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Influence de l'environnement (exercice et nutrition) durant la gestation sur l'état de stress oxydant et le métabolisme du glucose de la descendance

Siti Farida

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THÈSE

Pour obtenir le grade de

**DOCTEUR DE LA COMMUNAUTÉ UNIVERSITÉ
GRENOBLE ALPES**

**préparée dans le cadre d'une cotutelle entre la
Communauté Université Grenoble Alpes et l'Université
d'Indonésie**

Spécialité : **Physiologie, Physiopathologies et Pharmacologie**
Arrêté ministériel : le 6 janvier 2005 - 7 août 2006

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préparée au sein du **Laboratoire de Bioénergétique Fondamentale
et Appliquée - INSERM U1055 - UGA**
dans l'École Doctorale Chimie et Science du Vivant

Influence de l'environnement (exercice et nutrition) durant la gestation sur l'état de stress oxydant et le métabolisme du glucose chez les rats descendance

Thèse soutenue publiquement le « **26 Octobre 2016** »,
devant le jury composé de :

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**prepared as part of a joint supervision between
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Bioenergetics (LBFA) – INSERM U1055 - UGA**
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The influence of environment (exercise and diet) during gestation on oxidative stress status and glucose metabolism in rats offspring

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ABSTRACT

The influence of environment (exercise and diet) during gestation on oxidative stress status and glucose metabolism of offspring.

Prevalence of metabolic diseases is growing up in our modern societies and constitutes a major public health concern. Family history, environment and lifestyle play a role in the susceptibility to several metabolic disorders. Based on epidemiological data, a link has been established between the environment during the first stages of life and diseases occurrence in adulthood leading to the concept of "Developmental Origins of Health and Diseases" (DOHaD). A first aim of this work was to study, using a murine model, the effect of daily maternal exercise during gestation on offspring body composition, oxidative stress status (OS), liver and muscle mitochondrial and pancreatic functions and energy substrates handling. A second aim was to evaluate a high fructose diet (F) as an alternative experimental model of Gestational diabetes mellitus (GDM) on rats to test the effect of an iron-enrichment (FI) during pregnancy on glucose tolerance and OS status on mothers and offspring. Our results show a significant reduction in mitochondrial H_2O_2 release, as an index of reactive oxygen species (ROS) production and signalling, in liver and muscle of offspring from trained mothers. These changes are related to alterations in the mitochondrial oxygen consumption, mitochondrial membrane composition and in the activity of antioxidant enzymes. Moreover, maternal training before and during gestation is associated to changes in offspring pancreas structure and function and in energy substrates handling. We also confirmed that a fructose diet could be proposed as an experimental model of GDM. We then showed that in rat pups born from mothers fed a fructose iron-enriched diet (FI), the activities of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione-S-transferase in liver and GPx in brain were altered, depending on the gender. Thus, alterations in the glycemic and redox status in newborns suggest that fetuses are more sensitive than their mothers to the effect of an iron-enriched diet in the case of GDM. This work proposes a novel experimental model for GDM and confirms some studies conducted in the framework of the DOHaD concept and strengthens the idea that the environment during the early stages of life has an impact on the future health.

Key words: Exercise, Gestation, Offspring, Mitochondria, Oxidative Stress, Gestational Diabetes, Iron, High-Fructose Diet

Influence de l'environnement (exercice et nutrition) durant la gestation sur l'état de stress oxydant et le métabolisme du glucose de la descendance.

Les maladies métaboliques sont en pleine expansion dans nos sociétés actuelles et constituent un enjeu de santé publique majeur. Les antécédents familiaux, l'environnement et les habitudes de vie de l'individu vont jouer un rôle dans la susceptibilité à certains de ces désordres métaboliques. Sur la base de données épidémiologiques, un lien a été établi entre l'environnement durant les premières phases de la vie et survenue de pathologies à l'âge adulte conduisant au concept des "Developmental Origins of Health and Diseases" (DOHaD). Le premier objectif de ce travail était d'étudier, à partir d'un modèle murin, les conséquences de l'exercice physique quotidien de la mère pendant la gestation sur la composition corporelle, le statut oxydant, la fonction pancréatique et mitochondriale du foie et du muscle et la gestion des substrats énergétiques de la descendance. Un deuxième objectif était de valider un modèle de diabète gestationnel à partir d'un régime riche en fructose et d'étudier l'effet d'une supplémentation en fer durant ce diabète gestationnel sur le statut oxydant et la tolérance au glucose des mères et de la descendance. Nos résultats montrent une réduction significative de la production mitochondriale d' H_2O_2 , un indicateur de la production d'espèces réactives de l'oxygène, dans le foie et dans le muscle des petits de mères entraînées. Ces changements sont reliés à des altérations de la consommation d'oxygène mitochondriale, de la composition des membranes mitochondriales et de l'activité des enzymes antioxydantes. De plus, l'entraînement maternel avant et pendant la gestation est associé à des modifications de la structure et de la fonction du pancréas de la descendance et semble modifier sa gestion des substrats énergétiques.

Nous avons également confirmé qu'une diète riche en fructose durant la gestation peut être utilisée comme un modèle induisant un diabète gestationnel. Nous avons ainsi démontré chez les petits nés de mères nourries avec un régime riche en fructose et en fer, que les activités des enzymes antioxydantes comme la glutathion peroxidase (GPx), la glutathion-S-transferase dans le foie et la GPx dans le cerveau étaient altérées, les résultats étant différents selon le sexe des petits. Les différents résultats obtenus chez la descendance montrent que dans le cas d'un diabète gestationnel, les fœtus sont plus sensibles que leurs mères aux effets d'un régime riche en fer. Ce travail de thèse vient compléter les travaux menés dans le cadre des DOHaD et renforce l'idée que l'environnement lors des premières phases de la vie va avoir des conséquences sur la santé de l'individu.

Mots-clés : exercice, gestation, descendance, mitochondries, stress oxydant, diabète gestationnel, fer, régime riche en fructose.

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ABBREVIATIONS

| | |
|-------------|--|
| ADP | = adenosine diphosphate |
| Akt/PKB | = protein kinase B |
| ATP | = adenosine triphosphate |
| AMPK | = adenosine 5-monophosphate kinase |
| cAMP | = cyclic adenosine monophosphate |
| CAT | = catalase |
| CREB1 | = cyclic AMP response element-binding protein -1 |
| COX | = cytochrome oxidase |
| CPT1 | = carnitine palmitoyltransferase 1 |
| DAG | = diacylglycerol |
| DOHaD | = Developmental Origins of Health and Disease |
| ETC | = electron transport chain |
| FAD | = flavