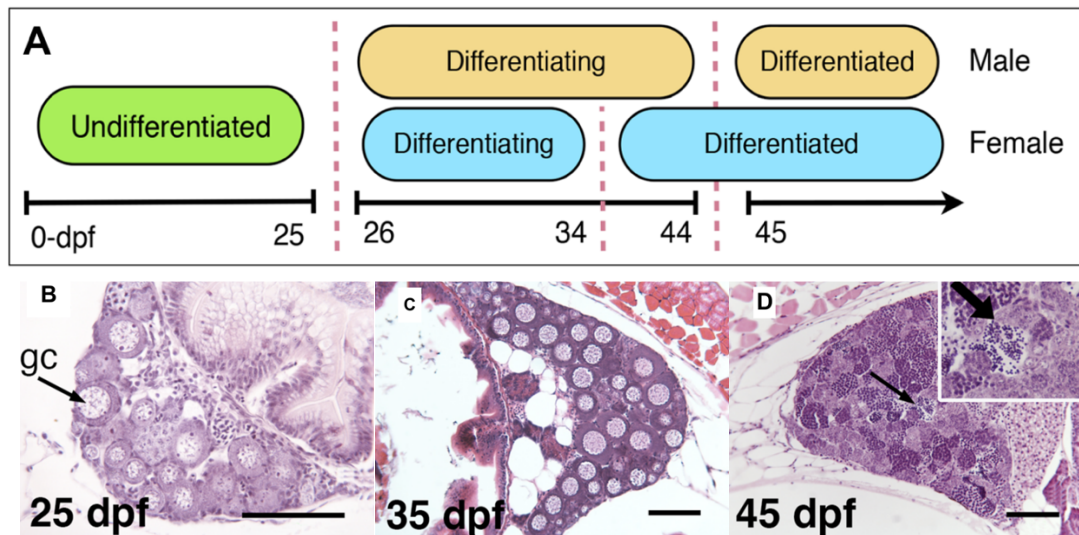


Figure 5: Timeline and histology of gonad differentiation in zebrafish. (B) Gonad differentiation began around 25 dpf; (C) ovarian differentiation is complete around 35 dpf, (C) whereas testis differentiation finishes around 45 dpf. Source: Adapted from CHEN; GE, 2013.

1.1.4 Regulation of spermatogenesis

Spermatogenesis is mainly regulated by the hypothalamic-pituitary-gonadal axis (HPG), known as the neuroendocrine axis of the reproduction, it is widely studied and well characterized in fish (TAKAHASHI et al., 2016) and in mammals (KAPRARA; HUHTANIEMI, 2018). Gonadotropin-releasing hormone (GnRH) plays a central role in this axis, which stimulates the biosynthesis and secretion of gonadotropins (GTH) follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (ZOHAR et al., 2010; TAKAHASHI et al., 2016). These play a crucial role in the reproductive physiology of fish and were initially called GTH I and GTH II, respectively, in fish (NAGAHAMA, 1994). FSH stimulates the proliferation of Sertoli cells and regulates the performance of their main functions. In this way, FSH regulates its structural, nutritional and energetic function, directly influencing the development of germ cells and successful spermatozoa production (JUTTE et al., 1983; SLAUGHTER; MEANS, 1983; SHARPE et al., 2003; HUHTANIEMI, 2015). LH acts on Leydig cells regulating the production of

androgens, which together with FSH regulate different phases of spermatogenesis, such as, the entry of spermatogonia into meiosis and spermiogenesis (YOUNG; KUSAKABE; NAKAMURA, 2005; CRESPO et al., 2016; GOLSHAN; ALAVI, 2019). Furthermore, another hormone that regulates spermatogenesis is 17 β -estradiol (E2), which plays an important role in stimulating the renewal of spermatogonial stem cells (MIURA et al., 1999; AMER et al. 2001). E2 in the male reproductive system will be further discussed in section 1.3.

1.1.5 Role of Sertoli cells and testicular energy metabolism in the regulation of spermatogenesis

The successful development of germ cells is closely related to the functioning of Sertoli cells, since they are responsible for providing physical, nutritional and energy support to the germ cells (SCHULZ et al., 2010; RATO et al., 2012b; ALVES et al., 2013a). Germ cells can only survive in close and continuous interaction with Sertoli cells. The number of Sertoli cells determines the spermatogenic capacity of the testis and is an important target of signaling systems that regulate spermatogenesis (MATTA et al. 2002). It is well reported that Sertoli secrete a wide variety of nutritional and energy factors needed for the metabolism, development and differentiation of germ cells. These factors include: transferrin (LE MAGUERESSE et al., 1988), ceruloplasmin (SKINNER; GRISWOLD, 1983); pyruvate and lactate (JUTTE et al., 1983; ROBINSON; FRITZ, 1981); androgen binding protein (HAGENÄS et al., 1975); activin and inhibin (YING, 1987); interleukins 1 and 6; insulin-like growth factor (IGF) I and II, transforming growth factor α and β (NEHAR et al., 1997; SKINNER, 2005).

As highlighted in the previous section, spermatogenesis is characterized by the development of spermatogonial stem cells into spermatozoa through processes of proliferation, division and cell differentiation. However, in addition to drastic morphofunctional changes, germ cells also undergo metabolic differentiation processes. Germ cells have peculiar nutritional needs during spermatogenesis, altering the metabolic profile throughout development (RATO et al., 2012b). There are differences in expression patterns of key proteins in the metabolism of glucose, glycogen and lipids, as well as in the preference for the use of different energy substrates by different germ cells. This can be explained by the need for a specific metabolic process for each stage such as supporting cell differentiation (LEIDERMAN;

MANCINI, 1969; RATO et al., 2012b; ALVES et al., 2013a; VILLARROEL-ESPÍNDOLA et al., 2013). Scientific evidences have already shown the expression and/or activity of glycolysis key-enzymes in spermatids and spermatozoa of humans, dogs, rats and mice, which are absent in spermatogonia and primary spermatocytes (YAÑEZ et al., 2003, 2007; ALBARRACÍN et al., 2004). It is also evidenced the activity of glycolytic enzymes in spermatozoa of different teleost fish species (LAHNSTEINER; PATZNER; WEISMAN 1993; MANSOUR; LAHNSTEINER; BERGER, 2003; ZIETARA et al., 2009).

Although fish are the most diverse and abundant group of vertebrates, the knowledge available in the scientific literature on energy metabolism at all stages of spermatogenesis is scarce. This knowledge is limited to spermatozoa bioenergetics. Spermatozoa of different species of teleost fish are dependent on triglycerides and glucose metabolism for energy generation by involving β -oxidation, glycolysis, Krebs cycle and oxidative phosphorylation (Figure 6) (LAHNSTEINER; PATZNER; WEISMAN 1993; MANSOUR; LAHNSTEINER; BERGER, 2003). However, during motility, the most energy-demanding situation for a fish spermatozoon, anaerobic glycolysis becomes the predominant pathway in energy generation, with a significant increase in lactate concentration (LAHNSTEINER; PATZNER; WEISMAN 1993; DREANNO et al., 2000). After motility is finished, triglyceride catabolism is used for the regeneration of ATP levels (LAHNSTEINER; PATZNER; WEISMAN 1993).

The influence of energy metabolism on spermatogenesis is further known in mammals. The importance of lactate as preferred substrate for spermatocytes and spermatids and for the differentiation and survival of these cells has been widely studied under *in vitro* conditions. It is reported that lactate provided by Sertoli cells is the main energy substrate of rodent and human spermatocytes and spermatids (MITA; HALL, 1982; JUTTE et al., 1983; GROOTEGOED; JANSEN; VAN DER MOLEN, 1984; BOUSSOUAR; BENAHMED, 2004; ALVES et al., 2013a). Production of pyruvate/lactate by rat Sertoli cells is stimulated by FSH. It was first suggested nearly four decades ago that these are the main energy substrates produced by Sertoli cells capable of maintaining the performance and survival of spermatocytes and spermatids, and regulating spermatogenesis (JUTTE et al., 1983; GROOTEGOED; JANSEN; VAN DER MOLEN, 1984). Furthermore, lactate is considered a testicular protective factor against apoptosis, and its availability has already been shown to inhibit germ cell apoptosis (ERKKILÄ et al., 2002; BUSTAMANTE-MARÍN et al., 2012). Therefore, the

reduction or inhibition of its production may impair spermatogenesis and contribute to male infertility (BUSTAMANTE-MARÍN et al., 2012; LUO et al., 2020).

1.1.5.1 Influence of testicular glucose metabolism

Glucose is reported to be the main source of pyruvate/lactate, which are crucial sources of energy (ATP) for successful spermatogenesis, which demands a high energy supply. Sertoli cells metabolize glucose to lactate through both aerobic and anaerobic pathways (Figure 6). Glucose enters the cell via glucose transporters (GLUT) and is then metabolized by a series of reactions catalyzed by glycolytic enzymes. The first limiting step of glycolysis is mediated by 6-phosphofructo-1-kinase (PFK-1) that catalyzes the irreversible conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. Fructose-2,6-bisphosphate is the most powerful allosteric activator of PFK-1 (SAKATA; ABE; UYEDA, 1991). The synthesis and degradation of fructose-2,6-bisphosphate is catalyzed by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) bifunctional enzyme (VAN SCHAFTINGEN, 1987; PILKIS et al., 1995). PFK-2/FBPase-2 isozymes are encoded by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB) genes and their expression already been reported in the rat testis (SAKATA; ABE; UYEDA, 1991), as well as mouse spermatogonia and mouse Sertoli (TM4) cell lines (GOMEZ et al., 2005).

The last and irreversible step of glycolysis is catalyzed by pyruvate kinase, which converts phosphoenolpyruvate (PEP) into pyruvate. Subsequently, pyruvate is converted into lactate by the action of lactate dehydrogenase (LDH) in the presence of reduced nicotinamide-adenine dinucleotide (NADH) (JUTTE et al., 1983; ROBINSON; FRITZ, 1991). It is reported that most of the pyruvate produced by Sertoli cells is converted into lactate (OLIVEIRA et al. 2015), then exported to the intratubular fluid via monocarboxylate transporter (MCT) 4, and later to the germ cells via MCT1 and MCT2 (SKINNER, 1991; RATO et al., 2012). In addition, pyruvate may also be converted into acetyl-CoA by the action of the pyruvate dehydrogenase complex (PDH), and enter the Krebs cycle (HUANG et al., 1998; RATO et al., 2012).

1.1.5.2 Influence of testicular glycogen metabolism

In addition to glucose, glycogen is also an important energy source for the production of lactate in Sertoli cells through glycogenolysis (RATO et al., 2012b). Glycogen is shown to play a crucial role during testicular development and also to act as a modulator of germ cell survival (VILLARROEL-ESPÍNDOLA et al. 2013). The complete breakdown of glycogen is catalyzed by the action of two key enzymes: glycogen phosphorylase and glycogen debranching enzyme (GDE) (Figure 6). Both muscle and liver glycogen phosphorylase isozymes have been found in the testes, and are encoded by glycogen phosphorylase liver form (PYGL) and glycogen phosphorylase muscle form (PYGM), respectively (DAVID; CRERAR, 1986). Glycogen phosphorylase is interconverted from an inactive form, phosphorylase b, into an active form, phosphorylase a, through phosphorylation by phosphorylase kinase (BIORN; BARTLESON; GRAVES, 2000). Once activated, glycogen phosphorylase cleaves glycosidic bonds from the non-reducing ends, releasing glucose-1-phosphate from the non-reducing ends of the glycogen, but is unable to remove glucose residues from the branch points (boundary dextrin). Glucose residues from the branch points are then removed by the GDE. GDE is a bifunctional enzyme, formally known as oligo($\alpha 1 \rightarrow 6$)-($\alpha 1 \rightarrow 4$)-glucan-transferase, catalyzes two successive reactions that remove branches. First, the transferase activity of the GDE transfers three glucose residues from the branch to a non-reducing end. The residue remaining at the branch point, linked ($\alpha 1 \rightarrow 6$), is then released as free glucose by the glucosidase activity ($\alpha 1 \rightarrow 6$) of the GDE (BATES et al., 1975; TAYLOR et al., 1975).

Although glycogen content and glycogen phosphorylase activity has been reported in the testes for over 50 years (ARZAC, 1950; LEIDERMAN; MANCINI 1969), the nongenomic and genomic regulation of glycogen metabolism in the testes is still unclear. The presence of glycogen is reported in Sertoli and germ cells of humans (ARZAC, 1950), mice (KANAI et al., 1989) and rats, as well as in spermatozoa of dogs (PALOMO et al., 2003) and in Sertoli cells of *Boleophthalmus pectinirostris* fish (CHUNG, 2008). In addition, glycogen phosphorylase has been reported to be activated by FSH, cAMP and Ca^{2+} -dependent mechanisms in rat Sertoli cells (SLAUGHTER; MEANS, 1983). Interestingly, the glycogen content (LEIDERMAN; MANCINI, 1969) and the phosphorylase activity are reported to be higher in immature

rat testes than in adult rat testes (SLAUGHTER; MEANS, 1983), which suggest a putative effect of glycogen metabolism on establishment of spermatogenesis.

1.1.5.3 Influence of amino acid metabolism

In addition to carbohydrate metabolism, amino acid metabolism is also involved in the energy metabolism of Sertoli cells (MATEUS et al., 2018). Studies that relate the influence of glucogenic amino acids to testicular energy metabolism are rare. However, it has been shown that Sertoli cells may maintain lactate production even in the total absence of glucose through amino acid metabolism or glycogen metabolism (RIERA et al., 2009; OLIVEIRA et al., 2011). Alanine is considered the main glucogenic amino acid, which can be converted into pyruvate (Figure 6) by alanine aminotransferase (ALT), which catalyzes the reversible interconversion of alanine and α -ketoglutarate into pyruvate and glutamate (SHERMAN, 1992; LIU et al., 2014; MATEUS et al., 2018). In addition, glucogenic amino acids or pyruvate may also be diverted to other pathways, including the Krebs cycle or gluconeogenesis (RATO et al., 2012a, 2012b). Pyruvate carboxylase catalyzes the carboxylation of pyruvate to oxaloacetate (OAA), which is a very important anaplerotic reaction that replenishes OAA for the Krebs cycle. In addition, pyruvate carboxylase participates in the first step of gluconeogenesis by providing OAA for subsequent conversion to PEP (JITRAPAKDEE ET AL., 2008; VALLE, 2017).

1.1.5.4 Influence of lipid metabolism

In addition to carbohydrate and amino acid metabolism, lipid metabolism is also involved in testicular energy metabolism (CRISÓSTOMO et al., 2017). Triacylglycerol (TAG) hydrolysis into fatty acids and glycerol also contributes to pyruvate production. Glycerol is an important metabolite for connecting the metabolic pathways of carbohydrates, including glycolysis (Figure 6) and gluconeogenesis, as well as lipids (CRISÓSTOMO et al., 2017). Glycerol is converted into glycerol-3-phosphate by glycerol-kinase (GK). GK presents two known isoforms, GK1 and GK2, while GK2 is the testis-specific isoform of GK (SARGENT et al., 1994; CHEN et al., 2017). As Sertoli cells sustain high metabolic activity, they are able to use several alternative substrates as their energy source. They may maintain viability in culture in

the complete absence of glucose, producing ATP for their own metabolism and lactate for the germ cells through lipid metabolism. Sertoli cells preferentially use lipids as their energy source, where β -oxidation seems to be the main metabolic pathway used by these cells (XIONG et al., 2009). Interestingly, they are also able to induce apoptosis of germ cells, phagocytize them and perform the degradation of residual bodies, converting them into lipids that are later metabolized to produce ATP (LEE et al., 1997; XIONG et al., 2009).

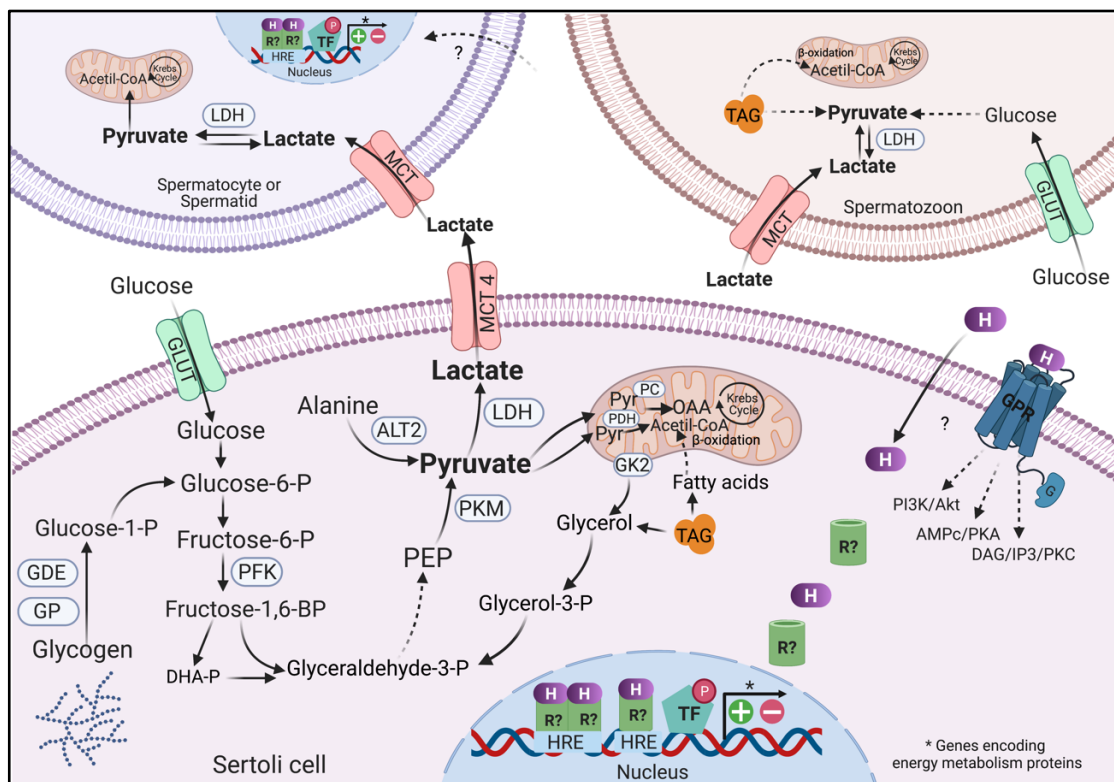


Figure 6: Schematic representation of the main metabolic pathways involved in energy production in the testis with emphasis on the glycolytic pathways in Sertoli cells. As knowledge about testicular energy metabolism is further known in mammals than in fish, the main metabolic pathways of Sertoli cell, spermatocyte and spermatid of mammals are represented. However, as this knowledge in fish is limited to spermatozoon bioenergetics, the main metabolic pathways of spermatozoon of teleost fish are represented. Therefore, glucose enters the cell from interstitial fluid through glucose transporters (GLUT) and is then metabolized by a series of enzymatic reactions through glycolysis, which results in the final production of pyruvate from phosphoenolpyruvate (PEP) by pyruvate kinase M1/2 (PKM). Most of the pyruvate is converted into lactate by the action of lactate dehydrogenase (LDH). Then, it is transported to the intratubular liquid through the monocarboxylate transporters 4 (MCT4) and subsequently to the spermatocytes and spermatids, which metabolize it and used it as the main source of energy. In addition, pyruvate can be generated through glycogen metabolism. Glycogen is degraded to glucose-1-phosphate by the action of the enzymes glycogen phosphorylase (GP) and glycogen reducing enzyme (GDE), which then enters into the glycolytic pathway. In addition, triacylglycerol (TAG) hydrolysis into fatty acids and glycerol also contributes to the formation of pyruvate. Glycerol is converted into glycerol-3-phosphate by the action of glycerol kinase 2 (GK2), which enters into the glycolytic pathway. Pyruvate can also be generated from alanine by the action of alanine aminotransferase (ALT). In addition to converting pyruvate into lactate, pyruvate can also be converted into Krebs cycle intermediates. It can be transported to the mitochondrial matrix and then be converted into acetyl-CoA by the action of pyruvate dehydrogenase complex (PDH), or to oxaloacetate (OAA) by

the action of pyruvate carboxylase (PC). In addition to Sertoli cell metabolism, energy metabolism was emphasis on the metabolism of Sertoli cell and fish spermatozoon. Spermatozoa of teleost fish are dependent on lipid catabolism, β -oxidation and glycolysis for energy generation. However, during motility, the most energy-demanding situation for a fish spermatozoon, anaerobic glycolysis becomes the predominant pathway in energy generation, with a significant increase in lactate concentration. In addition, energy metabolism can be regulated by hormones (H) and different receptors (R?) through nongenomic and/or genomic signaling pathways that culminate in the stimulation or suppression of the expression of genes encoding proteins associated with energy metabolism (*). Straight lines are direct pathways; dashed lines represent multi-step pathways; question marks (?) represent proposed/suggested effects, which are questions to be further investigated. DHA-P – Dihydroxyacetone-phosphate; TF – transcription factor. Source: Own construction.

1.2 CALCIUM SIGNALING

Calcium (Ca^{2+}) is considered the most important intracellular signaling ion, highly versatile that regulates the performance of a wide variety of cellular processes, which acts as a second messenger. Ca^{2+} signaling involves the participation of a variety of receptors, enzymes and ion channels. Different stimuli induce both the entry of external Ca^{2+} and the formation of second messengers that induce the release of Ca^{2+} from intracellular stores (BERRIDGE; BOOTMAN; RODERICK, 2003; CLAPHAM, 2007), which will be further discussed in the next sections.

1.2.1 Calcium influx from the extracellular medium through ion channels located at the plasma membrane

There are many ion channels located at the plasma membrane that control the entry of Ca^{2+} from the extracellular medium into the cells in response to various stimuli. Among them, membrane depolarization, harmful stimuli, extracellular agonists, intracellular messengers, as well as depletion of intracellular Ca^{2+} stores (BERRIDGE; BOOTMAN; RODERICK, 2003).

Plasma membrane voltage-dependent Ca^{2+} channels (VDCC) are one of the main channels involved in Ca^{2+} influx. Both Ca^{2+} concentrations difference and the electrical potential difference make up the electrochemical potential difference that moves the Ca^{2+} entry into the cell (AIRES, 2008). VDCC is a heterooligomeric complex that consists of an $\alpha 1$ and subunit and β , γ and $\alpha 2$ - δ accessory subunits (AIRES, 2008). VDCC are classified into two major functional classes: high voltage-activated (HVA) and low voltage-activated (LVA). HVA channels require strong depolarizations to open, but then they are slowly inactivated. Considering the biophysical and pharmacological characteristics of the currents, HVA are classified into L, N, P/Q and R types

(DARSZON et al., 2011). In addition, these channels may also be classified according to the $\alpha 1$ subunit structure (WANG; KOIDE; WELLMAN, 2017). On the other hand, LVA channels require weak depolarizations to open, and they inactivate faster. LVA channels are named T-type because they open transiently (FOX; NOWYCKY; TSIEN, 1987; DARSZON et al., 2011).

Furthermore, ligand-dependent channels, transient receptors potential (TRP) also contribute to the Ca^{2+} influx from the extracellular medium. Based on their structures, TRP are divided into seven groups: TRP canonical; TRP melastatin; TRP ankyrin; TRP mucolipin; TRP polycystin; TRP no mechanoreceptor potential C and TRP vanilloid (TRPV) (DARSZON et al., 2011). Most TRP are relatively non-selective Ca^{2+} channels. In contrast, TRPV are highly selective for Ca^{2+} (SEEBOHM; SCHREIBER, 2021). TRP vanilloid subfamily 1 (TRPV1) is one of the most studied TRP. It can be activated by temperatures above 42 °C, by vanilloid substances such as capsaicin (AIRES, 2008; SANZ-SALVADOR et al., 2012), phosphatidylinositol-4,5-bisphosphate (PIP2) (SUH; HILLE, 2008), among others. In addition, TRPV1 is a target of phosphorylation by protein kinase A (PKA) and protein kinase C (PKC) (POR et al., 2013).

1.2.2 Nongenomic signaling pathways and calcium release from intracellular calcium stores triggered by second messengers

In addition to the entry of Ca^{2+} from the extracellular medium, stimuli also lead to the formation of second messengers that induce the release of Ca^{2+} from sarco/endoplasmic reticulum or mitochondria, intracellular Ca^{2+} stores (BERRIDGE; BOOTMAN; RODERICK, 2003). The stored Ca^{2+} is complexed with buffering storage proteins such as calreticulin, calsequestrin, calnexins and binding protein (LEE; MICHALAK, 2010). The cytosolic Ca^{2+} concentrations in eukaryotic cells are maintained at very low levels, between 50 and 100 nM. However, when inositol triphosphate receptors (IP3R) and ryanodine receptors in the sarco/endoplasmic reticulum are activated, internal Ca^{2+} is released, which leads to a sudden increase in its cytosolic levels (BERRIDGE; BOOTMAN; RODERICK, 2003). Activation of G protein-coupled receptors may lead to the activation of phospholipase C (PLC), which in turn converts PIP2 into IP3 and diacylglycerol (DAG). IP3, in turn, binds to the IP3R of the endoplasmic reticulum and, together with the cytosolic Ca^{2+} itself, induce the

release of Ca^{2+} from the endoplasmic reticulum (LE MELLAY; GROSSE; LIEBERHERR, 1997; TAYLOR; TOVEY, 2010). In addition, the increased in the generation of the second messenger Ca^{2+} and DAG promotes the activation of PKC (MARINO; PALLOTTINI; TRENTALANCE, 1998; GONÇALVES et al., 2018). Moreover, the increased cytosolic Ca^{2+} levels and PKC activation may activate Ca^{2+} -dependent chloride (Cl^-) channels (CaCC), that trigger Cl^- efflux and membrane depolarization (KURUMA; HARTZELL, 2000; HARTZELL; PUTZIER; ARREOLA, 2005).

1.2.3 Role of Ca^{2+} in the regulation of cellular processes: a life and death signal

Ca^{2+} signaling plays a fundamental role in major testicular events. Among them, proliferation, differentiation and apoptosis of germ cells (GALARDO et al., 2014; GOLPOUR; SENICKA; NIKSIRAT, 2017); maturation and motility of spermatozoa (ALAVI et al., 2004; GOLPOUR; SENICKA; NIKSIRAT, 2017) and acrosome reaction (DE BLAS et al., 2002). In addition, Ca^{2+} signaling is also crucial for hormone exocytosis (WATSON; GAMETCHU, 1999; WOZNIAK; BULAYEVA; WATSON, 2005) and secretory vesicle release from Sertoli cells (MENEGAZ et al., 2010; ZANATTA et al., 2013).

The relationship between intracellular Ca^{2+} levels in response to glucose and lactate metabolism has already been evidenced in spermatids and pachytene spermatocytes (REYES et al., 2002). In addition to signaling ion, Ca^{2+} acts as a metabolism regulator acting as a cofactor in the performance of a variety of enzymatic reactions of energy metabolism (HANSFORD, 1985). Among them, the activation of phosphorylase kinase in hepatic glycogenolysis to supply glucose, coordinating the rates of glycogenolysis and glycogen synthesis during muscle contraction (PICTON; KLEE; COHEN, 1981). Ca^{2+} is also an essential cofactor for phospholipase A2, which cleaves cell membrane phospholipids into free fatty acids and lysophospholipids (DENNIS et al., 2011). Ca^{2+} also plays an important role in the regulation of the mitochondrial oxidative pathway, such as the Krebs cycle, electron transport chain (ETC) and oxidative phosphorylation (TRAASETH et al., 2004; GELLERICH et al., 2010). It also acts as a cofactor of pyruvate dehydrogenase phosphatase (PDP1) that dephosphorylates and activates the E1 domain of the PDH complex, which converts pyruvate into acetyl-CoA (BOGONEZ; GOMEZ-PUERTAS; SATRÚSTEGUI, 1992;

HUANG et al., 1998). In addition, Ca^{2+} also activates the Krebs cycle enzymes, such as isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (TRAASETH et al., 2004; DENTON, 2009). Additionally, it binds to the catalytic site of mitochondrial F1Fo-ATPase (ETC complex V) and activates ATP hydrolysis (NESCI et al., 2017; ALGIERI et al., 2019).

However, one of the paradoxes regarding Ca^{2+} is that it is a life and death signal (BERRIDGE; BOOTMAN; LIPP, 1998). It is well known that intracellular Ca^{2+} overload may lead to programmed cell death called apoptosis (Figure 7). The apoptosis is accompanied by a series of cellular events such as reticulum stress, mitochondrial dysfunction, activation of caspases, chromatin condensation, and DNA degradation (FAN et al., 2005; PINTON et al., 2008; WANG et al., 2017). The impaired Ca^{2+} flow from the endoplasmic reticulum to mitochondria with Bcl-2 family proteins involvement is reported to be a key factor to intrinsic apoptosis pathway (PINTON et al., 2008). Therefore, Ca^{2+} concentrations must be finely regulated in intra and extracellular media to keep cytosolic concentrations within physiological limits and its homeostasis (BERRIDGE; BOOTMAN; LIPP, 1998; DARSZON et al., 20011).

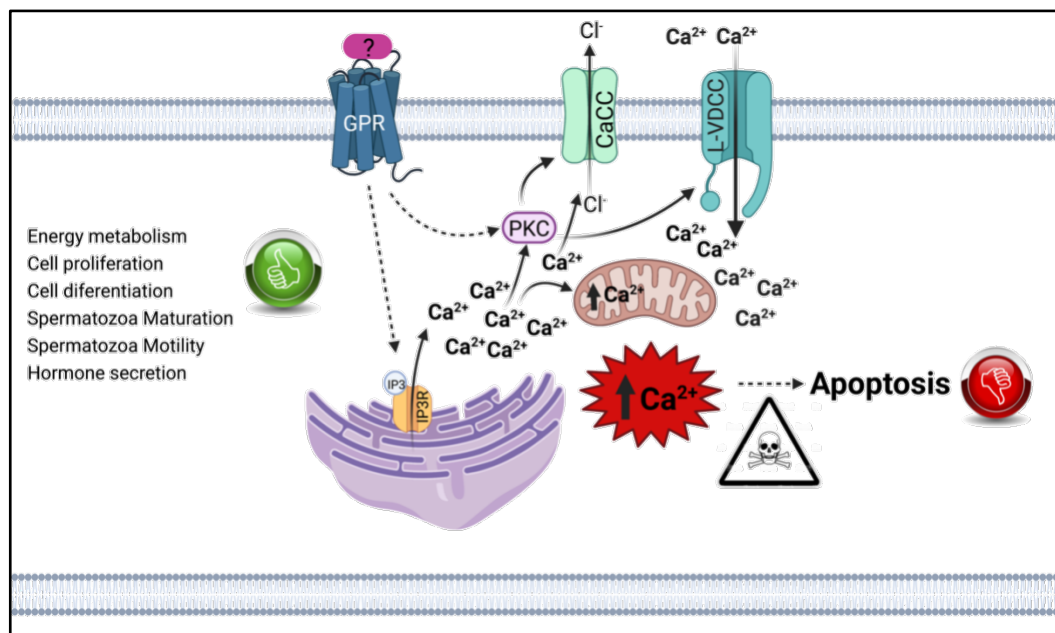


Figure 7: Schematic representation of the participation of calcium (Ca^{2+}) as a key second messenger in cellular processes as a life and death signal. Ca^{2+} as a life signal is involved in the performance of a wide variety of cellular processes, such as fertilization, hormone secretion, spermatozoa motility, muscle contraction, cell proliferation and differentiation, energy metabolism. Ca^{2+} as a death signal is triggered by the overload of its intracellular levels and programmed cell death, apoptosis. Question mark (?) represents a hypothetical G-protein coupled receptor (GPR) agonist; straight lines are direct pathways; dotted lines represent multi-step pathways. CaCC – Ca^{2+} -dependent chloride (Cl^-) channels; IP₃R – inositol triphosphate (IP₃) receptor; L-VDCC – L-type voltage-dependent Ca^{2+} channels; PKC – protein kinase C. Source: Own construction.

1.3 ESTRADIOL (E2) IN THE MALE REPRODUCTIVE SYSTEM

Although E2 and other estrogens are commonly known as “female hormones”, their production and their detectable plasma concentrations in males have been elucidated for many decades. High estrogen concentrations in stallion urine were first reported in the 1930s (ZONDEK, 1934). Testosterone is the main source of E2 in males (VERMEULEN et al. 2002). In male rats, serum and intratesticular testosterone concentrations are in the nM range, which is at least three orders of magnitude higher than E2 concentrations, which are in the pM range (JONG; UILENBROEK; MOLEN, 1975; COOKE et al., 2017). Testosterone is converted into E2 by cytochrome P450 aromatase and NADPH-cytochrome P450 reductase microsomal enzymes (CARREAU; WOLCZYNSKI; GALERAUD-DENIS, 2010). The transcription of this enzyme complex is stimulated by the hormones LH and FSH (LAMBARD et al., 2005). Leydig and germ cells are the main sources of aromatase and estrogens in male adult rodents (LAMBARD et al., 2005; BOIS et al., 2010). However, in male immature rats, Sertoli cells are the main source of aromatase and estrogens biosynthesis. Aromatase expression in immature rats Sertoli cells has a maximum expression at 20 DNP when compared to 10 and 30 DNP. However, aromatase transcripts are undetectable in adult rats Sertoli cells (BOIS et al., 2010).

1.3.1 Estrogen receptors expression in the testes of mammals and fish

The actions of E2 are primarily performed by its binding to nuclear estrogen receptors (ESR). Two ESRs subtypes are expressed in mammals: ESR α and ESR β , which are encoding by ESR1 and ESR2 genes, respectively (BOIS et al., 2010; FIETZ et al., 2014). ESR β has ubiquitous expression throughout the male reproductive system, while ESR α has greater specificity. However, the reports on ESR α and ESR β expression are highly variable, with large differences between species, as well as between gene or protein expression analysis techniques (CARREAU; HESS, 2010). Due variability of expression results of both ESRs led to the investigation and identification of the putative presence of ESR variants in the rat testis (BOIS et al., 2010). Overall, ESR α has been located in the interstitial space, while ESR β is more frequently located in the seminiferous epithelium. ESR1 or ESR α expression has been identified in Leydig cells and efferent ductal epithelium of different mammalian species

(FISHER et al., 1997; MOWA; IWAGANA, 2001; HESS; CARNES, 2004). However, Lucas et al. (2008) has solidly demonstrated that ESR α is also expressed in rat Sertoli cells and in some germ cells. In addition, a more recent study also demonstrated that ESR1 is highly expressed in stages VII to XIV of epithelium seminiferous of rats (BOIS et al., 2010). Furthermore, in adult rat testis, the same level of ESR2 expression has been evidenced in the seminiferous tubules and in Leydig cells of adult rats (BOIS et al., 2010). ESR2 or ESR β expression has already been identified in spermatogonia, pachytene spermatocytes, round spermatids (SAUNDERS et al. 1998; SHUGRUE et al. 1998; VAN PELT et al., 1999), as well as in mouse and rat Leydig and Sertoli cells (SAUNDERS et al., 1998; ZHOU et al. 2002; BOIS et al., 2010). However, it has been evidenced that both ESR1 and ESR2 expression levels are higher in round spermatids than in pachytene spermatocytes, suggesting a key role of estrogens in the haploid steps of spermatogenesis (BOIS et al., 2010).

However, three ER subtypes are expressed in fish: ESR α , ESR β 1 and ESR β 2, which are encoding by ESR1, ESR2b and ESR2a genes, respectively (MENUET et al., 2002; FILBY; TYLER, 2005). The third subtype, ESR β 2, was first identified in goldfish and Atlantic croaker fish, which was initially named ESR γ (HAWKINS et al., 2000). Later, this ESR was renamed as ESR β 2 when identified in zebrafish and, together with ESR β 1, they are the most expressed ESR subtypes in the testes (MENUET et al., 2002; FILBY; TYLER, 2005). The expression of ESR α has been detected in Leydig cells of rainbow trout fish (BOUMA; NAGLER, 2001). Moreover, the expression of ESR α and ESR β has been reported in Sertoli cells of *Acanthogobius flavimanus* fish (ITO; MOCHIDA; FUJII, 2007), in germ cells and spermatozoa of rainbow trout fish and catfish (BOUMA; NAGLER, 2001; WU et al., 2001). However, among all germ and somatic cells, the highest expression of ESR is reported in secondary spermatocytes, spermatids, spermatozoa (BOUMA; NAGLER, 2001; WU et al., 2001) and in Sertoli cells (ITO; MOCHIDA; FUJII, 2007). Overall, the expression pattern of ESR in fish testes indicates an important role for estrogens mainly in mature male germ cells. It has been reported that ESR2b (ESR β 1) knockout tilapia result in male and female infertility (YAN et al., 2019).

In addition to the classical estrogen receptors, estrogen action may also be performed through transmembrane receptors such as G-protein coupled estrogen receptors (GPER) (DAS et al., 2000). These receptors may trigger the generation of second messengers, such as intracellular cyclic adenosine monophosphate (cAMP)

and Ca^{2+} , which promotes an increase on Ca^{2+} influx and release of Ca^{2+} from intracellular stores, and therefore increases its cytosolic concentration (WATSON; GAMETCHU, 1999; JENG; KOCHUKOV; WATSON, 2010). GPER expression has already been detected in immature rats Sertoli cells, as well as in pachytene spermatocytes and round spermatids of adult rats (LUCAS et al., 2010; CHIMENTO et al., 2010; 2011). On the other hand, GPER has been reported to be highly expressed in early germ cells, such as spermatogonia and spermatocytes, as well as in Sertoli cells of adult male zebrafish (LIU et al., 2009).

1.3.2 Effects of estrogens on male reproductive system, spermatogenesis and energy metabolism in mammals and fish

E2 plays an important role in the regulation of gene expression in the testis and influences cellular processes crucial to spermatogenesis. Among them, the stimulation of spermatogonial stem cell renewal via ESR (MIURA et al., 1999), cell proliferation (PINTO et al. 2006) and cell division (ZHENG et al., 2019). Furthermore, it has been reported that E2 regulates the expression of genes encoding key proteins for cell proliferation, energy metabolism (PINTO et al. 2006), cell division, sexual differentiation, cell adhesion, drug metabolism, Ca^{2+} signaling (ZHENG et al., 2019), as well as maintenance of cell cytoskeleton and spermiation (BALASINOR et al. 2010). However, E2 effects can be beneficial or harmful, but this varies according to its concentration (physiological or supraphysiological), exposure time (acute or chronic), stage of sexual development and maturation of the animal. Thus, both the lack and the excess of estrogens lead to several harms to the male reproductive function. Male rats treated daily with estradiol benzoate (12.5 μg) from PND 1 to 15 resulted in a reduction in testis weight, number of Sertoli cells and pre-meiotic germ cells, in addition to an increase in cellular apoptosis in the seminiferous epithelium (WALCZAK-JĘDRZEJOWSKA et al., 2013). In addition, rats overexposed to micromolar concentrations of the oral contraceptive ethinylestradiol during fetal development up to PND 18 result in male reproductive tract dysfunctions and reduction in spermatozoa production in adulthood (HOWDESHELL et al. 2008). However, a close relationship between the decrease in intratesticular E2 in aged rats and the decrease in spermatozoa production has already been reported (CLARKE; PEARL, 2014). Moreover, at physiological levels, estrogens are essential for maintaining insulin

sensitivity and glucose homeostasis. However, increased E2 levels and estrogen-like endocrine disrupting chemicals (xenoestrogens) lead to impaired insulin signaling and loss of glucose homeostasis in rats and humans (GODSLAND, 2005; HAQ et al., 2020). In addition, fish exposed to low concentrations of E2 result in spermatogonial stem cells proliferation stimulation via ESR (MIURA et al., 1999; AMER et al. 2001), while high concentrations promote an inhibitory effect (SONG; GUTZEIT, 2003).

1.4 ESTROGENS RECEPTOR SIGNALING MECHANISMS

1.4.1 Direct genomic signaling: Classical pathway

Direct genomic signaling is known as the classical estrogen and nuclear estrogen receptors signaling mechanism. In addition to estrogens, there are other ESR agonists, such as xenoestrogens. ESRs belong to a family of nuclear receptors that act as ligand-activated transcription factors (O'MALLEY, 2005; KININIS; KRAUS, 2008). Ligand binding to the ligand-binding domain (LBD) of ESR in the cytoplasm results in the dissociation of heat shock proteins (HSP) from ESR (SANCHEZ et al., 1990) and then a conformational change occurs that induces receptor dimerization. This homodimer complex is then translocated to the nucleus, where it binds to chromatin in estrogen response element (ERE) sequences (KUMAR; CHAMBON, 1988; KLINGE, 2001). The homodimer then interacts with coregulators of gene transcription. These ESR coregulators are functionally divided into two subclasses: coactivators and corepressors. The first stimulates and the second suppresses the transcription of target genes (KININIS; KRAUS, 2008; DAVEREY et al., 2009). The most known and characterized ESR coactivators are those of the SRC/p160 family (steroid receptor coactivators), such as SRC-1/NCoA1, SRC-2/TIF2 and SRC-3 (LEO; CHEN, 2000; FENG; O'MALLEY, 2014). All these coactivators recruit histone acetyltransferases that break electrostatic bonds in DNA and thus promote chromatin relaxation, which increases the access of transcription factors to promoter regions in DNA, thereby stimulating mRNA expression (JOHNSON; O'MALLEY, 2012; FENG; O'MALLEY, 2014). On the other hand, ESR corepressors, such as NCoR (nuclear receptor corepressor) and RIP-140 (receptor-interacting protein 140) recruit histone deacetylases (HDACs) and repress gene transcription (LONARD; SMITH, 2002; DAVEREY et al., 2009; FENG; O'MALLEY, 2014).

1.4.2 Nongenomic signaling

The nongenomic signaling pathway is known as plasma membrane-initiated signaling involving signal transduction through the activation of intracellular signaling cascades. The nongenomic effects are also known as rapid response effects, which are defined as responses that do not directly and initially influence gene expression. The nongenomic actions of E2 and xenoestrogens involve the activation of signal transduction mechanisms with the subsequent generation of intracellular second messengers, such as Ca^{2+} and cAMP. Such mechanisms may involve the activation of GPER and ESR located at the plasma membrane (RAZANDI et al., 2004; THOMAS; DONG, 2006; BARTON et al., 2018). At the cell membrane, ESR may interact with scaffold proteins, such as caveolin-1 (SCHLEGEL et al., 2001) and nongenomic estrogen receptor activity modulator protein (MNAR/PELP-1) (CHESKIS et al., 2008). In addition, they may also interact with GPER, insulin-like growth factor 1 receptor (IGFR1), epidermal growth factor receptor (EGFR), as well as signaling molecules, including Ras, Src, phosphatidylinositol-3-kinase (PI3K) and Shc, which are located at or near the cell membrane (BJÖRNSTRÖM; SJÖBERG, 2005; SONG; ZHANG; SANTEN, 2005; BOONYARATANAKORNKIT; EDWARDS, 2007; SONG et al., 2010; FITZGERALD et al., 2015). In addition, there are plasma membrane ion channels that be involved in nongenomic signaling (GONÇALVES et al., 2018; BATISTA-SILVA et al., 2020), such as L-type VDCC (L-VDCC), which can be activated by PKA and PKC (KAMP; HELL, 2000).

Furthermore, GPER activation by estrogens or xenoestrogens may involve activation of PLC, which in turn converts PIP2 into IP3 and DAG (LE MELLAY; GROSSE; LIEBERHERR, 1997). IP3 activates the endoplasmic reticulum IP3R, which induces the Ca^{2+} release from the endoplasmic reticulum and transiently increases cytosolic Ca^{2+} levels (TAYLOR; TOVEY, 2010). This increase in the generation of the second messenger Ca^{2+} and DAG promotes the activation of PKC (MARINO; PALLOTTINI; TRENTALANCE, 1998; GONÇALVES et al., 2018). Furthermore, it may also involve the activation of adenylate cyclase, which in turn produces the second messenger cAMP, which exerts its effect mainly through PKA (SKROBLIN et al., 2010; GONÇALVES et al., 2018).

1.4.3 Crosstalk between nongenomic and genomic signaling pathways

A growing number of studies report a crosstalk between estrogen receptor signaling pathways involving nongenomic and genomic factors that converge on gene transcription (Figure 8). The intracellular signaling cascades that result in indirect changes in gene expression can be classified into four main ones: 1) PLC/PKC pathway; 2) cAMP/PKA pathway; 3) Ras/Raf/MAPK mitogen-activated protein kinase (MAPK) signaling cascade, and 4) PI3K/Akt (protein kinase B) cascade (Figure 8) (PICOTTO; MASSHEIMER; BOLAND, 1996; WATTERS et al., 1997; DUAN et al., 2002; KOUSTENI et al., 2003; MARINO; ACCONCIA; TRENTALANCE, 2003; LEONARD et al., 2015; GONÇALVES et al., 2018).

In one of the proposed mechanisms of crosstalk, after ligand binding to ERs and formation of homodimers, they translocate to the nucleus and bind to phosphorylated transcription factors through GPER-mediated signaling. The complexes then bind to ERE sequences via the ERs or to DNA-bound transcription factors, such as nuclear factor kappa B (NF- κ B), cAMP response element (CRE), activator protein-1 (AP-1), serum response element (SRE), signal transducer and activator of transcription (STAT), ATF-2/c-Jun and Sp1 (JAKACKA et al., 2001; LI et al., 2001; BJÖRNSTRÖM; SJÖBERG, 2005).

There is another crosstalk mechanism that involves the interaction of GPERs and ERs at the plasma membrane and subsequent activation of protein kinase cascades, including the p44/42 MAPK/extracellular signal-regulated kinases 1/2 (ERK 1/2), Akt, PKC and PKA pathways. Then, protein kinases phosphorylate transcription factors and thus, gene transcription is either stimulated or suppressed. Such transcription factors include: AP-1, STAT, Elk-1, serum response factor (SRF), cAMP response element binding protein (CREB) and NF- κ B (Figure 8) (DUAN et al., 2001; QUESADA et al., 2002; KOUSTENI et al., 2003; BJÖRNSTRÖM; SJÖBERG, 2005). In addition to transcription factors, ESR are also targets for phosphorylation by protein kinases (DE LEEUW; NEEFJES; MICHALIDES, 2011).

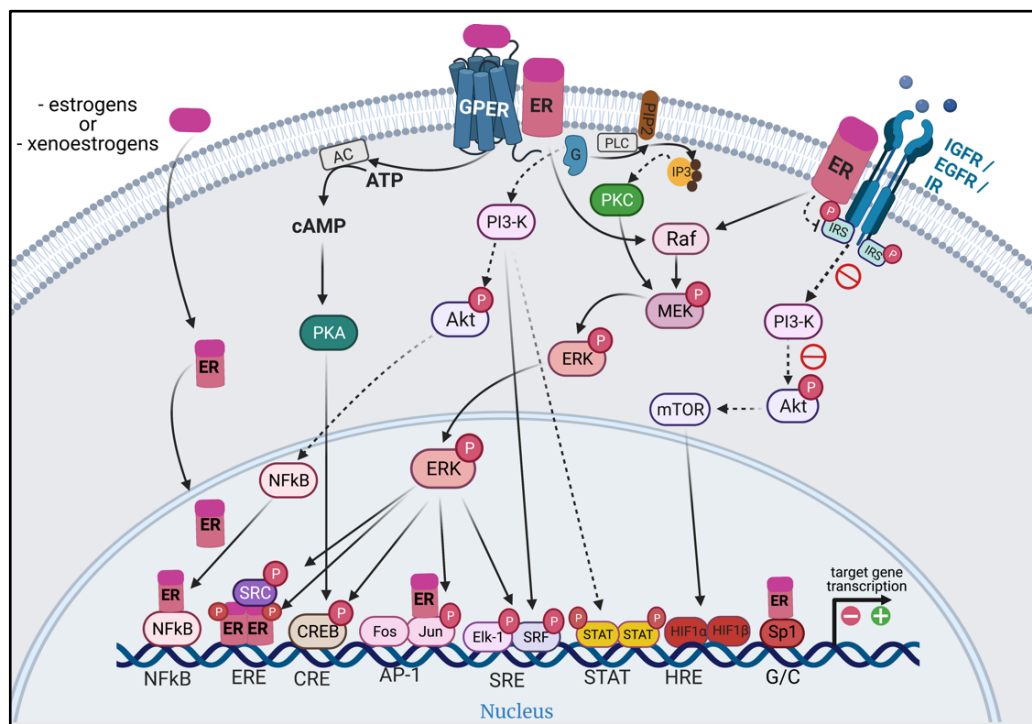


Figure 8: Schematic representation of the crosstalk between nongenomic and genomic estrogen receptor signaling pathways. Straight lines are direct pathways; dotted lines represent multi-step pathways. AP-1 – Activator protein-1; CRE – cAMP response element; DAG – Diacylglycerol; EGFR – Epidermal growth factor receptor; ERE – Estrogen response element; G/C – Regions rich in guanine (G) and cytosine (C); HIF – Hypoxia-induced factor; HRE – Hypoxia response element; IGFR – Insulin-like growth factor receptor 1; IP3 – Inositol triphosphate; IR – Insulin receptor; NF-κB – Nuclear factor kappa B; SRE – Serum response element; SRF – Serum response factor; STAT – Signal transducer and transcriptional activator. Source: Own construction based on BJÖRNSTRÖM; SJÖBERG, 2005.

1.5 ESTROGEN-LIKE ENDOCRINE DISRUPTING CHEMICALS: BISPHENOL A

A wide variety of exogenous compounds act as endocrine disrupting chemicals (EDC) and cause deleterious effects on the male reproductive system including effects on fertility (HILL; JANZ, 2003; BROUARD et al., 2016). Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA) is considered an estrogen-like endocrine disrupting chemical or xenoestrogen. Xenoestrogens are compounds that produce estrogenic or anti-androgenic responses in several animal species by mimicking the action of E2 and, therefore, interfering with endogenous endocrine regulation (MOLINA-MOLINA et al., 2013; URRIOLOA-MUNOZ et al., 2014). BPA is used for manufacturing epoxy resins to coat metal cans and for polymerizing polycarbonate plastic for the manufacture of food utensils, plastic containers, packaging, dental sealants, bottles and water supply tubes (KANG; KONDO; KATAYAMA, 2006; KOCH; CALAFAT, 2009). As such, human exposure to BPA is a frequent occurrence, since BPA is released from polycarbonate plastic at high temperatures (LE et al., 2008). Its presence in saliva (OLEA et al., 1996),

blood and breast milk (SUN et al., 2004); adult urine (CALAFAT et al., 2005) and infant urine (MENDONÇA et al., 2014) is already documented. Dietary exposure to BPA has been estimated to be up to 1.5 µg/kg/body weight (bw)/day in adults, 5.3 µg/kg/bw/day in children (EUROPEAN FOOD SAFETY AUTHORITY - EFSA, 2015), which may reach 10 µg/kg/bw/day when considering all sources of BPA exposure (NATIONAL TOXICOLOGY PROGRAM, 2008). However, the worst-case of BPA exposure scenario has been associated to 1-2 years-old babies (U.S. Food and Drug Administration, 2010; EFSA, 2015). Taking into account that this age range in humans is correlated with the development prepubertal stage similar to rats 8-21 DPN, studies in this animal model become even more relevant for investigate the impact of BPA on male reproduction. Thus, as spermatogenesis is not yet established in prepuberty stages, this is a critical stage on reproductive development that is highly vulnerable to changes caused by EDC (MOURITSEN et al., 2010), including BPA (BROUARD et al., 2016; OLIVEIRA et al., 2017).

Furthermore, the presence of BPA has been documented in rivers water and in fish including those in the marketplace, which suggest that this chemical may be a risk factor for aquatic ecosystems and for human health (YANG et al., 2014; WU et al., 2016). BPA is discharged into aquatic environment such as rivers and seas by migration of BPA-based products and by the effluents from wastewater treatment plants and landfills sites (KANG; AASI; KATAYAMA, 2007). BPA levels in surface water have been measured cross the globe and range from 0.0005 to 0.41 µg/L in Germany (FROMME et al., 2002), 0.004 to 0.38 µg/L in China (YANG et al., 2014), 0.003–1.9 µg/L in Canada (LALONDE; GARRON, 2020) and from 0.09 to 1.46 µg/L in Brazil (RAMOS et al., 2021). That is why studies in fish become even more relevant for understanding the effects of BPA and its impact on reproduction, since it is recognized that testes are one of the major targets of BPA toxicity (BATISTA-SILVA et al., 2020b).

BPA has a similar structure to the synthetic estrogen diethylstilbestrol (Figure 9), a potent ESR agonist (SEACHRIST et al., 2016). The molecular basis and mechanisms of action of the deleterious effects of BPA are poorly understood. The estrogenic activity of BPA by binding to mammalian ESR α and ESR β (GOULD et al., 1998; HIROI et al., 1999; MATTHEWS; TWOMEY; ZACHAREWSKI, 2001), as well as zebrafish ESR α , ESR β 1 and ESR β 2 (PINTO et al. al., 2019) have already been reported. In addition to ESR, BPA is also an agonist of other nuclear receptors, such

as zebrafish estrogen-related receptors (ERR) (TOHMÉ et al., 2014). In addition, human ERR γ is reported to be a high affinity binding protein for BPA (TAKAYANAGI et al., 2006). Furthermore, mechanisms of BPA toxicity in human neural stem cells dependent on ERR α were recently reported (DONG et al., 2022). In addition to nuclear receptors, BPA acts as a GPER agonist in mammalian (BOUSKINE et al., 2009; GE et al., 2014) and in zebrafish (GONZÁLEZ-ROJO et al., 2019). BPA alters gene expression involved in a wide variety of cellular processes, such as cell proliferation, division and differentiation in the testes of zebrafish (GONZÁLEZ-ROJO et al., 2019) and goldfish (HATEF et al., 2012), as well as in vascular smooth muscle (GAO et al., 2019) and human seminoma cells (BOUSKINE et al., 2009). These genomic responses promoted by BPA are a result of its binding to ER (GAO et al., 2019) and through genomic pathways initiated at the plasma membrane, such as the activation of GPER (GONZÁLEZ-ROJO et al., 2019), involving the participation of protein kinases and activation of gene transcription factors (BOUSKINE et al., 2009).

In addition, BPA may also act through nongenomic activation via activation of membrane-associated receptors, which lead to the generation of second messengers such as intracellular Ca²⁺ and cAMP (WOZNIAK; BULAYEVA; WATSON, 2005). BPA has demonstrated to trigger an increase on Ca²⁺ influx and its intracellular concentration by activating membrane-associated receptors, ion channels and protein kinases. These BPA effects are also involved in nongenomic responses/rapid responses in pituitary tumor cells (WOZNIAK; BULAYEVA; WATSON, 2005), pancreatic α cells (ALONSO-MAGDALENA et al., 2005), rat pituitary cells (JENG; KOCHUKOV; WATSON, 2010), brain microvascular endothelial cells (ALTMANN et al., 2015), and in immature rat testes (GONÇALVES et al., 2018).

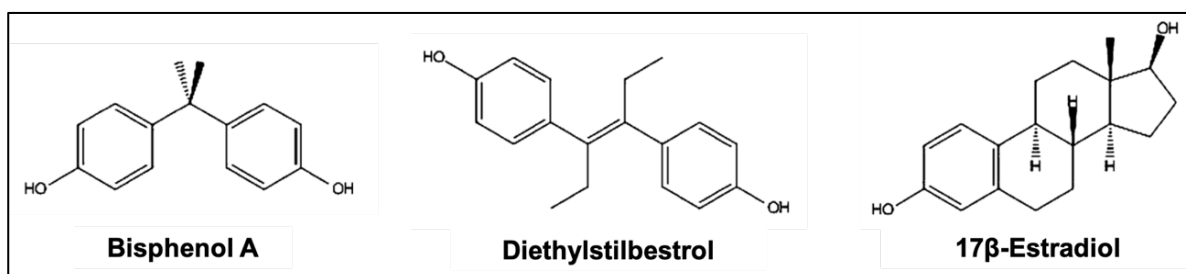


Figure 9: Chemical structures of BPA, diethylstilbestrol and E2. The structures of BPA and diethylstilbestrol are more similar to one another than to the endogenous E2, indicating that chemicals with variable structures are capable of binding to the ESR. Source: Adapted from JENKINS et al. 2012.

1.5.1 Effects of BPA on male reproductive system, spermatogenesis and energy metabolism in mammals and fish

A growing number of scientific reports obtained in animal models or human evidences that BPA promotes deleterious effects on male reproductive functions (THOMAS; DONG, 2006; ROUILLER-FABRE et al., 2015). *In vitro* exposure to low concentrations of BPA is reported to result in inhibition of key enzymes in testosterone synthesis in human and rat testicular microsomes (YEA et al., 2011). However, high concentrations of BPA impair glucose and lactate metabolism in rat Sertoli cells (HUANG et al., 2015) and induce rodent germ cell apoptosis through activation of mitochondrial apoptotic pathways (LI et al., 2009; WANG et al., 2010). Furthermore, *in vitro* exposure to BPA may also cause an increase in the production of reactive oxygen species (ROS), mitochondrial dysfunction, intracellular Ca^{2+} overload and apoptosis of rat Sertoli cells (WANG et al., 2017).

In addition to its effects on mammals, BPA has also been reported to cause deleterious effects at different stages of fish development. BPA has been shown to decrease the quantity, motility and velocity of spermatozoa, and testicular weight of zebrafish (CHEN et al., 2017) and of goldfish (HATEF et al., 2012). It also promotes embryonic malformations and mortality in zebrafish (CHEN et al., 2017). In addition, BPA causes morphological changes in the testes and in the expression of genes involved in steroidogenesis in zebrafish (LI et al., 2017). Exposure of fish to BPA may disrupt spermatogenesis through apoptosis of Leydig and germ cells, reduce androgen levels (WANG et al., 2019), and may increase vitellogenin concentration and intersexuality rates in male carp fish (MANDICH et al., 2007). In addition to its deleterious effects on male fish, BPA also disrupts the meiotic maturation of zebrafish oocytes through E2-like effects by a nongenomic estrogenic mechanism involving the activation of the GPER/EGFR/MAPK pathway (FITZGERALD et al., 2015).

1.5.2 BPA metabolism in rats and fish

BPA metabolism involves the production of two-phase II metabolites, glucuronic acid BPA (GA-BPA) and sulfate BPA (BPA-S). The oral route is the main route of exposure to BPA for mammals (World Health Organization, 2010). After BPA intake, the fraction present as conjugated BPA has been reported to be much higher

than others administration routes. This difference reinforces the prominent role of phase II first-pass metabolism in the intestine and liver after oral route of BPA administration (WILLHITE et al., 2008; DOERGE et al., 2010). Despite the effect of enterohepatic recirculation, which seems to maintain serum concentrations of BPA, the conjugated GA-BPA fraction has been reported to be about 99.5% in rats. GA-BPA elimination by biliary excretion in the intestine results in a slow rate of excretion in rats (DOERGE et al., 2010). On the other hand, in humans, BPA is eliminated by renal excretion. BPA is more rapidly conjugated and excreted in humans than in rats due to the lack of enterohepatic circulation in humans (VÖLKEL et al., 2002).

Unlike terrestrial vertebrates, in fish, the gills are the main gateway of entry for xenobiotic (DA CRUZ et al., 2015), therefore, they are the main site of BPA absorption. BPA is reported to be detected in the body of zebrafish after 2 hours of exposure, reaching 90% of steady state levels after 10 hours. BPA-GA levels in plasma and bile are reported be 100 and 22.600 times higher than those of BPA-S, respectively, which evidences that BPA is mainly conjugated to glucuronic acid. High BPA-GA concentrations found in bile indicate that biliary excretion in the intestine is the main route of elimination. The initial decline in BPA concentration is followed by a lower excretion rate, which results in a final BPA concentration after 7 days at 10.4% of initial values (LINDHOLST et al., 2003). BPA and BPA-S are likely to be eliminated in two phases, with two separate elimination compartments. The first rapid excretion rate is composed of muscle, liver and plasma, and the second slower rate of excretion is composed of the gallbladder (Figure 10) (LINDHOLST et al., 2003).

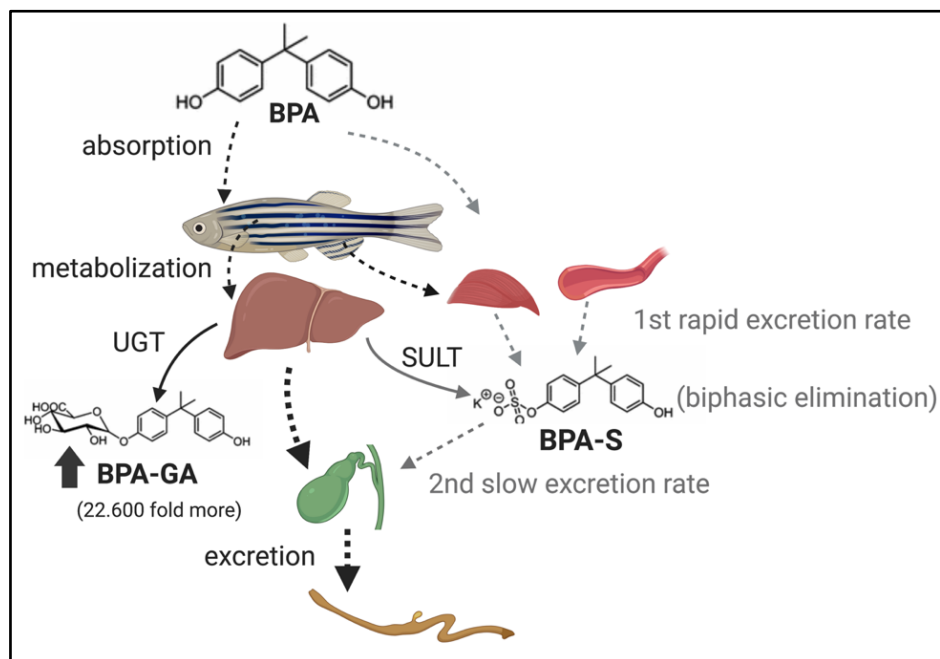


Figure 10: Schematic representation of the BPA metabolism in zebrafish. After being absorbed mainly by the gills, BPA is metabolized to glucuronic acid-BPA (BPA-GA) in the liver by the action of UDP-glucuronosyltransferase (UGT) and to BPA sulfate (BPA-S) by sulfotransferase (SULT). However, BPA is mainly conjugated to glucuronic acid, BPA-GA, which has bile levels 22.600 times higher than those of BPA-S. The high concentrations of BPA-GA found in bile indicate that biliary excretion in the intestine is the main route of elimination. BPA and BPA-S are eliminated in two phases, with two separate elimination compartments. The first rapid rate of excretion is made up of muscle, liver and plasma, and the second slower rate of excretion is made up of the gallbladder. Source: Own construction based on LINDHOLST et al., 2003.

JUSTIFICATION

Despite the extensive knowledge about the influence of energy metabolism on spermatogenesis in mammals, this knowledge is lacking in fish, the most abundant group of vertebrates. There are still no reports focusing on the mechanisms and modulators involved on testicular energy metabolism and its relationship with spermatogenesis in fish. Although BPA is reported to impair male reproduction, there are still no reports in fish focusing on the biochemical and molecular changes in testicular energy metabolism and the impact on spermatogenesis. Therefore, the study in fish becomes even more relevant for the discovery of new cellular and molecular targets and mechanisms by which BPA impact male reproduction. Thus, the use of zebrafish as an organism model is very relevant to the ecotoxicology and aquaculture fields, since fish are important for the balance of the aquatic ecosystem and are important sources of food and income for many communities.

It is reported that the main age range of human exposure to BPA has been associated to 1-2 years-old. Taking into account that this age range in humans is correlated with the development prepubertal stage similar to rats 8-21 DPN, studies in this animal model become relevant for investigate the impact of BPA on male reproduction. Such investigation becomes even more relevant, since spermatogenesis is not yet established and it is highly vulnerable to changes by hormones and xenobiotics, which can often be irreversible. Thus, given the extensive expertise and experience of the OeReCa laboratory in studying the role of estrogens in mammalian male reproductive function, interest emerged in studying the effects of E2 to correlate them with the effects of xenoestrogen BPA in rat testes. Recognizing the importance of energy metabolism for spermatogenesis successful in mammals, the influence of E2 and BPA on testicular energy metabolism in immature rats is worthy of study. Indeed, the influence of E2 and BPA on the expression of energy metabolism-associated genes in immature rat testis is pertinent for the discovery of new molecular targets by which BPA and E2 may impact male reproduction.

2 OBJECTIVES

2.1 GENERAL OBJECTIVE

To investigate the effects of BPA and/or E2 on calcium signaling, testicular energy metabolism and spermatogenesis in adult zebrafish and/or immature rats.

2.2 SPECIFIC OBJECTIVES

2.2.1 Specific objectives in zebrafish

1. To characterize the nongenomic BPA signaling pathways on calcium influx in the zebrafish testis;
2. To investigate the *in vitro* and *in vivo* nongenomic effects of BPA on testicular energy metabolism in zebrafish;
3. To study the *in vitro* genomic effects of BPA and the ESR involvement in the expression of genes and proteins associated to energy metabolism;
4. To analyze the *in vitro* effects of BPA on the expression of nuclear receptor genes and on expression of cell proliferation associated-proteins;
5. To study the *in vitro* effects of BPA and the involvement of ESR in the expression of germ cell marker genes and in the proportion of testicular cells in zebrafish testis;
6. To analyze the *in vivo* effects of BPA on testicular histological parameters on apoptosis and on proportion of testicular cells in zebrafish.

2.2.2 Specific objectives in rats

1. To investigate the *in vitro* effects of E2 and BPA, and the ESR involvement in the expression of energy metabolism-associated genes in immature rat testis;
2. To investigate the *in vitro* effects of E2 and BPA in the expression of germ cell marker genes in immature rat testis.

3 MATERIALS AND METHODS

3.1 CHEMICALS

BPA (Ref: 239658-50G), E2 (Ref: E8875), ICI 182,780 / Fulvestrant (Ref: I4409-25MG), 9-anthracene carboxylic acid, bovine serum albumin, capsazepine, CFTR (inh)-172, H-89 dihydrochloride hydrate, nifedipine, PD 98059, Ro 31-0432, thapsigargin, HEPES, serum replacement 3 50x (Ref: S2640-100ML), retinoic acid (Ref: R2625-100MG), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ were purchased from Sigma-Aldrich (St. Louis, USA). Dimethyl sulfoxide (DMSO) (Ref: 25-950-CQC) was purchased from Corning Incorporated (Manassas, USA). KCl was purchased from Merck (Darmstadt, Germany). In Situ Cell Death Detection kit (Ref: 11684817910) for TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) analysis and bovine serum albumin fraction V were purchased from Roche (Basel, Switzerland). D-(+)-Glucose and NaCl were purchased from Biosolve Chimie (Dieuze, France). Dulbecco's Phosphate Buffered Saline (DPBS) 10x with $\text{Mg}^{2+}/\text{Ca}^{2+}$ (Ref: CS3PBS00-01) was purchased from Eurobio Scientific (Les Ulis, France). DPBS 10x without $\text{Mg}^{2+}/\text{Ca}^{2+}$ (Ref: X0515-500), amphotericin B, penicillin and streptomycin (Ref: L0022-020) were purchased from Dominique Dutscher (Tourgéville, France). Leibovitz's L-15 medium with L-glutamine (Ref: AT207-1L) was purchased from HiMedia (Mumbai, India). 5% Sodium hypochlorite (Ref: HYPO-05P-1K0) was purchased from Labkem (Barcelona, Spain). Dulbecco's modified eagle medium/F-12 (Ref: 2104-025) was purchased from GIBCO (UK). $^{45}\text{Ca}[\text{CaCl}_2]$ (specific activity, 321 KBq/mg Ca^{2+}), ^{14}C -2-Deoxy-D-glucose (^{14}C -D-deoxy-D-glucose) (specific activity 9.25 GBq/mmol) and Optiphase Hisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Boston, USA). Kits for ALT (Ref: 108-2/100), aspartate aminotransferase (AST) (Ref: 109-2/100), lactate (Ref: 138-1/50) and LDH (Ref: 86-1/100) were purchased from Labtest Liquiform (Lagoa Santa, Brazil).

For RNA extraction, NucleoZOL reagent (Ref: 740404.200) was purchased from Macherey-Nagel (Düren, Germany). For molecular biology, agarose (Ref: V3125), M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) 5x buffer (Ref: M531A), RNasin (Ref: N2511), random primers (Ref: C1181), M-MLV RT (Ref: M1705), deoxyribose nucleotide triphosphate (dNTP): dATP (Ref: U120D), dCTP (Ref: U122D), dGTP (Ref: U121D), dTTP (Ref: U123D), and GoTaq® qPCR Master Mix

(Ref: A6001), RQ1 DNase 10x Reaction Buffer, RQ1 DNase and stop solution were purchased from Promega (Madison, USA). For histology, paraffin (Ref: 39602004) was purchased from Leica Biosystems (Richmond, USA) and Harris's hematoxylin (Ref: 400802010) was purchased from ProTaqS (Potsdam, Germany). For immunohistochemistry (IHC), DAB (3,3'-diaminobenzidine) solution was purchased from R&D Systems (Minneapolis, USA); Mayer's hematoxylin (Ref: 400802210) was purchased from ProTaqS (Potsdam, Germany). Anti-LDH (D-9; Ref: sc-374097) and anti-PCNA (PC10; Ref: sc-56) primary antibodies, as well as goat anti-mouse IgG-HRP (Ref: sc-2005) were purchased from Santa Cruz Biotechnology (Dallas, USA). In addition, anti-PKM (pyruvate kinase M1/2) (Ref: ab38237) primary antibody was purchased from Abcam (Cambridge, UK). Anti- β -actin clone C4 (Ref: MAB1501) was purchased from Millipore (Billerica, USA). Anti-phospho-ERK 1/2 (Ref: 4370) and anti-total-ERK 1/2 (Ref: 9102) primary antibodies, as well as goat anti-rabbit IgG-HRP (Ref: 7074) secondary antibody goat anti-rabbit IgG-HRP were purchased from Cell Signaling Technology (Danvers, USA).

3.2 ANIMALS

3.2.1 Fish

Adult male zebrafish (*Danio rerio*) were obtained commercially (Belo Horizonte, Brazil) and by the *Institut National de la Recherche Agronomique* – INRA (Rennes, France). Fish with lengths from 2.5 to 3.5 cm and weighing 200–300 mg were kept in aquarium on a 12 h light/dark cycle and fed twice a day with feed for ornamental fish. Aquarium water was maintained at 27 ± 1 °C, pH 7 ± 0.3 and salinity 0.7 ± 2 g/L. All fish and aquarium water parameters were carefully monitored and maintained according to recommendations of the Ethics Committee for the Use of Animals of the Federal University of Santa Catarina (Protocol CEUA/UFSC PP00968) and the regulations of the French government (*Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture*).

3.2.2 Rats

20-day-old immature male Wistar rats (*Rattus norvegicus*) were obtained from the *Centre Universitaire des Ressources Biologiques* (CURB) at the University of Caen Normandie (France). The animals were kept under standard conditions, 12 h light/12 h dark cycle at room temperature (21 ± 1 °C) with food and water *ad libitum*. All procedures with the animals were carried out in accordance with French government regulations (*Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture*).

3.3 TREATMENTS AND EXPERIMENTAL PROCEDURES IN ZEBRAFISH

3.3.1 Acute *in vitro* treatments of zebrafish testes

Stock solution of BPA (0.01 M) diluted in ethanol (0.1% into the assay solutions) was used to obtain the final concentrations of BPA in the *in vitro* assays of 30 minutes and 1 hour of incubation. Zebrafish were immobilized in an ice bath and euthanized by an overdose of tricaine (0.8 mg/L, dissolved in NaOH) and spinal transection, and the testes were then dissected. Subsequently, the testes were incubated with different concentrations of BPA in Cortland buffer (124 mM NaCl, 5 mM KCl, 1.7 mM CaCl₂, 3.4 mM NaH₂PO₄, 2.1 mM MgCl₂, 1.91 mM MgSO₄, 11.9 mM NaHCO₃ and 1 g/L glucose) in an atmosphere gassed with O₂:CO₂, 95:5 v/v, 28 °C for 30 minutes to perform the calcium influx studies, which will be described in section 3.4.1. In addition, the testes were incubated with 10 pM, 10 nM or 10 µM BPA in Cortland buffer in an atmosphere gassed with O₂:CO₂, 95:5 v/v, 28 °C for 1 hour to perform the biochemical analyses (BATISTA-SILVA et al., 2020), which will be presented in the following sections. These short-term incubation periods were chosen based on our previous work (BATISTA-SILVA et al., 2020).

3.3.2 Organotypic culture of zebrafish testis

The contact between somatic and germ cells is believed to be essential for the development of zebrafish male germ cells in culture. Thus, the organotypic culture of testis is widely used since the physiological relationship between somatic and germ

cells remains functionally preserved. Therefore, *in vitro* organotypic culture system of testis was performed according to Leal et al. (2009b) in order to study medium-term effects of BPA on molecular and histological parameters in the testis of zebrafish. The culture medium consisted in Leibovitz's L-15 medium with L-glutamine supplemented with 10 mM HEPES, 0.5 % w/v bovine serum albumin fraction V, 10 nM retinoic acid, 0.4 µg/mL amphotericin B, 200 U/mL penicillin and 200 µg/mL streptomycin, and pH was adjusted to 7.4. Briefly, zebrafish were euthanized with an overdose of tricaine (0.8 mg/L, dissolved in NaOH) and spinal transection, posteriorly testes were dissected and immersed in the basal culture medium (without retinoic acid) until the end of the dissection. Then, they were incubated in 0.5 % v/v hypochlorite de sodium in PBS containing CaCl₂ and MgCl₂ for 1 minute, and then put them in the basal culture medium (without retinoic acid). After, each testis was placed under a 1.5% w/v agarose cylinder/Ringer's solution (154 mM NaCl, 3 mM KCl, 1 mM HEPES, 5 mM CaCl₂·2H₂O, 4 mM MgCl₂·6H₂O and 1 g/L glucose; pH 7.4) positioned in 6-well flat-bottom plates containing the culture medium and the treatment. Thus, the testes were incubated with 10 pM and 10 µM BPA, as well as 0.1% and 0.0000001% DMSO (control groups; solvent used in BPA dilution) for 6 and 72 hours (Figure 11) in a humidified air atmosphere at 26 °C.

Thus, testes were incubated in the presence or absence of BPA for 6 and 72 hours in order to analyze their genomic effects through gene expression analysis. In addition, testes were incubated with BPA for 72 hours in order to analyze immunocontent of proteins, as well as histological and immunohistochemical parameters. These incubation periods were chosen based on histological parameters from previous experiments results (not shown) carried out in different incubation periods (6 hours; 3, 5 and 7 days) for implantation of the organotypic culture of zebrafish testis in our laboratory. Furthermore, for the 72-hour BPA treatment groups, the possible involvement of ESRα/β was investigated. For this, the testes were pre-incubated in the presence or absence of 10 µM ICI 182,780 (an ESRα/β antagonist) (ULHAQ; KISHIDA, 2018) for 2 hours before the 72-hour DMSO or BPA incubations (Figure 11).

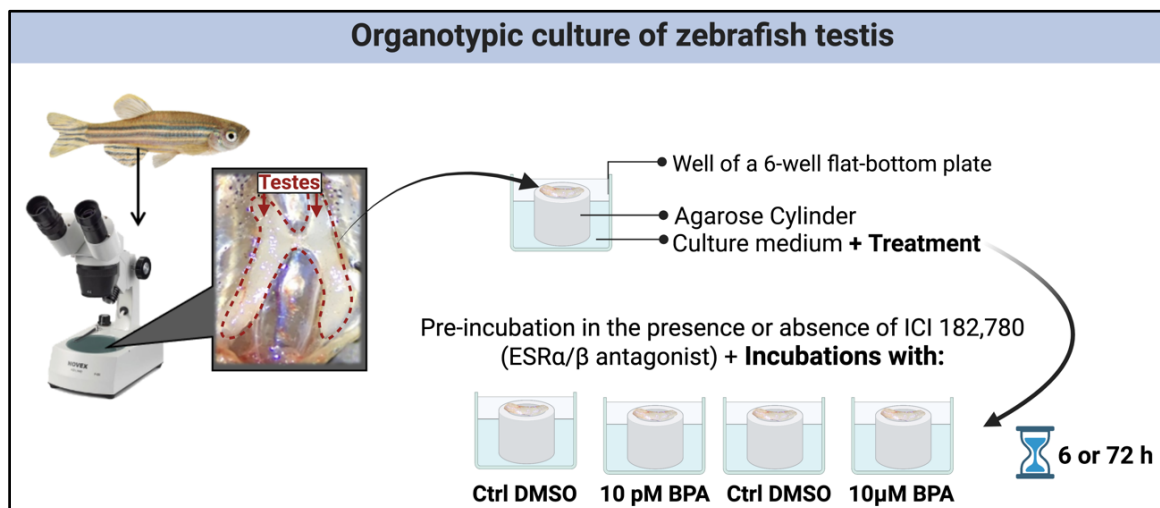


Figure 11: Schematic overview of organotypic culture of zebrafish testis. The testes were dissected and, subsequently, each testis was placed under a 1.5% w/v agarose cylinder in Ringer's solution positioned in 6-well flat-bottom plates containing the culture medium and the treatment. Thus, the testes were incubated with DMSO (control group) or BPA at 10 pM and 10 μM for 6 and 72 hours (h). In addition, the testes were pre-incubated in the presence or absence of 10 μM ICI 182,780 (an ESRα/β antagonist) for 2 h before the 72-h DMSO or BPA incubations. Ctrl – Control. Source: Own construction.

3.3.3 Acute *in vivo* treatments with BPA

Fish were treated with BPA to study its effects (*in vivo*) on biochemical and histological parameters in the testes which will be further presented below. Thus, fish were exposed to 10 pM or 10 μM BPA in the aquarium water for 12 hours. The control groups were exposed to the highest concentration (0.1%) of the solvent (ethanol) used to dilute BPA. This *in vivo* short-term period was chosen based on our previous work (BATISTA-SILVA et al., 2020).

3.4 METHODOLOGICAL APPROACHES USED IN ZEBRAFISH EXPERIMENTS

3.4.1 Calcium ($^{45}\text{Ca}^{2+}$) influx

In vitro Ca^{2+} influx studies were based on a standardized protocol for zebrafish (BATISTA-SILVA et al., 2020). The whole testes of zebrafish were dissected and quickly placed in microtubes containing Cortland's buffer (124 mM NaCl, 5 mM KCl, 1.7 mM CaCl_2 , 3.4 mM NaH_2PO_4 , 2.1 mM MgCl_2 , 1.91 mM MgSO_4 , 11.9 mM NaHCO_3 and 1 g/L glucose) and kept on ice. Testes were pre-incubated in Cortland's buffer gassed with $\text{O}_2:\text{CO}_2$, 95:5 v/v, pH 7.4 at $28 \pm 1^\circ\text{C}$ for 15 minutes. After that, the medium was replaced with fresh Cortland's buffer containing 0.1 μCi/mL radioactive Ca^{2+} .

($^{45}\text{Ca}^{2+}$) (an input about 20.000 counts per minute - CPM) in the absence (control) or presence of BPA at different concentrations: 10^{-15} M (1 fM), 10^{-12} M (1 pM), 10^{-11} M (10 pM), 10^{-9} M (1 nM), 10^{-8} M (10 nM) and 10^{-6} M (1 μM) for 30 minutes (incubation time). Additionally, to determine the time-course of 10 pM BPA action, testes were incubated for 5, 10, 15, 20 and 30 minutes.

In addition, in order to investigate BPA's mechanism of action on $^{45}\text{Ca}^{2+}$ influx, channel blockers, receptor antagonists and enzyme inhibitors were added 15 minutes before $^{45}\text{Ca}^{2+}$ and BPA incubation. The concentration of drugs nifedipine (100 μM), a blocker of L-VDCC; 9-anthracene (1 μM) and CFTR (inh)-172 (5 μM), blockers of CaCC and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels, respectively; thapsigargin (10 μM), an inhibitor of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA); ICI 182,780 (10 μM); an $\text{ESR}\alpha/\beta$ antagonist; H-89 (10 μM) an inhibitor of PKA; Ro 31-0432 (1 μM) an inhibitor of PKC and PD 98059, an inhibitor of MEK 1/2 were chosen based on a similar approach used for studies on $^{45}\text{Ca}^{2+}$ influx in immature rat testis (GONÇALVES et al., 2018) and in zebrafish testis (BATISTA-SILVA et al., 2020). Furthermore, capsazepine (10 μM), an antagonist of TRPV1 (AUZANNEAU et al., 2008); 2-aminoethoxydiphenyl borate (2-APB) (50 μM), an antagonist of IP3R (PETERS; PIPER, 2007) and LY 294002 (10 μM), an inhibitor of PI3K (CAVALLI et al., 2013) were also tested in this study.

At the end of the incubation period, lanthanum chloride solution (127.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO_4 , 10 mM HEPES, 11 mM glucose, 10 mM LaCl_3 , pH 7.3; 2 °C), was used to stop the Ca^{2+} flow. Subsequently, two lanthanum washes were performed to remove the extracellular Ca^{2+} . The cold washing solution containing lanthanum is essential to prevent the Ca^{2+} efflux and/or active extrusion and removal of any surface-bound calcium (BATRA, 1982). Finally, the samples were transferred to a new series of tubes and homogenized with NaOH (0.5 M) and aliquots (duplicates) were taken from each sample for radioactivity measurement using a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman, Boston, USA) (BATISTA-SILVA et al., 2020) and for total protein measurements (LOWRY et al., 1951). The results were expressed as pmol of $^{45}\text{Ca}^{2+}$ per μg of protein or as % of control of 4–7 fish per group from three independent experiments.

3.4.2 ¹⁴C-D-deoxy-D-glucose uptake

In vitro studies on ¹⁴C-D-deoxy-D-glucose uptake were based on previous work which was standardized for zebrafish (BATISTA-SILVA et al., 2020). Testes of zebrafish were dissected and quickly placed in microtubes containing Cortland buffer. The medium was replaced with Cortland buffer containing 0.1 µCi/mL ¹⁴C-deoxy-D-glucose in the absence (control) or presence of BPA (10 pM and 10 µM) for 1 hour in atmosphere gassed with O₂:CO₂, 95:5 v/v, 28 °C. After, the samples were washed with Cortland buffer and homogenized in NaOH (0.5 M). Aliquots were taken from each sample for radioactivity measurements in scintillation liquid in an LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman, Boston, USA). Another aliquot of the sample was used for protein determination (LOWRY et al., 1951). The results were expressed as nmol of glucose *per* mg of proteins.

3.3.3 Measurement of lactate content

Lactate content was measured in the zebrafish testes after *in vitro* exposure for 1 hour and *in vivo* exposure for 12 hours with BPA (10 pM and 10 µM). For this, testes were dissected and homogenized in cold 0.1 M Tris buffer (pH 7.3) and lactate content was measured through the lactate oxidase method (HUTCHESSON et al., 1997) according to manufacturer's instructions (Labtest Liquiform; Lagoa Santa, Brazil). Results were expressed as mg of lactate *per* µg of proteins (LOWRY et al., 1951).

3.3.4 Extraction and measurement of glycogen content

Glycogen content was measured in the zebrafish testes after *in vitro* exposure for 1 hour and *in vivo* exposure for 12 hours with BPA (10 pM and 10 µM). For this, the testes were first incubated in 33% KOH (potassium hydroxide) and heated at 100 °C for 15 minutes. After cooling, 95% ethanol was added and the samples were incubated at 70°C for 5 minutes. Then, they were cooled down on an ice bath to precipitate the glycogen. The homogenates were then centrifuged at 1300g for 15 minutes. The resulting supernatant was then discarded and the pellet was resuspended with

deionized water. Glycogen content was determined using iodine reagent (Calcium chloride saturated solution mixed with iodine solution + potassium iodide solution) for colorimetric estimation of glycogen at 460 nm according to Krisman (1962). The results were expressed as μg of glycogen *per* μg of proteins (LOWRY et al., 1951).

3.3.5 Activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Activities of LDH, ALT and AST were analyzed in the zebrafish testes after *in vitro* exposure for 1 hour and *in vivo* exposure for 12 hours with BPA (10 pM and 10 μM). For that, testes were homogenized in cold 0.1 M Tris buffer (pH 7.3) and LDH, ALT and AST activities were measured through the NADH oxidation method according to the manufacturer's instructions (Labtest Liquiform; Lagoa Santa, Brazil). The consequent reduction of absorbance at 340 nm on this kinetic reading is directly proportional to enzyme activity in the sample (NEHAR et al., 1997). The results were expressed as U *per* μg of proteins (LOWRY et al., 1951).

3.3.6 Measurement of triacylglycerol (TAG) content

TAG content was measured in zebrafish testes after *in vitro* exposure for 1 hour and *in vivo* exposure for 12 hours with BPA (10 pM and 10 μM). To determine the involvement of intracellular Ca^{2+} on *in vitro* effects of BPA (10 μM) on TAG content, the IP3R antagonist 2-APB was added 15 minutes before BPA incubation. Testicular TAG content was then measured through the glycerol phosphate oxidase method according to the manufacturer's instructions (Labtest Liquiform; Lagoa Santa, Brazil). The results were expressed as mg of TAG *per* μg of proteins (LOWRY et al., 1951).

3.3.7 Western Blot

3.3.7.1 Extraction and denaturation of proteins

Protein extraction was performed from samples of zebrafish testes from the organotypic culture of 72 hours of incubation with DMSO (control) and BPA. For this, the testes were homogenized with RIPA extraction buffer (50 mM Tris-HCl, 150 mM

NaCl, 5 mM EDTA, 1% Triton and 1% Sodium Dodecyl Sulfate - SDS) and a cocktail of antiproteases (1/100) (Sigma, #P8340) followed by an incubation on ice for 2 hours. The samples were then centrifuged at 12.000 rpm (Eppendorf 5810R refrigerated centrifuge) for 20 minutes, at 4 °C. After this, the supernatant containing the proteins was recovered and the determination of the protein concentration was performed at 595 nm by the Bradford method (BRADFORD, 1976).

Posteriorly, the proteins are denatured by heating at 95°C for 10 minutes in the presence of a loading buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 1% β -mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue). The proteins denaturation is done in order to separate them only based on their size/molecular weight, without any influence on their structures or their charges (due to the composition of each type of amino acid that composes them). After that, proteins negatively charged by the anionic detergent SDS may migrate to the positive pole in an electric field (electrophoresis).

3.3.7.2 Electrophoresis, transfer of proteins and antibody incubations

30 or 40 μ g of proteins were then added to the wells of the polyacrylamide gel (4% concentration gel), where the proteins were separated as a function of molecular weight by electrophoresis. Protein migration was performed in the presence of electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS; pH 8.5) at 150 V until the samples reached the separating gel (10%), and then at 200 V, totaling a migration duration of around 1 hour.

Subsequently, the proteins were transferred from the gels to nitrocellulose membranes (GE Healthcare). The transfer was performed at 100 V for 1 hour in the presence of transfer buffer (25 mM Tris, 192 mM glycine, 20% ethanol, 0.1% SDS; pH 8.5), with refrigerated system. After, the transfer of proteins was confirmed by staining with Ponceau red (0.1% ponceau red, 5% acetic acid). Then, membranes were washed with Tris-buffered saline (TBS - 10 mM Tris, 15 mM NaCl; pH 7.6).

The membranes were incubated with TBS-0.01% Tween-20 (TBS-T)/5% milk for 1 hour at room temperature in order to block non-specific sites. After that, the membranes were incubated overnight at 4°C with the primary antibody reference protein anti- β -actin clone C4 (1/2000), anti-PKM (pyruvate kinase M1/2) (1/1000), anti-PCNA (1/200), anti-p-ERK 1/2 (1/1000) and t-ERK 1/2 (1/1000) diluted in TBS-T/5%

milk (Table 3). After, the membranes were washed three times with TBS-T and then incubated for 1 hour at room temperature with secondary antibodies (1/2000) conjugated with horseradish peroxidase (IgG-HRP) (Table 3) diluted in TBST-T/5% milk. The membranes were then washed three times with TBS-T. Subsequently, enhanced luminol-based chemiluminescent substrate - ECL (Promega) was added over the membranes for 2 minutes in the dark followed by exposure to an X-ray film, developer and fixer in order to perform the revelation of proteins.

The films were scanned and the proteins bands were quantified through the ImageJ software. The immunocontent of PKM and PCNA proteins was normalized by β -actin reference protein immunocontent, and the results were expressed as % of control. In addition, p-ERK 1/2 levels were calculated in relation to t-ERK 1/2 immunocontent, and the results were expressed as % of control.

Antibodies	Dilution	Brand / (reference)	Weight molecular	Secondary antibodies	Dilution	Manufacturer / (Reference)
anti-PKM	1/1000	Abcam / (ab38237)	58 kDa	goat anti-rabbit IgG-HRP	1/2000	Cell Signaling Technology / (7074)
anti-PCNA (PC10)	1/200	Santa Cruz Biotechnology / (sc-56)	36 kDa	goat anti-mouse IgG-HRP	1/2000	Santa Cruz Biotechnology / (sc-2005)
anti- β -actin clone C4	1/2000	Millipore / (MAB1501)	43 kDa	goat anti-mouse IgG-HRP	1/2000	Santa Cruz Biotechnology / (sc-2005)
anti-phospho-p44/42 MAPK (p-ERK 1/2)	1/1000	Cell Signaling Technology / (4370)	42, 44 kDa	goat anti-rabbit IgG-HRP	1/2000	Cell Signaling Technology / (7074)
anti-total-p44/42 MAPK (t-ERK 1/2)	1/1000	Cell Signaling Technology / (9102)	42, 44 kDa	goat anti-rabbit IgG-HRP	1/2000	Cell Signaling Technology / (7074)

Table 3: Primary and secondary antibodies used in Western Blot.

3.3.8 Processing of zebrafish testes and acquisition of histological images

After pre-incubations in the presence or absence of ICI 182,780 and incubations with BPA and *in vivo* treatment with BPA, the testes were fixed in 4% paraformaldehyde/PBS (phosphate buffered saline) for 24 hours. After, the samples were washed with PBS. The tissues were dehydrated in graded ethanol solutions, then infiltrated in xylol or butanol solvents and finally were embedded in paraffin. Serial sections (5 μ m thickness) were performed using a rotary microtome (Jung RM 2035, Leica) and deposited on slides. After that, histological sections were deparaffinized in two xylene changes, then rehydrated by dipping them in 100%, 95% and 70% ethanol solutions and subsequently, they were immersed in distilled water and in PBS.

Histological sections were then stained with Harris's hematoxylin to analyze the proportion of spermatocytes and haploid cells (spermatids and spermatozoa), which will be further discussed in section 3.3.10. In addition, some histological sections of the *in vitro* exposure groups were used for the immunohistochemical analysis, which is discussed in the next section 3.3.9. Moreover, some histological sections of the *in vivo* exposure groups were also used for the TUNEL analysis, which is presented in section 3.3.11.

To obtain histological images, whole histological slice slides were scanned at 40x with an optical duplicator (0.25 $\mu\text{m}/\text{pixel}$) using the ScanScope CS microscope slide scanner (Leica Biosystems, Nussloch, Germany). They were recorded as tiled tagged image file format (TIFF) images.

3.3.9 Immunohistochemistry (IHC)

Deparaffinized and rehydrated histological sections were incubated with a 3% hydrogen peroxide/PBS solution for 10 minutes in order to block endogenous peroxidases, and then the slides were rinsed. Subsequently, the slides were deposited in a plastic container, dipped in 0.1 M citrate buffer (pH = 6) and heated in a microwave oven (90 W, for 10 minutes). Then the slides were washed with PBS and later dipped in 0.2% Triton/PBS solution for 20 minutes (membrane permeability). Subsequently, the slides were incubated with 3% bovine serum albumin /PBS for 20 minutes in order to block non-specific sites.

After that, the sections were incubated overnight with the 1/50 primary antibody anti-LDH (D-9) diluted in 3% bovine serum albumin/PBS, in a humid chamber at 4°C. Subsequently, the sections were washed with 0.2% Triton/PBS and then incubated with 1/200 anti-mouse IgG-HRP secondary antibody for 90 minutes at temperature room. Afterwards, the slides were washed with PBS and then incubated with DAB (monitoring under a light microscope). After revelation, the slides were counterstained with Mayer's hematoxylin for 1 minute in order to stain the nuclei. After rinsing them with water, they were immersed in 95% and 100% ethanol solutions, then in xylene and finally mounted with Eukitt® mounting medium and a coverslip. The slides were scanned as previously described in order to analyze the positive brown-stained) LDH cells through stereology, which is described below.

3.3.9.1 Stereology: Quantitative analysis of the positive (brown-stained) LDH cells

The counting of the positive (brown-stained) LDH cells was performed through QuPath software (BANKHEAD et al., 2017) using a groovy script to apply a stereology grid and user interface. The stereological method is a precise tool for obtaining quantitative information about histological data to be further analyzed by statistical methods (NOORAFSHAN, 2014). Thus, in order to obtain the density of positive LDH cells, brown-stained testicular cells were counted in a grid area of systematically distributed frames with a size of 50 μm and the space between them of 125 μm . Therefore, an average of 90 frames were analyzed for each sample. The results were expressed as number of positive LDH cells *per* testicular area (μm^2)

3.3.10 Processing and digital analysis of histological images of zebrafish testis

Histological sections slides were scanned as previously described. For each image a down-sampling by 4 was applied to allow an analysis with the innovative digital image analysis method through Ilastik software and the Pixel Classification module according to Berg et al. (2019). The first step was to select images from histological sections for machine training. Then, labels to pixels were assigned based on pixel features (smoothed pixel intensity, edge filters and texture descriptors) and user annotations. The pixel classification produces a user-defined category label to each pixel in the image. For this, five categories were labelled: background, artefact areas, spermatocytes, haploid cells (spermatids and spermatozoa) and tissue (cells other than spermatocytes, spermatids and spermatozoa). Spermatids and spermatozoa were counted in the same surface, since it would not be possible to distinguish these two categories in sections stained only with hematoxylin (which stains the nuclei) and perform analysis by pixel classification. Then, the training and learning step of the machine was carried out to identify and distinguish the different categories through the pixel classification by means of clicks/brush strokes in the areas of interest on the training images. These parameters were calculated and a probability map of each category was computed after all the histological images were exported to the program.

For each pixel in the image, Ilastik estimates the probability that the pixel belongs to each of the semantic categories using a nonlinear algorithm, Random Forest classifier, which operates in the resource space (BERG et al., 2019). To

consider that a pixel belongs to a specific category, it was established that at least 55 % probability was required. All images are compared between them, and if the values are equal, there is 100 % of probability that it is the same category. Therefore, more the values of reference (values in the areas obtained in the training and machine learning step) are different from the values of the new images, the less certain is the probability of indicating that it belongs to the same category, therefore, the value of the probability decreases. The probabilities map images were processed using a program in Python programming language with Scikit-image library according to Van Der Walt et al. (2014) in order to obtain the quantitative result of the digital analysis. For each image, the program calculated the surface area of spermatocytes, haploid cells and of tissue in order to determine the proportion of spermatocytes or haploid cells (spermatids and spermatozoa) in relation to the total surface of the testis. Total surface of the testis is the sum of the surfaces of all analyzed cells in the labelled categories: spermatocytes, haploid cells and tissue (cells other than spermatocytes, spermatids and spermatozoa).

3.3.11 TUNEL assay

Immunohistochemical detection and quantification of apoptosis were determined on labelling of DNA strand breaks through the colorimetric TUNEL assay according to the manufacturer's instructions (Roche; Basel, Switzerland). Some slides were incubated with DNase I (100 U/mL) for 10 minutes as a positive control or without Terminal Deoxynucleotidyl transferase recombinant enzyme as a negative control. The slides were incubated in the presence of DAB solution until a brown staining appears (monitoring under a light microscope), then they were rinsed with distilled water. Subsequently, the slides were counterstained with Mayer's hematoxylin for 1 minute in order to stain the nuclei. After rinsing them with water, they were immersed in 95% and 100% ethanol solutions, then in xylene and finally mounted with Eukitt® mounting medium and a coverslip. For analysis of the positive TUNEL cells (brown-stained cells), the slides were scanned as previously described.

3.3.11.1 Stereology: Quantitative stained cells analysis

In order to perform a quantitative analysis and determine the proportion of marked nuclei (in apoptosis) in relation to the unmarked nuclei, the counting was carried out using the Aperio Image Scope® through the stereology tool kit module. Taking into account a greater prevalence of spermatocytes undergoing apoptosis, these were chosen for analysis. For this, brown-stained (apoptotic) and unstained spermatocytes were counted in a grid area of systematically distributed frames with a size of 40 µm and the space between them of 80 µm. The percentage of apoptotic spermatocytes (positive TUNEL) was calculated as: $100 \times (\text{positive TUNEL spermatocytes brown-stained}) / (\text{positive TUNEL spermatocytes brown-stained} + \text{negative TUNEL spermatocytes unstained})$ (LAURINAVICIUS et al., 2014).

3.4 TREATMENTS AND EXPERIMENTAL PROCEDURES IN IMMATURE RATS

3.4.1 Organotypic culture of testis explants from immature rats

The organotypic culture of testis explants from immature rats was implemented and used in our experiments to maintain the physiological relationship of the main cellular interactions between Sertoli and germ cells. Thus, it was performed in order to study the *in vitro* effects of E2 and BPA on gene expression in the testis of immature rats. The culture medium consisted in DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) with L-glutamine and without phenol red, supplemented with serum replacement 3 (1/50), 0.25 µg/mL amphotericin B, 50 U/mL penicillin and 50 µg/mL streptomycin.

Three 20-day-old immature rats were used in each independent organotypic culture experiment. Briefly, the rats were euthanized by inhalation of CO₂ (4 L/min for 2 min; 6L/min for 2 min and 9L/min for 2 min), and the testes were then dissected and immersed in the basal culture medium. Then, in a laminar flow hood, the testes were cut into several small pieces ($\approx 1.5 \text{ mm}^3$ in size). Two small pieces of testis from each rat were distributed to all experimental groups, placed on flat-based inserts (Sarstedt; Germany) with a pore size of 1 µm positioned in wells of a 24-well flat-bottom plates containing the culture medium and the treatment. Thus, the testis explants were incubated with E2 at 10 pM, 1 nM and 1 µM, ethanol (control group); BPA at 10 pM, 1 nM and 1 µM, and DMSO (control group) for 6 hours (Figure 12) in a humidified air

atmosphere and gassed with O₂:CO₂, 95:5 v/v, at 32 °C. The concentrations of ethanol and DMSO solvents were 0.01%, corresponding to their highest concentration at the dilution of 1 µM E2 and BPA, respectively. In addition, the possible involvement of ESRα/β was also investigated. For this, testis explants were pre-incubated in the presence or absence of 1 µM ICI 182,780 (WANG et al., 2014), an ESRα/β antagonist for 1 hour before incubations with E2, BPA and solvents.

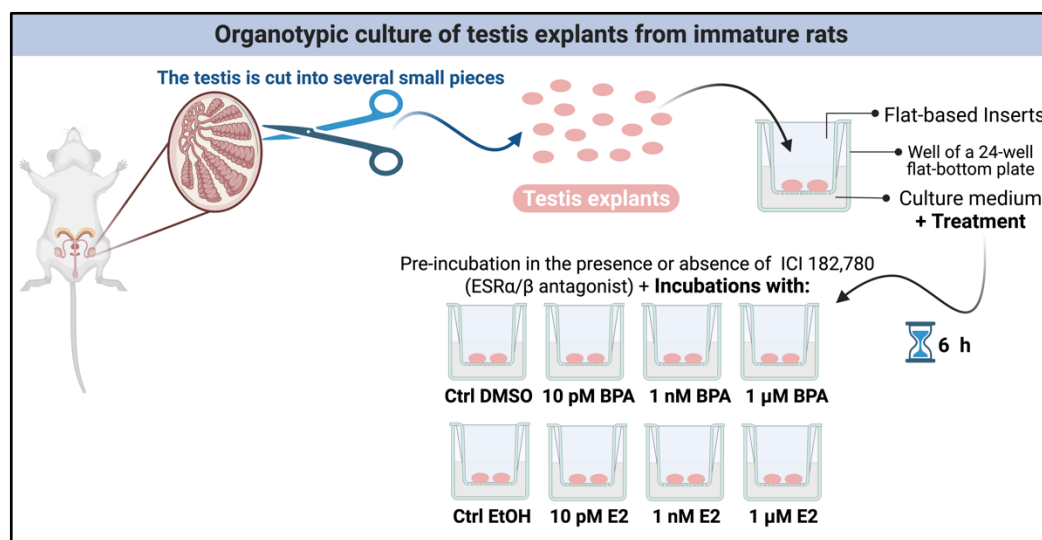


Figure 12: Schematic overview of organotypic culture of rat testis explants. The testis was dissected and cut into several small pieces ($\approx 1.5 \text{ mm}^3$ in size). Then, two small pieces of testis from each rat were distributed to all experimental groups, placed on flat-based inserts positioned in wells of a 24-well flat-bottom plates containing the culture medium and the treatment. Thus, the testis explants were pre-incubated in the presence or absence of 1 µM ICI 182,780 (ESRα/β antagonist) for 1 hour and incubated with DMSO or BPA at 10 pM, 1 nM and 1 µM; ethanol (EtOH) or E2 at 10 pM, 1 nM and 1 µM for 6 h (hours). Ctrl – Control. Source: Own construction.

3.5 METHODOLOGICAL APPROACHES USED IN ZEBRAFISH AND IMMATURE RATS EXPERIMENTS

3.5.1. Extraction and quantification of total RNA

After *in vitro* exposures of zebrafish testes (6- and 72-hour groups) and immature rat testis explants (6-hour group) to BPA or E2, total RNA was extracted. RNA extraction was performed using the NucleoZOL reagent protocol according to the manufacturer's instructions (Macherey-Nagel; Düren, Germany). The concentrations of RNA samples were determined by absorbance at 260 nm through NanoDrop 2000 (ThermoFisher) spectrophotometer, and RNA quality was verified by the ratio of the

optical density 260nm/280nm which must be between 1.8 - 2.1. Posteriorly, total RNA samples were diluted at 50 ng/ μ L in nuclease-free water.

After that, RNA samples of immature rat testis explants from organotypic culture were treated with DNase I in order to eliminate genomic DNA contamination (GREEN; SAMBROOK, 2019). RNA (5 μ L) were incubated with 5 μ L DNase MIX (1 μ L DNase 10x reaction buffer, 0.25 μ L DNase I and 3.75 μ L nuclease-free water for each sample) at 37°C for 30 minutes. Subsequently, each sample was incubated with 1 μ L stop solution and incubated at 65°C for 10 minutes for the enzyme to be deactivated.

3.5.2 Reverse Transcription (RT)

Reverse transcription was realized in order to obtain the complementary DNA (cDNA). First, mixes in a total volume of 20 μ L were prepared with 250 ng of total RNA, M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) 5x buffer (diluted 1/5), 12 IU RNasin, 0.1 μ g random primers, 500 μ mol/L deoxynucleotide triphosphate (dNTP) - dATP, dCTP, dGTP e dTTP dNTP, 100 IU M-MLV RT (0.5 μ L) and nuclease-free water. After, these mixes were incubated at 37 °C for 1 hour in the thermal cycler in order to perform the RT. MMLV-RT was then inactivated by a final step of 10 minutes at 72 °C. Negative control was realized by adding nuclease-free water instead of RNA, and RT negative samples were prepared without MMLV-RT in order to have a control to confirm whether the samples were not contaminated with genomic DNA. At the end, the cDNA samples were diluted (1/10) in nuclease-free water (180 μ L).

3.5.3 Relative gene expression analysis by real-time Polymerase Chain Reaction

Real-time PCR was performed in order to analyze the relative expression of target genes in the zebrafish testis (Table 4) and in the testis explants from immature rats (Table 5) after 6 and/or 72 hours of incubation with BPA or E2. The zebrafish and rat specific genes analyzed, as well as the sequence of their primers are shown in Tables 2 and 3, respectively. Thus, real-time PCR was performed in a final volume of 20 μ L containing: 5 μ L of cDNA diluted 1/10, 10 μ L of GoTaq® qPCR Master Mix 2X, 0.5 μ L of 15 μ M forward (F) and reverse (R) primers (Tables 4 and 5), 0.2 μ L of CXR reference dye and 3.8 μ L of nuclease-free. The negative control was realized by adding nuclease-free water instead of cDNA. PCR efficiency was determined for each set of

primers by performing a series of cDNA dilutions (1/10, 1/20, 1/50 and 1/100). Thus, the PCR efficiency of the primers used is comprised between 80% and 104%.

PCR was carried out in a CFX96 touch real-time PCR detection system from Bio-Rad (Hercules, CA, USA) as follows: an initial step of 3 minutes at 95 °C, followed by 39 cycles of 30 seconds at 95 °C (denaturation) and 60 seconds at 60 °C (amplification). At the end of PCR amplification, an increase of 0.5 °C every 5 seconds from 65 °C to 95 °C was performed to obtain the melting curve of the amplification products. The melting curve is considered a checkpoint of the PCR, allowing the monitoring of the specificity of the reaction. If there is any genomic DNA contaminant, some nonspecific dimerization, not being the target product of amplification, will appear different temperature values, not having the same melting temperature (T_m) pattern.

The quantification of gene expression by real-time PCR is made from the cycle threshold (C_t) values. C_t represents the number of cycles required to initiate amplification of target genes from each sample (WONG; MEDRANO, 2005). Therefore, to analyze the relative gene expression, C_t of target genes was normalized with the reference gene using the $2^{-\Delta C_t(\text{target gene} - \text{reference gene})}$ method (LIVAK; SCHMITTGEN, 2001). The ribosomal protein L8 (RPL8) gene was the reference gene used for the relative gene expression analysis in zebrafish testis, since its expression is stable and unaltered by xenoestrogens treatment (ZHU et al., 2016; MA et al., 2018). However, the most stable reference gene in immature rat testis was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used for the analysis of relative gene expression in the testis explants. Thus, gene expression of the treated groups was calculated relative to gene expression of the control group (fixed at 100%) and the results were expressed in % according to Brouard et al. (2016).

<i>Danio rerio</i> specific Genes	Primer Sequences (5' –3')	GenBank accession Number
RPL8	F: TTGTTGGTGTGTTGCTGGT R: GGATGCTCAACAGGGTTCAT	XM_005167684.1
PYGL	F: TCTGCTATGAAGCTGACCCG R: AGGCCTCCATTGCCTAATCC	NM_001008538.1
PFKFB2a	F: AGGTGCAGGACTGACCTATGA R: TGTGGGCGAGTTTGTCTATGT	NM_201008.1
PKMA	F: GTTCTGACCCGTGACCCCTTC R: GTCACCGCTAGGATTGGAGC	NM_199333.1
LDHBa	F: TGA CTG CATTCTCGTCGTGG R: TTTGGCAGGCCACTCAACTT	NM_131247.1
GPT2	F: GGGACAGCAACCAATCACCT R: GATACGCCGTGCTCTCCATT	NM_001098757.2
PCXA	F: CAGATGTGGTGCGCAAGATG R: CAAACAGGAGATGGGAGCGT	NM_001328356.1
MCT4	F: GACACGGCTTGGATCTCCTCTA R: TGCCAAGACCATAACCAATGA	NM_212708.1
ESR2b	F: TCATGTGAAGGGTGCAAGGC R: TCCCTCCTCACACCACACTT	NM_174862.3
ESR2a	F: GCCGACTCCGAAAGTGCTAT R: TGAGACCTCGGACCAGTCAT	NM_180966.2
ESRR α	F: TCTCTGTGACCTGGCCGATA R: AACACCAACACTTCCAGCCA	NM_212955.1
ESRR δ	F: GCCGACAGAAATACAAGCGG R: CGCAGAGGAGTCACTTCACA	XM_001921058.7
PIWIL-1	F: CGATGCACTGAAGGGGTCAT R: GGGCTCATAATCTTGCCCCA	NM_183338.1
SYCP-3	F: TGGTGTGGGGAATGAGGTTTC R: GGCGTTTCCTTTTGGTCTGC	NM_001040350.1
ODF3b	F: GATGCCTGGAGACATGACCA R: ACCATGACTAGCAATATTAAGACCT	XM_021467596.1

Table 4: Zebrafish-specific primer sequences for gene expression analysis by real-time RT-PCR. F – Forward; R – Reverse; RPL8 – Ribosomal protein L8; PYGL – Glycogen phosphorylase liver form; PFKFb2a – 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2a; PKMA – Pyruvate kinase M1/2a; LDHBa – Lactate dehydrogenase Ba; GPT2 – Alanine aminotransferase 2; PCXA – Pyruvate carboxylase A; MCT4 – Monocarboxylate transporter 4; ESR2b – Estrogen receptor β 1; ESR2a – Estrogen receptor β 2; ESRR α – Estrogen-related receptor α ; ESRR δ – Estrogen-related receptor δ ; PIWIL-1 – P-element induced wimpy testis-like; SYCP-3 – Synaptonemal complex protein 3; ODF3b – Outer dense fiber protein 3b.

<i>Rattus norvegicus</i> specific Genes	Primer Sequences (5' –3')	GenBank accession Number
GAPDH	F: AGTGCCAGCCTCGTCTCATA R: GACTGTGCCGTTGAACTTGC	NM_017008.4
PYGM	F: AACGTTGTCAACACCATGCG R: TTCTCAGCCAGGTTTCGGTC	NM_012638.1
PKM	F: TGAAGTACGCCCAGGATCT R: GAGCTGCTGGGTCTGAATGA	NM_053297.2
LDHc	F: TCCACCTGTGAAGGCTCAAC R: GCCATGCCACATTTCACAC	NM_017266.2
MCT1	F: GCCGCGAGATACACATAACG R: ATCGCAGGTGGCATCTTAGG	NM_012716.2
GPT2	F: CAGCCCAGGCTCACCATAAA R: TCGCGAATCTTCCGAGGTTA	XM_008772405.2
GK2	F: TTCCGAGATCTACGGCCAGA R: GCAGACTGATCCCCCAACA	NM_001004077.1
ITGa6	F: ACGATGCCACCTACCACAAG R: GCATGGTATCGGGGAACACT	NM_053725.2
PCNA	F: TCCCAGAACAGGAGTACAGC R: AAGGTCCCGGCATATACGTG	NM_022381.3
DMC1	F: GATTTCACTGGCCGTGGAGA R: TGAAGCGTGAGCCAGAAATGT	NM_001130567.1

Table 5: Rat-specific primer sequences for gene expression analysis by real-time RT-PCR. F –Forward; R – Reverse; GAPDH – Glyceraldehyde-3-phosphate dehydrogenase; PYGM – Glycogen phosphorylase muscle isoform; PKM – Pyruvate kinase M1/2; LDHc – Lactate dehydrogenase C; MCT1 – Monocarboxylate transporter 1; GPT2 – Alanine aminotransferase 2; GK2 – Glycerol kinase 2; ITGa6 – Integrin $\alpha 6$; PCNA – Proliferating cell nuclear antigen; DMC1 – DNA meiotic recombinase 1.

3.6 STATISTICAL ANALYSIS

The results were analyzed using the GraphPad Prism program (versions 5 and 9). The Shapiro–Wilk normality test was performed to confirm that the data have a normal distribution. Data are represented as mean \pm standard error of the mean (S.E.M.) of 3–7 fish *per* group (chapter I) or 5-9 rats *per* group (chapter II) from two or three independent experiments. Results were evaluated by *t*-Student test and one-way analysis of variance (ANOVA). The *post hoc* Bonferroni test was used to compare the different groups when there was a significant difference. Statistical differences between groups were considered when $p \leq 0.05$.

4 RESULTS, DISCUSSION AND CONCLUSIONS

4.1 CHAPTER I: STUDY OF THE NONGENOMIC AND GENOMIC EFFECTS OF BPA ON TESTICULAR ENERGY METABOLISM AND SPERMATOGENESIS IN ADULT ZEBRAFISH TESTIS

4.1.1 Results of Part I of Chapter I: Study of the *in vitro* effects of BPA on Ca^{2+} signaling in the testis of zebrafish

4.1.1.1 *In vitro* acute incubation of zebrafish testis with low-concentration BPA increases calcium influx

To study the effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish, a concentration-response curve of BPA at 10^{-15} M (1 fM), 10^{-12} M (1 pM), 10^{-11} M (10 pM), 10^{-9} M (1 nM), 10^{-8} M (10 nM) and 10^{-6} M (1 μM) was assayed with 30 minutes of incubation. BPA treatment at 10 pM and 10 nM stimulated $^{45}\text{Ca}^{2+}$ influx, compared to the control group (Figure 13A). Subsequently, a time-course protocol was used to examine the effect of BPA on $^{45}\text{Ca}^{2+}$ influx after 5, 10, 15, 20 and 30 minutes of incubation. For the first 5–20 minutes of incubation, there was no difference between control and BPA treated group. However, BPA significantly stimulated $^{45}\text{Ca}^{2+}$ influx at 30 minutes (Figure 13B). Consequently, for the further experiments, 10 pM BPA and 30 minutes of incubation were used for studies of $^{45}\text{Ca}^{2+}$ influx.

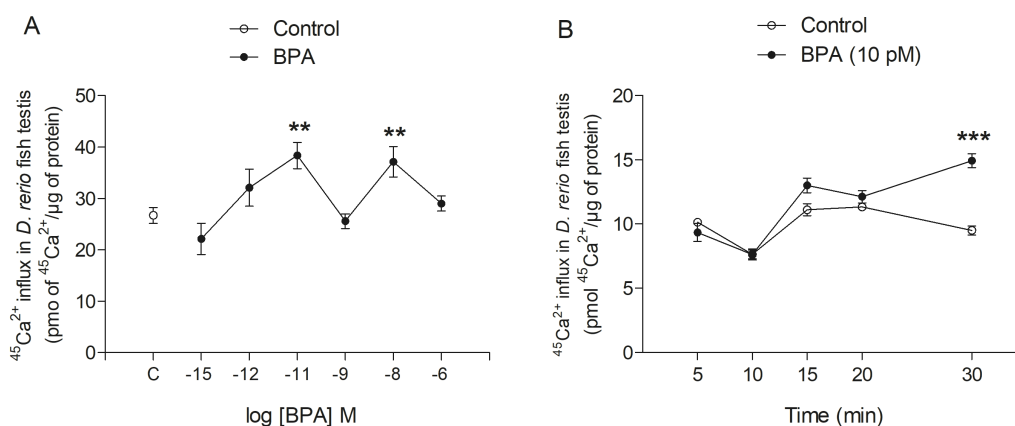
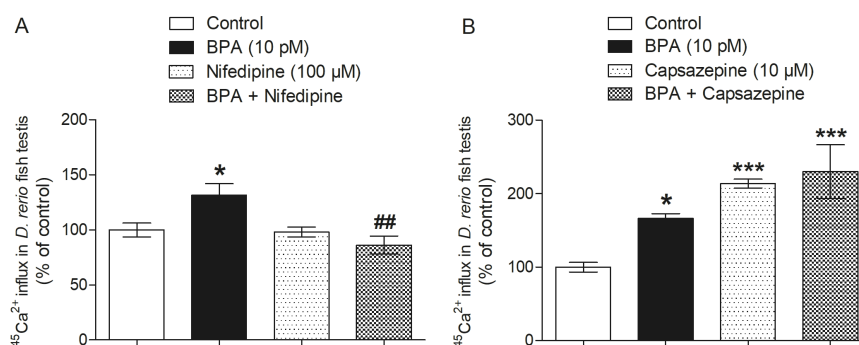


Figure 13: Concentration-response curve and time-course of $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish incubated with BPA. Effects of BPA on $^{45}\text{Ca}^{2+}$ influx: (A) concentration-response curve; (B) time-response curve. The data are expressed as mean \pm S.E.M. of 4-7 fish in each group from three independent experiments. ** $p \leq 0.01$ when compared to control group; *** $p \leq 0.001$ when compared to the respective control group at 30 minutes.

4.1.1.2 *In vitro* BPA increases $^{45}\text{Ca}^{2+}$ influx via L-VDCC and CaCC in the testis of zebrafish

The mechanism of action of BPA on Ca^{2+} influx in the testis of zebrafish was studied using a pharmacological approach employing channel blockers, receptor antagonists and enzyme inhibitors. As illustrated in Figure 14A, the increase on $^{45}\text{Ca}^{2+}$ influx promoted by BPA was prevented by the addition of nifedipine, a blocker of L-VDCC, suggesting that the increase on $^{45}\text{Ca}^{2+}$ influx induced by BPA was mediated by the activity of these channels. The stimulatory effect of BPA was similar to that observed for testes that were incubated with capsazepine, an antagonist of TRPV1. However, when testes were co-incubated with capsazepine and BPA, $^{45}\text{Ca}^{2+}$ influx was not potentiated, indicating that TRPV1 does not participate in the stimulatory effect of BPA on Ca^{2+} influx in the testis (Figure 14B). Subsequently, the increase on $^{45}\text{Ca}^{2+}$ influx promoted by BPA was abolished by the addition of 9-anthracene, a CaCC blocker, suggesting that the increase on $^{45}\text{Ca}^{2+}$ influx induced by BPA was also mediated by the activity of these channels (Figure 14C). Additionally, the $^{45}\text{Ca}^{2+}$ influx stimulated by BPA was similar to that observed in the presence of CFTR(inh)-172, an inhibitor of cystic fibrosis transmembrane conductance regulator Cl^- channels. Notably, this response was not potentiated by co-incubation with the two compounds, indicating that these Cl^- channels does not participate in the mechanism of action of BPA on the testis of zebrafish (Figure 14D).



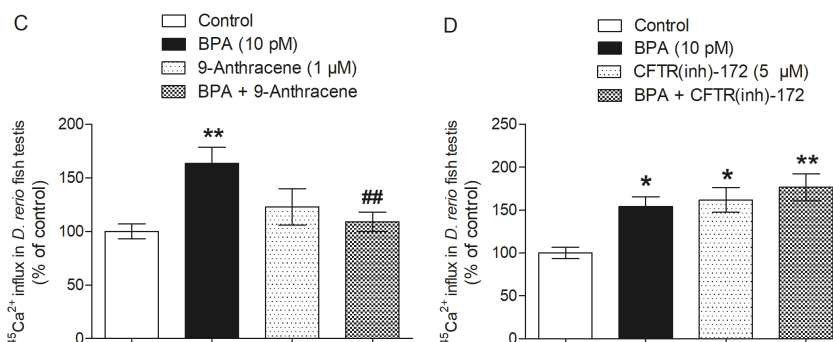


Figure 14: Membrane ion channels involvement in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish. Involvement of (A) L-type VDCC, (B) TRPV1, (C) CaCC and (D) CFTR channels on the effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish. (A) L-type VDCC blocker nifedipine, (B) TRPV1 antagonist capsazepine, (C) CaCC blocker 9-anthracycline or (D) CFTR blocker CFTR(inh)-172 were added 15 minutes before *in vitro* BPA incubation (30 minutes). The data are expressed as mean \pm S.E.M. of 4-7 fish in each group from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$, when compared to control group; ## $p \leq 0.01$, when compared to BPA group.

4.1.1.3 *In vitro* BPA increases $^{45}\text{Ca}^{2+}$ influx via IP3R activation

The involvement of intracellular Ca^{2+} , IP3R and SERCA in the effects of BPA in the testis of zebrafish was also studied. As illustrated in Figure 15A, when the IP3R antagonist, 2-APB, was co-incubated with BPA, the stimulatory effect of BPA was abolished, indicating that IP3R activation is involved in the mechanism of action of BPA in stimulating $^{45}\text{Ca}^{2+}$ influx. In addition, Figure 15B shows that the BPA-induced increase on $^{45}\text{Ca}^{2+}$ influx was abrogated when testes were co-incubated with BPA and the SERCA inhibitor, thapsigargin.

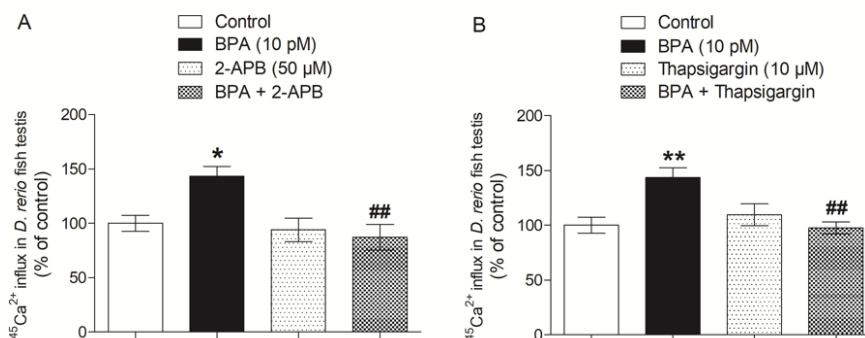


Figure 15: Involvement of Ca^{2+} release from intracellular stores in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish. Involvement of (A) IP3R and (B) SERCA in the effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis. The (A) IP3R antagonist, 2-APB, or (B) SERCA inhibitor, thapsigargin, were added 15 minutes before *in vitro* BPA incubation (30 minutes) of the testes. The data are expressed as mean \pm S.E.M. of 4-6 fish in each group from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$, when compared to control group; ## $p \leq 0.01$, when compared to BPA group.

4.1.1.4 PKC, MEK 1/2, PI3K and nuclear estrogen receptors are involved in the mechanism of action of BPA on $^{45}\text{Ca}^{2+}$ influx

Intracellular targets mediating the effects of BPA on $^{45}\text{Ca}^{2+}$ influx, such as kinase proteins, were also studied in the testis of zebrafish. Figure 16A shows that when testes were incubated with both BPA and H-89, a PKA inhibitor, the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx was not modified, indicating that PKA activation does not participate in this event. In contrast, Figure 16B shows that when the testis of zebrafish was co-incubated with Ro 31-0432, a PKC inhibitor, and BPA, the stimulatory effect of BPA was abolished, indicating that PKC is involved in the mechanism of action of BPA. Furthermore, as demonstrated in Figure 16C, when testes were co-incubated with PD 98059, a MEK 1/2 inhibitor, and BPA, the increase on $^{45}\text{Ca}^{2+}$ influx promoted by BPA was abolished, showing that MEK is involved in the downstream mechanism of action of BPA in the testis. In addition, as can be seen in Figure 16D, LY 294002, a PI3K, abolished the effects of BPA in the increase on $^{45}\text{Ca}^{2+}$ influx, indicating that PI3K activation is also involved in BPA's mechanism of action.

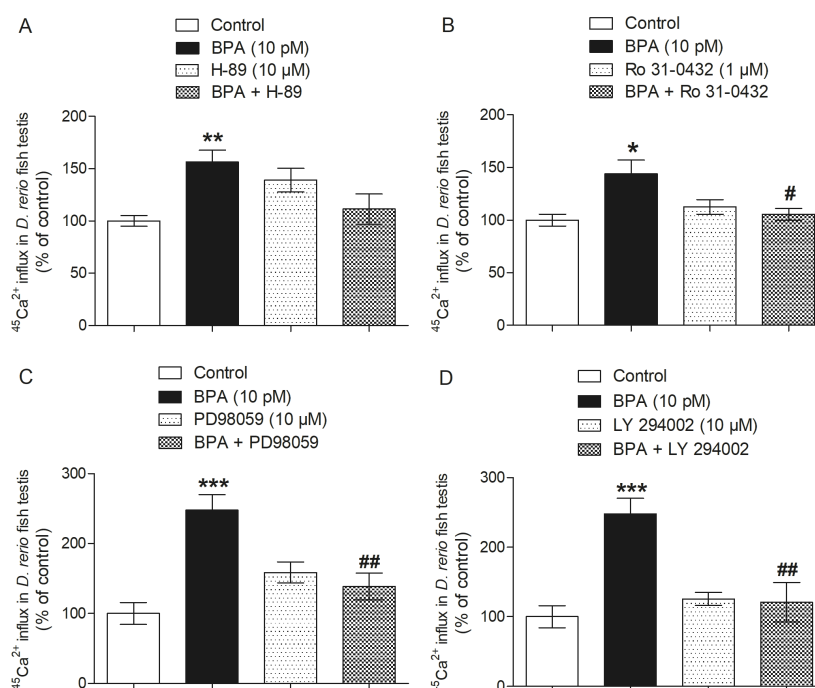


Figure 16: Involvement of protein kinases in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish. Involvement of (A) PKA, (B) PKC, (C) MEK 1/2 and (D) PI3K in the effects of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis. The (A) PKA inhibitor H-89, (B) PKC inhibitor Ro 31-0432, (C) MEK 1/2 inhibitor PD 98059 or (D) PI3K inhibitor LY 294002 were added 15 minutes before *in vitro* incubation of the testes with BPA (30 minutes). The data are expressed as mean \pm S.E.M. of 4-7 fish in each group from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$, when compared to control group; # $p \leq 0.05$ and ## $p \leq 0.01$, when compared to BPA group.

Furthermore, the involvement of ESR α/β in the mechanism of action of BPA was also investigated in the zebrafish testes. Figure 17 shows that the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in zebrafish testes was completely abolished when the ESR α/β antagonist ICI 182,780 was co-incubated with BPA.

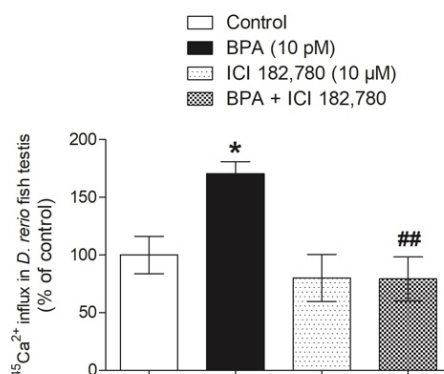


Figure 17: Involvement of ESR α/β in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of zebrafish. ICI 182,780 (ESR α/β antagonist) was added 15 minutes before *in vitro* incubation of the testes with BPA (30 minutes). The data are expressed as mean \pm S.E.M. of 4-7 fish in each group from three independent experiments. * $p \leq 0.05$, when compared to control group; ## $p \leq 0.01$, when compared to BPA group.

4.1.1.5 BPA-induced plasma membrane damage is dependent on activation of IP3R

In order to investigate whether *in vitro* acute BPA exposure induces plasma membrane damage, the release of LDH to the extracellular medium was investigated. LDH activity was analyzed after incubation of the zebrafish testes for 1 hour with BPA at different concentrations (10 pM, 10 nM and 10 μM). Figure 18A shows that only 10 μM BPA caused an increase in the extracellular activity of LDH compared to the control group, suggesting an increase in its release from the intra to the extracellular medium. Figure 18B shows that when testes were co-incubated with 2-APB and BPA, the BPA-induced LDH release was completely abolished, indicating that IP3R activation and Ca^{2+} release from intracellular Ca^{2+} stores are involved in this event. Subsequently, the involvement of Ca^{2+} release from intracellular stores in the BPA-induced increase in TAG content in the testes was also investigated. Figure 18C shows that testes incubated with 10 μM for 1 hour caused an increase on testicular TAG content. As can be seen in Figure 18D, when testes were co-incubated with 2-APB and BPA, the stimulatory effect of BPA on testicular TAG content was also completely abolished.

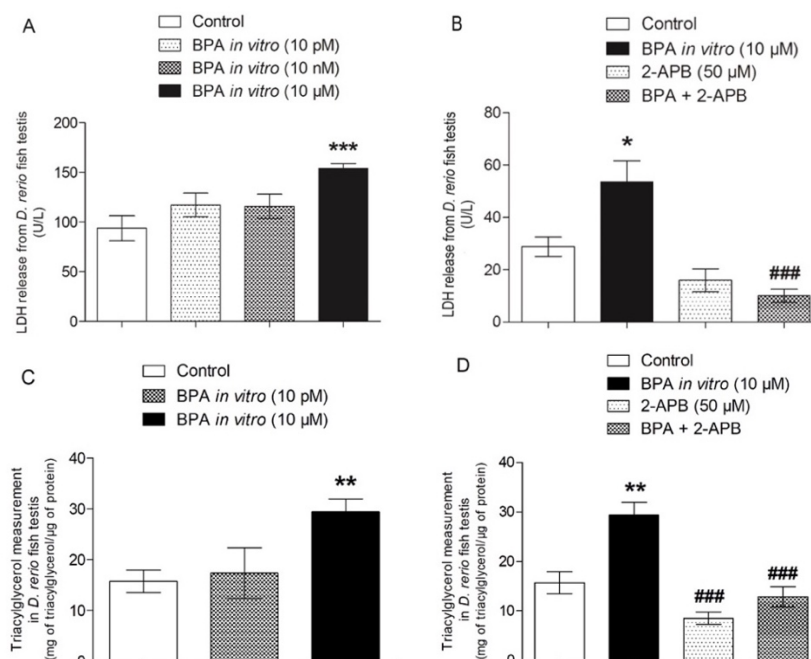


Figure 18: IP3R activation induces plasma membrane damage in the testis of zebrafish. (A) Effects of exposure of testes with different concentrations of BPA on LDH release after 1 hour of incubation. (B) Involvement of IP3R in LDH release after incubation with BPA. (C) Effects of BPA exposure for 1 hour on TAG content. (D) Involvement of IP3R in the BPA-induced increase in TAG content in the testis of zebrafish. The IP3R antagonist 2-APB was added 15 minutes before BPA incubation (1 hour). The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$, when compared to control group; ### $p \leq 0.001$, when compared to BPA group.

4.1.2 Discussion of Part I of Chapter I

The present study demonstrated the acute effect of BPA at a low concentration on Ca^{2+} signaling with the participation of the signaling pathways involving the nuclear estrogen receptor, proteins kinases MEK 1/2, PI3K and PKC, as well as IP3R. Additionally, testes exposed to a higher concentration of BPA have been shown to induce Ca^{2+} -dependent plasma membrane damage. In this study, some targets involved in Ca^{2+} signaling at the plasma membrane were studied, including L-VDCC, TRPV1, CaCC and the CFTR chloride channel. Also, targets involved in the intracellular Ca^{2+} -stores, such as IP3R and SERCA. We used a pharmacological approach and based the concentrations of the various drugs tested in studies from mammalian species (GONÇALVES et al., 2018) and in the testis of zebrafish (BATISTA-SILVA et al., 2020).

This study has shown in an unprecedented way that BPA activates L-VDCC in the testis of zebrafish. Consistent with our findings, BPA may also activate VDCC and voltage-dependent K^{+} channels in human and canine coronary smooth muscle, but at

concentrations 10^6 times more elevated than those used in our study (ASANO; TUNE; DICK, 2010). In addition, chronic BPA exposure (100 $\mu\text{g/kg/day}$) promotes up-regulation of T-VDCC mRNA expression that is dependent on ESR activation in the mouse testis and epididymis (WANG et al., 2015). Furthermore, we have been reported that bis(2-ethylhexyl)phthalate, another EDC from plastic industry, also exhibits similar signal transduction on $^{45}\text{Ca}^{2+}$ influx mediated by L-VDCC (BATISTA-SILVA et al., 2020), providing evidence that L-VDCC is a potent target of EDC action in the testis of zebrafish. Tran et al. (2018) showed that *in utero* BPA (100 mg/kg/day) in mice impairs embryo implantation through alterations in Ca^{2+} transport and the gene expression of channels, such as TRPV 5 and TRPV 6. However, we showed that the induction of Ca^{2+} influx by BPA is independent of TRPV1 activation in the testis of zebrafish.

Since the increase in Ca^{2+} influx, mediated by the plasma membrane VDCC, may occur via CaCC-dependent membrane depolarization (HARTZELL et al., 2005; VERKMAN; GALIETTA, 2009), the involvement of CaCC in the mechanism of action of BPA was studied. CaCCs is described in many different cell types, such as the Sertoli cells, where these channels are widely involved in cellular secretion (MENEZAS et al., 2010). Rapid exposure to very low concentration of BPA (1 pM) has been shown to trigger immediate increases in Ca^{2+} influx in the immature rat testis that is CaCC dependent, suggesting that BPA may influence secretory activity in the testis (GONÇALVES et al., 2018). Our findings are in agreement with these previous data, demonstrating that BPA-stimulated Ca^{2+} influx increased was also associated with CaCC participation in the testis of zebrafish. In addition, it has been reported that CaCC-induced depolarization is fundamental to fertilization in amphibian oocytes. Cytosolic Ca^{2+} increase due to Ca^{2+} release from stores, via IP3R activation, occurs upon fertilization and causes opening of the CaCC to produce membrane depolarization and prevent the polyspermy (HARTZELL, 1996; MACHACA et al., 2002). CFTR is a cAMP-activated Cl^- channel found in the apical cell plasma membrane of fluid-transporting tissues, such as airways, intestine, pancreas and testis (VERKMAN; GALIETTA, 2009). However, our data indicate that the mechanism of action of BPA is not dependent on the CFTR Cl^- channel in the testis of zebrafish. On the other hand, CFTR Cl^- channel was shown to be involved in the mechanism of action of BPA on $^{45}\text{Ca}^{2+}$ in the rat testis (GONÇALVES et al., 2018).

Having defined the involvement of L-VDCC and CaCC in the mechanism of action of BPA, the role of the IP3 pathway in modulating the release of intracellular Ca^{2+} from stores was studied, since CaCC activation can also be mediated by IP3R signaling and store-operated Ca^{2+} channels (SOC) activation (KURUMA; HARTZELL, 2000; HARTZELL et al., 2005). Additionally, Ca^{2+} influx through the L-VDCC shown in our study could also have induced plasma membrane depolarization via CaCC activation. In some cell types, membrane depolarization activates VDCC, which results in additional Ca^{2+} influx and further depolarization (VERKMAN; GALIETTA, 2009) and Ca^{2+} store depletion stimulates Ca^{2+} influx by SOC, which also contributes to CaCC activation (KURUMA; HARTZELL, 2000; HARTZELL et al., 2005). The activation of PLC, PKC and IP3R has been associated with activation of the GPER membrane-initiated signaling pathway (LE MELLAY et al., 1997); BPA-induced Ca^{2+} influx is reported to involve PLC activation in the testis of rats (GONÇALVES et al., 2018) and our results demonstrate that BPA-induced Ca^{2+} influx involves IP3R. Furthermore, as shown in our study, PKC activation was also crucial to the mechanism of action of BPA and these data are in agreement with previous studies in the rat testis (GONÇALVES et al., 2018).

In addition to increasing Ca^{2+} influx involving external Ca^{2+} and activation of plasma membrane ion channels, BPA also affects intracellular Ca^{2+} from stores. Gonçalves et al. (2018) reinforce our data suggesting that BPA inhibits SERCA in the rat testis reporting the same effect of BPA and thapsigargin on $^{45}\text{Ca}^{2+}$ influx as observed in our study. Furthermore, it has also been reported that BPA induces an increase in cytosolic Ca^{2+} levels by inhibiting SERCA in rat testicular microsomes (HUGHES, 2000) and in human skin fibroblasts (WOESTE et al., 2013). Thus, we could suggest that BPA also inhibits SERCA and contributes to increased cytosolic Ca^{2+} levels in the zebrafish testis in support of these previous studies. However, IP3R activation and SERCA inhibition led to intracellular Ca^{2+} store depletion in the endoplasmic reticulum. The depletion of Ca^{2+} stores is the trigger for plasma membrane SOC activation and stimulation of Ca^{2+} influx, which can be sustained for minutes to hours. This occurs as a compensatory mechanism for the Ca^{2+} supply to refill the endoplasmic reticulum, and to drive a wide assortment of biological processes, such as secretion, gene transcription and modulation of enzymatic activity (PRAKRIYA; LEWIS, 2015).

Consistent with our findings, previous works have reported the activation and participation of protein kinases in the mechanism of action of nongenomic effects of BPA, but in other animal models, other organ/cells target and times of exposure. It has been demonstrated that BPA, at very low concentrations, can rapidly (within a few minutes) activate ERK 1/2 phosphorylation in rat pituitary cells (JENG; WATSON, 2011). In addition, BPA (30 minutes – 6 hours of incubation) has also been shown to activate ERK1/2 in rat Sertoli cells (IZUMI et al., 2011). Furthermore, BPA exposure causes rapid activation of ERK 1/2 via both the GPER30 and ESR α/β pathways in the TM4 Sertoli cell and BPA up-regulates mRNA and protein expression of GPER30 (GE et al., 2014). BPA also triggers significant Ca²⁺ oscillations in TM4 Sertoli cells and increases intracellular Ca²⁺ within a few seconds. The ERK1/2 pathway participates in BPA-induced genomic pathways, inducing Sertoli cell damage and apoptosis (QIAN et al., 2014). Additionally, Li et al. (2006) demonstrated that BPA may activate ESR α -mediated nongenomic signaling pathways in MCF-7 cells within minutes via activation of MAPK, PI3K and PKC (LI et al., 2006). In addition, the role of BPA-induced ESR β activation in Ca²⁺ influx has been previously described in human oral mucosa-derived gingival keratinocytes (EHRENMANN et al., 2017). Previously published studies have also demonstrated the ability of BPA to bind to ESR and act as an agonist. *In vitro* BPA incubation efficiently activates both ESR α/β , showing a higher binding affinity to ESR β in MCF-7 cells (MOLINA-MOLINA et al., 2013). The *in vivo* estrogenic activity of BPA has also been demonstrated in the developing larvae brain of zebrafish (LE FOL et al., 2017). Overall, those previously published data are consistent with our findings, which evidence the involvement of ERK 1/2, PI3K, PKC and IP3R in the mechanism of action of BPA-induced Ca²⁺ influx, as well as the participation of ESR α/β . This could indicate a crosstalk between nongenomic and genomic pathways, since both kinases may activate transcription factors and induce gene transcription (DUAN et al., 2002; LEONARD et al., 2015).

Based on our present data, we propose a mechanism of action for BPA, following acute *in vitro* exposure of zebrafish testes to this compound (Figure 19). The disruption of intracellular Ca²⁺ homeostasis is a potent trigger of endoplasmic reticulum stress and apoptosis (GROENENDYK et al., 2010). BPA has already been demonstrated to decrease Sertoli cell viability and induce intracellular Ca²⁺-dependent cell death via apoptosis by disturbing testicular Ca²⁺ homeostasis (HUGHES, 2000). In addition, BPA suppresses TM4 Sertoli cell viability in a dose- and time-dependent

manner and induces mitochondrial apoptotic pathways that are dependent on calmodulin, phosphorylation of Ca^{2+} /calmodulin dependent kinase II and ERK 1/2 activation (QIAN et al., 2014). In agreement with these data, acute *in vitro* exposure of testes to BPA (10 μM) resulted in plasma membrane injury indicated by the release of LDH to the extracellular medium. This cell damage was also mediated by an IP3R-dependent intracellular Ca^{2+} increase, since the IP3R antagonist, 2-APB, abolished the BPA-stimulated effect. Based on our findings, we hypothesized that the integrity of the plasma membrane was compromised. Although the variability between the controls in different samples, the standard on LDH increase by BPA was maintained at an average of 1.75 fold keeping the proportionality. Furthermore, the incubation medium in which the testes were incubated and LDH evaluation are directly related to the size of the testes used, their quantity of proteins and consequently of enzymes. Cytosolic LDH release to extracellular medium is used to indicate the loss of membrane integrity and cell viability, since it is caused by membrane leaking and cell injury (KORZENIEWSKI; CALLEWAERT, 1983; GAUCHER; JARRAYA, 2015).

Additionally, the increase in testicular TAG after acute *in vitro* exposure to 10 μM BPA was also mediated by the IP3R-dependent intracellular Ca^{2+} increase, since the IP3R antagonist abolished the effect of BPA. Phospholipids, glycolipids, TAG and cholesterol are reported to be components of the spermatozoa plasma membrane, which are required for its maturation (LENZI et al., 1996). Changes in lipid metabolism are commonly present in several testicular conditions or diseases, such as cryptorchidism, which displays accumulation of lipids, such as TAG, alkyl-DAG and cholesterol esters in the rat testes (FURLAND et al., 2007). In addition, exposure of Sertoli cells to stressful conditions increases the content of TAG, DAG and phosphatidylinositol, as well as induces accumulation of lipid droplets and mitochondrial uncoupling (VALLÉS et al., 2014). The formation and increase in these lipids may be due to lipid-rich particle production (residual bodies) and apoptotic spermatogenic phagocytosis by Sertoli cells that die during harmful conditions (CHEMES, 1986; VALLÉS et al., 2014). Furthermore, the testis is very sensitive to glycerol accumulation, and high glycerol concentrations may cause leaking of the blood testicular barrier, promoting the apoptosis of the germ cell line (WIEBE et al., 2000). This phenomenon may cause the temporary arrest of spermatogenesis (WIEBE and BARR, 1984), oligospermia, and even azoospermia (WIEBE et al., 2000). Furthermore, the accumulations of TAG and lipid droplets are observed in

Saccharomyces cerevisiae yeast in a stress condition induced by chemicals. The lipid dysregulation induced by the interruption of Ca^{2+} homeostasis, leading to endoplasmic reticulum stress, has been previously reported (GARDARIN, 2010; RAJAKUMAR et al., 2016) and endoplasmic reticulum stress results in phospholipid accumulation (MUTHUKUMAR et al., 2011). Toxic molecules or chemicals can induce endoplasmic reticulum stress by altering the cytosolic Ca^{2+} that comes from internal stores, by modulating endoplasmic reticulum lipid composition and by stimulating ROS production (BIAGIOLI et al., 2008; FU et al., 2011). The findings reported herein are in agreement and indicate an intimate relationship between the disruption of intracellular Ca^{2+} homeostasis, Ca^{2+} leakage from the endoplasmic reticulum, plasma membrane damage, TAG increase and cell injury.

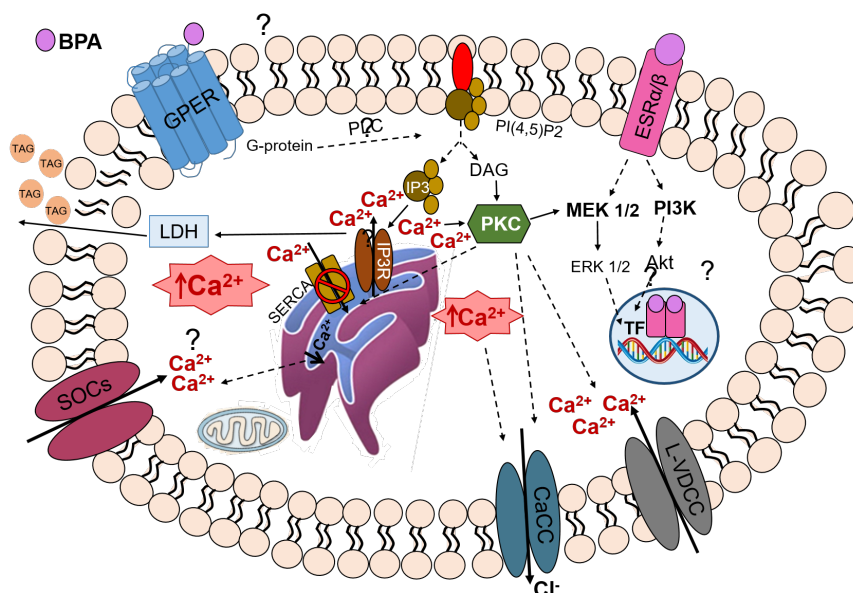


Figure 19: Schematic representation of the effects and mechanism of action of BPA on Ca^{2+} influx in the testis of zebrafish. GPER (?) may have been activated by BPA, which then induced activation of PLC (?) which converts PIP₂ to IP₃ and DAG. IP₃ binds to and activates IP₃R, inducing the release of Ca^{2+} from the endoplasmic reticulum and are involved on Ca^{2+} influx stimulated by BPA, such as L-VGCC, which can be activated by PKC, resulting in the entry of Ca^{2+} from the extracellular medium. Increased cytosolic Ca^{2+} and PKC may activate CaCC, which trigger Cl^- efflux. In addition, ESR α/β , MEK 1/2 and PI3K are also involved in the mechanism of action of BPA, which could indicate a crosstalk between nongenomic initiation effects mediated by PLC/PKC/IP₃R signaling and genomic responses (?). Hence, BPA signaling may modulate ion channels, receptors, and protein kinases, resulting in intracellular Ca^{2+} overload. Furthermore, protein kinases and ESR α/β involved in this signaling may activate transcription factors and induce gene expression. Straight lines are direct pathways; dotted lines represent multi-step pathways; question marks (?) represent suggested effects (hypotheses) based on our study and on literature, which are questions to be further investigated. Source: Own construction.

4.1.3 Conclusions of Part I of Chapter I

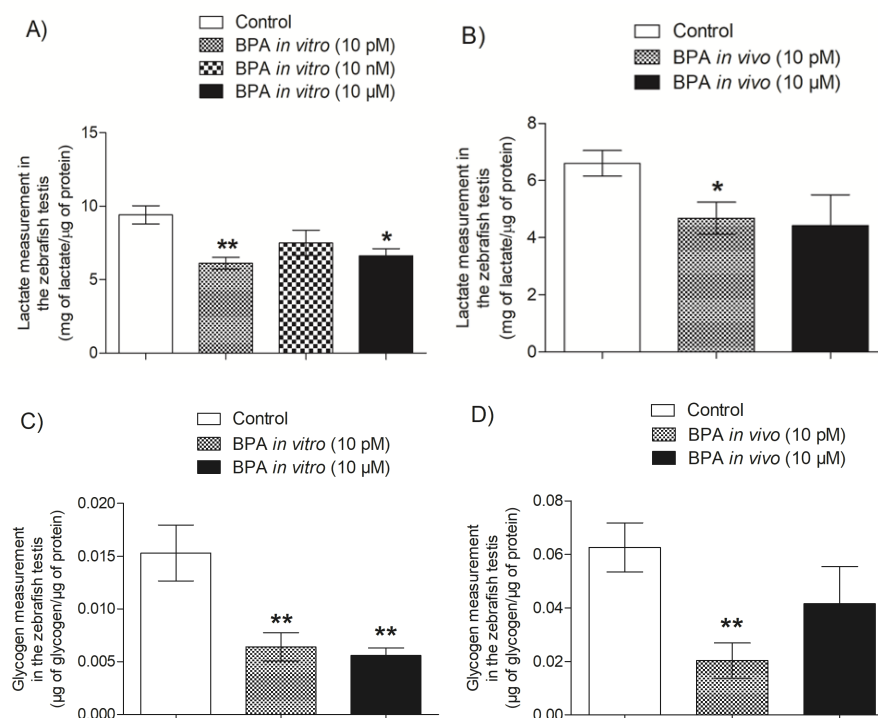
In general, from zebrafish testis incubations *in vitro* to BPA, it was evidenced that BPA:

- Increases $^{45}\text{Ca}^{2+}$ influx involving the nongenomic signaling pathways with the participation of plasma membrane ion channels L-VDCC and CaCC.
- Increases $^{45}\text{Ca}^{2+}$ influx and cytosolic Ca^{2+} overload involving the release of Ca^{2+} from endoplasmic reticulum by IP3R activation and SERCA inhibition.
- Increases $^{45}\text{Ca}^{2+}$ influx involving crosstalk between nongenomic and genomic signaling pathways with the participation of ESR, MEK, PI3K and PKC.
- Induces Ca^{2+} -dependent damage to the plasma membrane by increasing extracellular LDH activity and TAG content involving the release of Ca^{2+} from endoplasmic reticulum

4.1.4 Results of Part II of Chapter I: Study of the *in vitro* and *in vivo* effects of BPA on testicular energy metabolism and spermatogenesis in zebrafish

4.1.4.1 *In vivo* and *in vitro* BPA disturbs testicular energy metabolism by modulating enzymatic activities and reducing energy substrates

To study BPA effects on the main testicular energy substrates, lactate and glycogen contents and ^{14}C -deoxy-D-glucose uptake were analyzed. From *in vitro* incubations with 10 pM, 10 nM and 10 μM BPA, it was found that the lowest and highest concentration of BPA decreased testicular lactate (Figure 20A). Therefore, based on this result, the concentrations chosen for the other tests were 10 pM and 10 μM BPA. As can be seen in Figure 20C, 10 pM and 10 μM BPA also reduced glycogen content compared to the control group. However, ^{14}C -deoxy-D-glucose uptake did not change in the presence of *in vitro* BPA (Figure 20E). Surprisingly, the *in vivo* treatment with the lowest BPA concentration decreased lactate and glycogen content in the testis of zebrafish in relation to the control group (Figures 20B and 20D).



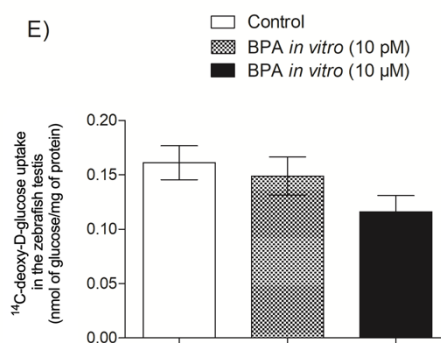
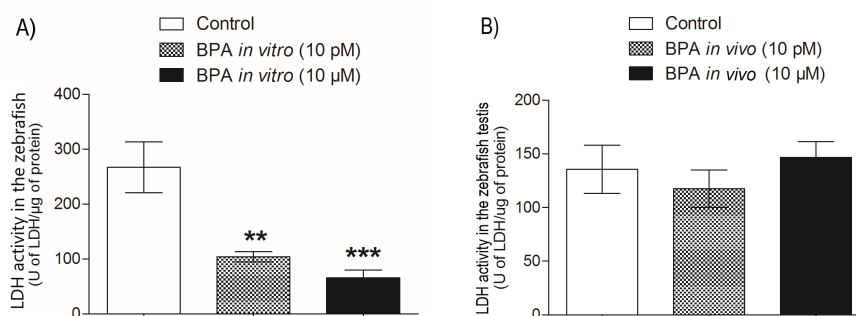


Figure 20: Effects of BPA on lactate content, glycogen content and ¹⁴C-deoxy-D-glucose uptake in the testis of zebrafish. (A and B) Lactate content, (C and D) glycogen content and (E) ¹⁴C-deoxy-D-glucose uptake were evaluated in testes incubated (*in vitro*) for 1 hour and in testes of zebrafish exposed (*in vivo*) for 12 hours to 10 pM and 10 μM BPA. The data are expressed as mean as mean ± S.E.M. of 4-6 fish in each group from two independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to control group.

LDH, ALT and AST activities were analyzed following BPA treatment to determine effects on enzymes involved in testicular energy metabolism. LDH activity was decreased after *in vitro* exposure of testes to both concentrations of BPA (Figure 21A), whereas *in vivo* exposure to BPA did not change its activity (Figure 21B). The lowest *in vitro* concentration of BPA (Figure 21C) and its highest *in vivo* concentration (Figure 21D) significantly decreased testicular ALT activity in relation to the control groups. In addition, *in vitro* incubation with both concentrations of BPA caused an increase in testicular AST activity (Figure 21E). On the other hand, *in vivo* exposure with both concentrations of BPA reduced testicular AST activity compared to the control group (Figure 21F).



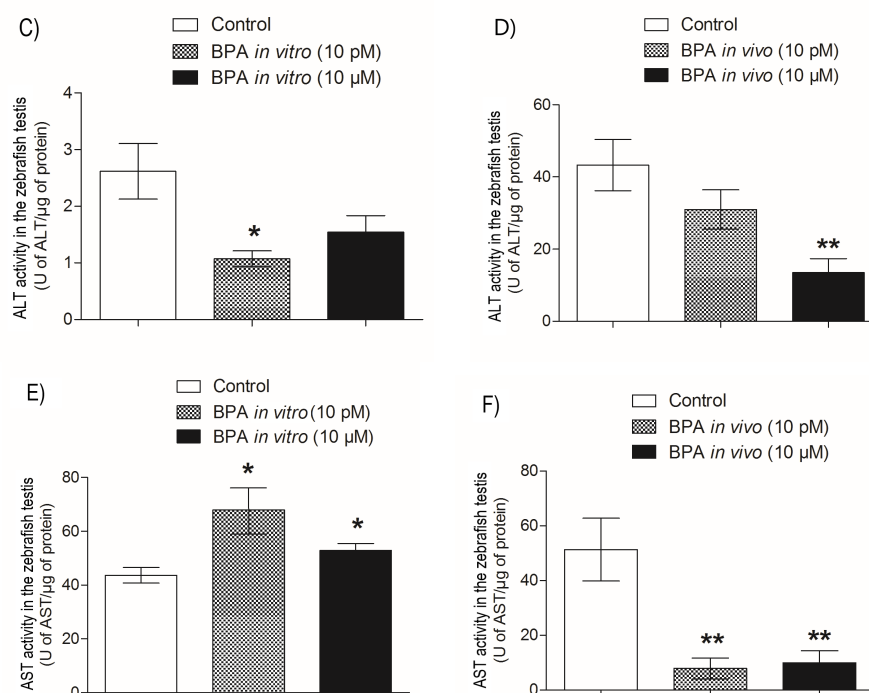


Figure 21: Effects of BPA on LDH, ALT and AST activities in the testis of zebrafish. (A and B) LDH; (C and D) ALT and (E and F) AST activities were evaluated in testes incubated (*in vitro*) for 1 hour and in testes of zebrafish exposed (*in vivo*) for 12 hours to 10 pM and 10 μM BPA. The data are expressed as mean ± S.E.M. of 4-6 fish in each group from two independent experiments. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001 when compared to control group.

4.1.4.2 *In vivo* BPA impairs zebrafish spermatogenesis by decreasing the proportion of the haploid cells

The impact of BPA on zebrafish spermatogenesis was determined by measuring the proportion of the surface of spermatocytes and haploid cells (spermatids and spermatozoa) in relation to the total surface of the testes after *in vivo* exposure to 10 pM or 10 μM BPA for 12 hours. Overlay images illustrate the image analysis of the proportion of spermatocytes or haploid cells superimposed on the testicular histological section image obtained following control (Figure 22A), 10 pM BPA (Figure 22B) and 10 μM BPA (Figure 22C) exposures, respectively. Additionally, Figures 22D and 22E show in a quantitative way the proportion of these cells in relation to the total testicular surface. Figure 22A shows the profile of distribution and proportion of the testicular cells in the control group by highlighting a normal distribution of spermatids and spermatozoa (in green) and spermatocytes (in red). Figures 22B and 22C show that there was no change in the proportion of the surface of spermatocytes (in red) in relation to the total testicular surface compared to the control group (Figure 22A) after *in vivo* exposures to BPA, which was confirmed by the quantitative analysis

(Figure 22D). However, Figures 22B, 22C and 22E highlight a reduction in the proportion of the surface of spermatids and spermatozoa (in green) in relation to the total testicular surface caused by *in vivo* exposures to 10 pM and 10 µM BPA.

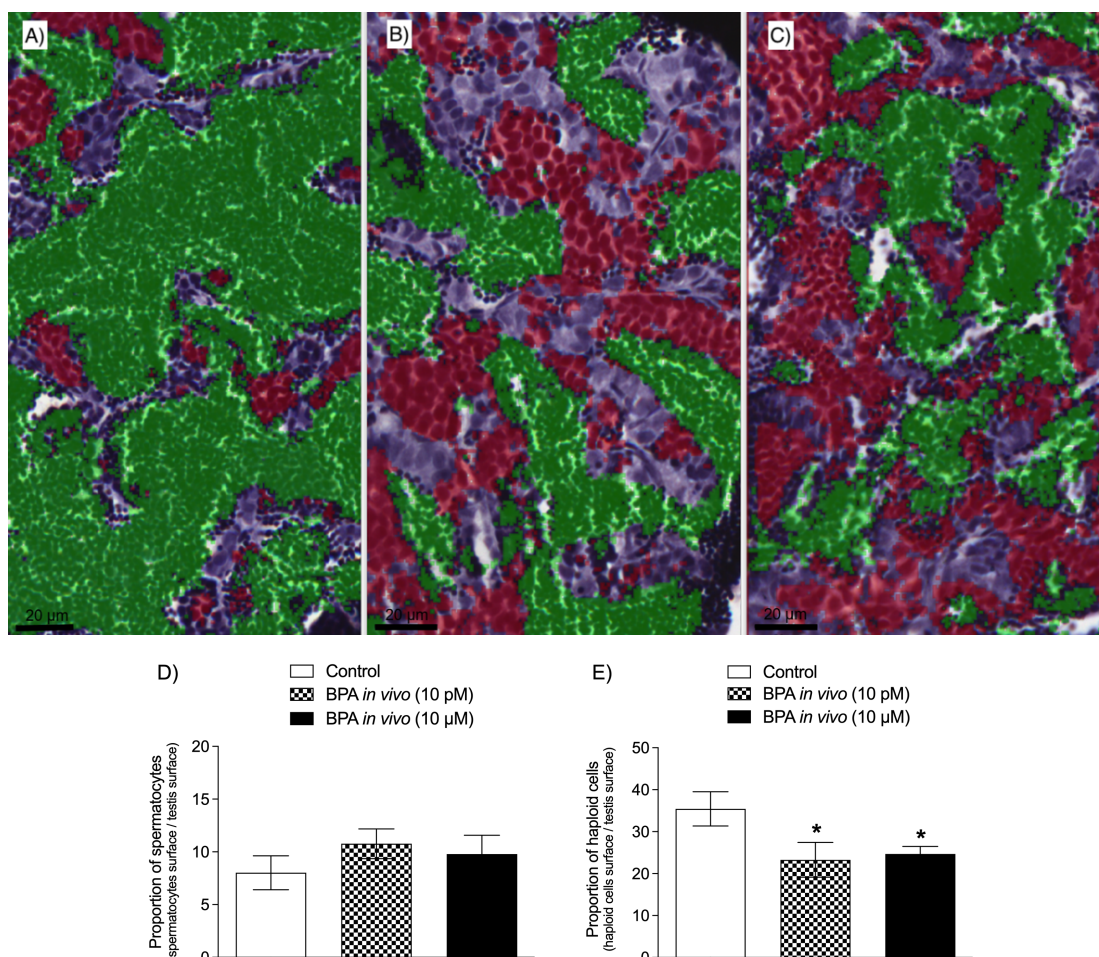


Figure 22: Effects of BPA on the proportion of spermatocytes, spermatids and spermatozoa in the zebrafish testis. The surface of the proportion of spermatocytes (in red), spermatids and spermatozoa (in green) were analyzed in testicular histological sections of zebrafish treated *in vivo* for 12 hours with (A) vehicle (control), (B) 10 pM or (C) 10 µM BPA. (A – C) Overlay images of the image analysis of the proportion of spermatocytes (in red), spermatids and spermatozoa (in green) superimposed on the testicular histological section image. Representation of the quantitative analysis of the proportion of (D) spermatocytes and (E) haploid cells (spermatids and spermatozoa) in relation to the total testicular surface. The data are expressed as mean \pm S.E.M. of 4-5 fish in each group from two independent experiments. * $p \leq 0.05$ when compared to control group.

4.1.4.3 *In vivo* BPA impairs spermatogenesis by increasing the spermatocytes apoptosis

Figures 23A (control), 23B (10 pM BPA) and 23C (10 BPA µM) show the detection of apoptosis by TUNEL immunostaining in testicular histological sections. As can be seen in Figures 23B and 23C, brown-stained cells (positive TUNEL cells) counted as apoptotic cells, were more prevalent in the spermatocytes after exposure

to 10 pM and 10 μ M BPA, respectively. Quantification of spermatocytes in apoptosis (Figure 23D) show a higher level in response to both concentrations of BPA tested compared to control group.

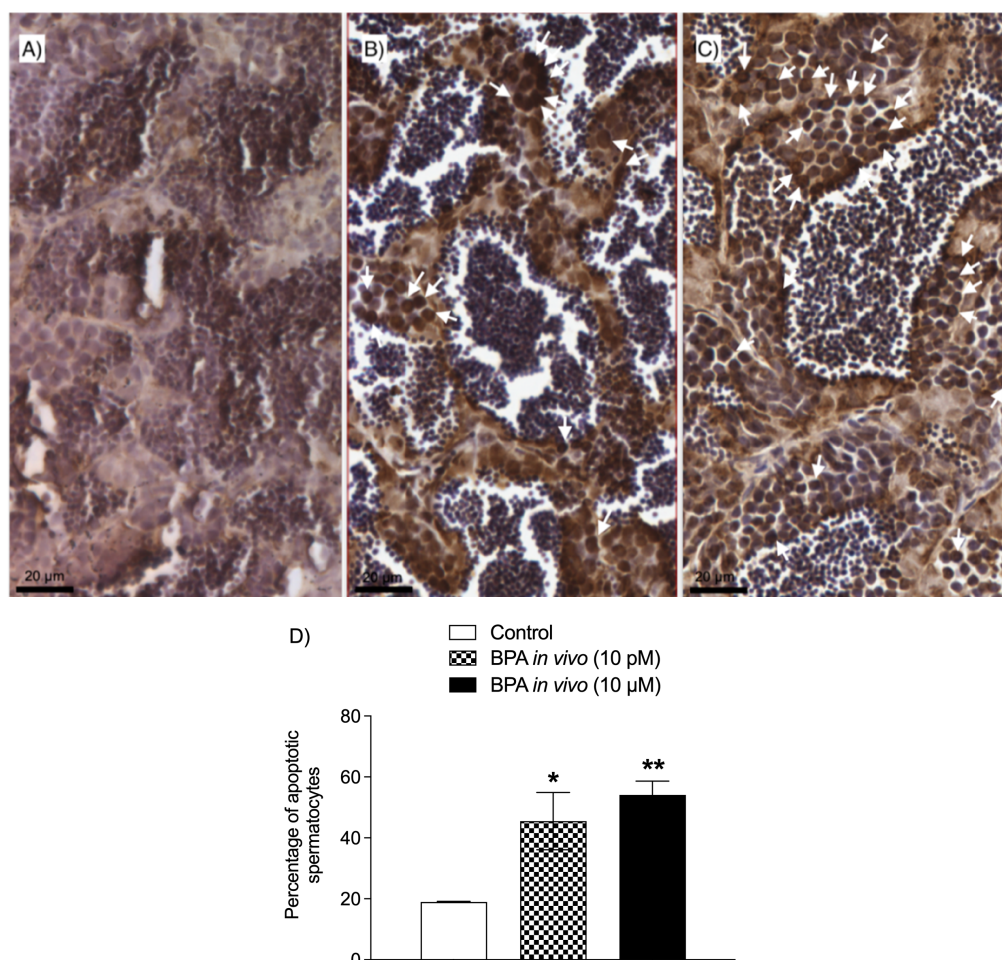


Figure 23: Effects of BPA on apoptosis in spermatocytes. Apoptosis by TUNEL immunostaining was evaluated in testicular histological sections of zebrafish exposed *in vivo* for 12 hours to (A) vehicle (control), (B) 10 pM or (C) 10 μ M BPA. (D) Quantitative analysis of the percentage of apoptotic spermatocytes. The results are expressed as mean \pm S.E.M. of 3-4 fish in each group from two independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to control group.

4.1.4.4 *In vitro* BPA disturbs testicular energy metabolism by modulating the expression of genes involved in carbohydrate and amino acid metabolism

In order to study the genomic effects of BPA on the testicular energy metabolism, the relative expression of genes encoding key proteins on carbohydrate and amino acid metabolism was analyzed in the testis of zebrafish after 6 and 72 hours of incubations with BPA. Incubations for 6 hours allow to assess the short-term effects on gene expression. However, incubations for 72 hours, in addition to allowing the

evaluation of the longer-term effects on gene expression, also allow the evaluation and correlation of the effects on the immunocontent of proteins and histological parameters. Thus, the relative expression of glycogen phosphorylase liver form (PYGL), 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2a (PFKFB2a), pyruvate kinase M1/2a (PKMA), lactate dehydrogenase Ba (LDHBA), alanine aminotransferase (GPT2), pyruvate carboxylase A (PCXA) and monocarboxylate transporter 4 (MCT4) was analyzed. As can be seen in Figure 24A, the relative expression of LDHBA, GPT2 and PCXA was increased in the testes incubated with 10 pM BPA for 6 hours, whereas the relative expression of PYGL, PFKFB2a, PKMA and MCT4 was not changed in relation to the control groups. On the other hand, PKMA relative expression was reduced in the testis after 72 hours of incubation with 10 pM BPA in relation to the control group, whereas the relative expression of no other genes was significantly changed (Figure 24B).

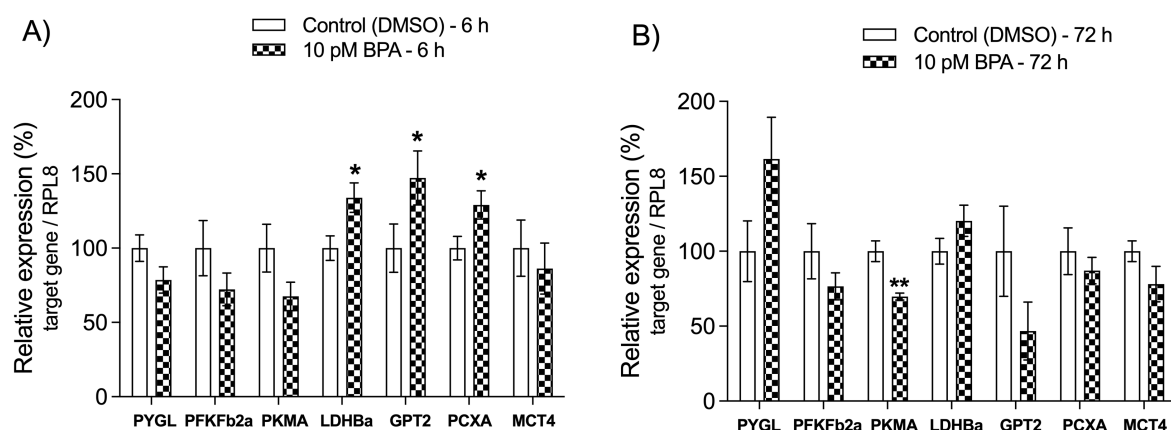


Figure 24: Effects of 10 pM BPA on the relative expression of energy metabolism-associated genes in the zebrafish testis. The relative expression of PYGL, PFKFB2a, PKMA, LDHBA, GPT2, PCXA and MCT4 was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA for (A) 6 or (B) 72 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to respective control group.

In addition to BPA at 10 pM, the relative expression of genes was also analyzed in zebrafish testes after 6 and 72 hours of incubations with 10 μ M BPA (Figures 25A and 25B). Figure 25A shows that there was no change in gene expression after 6 hours of incubation with the highest concentration, 10 μ M BPA, but only a tendency to increase GPT2 expression in relation to control group ($p = 0.057$). On the other hand, Figure 25B shows that the relative expression of PFKFB2a, LDHBA and GPT2 was decreased in zebrafish testes after 72 hours of incubation with 10 μ M

BPA, whereas the relative expression of PYGL and MCT4 genes was increased compared to the control groups. However, there was no change in the relative expression of PKMA and PCXA genes in the testes after 72 hours of incubation with 10 μ M BPA (Figure 25B).

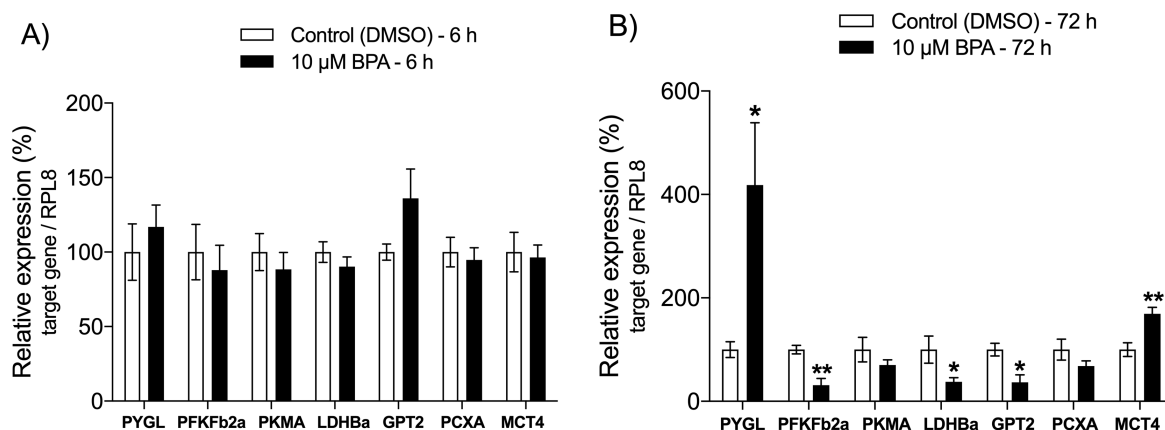


Figure 25: Effects of 10 μ M BPA on the relative expression of energy metabolism-associated genes in the zebrafish testis. The relative expression of PYGL, PFKFb2a, PKMA, LDHBa, GPT2, PCXA and MCT4 was analyzed after *in vitro* exposure of zebrafish testes to 10 μ M BPA for (A) 6 and (B) 72 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 4-6 fish in each group from three independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to respective control group.

4.1.4.5 Nuclear estrogen receptors (ESR α/β) are involved in the mechanism of action of 10 μ M BPA in modulating the relative expression of PYGL, PFKFb2a and LDHBa

The involvement of the ESR in the mechanism of action of 10 μ M BPA in modulating PKMA expression was evaluated. Figure 26 shows that the decrease in the relative expression of PKMA gene was not reversed by pre-incubation with ICI 182,780. Moreover, the ICI 182,780 group *per se* also significantly reduced PKMA relative expression, similarly to the effect shown by the BPA group alone. However, these effects are not observed when testes were pre-incubated with ICI 182,780 prior to incubation with 10 μ M BPA (Figure 26).

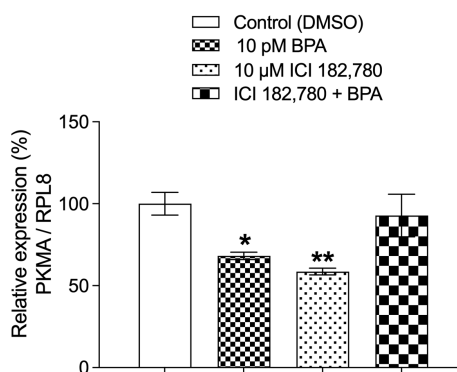


Figure 26: Analysis of the involvement of ESR α/β in the mechanism of action of 10 pM BPA in modulating PKMA relative expression in the zebrafish testis. The relative expression of PKMA was analyzed in the testes after pre-incubations in the absence (control) or presence of 10 μ M ICI 182,780 (ESR α/β antagonist) for 2 hours and incubations in the absence or presence of 10 pM BPA for 72 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of PKMA normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 4-6 fish in each group from three independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to control group.

In addition, the involvement of ESR α/β in the mechanism of action of 10 μ M BPA in modulating the expression of testicular energy metabolism-associated genes was also investigated. As can be seen in Figure 27A, the increase in the relative expression of PYGL by 10 μ M BPA was completely abolished by pre-incubation with ICI 182,780, indicating that the increase in PYGL expression by BPA is via ESR. Furthermore, the decreased relative expression of PFKFb2a (Figure 27B) and LDHBa (Figure 27C) by 10 μ M BPA was also completely abolished by pre-incubation with ICI 182,780, indicating that these BPA-mediated genomic effects are also ESR-dependent.

On the other hand, the decreased relative expression of GPT2 (Figure 27D) and the increased relative expression of MCT4 (Figure 27E) by 10 μ M BPA were not reversed by pre-incubation with ICI 182,780, indicating that ERs are not involved in these effects of BPA. However, the ICI 182,780 *per se* increased the expression of GPT2 in relation to the control and BPA groups.

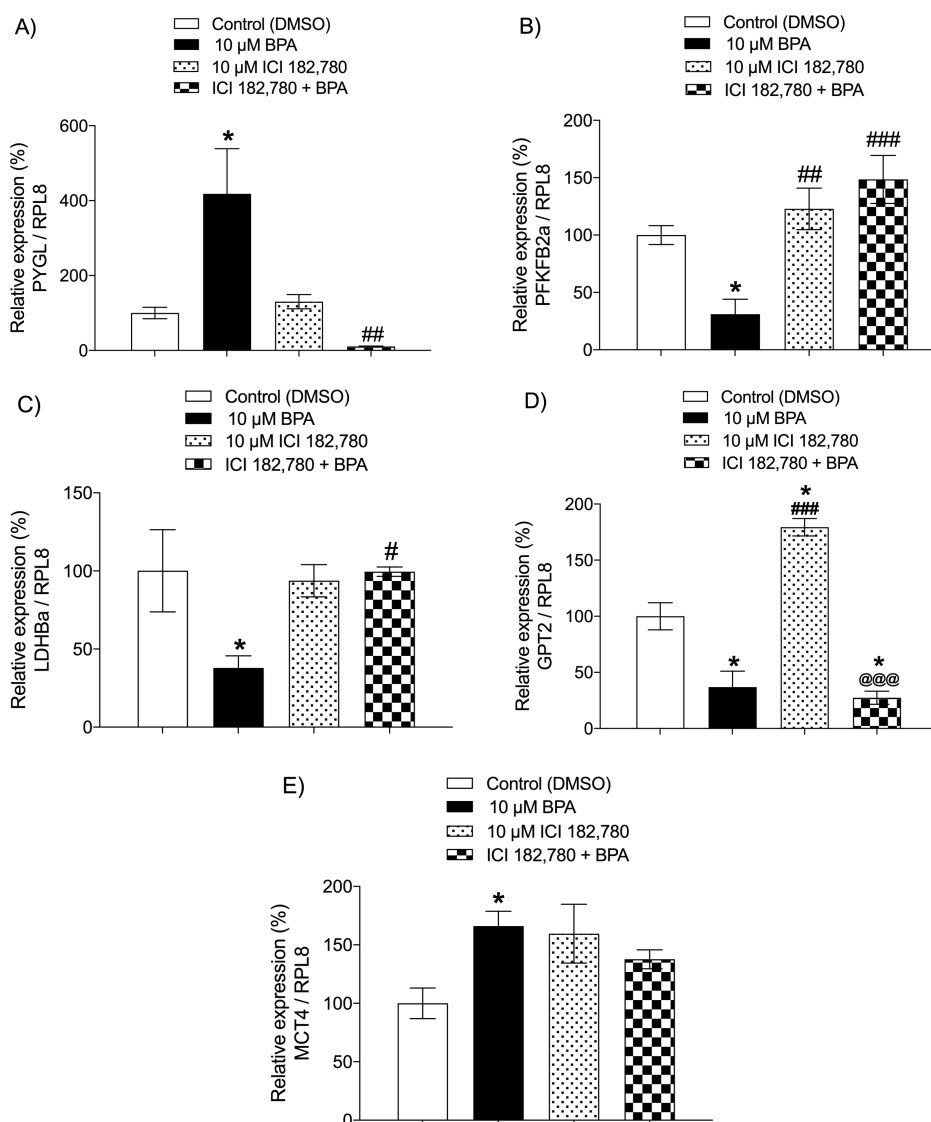


Figure 27: Involvement of ESR α/β in the mechanism of action of 10 μ M BPA on the relative expression of testicular energy metabolism-associated genes in zebrafish. The relative expression of (A) PYGL, (B) PFKFB2a, (C) LDHBa, (D) GPT2 and (E) MCT4 was analyzed in the testes after pre-incubations in the absence (control) or presence of 10 μ M ICI 182,780 (ESR α/β antagonist) for 2 hours and incubations in the absence or presence of 10 μ M BPA for 72 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 4-6 fish in each group from three independent experiments. * $p \leq 0.05$ when compared to control group; # $p \leq 0.05$, ## $p \leq 0.01$ and ### $p \leq 0.001$ when compared to BPA group; @@@ $p \leq 0.001$ when compared to ICI 182,780 group.

4.1.4.6 Immunocontent of pyruvate kinase M1/2 (PKM) protein is decreased in zebrafish testes exposed *in vitro* to the highest BPA concentration

Western blot analysis revealed a reduction in the immunocontent of PKM in the testes incubated with 10 μ M BPA for 72 hours in relation to control group, whereas it was no change by 10 pM BPA (Figure 28).

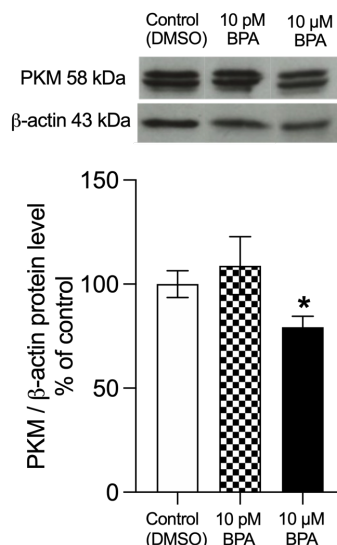


Figure 28: Effects of BPA on the immunocontent of pyruvate kinase M1/2 (PKM). PKM immunocontent was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA and 10 μM BPA for 72 hours. The results are expressed as % of control of PKM immunocontent normalized by β-actin reference protein immunocontent. The data are expressed as mean ± S.E.M. of 4 fish in each group from two independent experiments. * $p \leq 0.05$ when compared to control group.

4.1.4.7 Immunolocalization and positive LDH cells density: *In vitro* exposure to 10 μM BPA results in increased number of testicular cells expressing LDH in the zebrafish

Immunohistochemical and quantitative analyzes revealed that there was an increase in the number of testicular cells expressing LDH (density of positive LDH cells) in histological sections of testes incubated with 10 μM BPA for 72 hours (Figures 29C and 29D). However, there was no change in the number of positive LDH testicular cells after incubation with 10 pM BPA when compared to control group (Figures 29B and 29D). The immunolocalization of LDH in different types of testicular cells has not been previously established in fish. Hence, as a first approach, LDH protein was identified in the zebrafish testis by immunohistochemical analysis after *in vitro* treatments through organotypic culture. Therefore, in view of the experimental conditions of the present study, LDH in the zebrafish testis seems to be more abundant in mature germ cells, mainly in spermatids (black arrows). Furthermore, LDH protein was not identified in early germ cells, spermatogonia.

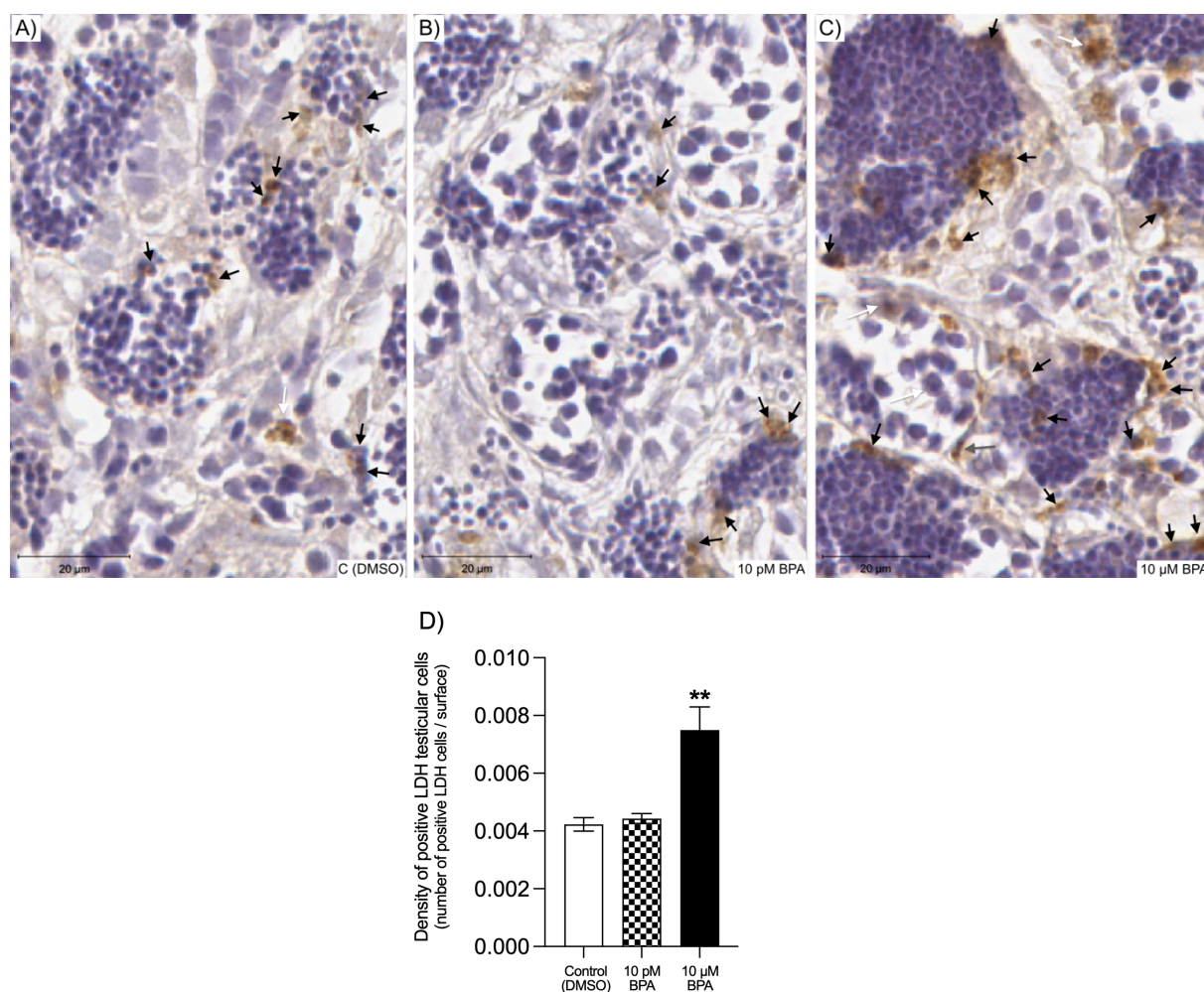


Figure 29: Immunolocalization of LDH and effects of BPA on density of LDH-expressing cells in testicular histological sections of zebrafish. The number of cells expressing LDH was analyzed in histological sections of testes incubated with (A) DMSO (C = Control), (B) 10 pM BPA and (C) 10 μM BPA for 72 hours. (D) Quantitative analysis of the density of positive LDH testicular cells (number of positive LDH cells / testicular surface). The results are expressed as mean \pm S.E.M. of 3-4 fish in each group from two independent experiments. ** $p \leq 0.01$ when compared to control group. (A – C) Black arrows represent positive LDH spermatids; White arrows represent positive LDH spermatocytes, and gray arrow represents positive LDH Sertoli cell.

4.1.4.8 The increase in the number of testicular cells expressing LDH after incubation with 10 μM BPA is dependent on nuclear estrogen receptors (ESR α/β)

After detecting the involvement of the highest concentration of BPA in the increase of positive LDH testicular cells, the involvement of ESR α/β in the mechanism of action of BPA was investigated. Thus, the increase in the number of positive LDH cells observed in histological sections of testes incubated with 10 μM BPA (Figures 30B and 30E) was completely abolished by pre-incubation with ICI 182,780 (Figures 30D and 30E), indicating that this effect of BPA is dependent on ESR α/β .

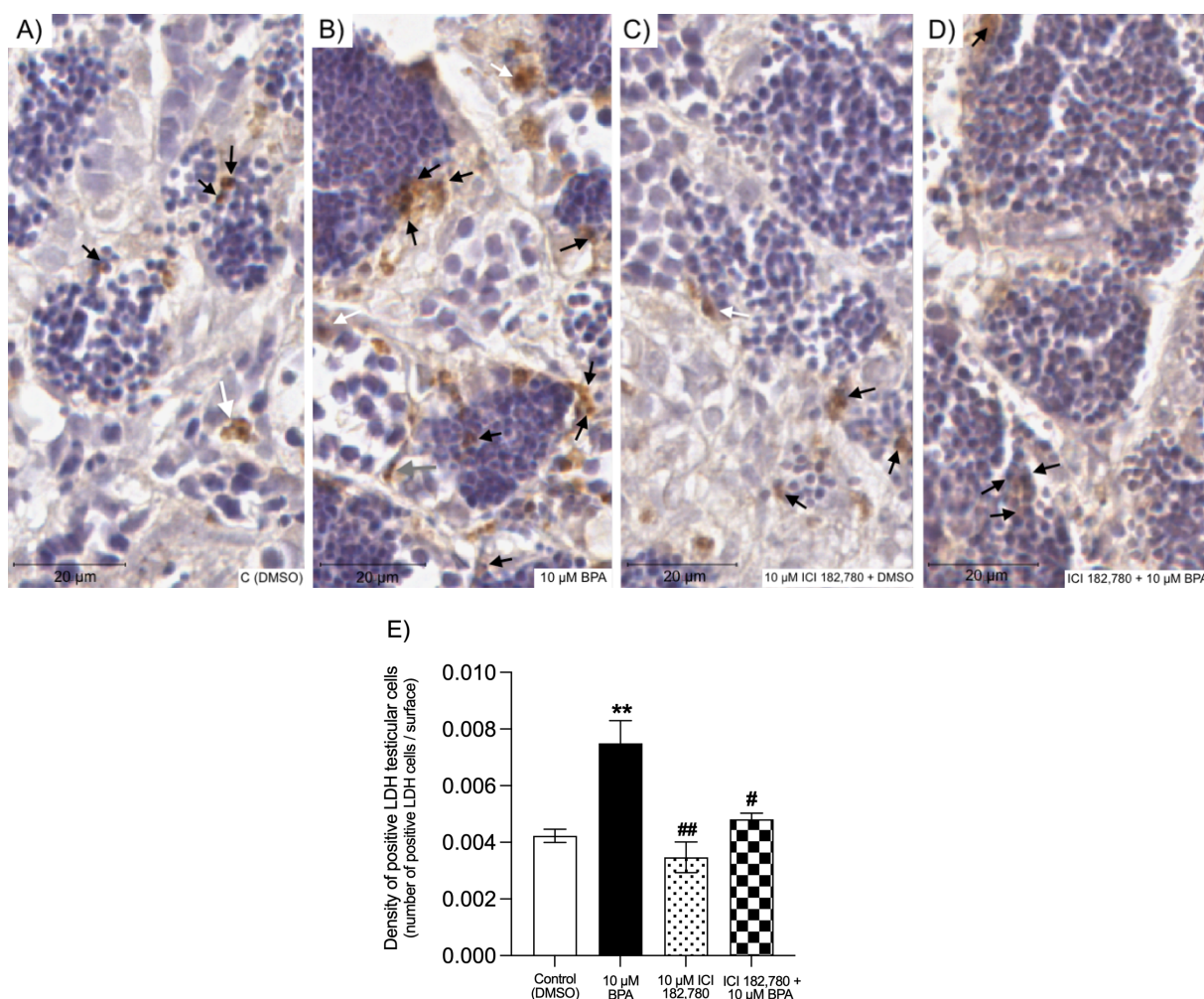


Figure 30: Involvement of ESR α/β in the increase in density of LDH-expressing cells mediated by BPA in testicular histological sections of zebrafish. Immunohistochemical expression of LDH in histological sections of testis was performed after pre-incubations in the absence (control) or presence of (C and D) 10 μ M ICI 182,780 (ESR α/β antagonist) for 2 hours and incubations with (A and C) DMSO or (B and D) BPA 10 μ M for 72 hours. (E) Quantitative analysis of the density of positive LDH testicular cells (number of LDH positive cells / testicular surface). The results are expressed as mean \pm S.E.M. of 3-4 fish in each group from two independent experiments. ** $p \leq 0.01$ when compared to control group; # $p \leq 0.05$ and ## $p \leq 0.01$ when compared to BPA group. (A – D) Black arrows represent positive LDH spermatids; White arrows represent positive LDH spermatocytes, and gray arrow represents positive LDH Sertoli cell.

4.1.4.9 *In vitro* BPA modulates gene expression of nuclear estrogen receptors and estrogen-related receptors in zebrafish testis

After detecting the involvement of ESR in the mechanism of action of some effects of BPA and since it is recognized that BPA may bind to nuclear receptors other than classical ESR (TOHME et al., 2014), the gene expression of ESR and ESRR was investigated. Thus, the relative expression of estrogen receptor $\beta 2$ (ESR2a), estrogen receptor $\beta 1$ (ESR2b), estrogen-related receptor α (ESRR α) and estrogen-related receptor δ (ESRR δ) genes was analyzed after 6 and 72 hours of incubations with BPA.

As can be seen in Figure 31A, the ESR2b relative gene expression was increased in zebrafish testes incubated with 10 pM BPA for 6 hours, whereas the gene expression of other receptors was not significantly changed compared to the control group. However, the relative gene expression of none of the investigated receptors was significantly altered after 72 hours of incubation with 10 pM BPA (Figure 31B).

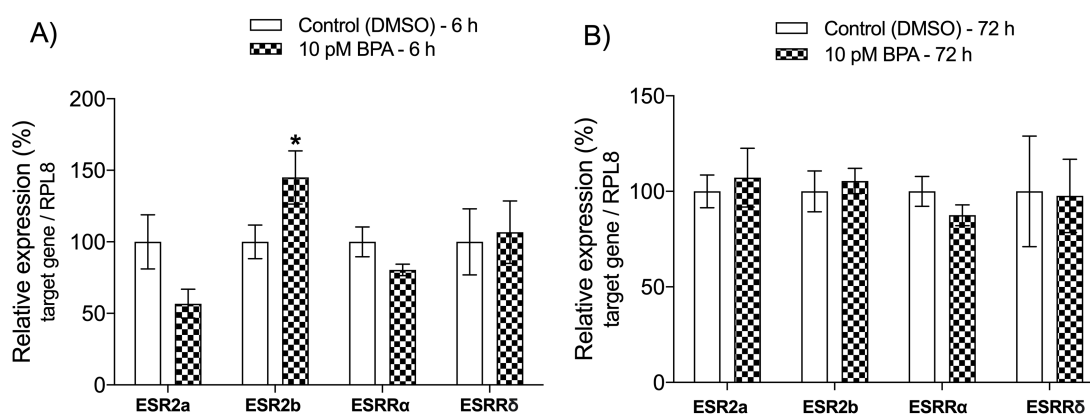


Figure 31: Effects of 10 pM BPA on the gene expression of nuclear estrogen receptors and estrogen-related receptors in the zebrafish testis. The relative expression of ESR2a, ESR2b, ESRRα and ESRRδ was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA for (A) 6 and (B) 72 hours. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$ when compared to respective control group.

Furthermore, as can be seen in Figure 32A, the expression of none of the investigated receptors was significantly altered in zebrafish testes incubated with 10 μ M BPA for 6 hours in relation to the control groups. However, surprisingly, the relative expression of the ESR2a and ESR2b genes was significantly upregulated after 72 hours of incubation with 10 μ M BPA (Figure 32B). Furthermore, the relative expression of the ESRRα gene was downregulated after 72 hours of incubation with 10 μ M BPA (Figure 32B).

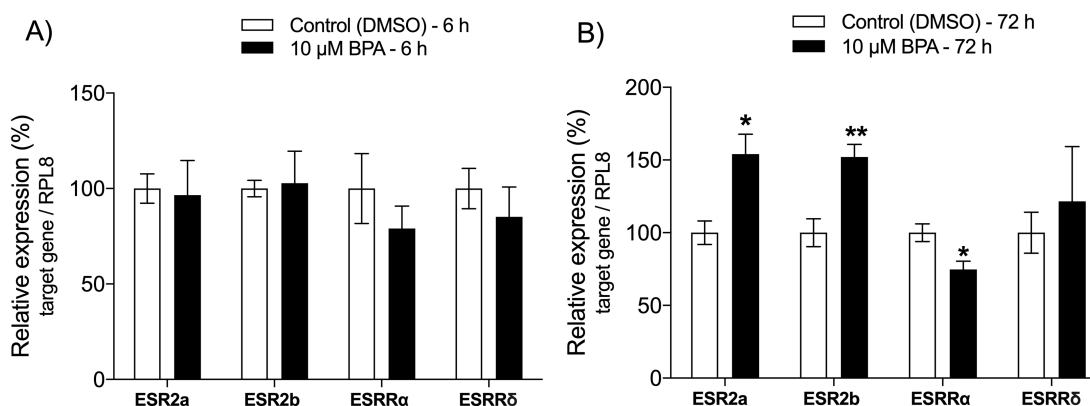


Figure 32: Effects of 10 μ M BPA on the gene expression of nuclear estrogen receptors and estrogen-related receptors in the zebrafish testis. The relative expression of ESR2a, ESR2b, ESR α and ESR δ was analyzed after *in vitro* exposure of zebrafish testes to 10 μ M BPA for (A) 6 and (B) 72 hours. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to respective control group.

4.1.4.10 *In vitro* BPA modulates the expression of PIWIL-1, SYCP-3 and ODF3b, respective marker genes of spermatogonia, spermatocytes and spermatids

In order to correlate changes in testicular energy metabolism caused by BPA with possible changes in spermatogenesis, the expression of specific genes of germ cells was analyzed. The expression of P-element-induced wimpy-like protein 1 (PIWIL-1), synaptonemal complex protein 3 (SYCP-3) and outer dense fiber protein 3b (ODF3b) genes, respective markers of spermatogonia, spermatocytes and spermatids were analyzed after 6 and 72 hours of incubations with BPA. As can be seen in Figure 33A, the relative PIWIL-1 and SYCP-3 genes expression was increased in zebrafish testes incubated with 10 pM BPA for 6 hours, whereas the expression of ODF3b was not changed. However, the relative ODF3b gene expression was significantly reduced after 72 hours of incubation with 10 pM BPA (Figure 33B).

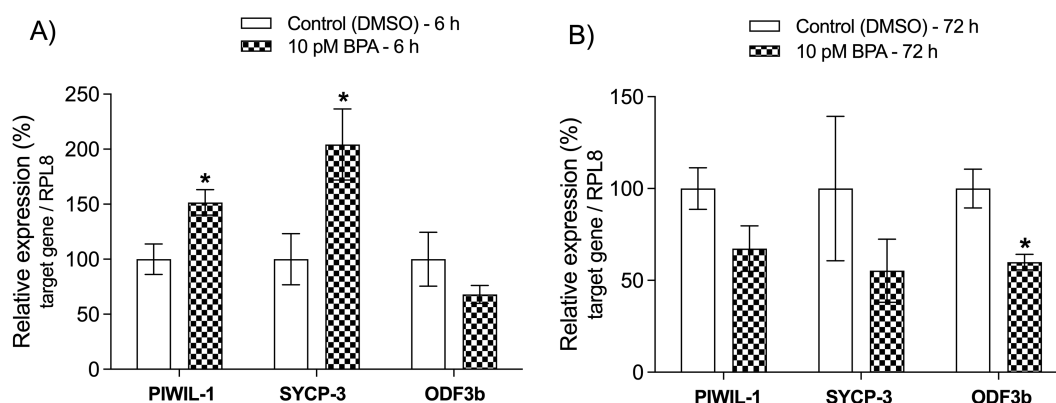


Figure 33: Effects of 10 pM BPA on the expression of specific marker genes of testicular cells in zebrafish. PIWIL-1, SYCP-3 and ODF3b are respective markers of spermatogonia, spermatocytes and spermatids. The relative expression of PIWIL-1, SYCP-3 and ODF3b genes was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA for (A) 6 and (B) 72 hours. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$ when compared to respective control group.

On the other hand, as can be seen in Figure 34A, the relative expression of PIWIL-1, SYCP-3 and ODF3b genes was not changed in the zebrafish testes incubated with 10 μ M BPA for 6 hours. However, the relative SYCP-3 gene expression,

a spermatocyte marker gene, was reduced after 72 hours of incubation with 10 μ M BPA, whereas the relative expression of PIWIL-1 and ODF3b was not changed (Figure 34B).

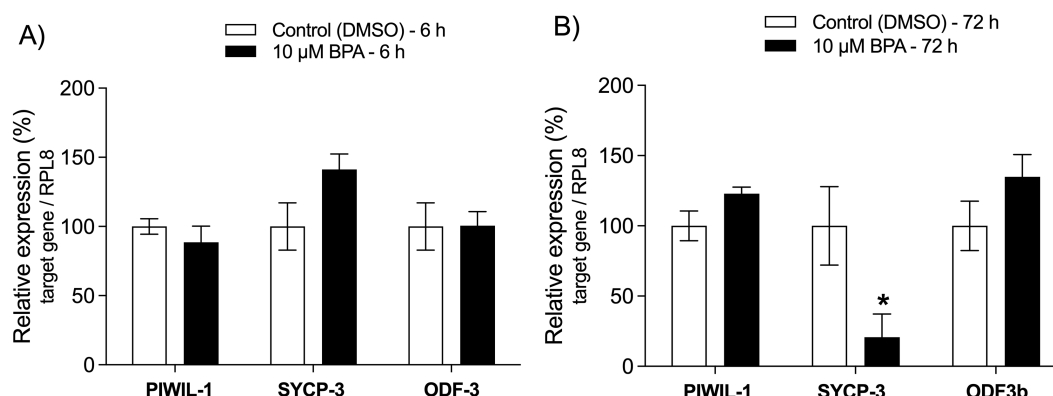


Figure 34: Effects of 10 μ M BPA on the expression of specific marker genes of testicular cells in zebrafish. PIWIL-1, SYCP-3 and ODF3b are respective markers of spermatogonia, spermatocytes and spermatids. The relative expression of PIWIL-1, SYCP-3 and ODF3b genes was analyzed after *in vitro* exposure of zebrafish testes to 10 μ M BPA for (A) 6 and (B) 72 hours. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 5-6 fish in each group from three independent experiments. * $p \leq 0.05$ when compared to respective control group.

4.1.4.11 The decreased expression of ODF3b (spermatid marker gene) mediated by the lowest concentration of BPA is dependent on nuclear estrogen receptors activation

The involvement of ESR α/β in the mechanism of action of 10 μ M BPA in modulating the expression of spermatid marker ODF3b gene was also investigated. The decreased relative expression of ODF3b by the lowest concentration of BPA was completely abolished by pre-incubation with ICI 182,780, indicating that this BPA-mediated genomic effect is dependent on ESR α/β (Figure 35A).

Furthermore, the involvement of ESR α/β in the mechanism of action of 10 μ M BPA in modulating the expression of spermatocyte marker SYCP-3 gene was also evaluated. However, the decreased relative expression of SYCP-3 was not reversed by pre-incubation with ICI 182,780. Thus, when testes were pre-incubated with ICI 182,780 prior to incubation with 10 μ M BPA, the expression SYPC-3 levels were the same when compared to BPA group alone (Figure 35B).

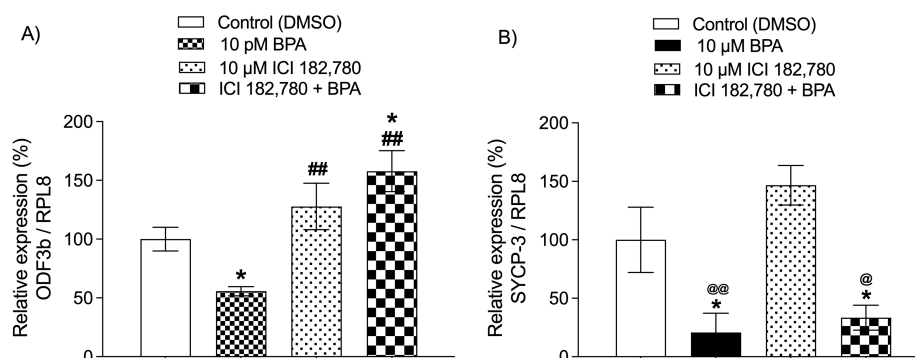


Figure 35: Involvement of ESR α/β in the mechanism of action of BPA in modulating the expression of ODF3b and SYCP-3 in the zebrafish testis. The relative expression of (A) ODF3b and (B) SYCP-3 was analyzed in the testes after pre-incubations in the absence (control) or presence of 10 μ M ICI 182,780 (ESR α/β antagonist) for 2 hours and incubations in the absence or presence of (A) 10 μ M or (B) 10 μ M BPA for 72 hours. The results are expressed as relative expression (%) of target genes normalized by RPL8 reference gene. The data are expressed as mean \pm S.E.M. of 3-5 fish in each group from two independent experiments. * $p \leq 0.05$ when compared to control group; ## $p \leq 0.01$ when compared to BPA group; @ $p \leq 0.05$ and @@ $p \leq 0.01$ when compared to ICI 182,780 group.

4.1.4.12 *In vitro* exposure to BPA affects cell proliferation by decreasing PCNA immunocontent and activating ERK 1/2

PCNA immunocontent was analyzed after *in vitro* exposure to 10 μ M and 10 μ M BPA for 72 hours in order to investigate whether BPA also affects cell proliferation, which is linked to spermatogonial proliferation in the testis. PCNA immunocontent was reduced in the testes of zebrafish incubated with the highest BPA concentration for 72 hours, whereas its expression was not altered by the lowest BPA concentration in relation to the control group (Figure 36).

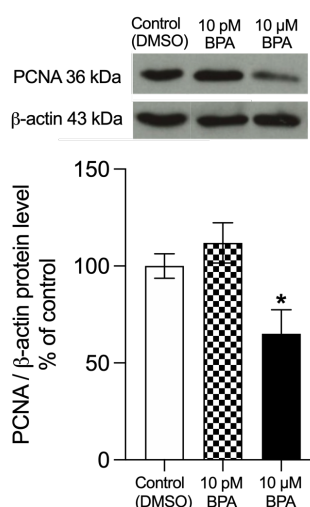


Figure 36: Effects of BPA on the immunocontent of proliferating cell nuclear antigen (PCNA). PCNA immunocontent was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA and 10 μ M BPA for 72 hours. The results are expressed as % of control of PCNA immunocontent normalized by β -actin

reference protein immunocontent. The data are expressed as mean \pm S.E.M. of 4 fish in each group from two independent experiments. * $p \leq 0.05$ when compared to control group.

After detecting the influence of BPA on the immunocontent of PCNA, the involvement of BPA in one of the signaling pathways of cell proliferation was investigated by analyzing the immunocontent of t-ERK 1/2 and phosphorylation of ERK 1/2. Western blot analysis revealed an increase in ERK 1/2 phosphorylation (p-ERK / t-ERK) in the zebrafish testes by the 10 pM and 10 μ M BPA after 72 hours of *in vitro* exposure (Figure 37).

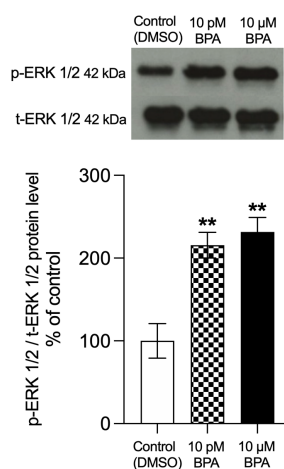


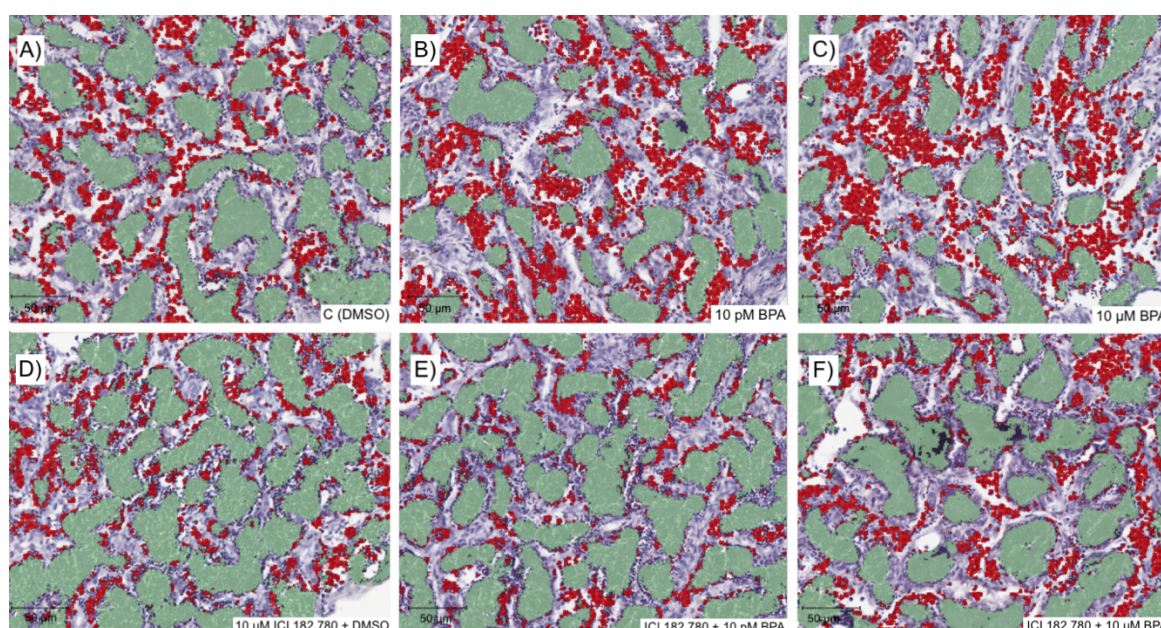
Figure 37: Effects of BPA on the ERK 1/2 phosphorylation. The immunocontent of p-ERK 1/2 and t-ERK 1/2 was analyzed after *in vitro* exposure of zebrafish testes to 10 pM BPA and 10 μ M BPA for 72 hours. The data (p-ERK 1/2 / t-ERK 1/2) are expressed as mean \pm S.E.M. of 4 fish in each group from two independent experiments. ** $p \leq 0.01$ when compared to control group.

4.1.4.13 Impaired spermatogenesis by decreasing the proportion of haploid cells in testes mediated by the lowest concentration of BPA (*in vitro*) is dependent on nuclear estrogen receptors activation

The impact of BPA on zebrafish spermatogenesis was determined by measuring the proportion of the spermatocytes and haploid cells (spermatids + spermatozoa) in the testes after *in vitro* exposures to BPA for 72 hours. In addition, the involvement of ESR α/β in the mechanism of action of BPA in impairing spermatogenesis was also investigated. Thus, the proportion of the spermatocytes and haploid cells was investigated in the testes after pre-incubations in the presence or absence of ICI 182,780 (ESR α/β antagonist), and incubations with DMSO, 10 pM or 10 μ M BPA for 72 hours. Overlay images illustrate the image analysis of the proportion of spermatocytes (in red) and haploid cells (in green) superimposed on the image of

the testicular histological section obtained after *in vitro* exposures. Thus, overlay images were obtained after pre-incubation in the presence (Figures 38C, 38D and 38E) or absence (Figures 38A, 38B and 38C) of 10 μ M of ICI 182,780 and incubations with DMSO (Figures 38A and 38D), 10 pM BPA (Figures 38B and 38E) or 10 μ M BPA (Figures 38C and 38F). Additionally, Figures 38G and 38H show in a quantitative way the proportion of spermatocytes and haploid cells in relation to the total testicular surface.

Figure 38A shows the profile of distribution and proportion of the testicular cells in the control group by highlighting a normal distribution of spermatids and spermatozoa (in green) and spermatocytes (in red). However, there was no change in the proportion of spermatocytes (in red) after *in vitro* exposures to 10 pM (Figures 38B and 38G) or 10 μ M BPA (Figures 38C and 38G). On the other hand, Figures 38B and 38H show a reduction in the proportion of the surface of haploid cells (in green) in relation to the total testicular surface caused by *in vitro* exposure to the lowest concentration of BPA. Interestingly, Figures 38E and 38H show that this reduction (in green) caused by the lowest concentration of BPA was abolished by pre-incubation with ICI 182,780, indicating that this BPA-mediated damage to spermatogenesis is dependent on ESR activation. However, there was no significant change, but only a tendency ($p = 0.08$) to reduce the proportion of haploid cells (in green) after *in vitro* exposure to the highest concentration of BPA (Figures 38C and 38H).



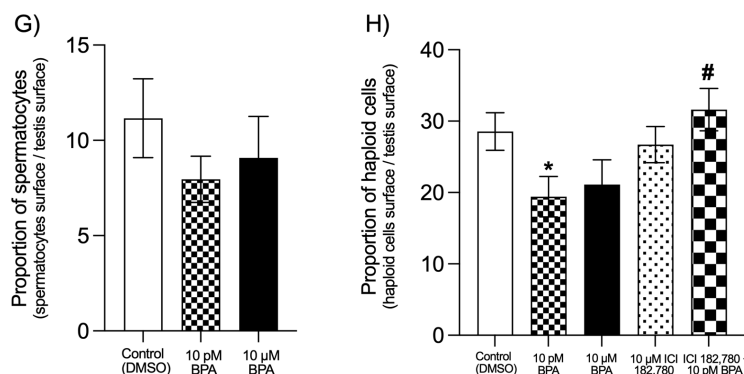


Figure 38: Effects of BPA and involvement of ESR α/β on the proportion of spermatocytes, spermatids and spermatozoa in the zebrafish testis. The surface of the proportion of spermatocytes (in red), spermatids and spermatozoa (in green) were analyzed in histological sections of zebrafish testes incubated in the (A) absence or presence of (B) 10 pM or (C) 10 μ M BPA. Overlay images of the digital analysis of the proportion of spermatocytes (in red) and haploid cells (in green) superimposed on the testicular histological sections were obtained after pre-incubations for 2 hours in the (C – E) presence or (A – C) absence of 10 μ M of ICI 182,780 (ESR α/β antagonist) and incubations for 72 hours with (A and D) DMSO, (B and E) 10 pM BPA or (C and F) 10 μ M BPA. Representation of the quantitative analysis of the proportion of (G) spermatocytes and (H) haploid cells in relation to the total testicular surface after pre-incubation in the (G) absence or (H) presence of 10 μ M ICI 182,780 prior to incubations DMSO, 10 pM or 10 μ M BPA. The data are expressed as mean \pm S.E.M. of 3–4 fish in each group from two independent experiments. * $p \leq 0.05$ when compared to control group; # $p \leq 0.05$ when compared to BPA group.

4.1.5 Discussion Part II of Chapter I

Different concentrations of BPA, ranging from picograms to micrograms *per* liter, have already been detected in rivers and surface waters and effluents in different countries (FROMME et al., 2002; HUANG et al., 2012; RAMOS et al., 2021). A recent study reported that on an annual basis that BPA concentrations ranged from 0.09 to 1.46 μ g/L BPA in river water and 0.36–3.57 μ g/L BPA in drinking water in Minas Gerais/Brazil (RAMOS et al., 2021). However, worryingly, concentrations at 30.1 μ g/L and 240 μ g/L BPA have already been detected in the bile of wild and market fish, respectively (WU et al., 2016). Thus, 10 pM BPA used in the present study (2.3 ng/L) is below the reported concentrations in untreated water, drinking water, water from rivers and sea. And, the highest concentration, 10 μ M (2.3 mg/L), is close to that reported in leachate water and effluents generated by municipal solid waste plants (MORIN; ARP; HALE, 2015; FUDALA-KSIAZEK; PIERPAOLI; LUCZKIEWICZ, 2017). Therefore, considering that BPA is found in different concentrations in aquatic environments and organisms, and that this is mainly produced by human-generated pollution, studies with fish become even more relevant for understanding the potential for effects on male reproduction.

Through the use of *in vitro* and *in vivo* testing our studies evidenced that BPA induces biochemical and molecular changes on testicular energy metabolism in zebrafish. Our findings showed that a short-term *in vivo* exposure of zebrafish to lowest BPA concentration for 12 hours caused a reduction on lactate content, whereas LDH activity was not changed. This reduction may have been mediated by the reduction on ALT activity (converts alanine into pyruvate) or on glycogen content, which may consequently lead to reduced pyruvate levels (Figure 39). However, *in vitro* exposure of zebrafish testes to BPA (10 pM and 10 μ M) for 1 hour resulted in a reduction in lactate content, which may have been a result of reduced LDH and ALT activities or reduced glycogen content, which may consequently lead to reduced pyruvate levels (Figure 40).

LDH catalyzes the reduction of pyruvate to lactate (GRANCHI et al., 2010), which is then transported to the mature germ cells through MCT (SKINNER, 1991; RATO et al., 2012a). Lactate has been reported for decades as the preferred energy substrate for rat spermatocytes and spermatids (MITA; HALL, 1982; GROOTEGOED; JANSEN; VAN DER MOLEN, 1984). In fact, the energy production by rat spermatids has been reported to be more efficient when lactate is present in high concentrations and pyruvate in low concentrations (MITA; HALL, 1982). Although the role of lactate for each testicular cell of fish is still unclear, its energy importance for spermatozoa at the end of the motility phase is known (DREANNO et al., 2000). To date, the present study was the first to evidence LDH immunolocalization in the testis of a fish species. In view of our experimental conditions, LDH expression in the zebrafish testis seems to be more abundant in mature germ cells, mainly in spermatids, whereas was not identified in early germ cells, spermatogonia. It has been reported that reduction or inhibition of lactate production may impair spermatogenesis and contribute to male infertility (BUSTAMANTE-MARÍN et al., 2012; LUO et al., 2020). Thus, the increased relative expression of LDHBa, as well as decreased relative expression of PKMA in the testis incubated with 10 pM BPA for 6 and 72 hours, respectively, could suggest a compensatory genomic response by the need for lactate production (Figure 40). However, PKM immunocontent was reduced by 10 μ M BPA, whereas it was not changed by incubation with 10 pM BPA for 72 hours. In addition, interestingly, the density of testicular cells expressing LDH was increased in the testes incubated with 10 μ M BPA for 72 hours, whereas LDHBa gene expression was reduced. These differences in gene and protein expressions are in agreement with previous studies.

Differences in gene expression and immunocontent of LDH and MCT have already been evidenced with the suggestion that this occurs because they are regulated at different time intervals and/or regulated by post-transcriptional mechanisms (ALVES et al. al., 2013b). Taking into account that mRNA transcription and protein synthesis are regulated and occur at different time intervals, it is plausible that differences in the pattern of gene and protein expressions are observed after being analyzed in the same treatment period. However, glycolysis and lactate production can be impaired in the testes these changes in the decrease in LDH expression, PKM if prolonged, can significantly reduce glycolysis and therefore inhibit lactate production.

However, if the changes in PKM and LDHBa expression are prolonged, they may impair glycolysis and lactate production. Furthermore, increased MCT4 gene expression in the testes incubated with 10 μ M BPA for 72 hours could suggest a greater need for lactate availability in testicular cells exposed to BPA. Studies with BPA, its analogues or other EDC obtained in different animal models, isolated cells or other tissues reinforce our data. *In vitro* exposure to BPA (10 μ M and 1 mM) for 48 hours has also been shown to impair glucose metabolism by decreasing the lactate production, gene expression and immunocontent of LDH in rat Sertoli cells (HUANG et al., 2015). Another EDC, bis(2-ethylhexyl)phthalate, was also reported to decrease LDH activity and lactate content in the zebrafish testis after short-term exposures (BATISTA-SILVA et al., 2020a). In addition, similar effects in terms of the reduction on lactate content and LDH activity were seen in the zebrafish testis after acute *in vitro* exposure to triterpene betulin (DE OLIVEIRA et al., 2021). Furthermore, testicular glycolysis impairment BPA-mediated could be reinforced by reduction in PFKFb2a gene expression in the testis after 72 hours of *in vitro* exposure to 10 μ M BPA. It has been reported that decreased liver PFKFb expression during starvation and diabetes is restored and stimulated by high-carbohydrate diet refeeding or insulin administration (COLOSIA et al., 1988). The exposure of rats to BPA was reported to disrupt glucose homeostasis and testicular insulin signaling by decreasing hexokinase and PFK-1 activities, insulin levels, as well as the expression of GLUT, insulin receptor, insulin receptor substrates and PI3K (D'CRUZ et al. 2012; D'CRUZ; JUBENDRADASS; MATHUR, 2012). Therefore, these previous studies corroborate our findings, which leads us to suggest that the BPA-mediated changes in the expression of glycolysis-related genes observed in our study were due to impaired insulin signaling or lacking energy substrate.

In addition to lactate, glycogen homeostasis is also important for maintaining the integrity of male germinal epithelium in rats (KURAMORI et al., 2009). Studies obtained in rodents also indicate that an imbalance in glycogen homeostasis in the testes may induce apoptosis and germ cells degeneration (THAKUR et al., 2003; KURAMORI et al., 2009; VILLARROEL-ESPÍNDOLA et al., 2013). However, there are no reports which show the participation of glycogen as a testicular energy source, nor the glycogen phosphorylase gene expression in the testes of zebrafish. Our findings showed that after *in vitro* exposure to BPA (10 pM and 10 μ M) for 1 hour, as well as *in vivo* exposure to BPA (only 10 pM) for 12 hours, caused a significant reduction on testicular glycogen content. Interestingly, the relative expression of PYGL gene encoding glycogen phosphorylase was increased in the testis after 72 hours of *in vitro* exposure to 10 μ M BPA, and this increase was dependent on the ESR. Glycogen phosphorylase participates in glycogenolysis by releasing glucose-1-phosphate from the non-reducing ends of glycogen. Thus, our findings evidenced that testicular glycogenolysis is stimulated by BPA in zebrafish. Zhao et al. (2018) reinforce our hypothesis by reporting that liver and muscle glycogenolysis and gluconeogenesis are stimulated by exposure of zebrafish to bisphenol S, an analogue of BPA.

The observed decrease of energy substrates after *in vitro* and *in vivo* exposures to BPA may also be due to decreased ALT activity and consequently lower pyruvate production. Our studies demonstrate a BPA-induced decrease in ALT activity. However, reduction in ALT activity was demonstrated after *in vitro* exposure to the lowest BPA concentration, whereas after *in vivo* exposure it was due to the highest concentration of BPA. Thus, the increased relative expression of GPT2 gene encoding ALT after 6 hours of incubation of the testes with 10 pM BPA seems to have been a compensatory response (Figure 40). However, the relative expression of GPT2 was decreased after 72 hours of exposure to the highest concentration of BPA. To date, this is the first study that evidenced GPT2 gene expression in the testis of a fish species. In addition, the present study was the first to identify GPT2 as target of the genomic effect of BPA in the testes. In addition to being the end product of glycolysis, pyruvate may also be generated by ALT, which catalyzes the reversible interconversion of alanine and α -ketoglutarate into pyruvate and glutamate (SHERMAN, 1992; LIU et al., 2014). Therefore, the BPA-induced changes in ALT activity and GPT2 expression shown in the present study may impair alanine and/or pyruvate production. Studies related to the action of ALT in the testis are rare even in

mammals. Alanine is the main glucogenic amino acid that can be used as an energy substrate by Sertoli cells for several biochemical pathways, including the Krebs cycle and gluconeogenesis (RATO et al., 2012a, 2012b). It has been shown that Sertoli cells may maintain lactate production even in the total absence of glucose through amino acid or glycogen metabolism (RIERA et al., 2009). Furthermore, high levels of ALT immunocontent in rat Sertoli cells were previously reported, suggesting that a significant part of the pyruvate produced by these cells has its origin in the conversion of alanine by ALT (MATEUS et al., 2018).

The present study was the first to identify testicular AST and pyruvate carboxylase as targets for BPA. In addition, this is the first study that evidences the expression of the PCXA gene in the testes. AST catalyzes the interconversion of aspartate and α -ketoglutarate to OAA and glutamate (SCHWARTZ, 1971). Therefore, the drastic reduction in AST activity in the testes of zebrafish exposed to BPA (10 pM and 10 μ M) could indicate a decrease in aspartate and Krebs cycle intermediates, α -ketoglutarate and OAA. Furthermore, it could also be suggested that a reduction in AST activity may compromise the malate-aspartate transport system for the reoxidation of reduction equivalents such as NADH, which is produced when lactate is oxidized to pyruvate by LDH (CETICA; PINTOS; DALVIT, 1999). On the other hand, AST activity was increased in the zebrafish testes after 1 hour of incubation with BPA. The increase on mitochondrial enzyme activities may be an indicator of mitochondrial damage (GIANNINI; TESTA; SAVARINO, 2005; LO; WANG; WU, 2014). Furthermore, the relative expression of the PCXA gene encoding pyruvate carboxylate was increased in the testes after 6 hours of incubation with 10 pM BPA. Pyruvate carboxylase catalyzes the carboxylation of pyruvate to OAA. This is a very important anaplerotic reaction that replenishes OAA for the Krebs cycle, and provides OAA for subsequent conversion to PEP in the first step of gluconeogenesis (JITRAPAKDEE et al., 2008; VALLE, 2017). Therefore, BPA-induced changes in AST activity and PCXA expression in the testis could suggest a high energy demand and need for Krebs cycle intermediates.

In addition to testicular energy metabolism, spermatogenesis was also affected by BPA. Gene expression analysis in the zebrafish testes showed a different expression pattern of testicular cell markers in relation to exposure times and concentrations of BPA. The relative expression of PIWIL-1 and SYCP-3 was increased after 6 hours of incubation with 10 pM BPA. However, the relative expression of the

spermatid marker gene, ODF3b, was reduced in the testes incubated with 10 pM BPA for 72 hours. These findings are in agreement with our histological data. A reduction in the proportion of haploid cells (spermatids and spermatozoa) in relation to the total testicular surface was also observed after 72 hours of exposure to 10 pM BPA. According to a previous study, ODF3b gene expression has already been directly related to the proportion of haploid cells in the zebrafish testes (TOVO-NETO et al., 2020). In addition to *in vitro* BPA, *in vivo* acute exposure to 10 pM and 10 µM BPA also caused impairment on spermatogenesis by increasing the percentage of apoptotic spermatocytes and reducing the proportion of haploid cells in relation to testicular surface. Thus, based on these findings, the influence of impaired testicular energy metabolism BPA-mediated could be proposed as one of the causes that lead to impaired spermatogenesis in zebrafish (Figure 39). In agreement with our findings, it has been reported that a mixture of xenoestrogens containing BPA results in a reduction in the number of spermatozoa accompanied by an increase in the number of testicular apoptotic cells in zebrafish (WANG et al., 2019). In addition, exposure of mice to BPA during puberty has also been reported to increase the percentage of testicular apoptotic cells by mitochondrial apoptotic pathways (WANG et al., 2010). However, the present study is the first to use the innovative approach to digital image analysis by pixel classification module through the Ilastik software to perform the proportion of spermatocytes and haploid cells in histological sections of zebrafish testis. Quantitative analyzes on histological sections are a time-consuming, tedious process, subject to errors when performed manually (BERG et al., 2019), and may still generate biased results, since the areas of analysis are chosen by the person performing the analysis. Thus, the results from digital analysis are not affected by the choice of section areas in the digital image analysis, which allows us to have an automatic, standardized and controlled analysis of the entire testicular histological sections, generating accurate and reproducible data (AEFFNER et al., 2018).

In addition, our findings evidencing the reduction of SYCP-3 gene expression triggered by *in vitro* exposure to 10 µM BPA for 72 hours are in agreement with previous reports. On the other hand, our histological data on the proportion of spermatocytes in the testis are not in agreement with data on the SYCP-3 expression. The reduction of SYCP-3 gene expression was also reported in the zebrafish testes after *in vivo* exposure to higher concentrations of BPA (2000 mg/L) for 21 days (GONZÁLEZ-ROJO et al., 2019). In addition, *in vitro* exposures of rat seminiferous

tubules to BPA (1 nM and 10 nM) for 8, 14 and 21 days led to partial arrest of meiosis due to changes in the expression of the meiosis and spermatocytes marker genes, and histological changes (ALI et al., 2014). It has been revealed that SYCP-3 knockout mice have an unformed synaptonemal complex with arrest of meiosis in zygotic spermatocytes, rendering the mice infertile (YUAN et al., 2000).

In addition to the changes in meiotic and post-meiotic phases, cell proliferation also appears to be affected by BPA, contributing to the BPA mechanism in impairing spermatogenesis. Our findings evidencing the reduction in PCNA immunocontent triggered by *in vitro* exposure to 10 μ M BPA for 72 hours are in agreement with previous reports. BPA-mediated reduction in PCNA protein expression was recently reported in mouse testes, suggesting that germ cell proliferation is impaired by BPA (LIU; WANG; LI, 2020). PCNA is a nuclear protein which is differentially regulated during the cell cycle, reaching maximum expression at the G1/S phase transition (CELIS; CELIS, 1985). However, in addition to the DNA synthesis, PCNA participates also of other important cellular processes, such as DNA repair and apoptosis (JÓNSSON; HÜBSCHER, 1997). It can be suggested that the reduction in PCNA mediated by BPA may have resulted in spermatogonial proliferation inhibition or apoptosis, since it has been reported that cells expressing little or no PCNA undergo apoptosis (PAUNESKU et al. 2001). A previous study reinforces our data by reporting anti-proliferative effects of BPA on primary and prostate cancer cells, which were observed after the same exposure period to the same concentration of BPA as in our study. Micromolar concentrations of BPA increase the expression and phosphorylation of p53 and trigger cell cycle arrest through ERK activation via EGFR/ESR β /ERK signaling pathways crosstalk (BILANCO et al., 2017). Furthermore, high p53 levels have been reported to inhibit PCNA expression (MERCER et al., 1991; YAMAGUCHI et al. 1994; PAUNESKU et al. 2001). Thus, these previous data are in agreement with our findings. A significant increase in ERK 1/2 phosphorylation was also demonstrated in testes incubated with both concentrations of BPA for 72 hours. Therefore, it can be suggested that the reduction in PCNA expression caused by BPA may have been mediated by the increase in p53 expression and phosphorylation through the activation of ERK 1/2, with possible involvement of ESR β , which were upregulated by 72 hours-incubation to 10 μ M BPA. Furthermore, previous reports also show that BPA stimulates the phosphorylation/activation of ERK 1/2 in rat Sertoli cells (IZUMI et al., 2011) and in TM4 Sertoli via ESR α / β (GE et al., 2014) after short-term *in vitro* exposure. Moreover,

the involvement of ERK 1/2 in BPA-mediated genomic signaling pathways has been shown to induce Sertoli cell damage and apoptosis (QIAN et al., 2014).

The present study showed that only after 72 hours of *in vitro* exposure to the highest concentration of BPA resulted in different genomic responses. The most of the BPA-mediated genomic effects on energy metabolism were ESR-dependent (Figure 41). The present study showed for the first time that the decreased expression of PFKFb2a and LDHBa, as well as increased PYG expression and the number of cells expressing LDH, were totally reversed by pre-incubation with ICI 182,780, an ESR α/β antagonist. The increased expression of ESR2a and ESR2b after 72 hours of incubation with 10 μ M BPA evidences that these ESR are targets of the genomic effects of BPA in the testis. Our findings are consistent with other previous studies which reported that the gene expression modulation by micromolar concentrations of BPA is mediated via ESR after long-term exposures, from hours to days (GAIDO et al. al. 1997; LÖSEL; WEHLING, 2003; MOLINA-MOLINA et al., 2013; LE FOL et al., 2017). Furthermore, another previously published study reinforces our data by reporting that chronic exposure to BPA increases ESR2b expression in the liver and ESR2a expression in the testis of male goldfish (HATEF et al., 2012). However, changes in the relative expression of GPT2, MCT4 and SYCP-3 by 10 μ M BPA were not reversed by pre-incubation with ICI 182,780. Therefore, these differences in gene expression BPA-mediated dependent or non on ESR α/β indicate that GPT2, MCT4 and SYCP-3 are also transcriptional targets of nuclear receptors other than ESR α/β . However, the decreased ODF3b expression (spermatid marker gene) and decreased proportion of spermatids and spermatozoa mediated by 10 pM BPA were dependent on ESR activation, was also reinforced by ESR2b up-regulation. Nevertheless, as all effects of BPA presented in this study were evaluated in the whole testis, it is reinforced the need to consider multiple pathways of the BPA actions according to each cell type. Overall, our findings indicate that most of the ESR-dependent effects mediated by BPA were mainly targeted at Sertoli and haploid cells (Figure 41), which is reinforced by the fact that these are the testicular cells that have the highest expression of the ESR in fish species (BOUMA; NAGLER, 2001; WU et al. al., 2001; ITO; MOCHIDA; FUJII, 2007). Thus, a cause-effect relationship between changes on testicular energy metabolism and defective spermatogenesis with reduced haploid cells can be proposed (Figure 41).

Furthermore, nuclear receptors other than ESR are also targets of BPA genomic responses in the zebrafish testis. The reduced expression of ESRR α gene mediated by 10 μ M BPA may be linked to the reduced expression of PFKfb2a and PKMA genes. ESRR encode ERR which are orphan nuclear receptors and key regulators of energy metabolism, stimulating mitochondrial biogenesis, glycolysis, fatty acid oxidation, the Krebs cycle, oxidative phosphorylation and ATP synthesis. ERRs directly bind and activate the promoters of many genes that encode glycolytic enzymes, such as PKM and LDH (GIGUÈRE, 2008; CAI et al., 2013). Furthermore, the *Drosophila* ERR is required for the induction of glycolysis to support cell proliferation during embryonic development (TENNESSEN et al., 2011). PFK gene is a direct transcriptional target of the ERR, and most genes encoding glycolytic enzymes and lactate levels are significantly reduced in mutant ERRs (TENNESSEN et al., 2011).

Some results of the *in vitro* exposure BPA group had not the same effects to those reported in the *in vivo* BPA exposure group, as observed in AST and LDH activities, as well as in the proportion of haploid cells. Different biological effects in the testes promoted by *in vivo* and *in vitro* exposures to BPA have also been observed in a previous study in zebrafish (GONZÁLEZ-ROJO et al., 2019). Although there may be several mechanisms that contribute to this response. Among them, when the animal is exposed *in vivo* to BPA, it undergoes phase II metabolism, resulting in the production of its metabolites BPA-GA and BPA-S (LINDHOLST et al., 2003). In addition, studies with BPA present an important challenge to traditional concepts in toxicology (known as “dose makes poison”). Non-monotonic and biphasic dose-response patterns are reported for BPA (WETHERILL et al., 2007; VANDENBERG et al., 2012; GONÇALVES et al., 2018).

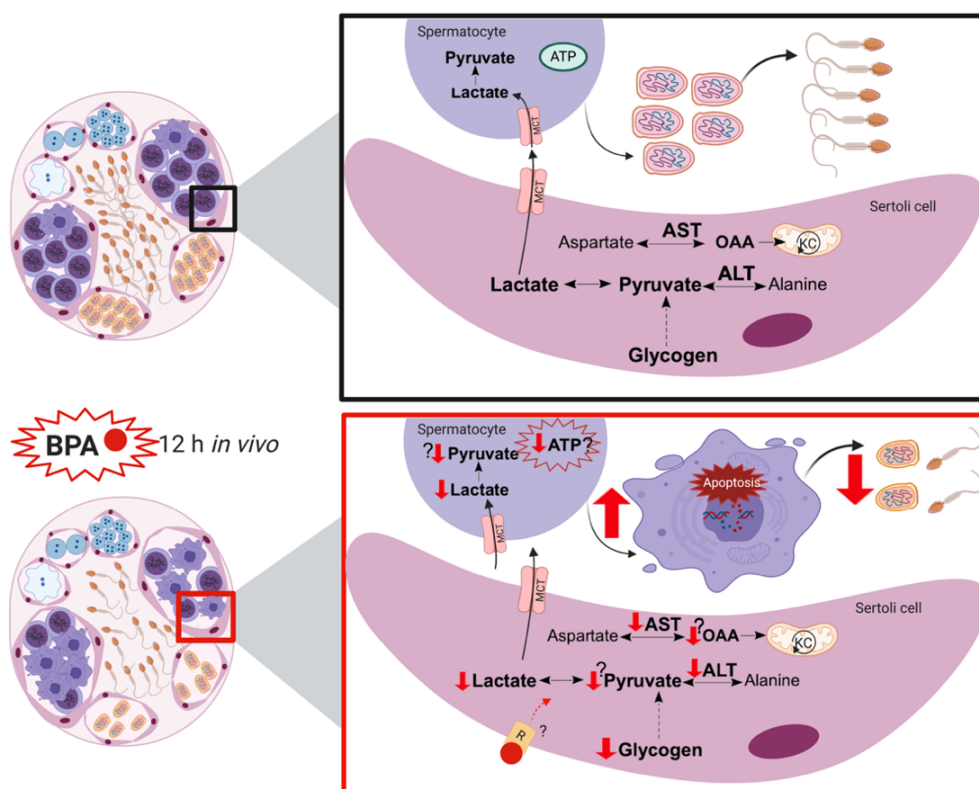


Figure 39: Schematic representation of the proposed mechanism of the *in vivo* effects of BPA on testicular energy metabolism and spermatogenesis in zebrafish. After 12 hours (h) of exposure, BPA resulted in a reduction on testicular lactate, the main energy substrate for the mature germ cells. This reduction may have been mediated by the reduction on ALT activity (converts alanine into pyruvate) or on glycogen content, which may consequently lead to reduced pyruvate levels. In addition, AST activity was inhibited and consequently led to a reduction on OAA production, intermediate in the Krebs cycle in Sertoli cells. If less lactate is produced by the Sertoli cells, therefore less lactate is transported to the spermatocytes, which would result in a lack of pyruvate, and consequently ATP levels. This lack of energy could have caused an increase of apoptosis in spermatocytes and, consequently, a reduction in the proportion of spermatids and spermatozoa. Straight lines are direct pathways; dashed lines represent multi-step pathways; question marks (?) represent suggested effects (hypotheses) based on our study and on literature, which are questions to be further investigated. ALT – Alanine aminotransferase; AST – Aspartate aminotransferase; KC – Krebs Cycle; LDH – Lactate dehydrogenase; OAA – Oxaloacetate. Source: Own construction.

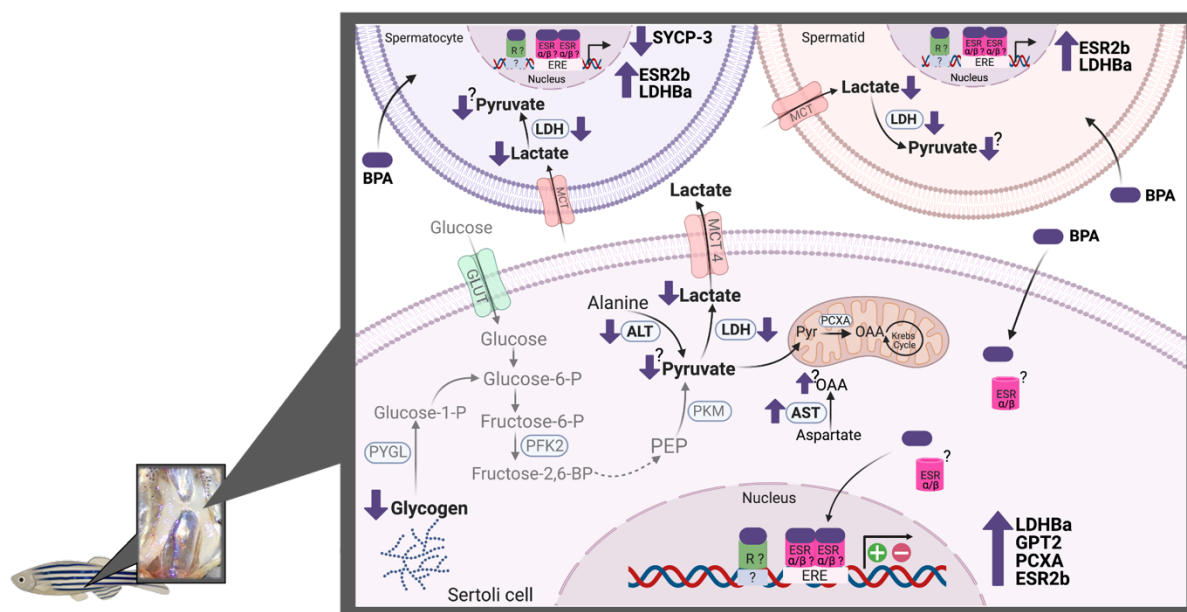


Figure 40: Schematic representation of the proposed mechanism of the 1-hour and 6-hour *in vitro* effects of BPA on testicular energy metabolism. All 1-hour and 6-hour *in vitro* effects of BPA analyzed in our study are shown in bold. After 1 hour of incubation, BPA resulted in a reduction on testicular lactate, the main energy substrate for the mature germ cells. This reduction may have been mediated by the reduction on ALT activity (converts alanine to pyruvate) and LDH activity (converts pyruvate to lactate), which consequently led to a reduction on pyruvate levels. This could contribute to the higher energy demand and the observed reduction in testicular glycogen stores. In addition, the increased relative expression of LDHBa and GPT2 mediated by BPA after 6 hours of incubation may have been a compensatory response by the need for pyruvate/lactate production. In addition, AST (converts aspartate to OAA) activity, as well as PCXA relative expression were increased by BPA after 1 and 6 hours of incubation, respectively, which may suggest a high production of OAA, an intermediate of the Krebs cycle and of the first step of gluconeogenesis, or mitochondrial damage. If less lactate is produced by the Sertoli cells, therefore less lactate is transported to the mature germ cells, which would result in a lack of pyruvate and ATP levels. Straight lines are direct pathways; dashed lines represent multi-step pathways; question marks (?) represent suggested effects (hypotheses) based on our study and on literature, which are questions to be further investigated. ALT/GPT2 – Alanine aminotransferase; AST – Aspartate aminotransferase; BPA – Bisphenol A; ERE – Estrogen response element; ESR2b – Estrogen receptor β 1; KC – Krebs Cycle; LDH – Lactate dehydrogenase; MCT – Monocarboxylate transporter; OAA – Oxaloacetate; PCXA – Pyruvate carboxylase A; R – Receptor. Source: Own construction.

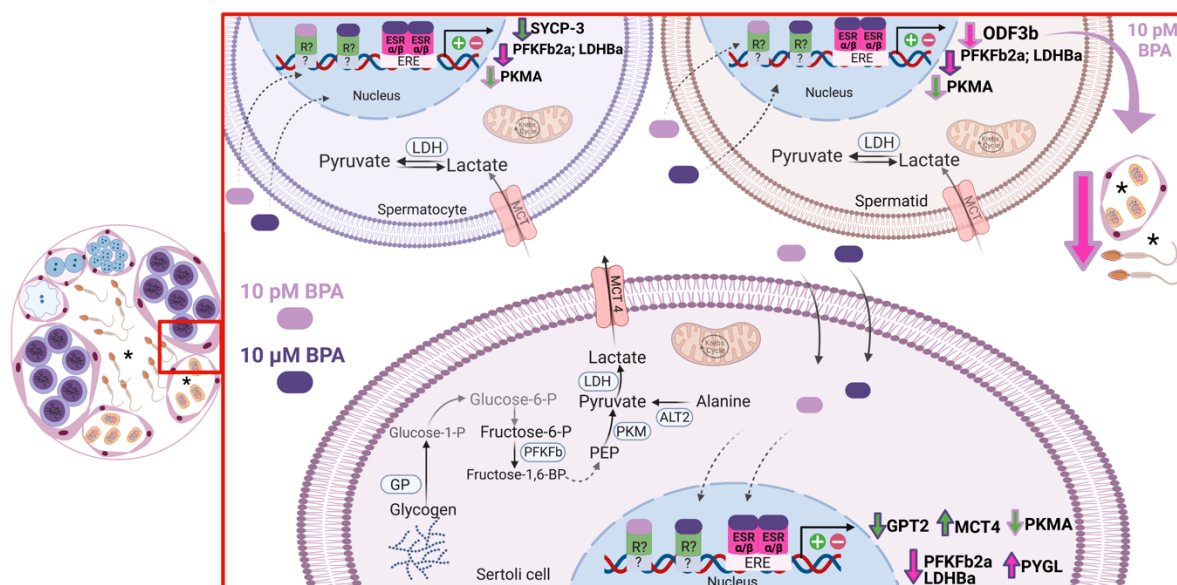


Figure 41: Schematic representation of an overview of the involvement of ESR α/β in 72-hour genomic effects of BPA (*in vitro*) in the zebrafish testes. All 72-hour effects of BPA reversed or not by the ESR α/β antagonist (ICI 182,780) analyzed in our study are shown in bold. However, as all effects of BPA presented in this study were evaluated in the whole testis, it is reinforced the need to consider multiple pathways of the BPA actions according to each cell type. The decrease in PFKFb2a and LDHBa expression, as well as the increase in PYGL expression by 10 μ M BPA were dependent on ESR activation (pink arrows with purple stroke). On the other hand, the decrease in PKMA gene expression mediated by 10 pM BPA involved the action of receptors (R?) other than ESR (green arrows with lilac stroke). As PFKFb, LDH and PKM are widely expressed in male germ and somatic cells, it is suggested that the modulation of their expression was mediated in both spermatocytes, spermatids and Sertoli cells. In addition, the decrease in GPT2 expression, as well as the increase in MCT4 expression were also not dependent on ESR (green arrows with purple stroke). According to the literature, glycogen phosphorylase (GP), MCT4 and ALT2/GPT2 are expressed in mammalian Sertoli cells, therefore, is suggested that the modulation of their expression was mediated in Sertoli cells. Furthermore, the decrease in expression of the spermatocyte and meiosis specific gene, SYCP3, mediated by 10 μ M BPA was also not dependent on ESR α/β (green arrows with purple stroke) in spermatocytes. On the other hand, the decrease in ODF3b expression (spermatid marker gene) and the decrease in the proportion of spermatids and spermatozoa in the testis mediated by the lowest concentration of BPA were dependent on ESR α/β activation (pink arrows with lilac stroke). Therefore, a cause-effect relationship is suggested between changes on testicular energy metabolism and defective spermatogenesis with reduced haploid cells. Overall, our findings indicate that most of the ESR α/β -dependent effects mediated by BPA were mainly targeted at Sertoli and haploid cells, which is reinforced by the fact that these are the testicular cells that have the highest expression of the ESR α/β . Dashed lines represent multi-step pathways; question marks (?) are questions to be further investigated. Source: Own construction.

4.1.6 Conclusions of Part II of Chapter I

In general, from zebrafish testis incubations to BPA and different methodological approaches, it was evidenced that BPA *in vitro*:

- Affects testicular energy metabolism by reducing the contents of lactate and glycogen, as well as modulating the activities of LDH, ALT and AST.
- Impairs testicular energy metabolism by altering the relative expression of genes associated to energy metabolism dependent or/and not on ESR α/β participation: PYGL, PFKFb2a, LDHBa, GPT2, MCT4, PKMA and PCXA.
- In addition, it impairs energy metabolism by reducing PKM immunocontent and increasing immunohistochemical expression of LDH dependent on ESR α/β participation.
- Impairs cell proliferation and spermatogenesis by reducing the relative expression (dependent or not on ESR α/β) of germ cell marker genes and the PCNA immunocontent, as well as activating ERK 1/2.
- In addition, it impairs spermatogenesis by reducing ESR α/β -dependent the relative expression of ODF3b (a spermatid marker gene) and the proportion of haploid cells dependent on ESR α/β participation.

In addition, acute exposure of zebrafish to BPA:

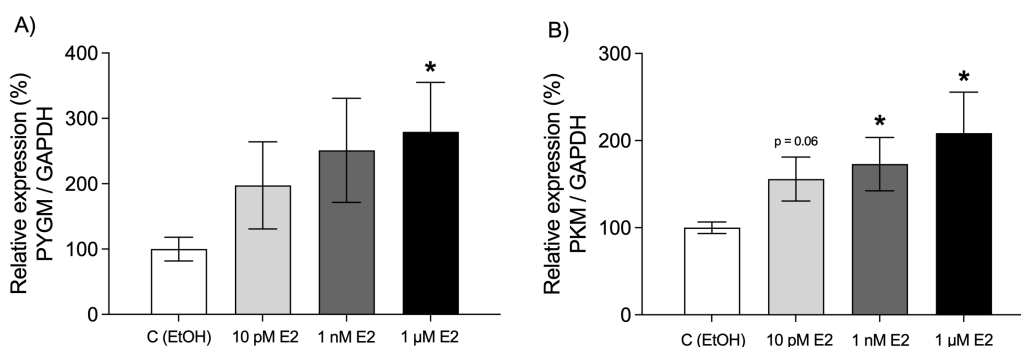
- Affects testicular energy metabolism by reducing the contents of glycogen and lactate, as well as ALT and AST activities.
- Impairs spermatogenesis by increasing apoptosis and reducing the proportion of haploid cells.

4.2 CHAPTER II: STUDY OF THE *IN VITRO* GENOMIC EFFECTS OF E2 AND BPA ON TESTICULAR ENERGY METABOLISM AND SPERMATOGENESIS IN IMMATURE RAT TESTIS

4.2.1 Results E2 of Chapter II

4.2.1.1 Acute *in vitro* exposure to E2 disturbs testicular energy metabolism of immature rats by modulating the expression of genes involved in carbohydrate and amino acid metabolism

In order to study the genomic effects of E2 on the testicular energy metabolism of immature rats, the relative expression of energy metabolism-associated genes was analyzed in testis explants after 6 hours of incubations with E2 at 10 pM, 1 nM and 1 μ M. Thus, the relative expression of glycogen phosphorylase muscle form (PYGM), pyruvate kinase M1/2 (PKM), lactate dehydrogenase C (LDHc), alanine aminotransferase (GPT2), glycerol kinase 2 (GK2) and monocarboxylate transporter 1 (MCT1) was analyzed. As can be seen in Figure 42A, the relative expression of PYGM was stimulated in the testis explants incubated only with 1 μ M E2 compared to the control group. Furthermore, the relative expression of PKM was significantly increased in the testis explants incubated with E2 at 1 nM and 1 μ M (Figure 42B), as well as a tendency ($p = 0.06$) to increase it by 10 pM E2 (Figure 42B). In addition, the relative expression LDHc was significantly increased in the testis explants incubated with 1 nM E2 (Figure 42C). On the other hand, *in vitro* exposure to E2 for 6 hours did not significantly change the relative expression of MCT1 (Figure 42D), GPT2 (Figure 42E) and GK2 (Figure 42F) compared to the control group. However, there was a tendency ($p = 0.061$) to increase GPT2 relative expression by 10 pM E2 (Figure 42E).



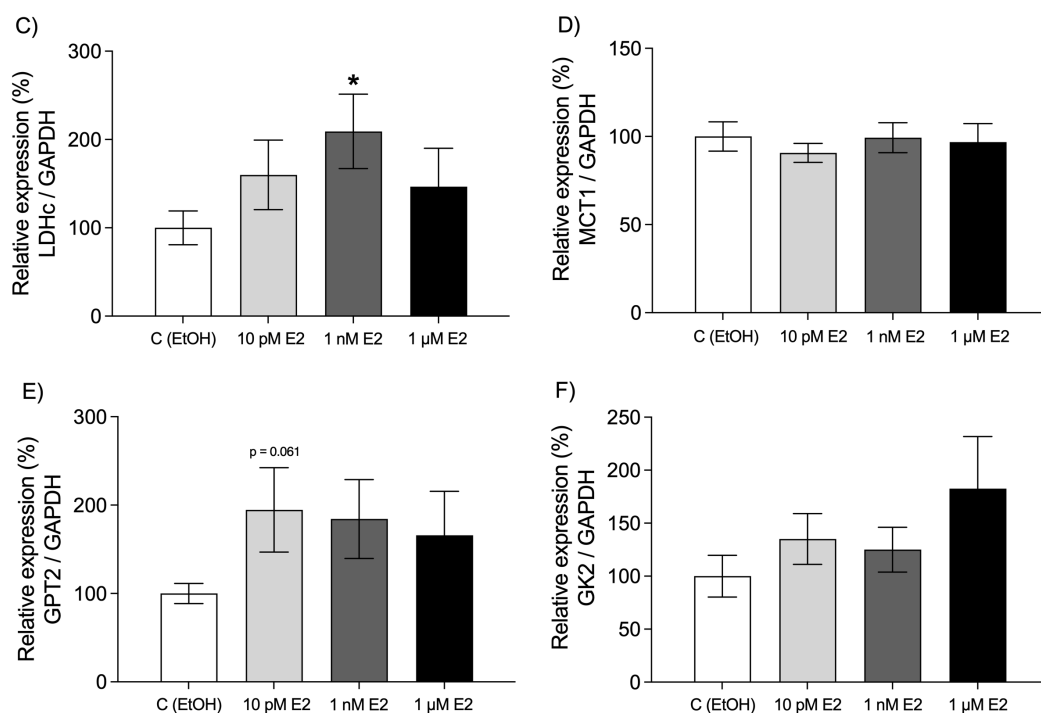


Figure 42: Effects of 10 pM, 1 nM and 1 μ M E2 on the relative expression of energy metabolism-associated genes in immature rats. The relative expression of (A) PYGL, (B) PKMA, (C) LDHc, (D) MCT1, (E) GPT2 and (F) GK2 was analyzed after *in vitro* exposure of testis explants from 20-day-old immature rats to 10 pM, 1 nM and 1 μ M E2 for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean \pm S.E.M. of 6-8 animals in each group from three independent experiments. * $p \leq 0.05$ when compared to control group. C = control; EtOH = ethanol (vehicle).

4.2.1.2 Changes in the expression of PYGM, PKM and LDHc in immature rat testis after 6 hours of incubations with E2 are not dependent on nuclear estrogen receptors

In order to investigate the participation of ESR α/β in the mechanism of action of E2 in modulating the expression of testicular energy metabolism-associated genes, testis explants were pre-incubated with ICI 182,780 (ESR α/β antagonist) 1 hour prior to E2 incubations for 6 hours. The increased relative expression of PYGM (Figure 43A) and PKM (Figure 43C) by E2 at 1 μ M was not reversed by pre-incubation with ICI 182,780, indicating that these genomic effects of E2 are independent of ESR α/β activation. In addition, the increased relative expression of PKM (Figure 43B) and LDHc (Figure 43D) by E2 at 1 nM was also not reversed by pre-incubation with ICI 182,780, indicating that these genomic effects of E2 are also not via ESR α/β activation. Moreover, ICI 182,780 group *per se* increased PKM expression in relation to the control group (Figures 43B and 43C). This effect was also observed when testis explants were

pre-incubated with ICI 182,780 prior to incubation with 1 μ M E2 (Figure 43C), but not before incubation with 1 nM E2 (Figure 43B).

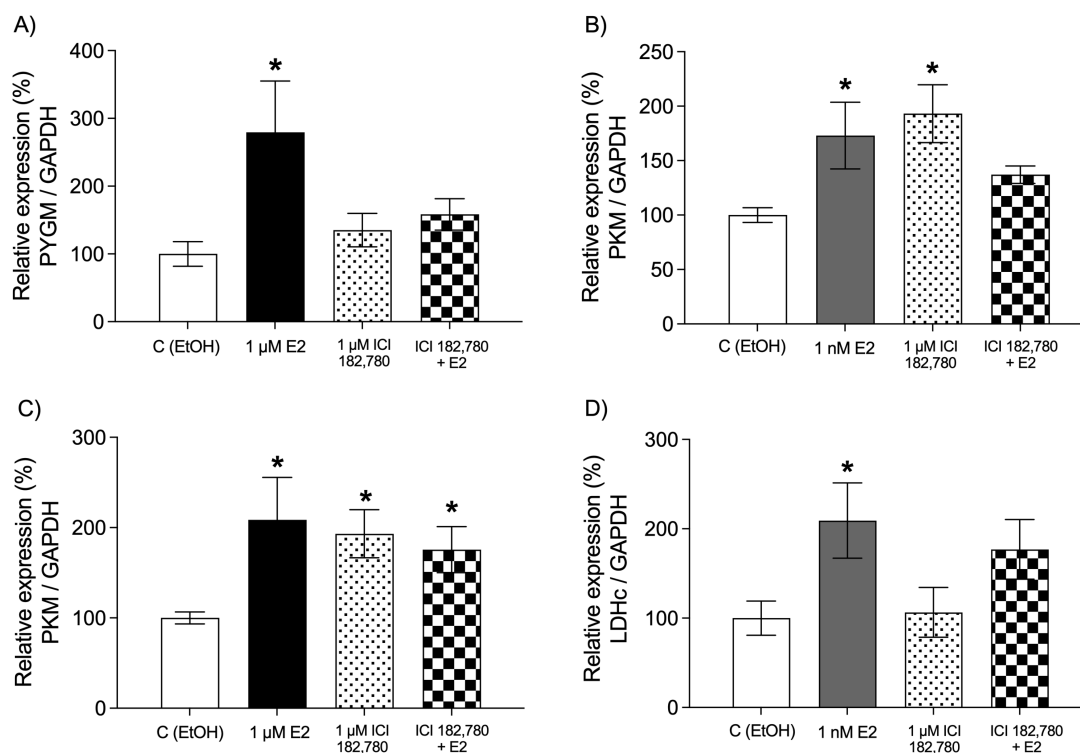


Figure 43: Involvement of ESR α / β in the mechanism of action of E2 on the relative expression of testicular energy metabolism-associated genes in immature rats. The relative expression of (A) PYGM, (B and C) PKM and (D) LDHc was analyzed in the testis explants from 20-day-old immature rats after pre-incubation with 1 μ M ICI 182,780 (ESR α / β antagonist) for 1 hour and incubations with (A and C) 1 μ M and (B and D) 1 nM E2 for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean \pm S.E.M. of 6-9 animals in each group from two or three independent experiments. *p \leq 0.05 compared to the control group. C = control; EtOH = ethanol (vehicle).

4.2.1.3 Acute *in vitro* exposure to E2 does not modulate the expression of spermatogonia and spermatocytes marker genes in immature rats

In order to correlate changes in testicular energy metabolism caused by E2 with possible changes in spermatogenesis, the expression of specific genes of germ cells was analyzed. The relative expression of integrin α 6 (ITGa6), proliferating cell nuclear antigen (PCNA) and DNA meiotic recombinase 1 (DMC1) was analyzed in testis explants from immature rats after 6 hours of incubations with E2 at 10 pM, 1 nM and 1 μ M. However, *in vitro* exposure to different E2 concentrations for 6 hours did not change the relative expression of ITGa6 (Figure 44A), PCNA (Figure 44B) and DMC1 (Figure 44C) in relation to the control group.

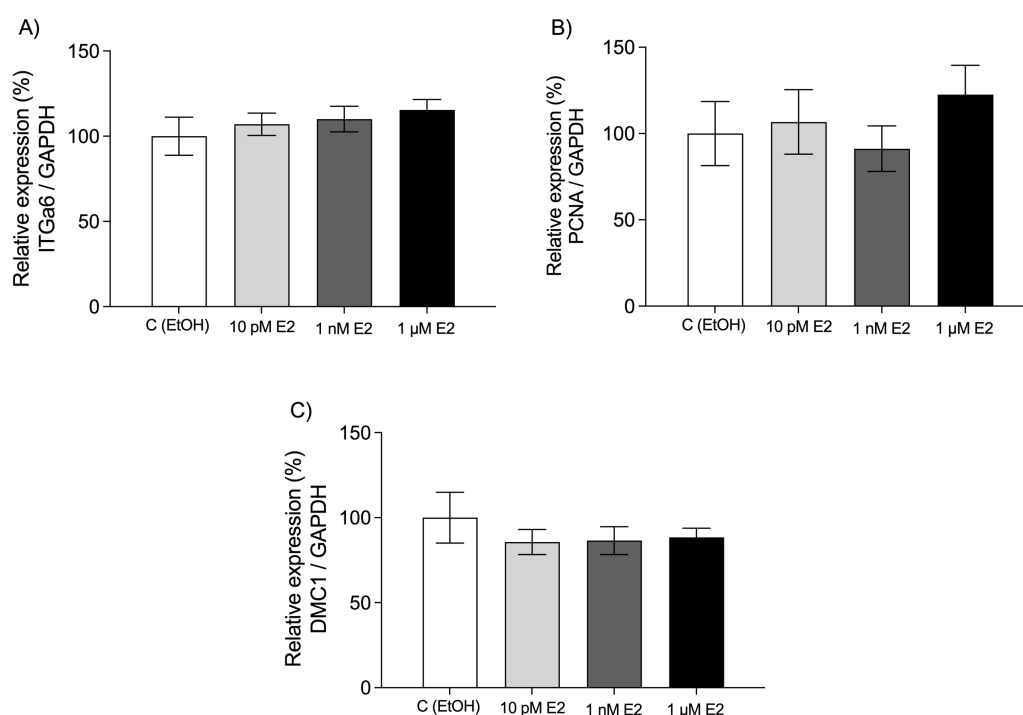


Figure 44: Effects of 10 pM, 1 nM and 1 μM E2 on the relative expression of spermatogonia and spermatocytes cell marker genes in immature rats. The relative expression of (A) ITGa6, (B) PCNA and (C) DMC1 was analyzed after *in vitro* exposure of testis explants from 20-day-old immature rats to 10 pM, 1 nM and 1 μM E2 for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean \pm S.E.M. of 6 animals in each group from two independent experiments. C = control; EtOH = ethanol (vehicle).

4.2.2 Results BPA of Chapter II

4.2.2.1 Acute *in vitro* exposure to BPA disturbs testicular energy metabolism of immature rats by modulating the expression of genes involved in carbohydrate and amino acid metabolism

In order to study the genomic effects of BPA on the testicular energy metabolism of immature rats, the relative expression of energy metabolism-associated genes was analyzed in testis explants after 6 hours of incubations with BPA at 10 pM, 1 nM and 1 μM. Thus, the relative expression of PYGM, PKM, LDHc, GPT2, GK2 and MCT1 was analyzed. The relative expression of PYGM (Figure 45A) and LDHc (Figure 45C) was stimulated after 6 hours of incubation only with 1 μM BPA in relation to the control group. Moreover, the relative expression of PKM (Figure 45B) and GPT2 (Figure 45E) was significantly increased in testis explants incubated with BPA at 1 nM and 1 μM compared to the control groups. Besides, there was a tendency to increase

PKM relative expression by 10 pM BPA ($p = 0.064$) *in vitro* exposure (Figure 45B). On the other hand, *in vitro* exposure to different BPA concentrations for 6 hours did not change the MCT1 relative expression (Figure 45D). However, GK2 relative expression was significantly augmented in testis explants after 6 hours of incubation with 1 nM BPA in relation to the control group (Figure 45F).

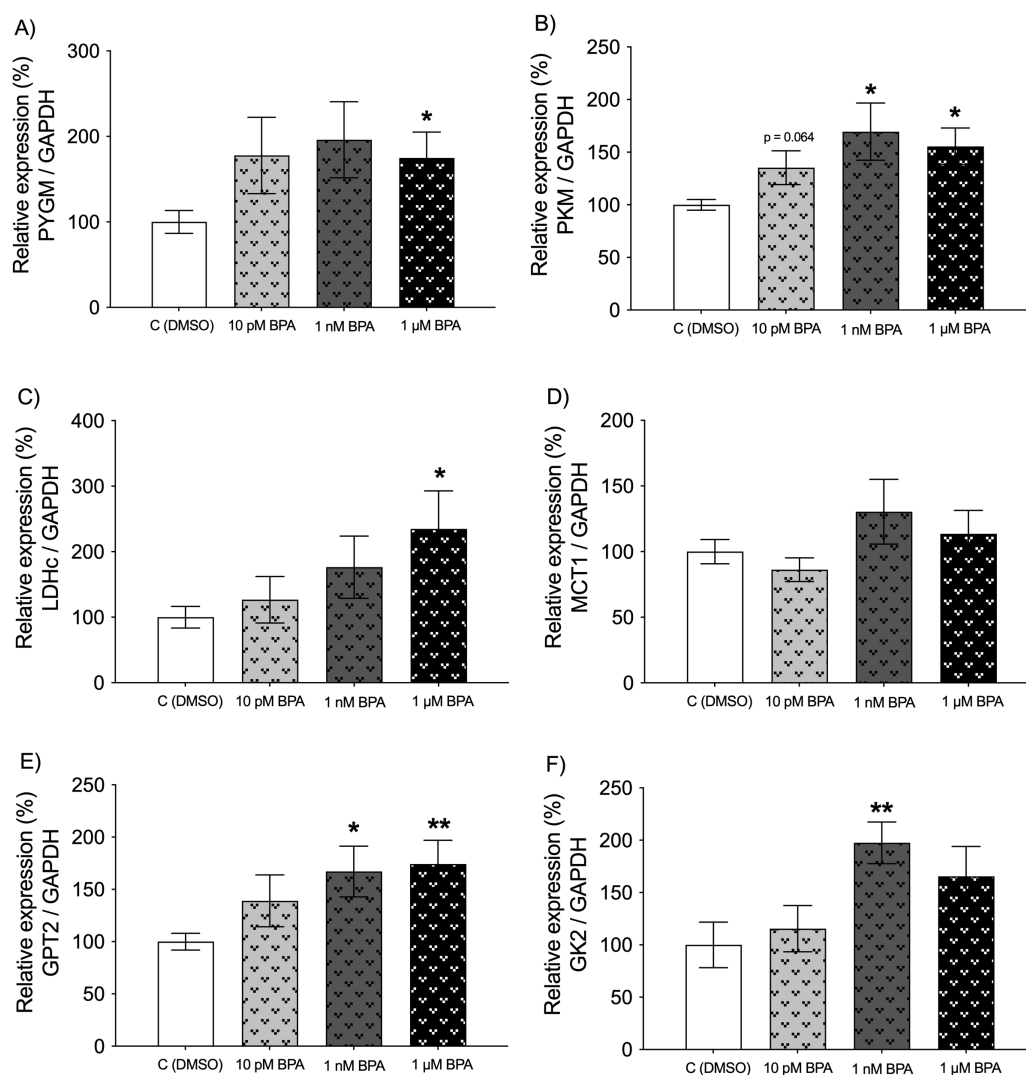
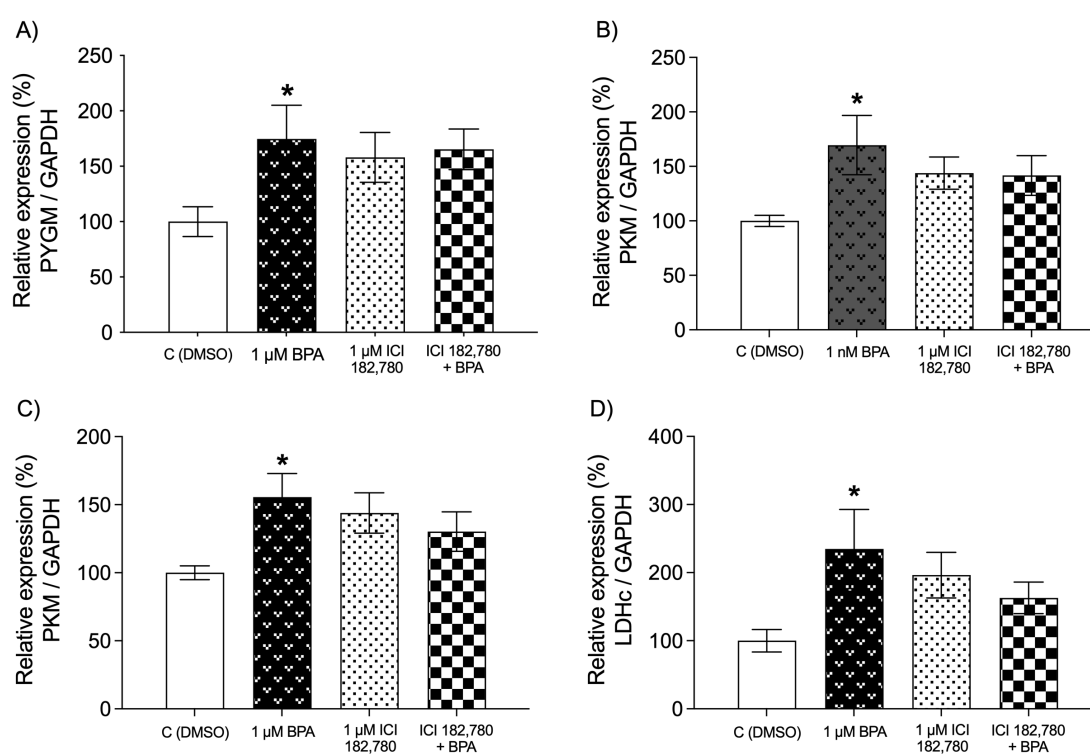


Figure 45: Effects of 10 pM, 1 nM and 1 μM BPA on the relative expression of energy metabolism-associated genes in immature rats. The relative expression of (A) PYGL, (B) PKMA, (C) LDHc, (D) MCT1, (E) GPT2 and (F) GK2 was analyzed after *in vitro* exposure of testis explants from 20-day-old immature rats to 10 pM, 1 nM and 1 μM BPA for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean \pm S.E.M. of 6-9 animals in each group from two or three independent experiments. * $p \leq 0.05$ and ** $p \leq 0.01$ when compared to control group. C = control.

4.2.2.2 Changes in the expression of PYGM, PKM, LDHc, GPT2 and GK2 in immature rat testis after 6 hours of incubations with BPA are not dependent on nuclear estrogen receptors

In order to investigate the participation of ESR α / β in the mechanism of action of BPA in modulating the expression of testicular energy metabolism-associated genes, testis explants were pre-incubated with the antagonist ICI 182,780 1 hour prior to BPA incubations for 6 hours. The increase in the relative expression of PYGM (Figure 46A), PKM (Figure 46C), LDHc (Figure 46D) and GPT2 (Figure 46F) by incubation with 1 μ M BPA was not reversed by pre-incubation with ICI 182,780. In addition, the increase in the relative expression of PKM (Figure 46B), GPT2 (Figure 46E) and GK2 (Figure 46G) by incubation with 1 nM BPA was also not reversed by pre-incubation with ICI 182,780, indicating that these genomic effects of BPA are independent of ESR α / β activation.



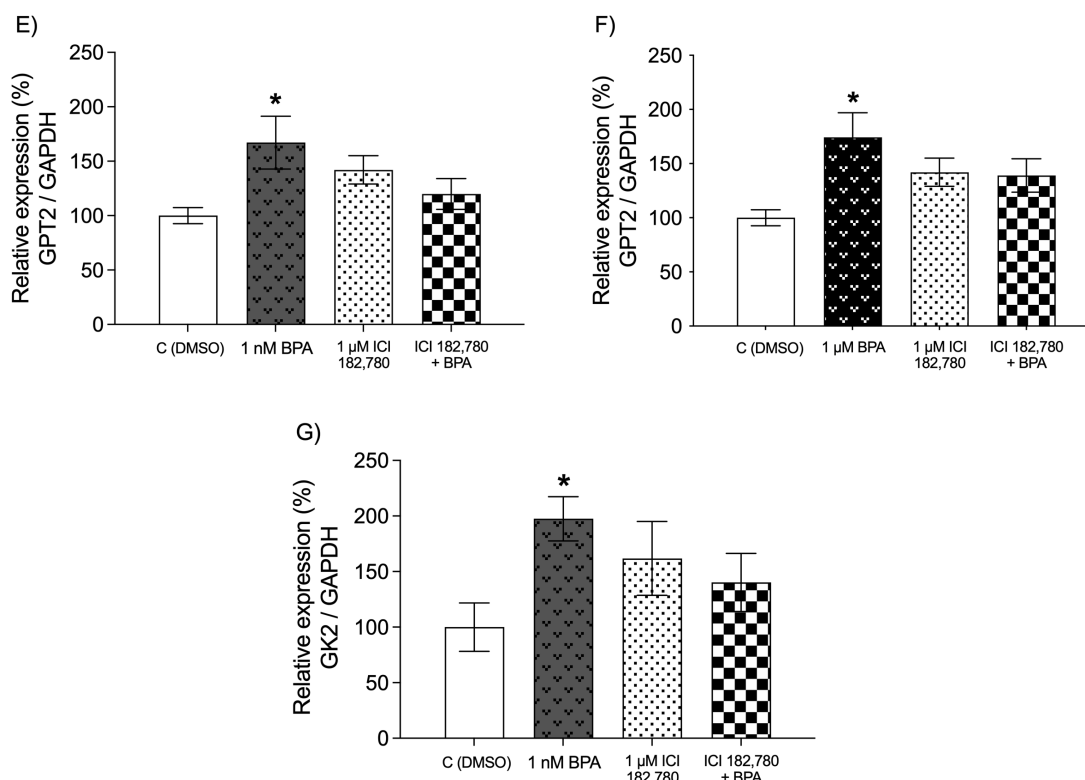


Figure 46: Involvement of nuclear estrogen receptors (ESR) in the mechanism of action of BPA on the relative expression of testicular energy metabolism-associated genes in immature rats. The relative expression of (A) PYGM, (B and C) PKM, (D) LDHc, (E and F) GPT2, (G) GK2 was analyzed in the testis explants from 20-day-old immature rats after pre-incubation with 1 μ M ICI 182,780 (ESR α/β antagonist) for 1 hour and incubations with (A, C, D and F) 1 μ M, and (B, E and G) 1 nM BPA for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean \pm S.E.M. of 6-9 animals in each group from two or three independent experiments. * $p \leq 0.05$ compared to the control group.

4.2.2.3 Acute *in vitro* exposure to BPA does not modulate the expression of spermatogonia and spermatocytes marker genes in immature rat testis

In order to correlate changes in testicular energy metabolism caused by BPA with possible changes in spermatogenesis, the expression of specific genes of germ cells was analyzed. The relative expression of ITGa6, PCNA and DMC1 was analyzed in testis explants from immature rats after 6 hours of incubations with BPA at 10 pM, 1 nM and 1 μ M. However, *in vitro* exposure to different BPA concentrations for 6 hours did not change the relative expression of ITGa6 (Figure 47A), PCNA (Figure 47B) and DMC1 (Figure 47C) in relation to the control group. However, there was only a tendency to increase PCNA relative expression ($p = 0.076$) by 1 nM BPA *in vitro* exposure (Figure 47B).

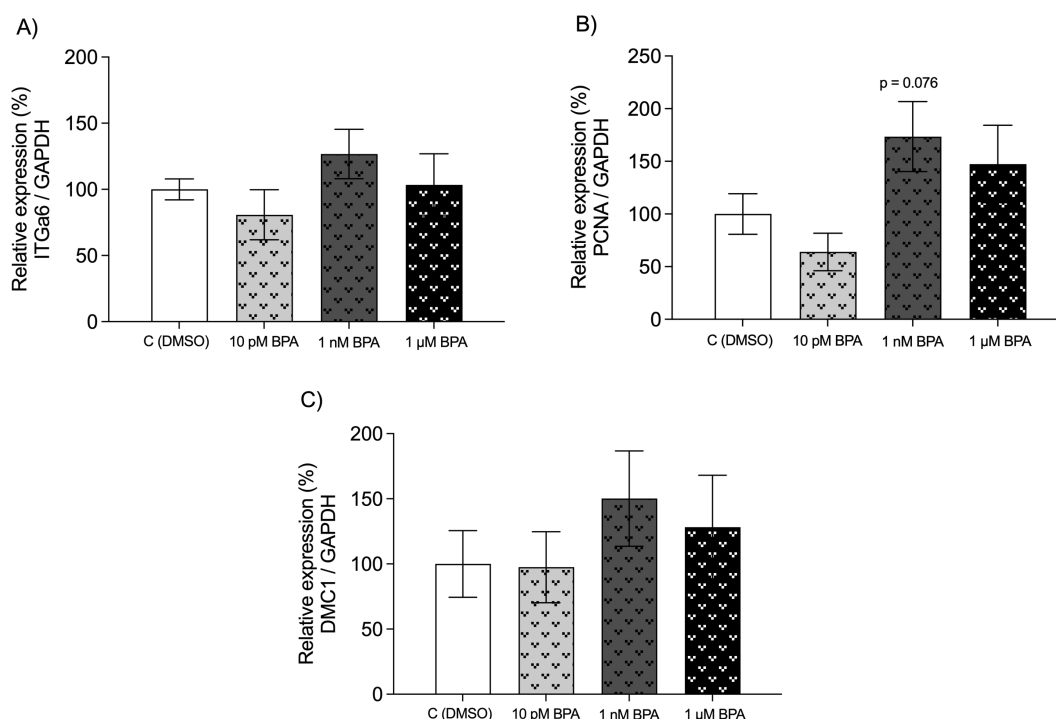


Figure 47: Effects of 10 pM, 1 nM and 1 μM BPA on the relative expression of spermatogonia and spermatocytes cell marker genes in immature rats. The relative expression of (A) ITGa6, (B) PCNA and (C) DMC1 was analyzed after *in vitro* exposure of testis explants from 20-day-old immature rats to 10 pM, 1 nM and 1 μM BPA for 6 hours. The gene expression of the control group is fixed at 100%. The results are expressed as relative expression (%) of target genes normalized by GAPDH reference gene. The data are expressed as mean ± S.E.M. of 6 animals in each group from two independent experiments. C = control.

4.2.3 Discussion of Chapter II

It has reported that E2 regulates the expression of genes involved in sexual differentiation, different phases of spermatogenesis, energy metabolism and Ca^{2+} signaling (PINTO et al. 2006; BALASINOR et al. 2010; ZHENG et al., 2019). E2 at physiological levels is essential for the correct performance of normal spermatogenesis, while its excess is associated with impaired spermatogenesis and infertility (PAVLOVICH et al., 2001; WALCZAK-JĘDRZEJOWSKA et al., 2013; LE et al., 2019). Furthermore, a relationship between estradiol-induced azoospermia and disturbance of testicular energy metabolism has already been shown (LE et al., 2019). However, the human population, especially during early childhood, is exposed to xenoestrogens such as BPA (NATIONAL TOXICOLOGY PROGRAM, 2008; EFSA, 2015). A growing number of scientific reports reveal that exposure to xenoestrogens leads to a decline in male reproductive capacity, associated with testicular atrophy, reduced number and quality of spermatozoa (JENSEN et al., 1995). High plasma and

seminal fluid of E2 (PAVLOVICH et al., 2001; VITKU et al., 2015) and BPA were found in infertile men, and negatively correlated with number of spermatozoa (VITKU et al., 2015).

Through the use of short-term *in vitro* testing, our studies show that E2 and BPA, but mainly BPA, induce changes in the expression of energy metabolism-associated genes in testis explants from 20-day-old rats. The present study was the first to report that PYGM expression was stimulated by both E2 and BPA at 1 μ M in the testis of rats. PYGM mRNA levels have already been reported in adult rat testes (DAVID; CRERAR, 1986). Glycogen phosphorylase has been reported to be activated by FSH, cAMP and Ca^{2+} -dependent mechanisms in rat Sertoli cells (SLAUGHTER; MEANS, 1983). Interestingly, glycogen content was reported to be more pronounced in the testes of immature rats than in adult rats (LEIDERMAN; MANCINI, 1969). In addition, the high ratio of phosphorylase a to the total phosphorylase activity observed in immature male rats has been comparable to the ratio found when the enzyme from skeletal muscle or adipose tissue is fully activated in response to hormonal or nutritional stimuli (SLAUGHTER; MEANS, 1983). Thus, these data may suggest a putative effect of glycogen metabolism on establishment of spermatogenesis. Therefore, the increased PYGM expression may be due to the need for the energy source supplied by glycogenolysis. As glycogen can be used during periods when the availability of other preferred energy sources decreases, it is suggested that both E2 and BPA induced increased use of other energy substrates. Previous studies reinforce our findings showing that Sertoli cells from humans (OLIVEIRA et al., 2011) and from 20-day-old rats (RATO et al., 2012b) cultured under different experimental conditions, including in the presence of E2, have a high pyruvate consumption rate. Almost all available pyruvate is consumed by Sertoli cells within the first 5 hours of incubation (RATO et al., 2012b). In addition, another study reported that mice treated with estradiol benzoate showed a decrease in ATP content, hexokinase and pyruvate kinase activities (LE et al., 2019). Therefore, the increased relative expression of PKM in the testis after 6 hours of incubation with E2 and BPA at 1 nM and 1 μ M may have been due to the need to increase pyruvate production. Pyruvate kinase catalyzes the last and irreversible step of glycolysis by converting PEP into pyruvate. It is reported that most of the pyruvate produced by Sertoli cells is converted into lactate (OLIVEIRA et al. 2015) by the action of LDH (JUTTE et al., 1983; ROBINSON; FRITZ, 1991).

Lactate has been reported for decades as the preferred energy substrate for spermatocytes and spermatids (MITA; HALL, 1982; GROOTEGOED; JANSEN; VAN DER MOLEN, 1984; ALVES et al., 2013a). Thus, the increased relative expression of LDHc in the testis after 6 hours of incubation with E2 and BPA observed in the present study could suggest a compensatory genomic response by the need for lactate production. Previous data support our findings reporting that lactate production is suppressed by E2 and BPA, however, obtained over longer periods of exposure to higher concentrations of E2 and BPA than those in the present study. A reduced lactate production rate is reported in Sertoli cells from 20-day-old rats incubated with 100 nM E2 between 15 and 25 hours of culture (RATO et al., 2012b). In addition, *in vitro* exposure to E2 for 24 hours has also been reported to suppresses the lactate production in a dose-dependent manner in Sertoli cells of *Hemidactylus flaviviridi* lizard (KHAN; RAI, 2004). Furthermore, *in vitro* exposure to high concentrations of BPA (10 μ M and 1 mM) for 48 hours has been impaired glucose metabolism by decreasing the lactate production, gene expression and immunocontent of LDH in rat Sertoli cells (HUANG et al., 2015).

Most of the pyruvate produced by Sertoli cells is converted into lactate (OLIVEIRA et al. 2015), then exported to the intratubular fluid via MCT4 and later to the germ cells via MCT1 or MCT2 (SKINNER, 1991; RATO et al., 2012). It has been reported that although MCT1 is expressed in spermatogonia, spermatocytes and spermatids, its predominant expression is in rat and mouse spermatogonia (KISHIMOTO et al., 2015). However, under our experimental conditions, the expression of MCT1 was not altered by either E2 or BPA in the immature rat testis, at least after 6 hours of incubation. To date, only a previous study obtained in adult rat brain have been reported that MCT1 expression is reduced by BPA. However, this reduction was mediated by early *in vivo* exposure to BPA from birth to postnatal day 21 (XU et al., 2017). Nevertheless, the expression of MCT4 was reduced in Sertoli cells from 20-day-old immature rats (RATO et al. 2012b), while it was increased in human Sertoli cells (OLIVEIRA et al. 2011) after 50 hours of *in vitro* treatment with 100 nM E2. In addition, a decreased expression of MCT2 and MCT4 was also observed in the testes of mice treated with estradiol benzoate for 4 weeks (LE et al., 2019). Therefore, these data indicate that short *in vitro* exposure to E2 and BPA does not interfere with lactate transport via MCT1, while their long-term effects interfere with lactate transport by MCT1, MCT2 and MCT4-dependent mechanisms.

In addition to glucose and glycogen metabolism, amino acid metabolism is also involved in the energy metabolism of Sertoli cells (MATEUS et al., 2018). However, studies related to the expression and activity of ALT in the testis are rare even in mammals. Our findings demonstrated for the first time that the relative expression of GPT2, which encodes ALT2, was significantly stimulated in the immature rat testis after 6 hours of incubation with BPA (1 nM and 1 μ M). Thus, we show unprecedented evidence that GPT2 is a molecular target of the genomic effect of BPA in the testis of immature rats. On the other hand, E2 did not significantly alter GPT2 expression, it only showed a tendency ($p = 0.061$) to increase it. In addition to being the end product of glycolysis, pyruvate may also be generated by ALT, which catalyzes the reversible interconversion of alanine and α -ketoglutarate into pyruvate and glutamate (SHERMAN, 1992; LIU et al., 2014). Thus, the increased BPA-mediated GPT2 expression in the immature rat testis could suggest a compensatory genomic response by the need for pyruvate or alanine production, as already suggested by the high pyruvate consumption rate by Sertoli cells (OLIVEIRA et al., 2011; RATO et al., 2012b). Those previous data reinforce our findings reporting that Sertoli cells from immature rats (RATO et al., 2012b) and from humans (OLIVEIRA et al., 2011) have a drop in alanine production within the first 5 hours of incubation, including in the presence of E2. However, a significant increase in alanine production by immature rat Sertoli cells is reported after 25 hours of incubation with 100 nM E2, indicating a lower redox/higher oxidative state for the cells under these conditions (RATO et al., 2012b). Similar data on E2-induced alanine production have also been reported in human Sertoli cells after 35 hours of incubation (OLIVEIRA et al. 2011). As discussed above, pyruvate is preferentially converted into lactate by NADH-dependent reduction in Sertoli cells. However, some of the pyruvate can be converted into alanine. As the reduction of pyruvate into lactate or its conversion into alanine is related to the reoxidation of cytosolic NADH into NAD⁺, the lactate/alanine ratio reflects the NADH/NAD⁺ ratio (RATO et al., 2012b).

The generation of energy from precursors other than glucose, mainly in states of low energy supply, is crucial for maintaining the function of testicular functions. Hence, in addition to glycogen and alanine, glycerol also contributes to pyruvate production in Sertoli cells. Glycerol converted to glycerol-3-phosphate by the action of GK2 enters the glycolytic pathway (CRISÓSTOMO et al., 2017). Under our experimental conditions, E2 did not change GK2 expression. However, we showed for

the first time that the relative expression of GK2 was significantly stimulated in the immature rat testis after 6 hours of incubation with BPA (1 nM). Thus, we show unprecedented evidence that GK2 is a molecular target of the genomic effect of BPA in the testis of immature rats. This data is in agreement with our previous findings, suggesting that *in vitro* BPA leads to a high energy consumption. However, interestingly, a relationship between high levels of GK2 and disturbances in the motility of spermatozoa has already been reported. GK2 protein levels were higher in asthenozoospermic human spermatozoa (defined as those with <25% progressive motility) than in normozoospermic human spermatozoa (SIVA et al., 2010).

However, none of the genomic effects of BPA observed in the present study were reversed by pre-incubation with the ESR α/β antagonist. Therefore, previous studies are in agreement with our findings, in which reported that the gene expression modulation by BPA is mediated via ESR after long-term exposures, from hours to days (GAIDO et al. al. 1997; LÖSEL; WEHLING, 2003; MOLINA-MOLINA et al., 2013). Hence, increased relative expression of PYGM, PKM and LDHc mediated by E2 and BPA was not dependent on ESR activation. This could suggest that under our experimental conditions, BPA *in vitro* has the same mechanism of action as E2 in inducing the expression of PYGM, PKM and LDHc. Therefore, our findings indicate that short-term E2- and BPA-mediated genomic responses on metabolism-associated genes are transcriptional targets of receptors other than ESR of the classical genomic pathway. A crosstalk is reported between nongenomic and genomic GPER-dependent signaling pathways that converge on gene transcription after short-term exposure to E2 and BPA (CHIMENTO et al., 2010; 2011; GE et al., 2014; HERZ et al., 2017). In addition, it has already been reported that the expression of glycolysis-associated genes is dependent on ERR (GIGUÈRE, 2008; TENNESSEN et al., 2011; CAI et al., 2013).

Furthermore, *in vitro* exposures to E2 and BPA for only 6 hours did not change the expression of none of the germ cell marker gene evaluated in the present study, under our experimental conditions. Nevertheless, only a tendency to increase PCNA relative expression ($p = 0.076$) by 1 nM BPA was observed. PCNA is one of the most used spermatogonial cell proliferation markers (SCHLATT; WEINBAUER, 1994; FRANCO et al., 2010). However, previous studies reinforce our data reporting that modulation in the expression of the germ cell marker genes accompanied by changes in spermatogenesis are mediated by BPA or estradiol over periods of exposure longer

than 6 hours (ALI et al., 2014; ZHANG et al., 2018; LIU; WANG; LIU, 2021). *In vitro* exposure of TM4 Sertoli cells to nanomolar concentrations of BPA for 48 hours resulted in increased PCNA immunocontent, as well as decreased expression of p21 and p53 (GE et al., 2014). On the other hand, *in vivo* chronic treatment with BPA was recently reported to decrease PCNA protein expression in mice testes (LIU; WANG; LIU, 2021). In addition to BPA, *in vivo* chronic treatment with estradiol benzoate has also been reported to decrease PCNA gene expression in the mice seminiferous tubules (ZHANG et al., 2018).

4.2.4 Conclusions of Chapter II

It was evidenced that short-term incubations *in vitro* of testis explants from immature rats to E2:

- Affect testicular energy metabolism by altering the relative expression of genes associated with carbohydrate metabolism PYGM, PKM and LDHc, independent of the participation of ESR α / β .
- Do not affect the expression of germ cells marker genes: ITGa6, PCNA and DMC1.

In addition, it was evidenced that short-term incubations *in vitro* of testis explants from immature rats to BPA:

- Affect testicular energy metabolism by altering the relative expression of genes associated with energy metabolism PYGM, PKM, LDHc, GPT2 and GK2, independent of the ESR α / β -participation.
- Do not affect the expression of germ cells marker genes: ITGa6, PCNA and DMC1.

5 CONCLUSIONS

Overall, our data collectively evidence that short-term exposures to BPA impair Ca^{2+} homeostasis, energy metabolism and spermatogenesis. The acute *in vitro* exposure of zebrafish testis to a low concentration of BPA increases Ca^{2+} influx and cytosolic Ca^{2+} overload involving crosstalk between non-genomic and genomic signaling pathways. In addition, our study evidenced through different methodological approaches that *in vitro* and *in vivo* exposures to BPA affect testicular metabolism of carbohydrates and impair spermatogenesis in zebrafish through its nongenomic and genomic effects dependent or not on ESR α/β . Therefore, this work showed unprecedented evidence of the influence of impaired testicular energy metabolism as one of the possible causes that may lead to impaired spermatogenesis in fish, which is the most abundant group of vertebrates. However, additional studies will be needed to reinforce this hypothesis.

Furthermore, *in vitro* exposures to E2 and BPA impair energy metabolism by altering gene expression in the testis of immature rats. BPA seems to have the same mechanism of action as E2 in the ESR α/β -independent induction of the expression of genes associated with carbohydrates metabolism. However, additional studies will be needed to reinforce this hypothesis.

Overall, these results collectively evidence that BPA impairs testicular energy metabolism at different stages of sexual maturation: in adult fish, as an aquatic animal model organism, as well as in immature rat, as a mammalian animal model. In addition, BPA impairs the establishment and correct progression of spermatogenesis in zebrafish. This may affect male fertility and reproduction, which could impact the aquaculture field, since fish are important for the balance of the aquatic ecosystem and are important sources of food and income for many communities. In addition, our findings evidenced new molecular targets by which BPA and E2 may impact male reproduction of both aquatic and terrestrial vertebrate species.

6 FUTURE PERSPECTIVES

In relation to Chapter I, we highlight the importance of further studies to investigate the involvement of Ca^{2+} signaling and correlate it with the *in vitro* effects of BPA on testicular energy metabolism. Moreover, we highlight the importance of additional studies evaluating long-term effects of BPA on energy substrates and activities of key enzymes of energy metabolism for a further understanding of its impacts on testicular energy metabolism. In addition, additional studies investigating the involvement of other receptors in its mechanism of action would be needed to understanding the mechanisms by which BPA impact energy metabolism and spermatogenesis. Furthermore, we highlight the importance of *in vitro* approach of spermatogenesis using inhibitors of key enzymes of energy metabolism to investigate the participation of such target enzymes in the BPA-mediated impairment in the progression of spermatogenesis in zebrafish.

In relation to Chapter II, it is intended to continue the quantitative analysis of the immunohistochemical expression of LDH and PKM in testicular histological from immature rats exposed *in vitro* with E2 and BPA. In addition, it is also intended to perform morphological analyses on spermatogenesis parameters to correlate the impact of the genomic effects of E2 and BPA on energy metabolism with the establishment of spermatogenesis in immature rats. Furthermore, we highlight the importance of further studying the long-term effects of BPA and E2 on the measurement of energy substrates, gene expression and activities of key enzymes of energy metabolism for a better understanding of their impacts on testicular energy metabolism. In addition, additional studies investigating the involvement of other receptors in their mechanism of action will also be needed to understand the mechanisms by which BPA and E2 impact energy metabolism and spermatogenesis. Furthermore, we highlight the importance of an *in vitro* approach of spermatogenesis using inhibitors of key enzymes of energy metabolism to investigate the participation of such target enzymes in the BPA-mediated impairment of establishment of spermatogenesis in immature rats.

RÉSUMÉ SUBSTANTIEL

Introduction

Le testicule possède deux fonctions majeures : la production de spermatozoïdes (spermatogenèse) et d'hormones stéroïdes (stéroïdogenèse) (MATTA, 2002). En ce qui concerne à sa morphologie, le testicule est constitué de deux compartiments principaux : l'interstitiel et le tubulaire (KOULISH ; KRAMER ; GRIER, 2002). Le compartiment interstitiel est principalement composé de cellules de Leydig, tandis que l'épithélium tubulaire est composé de cellules de Sertoli et de cellules germinales. La spermatogenèse est un événement biologique complexe hautement arrangée dans laquelle les cellules souches spermatogoniales deviennent des spermatozoïdes par une série de processus impliquant la prolifération, la division et la différenciation cellulaires. Cependant, la spermatogenèse chez les poissons présente certaines différences par rapport à la spermatogenèse chez les mammifères en ce qui concerne les paramètres morphologiques et physiologiques. Parmi eux, la principale différence est que les poissons téléostéens présentent une spermatogenèse kystique (SCHULZ et al., 2010 ; URIBE ; GRIER ; MEJÍA-ROA, 2015), qui se produit dans les kystes qui se forment dans les tubules séminifères lorsque des spermatogonies indifférenciées sont complètement enveloppées par les extensions cytoplasmiques des cellules de Sertoli (MATTA, 2002 ; SCHULZ et al., 2005, 2010 ; LEAL et al., 2009a). La durée combinée des phases de méiose et spermiogenèse de la spermatogenèse, chez le poisson *Danio rerio* (zebrafish) est d'environ 6 jours (LEAL et al., 2009a), tandis que la durée estimée de la spermatogenèse chez le rat est d'environ 52 jours (CLERMONT ; HARVEY, 1965 ; CLERMONT, 1972). De plus, une autre différence importante est que les cellules de Sertoli des poissons conservent leur capacité à proliférer même au stade adulte (SCHULZ et al., 2005 ; LEAL et al., 2009a), contrairement aux cellules de Sertoli des rats, qui ne prolifèrent que jusqu'au stade infantile ou juvénile, avant d'atteindre la puberté (ORTH et al., 1982). Ainsi, les testicules des mammifères adultes contiennent un nombre fixe de cellules de Sertoli définies avant la puberté qui déterminent la capacité de production de spermatozoïdes et la taille du testicule (SHARPE et al., 2003 ; JOHNSON ; THOMPSON ; VARNER, 2008). Le bon déroulement de la spermatogenèse est donc étroitement lié au fonctionnement des cellules de Sertoli, car elles sont chargées de fournir les soutiens physique, nutritionnel et énergétique aux cellules germinales (SCHULZ et al., 2010 ; RATO et al., 2012b ; ALVES et al., 2013a). Le lactate métabolisé et fourni par les cellules de Sertoli est considéré comme la principale source d'énergie des spermatocytes et des spermatides de rongeurs et humains. Les cellules de Sertoli métabolisent principalement le glucose pour produire du lactate par la glycolyse, où le pyruvate est converti en lactate par l'enzyme lactate déshydrogénase (LDH) (MITA ; HALL, 1982 ; JUTTE et al., 1983 ; GROOTEGOED ; JANSEN ; VAN DER MOLEN, 1984 ; ALVES et al., 2013a). En plus du glucose, les cellules de Sertoli peuvent également utiliser du glycogène, de l'alanine et du glycérol

comme substrats énergétiques pour la production de pyruvate/lactate (RATO et al., 2012b ; ALVES et al., 2013a).

L'ion calcium (Ca^{2+}) joue un rôle important dans la régulation du métabolisme agissant comme cofacteur dans plusieurs réactions enzymatiques du métabolisme énergétique (PICTON ; KLEE ; COHEN, 1981 ; HANSFORD, 1985 ; BOGONEZ ; GOMEZ-PUERTAS ; SATRÚSTEGUI, 1992 ; TRAASETH et al., 2004). De plus, Ca^{2+} est un important second messenger cellulaire impliqué dans la prolifération, la différenciation et l'apoptose des cellules germinales (GALARDO et al., 2014 ; GOLPOUR ; SENICKA ; NIKSIRAT, 2017) ; ainsi que dans la maturation et motilité des spermatozoïdes (ALAVI et al., 2004 ; GOLPOUR ; SENICKA ; NIKSIRAT, 2017) et la réaction acrosomique (DE BLAS et al., 2002). Cependant, l'un des paradoxes concernant le Ca^{2+} est qu'il est un signe de vie ou de mort, car il est bien connu qu'une surcharge intracellulaire en Ca^{2+} peut entraîner une mort cellulaire programmée appelée apoptose (BERRIDGE ; BOOTMAN ; LIPP, 1998 ; PINTON et al., 2008). De plus, 17β -estradiol (E2) joue un rôle important dans la régulation de processus cellulaires cruciaux pour la spermatogenèse, tels que le renouvellement des cellules souches spermatogoniales (MIURA et al., 1999), la prolifération cellulaire (PINTO et al. 2006) et la division cellulaire (ZHENG et al., 2019). Les actions de l'E2 sont réalisées via les récepteurs membranaires ou récepteurs nucléaires (voie classique), notamment en se liant aux récepteurs nucléaires aux œstrogènes (ESR) α et β (ESR α/β). Ainsi, l'E2 module l'expression d'une large variété de gènes impliqués dans la prolifération et la division cellulaire, le métabolisme énergétique, la signalisation calcique (PINTO et al. 2006 ; ZHENG et al., 2019) ainsi que le maintien du cytosquelette cellulaire et de la spermiation (BALASINOR et al. 2010).

Cependant, les effets de l'E2 peuvent être bénéfiques ou néfastes, ce qui dépend principalement en fonction de sa concentration (physiologique ou supra-physiologique). Il est démontré que des niveaux accrus d'œstrogènes naturels et environnementaux (xénoœstrogènes), tels que le bisphénol A (BPA), affectent négativement les systèmes reproducteur et endocrinien et, par conséquent, la fertilité masculine (GODSLAND, 2005 ; THOMAS ; DONG, 2006 ; ROUILLER-FABRE et al., 2015 ; HAQ et al., 2020). Ce qui le rend encore plus préoccupant, c'est que les humains et les animaux aquatiques sont susceptibles d'être exposés au BPA. L'exposition humaine au BPA est un phénomène fréquent, car le BPA est libéré du plastique polycarbonate à des températures élevées (LE et al., 2008) et sa présence a été déjà détectée dans de nombreux fluides biologiques (OLEA et al., 1996 ; SUN et al., 2004 ; MENDONÇA et al., 2014). Cependant, le pire scénario d'exposition au BPA est associé à la petite enfance (U.S. Food and Drug Administration, 2010 ; EFSA, 2015). Tenant compte du fait que cette période chez l'homme correspond au stade de développement prépubère similaire à celui du rat (OJEDA ; ADVIS ; ANDREWS 1980), les études dans ce modèle animal deviennent encore plus pertinentes pour étudier l'impact du BPA sur la reproduction masculine. Ainsi, comme la spermatogenèse n'est pas encore établie dans la phase prépubère, il s'agit d'une phase critique du développement reproducteur qui est très vulnérable aux changements causés par les œstrogènes et les xénoœstrogènes (NORIEGA et al. 2009 ; MOURITSEN et al., 2010).

; OLIVEIRA et al., 2017). De plus, la présence de BPA a également été documentée dans l'eau des rivières et les poissons (YANG et al., 2014 ; WU et al., 2016), ce qui indique que ce produit chimique est un facteur de risque pour les écosystèmes aquatiques et la santé humaine. Le BPA est rejeté dans les milieux aquatiques, tels que les rivières et les mers par la migration des produits à base de BPA et des effluents des usines de traitement des eaux usées et des décharges (KANG ; AASI ; KATAYAMA, 2007). Bien que le BPA soit signalé comme un perturbateur endocrinien qui affecte la reproduction mâle, il n'y a toujours pas de rapports chez les poissons se concentrant sur les changements biochimiques et moléculaires du métabolisme énergétique testiculaire et l'impact sur la spermatogenèse. Par conséquent, l'étude chez les poissons devient encore plus pertinente pour la découverte de nouvelles cibles et mécanismes cellulaires et moléculaires par lesquels le BPA affecte la reproduction mâle. Ainsi, l'utilisation du zebrafish comme organisme modèle est très pertinente dans les domaines de l'écotoxicologie et de l'aquaculture, car les poissons sont importants pour l'équilibre de l'écosystème aquatique et sont d'importantes sources de nourriture et de revenus pour de nombreuses communautés. Par conséquent, l'objectif général de cette thèse a été d'étudier les effets du BPA et/ou de l'E2 sur la signalisation calcique, le métabolisme énergétique testiculaire et la spermatogenèse chez le zebrafish adulte et/ou chez le rat immature.

Objectifs spécifiques – Chapitre I

- Caractériser les voies de signalisation du BPA dans l'influx calcique, évaluer des paramètres biochimiques des effets *in vitro* et *in vivo* du BPA sur le métabolisme énergétique testiculaire, ainsi que ses effets *in vivo* sur des paramètres histologiques et d'apoptose sur la spermatogenèse dans les testicules de zebrafish.
- Analyser les effets génomiques *in vitro* du BPA et l'implication des ESR α/β sur l'expression des gènes impliqués au métabolisme énergétique testiculaire et des gènes marqueurs des cellules germinales chez le zebrafish.
- Évaluer les effets *in vitro* du BPA sur l'expression de gènes codant pour des récepteurs nucléaires et sur l'expression de protéines impliquées dans le métabolisme énergétique et dans la prolifération cellulaire dans des testicules de zebrafish.

Objectifs spécifiques – Chapitre II

- Étudier les effets *in vitro* de l'E2 et du BPA, ainsi que l'implication des ESR α/β sur l'expression des gènes impliqués au métabolisme énergétique et des gènes marqueurs de cellules germinales dans des testicules de rats immatures.

Pour une meilleure compréhension de ce travail et ce résumé, les méthodes, résultats et discussion ont été divisés en chapitre I (parties I et II) qui correspondent aux études chez les poissons *Danio rerio* (zebrafish) adultes, et chapitre II qui correspond aux études chez les rats Wistar immatures de 20 jours post-partum.

Matériels et Méthodes – Partie I du Chapitre I

Des études *in vitro* sur l'influx calcique ont été réalisées conformément à Batista-Silva et al. (2020) dans des testicules de zebrafish. Pour cela, les testicules ont été incubés avec du tampon Cortland contenant 0,1 $\mu\text{Ci/ml}$ de Ca^{2+} radioactif ($^{45}\text{Ca}^{2+}$) en l'absence (contrôle) ou en présence de BPA à différentes concentrations (1 fM, 1 pM, 10 pM, 1 nM, 10 nM et 1 μM) pendant 30 minutes pour obtenir une courbe concentration-réponse. Ensuite, pour étudier le mécanisme d'action du BPA sur l'influx calcique, des bloqueurs, des antagonistes et des inhibiteurs ont été ajoutés 15 minutes avant l'incubation du $^{45}\text{Ca}^{2+}$ et du BPA (10 pM). Les bloqueurs utilisés et les canaux évalués étaient : nifédipine, un bloqueur des canaux calciques de type L dépendants du voltage (L-VDCC) (BATISTA-SILVA et al., 2020) ; 9-anthracène et CFTR (inh)-172, des bloqueurs des canaux chlorures dépendants du Ca^{2+} (CaCC) et régulateur de la conductance transmembranaire de la fibrose kystique (CFTR) (GONÇALVES et al., 2018), respectivement. Les antagonistes utilisés et les récepteurs évalués étaient : capsazépine, un antagoniste du membre 1 de la sous-famille vanilloïde des canaux cationiques potentiels de récepteur transitoire (TRPV1) (AUZANNEAU et al., 2008) ; 2-APB, un antagoniste des récepteurs de l'inositol triphosphate (IP3R) (PETERS ; PIPER, 2007), et ICI 182,780, un antagoniste des $\text{ESR}\alpha/\beta$. Les inhibiteurs utilisés et les enzymes évaluées : thapsigargine, un inhibiteur de la Ca^{2+} -ATPase du réticulum sarco/endoplasmique (SERCA) ; H-89, un inhibiteur de la protéine kinase A (PKA) ; Ro 31-0432, un inhibiteur de la PKC ; LY 294002, un inhibiteur de la phosphatidylinositol 3-kinase (PI3K), et PD 98059, un inhibiteur de MEK 1/2 (*Mitogen activated protein kinase kinase*) (GONÇALVES et al., 2018).

À la fin de la période d'incubation, une solution de chlorure de lanthane a été utilisée pour arrêter le flux de Ca^{2+} (BATRA, 1982). Après, la mesure de la radioactivité a été réalisée dans un spectromètre à scintillation liquide LKB rack bêta. De plus, afin d'étudier si l'exposition aiguë *in vitro* au BPA induit des dommages à la membrane cellulaire, la libération de LDH dans le milieu extracellulaire a été évaluée. Ainsi, l'activité de la LDH a été analysée après des incubations des testicules de zebrafish pendant 1 heure avec du BPA à différentes concentrations (10 pM, 10 nM et 10 μM). De plus, l'implication de l'activation des IP3R et de la libération de Ca^{2+} des réserves calciques intracellulaires dans le mécanisme de libération de la LDH a également été analysée. Par la suite, l'implication de la libération de Ca^{2+} à partir des réserves calciques intracellulaires dans l'augmentation induite par le BPA de la teneur en triglycérides (TAG) dans des testicules a également été étudiée.

Matériels et Méthodes – Partie II du Chapitre I

Cette partie du Chapitre I a été consacrée à l'analyse des paramètres biochimiques, moléculaires et histologiques des effets *in vitro* et/ou *in vivo* du BPA sur le métabolisme énergétique et la spermatogenèse du zebrafish. Premièrement, des testicules des poissons ont été incubés pendant 1 heure ou les poissons ont été traités *in vivo* pendant 12 heures avec 10 pM et 10 μM de BPA. Après, la teneur en lactate et

et les activités de la LDH, de l'alanine aminotransférase (ALT) et de l'aspartate aminotransférase (AST) ont été mesurées dans des testicules de zebrafish conformément aux instructions du fabricant (Labtest Liquiform ; Brésil). De plus, la teneur en glycogène a été mesurée par la méthode de Krisman (KRISMAN, 1962), ainsi que l'absorption du ^{14}C -désoxy-D-glucose dans des testicules a été réalisée selon Batista-Silva et al. (2020).

De plus, la culture organotypique de testicule de zebrafish a été réalisée selon Leal et al. (2009b) afin d'étudier les effets *in vitro* du BPA sur l'expression des gènes, l'immunocontenu et l'expression des protéines, ainsi que des paramètres histologiques de la spermatogenèse et apoptose. Les testicules ont été incubés dans du milieu de culture L-15 Leibovitz additionné d'acide rétinoïque, avec les traitements : 10 pM et 10 μM de BPA, ou 0,1% et 0,0000001% de DMSO (contrôle) pendant 6 et/ou 72 heures. De plus, afin d'étudier l'implication des ESR α/β dans le mécanisme d'action du BPA, les testicules du groupe de 72 heures ont été pré-incubés en l'absence ou en présence de 10 μM d'ICI 182,780 (ULHAQ ; KISHIDA, 2018), un antagoniste des ESR α/β , pendant 2 heures. Après, pour analyser l'expression relative d'une variété de gènes, l'extraction des ARN testiculaire, la rétrotranscription pour obtenir de l'ADN complémentaire et la réaction en chaîne par polymérase (PCR) en temps réel ont été réalisées. Les gènes analysés étaient : 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2a (PFKFB2a), pyruvate kinase M1/2a (PKMA), LDHBa, alanine aminotransférase (GPT2), l'isoforme hépatique de la glycogène phosphorylase (PYGL), pyruvate carboxylase (PCXA), transporteur de monocarboxylate 4 (MCT4) ; les ESR β 2 (ESR2a) et ESR β 1 (ESR2b), les récepteurs liés aux œstrogènes α (ESRR α) et δ (ESRR δ) ; PIWIL-1 (*P-element induced wimpy testis-like*), protéine du complexe synaptonémal 3 (SYCP-3); la protéine de fibre dense externe 3b (ODF3b) et la protéine ribosomique L8 (RPL8). De plus, l'extraction de protéines et Western Blot ont été réalisées pour analyser l'immunocontenu de PKM, l'antigène nucléaire des cellules proliférantes (PCNA), le phospho-ERK 1/2 (p-ERK 1/2) et le total-ERK 1/2 (t-ERK 1/2). Les membranes ont été incubées pendant une nuit avec les anticorps primaires l'anti- β -actine clone C4 (protéine de référence), l'anti-PKM, l'anti-PCNA, l'anti-p-ERK 1/2 ou l'anti-t-ERK 1/2, puis avec les anticorps secondaires IgG-HRP. La révélation des bandes protéiques a été réalisé par l'ajout d'un substrat chimioluminescent amélioré (ECL) à base de luminol, suivi d'une exposition du film radiographique, du révélateur et du fixateur.

De plus, les traitements *in vitro* (72 heures) des testicules et *in vivo* (12 heures) des poissons avec du BPA, les testicules ont été fixés dans du paraformaldéhyde à 4 %, déshydratés dans des solutions graduées d'éthanol, infiltrés dans du solvant xylène et enfin inclus dans de la paraffine. Des coupes en série (5 μm d'épaisseur) ont été effectués à l'aide d'un microtome rotatif pour subséquents analyses histologiques. La proportion de surface de spermatocytes et de cellules haploïdes (spermatides et spermatozoïdes) a été évaluée à l'aide du logiciel Ilastik par la méthode de classification des pixels et du programme en langage de programmation Python (VAN DER WALT et al., 2014) après les traitements *in vitro* et *in vivo*. De plus, après le traitement *in vivo*, le pourcentage de spermatocytes apoptotiques a été analysé par le

dosage de TUNEL (*Terminal deoxynucleotidyl transferase dUTP nick end labeling*) conformément aux instructions du fabricant (Roche ; Switzerland) et le comptage de cellules TUNEL positives a été effectué à l'aide de l'Aperio Image Scope® via le module de kit d'outils de stéréologie. De plus, l'expression et l'immunolocalisation de la LDH dans les testicules de zebrafish ont été analysées par la technique d'immunohistochimie (IHC). Pour cela, les coupes histologiques ont été incubés pendant une nuit avec l'anticorps primaire anti-LDH (D-9), puis avec l'anticorps secondaire anti-souris IgG-HRP et ensuite avec du DAB (3,3'-diaminobenzidine). Le comptage de cellules LDH positives par surface testiculaire (densité) a été effectué à l'aide du logiciel QuPath.

Matériels et Méthodes – Chapitre II

La culture organotypique d'explants de testicules de rats immatures a été réalisée afin d'étudier les effets *in vitro* de l'E2 et du BPA sur l'expression des gènes. Les explants de testicule ont été incubés avec du milieu de culture DMEM/F-12 (*Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12*) avec les traitements : E2 à 10 pM, 1 nM et 1 µM, ou 0,01 % d'éthanol (groupe contrôle) ; BPA à 10 pM, 1 nM et 1 µM, ou avec 0,01% DMSO (groupe contrôle) pendant 6 heures. De plus, afin d'étudier l'implication des ESRα/β dans le mécanisme d'action de l'E2 et du BPA, les explants de testicule ont été pré-incubés en l'absence ou en présence de 1 µM d'ICI 182,780 (WANG et al., 2014), un antagoniste des ESRα/β, pendant 1 heure. Après, pour analyser l'expression relative d'une variété de gènes, l'extraction et le traitement des ARN testiculaires avec de la DNase I (GREEN ; SAMBROOK, 2019), la rétrotranscription pour obtenir de l'ADN complémentaire et la PCR en temps réel ont été effectués. Les gènes analysés étaient : l'isoforme musculaire de la glycogène phosphorylase (PYGM) ; PKM ; LDHc ; MCT1 ; GPT2 ; glycérol kinase 2 (GK2) ; intégrine α6 (ITGa6) ; PCNA ; ADN recombinase méiotique 1 (DMC1) et glycéraldéhyde-3-phosphate déshydrogénase (GAPDH).

Résultats et discussion – Part I du Chapitre I

Nos résultats ont montré que les traitements *in vitro* des testicules de zebrafish au BPA à 10 pM et à 10 nM pendant 30 minutes ont stimulé l'influx calcique par rapport au groupe contrôle. Par la suite, le mécanisme d'action du BPA à la plus faible concentration sur l'influx calcique a été étudié à l'aide d'une approche pharmacologique utilisant des bloqueurs, des antagonistes et des inhibiteurs. Ainsi, nos résultats ont mis en évidence que l'augmentation de l'influx calcique induite par le BPA à 10 pM a été abolie par les préincubations avec de la nifédipine et du 9-anthracène, suggérant que cette augmentation de l'influx calcique a été induite par les canaux L-VDCC et CaCC. Cependant, l'augmentation de l'influx calcique induite par le BPA n'a pas été reversée par les préincubations avec de la capsazépine et du CFTR(inh)-172, indiquant que ni les récepteurs TRPV1 ni les canaux CFTR ne participent au mécanisme d'action du BPA pour stimuler l'influx calcique. De plus,

l'implication des réserves calciques intracellulaires dans les effets du BPA a également été investiguée. Ainsi, nos résultats ont montré que l'augmentation de l'influx calcique induite par le BPA (10 pM) a été abolie par les préincubations avec du 2-APB et de la thapsigargine, suggérant que cette augmentation de l'influx calcique a également été induite par l'activation de l'IP3R du réticulum endoplasmique et l'inhibition de la SERCA. De plus, des cibles intracellulaires, telles que les protéines kinases, ont également été investiguées dans des testicules de zebrafish. Ainsi, nos résultats ont montré que l'augmentation de l'influx calcique induite par le BPA (10 pM) a été abolie par les préincubations avec les inhibiteurs Ro 31-0432, PD 98059, LY 294002, et l'antagoniste des ESR α/β , ICI 182,780, indiquant que les protéines kinases PKC, MEK 1/2 et PI3K, ainsi que les ESR sont impliqués dans le mécanisme d'action du BPA. En revanche, l'augmentation de l'influx calcique induite par le BPA (10 pM) n'a pas été reversée par la préincubation avec du H-89, indiquant que l'activation de la PKA ne participe pas à cet événement.

De plus, afin d'étudier si l'exposition aiguë *in vitro* au BPA induit des dommages à la membrane cellulaire, la libération de LDH dans le milieu extracellulaire, ainsi que la teneur en TAG ont été analysées. L'incubation des testicules avec 10 μ M de BPA pendant 1 heure a induit une augmentation de l'activité extracellulaire de la LDH par rapport au groupe contrôle, suggérant une augmentation de sa libération du milieu intra vers le milieu extracellulaire. De plus, 10 μ M de BPA a également induit une augmentation de la teneur en TAG testiculaire. Par conséquent, la libération de LDH et l'augmentation de la teneur en TAG induite par la concentration la plus élevée de BPA ont été complètement abolies par la préincubation avec du 2-APB. Par conséquent, cela indique que l'activation de l'IP3R et la libération de Ca²⁺ des réserves calciques intracellulaires sont impliquées dans le mécanisme d'endommagement de la membrane cellulaire induit par 10 μ M de BPA.

Discussion – Part I du Chapitre I

La présente étude a démontré l'effet aigu du BPA à une faible concentration sur la signalisation calcique avec la participation des voies de signalisation impliquant les ESR, protéines kinases MEK 1/2, PI3K et PKC, ainsi que l'IP3R. Cette étude a montré de manière inédite que le BPA active le L-VDCC dans les testicules de zebrafish. Des études avec du BPA ou d'autres xénoestrogènes obtenus dans différents modèles animaux, cellules isolées ou d'autres tissus renforcent nos données. Il a été rapporté que le BPA peut également activer les L-VDCC et canaux de K⁺ voltage-dépendants dans le muscle lisse coronaire humain et canin, mais à des concentrations 10⁶ fois plus élevées que celles utilisées dans notre étude (ASANO ; TUNE ; DICK, 2010). De plus, il a été rapporté que l'exposition chronique au BPA favorise l'augmentation de l'expression de l'ARNm du T-VDCC qui dépend de l'activation des ESR dans des testicules et l'épididyme de la souris (WANG et al., 2015). En outre, il a été rapporté que le bis(2-éthylhexyle)phtalate, un autre xénoestrogène de l'industrie du plastique, présente également une transduction de signal similaire sur l'influx calcique via le L-VDCC (BATISTA-SILVA et al., 2020), ce

qui indique que le L-VDCC est une cible puissante de l'action de xénoestrogènes dans les testicules de zebrafish. Tran et al. (2018) ont montré que le BPA *in utero* chez la souris altère l'implantation embryonnaire par des altérations du transport du Ca^{2+} et de l'expression des gènes des canaux calciques, tels que TRPV 5 et TRPV 6. Cependant, nous avons montré que l'induction de l'influx calcique induite par le BPA est indépendant de l'activation de TRPV1 dans des testicules de zebrafish. Par conséquent, comme l'augmentation de l'influx calcique via le L-VDCC peut se produire par une dépolarisation membranaire dépendante du CaCC (VERKMAN ; GALIETTA, 2009), l'implication du CaCC dans le mécanisme d'action du BPA a été étudiée. CaCC est décrit dans de nombreux types cellulaires différents, tels que les cellules de Sertoli, où ces canaux sont largement impliqués dans la sécrétion cellulaire (MENEGAZ et al., 2010). Il a été démontré qu'une exposition rapide à une très faible concentration de BPA (1 pM) déclenche une augmentation immédiate de l'influx calcique dans des testicules de rats immatures qui dépend du CaCC, ce qui suggère que le BPA peut influencer l'activité sécrétoire dans le testicule (GONÇALVES et al., 2018). Nos résultats sont en accord avec ces données précédentes, démontrant que l'augmentation de l'influx calcique stimulé par le BPA a été également associée à la participation du CaCC dans les testicules de zebrafish. De plus, il a été rapporté que la dépolarisation induite par le CaCC est fondamentale pour la fécondation des ovocytes d'amphibiens. L'augmentation des niveaux intracellulaires de Ca^{2+} due la libération de Ca^{2+} des réserves calciques intracellulaires via l'activation de l'IP3R se produit lors de la fécondation et provoque l'ouverture des CaCC pour produire une dépolarisation membranaire et empêcher la polyspermie (HARTZELL, 1996 ; MACHACA et al., 2002).

Ensuite, le rôle de la voie IP3/IP3R dans la modulation de la libération du Ca^{2+} des réserves calciques intracellulaires a également été étudié, puisque l'activation du CaCC peut également être induite par l'IP3R et l'activation des SOC (*store-operated Ca^{2+} channels*) (KURUMA ; HARTZELL, 2000 ; HARTZELL et al., 2005). De plus, l'influx calcique à travers le L-VDCC démontré dans notre étude pourrait également avoir induit une dépolarisation de la membrane plasmique via l'activation du CaCC. Dans certains types de cellules, la dépolarisation membranaire active le VDCC, ce qui entraîne l'influx calcique et la dépolarisation supplémentaires (VERKMAN ; GALIETTA, 2009), et l'épuisement des réserves intracellulaires de Ca^{2+} stimule l'influx calcique par le SOC, ce qui contribue aussi à l'activation du CaCC (HARTZELL et al., 2005). L'activation de PLC, PKC et IP3R a été associée à l'activation de la voie signalisation du GPER initiée par la membrane (LE MELLAY et al., 1997). Il a été rapporté par Gonçalves et al. (2018) que l'influx calcique induit par le BPA implique l'activation de la PLC dans des testicules de rats (GONÇALVES et al., 2018) et nos résultats ont démontré que l'influx calcique induit par le BPA implique IP3R. De plus, l'activation de la PKC a été également cruciale pour le mécanisme d'action du BPA et ces données sont en accord avec des études antérieures dans le testicule de rat (GONÇALVES et al., 2018). De plus, il pourrait être suggéré sur la base de nos données et d'études précédentes que le BPA inhibe SERCA et contribue à l'augmentation des niveaux de Ca^{2+} cytosolique dans des testicules de zebrafish.

Gonçalves et al. (2018) renforcent nos données suggérant que le BPA inhibe SERCA dans les testicules de rats rapportant le même effet du BPA et de la thapsigargine sur l'influx calcique comme observé dans notre étude. De plus, il a également été rapporté que le BPA induisait une augmentation des niveaux de Ca^{2+} cytosolique en inhibant SERCA dans des microsomes testiculaires de rat (HUGHES, 2000) et dans des fibroblastes de peau humaine (WOESTE et al., 2013). L'activation d'IP3R et l'inhibition de SERCA entraînent l'épuisement des réserves calciques intracellulaires dans le réticulum endoplasmique. L'épuisement des réserves calciques intracellulaires est le déclencheur de l'activation des SOC de la membrane plasmique et de la stimulation de l'influx calcique, qui peut être maintenu pendant des minutes à des heures. Cela se produit en tant que mécanisme compensatoire de l'apport calcique pour remplir le réticulum endoplasmique et gérer de nombreux processus biologiques, tels que la sécrétion, la transcription de gènes et la modulation des activités des enzymes (PRAKRIYA ; LEWIS, 2015).

Conformément à nos résultats, des études publiées précédemment ont rapporté des réponses rapides des effets du BPA sur des voies non-génomiques dans d'autres modèles animaux, d'autres organes/cellules et différents temps d'exposition au BPA. Il a été démontré que le BPA, à de très faibles concentrations, peut activer rapidement la phosphorylation de l'ERK 1/2 dans les cellules hypophysaires de rat (JENG ; WATSON, 2011). En outre, il a également été démontré que le BPA active ERK1/2 dans les cellules de Sertoli de rat (IZUMI et al., 2011). De plus, il a été rapporté que l'exposition au BPA provoque une activation rapide de ERK1/2 par les voies de signalisation de GPER30 et ESR α/β dans les cellules de Sertoli TM4 (GE et al., 2014). Qian et al. (2014) ont démontré que le BPA déclenche également d'importantes oscillations de Ca^{2+} dans les cellules de Sertoli TM4 et augmente rapidement les niveaux calciques intracellulaires. La voie ERK1/2 participe aux voies génomiques induites par le BPA, induisant des dommages aux cellules de Sertoli et l'apoptose (QIAN et al., 2014). De plus, Li et al. (2006) ont mis en évidence que le BPA peut activer en quelques minutes des voies de signalisation non-génomiques médiées par ESR α dans les cellules MCF-7 via l'activation de MAPK, PI3K et PKC. De plus, le rôle de l'activation de ESR β induite par le BPA dans l'influx calcique a déjà été décrit dans les kératinocytes gingivaux humains dérivés de la muqueuse buccale (EHRENMANN et al., 2017). Des études publiées précédemment ont également démontré la capacité du BPA à se lier aux ESR et à agir comme un agoniste. L'incubation *in vitro* de BPA active efficacement ESR α et ESR β (MOLINA-MOLINA et al., 2013). De plus, l'activité œstrogénique *in vivo* du BPA a également été démontrée dans le cerveau des larves de zebrafish en développement (LE FOL et al., 2017). Dans l'ensemble, ces données publiées précédemment sont cohérentes avec nos résultats, qui mettent en évidence l'implication de l'ERK 1/2, de la PI3K, de la PKC et de l'IP3R dans le mécanisme d'action de l'influx calcique induit par le BPA, ainsi que la participation des ESR α/β . Cela pourrait indiquer une *crosstalk* entre les voies non-génomiques et génomiques, puisque ces protéines kinases et les ESR α/β peuvent induire la transcription des gènes (DUAN et al., 2002 ; LEONARD et al., 2015). Cependant, la perturbation de l'homéostasie calcique est un puissant déclencheur du stress du réticulum

endoplasmique et de l'apoptose (GROENENDYK et al., 2010). Il a déjà été démontré que le BPA diminuait la viabilité des cellules de Sertoli et induisait la mort cellulaire dépendante du Ca^{2+} intracellulaire via l'apoptose en perturbant l'homéostasie testiculaire du Ca^{2+} (HUGHES, 2000).

De plus, il a déjà été rapporté que le BPA affecte la viabilité des cellules de Sertoli TM4 de manière dépendante de la concentration en BPA et du temps et induit des voies apoptotiques mitochondriales qui dépendent de l'activation de ERK 1/2 (QIAN et al., 2014). En accord avec ces données, l'exposition aiguë *in vitro* des testicules au BPA (10 μM) a entraîné une lésion de la membrane plasmique indiquée par une activité extracellulaire accrue de LDH, ce qui indique sa libération du milieu intra au milieu extracellulaire. Cette lésion a été de manière surprenante médiée par une augmentation de Ca^{2+} intracellulaire dépendante de l'IP3R, puisque l'antagoniste de l'IP3R (2-APB) a aboli l'effet stimulé par le BPA. De plus, l'augmentation du TAG testiculaire après une exposition aiguë *in vitro* à 10 μM de BPA a été également médiée par l'augmentation du Ca^{2+} intracellulaire dépendant de l'IP3R, puisque l'antagoniste de l'IP3R a aboli l'effet du BPA. Les phospholipides, les glycolipides, le TAG le cholestérol sont signalés comme des composants de la membrane cellulaire des spermatozoïdes, qui sont nécessaires à leur maturation (LENZI et al., 1996). Des modifications du métabolisme des lipides sont couramment présentes dans plusieurs conditions ou maladies testiculaires, telles que la cryptorchidie, qui présente une accumulation de lipides, tels que de TAG, d'alkyl-DAG et d'esters de cholestérol dans les testicules de rat (FURLAND et al., 2007). De plus, l'exposition des cellules de Sertoli à des conditions stressantes augmente la teneur en TAG, DAG et phosphatidylinositol, ainsi que l'accumulation de gouttelettes lipidiques et le découplage mitochondrial (VALLÉS et al., 2014). La formation et l'augmentation de ces lipides peuvent être dues à la production de particules riches en lipides (corps résiduels) et à la phagocytose par les cellules de Sertoli des cellules germinales apoptotiques qui meurent dans des conditions nocives (CHEMES, 1986 ; VALLÉS et al., 2014). De plus, le testicule est très sensible à l'accumulation de glycérol, et des concentrations élevées de glycérol peuvent provoquer une fuite de la barrière hémato-testiculaire, ce qui favorise l'apoptose de la lignée germinale (WIEBE et al., 2000). Ce phénomène peut entraîner l'arrêt temporaire de la spermatogenèse (WIEBE et BARR, 1984), l'oligospermie, voire l'azoospermie (WIEBE et al., 2000). De plus, des accumulations de TAG et de gouttelettes lipidiques sont observées chez la levure *Saccharomyces cerevisiae* dans un état de stress induit par des produits chimiques. Il a été rapporté précédemment que la dérégulation des lipides induite par l'interruption de l'homéostasie calcique conduit à un stress du réticulum endoplasmique (GARDARIN, 2010 ; RAJAKUMAR et al., 2016) qui conduit à une accumulation de phospholipides (MUTHUKUMAR et al., 2011). Par conséquent, les résultats rapportés ici sont en accord et indiquent une relation entre la perturbation de l'homéostasie calcique, la fuite de Ca^{2+} du réticulum endoplasmique, les lésions à la membrane cellulaire et l'augmentation de la teneur en TAG.

Résultats et discussion – Part II du Chapitre I

Pour étudier les effets du BPA sur les principaux substrats énergétiques testiculaires, les teneurs en lactate et en glycogène et l'absorption du ^{14}C -désoxy-D-glucose ont été analysées dans des testicules de zebrafish. À partir d'incubations *in vitro* avec 10 pM, 10 nM et 10 μM de BPA pendant 1 heure, il a été constaté que la concentration la plus faible et la plus élevée de BPA ont réduit la teneur testiculaire en lactate par rapport au groupe contrôle. Ainsi, sur la base de ce résultat, les concentrations choisies pour les autres analyses ont été 10 pM et 10 μM de BPA. Par conséquent, 10 pM et 10 μM de BPA ont également réduit la teneur en glycogène par rapport au groupe contrôle. Cependant, l'absorption de ^{14}C -désoxy-D-glucose n'a pas été modifiée en présence de BPA *in vitro*. Par ailleurs, à partir des traitements *in vivo* avec 10 pM et 10 μM de BPA pendant 12 heures, il a été constaté que la plus faible concentration de BPA a diminué la teneur en lactate et en glycogène dans des testicules de zebrafish par rapport au groupe contrôle. De plus, les activités de LDH, ALT et AST ont également été analysées après des traitements *in vitro* et *in vivo* au BPA afin de déterminer les effets sur les enzymes impliquées dans le métabolisme énergétique testiculaire. Le traitement aigu au BPA *in vitro* a diminué l'activité de la LDH, accentuant la diminution de la teneur en lactate. Cependant, l'activité de la LDH n'a pas été modifiée après des traitements *in vivo* en présence de BPA. Par ailleurs, 10 pM de BPA *in vitro*, ainsi que 10 μM de BPA *in vivo* ont significativement réduit l'activité de l'ALT dans les testicules par rapport aux groupes contrôles. De plus, les incubations *in vitro* avec 10 pM et 10 μM de BPA ont provoqué une augmentation de l'activité de l'AST dans les testicules. En revanche, l'exposition *in vivo* aux deux concentrations de BPA a réduit l'activité de l'AST par rapport le groupe contrôle.

Par ailleurs, l'impact du BPA sur la spermatogenèse et l'apoptose ont également été investigués chez le zebrafish. La proportion de la surface des spermatocytes et des cellules haploïdes (spermatides + spermatozoïdes) par rapport à la surface totale testiculaire a été mesurée après l'exposition *in vivo* des poissons à 10 pM ou 10 μM de BPA pendant 12 heures. Le groupe contrôle a montré un profil de distribution et de proportion des cellules testiculaires mettant en évidence une proportion normale de spermatocytes, spermatides et spermatozoïdes. Cependant, l'exposition au BPA (10 pM et 10 μM) pendant 12 heures n'a provoqué aucun changement dans la proportion de surface des spermatocytes par rapport au groupe contrôle. En revanche, l'exposition au BPA à 10 pM et à 10 μM a entraîné une diminution de la proportion de la surface des cellules haploïdes (spermatides et spermatozoïdes) par rapport à la surface testiculaire totale comparé au groupe contrôle. De plus, l'exposition des poissons à 10 pM et 10 μM de BPA a entraîné une augmentation significative de l'apoptose des spermatocytes (cellules marquées/positives pour TUNEL) par rapport au groupe contrôle.

De plus, afin d'étudier les effets génomiques du BPA sur le métabolisme énergétique testiculaire, l'expression relative de gènes codant pour des protéines clés du métabolisme des glucides et des acides aminés a été analysée dans des testicules de zebrafish après 6 et 72 heures d'incubations au BPA. Ainsi, l'expression relative de LDHBa, GPT2 et PCXA a été augmentée dans des testicules incubés avec 10 pM de

BPA pendant 6 heures, alors que l'expression relative de PYGL, PFKFb2a, PKMA et MCT4 n'a pas été modifiée par rapport aux groupes contrôles. D'autre part, l'expression relative de PKMA a été réduite après 72 heures d'incubation avec 10 μ M de BPA par rapport au groupe contrôle, alors que l'expression relative d'aucun autre gène n'a été significativement modifiée. En revanche, l'analyse par Western blot a révélé une réduction de l'immunocontenu en PKM dans les testicules incubés avec 10 μ M de BPA pendant 72 heures par rapport au groupe contrôle, alors qu'il n'y a pas eu de changement par le BPA à 10 μ M. En plus du BPA à 10 μ M, l'expression relative des gènes a également été analysée dans des testicules de zebrafish après 6 et 72 heures d'incubation avec 10 μ M de BPA. Cependant, nos résultats ont montré qu'il n'y a pas eu de changement dans l'expression des gènes après 6 heures d'incubation avec la concentration la plus élevée de BPA, mais seulement une tendance à augmenter l'expression de GPT2 par rapport au groupe contrôle ($p = 0.057$). D'autre part, nos résultats ont mis en évidence que l'expression relative de PFKFb2a, LDHBa et GPT2 a diminué dans les testicules après 72 heures d'incubation avec 10 μ M de BPA, tandis que l'expression relative de PYGL et MCT4 a été augmentée par rapport aux groupes contrôles. Cependant il n'y a pas eu de changement dans l'expression relative des gènes PKMA et PCXA dans les testicules après 72 heures d'incubation avec 10 μ M de BPA. Par conséquent, l'implication des ESR α/β dans le mécanisme d'action du BPA dans la modulation de l'expression des gènes a été investiguée. Cependant, il a été montré que la diminution de l'expression relative du gène PKMA induite par 10 μ M de BPA n'a pas été reversée par la préincubation avec ICI 182,780, indiquant que cette diminution n'implique pas les ESR α/β . De plus, nos résultats ont mis en évidence que l'augmentation de l'expression relative de PYGL par 10 μ M de BPA a été complètement abolie par la préincubation avec ICI 182 780, indiquant que l'augmentation de l'expression de PYGL par BPA est médiée par les ESR α/β . En outre, la diminution de l'expression relative de PFKFb2a et de LDHBa par 10 μ M de BPA a également été supprimée par la préincubation avec ICI 182 780, indiquant que ces effets génomiques médiés par le BPA sont également dépendants des ESR α/β . D'autre part, la diminution de l'expression de GPT2 et l'augmentation de l'expression de MCT4 par 10 μ M de BPA n'ont pas été reversées par l'ICI 182,780, indiquant que les ESR α/β ne sont pas impliqués dans ces effets médiés par le BPA à 10 μ M.

Des analyses quantitatives d'IHC ont mis en évidence que les coupes histologiques de testicules incubés avec du BPA 10 μ M pendant 72 heures présentaient une augmentation du nombre de cellules testiculaires exprimant la LDH (densité des cellules positives pour la LDH). Cependant, il n'y a pas eu de changement dans le nombre de cellules testiculaires exprimant la LDH après l'incubation avec 10 μ M de BPA par rapport au groupe contrôle. L'immunolocalisation de la LDH dans différents types de cellules testiculaires n'avait pas été établie auparavant chez les poissons. Ainsi, en première approche, la protéine LDH a été identifiée dans les testicules de zebrafish par l'analyse immunohistochimique après des traitements *in vitro* par culture organotypique. Par conséquent, au vu des conditions expérimentales de la présente étude, la LDH dans les testicules de zebrafish semble être plus abondante dans les cellules germinales matures, principalement dans les

spermatides. De plus, la protéine LDH n'a pas été identifiée dans les cellules germinales précoces, les spermatogonies. Après avoir détecté l'implication de la concentration la plus élevée de BPA dans l'augmentation des cellules testiculaires positives à la LDH, l'implication des ESR α/β dans le mécanisme d'action du BPA a été étudiée. Ainsi, l'augmentation du nombre de cellules LDH positives observée dans les coupes histologiques de testicules incubées avec du BPA 10 μ M a été complètement abolie par la préincubation avec ICI 182,780, indiquant que cet effet du BPA est dépendant de l'activation des ESR.

Par conséquent, après avoir mis en évidence l'implication de l'ESR dans le mécanisme d'action de certains effets du BPA, et puisqu'il est reconnu que le BPA peut se lier à des récepteurs nucléaires autres que l'ESR classique (TOHMÉ et al., 2014), l'expression des gènes ESR et ESRR a été investiguée. L'expression relative du gène ESR2b a été augmentée dans des testicules incubés avec 10 pM de BPA pendant 6 heures, alors que l'expression des autres récepteurs n'a pas été significativement modifiée par rapport au groupe contrôle. Cependant, l'expression relative d'aucun des récepteurs étudiés n'a été significativement modifiée dans des testicules après 72 heures d'incubation avec 10 pM de BPA. De plus, l'expression d'aucun des récepteurs étudiés n'a été significativement altérée dans des testicules de zebrafish incubés avec 10 μ M de BPA pendant 6 heures par rapport aux groupes contrôles. En revanche, l'expression relative des gènes ESR2a et ESR2b a été significativement augmentée après 72 heures d'incubation avec 10 μ M de BPA. De plus, l'expression relative du gène ESRR α a été régulée négativement après 72 heures d'incubation avec 10 μ M de BPA.

Afin de corréliser les modifications du métabolisme énergétique testiculaire causées par le BPA avec d'éventuelles modifications de la spermatogenèse, l'expression de gènes spécifiques/marqueurs des cellules germinales a été analysée après 6 et 72 heures d'incubations avec du BPA. Ainsi, l'expression relative des gènes PIWIL-1 et SYCP-3 a été augmentée dans des testicules de zebrafish incubés avec 10 pM de BPA pendant 6 heures, tandis que l'expression relative d'ODF3b n'a pas été modifiée. Cependant, l'expression relative du gène ODF3b a été significativement réduite après 72 heures d'incubation avec 10 pM de BPA. En revanche, l'expression relative des gènes PIWIL-1, SYCP-3 et ODF3b n'a pas été modifiée dans des testicules incubés avec 10 μ M de BPA pendant 6 heures. Cependant, l'expression relative de SYCP-3, un gène marqueur des spermatocytes, a été réduite après 72 heures d'incubation avec 10 μ M de BPA, alors que l'expression relative de PIWIL-1 et ODF3b n'a pas été modifiée. De plus, l'implication des ESR dans le mécanisme d'action du BPA dans la modulation de l'expression des gènes ODF3b et SYCP3 a également été étudiée. La diminution de l'expression relative d'ODF3b par la plus faible concentration de BPA a été complètement supprimée par la préincubation avec ICI 182 780, indiquant que le BPA augmente l'expression d'ODF3b via ESR α/β . Par ailleurs, la diminution de l'expression relative de SYCP-3 n'a pas été reversée par la préincubation avec ICI 182,780. Ainsi, lorsque les testicules ont été pré-incubés avec de l'ICI 182,780 avant l'incubation avec 10 μ M de BPA, les niveaux d'expression de

SYPC-3 ont été les mêmes par rapport au groupe BPA, en montrant que les ESR α/β ne sont pas impliqués dans cet effet du BPA.

Par ailleurs, l'immunocontenu en PCNA a été analysé après l'exposition *in vitro* des testicules de zebrafish avec du BPA pendant 72 heures afin de déterminer si le BPA affecte également la prolifération cellulaire, qui est liée à la prolifération des spermatogonies dans les testicules. Ainsi, l'immunocontenu en PCNA a été réduit dans des testicules incubés avec la plus forte concentration de BPA pendant 72 heures, alors que son expression n'a pas été altérée par la plus faible concentration de BPA par rapport au groupe contrôle. Après avoir détecté l'influence du BPA sur l'expression de la protéine PCNA, l'implication du BPA dans l'une des voies de signalisation de la prolifération cellulaire a été étudiée en analysant l'immunocontenu de t-ERK 1/2 et la phosphorylation de ERK 1/2. L'analyse par Western blot a révélé une augmentation de la phosphorylation de ERK 1/2 (p-ERK / t-ERK) dans les testicules par le BPA à 10 pM et à 10 μ M après 72 heures d'exposition *in vitro*. De plus, l'impact du BPA sur la spermatogenèse a également été investigué après 72 heures d'exposition des testicules au BPA. En outre, l'implication des ESR α/β dans le mécanisme d'action du BPA dans l'altération de la spermatogenèse a également été étudiée. Ainsi, la proportion de spermatocytes et de cellules haploïdes (spermatides et spermatozoïdes) a été analysée dans des testicules après des préincubations en présence ou pas de l'antagoniste ICI 182,780 et des incubations avec du DMSO, 10 pM ou 10 μ M BPA pendant 72 heures. Par conséquent, le groupe contrôle a montré un profil de distribution et de proportion des cellules testiculaires mettant en évidence une proportion normale de spermatocytes, spermatides et spermatozoïdes. Cependant, l'exposition *in vitro* au BPA (10 pM et 10 μ M) pendant 72 heures n'a pas provoqué de changement dans la proportion de surface des spermatocytes par rapport au groupe contrôle. En revanche, l'exposition à la plus faible concentration de BPA a entraîné une diminution de la proportion de la surface des cellules haploïdes par rapport à la surface testiculaire totale comparé au groupe contrôle. Fait intéressant, nos résultats ont mis en évidence que cette réduction causée par la plus faible concentration de BPA a été abolie par la préincubation avec de l'ICI 182,780, ce qui indique que ces dommages à la spermatogenèse induits par le BPA dépendent de l'activation de l'ESR. Cependant, il n'y a pas eu de changement significatif, mais seulement une tendance ($p = 0,08$) à réduire la proportion de cellules haploïdes après exposition *in vitro* à la plus forte concentration de BPA.

Discussion – Part II du Chapitre I

Grâce à l'utilisation d'approches *in vitro* et *in vivo*, nos résultats ont mis en évidence que le BPA induit des changements biochimiques et moléculaires sur le métabolisme énergétique testiculaire chez le zebrafish. Nos résultats ont montré qu'une exposition *in vivo* à court terme des poissons à la plus faible concentration de BPA pendant 12 heures a provoqué une réduction de la teneur en lactate, alors que l'activité de la LDH n'a pas été modifiée. Cependant, l'exposition *in vitro* des testicules de zebrafish au BPA (10 pM et 10 μ M) pendant 1 heure a entraîné une réduction

concomitante de la teneur en lactate et de l'activité de la LDH. La LDH catalyse la réduction du pyruvate en lactate (GRANCHI et al., 2010), qui est ensuite exporté vers les cellules germinales matures via MCT (SKINNER, 1991 ; RATO et al., 2012a). Le lactate est la principale source d'énergie des spermatocytes et des spermatides des rongeurs et des humains (RATO et al., 2012b). Bien que le rôle du lactate pour chaque cellule testiculaire de poisson soit encore mal connu, son importance énergétique pour les spermatozoïdes en fin de phase de motilité est déjà rapportée (DREANNO et al., 2000). En effet, la présente étude a été la première à mettre en évidence l'immunolocalisation de la LDH dans les testicules d'une espèce de poisson. Au vu de nos conditions expérimentales, l'expression de la LDH dans des testicules de zebrafish semble être plus abondante dans les cellules germinales matures, principalement dans les spermatides, alors qu'elle n'a pas été identifiée dans les cellules germinales précoces, les spermatogonies. Il a été rapporté que la réduction ou l'inhibition de la production de lactate peut altérer la spermatogenèse et contribuer à l'infertilité masculine (BUSTAMANTE-MARÍN et al., 2012 ; LUO et al., 2020). Ainsi, l'augmentation de l'expression relative de LDHBa, ainsi que la diminution de l'expression relative de PKMA dans les testicules incubés avec 10 pM de BPA pendant 6 et 72 heures, respectivement, pourraient suggérer une réponse génomique compensatoire par le besoin de production de lactate. Cependant, l'immunocontenu en PKM a été réduit par 10 µM de BPA, alors qu'il n'a pas été modifié par une incubation avec 10 pM de BPA pendant 72 heures. De plus, fait intéressant, la densité des cellules testiculaires exprimant la LDH a été augmentée dans les testicules incubés avec 10 µM de BPA pendant 72 heures, alors que l'expression du gène LDHBa a été réduite. Ces différences entre l'expression des gènes et des protéines sont en accord avec des études antérieures. Des différences dans l'expression des gènes et des protéines LDH et MCT ont déjà été mises en évidence, suggérant que cela se produit parce qu'elles sont régulées à des intervalles de temps différents et/ou régulées par des mécanismes post-transcriptionnels (ALVES et al., 2013). Compte tenu du fait que la transcription de l'ARNm et la synthèse des protéines sont régulées et se produisent à des intervalles de temps différents, il est plausible que des différences dans l'expression des gènes et des protéines soient observées après avoir été analysées au cours de la même période de traitement.

Par conséquent, si les modifications de la diminution de l'expression de la LDH et de la PKM sont prolongées, elles peuvent réduire considérablement la glycolyse et donc inhiber la production de lactate. De plus, une expression accrue du gène MCT4 dans des testicules incubés avec 10 µM de BPA pendant 72 heures pourrait suggérer un besoin accru de disponibilité de lactate aux cellules testiculaires exposées au BPA. Des études publiées précédemment avec du BPA, ses analogues ou d'autres xénoestrogènes obtenus dans différents modèles animaux, cellules isolées ou d'autres tissus renforcent nos données. Il a également été démontré qu'une exposition *in vitro* au BPA (10 µM et 1 mM) pendant 48 heures altère le métabolisme du glucose en diminuant la production de lactate, l'expression du gène et de la protéine LDH dans les cellules de Sertoli de rat (HUANG et al., 2015). En outre, il a été rapporté que le bis(2-éthylhexyle)phthalate, un autre xénoestrogène de l'industrie du plastique, réduit

également la teneur en lactate et l'activité de la LDH dans des testicules de zebrafish après des expositions à court terme (BATISTA-SILVA et al., 2020). De plus, l'altération de la glycolyse testiculaire médiée par le BPA peut être renforcée par la réduction observée dans notre étude de l'expression du gène PFKFb2a dans des testicules après 72 heures d'incubation au BPA à 10 μ M. Il a été rapporté qu'une diminution de l'expression de PFKFb dans le foie pendant le jeûne prolongé et le diabète est restaurée et stimulée par une alimentation riche en glucides ou par l'administration d'insuline (COLOSIA et al., 1988). Il a été rapporté que l'exposition des rats au BPA perturbe l'homéostasie du glucose et la signalisation de l'insuline testiculaire en diminuant les activités de l'hexokinase et de la PFK-1, les niveaux d'insuline, ainsi que l'expression du récepteur de l'insuline, des substrats des récepteurs de l'insuline et de la PI3K (D'CRUZ et al. 2012 ; D'CRUZ ; JUBENDRADASS ; MATHUR, 2012). Par conséquent, ces études antérieures corroborent nos résultats, ce qui nous amène à suggérer que les changements médiés par le BPA dans l'expression des gènes liés à la glycolyse observés dans notre étude étaient dus à une altération de la signalisation de l'insuline ou à un manque de substrat énergétique.

En plus du lactate, l'homéostasie du glycogène est également importante pour maintenir l'intégrité de l'épithélium germinal chez les rats mâles (KURAMORI et al., 2009). Cependant, aucun rapport ne montre la participation du glycogène en tant que source d'énergie testiculaire, ni l'expression du gène de la glycogène phosphorylase dans des testicules de zebrafish. Nos résultats ont montré qu'après une exposition *in vitro* au BPA pendant 1 heure, ainsi qu'une exposition *in vivo* au BPA pendant 12 heures, provoquait une réduction significative de la teneur testiculaire en glycogène. Fait intéressant, l'expression relative du gène PYGL codant pour la glycogène phosphorylase a augmenté dans des testicules après 72 heures d'exposition *in vitro* à 10 μ M de BPA, et cette augmentation était dépendante des ESR. La glycogène phosphorylase participe à la glycogénolyse en libérant du glucose-1-phosphate à partir des extrémités non réductrices du glycogène. Ainsi, nos résultats ont mis en évidence que la glycogénolyse testiculaire est stimulée par le BPA chez le zebrafish. Zhao et al. (2018) renforcent notre hypothèse en rapportant que la glycogénolyse et la gluconéogenèse hépatiques et musculaires sont stimulées par l'exposition du zebrafish au bisphénol S. La diminution observée des substrats énergétiques après des expositions *in vitro* et *in vivo* au BPA peut également être due à une diminution de l'activité de l'ALT et par conséquent à une production de pyruvate plus faible. Nos études démontrent une diminution de l'activité ALT induite par le BPA. Ainsi, l'augmentation de l'expression relative de GPT2 codant pour l'ALT après 6 heures d'incubation des testicules avec 10 μ M de BPA semble avoir été une réponse compensatoire. Cependant, l'expression relative de GPT2 a diminué après 72 heures d'exposition à la plus forte concentration de BPA. Notre étude est la première étude mettant en évidence l'expression de GPT2 dans des testicules d'une espèce de poisson. De plus, la présente étude a été la première à identifier GPT2 comme cible de l'effet génomique du BPA dans les testicules. En plus d'être le produit final de la glycolyse, le pyruvate peut également être généré par l'ALT, qui catalyse l'interconversion réversible de l'alanine et de l' α -cétooglutarate en pyruvate et glutamate

(SHERMAN, 1992 ; LIU et al., 2014). Par conséquent, les changements induits par le BPA dans l'activité de l'ALT et l'expression de GPT2 montrés dans la présente étude peuvent altérer la production d'alanine et/ou de pyruvate. Les études liées à l'action de l'ALT dans les testicules sont rares même chez les mammifères. Il a été démontré que les cellules de Sertoli peuvent maintenir la production de lactate même en l'absence totale de glucose par le métabolisme des acides aminés ou du glycogène (RIERA et al., 2009). De plus, des niveaux élevés de l'immunocontenu en ALT dans les cellules de Sertoli de rats ont déjà été signalés, ce qui suggère qu'une partie importante du pyruvate produit par ces cellules provient de la conversion de l'alanine par l'ALT (MATEUS et al., 2018).

De plus, notre étude a également été la première à identifier l'AST et la pyruvate carboxylase comme cibles du BPA dans les testicules. De plus, il s'agit de la première étude qui met en évidence l'expression du gène PCXA dans les testicules. L'AST catalyse l'interconversion de l'aspartate et de l' α -cétoglutarate en oxaloacétate (OAA) et en glutamate (SCHWARTZ, 1971). Par conséquent, la réduction drastique de l'activité AST dans les testicules de zebrafish exposés *in vivo* au BPA pourrait indiquer une diminution de l'aspartate et des intermédiaires du cycle de Krebs, de l' α -cétoglutarate et de l'OAA. En outre, il pourrait également être suggéré qu'une réduction de l'activité de l'AST peut compromettre le système de transport malate-aspartate pour la réoxydation des équivalents de réduction tels que le NADH (CETICA ; PINTOS ; DALVIT, 1999). De plus, l'expression relative du gène PCXA codant pour la pyruvate carboxylase a été augmentée dans les testicules après 6 heures d'incubation avec 10 pM de BPA. La pyruvate carboxylase catalyse la carboxylation du pyruvate en OAA. Il s'agit d'une réaction anaplérotique très importante qui reconstitue de l'OAA pour le cycle de Krebs et fournit de l'OAA pour une conversion ultérieure en phosphoenolpyruvate dans la première étape de la gluconéogenèse (JITRAPAKDEE ET AL., 2008 ; VALLE, 2017). Par conséquent, les changements induits par le BPA dans l'activité de l'AST et l'expression de PCXA dans les testicules pourraient suggérer une forte demande énergétique et un besoin d'intermédiaires du cycle de Krebs. En plus du métabolisme énergétique testiculaire, la spermatogenèse a également été affectée par le BPA.

L'analyse de l'expression des gènes dans des testicules de zebrafish a montré des différences dans l'expression des marqueurs de cellules testiculaires en fonction du temps d'exposition et des concentrations de BPA. L'expression relative de PIWIL-1 et SYCP-3 a été augmentée après 6 heures d'incubation avec 10 pM de BPA. Cependant, l'expression relative du gène marqueur des spermatides, ODF3b, a été réduite dans les testicules incubés avec 10 pM de BPA pendant 72 heures. Fait intéressant, ces résultats sont en accord avec nos données histologiques. Une diminution de la proportion de cellules haploïdes (spermatides et spermatozoïdes) par rapport à la surface testiculaire totale a également été observée après 72 heures d'exposition à 10 pM de BPA. Selon une étude précédente, l'expression du gène ODF3b a déjà été directement liée à la proportion de cellules haploïdes dans les testicules de zebrafish (TOVO-NETO et al., 2020). En plus du BPA *in vitro*, une exposition aiguë *in vivo* à 10 pM et 10 μ M de BPA a également provoqué une altération

de la spermatogenèse en augmentant le pourcentage de spermatocytes apoptotiques et en réduisant la proportion de cellules haploïdes par rapport au groupe contrôle. Ainsi, sur la base de ces résultats, l'influence de l'altération du métabolisme énergétique testiculaire médiée par le BPA pourrait être proposée comme l'une des causes qui conduisent à une altération de la spermatogenèse chez le zebrafish. En accord avec nos résultats, il a été rapporté qu'un mélange de xénoestrogènes contenant du BPA entraîne une réduction du nombre de spermatozoïdes accompagnée d'une augmentation du nombre de cellules apoptotiques testiculaires chez le zebrafish (WANG et al., 2019). De plus, il a également été rapporté que l'exposition des souris au BPA pendant la puberté a augmenté le pourcentage de cellules testiculaires apoptotiques par les voies apoptotiques mitochondriales (WANG et al., 2010). En outre, nos résultats ont montré que la réduction de l'expression du gène SYCP-3 déclenchée par une exposition *in vitro* à 10 μ M de BPA pendant 72 heures sont en accord avec des rapports précédents. En revanche, nos données histologiques sur la proportion de spermatocytes dans les testicules ne sont pas en accord avec les données sur l'expression de SYCP-3. La réduction de l'expression de SYCP-3 a également été rapportée dans les testicules de zebrafish après une exposition *in vivo* à des concentrations plus élevées de BPA (2000 mg/L) pendant 21 jours (GONZÁLEZ-ROJO et al., 2019). De plus, des expositions *in vitro* de tubules séminifères de rats au BPA (1 nM et 10 nM) pendant 8, 14 et 21 jours ont entraîné un arrêt partiel de la méiose en raison de modifications de l'expression des gènes marqueurs de la méiose et des spermatocytes, et de modifications histologiques (ALI et al., 2014).

En plus des changements dans les phases méiotiques et post-méiotiques, la prolifération cellulaire semble également être affectée par le BPA, contribuant au mécanisme du BPA en altérant la spermatogenèse. Nos résultats ont mis en évidence que la réduction de l'expression de la protéine PCNA déclenchée par l'exposition *in vitro* à 10 μ M de BPA pendant 72 heures sont en accord avec des travaux précédents. La réduction de l'expression de PCNA a également été récemment rapportée, suggérant que la prolifération des cellules germinales est altérée par le BPA (LIU ; WANG ; LI, 2020). La PCNA est une protéine nucléaire qui est régulée de manière différentielle au cours du cycle cellulaire, atteignant une expression maximale à la transition de phase G1/S (CELIS ; CELIS, 1985). En plus de la synthèse de l'ADN, le PCNA participe également à d'autres processus cellulaires importants, tels que la réparation de l'ADN et l'apoptose (JÓNSSON ; HÜBSCHER, 1997). Une étude précédente renforce nos données en rapportant des effets antiprolifératifs du BPA sur les cellules cancéreuses primaires et de la prostate, qui ont été observés après la même période d'exposition à la même concentration de BPA que dans notre étude. Bilanco et al. (2017) ont rapporté que le BPA a entraîné une augmentation de l'expression et de la phosphorylation de p53 et a déclenché l'arrêt du cycle cellulaire par l'activation de ERK via une *crosstalk* des voies de signalisation EGFR/ESR β /ERK. De plus, une augmentation significative de la phosphorylation de ERK 1/2 dans des testicules incubés avec du BPA pendant 72 heures a été démontrée dans notre étude. Par conséquent, il pourrait être suggéré que la réduction de l'expression de la protéine

PCNA causée par le BPA peut avoir été médiée par l'augmentation de l'expression ou de la phosphorylation de p53 via ERK 1/2 avec une implication possible de ESR β , lesquels ont été positivement régulés par le BPA à 10 μ M. De plus, des rapports antérieurs montrent également que le BPA stimule la phosphorylation de ERK 1/2 dans les cellules de Sertoli de rat (IZUMI et al., 2011) et TM4 via ESR α/β (GE et al., 2014) après une courte exposition *in vitro*.

De plus, notre étude a montré que l'expression relative d'aucun des gènes étudiés n'a été altérée dans les testicules de zebrafish après 6 heures d'incubation avec 10 μ M de BPA. En revanche, la modulation de l'expression relative des gènes par le BPA à 10 μ M n'a été observée qu'après 72 heures. La présente étude a montré pour la première fois que la diminution de l'expression de PFKFb2a et de LDHBa, ainsi que l'augmentation de l'expression de PYGL et du nombre de cellules exprimant la LDH, ont été reversées par la préincubation avec de l'antagoniste des ESR α/β , ICI 182,780. L'expression accrue d'ESR2a et d'ESR2b après 72 heures d'incubation avec 10 μ M de BPA mets en évidence que ces ESR sont des cibles des effets génomiques du BPA dans le testicule. Nos résultats sont cohérents avec des études antérieures qui ont rapporté que la modulation de l'expression des gènes par des concentrations micromolaires de BPA est médiée par les ESR après des expositions à long terme, de quelques heures à quelques jours (GAIDO et al., 1997 ; LÖSEL ; WEHLING, 2003 ; MOLINA -MOLINA et al., 2013 ; LE FOL et al., 2017). De plus, une autre étude publiée précédemment renforce nos données en rapportant que l'exposition chronique au BPA augmente l'expression d'ESR2b dans le foie et l'expression d'ESR2a dans des testicules de goldfish mâles (HATEF et al., 2012). Cependant, les changements dans l'expression relative de GPT2, MCT4 et SYCP-3 par 10 μ M de BPA n'ont pas été dépendants des ESR. Par conséquent, les différences observées dans les réponses génomiques médiées par le BPA indiquent que les gènes associés au métabolisme énergétique sont également des cibles transcriptionnelles de récepteurs autres que les ESR α/β . Cependant, la diminution de l'expression d'ODF3b (gène marqueur de spermatides) et de la proportion de spermatides et de spermatozoïdes induites par 10 μ M de BPA ont été dépendants de l'activation des ESR. Néanmoins, comme tous les effets du BPA présentés dans cette étude ont été évalués dans l'ensemble du testicule, cela renforce la nécessité de considérer plusieurs voies d'action du BPA en fonction de chaque type cellulaire. Dans l'ensemble, nos résultats indiquent que la plupart des effets dépendants des ESR α/β médiés par le BPA ont été principalement ciblés sur les cellules de Sertoli et cellules haploïdes, ce qui est renforcé par le fait que ces cellules chez les poissons sont les cellules testiculaires qui ont la plus forte expression des ESR α/β (BOUMA ; NAGLER, 2001 ; WU et al., 2001 ; ITO ; MOCHIDA ; FUJII, 2007). Ainsi, une relation de cause à effet entre les altérations du métabolisme énergétique testiculaire et la spermatogenèse défectueuse pourrait être suggérée.

De plus, les récepteurs nucléaires autres que les ESR α/β sont également des cibles des réponses génomiques du BPA dans les testicules de zebrafish. L'expression réduite du gène ESRR α médiée par 10 μ M de BPA peut être liée à l'expression réduite des gènes PFKFb2a et PKMA. Les ESRR codent les ERR qui sont des récepteurs nucléaires orphelins et des régulateurs clés du métabolisme énergétique, stimulant la

biogénèse mitochondriale, la glycolyse, l'oxydation des acides gras, le cycle de Krebs, la phosphorylation oxydative et la synthèse d'ATP. Les ERR se lient et activent directement les promoteurs de nombreux gènes qui codent pour des enzymes glycolytiques, tels que la PKM et la LDH (GIGUÈRE, 2008 ; CAI et al., 2013). Le gène PFK est une cible transcriptionnelle directe de l'ERR. L'expression de la plupart des gènes codant pour des enzymes glycolytiques et les niveaux de lactate sont considérablement réduits dans les ERR mutants (TENNESSEN et al., 2011). De plus, l'ERR de la *Drosophile* a été signalé comme étant nécessaire à l'induction de la glycolyse pour soutenir la prolifération cellulaire au cours du développement embryonnaire (TENNESSEN et al., 2011).

Résultats et discussion – Chapitre II

Nos études *in vitro* après 6 heures d'incubation ont montré que l'expression relative de PYGM a été augmentée par l'E2 à 1 μ M et le BPA à 1 μ M dans des explants testiculaires de rats immatures. De plus, 1 nM et 1 μ M d'E2 et de BPA ont stimulé l'expression relative de PKM. Par ailleurs, l'expression relative de LDHc a également été stimulée par 1 nM d'E2 et de BPA. De plus, l'expression relative de GPT2 et GK2 a été augmentée par le BPA (1 nM ou 1 μ M), mais pas modifiée par l'E2. Cependant, aucun de ces effets génomiques du BPA et de l'E2 n'a été reversé par la préincubation avec de l'ICI 182,780, donc aucun de ces effets n'a été dépendant de l'activation des ESR. De plus, des expositions *in vitro* à l'E2 et au BPA pendant seulement 6 heures n'ont modifié l'expression d'aucun des gènes marqueurs des cellules germinales évalués dans la présente étude. Globalement, le BPA *in vitro* semble avoir le même mécanisme d'action que l'E2 dans l'induction de l'expression des gènes impliqués au métabolisme des glucides indépendante des ESR.

Discussion – Chapitre II

Grâce à l'utilisation d'une approche *in vitro* à court terme, nos études ont mis en évidence que l'E2 et le BPA, mais principalement le BPA, ont induit des changements dans l'expression des gènes impliqués au métabolisme énergétique dans des explants testiculaires de rats immatures. La présente étude a été la première à rapporter que l'expression de PYGM a été stimulée à la fois par l'E2 et le BPA à 1 μ M dans des testicules de rats. Des niveaux d'ARNm de PYGM ont déjà été rapportés dans des testicules de rats adultes (DAVID ; CRERAR, 1986). Fait intéressant, la teneur en glycogène a été signalée comme étant plus prononcée dans les testicules de rats immatures que chez les rats adultes (LEIDERMAN ; MANCINI, 1969). De plus, le rapport élevé entre la phosphorylase a et l'activité phosphorylase totale observé chez les rats mâles immatures est comparable au rapport trouvé lorsque l'enzyme du muscle squelettique ou du tissu adipeux est activée en réponse à des stimuli hormonaux ou nutritionnels (SLAUGHTER ; MEANS, 1983). Ainsi, ces données peuvent suggérer un effet putatif du métabolisme du glycogène sur l'établissement de la spermatogénèse. Par conséquent, l'expression accrue de PYGM observée dans

notre étude peut être due au besoin de la source d'énergie fournie par la glycogénolyse. Comme le glycogène peut être utilisé pendant les périodes où la disponibilité d'autres sources d'énergie préférées diminue, il est suggéré que l'E2 et le BPA aient induit une utilisation accrue d'autres substrats énergétiques. Des études antérieures renforcent nos résultats montrant que les cellules de Sertoli d'humains (OLIVEIRA et al., 2011) et de rats immatures (RATO et al., 2012b) cultivées dans différentes conditions expérimentales, y compris en présence d'E2, ont un taux élevé de consommation de pyruvate. Il a été démontré précédemment que presque tout le pyruvate disponible est consommé par les cellules de Sertoli dans les 5 premières heures d'incubation (RATO et al., 2012b).

De plus, une autre étude a rapporté que les souris traitées avec du benzoate d'estradiol présentaient une diminution de la teneur en ATP, ainsi qu'une diminution des activités de l'hexokinase et de la pyruvate kinase (LE et al., 2019). Par conséquent, l'augmentation de l'expression relative de PKM dans les testicules après 6 heures d'incubation avec E2 et BPA à 1 nM et 1 μ M peut avoir été due à la nécessité d'augmenter la production de pyruvate. Il est rapporté que la majeure partie du pyruvate produit par les cellules de Sertoli est convertie en lactate (OLIVEIRA et al., 2015) par l'action de la LDH (JUTTE et al., 1983). Le lactate est le principal substrat énergétique pour générer de l'ATP dans les spermatocytes et les spermatides des rongeurs et des humains (GROOTEGOED ; JANSEN ; VAN DER MOLEN, 1984 ; RATO et al., 2012b). Ainsi, l'augmentation de l'expression relative de LDHc dans les testicules après 6 heures d'incubation avec de l'E2 et du BPA observée dans la présente étude pourrait suggérer une réponse génomique compensatoire par le besoin de production de lactate. Des données précédentes confirment nos conclusions indiquant que la production de lactate est supprimée par l'E2 et le BPA, cependant, obtenues sur de plus longues périodes d'exposition à des concentrations plus élevées d'E2 et de BPA que celles de notre étude. Un taux de production de lactate réduit est rapporté dans les cellules de Sertoli de rats immatures incubés avec 100 nM d'E2 entre 15 et 25 heures de culture (RATO et al., 2012b). En outre, il a également été rapporté qu'une exposition *in vitro* à l'E2 pendant 24 heures supprime la production de lactate de manière dose-dépendante dans les cellules de Sertoli du lézard *Hemidactylus flaviviridi* (KHAN ; RAI, 2004). De plus, une exposition *in vitro* à de fortes concentrations de BPA (10 μ M et 1 mM) pendant 48 heures a altéré le métabolisme du glucose en diminuant la production de lactate, l'expression du gène et de la protéine LDH dans les cellules de Sertoli de rat (HUANG et al., 2015). La majeure partie du pyruvate produit par les cellules Sertoli est convertie en lactate (OLIVEIRA et al., 2015) pour l'exporter vers les cellules germinales via MCT4 et MCT1 ou MCT2 (SKINNER, 1991 ; RATO et al., 2012). Il a été rapporté que le MCT1 est exprimé dans les spermatogonies, les spermatocytes et les spermatides chez le rat et chez la souris (KISHIMOTO et al., 2015). Cependant, dans nos conditions expérimentales, l'expression de MCT1 n'a été altérée ni par l'E2 ni par le BPA dans les testicules de rats immatures au moins après 6 heures d'incubation. Il y a seule une étude précédente obtenue dans le cerveau de rats adultes dans laquelle a rapporté que l'expression de MCT1 est réduite par le BPA. Cependant, cette réduction a été médiée

par une exposition *in vivo* précoce au BPA depuis la naissance jusqu'au jour 21 post-partum (XU et al., 2017). Néanmoins, il a été précédemment rapporté que l'expression du MCT4 a été réduite dans les cellules de Sertoli de rats immatures (RATO et al. 2012b), alors qu'elle a été augmentée dans les cellules de Sertoli humaines (OLIVEIRA et al. 2011) après 50 heures de traitement *in vitro* avec 100 nM E2. De plus, une diminution de l'expression de MCT2 et MCT4 a également été observée dans les testicules de souris traitées avec du benzoate d'estradiol pendant 4 semaines (LE et al., 2019). Par conséquent, ces données indiquent qu'une courte exposition *in vitro* à l'E2 et au BPA n'interfère pas avec le transport du lactate via MCT1, tandis que leurs effets à long terme interfèrent avec le transport du lactate par les mécanismes dépendants de MCT1, MCT2 et MCT4.

En plus du métabolisme du glucose et du glycogène, le métabolisme des acides aminés est également impliqué dans le métabolisme énergétique des cellules de Sertoli (MATEUS et al., 2018). Cependant, les études liées à l'expression et à l'activité de l'ALT dans les testicules sont rares, même chez les mammifères. Nos résultats ont démontré pour la première fois que l'expression relative de GPT2, qui code pour ALT2, a été significativement stimulée dans des testicules de rats immatures après 6 heures d'incubation avec du BPA (1 nM et 1 μ M). Ainsi, nous avons mis en évidence sans précédent que le GPT2 est une cible moléculaire des effets du BPA dans des testicules de rats immatures. En revanche, l'E2 n'a pas modifié significativement l'expression de GPT2, il a seulement montré une tendance ($p = 0,061$) à l'augmenter. En plus d'être le produit final de la glycolyse, le pyruvate peut également être généré par l'ALT, qui catalyse l'interconversion réversible de l'alanine et de l' α -cétoglutarate en pyruvate et glutamate (SHERMAN, 1992 ; LIU et al., 2014). Ainsi, l'expression accrue de GPT2 médiée par le BPA dans des testicules de rats immatures pourrait suggérer une réponse génomique compensatoire par le besoin de production de pyruvate ou d'alanine, comme déjà suggéré par le taux de consommation élevé de pyruvate par les cellules de Sertoli (OLIVEIRA et al., 2011 ; RATO et al., 2012b). Ces données précédentes renforcent nos résultats indiquant que les cellules de Sertoli de rats immatures (RATO et al., 2012b) et d'humains (OLIVEIRA et al., 2011) ont une baisse de la production d'alanine dans les 5 premières heures d'incubation, y compris en présence de E2. Cependant, une augmentation significative de la production d'alanine par les cellules de Sertoli de rats immatures est rapportée après 25 heures d'incubation avec 100 nM d'E2, indiquant un état redox plus faible/oxydatif plus élevé pour les cellules dans ces conditions (RATO et al., 2012b). Des données similaires sur la production d'alanine induite par l'E2 ont également été rapportées dans des cellules humaines de Sertoli après 35 heures d'incubation (OLIVEIRA et al. 2011). Comme discuté ci-dessus, le pyruvate est préférentiellement converti en lactate par réduction dépendante du NADH dans les cellules de Sertoli. Cependant, une partie du pyruvate peut être convertie en alanine. Comme la réduction du pyruvate en lactate ou sa conversion en alanine est liée à la réoxydation du NADH cytosolique en NAD⁺, le rapport lactate/alanine reflète le rapport NADH/NAD⁺ (RATO et al., 2012b).

La génération d'énergie à partir de précurseurs autres que le glucose, principalement dans des états de faible apport énergétique, est cruciale pour le maintien de la fonction des fonctions testiculaires. Ainsi, en plus du glycogène et de l'alanine, le glycérol contribue également à la production de pyruvate dans les cellules de Sertoli. Le glycérol converti en glycérol-3-phosphate par l'action de GK2 entre dans la voie glycolytique (CRISÓSTOMO et al., 2017). Dans nos conditions expérimentales, l'E2 n'a pas modifié l'expression de GK2. Cependant, nous avons montré pour la première fois que l'expression relative de GK2 a été significativement stimulée dans des testicules immatures de rat après 6 heures d'incubation avec du BPA (1 nM). Ainsi, nous avons mis en évidence sans précédent que la GK2 est une cible moléculaire des effets du BPA dans des testicules de rats immatures. Ces données sont en accord avec nos résultats précédents, suggérant que le BPA *in vitro* entraîne une consommation d'énergie élevée. Cependant, fait intéressant, une relation entre des niveaux élevés de GK2 et des perturbations de la motilité des spermatozoïdes a déjà été rapportée. Les niveaux de la protéine GK2 dans les spermatozoïdes humains asthénozoospermiques (définis comme ceux ayant une motilité progressive < 25 %) ont été démontrés plus élevés que dans les spermatozoïdes humains normozoospermiques (SIVA et al., 2010).

Cependant, aucun des effets génomiques du BPA observés dans la présente étude n'a été réversé par la préincubation avec l'antagoniste des ESR. Par conséquent, des études antérieures sont en accord avec nos résultats, qui ont rapporté que la modulation de l'expression des gènes par le BPA est médiée par les ESR α/β après des expositions à long terme, de quelques heures à quelques jours (GAIDO et al. 1997 ; LÖSEL ; WEHLING, 2003 ; MOLINA-MOLINA et al., 2013). Par conséquent, l'augmentation de l'expression relative de PYGM, PKM et LDHc médiée par l'E2 et le BPA n'est pas dépendante de l'activation des ESR. Ceci pourrait suggérer que dans les conditions expérimentales de notre étude, le BPA *in vitro* a le même mécanisme d'action que l'E2 pour induire l'expression de PYGM, PKM et LDHc. Par conséquent, nos résultats indiquent que les réponses génomiques médiées par l'E2 et le BPA à court terme sur les gènes impliqués au métabolisme sont des cibles transcriptionnelles de récepteurs autres que les ESR α/β de la voie génomique classique. Une *crosstalk* a été déjà rapportée entre les voies de signalisation non-génomiques et génomiques dépendantes du GPER qui convergent vers la transcription génique après une exposition à court terme à l'E2 et au BPA (CHIMENTO et al., 2010 ; 2011 ; GE et al., 2014 ; HERZ et al., 2017). De plus, il a déjà été rapporté que l'expression des gènes impliqués à la glycolyse est dépendante des *estrogen-related receptors* (ERR) (GIGUÈRE, 2008 ; TENNESSEN et al., 2011 ; CAI et al., 2013).

En outre, des expositions *in vitro* à l'E2 et au BPA pendant seulement 6 heures n'ont modifié l'expression d'aucun des gènes marqueurs des cellules germinales évalués dans la présente étude, dans nos conditions expérimentales. Néanmoins, seule une tendance à augmenter l'expression relative de PCNA ($p = 0,076$) de 1 nM de BPA a été observée. Le PCNA est l'un des marqueurs de prolifération des spermatogonies les plus utilisés (SCHLATT ; WEINBAUER, 1994 ; FRANCO et al.,

2010). Cependant, des études antérieures renforcent nos données rapportant que la modulation de l'expression des gènes marqueurs des cellules germinales accompagnée de modifications de la spermatogenèse sont médiées par le BPA ou l'estradiol sur des périodes d'exposition supérieures à 6 heures (ALI et al., 2014 ; ZHANG et al., 2018 ; LIU ; WANG ; LIU, 2021). L'exposition *in vitro* de cellules TM4 Sertoli à des concentrations nanomolaires de BPA pendant 48 heures a entraîné une augmentation de l'expression de la protéine PCNA (GE et al., 2014). D'autre part, il a été récemment rapporté qu'un traitement chronique *in vivo* avec du BPA diminue l'expression du gène PCNA dans des testicules de souris (LIU ; WANG ; LIU, 2021). En plus du BPA, un traitement chronique *in vivo* avec du benzoate d'estradiol a également été rapporté pour diminuer l'expression de PCNA dans les tubules séminifères de souris (ZHANG et al., 2018).

Conclusions – Chapitre I

Globalement, à partir d'incubations des testicules de zebrafish au BPA et des différentes approches méthodologiques, il a été mis en évidence que le BPA *in vitro* altère l'homéostasie calcique, le métabolisme énergétique et la spermatogenèse. Donc, le BPA augmente l'influx calcique et la surcharge cytosolique de Ca^{2+} impliquant une *crosstalk* entre les voies de signalisation non-génomiques et génomiques. De plus, il affecte le métabolisme énergétique testiculaire en réduisant des substrats énergétiques et des activités enzymatiques, ainsi qu'en altérant l'expression relative des gènes et des protéines impliqués dans le métabolisme glucidique, dépendant ou pas des ESR. Il altère également la prolifération cellulaire et la spermatogenèse en induisant une réduction de la proportion de cellules haploïdes dépendantes des ESR α/β . De plus, à partir d'expositions à court terme des poissons au BPA, il a été mis en évidence que le BPA *in vivo* affecte le métabolisme énergétique et la spermatogenèse. Donc, il affecte le métabolisme testiculaire en réduisant des substrats énergétiques et des activités enzymatiques, et altère la spermatogenèse en induisant une augmentation de l'apoptose de spermatocytes et une réduction de la proportion de cellules haploïdes.

Conclusions – Chapitre II

Globalement, à partir d'incubations *in vitro* à court terme d'explants de testicules de rats immatures à l'E2 et au BPA, il a été mis en évidence qu'ils altèrent le métabolisme énergétique testiculaire en modifiant l'expression relative des gènes impliqués au métabolisme des glucides, des acides aminés et des lipides, indépendante des ESR α/β . En revanche, ils n'affectent pas l'expression des gènes marqueurs des cellules germinales après les incubations *in vitro* à court terme.

Mots clés : Bisphénol A, calcium, lactate, métabolisme énergétique, œstradiol, récepteurs aux œstrogènes, spermatogenèse, testicule.

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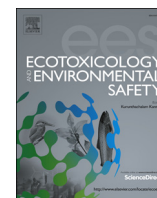
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APPENDIX

APPENDIX A - Article entitled "Role of bisphenol A on calcium influx and its potential toxicity on the testis of *Danio rerio*" published in 2020 in the *Ecotoxicology and Environmental Safety* journal (impact factor = 6.291).

APPENDIX B - Article entitled "*In vivo* and *in vitro* short-term bisphenol A exposures disrupt testicular energy metabolism and negative impact spermatogenesis in zebrafish" published in 2022 in the *Reproductive Toxicology* journal (impact factor = 3.143).



Role of bisphenol A on calcium influx and its potential toxicity on the testis of *Danio rerio*

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ABSTRACT

This study investigated the acute *in vitro* effect of low-concentration bisphenol A (BPA) on calcium ($^{45}\text{Ca}^{2+}$) influx in zebrafish (*Danio rerio*) testis and examined whether intracellular Ca^{2+} was involved in the effects of BPA on testicular toxicity. *In vitro* studies on $^{45}\text{Ca}^{2+}$ influx were performed in the testes after incubation with BPA for 30 min. Inhibitors were added 15 min before the addition of $^{45}\text{Ca}^{2+}$ and BPA to testes to study the mechanism of action of BPA. The involvement of intracellular calcium from stores on lactate dehydrogenase (LDH) release and on triacylglycerol (TAG) content were carried out after *in vitro* incubation of testes with BPA for 1 h. Furthermore, gamma-glutamyl transpeptidase (GGT) and aspartate aminotransferase (AST) activities were analyzed in the liver at 1 h after *in vitro* BPA incubation of *D. rerio*. Our data show that the acute *in vitro* treatment of *D. rerio* testes with BPA at very low concentration activates plasma membrane ionic channels, such as voltage-dependent calcium channels and calcium-dependent chloride channels, and protein kinase C (PKC), which stimulates Ca^{2+} influx. In addition, BPA increased cytosolic Ca^{2+} by activating inositol triphosphate receptor (IP_3R) and inhibiting sarco/endoplasmic reticulum calcium ATPase (SERCA) at the endoplasmic reticulum, contributing to intracellular Ca^{2+} overload. The protein kinases, PKC, MEK 1/2 and PI3K, are involved in the mechanism of action of BPA, which may indicate a crosstalk between the non-genomic initiation effects mediated by PLC/PKC/ IP_3R signaling and genomic responses of BPA mediated by the estrogen receptor (ESR). *In vitro* exposure to a higher concentration of BPA caused cell damage and plasma membrane injury with increased LDH release and TAG content; both effects were dependent on intracellular Ca^{2+} and mediated by IP_3R . Furthermore, BPA potentially induced liver damage, as demonstrated by increased GGT activity. In conclusion, *in vitro* effect of BPA in a low concentration triggers cytosolic Ca^{2+} overload and activates downstream protein kinases pointing to a crosstalk between its non-genomic and genomic effects of BPA mediated by ESR. Moreover, *in vitro* exposure to a higher concentration of BPA caused intracellular Ca^{2+} -dependent testicular cell damage and plasma membrane injury. This acute toxicity was reinforced by increased testicular LDH release and GGT activity in the liver.

1. Introduction

A wide variety of exogenous compounds function as Endocrine Disrupting Chemicals (EDC), and cause deleterious effects on the male reproductive system including effects on fertility (Hill and Janz, 2003; Brouard et al., 2016). Bisphenol A [2,2-bis(4-hydroxyphenyl)propane] (BPA) is an EDC that is considered to be a xenoestrogen. Xenoestrogens are compounds that produce estrogenic or anti-androgenic responses in

several animal species by mimicking the action of 17β -estradiol (E2) and, therefore, interfering with endogenous endocrine regulation (Molina-Molina et al., 2013; Urriola-Munoz et al., 2014). BPA is used for manufacturing epoxy resins to coat metal cans and for polymerizing polycarbonate plastic for the manufacture of food utensils, plastic containers, packaging, dental sealants, bottles and water supply tubes (Kang et al., 2006; Koch and Calafat, 2009). As such, human exposure to BPA is a frequent occurrence, since BPA is released from

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polycarbonate plastic at high temperatures (Le et al., 2008).

BPA has a similar structure to the synthetic estrogen diethylstilbestrol (DES), a potent nuclear estrogen receptor (ER) agonist (Seachrist et al., 2016). BPA can bind to classical nuclear ER α and ER β subtypes and trigger genomic responses by the activation of gene transcription factors (Bouskine et al., 2009). However, a third type of classical ER, initially denominated ER γ , was identified in goldfish (*Carassius auratus*) and Atlantic croaker fish (*Micropogonias undulatus*) (Hawkins et al., 2000). Subsequently, this ER was renamed as ER- β 2 when identified in *D. rerio* and, together with ER- β 1, it is strongly expressed in the testis (Menuet et al., 2002). This ER subtype has not been identified in any other vertebrate classes. Subsequently, the Official Zebrafish Nomenclature Guidelines standardized the nomenclature of ER subtypes from *D. rerio*, with the ER α subtype now designated as Esr1, the ER β 1 subtype now designated as Esr2b and the ER β 2 subtype now designated as Esr2a (Filby and Tyler, 2005).

BPA can also act through non-genomic activation via membrane-associated ERs, G protein-coupled receptor (GPR30) activation or stimulation of Ca²⁺ flux. This activation occurs within a time frame of seconds to minutes, via the activation of protein kinases, enzyme activity changes, Ca²⁺, Cl⁻ and K⁺ channel activation, secretory activity and generation of second messengers such as intracellular Ca²⁺, cAMP or changes in nitric oxide (Wozniak et al., 2005; Thomas and Dong, 2006; Watson et al., 2007; Ehrenmann et al., 2017; Gonçalves et al., 2018). The influence of ionic and channel activities on the physiological and biochemical processes of male reproductive functions has been investigated through pharmacological approaches (Alavi and Cosson, 2006; Darszon et al., 2011), where Ca²⁺ signaling has been shown to play a fundamental role in major testicular events, such as hormone exocytosis (Watson and Gametchu, 1999; Wozniak et al., 2005), secretory vesicle release from Sertoli cells (Menegaz et al., 2010; Zanatta et al., 2013) and the acrosome reaction (De Blas et al., 2002). In addition, Ca²⁺ is crucial for maturation and motility of spermatozoa (Alavi et al., 2004; Golpour et al., 2017). BPA, at picomolar to nanomolar concentrations, triggers Ca²⁺ and protein changes, which are mediated by non-genomic pathways through the plasma membrane estrogen receptor in pituitary tumor cells (Wozniak et al., 2005), pancreatic α -cells (Alonso-Magdalena et al., 2005), human testicular seminoma cells (Bouskine et al., 2009), rat pituitary cells (Jeng et al., 2010), brain microvascular endothelial cells (Altmann et al., 2015) and immature rat testes (Gonçalves et al., 2018).

A growing number of scientific reports of studies in animal models or humans indicate that BPA disrupts the endocrine environment and causes deleterious effects on male reproductive function (Thomas and Dong, 2006; Rouiller-Fabre et al., 2015). BPA exposure has been shown to negatively affect sperm quality and, consequently, male fertility by reducing the total number, volume, density, motility and velocity of the goldfish sperm (Hatef et al., 2012, 2013). High concentrations of BPA may also cause an increase in reactive oxygen species (ROS) production, mitochondrial dysfunction, intracellular Ca²⁺ overload and rat Sertoli cell apoptosis (Wang et al., 2017). Furthermore, a single dose of BPA rapidly induces the activation of caspases and germ cell apoptosis through the activation of p38 MAPK in male rats (Urriola-Munoz et al., 2014). In addition, BPA, at nanomolar concentrations, also disrupts oocyte meiotic maturation in *D. rerio*, mimicking the effects of E2 by a non-genomic estrogenic mechanism involving activation of the GPER/EGFR/MAPK pathway (Fitzgerald et al., 2015). As such, it is clear that the effect and the rapid response mechanism of action of BPA starts at the plasma membrane and involves crosstalk to alter unknown genomic responses in the testis of *D. rerio*. In addition to the toxic effects of BPA on testes, BPA is also closely associated with liver toxicity; which makes these two organs the main targets for EDC compounds. Furthermore, the liver is the main organ taken into consideration in toxicological studies, since it has an important role in the metabolism of endogenous and exogenous substances (Uzunhisarcikli and Aslanturk, 2019). Due to the high sensitivity of gamma-glutamyl transpeptidase (GGT) and

aspartate aminotransferase (AST) to liver dysfunction and toxicity, these enzymes are useful to assist in the identification of such disorders (Giannini et al., 2005).

We hypothesized that BPA alters Ca²⁺ homeostasis through activation of the membrane-initiated signaling pathway in the testis of *D. rerio*, which induces toxicity and disturbs crucial testicular functions and male fertility. In the present study, the acute *in vitro* effects of low-concentration BPA were investigated to understand the non-genomic responses and the crosstalk with genomic pathway triggered by Ca²⁺ influx in the testis of *D. rerio*. The effects of BPA on cell viability and the involvement of intracellular Ca²⁺ in the mechanism of toxicity of BPA in the testis were investigated. The acute toxic effects of BPA were also analyzed in the liver.

2. Materials and methods

2.1. Chemicals

(7,17 α)-7-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), 9-anthracene carboxylic acid, bisphenol A (BPA), bovine serum albumin, capsazepine, CFTR (inh)-172, H-89 dihydrochloride hydrate, nifedipine, PD 98,059, Ro 31-0432 and thapsigargin were purchased from Sigma-Aldrich (St. Louis, MO, USA). [⁴⁵Ca] CaCl₂ (sp. act. 321 KBq/mg Ca²⁺), specific activity 9.25 GBq/mmol, and OptiphasHisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Boston, USA). Kits for measuring the activities of aspartate aminotransferase (AST) (Ref: 109-2/100), gamma-glutamyl transpeptidase (GGT) (Ref: 105-2/50) and lactate dehydrogenase (LDH) (Ref: 86-1/100) were purchased from Labtest Liquiform (Lagoa Santa, MG, Brazil). All other chemicals were of analytical grade.

2.2. Animals

D. rerio were obtained commercially (Belo Horizonte, MG, Brazil). Male fish with lengths from 2.5 to 3.5 cm and weighing 200–300 mg were kept in aquarium on a 12 h light/dark cycle and fed twice a day with feed for ornamental fish. Aquarium water was maintained at 27 \pm 2 °C, pH 7 \pm 0.3 and salinity 0.7 \pm 2 g/L. All fish and the parameters of aquarium water were carefully monitored and maintained in accordance with the ethical recommendations of the local Ethical Committee for Animal Use of the Federal University of Santa Catarina (Protocol CEUA/UFSC/PP00968).

2.3. ⁴⁵Ca²⁺ influx

In vitro studies on Ca²⁺ influx were based on a standardized protocol for *D. rerio* (Batista-Silva et al., 2020). The whole testes from *D. rerio* were dissected and quickly placed in microtubes containing Cortland's buffer (124 mM NaCl, 5 mM KCl, 1.7 mM CaCl₂, 3.4 mM NaH₂PO₄, 2.1 mM MgCl₂, 1.91 mM MgSO₄, 11.9 mM NaHCO₃ and 1 g/L glucose) and kept on ice. Testes were preincubated in Cortland's buffer gassed with O₂: CO₂, 95: 5 v/v, pH 7.4 at 28 \pm 1 °C for 15 min. After that, the medium was replaced with fresh Cortland's buffer containing 0.1 μ Ci/mL ⁴⁵Ca²⁺ (an input about 20,000 counts per minute - cpm) in the absence (control) or presence of BPA (10⁻¹⁵ M (1 fM), 10⁻¹² M (1 pM), 10⁻¹¹ M (10 pM), 10⁻⁹ M (1 nM), 10⁻⁸ M (10 nM) and 10⁻⁶ M (1 μ M)) for 30 min (incubation time). Additionally, to determine the time-course of 10 pM BPA action, testes were incubated for 5, 10, 15, 20 and 30 min. To investigate BPA's mechanism of action on ⁴⁵Ca²⁺ influx, channel blockers, receptor antagonists and enzyme inhibitors were added 15 min before ⁴⁵Ca²⁺ and BPA incubation. The concentration of drugs nifedipine (100 μ M), a blocker L-type voltage-dependent Ca²⁺ channels (L-VDC); 9-anthracene (1 μ M) and CFTR (inh)-172 (5 μ M), blockers of Ca²⁺-dependent chloride channels (CaCC) and cystic fibrosis transmembrane conductance regulator

(CFTR) chloride channels, respectively; thapsigargin (10 μ M), an inhibitor of Ca^{2+} -ATPase of sarco/endoplasmic reticulum (SERCA); ICI 182780 (10 μ M); an ESR antagonist; H-89 (10 μ M) an inhibitor of protein kinase A (PKA); Ro 31-0432 (1 μ M) an inhibitor of protein kinase C (PKC) and PD 98059, an inhibitor of MAPK kinase (MEK 1/2) were chosen based on a similar approach used for studies on calcium influx in immature rat testis (Batista-Silva et al., 2020; Gonçalves et al., 2018). Furthermore, specific antagonist or inhibitors as capsazepine (10 μ M), an antagonist of transient receptor potential vanilloid sub-family 1 (TRPV1) (Auzanneau et al., 2008); 2-APB (50 μ M), an antagonist of inositol triphosphate receptor (IP_3R) (Peters and Piper, 2007) and LY 294002 (10 μ M) an inhibitor of phosphatidylinositol-3-kinase (PI3K) (Cavalli et al., 2013) were tested in this study.

At the end of the incubation period, lanthanum chloride solution (127.5 mM NaCl, 4.6 mM KCl, 1.2 mM MgSO_4 , 10 mM HEPES, 11 mM glucose, 10 mM LaCl_3 , pH 7.3; 2 $^\circ\text{C}$), was used to stop the Ca^{2+} flow. Subsequently, two lanthanum washes were performed to remove the extracellular Ca^{2+} . The cold washing solution containing lanthanum is essential to prevent the Ca^{2+} efflux and/or active extrusion and removal of any surface-bound calcium (Batra, 1982).

Finally, the samples were transferred to a new series of tubes and homogenized with NaOH (0.5 M) and aliquots (duplicates) were taken from each sample for radioactivity measurement using a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman, Boston, USA) (Batista-Silva et al., 2020) and for total protein measurements (Lowry et al., 1951). The results were expressed as pmol of $^{45}\text{Ca}^{2+}$ per μg of protein or as % of control of 4–7 fish per group from three independent experiments. These were obtained by the conversion of counts per minute (CPM) for each sample normalized by the total protein content.

2.4. Lactate dehydrogenase activity

The release of LDH to the extracellular medium was used to analyze the *in vitro* effect of BPA on cell viability (Korzeniewski and Callewaert, 1983; Gaucher and Jarraya, 2015). Testes from *D. rerio* were dissected and incubated *in vitro* for 1 h with Cortland buffer (control group) or 10 pM, 10 nM and 10 μ M BPA in atmosphere gassed with O₂: CO₂, 95: 5 v/v, 28 \pm 1 $^\circ\text{C}$. After incubation, LDH release was measured in the incubation medium. To analyze the intracellular involvement of Ca^{2+} in the effect of BPA (10 μ M), the inositol triphosphate receptor (IP_3R) antagonist 2-APB was added 15 min before BPA incubation and LDH activity was measured, according to the manufacturer's instructions, by the NADH oxidation method (Labtest Liquiform; Lagoa Santa, MG, Brazil). The resulting reduction of absorbance at 340 nm on this kinetic assay is directly proportional to LDH activity in the sample (Nehar et al., 1997). The results were expressed as U/L based on 5–6 fish per group from three independent experiments.

2.5. Triacylglycerol measurement

To analyze the *in vitro* effect of BPA on testicular triacylglycerol (TAG) content in *D. rerio*, testes were incubated for 1 h with 10 pM or 10 μ M BPA or with Cortland buffer (control group) in an atmosphere gassed with O₂: CO₂, 95: 5 v/v, 28 \pm 1 $^\circ\text{C}$. To determine the involvement of intracellular Ca^{2+} in the effects of BPA (10 μ M) on TAG content, the IP_3R antagonist 2-APB was added 15 min before BPA incubation. Testicular TAG content was then measured, according to the manufacturer's instructions by glycerol phosphate oxidase method (Labtest Liquiform; Lagoa Santa, MG, Brazil). The results were expressed as mg of TAG per μg of protein (Lowry et al., 1951) of 5–6 fish per group from three independent experiments.

2.6. Aspartate aminotransferase activity and gamma-glutamyl transpeptidase

In order to analyze the potential toxicity promoted by BPA on AST and GGT activities, these enzymes were measured in the liver of *D. rerio*. For this, livers were dissected and incubated *in vitro* for 1 h in Cortland buffer (control group) or 10 pM, 10 nM and 10 μ M BPA in atmosphere gassed with O₂: CO₂, 95: 5 v/v, 28 \pm 1 $^\circ\text{C}$. Subsequently, AST and GGT activities were measured using commercial kits and according to the manufacturer's instructions (Labtest Liquiform; Lagoa Santa, MG, Brazil). The reduction of absorbance at 340 nm in this kinetic assay is directly proportional to AST activity in the sample (Nehar et al., 1997). The activity of AST was expressed as U/ μg of protein (Lowry et al., 1951) of 5–6 fish per group from three independent experiments. For GGT measurement, a colorimetric and fixed-time kinetic test was employed using L- γ -glutamyl-p-nitroaniline as the substrate and glycylglycine as the acceptor molecule, where the amount of p-nitroaniline formed was directly proportional to GGT activity in the sample. The reading was performed at 405 nm and the GGT activity was expressed as U/ μg of protein (Lowry et al., 1951) of 4–6 fish per group from three independent experiments.

2.7. Statistical analyses

Data are represented as means \pm standard error of the mean (S.E.M.) of 4–7 fish per group from three independent experiments. The results were analyzed by the STATISTICA program and GraphPad Prism 5. One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or Student's *t*-test were used to identify significant differences between groups, when $p \leq 0.05$.

3. Results

3.1. *In vitro* acute incubation of *D. rerio* testis with low-concentration BPA increases calcium influx

To study the effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio*, a concentration-response curve of BPA at 10^{-15} M (1 fM), 10^{-12} M (1 pM), 10^{-11} M (10 pM), 10^{-9} M (1 nM), 10^{-8} M (10 nM) and 10^{-6} M (1 μ M) was assayed with 30 min of incubation. BPA treatment at 10 pM and 10 nM stimulated $^{45}\text{Ca}^{2+}$ influx, compared to the control group (Fig. 1A). Subsequently, a time-course protocol was used to examine the effect of BPA on $^{45}\text{Ca}^{2+}$ influx after 5, 10, 15, 20 and 30 min of incubation. For the first 5–20 min of incubation, there was no difference between control and BPA treated group. However, BPA significantly stimulated $^{45}\text{Ca}^{2+}$ influx at 30 min (Fig. 1B). Consequently for the further experiments, 10 pM BPA and 30 min of incubation was used for studies of $^{45}\text{Ca}^{2+}$ influx.

3.2. *In vitro* BPA incubation increases $^{45}\text{Ca}^{2+}$ influx via L-type voltage-dependent calcium channels and calcium-dependent chloride channels in the testis of *D. rerio*

The mechanism of action of BPA on rapid responses in the testis of *D. rerio* was studied using a pharmacological approach employing channel blockers, receptor antagonists and enzyme inhibitors. As illustrated in Fig. 2A, the increase in $^{45}\text{Ca}^{2+}$ influx promoted by BPA was prevented by the addition of nifedipine, a blocker of L-type voltage-dependent calcium channels (L-VDCC). The stimulatory effect of BPA was similar to that observed for testes that were incubated with capsazepine, an antagonist of transient receptor potential vanilloid sub-family 1 (TRPV1). However, when testes were co-incubated with BPA and capsazepine, $^{45}\text{Ca}^{2+}$ influx was potentiated, indicating that TRPV1 does not participate in the mechanism of BPA in this event in the *D. rerio* testis (Fig. 2B). Subsequently, the increase in $^{45}\text{Ca}^{2+}$ influx promoted by BPA was abolished by the addition of 9-anthracene, a Ca^{2+} -

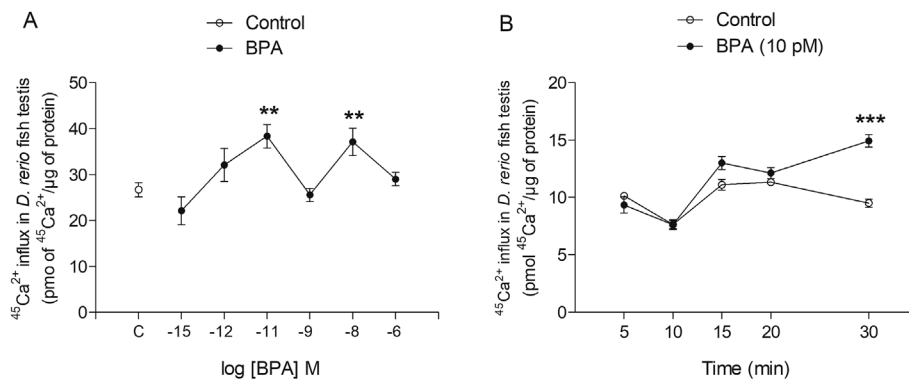


Fig. 1. Concentration-response curve and time-course of $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio* incubated with BPA. Effects of BPA on $^{45}\text{Ca}^{2+}$ influx: (A) concentration-response curve; (B) time-response curve. Results are expressed as mean \pm S.E.M. of 4–7 fish per group from three independent experiments. Data were analyzed by one-way ANOVA, followed by the Bonferroni *post hoc* test. **p < 0.01 when compared to control group; ***p < 0.001 when compared to the respective control group at 30 min.

dependent chloride channel (CaCC) blocker, suggesting that the increase in $^{45}\text{Ca}^{2+}$ influx induced by BPA was also mediated by the activity of this channel (Fig. 2C). Additionally, the $^{45}\text{Ca}^{2+}$ influx stimulated by BPA was similar to that observed in the presence of CFTR(inh)-172, an inhibitor of cystic fibrosis transmembrane conductance regulator chloride channels. Notably, this response was not potentiated by co-incubation with the two compounds, indicating that this type of chloride channels does not participate in the mechanism of action of BPA on the testis of *D. rerio* (Fig. 2D).

3.3. Acute *in vitro* incubation of the testis of *D. rerio* with BPA promotes intracellular calcium storage depletion through inositol triphosphate receptor activation and Ca^{2+} -ATPase inhibition, consequently leading to store-operated Ca^{2+} channels activation

The involvement of intracellular Ca^{2+} and store-operated Ca^{2+} channels (SOC), such as inositol triphosphate receptor (IP_3R) and sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), in the effects of BPA on the testis of *D. rerio* was studied. As illustrated in Fig. 3A, when the IP_3R antagonist, 2-APB, was co-incubated with BPA, the stimulatory effect of BPA was abolished. In addition, Fig. 3B shows that the BPA-induced increase in $^{45}\text{Ca}^{2+}$ influx was abrogated when testes were co-

incubated with BPA and the SERCA inhibitor, thapsigargin.

3.4 The effects of BPA in the testis of *D. rerio* are mediated by protein kinase C, mitogen-activated protein kinase, phosphatidylinositol-3-kinase and nuclear estrogen receptor, indicating a crosstalk between non-genomic and genomic responses.

Intracellular targets, such as kinase proteins, were also studied in the *D. rerio* testis. Fig. 4A shows that when testes were incubated with both BPA and H-89, a protein kinase A (PKA) inhibitor, the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx was not modified, indicating that PKA activation does not participate in this event. In contrast, Fig. 4B shows that when the testis of *D. rerio* was co-incubated with Ro 31-0432 a protein kinase C (PKC) inhibitor and BPA, the stimulatory effect of BPA was abolished, indicating that PKC is involved in the mechanism of action of BPA. Furthermore, as demonstrated in Fig. 4C, when testes were co-incubated with PD 98059, a mitogen-activated protein kinase (MEK 1/2) inhibitor, and BPA, the increase in $^{45}\text{Ca}^{2+}$ influx promoted by BPA was abolished, showing that MEK is involved in the downstream mechanism of action of BPA in the testis. In addition, as can be seen in Fig. 4D, LY 294002, a phosphatidylinositol-3-kinase (PI3K), abolished the effects of BPA on the increase in $^{45}\text{Ca}^{2+}$ influx, indicating that PI3K activation is also involved in BPA's mechanism of action.

Since BPA can bind to the nuclear ESR, the involvement of these

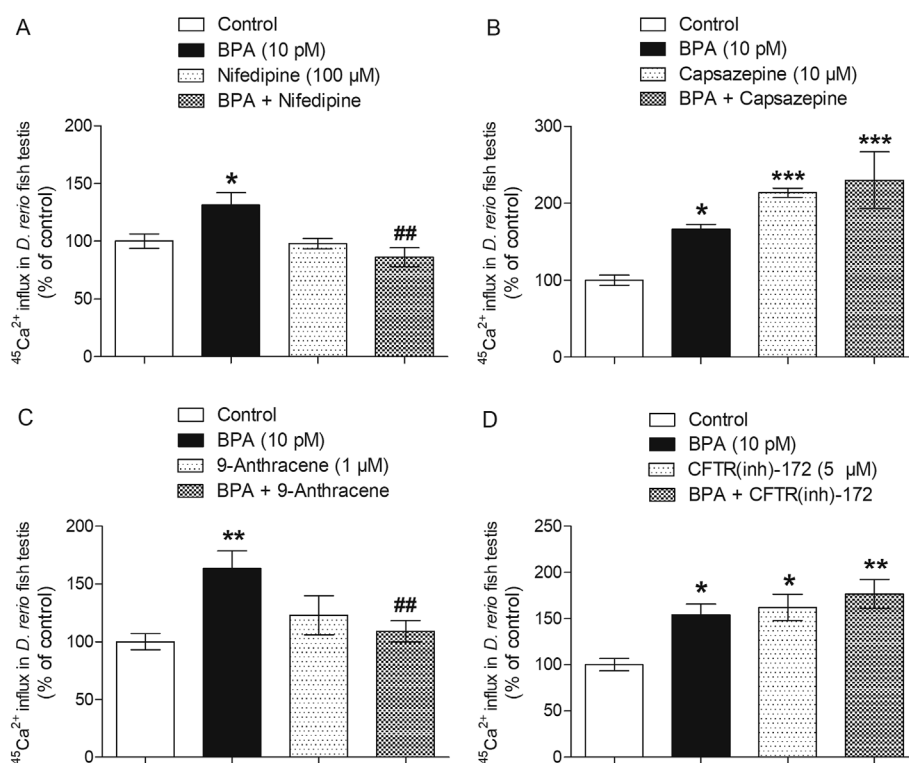
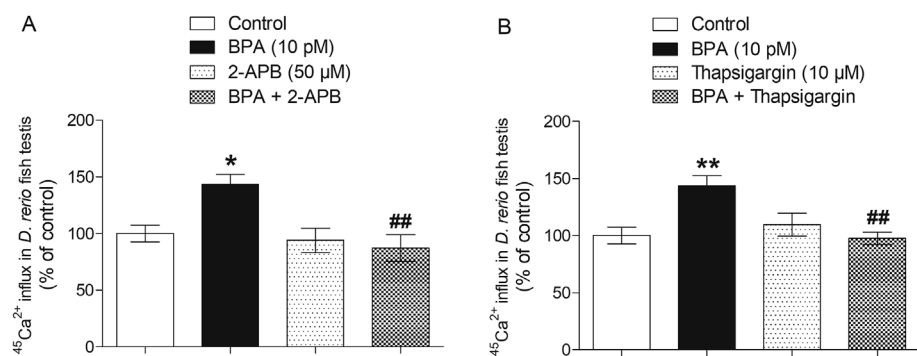


Fig. 2. Membrane ion channels involvement in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio*. Involvement of (A) L-type VDCC, (B) TRPV1, (C) CaCC and (D) CFTR channels on the effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio*. (A) L-type VDCC blocker nifedipine, (B) TRPV1 antagonist capsazepine, (C) CaCC blocker 9-anthracene or (D) CFTR blocker CFTR(inh)-172 were added 15 min before *in vitro* BPA incubation (30 min). The results are expressed as mean \pm S.E.M. of 4–7 fish per group from three independent experiments. Data were analyzed by one-way ANOVA, followed by the Bonferroni *post hoc* test. *p < 0.05, **p < 0.01 and ***p < 0.001, when compared to control group; ##p < 0.01, when compared to BPA group.



receptors in BPA-induced $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio* was investigated. Fig. 5 shows that the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in *D. rerio* testes was completely abolished when the classic ESR antagonist ICI 182,780 was co-incubated with BPA.

3.5. Involvement of intracellular calcium influx in the potential toxicity of BPA in the testis of *D. rerio*

In order to investigate whether *in vitro* acute BPA exposure induces plasma membrane damage and cell death induction, the release of LDH from the testis of *D. rerio* to the extracellular medium was investigated. LDH activity was analyzed after *in vitro* incubation of the *D. rerio* testes for 1 h with BPA at different concentrations (10 pM, 10 nM and 10 μM) (Fig. 6A). Only 10 μM BPA was able to induce LDH release to the extracellular medium, in relation to the control group. As such, the participation of IP₃R in BPA-induced Ca^{2+} influx was examined in order to analyze the role of the release of intracellular Ca^{2+} from stores. For this, the testis of *D. rerio* were incubated with the IP₃R antagonist 2-APB for 15 min before BPA incubation. Fig. 6B shows that when testes were co-incubated with 2-APB and BPA, the BPA-induced LDH release was completely abolished, indicating that IP₃R and the release of intracellular Ca^{2+} from stores were involved in the plasma membrane

injury that was triggered by the higher concentration of BPA. Subsequently, the involvement of Ca^{2+} release from intracellular stores in the BPA-induced increase in TAG content in testes was also investigated. Fig. 6C shows that when testes were incubated *in vitro* with the highest concentration of BPA (10 μM) for 1 h, testicular TAG content increased. As can be seen in Fig. 6D, when testes were co-incubated with BPA and the IP₃R antagonist, 2-APB, the stimulatory effect of BPA on testicular TAG content was also completely abolished.

3.6. Effects of acute *in vitro* incubation of liver from *D. rerio* with BPA on gamma-glutamyl transpeptidase and aspartate aminotransferase activities

To investigate the potential toxicity of BPA on *D. rerio* fish, livers of *D. rerio* were incubated with 10 pM, 10 nM and 10 μM BPA for 1 h and the activities of GGT and AST enzymes were analyzed. Fig. 7A shows that there was a significant increase in liver GGT activity, but not in liver AST activity (Fig. 7B) after incubation with BPA.

4. Discussion

To our knowledge, this is the first demonstration of the acute effects of low concentrations of BPA on Ca^{2+} signaling in the zebrafish testis

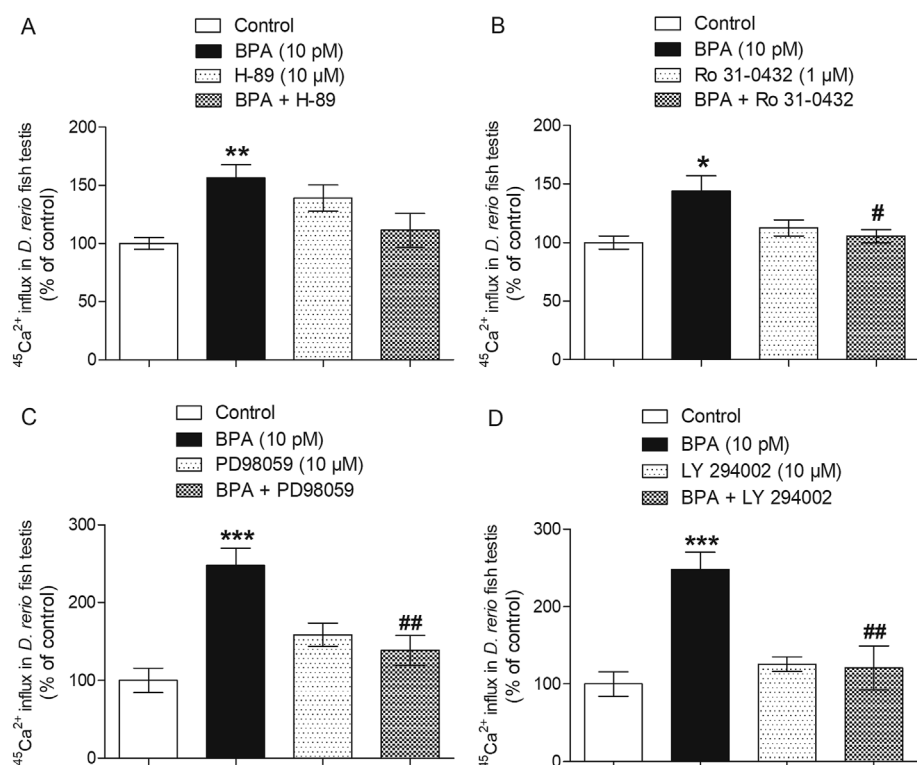


Fig. 4. Involvement of protein kinases in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio*. Involvement of (A) PKA, (B) PKC, (C) MAPK kinase, (D) PI3K in the effects of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis. The (A) PKA inhibitor H-89, (B) PKC inhibitor Ro 31-0432, (C) MEK 1/2 inhibitor PD 98059 or (D) PI3K inhibitor LY 294002 were added 15 min before *in vitro* incubation of testes with BPA (30 min). The results are expressed as mean \pm S.E.M. of 4–7 fish per group from three independent experiments. Data were analyzed by one-way ANOVA followed by the Bonferroni *post hoc* test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, when compared to control group; # $p < 0.05$ and ## $p < 0.01$, when compared to BPA group.

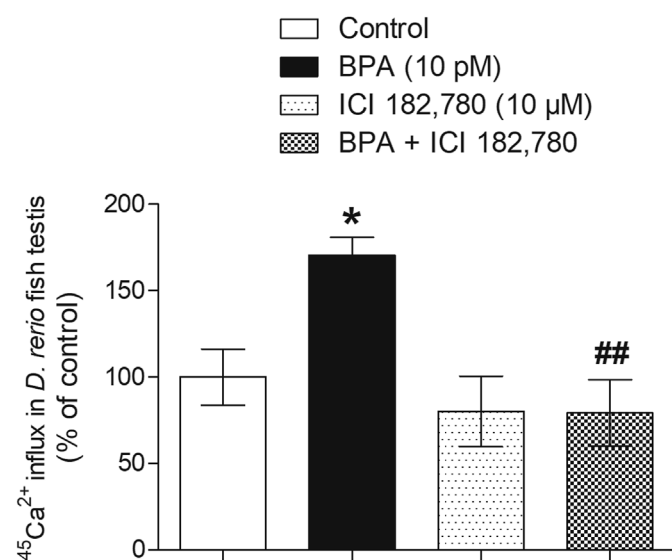


Fig. 5. Involvement of the estrogen receptor in the stimulatory effect of BPA on $^{45}\text{Ca}^{2+}$ influx in the testis of *D. rerio*. ICI 182780, an ESR antagonist was added 15 min before *in vitro* incubation of testes with BPA (30 min). The results are expressed as mean \pm S.E.M. of 4–7 fish per group from three independent experiments. Data were analyzed by one-way ANOVA followed by the Bonferroni *post hoc* test. * $p < 0.05$, when compared to control group; ## $p < 0.01$, when compared to BPA group.

which involves crosstalk between the non-genomic initiation effects mediated by PLC/PKC/IP₃R signaling and genomic responses of BPA mediated by the estrogen receptor. Additionally, we show that Ca^{2+} influx following exposure to a higher concentration of BPA induced *D. rerio* testis and liver toxicity. In the present study, some targets involved in Ca^{2+} signaling at the plasma membrane were studied, including L-VDCC, TRPV1, CaCC and the CFTR chloride channel. Also, targets involved in the intracellular Ca^{2+} -stores, such as IP₃R and SERCA. We used a pharmacological approach and based the concentrations of the various drugs tested in studies from mammalian species and in testis from *D. rerio* (Batista-Silva et al., 2020).

In the present study, we demonstrated, for the first time, that BPA activates L-VDCC in the testis of *D. rerio*. Consistent with our findings, BPA can also activate VDCC and voltage-dependent K^+ channels in human and canine coronary smooth muscle, but at concentrations 10^6 times more elevated than those used in our study (Asano et al., 2010). In addition, chronic BPA exposure (100 $\mu\text{g}/\text{kg}/\text{day}$) promotes up-regulation of T-VDCC mRNA expression that is dependent on nuclear ER activation in the mouse testis and epididymis (Wang et al., 2015). Furthermore, we recently reported that bis(2-ethylhexyl)phthalate, another EDC from plastic industry, also exhibits similar signal transduction on $^{45}\text{Ca}^{2+}$ influx mediated by L-VDCC (Batista-Silva et al., 2020), providing evidence that L-VDCC is a potent target of EDC action in the testis of *D. rerio*. Tran et al. (2018) showed that *in utero* BPA (100 mg/kg/day) in mice impairs embryo implantation through alterations in Ca^{2+} transport and the gene expression of channels, such as TRPV 5 and TRPV 6. However, we showed that the induction of Ca^{2+} influx by BPA is independent of TRPV1 activation in the testis of *D.*

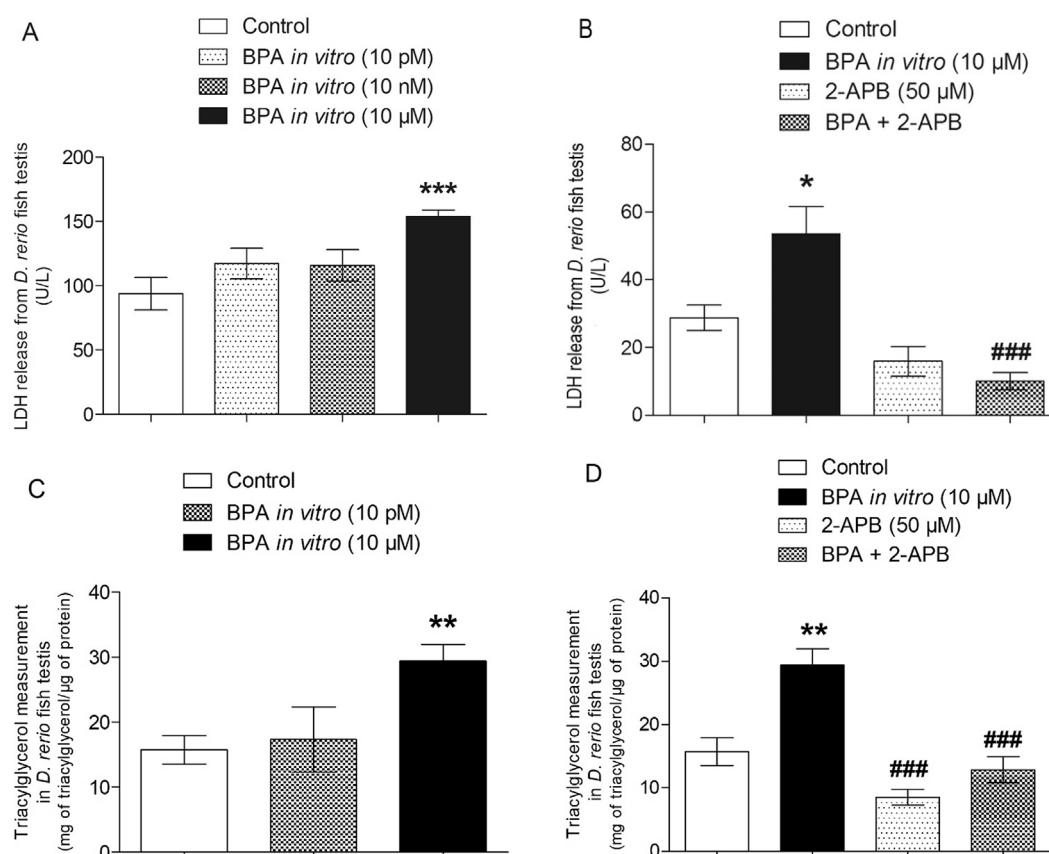


Fig. 6. Inositol triphosphate receptor (IP₃R) activation induces cell and plasma membrane damage in the testis of *D. rerio*. (A) Effects of exposure of testes with different concentrations of BPA on LDH release after 1 h of incubation. (B) Involvement of IP₃R in LDH release after incubation with BPA. (C) Effects of BPA exposure for 1 h on triacylglycerol (TAG) content. (D) Involvement of IP₃R in the BPA-induced increase in TAG content in the testis of *D. rerio*. The IP₃R antagonist 2-APB was added 15 min before BPA incubation (1 h). The results are expressed as mean \pm S.E.M. of 5–6 fish per group from three independent experiments. The data were analyzed by (A and C) *t*-Student test and (B and D) by one-way ANOVA, followed by the Bonferroni *post hoc* test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, when compared to control group; ### $p < 0.001$, when compared to BPA group.

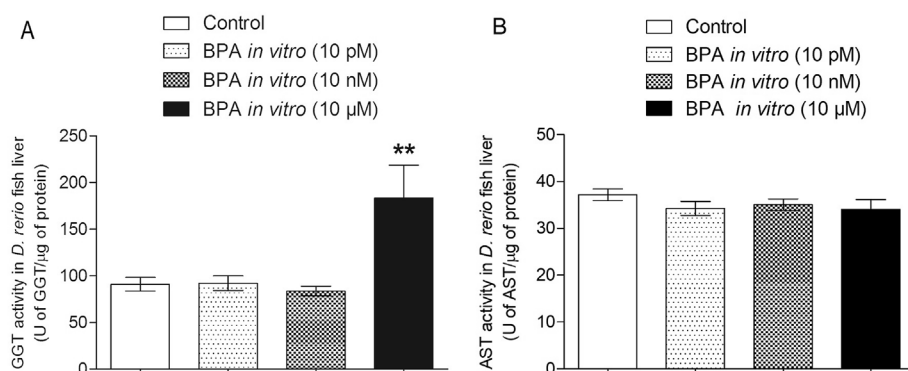


Fig. 7. BPA increases the activity of gamma-glutamyl transpeptidase (GGT) in the liver of *D. rerio*. Effects of BPA after 1 h of *in vitro* incubation of livers on (A) GGT and (B) aspartate aminotransferase (AST) activities. The results are expressed as means \pm S.E.M. of 4–6 fish per group from three independent experiments. The data were analyzed by *t*-Student test. ** $p < 0.01$, when compared to control group.

rerio.

Since the increase in Ca^{2+} influx, mediated by the plasma membrane VDCC, may occur via CaCC-dependent membrane depolarization (Hartzell et al., 2005; Verkman and Galletta, 2009), the involvement of CaCC in the mechanism of action of BPA was studied. CaCCs are described in many different cell types, such as the Sertoli cells, where these channels are widely involved in cellular secretion (Menegaz et al., 2010). Rapid exposure to very low concentration of BPA (1 pM) has been shown to trigger immediate increases in Ca^{2+} influx in the immature rat testis that is CaCC dependent, suggesting that BPA may influence secretory activity in the testis (Gonçalves et al., 2018). Our results are in agreement with these previous findings, demonstrating that BPA-stimulated Ca^{2+} influx increased was also associated with CaCC participation in the testis of *D. rerio*. In addition, it has been reported that CaCC-induced depolarization is fundamental to fertilization in amphibian oocytes. Cytosolic Ca^{2+} increase due to Ca^{2+} release from stores, via IP₃ receptor activation, occurs upon fertilization and causes opening of the CaCCs to produce membrane depolarization and prevent the fusion of additional sperm (polyspermy) (Hartzell, 1996; Machaca et al., 2002). CFTR is a cAMP-activated chloride channel found in the apical cell plasma membrane of fluid-transporting tissues, such as airways, the intestine, pancreas and testis; our data indicate that the mechanism of action of BPA is independent of the CFTR chloride channel and PKA activation in the *D. rerio* testis, consistent with the findings of other studies (Verkman and Galletta, 2009).

Having defined the involvement of L-VDCC and CaCC in the mechanism of action of BPA, the role of the IP₃ pathway in modulating the release of intracellular ionic Ca^{2+} from stores Ca^{2+} was studied, as CaCC activation can also be mediated by IP₃R signaling and SOC activation (Kuruma and Hartzell, 2000; Hartzell et al., 2005). Additionally, Ca^{2+} influx through the L-type VDCC shown in our study could also have induced plasma membrane depolarization via CaCC activation. In some cell types, membrane depolarization activates VDCC, which results in additional Ca^{2+} influx and further depolarization (Verkman and Galletta, 2009) and Ca^{2+} store depletion stimulates Ca^{2+} influx by SOCs, which also contributes to CaCC activation (Kuruma and Hartzell, 2000; Hartzell et al., 2005). The activation of phospholipase C (PLC), PKC and IP₃R has been associated with activation of the GPR membrane-initiated signaling pathway (Le Mellay et al., 1997); BPA-induced Ca^{2+} influx is reported to involve PLC activation in the rat testis (Gonçalves et al., 2018) and our results demonstrate that BPA-induced Ca^{2+} influx involves IP₃R. Furthermore, as shown in our study, PKC activation was also crucial to the mechanism of action of BPA and these data are in agreement with previous studies in the rat testis (Gonçalves et al., 2018).

Our data also suggest that SERCA inhibition by BPA may also contribute to an increase of cytosolic Ca^{2+} in the testis of *D. rerio* in support of previous studies showing that BPA also induces cytosolic Ca^{2+} augmentation by inhibiting SERCA in rat testis microsomes (Hughes, 2000) and in human skin fibroblasts (Woeste, 2013). IP₃R activation

and SERCA inhibition lead to intracellular Ca^{2+} store depletion in the endoplasmic reticulum. The depletion of Ca^{2+} stores is the trigger for plasma membrane SOCs activation and stimulation of Ca^{2+} influx, which can be sustained for minutes to hours. This occurs as a compensatory mechanism for the Ca^{2+} supply to refill the ER, and to drive a wide assortment of biological processes, such as secretion, gene transcription, modulation of enzymatic activity and motility (Prakriya and Lewis, 2015).

Consistent with our findings, previous works have reported rapid responses of BPA effects on non-genomic pathways, but in other animal models, other organ/cells target and times of exposure. It has been demonstrated that BPA, at very low concentrations, can rapidly (within a few minutes) activate ERK 1/2 phosphorylation in rat pituitary cells, via an oscillating temporal pattern similar to that seen in response to physiological estrogens (Jeng and Watson, 2011). In addition, BPA (30 min–6 h of incubation) also activates ERK1/2 in rat Sertoli cells, reduces transferrin secretion from germ cells and increases pro-inflammatory cytokine expression in an ERK1/2 pathway-dependent manner (Izumi et al., 2011). Furthermore, BPA exposure causes rapid (5 min) activation of ERK1/2 via both the GPR30 and ER α/β pathways in the TM4 Sertoli cell and BPA up-regulates mRNA and protein expression of GPR30 (Ge et al., 2014). BPA also triggers significant Ca^{2+} oscillations in TM4 Sertoli cells and increases intracellular Ca^{2+} within a few seconds. The ERK1/2 pathway participates in BPA-induced genomic pathways, inducing Sertoli cell damage and apoptosis (Qian et al., 2014), and within minutes can activate non-genomic signaling pathways mediated by ER α in MCF-7 cells via activation of MAPK, PI3-K and PKC (Li et al., 2006). These previously published data are consistent with those of the present study, in which we show the involvement of ERK 1/2, PI3-K, PKC and IP₃R in the mechanism of action of BPA-induced Ca^{2+} influx, as well as the participation of ER α/β . This could indicate a crosstalk between non-genomic and genomic pathways, since both kinases may induce genomic transcription (Duan et al., 2002; Leonard et al., 2015). The role of BPA-induced ER β activation in Ca^{2+} influx has been previously described in human oral mucosa-derived gingival keratinocytes (Ehrenmann et al., 2017). Previously published studies have also demonstrated the ability of BPA to bind to ERs and act as an agonist. *In vitro* BPA incubation efficiently activates both ERs, showing a higher binding affinity to ER β in MCF-7 cells (Molina-Molina et al., 2013). The *in vivo* estrogenic activity of BPA has also been demonstrated in the developing larvae brain of *D. rerio*. BPA-induced brain aromatase expression is ER-dependent and BPA efficiently transactivates all ERs subtypes in *D. rerio* hepatic cell lines (Le Fol et al., 2017). Chronic exposure to BPA increases mRNA transcription of ESR2b (ER β 1) in the liver, ESR2a (ER β 2) and androgen receptor in the testis and vitellogenin in the liver of male goldfish (Hatef et al., 2012). The BPA-mediated effects on the phosphorylation state of the STATs transcription factor suggests that BPA may change gene expression and induce a rapid increase in phosphorylation and activation of p38^{MAPK}, ERK, PI3-kinase and PKC in mussels (Canesi et al., 2004).

Based on our present data, we propose a mechanism of action for BPA, following acute *in vitro* exposure of *D. rerio* testes to this compound. The disruption of intracellular Ca^{2+} homeostasis is a potent trigger of endoplasmic reticulum stress and apoptosis (Groenendyk et al., 2010). BPA has already been demonstrated to decrease Sertoli cell viability and induce intracellular Ca^{2+} -dependent cell death via apoptosis by disturbing testicular Ca^{2+} homeostasis (Hughes, 2000). In addition, BPA suppresses TM4 Sertoli cell viability in a dose- and time-dependent manner and induces mitochondrial apoptotic pathways that are dependent on calmodulin, phosphorylation of Ca^{2+} /calmodulin dependent kinase II (CaMKII) and ERK 1/2 activation (Qian et al., 2014). In agreement with these data, acute *in vitro* exposure of testes to BPA at a higher concentration (10 μM) also resulted in decreased cell viability, promoting cell damage, plasma membrane injury and possibly cell death, indicated by the release of LDH, a cytosolic enzyme, to the extracellular medium. This cell damage was also mediated by an IP_3R -dependent intracellular Ca^{2+} increase, since the IP_3R antagonist, 2-APB, abolished the BPA-stimulated effect. Based on our findings, we hypothesized that the integrity of the plasma membrane was compromised. Ionic cytosolic Ca^{2+} is known to play a crucial role in apoptosis and cell differentiation in testicular somatic cells and germ cells (Pinton et al., 2008; Galardo et al., 2014). Although the variability between the controls in different samples, the standard on LDH increase by BPA was maintained at an average of 1.75 fold keeping the proportionality. Furthermore, the incubation medium in which the testes were incubated and LDH (U/L) evaluated are directly related to the size of the testes used, their quantity of proteins and consequently of enzymes. Cytosolic LDH release to extracellular medium is used to indicate the loss of membrane integrity and cell viability, since it is caused by membrane leaking and cell injury (Korzeniewski and Callewaert, 1983; Gaucher and Jarraja, 2015).

Additionally, the increase in testicular TAG after acute *in vitro* exposure to BPA at a high concentration was mediated by the IP_3R -dependent intracellular Ca^{2+} increase, since the IP_3R antagonist abolished the effect of BPA. TAG, cholesterol, phospholipids and glycolipids are components of the spermatozoa plasma membrane and are required for the maturation of spermatozoa (Lenzi et al., 1996). Changes in lipid metabolism are commonly present in several testicular conditions or diseases, such as cryptorchidism, which displays accumulation of lipids, TAG, alkyl-DAG and cholesterol esters in the rat testes (Furland et al., 2007). In addition, exposure of Sertoli cells to stress increases the TAG, DAG and phosphatidylinositol content, induces accumulation of lipid droplets and mitochondrial uncoupling, and reduces the expression of transferrin, a secretion marker of Sertoli cells (Vallés et al., 2014). The formation and increase in these lipids may be due to lipid-rich particle production (residual bodies) and apoptotic spermatogenic phagocytosis by Sertoli cells that die during harmful conditions (Chemes, 1986; Vallés et al., 2014), such as chemical exposure. Furthermore, the testis is very sensitive to glycerol accumulation, and high glycerol concentrations may cause leaking of the blood testicular barrier, promoting the apoptosis of the germ cell line (Wiebe et al., 2000). This phenomenon can cause the temporary arrest of spermatogenesis (Wiebe and Barr, 1984), oligospermia, and even azoospermia (Wiebe et al., 2000). Furthermore, the accumulations of TAG and lipid droplets are observed in *Saccharomyces cerevisiae* yeast in a stress condition induced by chemicals. The lipid dysregulation induced by the interruption of Ca^{2+} homeostasis, leading to endoplasmic reticulum stress, has been previously reported (Gardarin, 2010; Rajakumar et al., 2016) and ER stress results in phospholipid accumulation (Muthukumar et al., 2011). Toxic molecules or chemicals can induce ER stress by altering the ionic cytosolic Ca^{2+} that comes from internal stores, by modulating ER lipid composition and by stimulating ROS production (Biagioli et al., 2008; Fu et al., 2011). The findings reported herein are in agreement and indicate an intimate relationship between the disruption of intracellular Ca^{2+} homeostasis, Ca^{2+} leakage from the endoplasmic reticulum, plasma membrane damage, TAG increase and cell injury.

In addition, our findings corroborate the data previously reported by Sun et al. (2019), in which was shown that *D. rerio* exposed to BPA (100 and 2000 $\mu\text{g/L}$) and E2 (200 and 2000 ng/L) promoted the accumulation of lipids, increasing triglycerides in the liver. Furthermore, offspring from rats exposed to BPA (50 and 200 mg/kg/day) during the gestation, lactation and for 3 months after weaning had hepatic histopathological changes and increases in serum GGT, alkaline phosphatase (ALP), LDH, TAG and total cholesterol, which indicate hepatic toxicity (Moustafa and Ahmed, 2016). Our findings are in agreement with these previous data, but we also demonstrated that a rapid exposure *in vitro* with the highest concentration of BPA used at work caused an increase in GGT activity in the liver; however, AST activity was not changed. GGT is located at the plasma membrane in hepatocytes and it is well established that elevation of its activity is an indicator of liver injury (Whitfield, 2001). These data reinforced BPA's toxicity at the plasma membrane. In addition to impairment of plasma membrane integrity of testicular cells, it is shown that BPA also damaged the plasma membrane of hepatocytes. Surprisingly, higher urinary BPA concentrations of adult humans were associated with clinically abnormal concentrations of the liver enzymes GGT and ALP (Lang et al., 2008).

5. Conclusions

Our data show that the acute *in vitro* exposure of fish testes to very low concentrations of BPA activates plasma membrane ionic channels, such as L-VDCC and CaCC, and PKC, which stimulates Ca^{2+} influx. In addition, BPA increases ionic cytosolic Ca^{2+} by activating IP_3R and inhibiting SERCA at the endoplasmic reticulum, contributing to intracellular Ca^{2+} overload. Moreover, PKC, MEK 1/2 and PI3K are involved in the mechanism of action of BPA, which may indicate a crosstalk between non-genomic initiation effects mediated by PLC/PKC/ IP_3R signaling and genomic responses mediated by ESR. *In vitro* exposure to a higher concentration of BPA caused intracellular Ca^{2+} -dependent cell damage and plasma membrane injury with increased LDH release and increased TAG content, mediated by the IP_3R . Furthermore, *in vitro* exposure to a higher concentration of BPA reinforced its toxicity by inducing liver damage, as demonstrated by increased GGT activity.

Credit author statement

Silva FRMB, De Moura KRS, Van Der Kraak G and Delalande C proposed the specific aims of the project, analyzed data and wrote the paper. Batista-Silva H, Rodrigues K prepared and carried out the experiments, analyzed the data by statistical methods, revised the literature and prepared figures and legends and contributed to the discussion. All authors revised and edited the manuscript. F. R. M. B. S. was responsible for the acquisition of the financial support for the project (since reagents to the final manuscript for publication) and had access to all data of this study and takes responsibility for the integrity and accuracy of the data analysis.

Authors' contribution

Silva FRMB, De Moura KRS, Van Der Kraak G and Delalande C proposed the specific aims of the project, analyzed data and wrote the paper. Batista-Silva H, Rodrigues K prepared and carried out the experiments, analyzed the data by statistical methods, revised the literature and prepared figures and legends and contributed to the discussion. All authors revised and edited the manuscript. F. R. M. B. S. had access to all data of this study and takes responsibility for the integrity and accuracy of the data analysis.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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In vivo and *in vitro* short-term bisphenol A exposures disrupt testicular energy metabolism and negatively impact spermatogenesis in zebrafish

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ABSTRACT

This study investigated the *in vitro* and short-term *in vivo* effects of Bisphenol A (BPA) on testicular energy metabolism and morphology in the zebrafish (*Danio rerio*). Testes were incubated *in vitro* for 1 h or fish were exposed *in vivo* to BPA in the tank water for 12 h. Testicular lactate, glycogen and cholesterol were measured and ¹⁴C-deoxy-D-glucose uptake and activity of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. In addition, testis samples from the *in vivo* exposures were subject to digital analysis of testicular cells using Ilastik software and the Pixel Classification module and estimation of apoptosis by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) immunohistochemical analysis. Our results from *in vitro* studies showed that BPA at 10 pM and 10 μM decreased testicular lactate content, glycogen content and LDH activity, but increased testicular AST activity. In addition, only BPA at 10 pM significantly decreased testicular ALT activity and cholesterol content. However, ¹⁴C-deoxy-D-glucose uptake was not changed. Furthermore, our results from *in vivo* studies showed that 10 pM BPA but not 10 μM BPA reduced testicular content of lactate and glycogen. In addition, both BPA concentrations decreased AST activity, whereas only BPA at 10 μM reduced ALT activity. However, LDH activity was not changed. Additionally, both concentrations of BPA induced spermatocyte apoptosis and a decrease in the proportion of the surface area of spermatids and spermatozoa. Collectively these data suggest that short-term BPA exposure affects energy metabolism and spermatogenesis in male zebrafish.

1. Introduction

Bisphenol A (BPA) [2,2-bis(4-hydroxyphenyl)propane] is an environmental toxicant that causes deleterious impacts on male fertility as in many animal models including humans [1–3]. It is used mainly for the production of epoxy resins and polycarbonate plastics by the plastics industry. Epoxy resins are used to coat metal cans on the food-contact surface, coating for PVC pipe and the production of polycarbonate plastics used to improve resistance, hardness and toughness in food packaging, plastic bottles and household appliances [4,5]. The presence of BPA has been documented in water rivers and in fish including those in the marketplace, which suggest that this chemical may be a risk factor for aquatic ecosystems and for human health [6,7]. BPA is discharged into aquatic environment such as rivers and seas by migration

of BPA-based products and by the effluents from wastewater treatment plants and landfills sites [8]. BPA levels in surface water have been measured cross the globe and range from 0.0005 to 0.41 μg/L in Germany [9], 0.004 to 0.38 μg/L in China [7], 0.003–1.9 μg/L in Canada [10] and from 0.09 to 1.46 μg/L in Brazil [11]. Therefore, environmental quality standards (EQS) or water quality guidelines, have been proposed in order to protect all forms of aquatic life for indefinite periods of exposure. However, EQS for BPA has not yet established in all countries. EQS for BPA has been established at 0.24 μg/L in Switzerland [12] and at 3.5 μg/L in Canada [10]. That is why studies in fish become even more relevant for understanding the effects of BPA and its impact on reproduction, since it is recognized that testes are one of the major targets of BPA toxicity [13,14].

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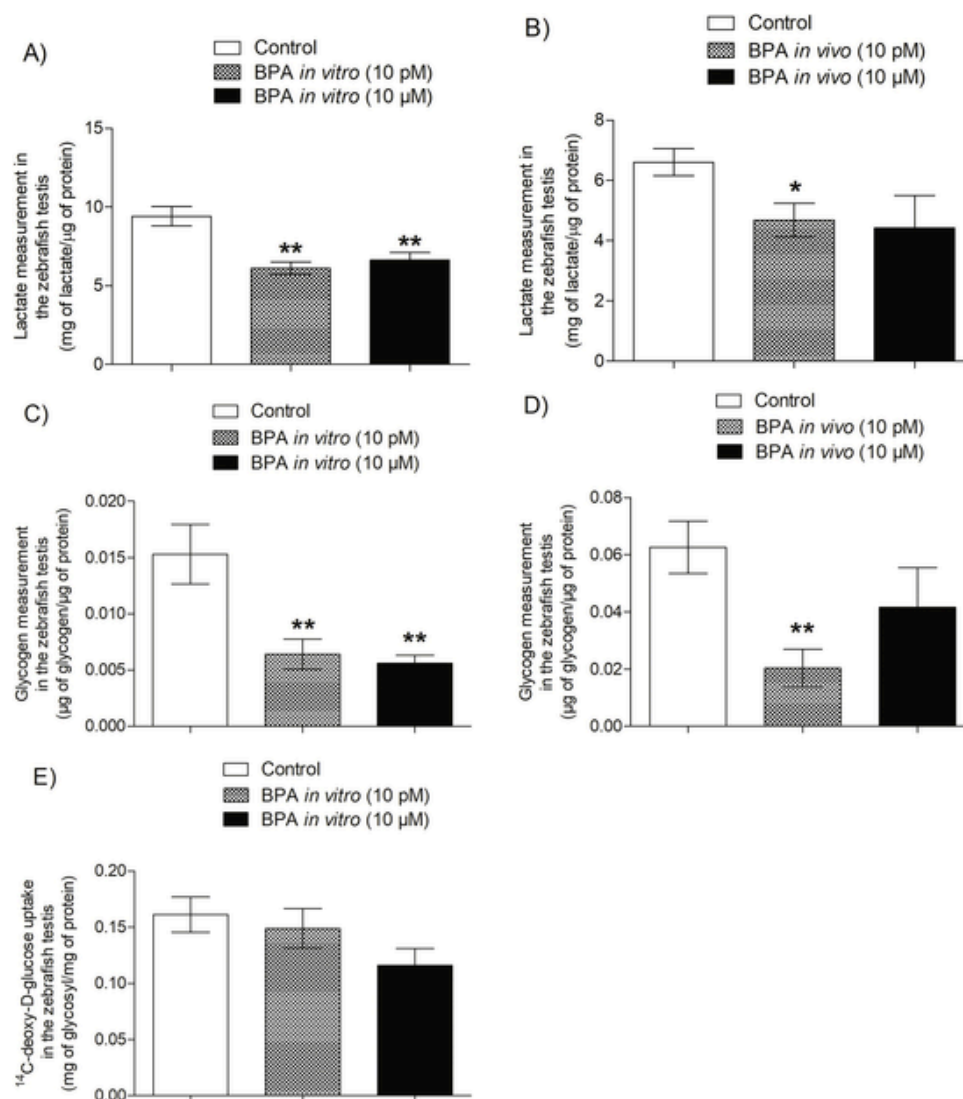


Fig. 1. Effects of BPA on (A and B) lactate, (C and D) glycogen content and (E) ^{14}C -deoxy-D-glucose uptake in testis incubated (*in vitro*) for 1 h and in testis from zebrafish exposed (*in vivo*) for 12 h to 10 pM and 10 μM BPA. The results were expressed as mean \pm S.E.M. of (A) $n = 5-6$; (B) $n = 5$; (C) $n = 4-6$; (D) $n = 4-5$ and (E) $n = 5-6$ fish in each group. * $p < 0.05$ and ** $p < 0.01$ when compared to control group.

Zebrafish has some advantages as an animal model for aquatic organisms, and has there is a wealth of information available about its reproductive physiology. The duration of the meiotic and spermiogenic phases of spermatogenesis in zebrafish is approximately 6 days, one of the shortest times among fish [15]. Due to this shorter duration, biological or toxic effects on spermatogenesis in the zebrafish may be observed even after a short-term exposure to estrogens or endocrine disrupting compounds (EDC) [16,17]. In addition, in adult fish there is proliferation of Sertoli cells resulting in approximately 100 spermatids being supported by one Sertoli cell [18], which is about 10-fold greater than mammals [19]. This is a determining factor for male fertility, since Sertoli cells are responsible for maintaining the physical, metabolic and energy support for the germ cells and thus, determine the size of the testis, spermatozoa production and consequently, fertility [20–22].

Although fish are the most diverse and abundant vertebrate group, available knowledge about the metabolic support by Sertoli cells and influence of testicular energy metabolism on spermatogenesis is scarce. This knowledge is better addressed in mammals. Sertoli cells metabolize mainly glucose to provide lactate for germ cells through glycolysis [23,24], in which lactate dehydrogenase (LDH) catalyses the reduction of the final product pyruvate to lactate, in the presence of NADH [25]. In addition, pyruvate may also be generated by alanine aminotrans-

ferase (ALT), which catalyses the reversible interconversion of alanine and α -ketoglutarate into pyruvate and glutamate [26,27]. Furthermore, aspartate aminotransferase (AST) activity is involved in the malate–aspartate shuttle system to the re-oxidation of reduction equivalents, such as NADH [28]. Additionally, in addition to glucose, glycogen is also an important energy source for the production of lactate in Sertoli cells through glycogenolysis. The lactate is then transported to the germ cells through monocarboxylate transporters, which is the main energy substrate used to generate ATP in spermatocytes and spermatids [29]. Thus, availability of lactate is essential for the differentiation and germ cells survival [30,31].

Decreased lactate production and availability can disrupt spermatogenesis and contribute to male infertility [30,31]. High BPA concentrations impair glucose and lactate metabolism in rat Sertoli cells [32] and induce apoptosis of rat and mice germ cells *via* activation of mitochondrial apoptotic pathways [33,34]. Batista-Silva et al. [14] reported rapid responses to low concentrations of BPA (10 pM) leading to calcium overload in the zebrafish testis with the involvement of protein kinases and oestrogen receptors. Additionally, *in vitro* treatment with a higher concentration (10 μM) of BPA caused a calcium-dependent testicular cell damage and hepatic toxicity [14].

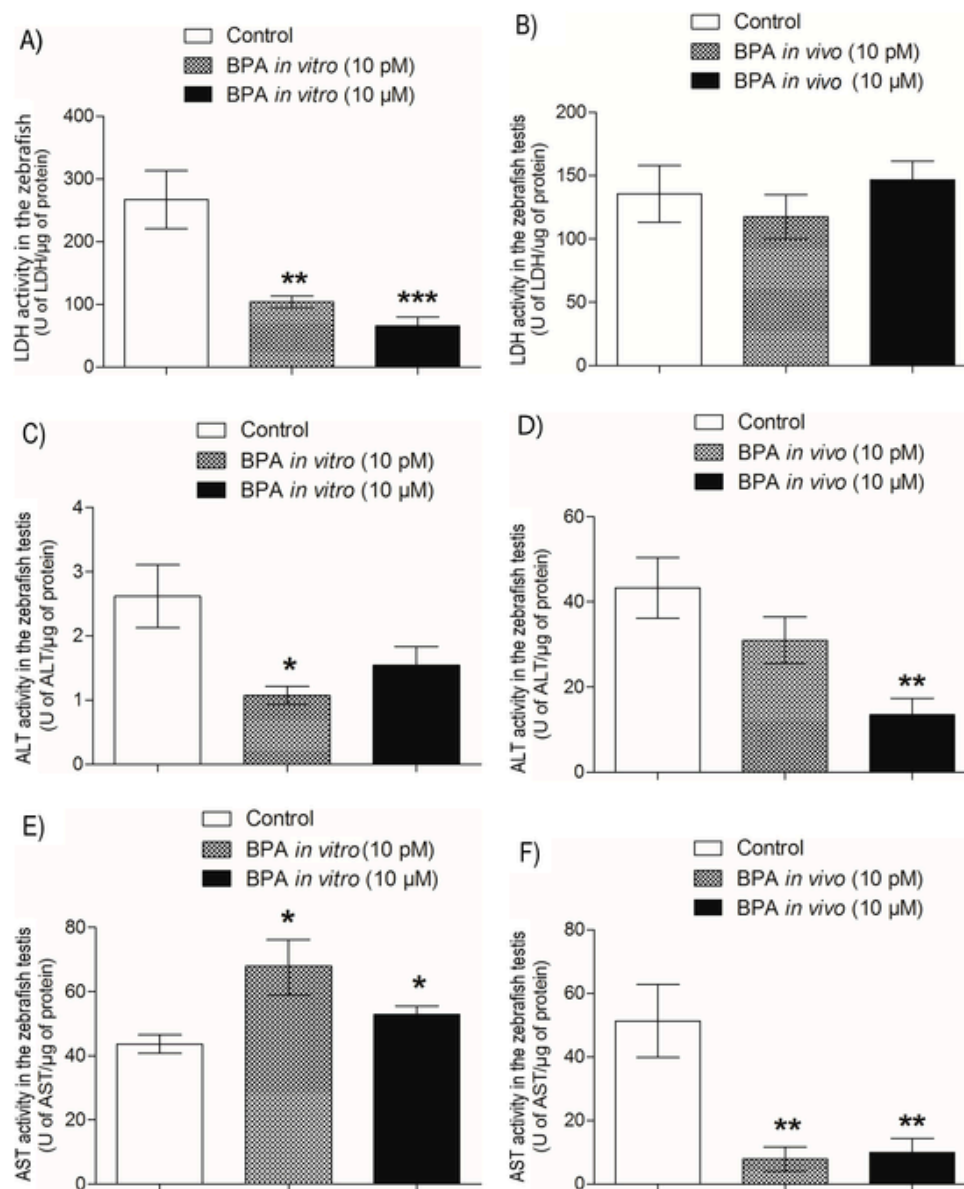


Fig. 2. Effects of BPA on (A and B) LDH; (C and D) ALT and (E and F) AST activities in testis incubated (*in vitro*) for 1 h and in testis from zebrafish exposed (*in vivo*) for 12 h to 10 pM and 10 μM BPA. The results were expressed as mean \pm S.E.M. of 4-6 fish in each group. * p < 0.05, ** p < 0.01 and *** p < 0.01 when compared to control group.

Chronic *in vivo* exposure to BPA impairs male reproductive development in fish with effects observed during the embryonic, larval and sexually mature periods. BPA has been shown to decrease testicular weight, volume, amount, motility and velocity of spermatozoa, as well as promote embryonic malformations and mortality in the zebrafish [35]. In addition, BPA causes morphological damage in the testes and changes in the expression of genes involved in steroidogenesis in zebrafish [36], as well as increased vitellogenin concentration and increased intersexuality rates in male carp [37]. Furthermore, BPA-exposure to mature goldfish disrupts testis maturation through apoptosis of germ cells and Leydig cells and reductions in 11-ketotestosterone levels [38].

Thus, this study used *in vitro* and *in vivo* approaches to investigate the impact of short-term exposure to BPA on testicular energy metabolism and its influence on spermatogenesis in zebrafish.

2. Materials and methods

2.1. Chemicals

BPA ($\geq 99\%$, CAS number 80-05-7) and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO, USA). [U- ^{14}C]-2-Deoxy-D-glucose (^{14}C -DG), specific activity 9.25 GBq/mmol and Optiphase Hisafe III biodegradable scintillation liquid were purchased from Perkin-Elmer (Boston, USA). For histology, paraffin was purchased from Merck (São Paulo, Brazil) and Harris's haematoxylin (Ref: 400802010) was purchased from ProTaq (Potsdam, Germany). For analysis of apoptosis by Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay, *In Situ* Cell Death Detection Kit, POD was purchased from Roche Applied Science (Mannheim, Germany). DAB (3,3'-diaminobenzidine) solution was purchased from R&D Systems (Minneapolis, USA) and Mayer's haematoxylin (Ref: 400802210) was purchased from ProTaq (Potsdam, Germany). Kits for alanine aminotransferase (ALT) (Ref: 108-2/100), aspartate aminotransferase (AST) (Ref: 109-2/100), lactate (Ref: 138-1/50) and LDH

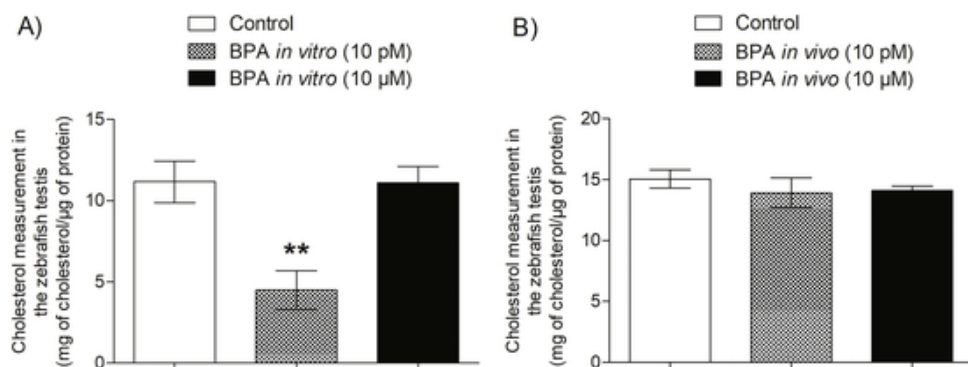


Fig. 3. Effects of BPA on total cholesterol measurements (A) in testis incubated *in vitro* for 1 h and (B) in testis from zebrafish exposed (*in vivo*) for 12 h to 10 pM and 10 μM BPA. The results are expressed as mean \pm S.E.M. of (A and B) $n = 4$ –5 fish in each group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to control group.

(Ref: 86–1/100) were purchased from Labtest Liquiform (Lagoa Santa, MG, Brazil). All other chemicals to prepare buffers were of analytical grade and purchased from Diprolab (Florianópolis, SC, Brazil).

2.2. Animals

Mature male zebrafish were obtained commercially (Belo Horizonte, MG, Brazil) and acclimatized for 7 days before experimental procedures in an air-conditioned room (21 ± 1 °C). Fish with lengths from 2.0–3.0 cm and weighing between 220–330 mg were kept in aquariums with dechlorinated tap water, pH 7.4 on a 12 h light/dark cycle. The water temperature of the aquarium was maintained at 27 ± 1 °C, pH 7.4 ± 0.3 and salinity 0.7 ± 2 g/L. Fish were fed twice a day with commercial feed containing a mixture of astaxanthin, kelp algae, spirulina, flaked with beta-glucan and crustaceans (Tropical®, Florianópolis, SC, Brazil). All fish were carefully monitored and maintained in accordance with the ethical recommendations of the local Ethical Committee for Animal Use of the Federal University of Santa Catarina (Protocol CEUA/UFSC/PP00968).

2.3. In vitro and in vivo BPA treatments

Stock solution of BPA (0.01 M) diluted in ethanol (0.01 % into the assay solutions) was used to obtain the final concentrations 10 pM and 10 μM BPA based on our earlier work [17].

For *in vitro* studies, testes were dissected and incubated with 10 pM and 10 μM BPA in Cortland buffer (124 mM NaCl, 5 mM KCl, 1.7 mM CaCl_2 , 3.4 mM NaH_2PO_4 , 2.1 mM MgCl_2 , 1.91 mM MgSO_4 , 11.9 mM NaHCO_3 and 1 g/L glucose) in an atmosphere gassed with O_2 : CO_2 , 95: 5 v/v, 28 °C for 1 h [14]. After 1 h of incubation, analysis of biochemical parameters (described in the next sections) was carried out.

For the *in vivo* studies, zebrafish were held in 7 L-aquaria (L x W x H: 25 x 14 x 20 cm) at a density of 2 fish per L. Two independent experiments were performed. Zebrafish were treated with 10 pM and 10 μM BPA in the aquarium water for 12 h. Control groups were exposed to the highest concentration (0.01 %) of the solvent (ethanol) used to dilute BPA. After the treatment period, the testes were dissected for further biochemical and histological analysis (described in the next sections).

The *in vitro* and *in vivo* short-term incubation periods were chosen based on our previous work [17]. For both studies, the two testes from each individual fish represents a single sample, and the sample size (n) corresponds to the number of fish analyzed.

2.4. Lactate content measurement

Testes were homogenized in cold 0.1 M Tris buffer, pH 7.3 and lactate content was measured according to the manufacturer's instructions

(Labtest Liquiform; Lagoa Santa, MG, Brazil) by the lactate oxidase method [39]. The results were expressed as mg of lactate *per* μg of proteins [40].

2.5. ^{14}C -deoxy-D-glucose uptake

In vitro studies on ^{14}C -deoxy-D-glucose uptake were based on previous work which was standardized for fish [17]. Testes from zebrafish were dissected and quickly placed in microtubes containing Cortland buffer. The medium was replaced with Cortland buffer containing 0.1 μCi/mL ^{14}C -deoxy-D-glucose in the absence (control) or presence of BPA (10 pM and 10 μM) for 1 h in atmosphere gassed with O_2 : CO_2 , 95: 5 v/v, 28 °C. After, the samples were washed with Cortland buffer and homogenized in NaOH (0.5 M). Aliquots were taken from each sample for radioactivity measurements in scintillation liquid in an LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman, Boston, USA). Another aliquot of the sample was used for protein determination [40]. The results were expressed as mg of glycosyl *per* mg of proteins.

2.6. Glycogen content measurement

Testes were incubated in 33 % KOH and heated at 100 °C for 15 min. After cooling, 95 % ethanol was added, and the samples were incubated at 70 °C for 5 min. Then, they were cooled down on an ice bath to precipitate the glycogen. The homogenates were then centrifuged at 1300 g for 15 min. The resulting supernatant was then discarded, and the pellet was resuspended with deionized water. Glycogen content was determined using iodine reagent (CaCl_2 saturated solution mixed with iodine + potassium iodide solution) for colorimetric estimation of glycogen at 460 nm according Krisman [41]. The results were expressed as μg of glycogen *per* μg of proteins [40].

2.7. Activities of lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Testes were homogenized in cold 0.1 M Tris buffer, pH 7.3 and LDH, ALT and AST activities were measured according to the manufacturer's instructions (Labtest Liquiform; Lagoa Santa, MG, Brazil) by the NADH oxidation method. The consequent reduction of absorbance at 340 nm on this kinetic reading is directly proportional to enzyme activity in the sample [42]. The results were expressed as U *per* μg of proteins [40].

2.8. Cholesterol measurements

Testes were homogenized in cold 0.1 M Tris buffer, pH 7.3 and total cholesterol content was measured according to the manufacturer's instructions by an enzymatic colorimetric method using the cholesterol

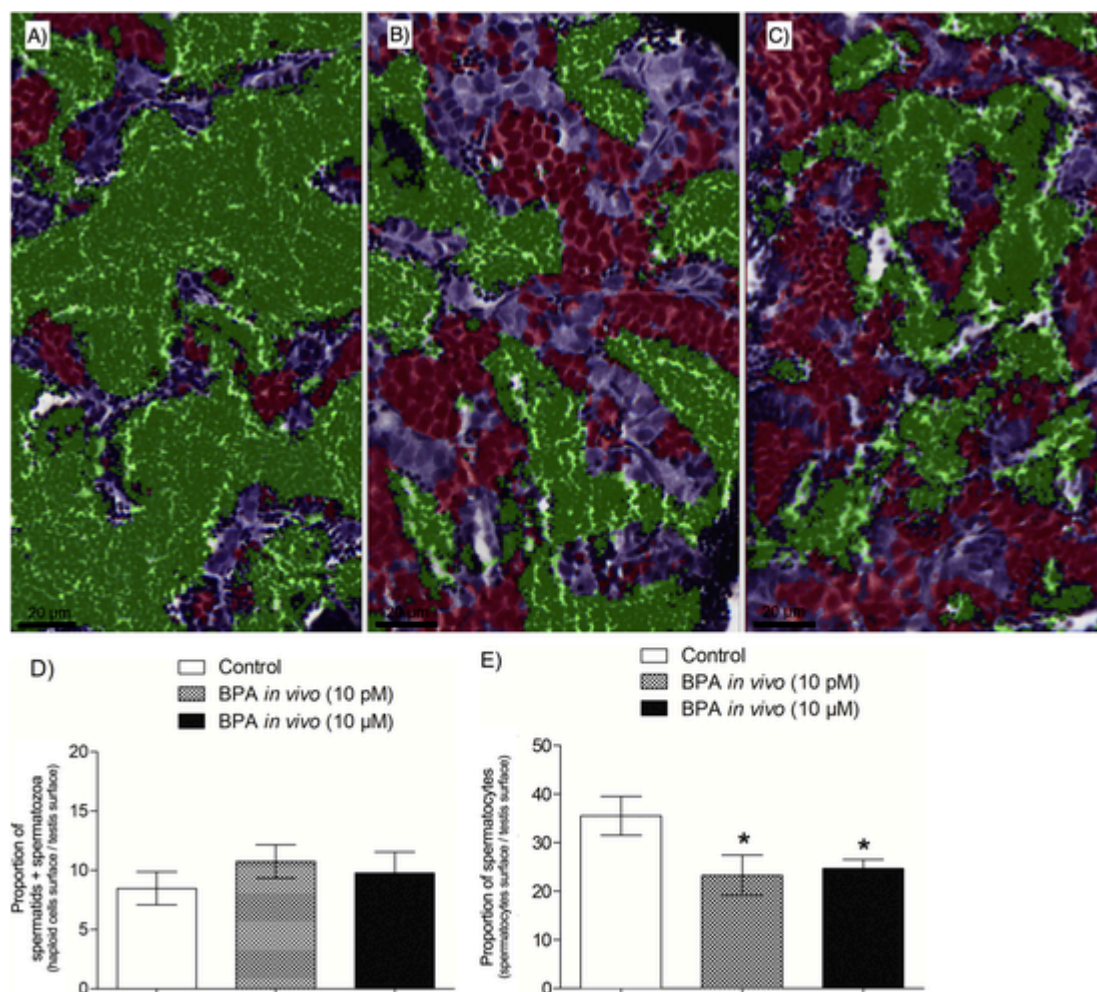


Fig. 4. Effects of BPA on the proportion of the surface area of spermatocytes (in red), spermatids and spermatozoa (in green) in zebrafish treated *in vivo* for 12 h with (A) vehicle (control), (B) 10 pM or (C) 10 μM BPA. (A, B and C) Overlay images of the image analysis of the proportion of spermatocytes, spermatids and spermatozoa superimposed on the testicular histological section image. Representation of the quantitative analysis of the proportion of (D) spermatocytes, (E) spermatids and spermatozoa in relation to the total testicular surface area. The results are expressed as mean \pm S.E.M. of 4-5 fish in each group. * $p < 0.05$ when compared to control group (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

oxidase method (Lagoa Santa, MG, Brazil). The results were expressed as mg of cholesterol *per* μg of proteins [40].

2.9. Histology

2.9.1. Tissue processing

After *in vivo* short-term exposure to BPA, testes were collected and fixed in 4% paraformaldehyde/PBS (phosphate buffered saline) for 24 h. After, the samples were washed with PBS. The tissues were dehydrated in graded ethanol solutions, then infiltrated in xylol solvent and finally were embedded in paraffin. Serial sections (5 μm thickness) were cut using a rotary microtome (Jung RM 2035, Leica). For spermatogenesis analysis, they were stained with Harris's haematoxylin to perform the quantitative analysis of the germ cells.

2.9.2. Image acquisition, processing and digital analysis

Whole slide images of histological sections were scanned at 40x with an optical duplicator (0.25 μm/pixel) using the ScanScope CS microscope slide scanner (Leica Biosystems, Nussloch, Germany). They were recorded as tiled tagged image file format (TIFF) images.

For each image a down-sampling by 4 was applied to allow an analysis with the innovative digital image analysis method through Ilastik software and the Pixel Classification module according to Berg et al. [43]. The first step was to select images from histological sections for

machine training. Then, labels to pixels were assigned based on pixel features (smoothed pixel intensity, edge filters and texture descriptors) and user annotations. The pixel classification produces a user-defined category label to each pixel in the image. For this, five categories were labelled: background, artefact areas, spermatocytes, spermatids + spermatozoa (haploid cells) and tissue (cells other than spermatocytes, spermatids and spermatozoa). Spermatids and spermatozoa were counted in the same surface, since it would not be possible to distinguish these two categories in sections stained only with haematoxylin (which stains the nuclei) and perform analysis by pixel classification. Then, the training and learning step of the machine was carried out to identify and distinguish the different categories through the pixel classification by means of clicks/brush strokes in the areas of interest on the training images. These parameters were calculated and a probability map of each category was computed after all the histological images were exported to the program. For each pixel in the image, Ilastik estimates the probability that the pixel belongs to each of the semantic categories using a non-linear algorithm, Random Forest classifier, which operates in the resource space [43]. To consider that a pixel belongs to a specific category, it was established that at least 55 % probability was required. All images are compared between them, and if the values are equal, there is 100 % of probability that it is the same category. Therefore, more the values of reference (values in the areas obtained in the training and machine learning step) are different from the

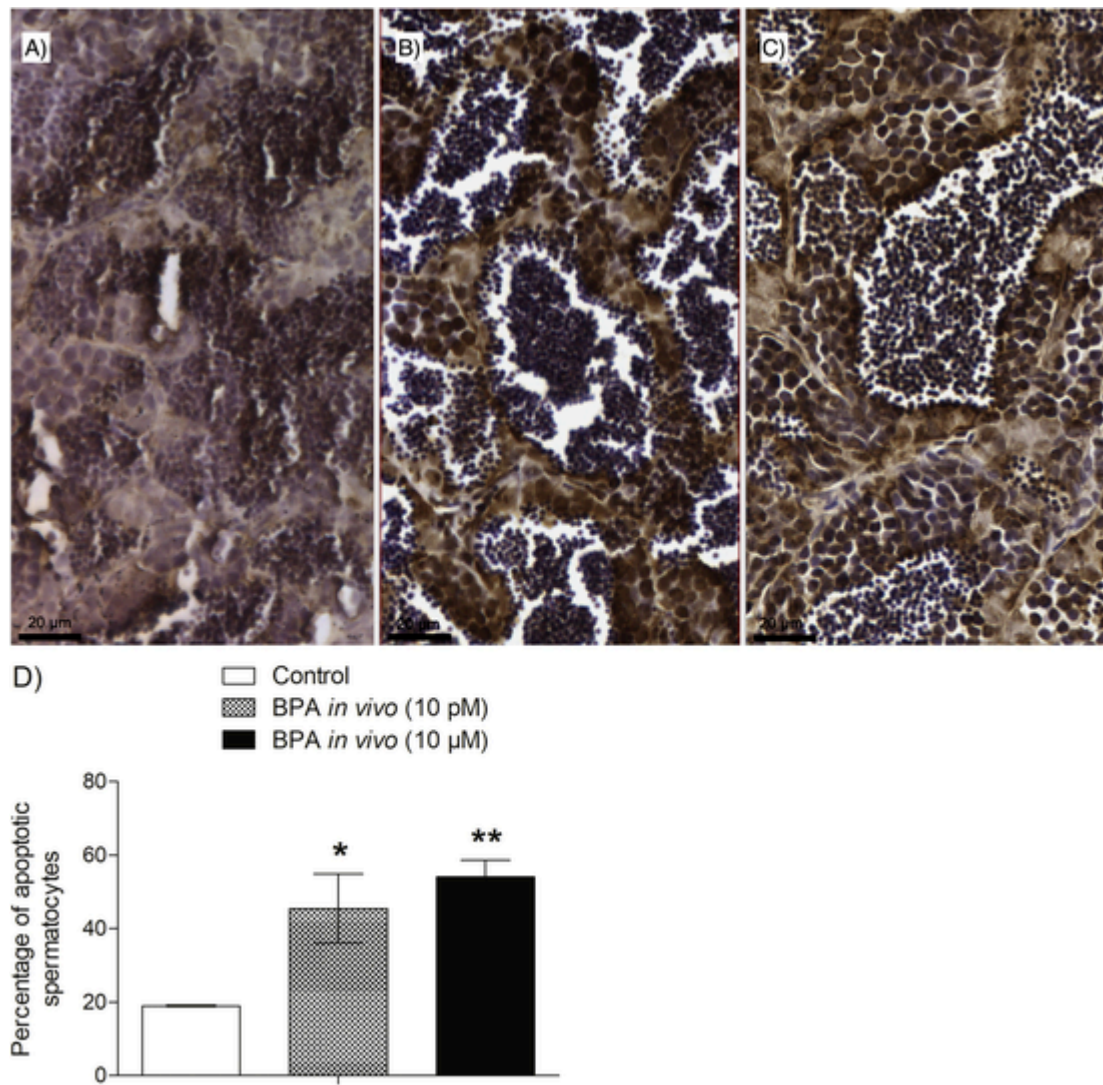


Fig. 5. Effects of BPA on apoptosis in spermatocytes of zebrafish exposed *in vivo* for 12 h to (A) vehicle (control), (B) 10 pM or (C) 10 μM BPA. (D) The quantitative analysis of the percentage of apoptotic spermatocytes. The results are expressed as mean \pm S.E.M. of 3–4 fish in each group. * $p < 0.05$ and ** $p < 0.01$ when compared to control group.

values of the new images, the less certain is the probability of indicating that it belongs to the same category, therefore, the value of the probability decreases. The probabilities map images were processed using a program in Python programming language with Scikit-image library according to Van Der Walt et al. [44] in order to obtain the quantitative result of the digital analysis. For each image, the program calculated the surface area of spermatocytes, of spermatids + spermatozoa and of tissue in order to determine the proportion of spermatocytes or spermatids + spermatozoa in relation to the total surface of the testis. Total surface of the testis is the sum of the surfaces of all analysed cells in the labelled categories: spermatocytes, spermatids + spermatozoa and tissue (cells other than spermatocytes and spermatids + spermatozoa).

2.9.3. TUNEL assay

Immunohistochemical detection and quantification of apoptotic cells were determined on labelling of DNA strand breaks through the colorimetric TUNEL assay according to the manufacturer's instructions (Roche Applied Science). Some slides were incubated with DNase I (100 U/mL) for 10 min as a positive control or without Terminal Deoxynucleotidyl transferase recombinant enzyme as a negative control. The slides were incubated in the presence of DAB solution until a brown staining appears (approximately 2 min), then they were rinsed with dis-

tilled water. Counter-staining with Mayer's haematoxylin for 1 min was performed to stain cell nuclei. Subsequently, 95 % and 100 % (2x for 5 min each) ethanol baths and a xylene bath (5 min) were carried out. Finally, slides were mounted with Eukitt®. For analysis of the positive TUNEL cells (brown-stained cells), the slides were scanned as described in the section 2.9.4.

2.9.4. Stereology: quantitative stained cells analysis

In order to perform a quantitative analysis and determine the proportion of marked nuclei (in apoptosis) in relation to the unmarked nuclei of a certain cell type, the counting was carried out using the Aperio Image Scope® through the Stereology Tool Kit module. Taking into account a greater prevalence of spermatocytes undergoing apoptosis, these were chosen for analysis. The stereological method is a precise tool for obtaining quantitative information about histological data to be further analysed by statistical methods [45]. For this, brown-stained (apoptotic) and unstained spermatocytes were counted in a grid area of systematically distributed frames with a size of 40 μm and the space between them of 80 μm. The percentage of apoptotic spermatocytes (positive TUNEL) was calculated as: $100 \times (\text{positive TUNEL spermatocytes brown-stained}) / (\text{positive TUNEL spermatocytes brown-stained} + \text{negative TUNEL spermatocytes unstained})$ [46].

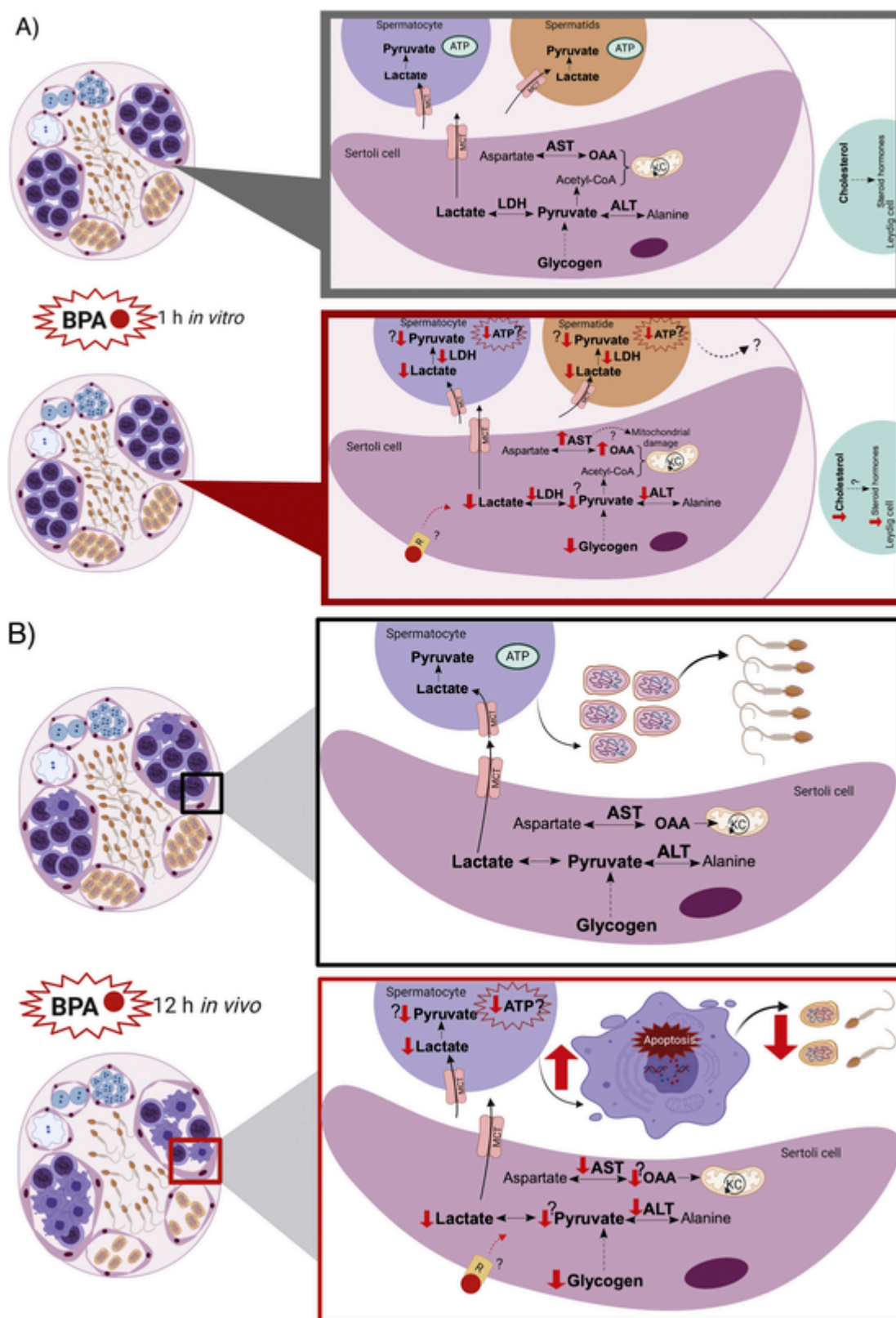


Fig. 6. Schematic representation of the proposed mechanism of the (A) *in vitro* and (B) *in vivo* effects of BPA on biochemical pathways in the zebrafish testis and its relationship with spermatogenic wave. (A) BPA caused a reduction in testicular lactate, the main energy substrate for the mature germ cells. This reduction was mediated by the reduction on activities of ALT (converts alanine to pyruvate) and LDH (converts pyruvate to lactate), and which is assumed to lead to a reduction on pyruvate. This could contribute to the higher energy demand and the observed reduction in testicular glycogen stores. In addition, AST (converts aspartate to OAA) activity was increased, which may suggest a high production of OAA, an intermediate in the Krebs cycle, or damage to mitochondria. If less lactate is produced by the Sertoli cells, therefore less lactate is transported to the mature germ cells, which would result in a lack of pyruvate and ATP. In addition, BPA caused a reduction on con-

tent of testicular cholesterol (precursor to steroid hormones), which could suggest a reduction in the production of steroid hormones. (B) BPA resulted in a reduction on testicular lactate, the main energy substrate for the mature germ cells. This reduction was mediated by the reduction on ALT activity (which converts alanine to pyruvate) or on glycogen content, which consequently led to a reduction on pyruvate. In addition, AST activity was inhibited and consequently led to a reduction of OAA production, intermediate in the Krebs cycle in Sertoli cells. If less lactate is produced by the Sertoli cells, therefore less lactate is transported to the spermatocytes, which would result in a lack of pyruvate, and consequently ATP. This lack of energy could have caused an increase of apoptosis in spermatocytes and, consequently, a reduction in the number of spermatids and spermatozoa. Straight lines are direct pathways; dotted lines represent multi-step pathways; question mark are questions to be further investigated. ALT – alanine aminotransferase; AST – aspartate aminotransferase; BPA – bisphenol A; KC – Krebs Cycle; LDH – lactate dehydrogenase; OAA – oxaloacetate.

2.10. Statistical analyses

The data are represented as mean \pm standard error of the mean (S.E.M.) of 3–6 fish *per* group from two independent experiments. The results were analysed by GraphPad Prism 5. One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test or Student's *t*-test were used to identify significant differences between groups. Statistical differences between groups were considered when $p \leq 0.05$.

3. Results

3.1. BPA disrupts testicular energy metabolism

To study BPA effects on the main testicular energy substrates, lactate and glycogen contents and ^{14}C -deoxy-D-glucose uptake were analysed. *In vitro* incubations with 10 pM and 10 μM BPA decreased both testicular lactate (Fig. 1A) and glycogen (Fig. 1C). However, ^{14}C -deoxy-D-glucose uptake did not change in the presence of BPA (Fig. 1E). Surprisingly, the *in vivo* treatment with the lowest BPA concentration decreased lactate and glycogen content in the testis of zebrafish (Fig. 1B and D).

LDH, ALT and AST activities were analysed following BPA treatment to determine effects on enzymes involved in testicular energy metabolism. *In vitro* BPA acute treatment decreased LDH activity (Fig. 2A) emphasizing the diminished lactate content showed in Fig. 1A. However, LDH activity was not modified after *in vivo* treatments in the presence of BPA (Fig. 2B). The lowest *in vitro* concentration of BPA (Fig. 2C) and its highest *in vivo* concentration (Fig. 2D) significantly decreased testicular ALT activity. In addition, *in vitro* incubation with both concentrations of BPA caused an increase in testicular AST activity (Fig. 2E). On the other hand, *in vivo* exposure with both concentrations of BPA reduced testicular AST activity (Fig. 2F).

3.2. BPA *in vitro* decreases testicular cholesterol

Fig. 3A shows that the lowest *in vitro* concentration of BPA decreased the total testicular cholesterol whereas there was no effect of *in vivo* BPA exposure on testicular cholesterol content (Fig. 3B).

3.3. BPA impairs spermatogenesis by decreasing the proportion of the surface of spermatids and spermatozoa

The impact of BPA on zebrafish spermatogenesis was determined by measuring the proportion of the surface of spermatocytes and haploid cells (spermatids + spermatozoa) in relation to the total surface of the testis after *in vivo* treatment with 10 pM or 10 μM BPA for 12 h. Overlay images illustrating the image analysis of the proportion of spermatocytes or spermatids and spermatozoa superimposed on the testicular histological section image obtained following control (Fig. 4A), 10 pM BPA (Fig. 4B) and 10 μM BPA (Fig. 4C) treatments, respectively. Additionally, Fig. 4D and 4E show in a quantitative way the proportion of these cells in relation to the total testicular surface. Fig. 4A shows the profile of distribution and proportion of the testicular cells in the control group by highlighting a normal distribution of spermatids and spermatozoa (in green) and spermatocytes (in red). Fig. 4B and C show that there was no change in the proportion of the surface of spermatocytes (in red) in relation to the total testicular surface compared to the con-

trol group (Fig. 4A) after *in vivo* treatments with BPA, which was confirmed by the quantitative analysis (Fig. 4D). However, Fig. 4B, 4C and 4E highlight a reduction in the proportion of the surface of spermatids and spermatozoa (in green) in relation to the total testicular area caused by *in vivo* treatments with 10 pM and 10 μM BPA.

3.4. BPA impairs spermatogenesis by increasing the spermatocytes apoptosis

Fig. 5A (control), 5B (10 pM BPA) and 5C (10 μM BPA) show the detection of apoptosis by TUNEL immunostaining in testicular histological section. As can be seen in Fig. 5B and 5C, brown-stained cells (positive TUNEL cells) counted as apoptotic cells, were more prevalent in the spermatocytes after exposure to 10 pM and 10 μM BPA, respectively. Quantification of spermatocytes in apoptosis (Fig. 5D) show a higher level in response to both concentrations of BPA tested compared to control group.

4. Discussion

Different concentrations of BPA, ranging from picograms to micrograms *per* litre, have already been detected in rivers and surface waters and effluents in different countries [9,11,47]. A recent study reported that on an annual basis that BPA concentrations ranged from 0.09 to 1.46 $\mu\text{g/L}$ BPA in river water and 0.36–3.57 $\mu\text{g/L}$ BPA in drinking water in Minas Gerais/Brazil [11]. However, worryingly, concentrations at 30.1 $\mu\text{g/L}$ and 240 $\mu\text{g/L}$ BPA have already been detected in the bile of wild and market fish, respectively [6]. Thus, 10 pM BPA used in the present study (2.3 ng/L) is below the reported concentrations in untreated water, drinking water, water from rivers and sea. And, the highest concentration, 10 μM (2.3 mg/L), is close to that reported in leachate water and effluents generated by municipal solid waste plants [48,49]. Therefore, considering that BPA is found in different concentrations in aquatic environments and organisms, and that this is mainly produced by human-generated pollution, studies with fish become even more relevant for understanding the potential for effects on male reproduction.

Through the use of short-term *in vitro* and *in vivo* testing our studies show that BPA induces changes on testicular energy metabolism. In addition, short-term *in vivo* approach demonstrated that BPA mediates morphological changes in spermatogenesis. Lactate content and LDH activity in the testis were reduced after 1 h of *in vitro* exposure to BPA (10 pM and 10 μM). LDH catalyses the reduction of pyruvate to lactate [25], which is the main energy substrate to generate ATP in spermatocytes and spermatids [29]. Therefore, the reduction or inhibition in its production may impair spermatogenesis and contribute to male infertility [30,31]. Studies obtained with other animal models or isolated cells reinforce our data. *In vitro* exposure to BPA for 48 h has also been shown to impair glucose metabolism, lactate production, LDH gene expression and protein transduction in rat Sertoli cells [32]. Another EDC, bis(2-ethylhexyl)phthalate, was reported to induce testicular toxicity by altering LDH and gamma-glutamyl transpeptidase activities and lactate content in zebrafish testis following short-term treatment at low concentrations [17]. Additionally, a change in testicular LDH activity induced by pollutants present in the pulp and paper mill effluents was reported to be associated with disruption of spermatogenesis in the zebrafish [50]. Similar effects in terms of the reduction in LDH activity and diminished glucose uptake were seen in the rat testis after acute

treatment with pulp and paper mill effluent [51]. Furthermore, our findings showed that a short-term *in vivo* exposure of zebrafish to lowest BPA concentration for 12 h caused a reduction on lactate content, whereas LDH activity was not changed. Other studies obtained with other animal models and longer times of exposure to BPA are in accordance with our data. Testicular energy metabolism and steroidogenesis declined following chronic *in vivo* exposure of rats to high concentrations of BPA [52]. BPA also triggered a decrease in the activities of glycolytic enzymes hexokinase and phosphofructokinase, glucose transporters, insulin receptor and PI-3K, as well as a decrease in testicular glucose [53].

Glycogen homeostasis is important for maintaining the integrity of male germinal epithelium in rats [54]. Studies in rats also indicate that an imbalance in glycogen homeostasis in the testes may induce apoptosis and germ cells degeneration [54–56]. However, there are no reports of the involvement of testicular energy metabolism which show the participation of glycogen as an energy substrate in the testes of zebrafish. Our findings provide evidences that *in vitro* short-term exposure to BPA (10 pM and 10 μ M), as well as *in vivo* exposure to BPA (only the lowest concentration), caused a significant reduction in testicular glycogen content. Thus, our findings provide the first evidence for a possible correlation between the reduction on glycogen and lactate content and increase in apoptotic spermatocytes and a reduction in the percentage of spermatids and spermatozoa in zebrafish. Other studies suggest that lactate is a protective factor testicular against apoptosis [57], since it increases germ cell survival and inhibits rat spermatocytes apoptosis [30].

The observed decrease of energy substrates after *in vitro* and *in vivo* exposures to BPA may also be due to decreased ALT activity and consequently lower pyruvate production. Our studies demonstrate a BPA-induced decrease in ALT activity. However, reduction on ALT activity was demonstrated after *in vitro* exposure to the lowest BPA concentration, whereas after *in vivo* exposure it was due to the highest concentration of BPA. The main product of glycolysis, pyruvate, may also be generated by ALT activity, which catalyses the reversible interconversion of alanine and α -ketoglutarate into pyruvate and glutamate [26,27]. It has been shown that Sertoli cells may maintain lactate production even in the total absence of glucose through the metabolism originating from amino acids or alternatively by glycogen metabolism [58]. Studies related to the action of ALT in the testis are rare even in mammals. However, for the first time we have shown the involvement of ALT in testicular energy metabolism of zebrafish and as a target for BPA. In addition, high levels of ALT expression in rat Sertoli cells have recently been reported, suggesting that a significant part of the pyruvate produced by these cells has its origin in the conversion of alanine by ALT [59]. Alanine is the main glucogenic amino acid which may be used as an energy substrate by Sertoli cells for several biochemical pathways, including the Krebs cycle and gluconeogenesis [60]; [29]. Additionally, it has already been reported that the metabolism of amino acids and glycogen contribute to mammals testicular energy sources and Sertoli cells metabolism [58,61,62].

The present study was the first to identify testicular AST as a target for BPA. Testicular AST activity was decreased dramatically after zebrafish *in vivo* exposure to BPA (10 pM and 10 μ M), likely indicating a decrease in aspartate and the Krebs cycle intermediates, α -ketoglutarate and oxaloacetate (OAA), since AST mediates the interconversion of aspartate to OAA [63]. In addition, it could also be suggested that a reduction in AST activity may compromise the malate–aspartate shuttle system to the re-oxidation of reduction equivalents, such as NADH, which is produced when lactate is oxidized to pyruvate by LDH [28]. On the other hand, *in vitro* exposure of zebrafish testis to both BPA concentrations increased AST activity which is an indicator of mitochondrial damage as reported by Giannini et al. [64] and Lo et al. [65]. Some results of the *in vitro* exposure group were not identical or were even inverse to those reported in the *in vivo* exposure group, as observed in AST

and LDH activities. Different biological effects promoted by *in vivo* and *in vitro* exposure to BPA have also been observed in a previous study in zebrafish [66]. Such differences can be expected, since *in vivo* treatment, BPA consequently undergoes phase II metabolism, resulting in the production of its metabolites: BPA conjugated with glucuronic acid and BPA conjugated with sulphate [67].

It is known that testicular cholesterol homeostasis is also an important requirement steroidogenesis and therefore for spermatogenesis [68]. Our data showed that the lowest concentration of BPA *in vitro* resulted in decreased testicular cholesterol, suggesting an impairment of steroidogenesis, since cholesterol is the precursor of the steroid hormones [69,70]. These reports are in line with a study in mice showing that chronic oral administration of BPA led to a dose-dependent decrease of testicular total lipid and cholesterol content and reductions in the activity of key steroidogenic enzymes and testosterone levels [70].

It is important to note that some of the biochemical changes caused by BPA were only observed at the lowest concentration tested. This included changes in lactate and glycogen content after *in vivo* exposure, as well as ALT activity and cholesterol content after *in vitro* exposure. Studies with BPA present an important challenge to traditional concepts in toxicology, which assume that the dose-response curve is monotonic (dogma known as “the dose makes the poison”), since BPA may cause effects at lower concentrations that are not observed by effects at higher concentrations. This response pattern may be related to non-monotonic dose-response curves described by [71] although there may be several mechanisms contributing to this response. Biochemical changes caused by BPA shown in this study were evaluated in the whole testis, which reinforces the need to consider multiple pathways mediating chemical actions. This kind of non-selectivity to a specific receptor is quite common for EDCs, and it may be proposed that binding to different receptors could be an explanation for the differences in biochemical responses shown in the present study. At lower concentrations, BPA is reported to bind almost exclusively to the oestrogen receptors, but at higher concentrations it may also bind to other receptors such as the androgen or thyroid hormone receptors that may act in an antagonistic manner to modify BPA actions [71–73]. Kohn and Melnick (2002) proposed through mathematical models that when exposures to EDCs occur in the presence of endogenous hormones and unoccupied hormone receptors, at low concentrations, both the endogenous hormone and the EDC bind to the receptors and activate this response. However, at higher concentrations, EDCs may overcome the natural binder (Kohn and Melnick, 2002). It is suggested that low concentrations of BPA may influence the response of fish or testes to oestrogens, thus inducing an additive or synergistic response, which is characteristic of oestrogen receptors agonists. Thus, it is possible that some of the effects of low concentration of BPA are actually its effects added to the effects of endogenous oestrogen [71,74].

In addition to biochemical changes, BPA was also able to directly affect the spermatogenic wave by inducing apoptosis (positive TUNEL cells) in spermatocytes and reducing the percentage of spermatids and spermatozoa. The interest in analysing the BPA-mediated apoptosis of spermatocytes in zebrafish was due to the previous evidence that these cells are susceptible to BPA-induced apoptosis in zebrafish [66] and in mammals [33], as well as by the absence of lactate [30]. The work from González-Rojo et al. [66] provide evidence that the meiotic phase of spermatogenesis was disrupted after exposure of zebrafish to 8.76 μ M BPA for 21 days, an exposure time longer than that shown in our study. In another study, a mixture of xenoestrogens containing BPA resulted in a reduced number of spermatozoa and an increased number of apoptotic (positive TUNEL cells) and proliferating germ cells in zebrafish [38]. In addition, the percentage of apoptotic germ cells (positive TUNEL cells) was also increased in the testes of mice treated with BPA during puberty, as well as activation of caspases and translocation of cytochrome c from mitochondria into cytosol [34]. However, our study is the first that used the innovative approach for digital image analysis,

pixel classification module, through Ilastik software to perform a quantitative analysis of testicular germ cells in histological sections of testis from zebrafish which were exposed to BPA. This digital quantitative analysis, as well as stereology (for counting positive TUNEL spermatocytes) carried out in our study, present several advantages over manual analysis. Quantitative analyses in histological sections are a time-consuming, tedious process, subject to errors when performed manually [43], and can still generate questionable results, since the areas of analysis are chosen by those who perform the analysis. On the other hand, the results are not affected by the choice of the area of the sections on digital image analysis, which allows us to have an automatic, standardized and controlled analysis of the whole histological section, generating accurate and high-quality data and reproducible results [75].

5. Conclusion

In conclusion, as schematically represented in Fig. 6, the *in vitro* and *in vivo* short-term exposure to a low and high concentrations of BPA alters testicular energy metabolism and promptly induces spermatocyte apoptosis and impairs the ongoing spermatogenesis in zebrafish. Acute *in vitro* exposure to BPA decreases lactate, glycogen and cholesterol content, as well as in the activities of LDH and ALT. In addition, *in vitro* exposure to BPA increases AST activity. Furthermore, acute *in vivo* exposure to BPA decreases lactate and glycogen content, as well as in the activities of ALT and AST. These studies reinforce the concept that BPA has multiple biochemical targets in the testis. BPA also triggers changes in spermatogenesis by increasing the percentage of apoptotic spermatocytes and decreasing the proportion of the surface of spermatids and spermatozoa.

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Authors' contributions

HBS, KR, KRSM performed the experimental procedures. HBS, NE and CD performed all statistical analyses. HBS, GVK and FRMBS designed the study and wrote the manuscript. CD, KRSM and GVK reviewed the manuscript, providing criticism of the study. All authors reviewed the final version of the manuscript. FRMBS and CD provided financial support and had access to all data of this study and takes responsibility for the integrity and accuracy of the data analysis.

Uncited references

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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NONGENOMIC AND GENOMIC EFFECTS OF ESTRADIOL AND XENOESTROGENS AND THEIR MECHANISMS OF ACTION IN ZEBRAFISH AND RAT TESTICULAR CELLS

ABSTRACT: The correct performance of spermatogenesis is linked to the functioning of Sertoli cells, as they are responsible for providing physical, nutritional and energy support to the germ cells. However, aquatic and terrestrial animals are susceptible to exposure to the xenoestrogen bisphenol A (BPA), which negatively affect the endocrine and reproductive systems, and therefore male fertility. Therefore, our objective was to investigate the effects and mechanism of action of BPA and/or 17 β -estradiol (E2) on calcium (Ca²⁺) signaling, testicular energy metabolism and spermatogenesis in adult zebrafish and/or immature rats. Thus, we studied non-genomic and genomic effects of BPA (10 pM and 10 μ M) on Ca²⁺ signaling, testicular energy metabolism and spermatogenesis in zebrafish (*Danio rerio*) after short- and medium-term *in vitro* and/or *in vivo* exposures. Furthermore, we studied the *in vitro* genomic effects of E2 and BPA (10 pM, 1 nM and 1 μ M) on testicular energy metabolism after short-term exposure of testis explants from 20-day-old immature rats (Wistar). The acute *in vitro* exposure of zebrafish testes to BPA increased the Ca²⁺ influx and cytosolic Ca²⁺ overload involving crosstalk between non-genomic and genomic signaling pathways. In addition, our study evidenced through different methodological approaches that *in vitro* and *in vivo* exposures to BPA triggered changes on testicular carbohydrate metabolism and damage to spermatogenesis in zebrafish through its nongenomic and genomic effects dependent or not on ESR α/β in fish. In addition, *in vitro* exposures to E2 and BPA induced changes on energy metabolism, and BPA appears to have the same mechanism of action as E2 in the ESR α/β -independent induction of the expression of genes associated to carbohydrate metabolism.

RÉSUMÉ : Le bon fonctionnement de la spermatogenèse est lié au fonctionnement des cellules de Sertoli, car elles sont chargées de fournir le soutien physique, nutritionnel et énergétique aux cellules germinales. Cependant, les animaux aquatiques et terrestres sont susceptibles d'être exposés au xénoestrogène bisphénol A (BPA), qui affecte négativement les systèmes reproducteur et endocrinien, et par conséquent, la fertilité mâle. Par conséquent, notre objectif a été d'étudier les effets et le mécanisme d'action du BPA et/ou du 17 β -estradiol (E2) sur la signalisation calcique, le métabolisme énergétique testiculaire et la spermatogenèse chez le zebrafish adulte et/ou chez le rat immature. Ainsi, nous avons étudié les effets non-génomiques et génomiques du BPA (10 pM et 10 μ M) sur la signalisation calcique, métabolisme énergétique testiculaire et la spermatogenèse chez le zebrafish (*Danio rerio*) après des expositions *in vitro* et/ou *in vivo* à court et moyen terme. De plus, nous avons étudié les effets génomiques *in vitro* de l'E2 et du BPA (10 pM, 1 nM et 1 μ M) sur le métabolisme énergétique après des expositions à court terme d'explants de testicule de rats Wistar immatures de 20 jours post-partum. Par conséquent, nos résultats ont montré que l'incubation des testicules de zebrafish au BPA a augmenté l'influx calcique et la surcharge cytosolique de Ca²⁺ impliquant une *crosstalk* entre les voies de signalisation non-génomiques et génomiques. De plus, notre étude a mis en évidence à travers différentes approches méthodologiques que des expositions *in vitro* et *in vivo* au BPA ont provoqué des changements au métabolisme testiculaire des glucides et des dommages à la spermatogenèse chez le zebrafish par ses effets non-génomiques et génomiques dépendants ou pas des ESR α/β . De plus, des expositions *in vitro* à l'E2 et au BPA ont provoqué des changements au métabolisme énergétique, et le BPA semble avoir le même mécanisme d'action que l'E2 dans l'induction indépendante de l'ESR α/β de l'expression des gènes associés au métabolisme des glucides.

Keywords: Bisphenol A, calcium, energy metabolism, estrogen receptors, spermatogenesis.

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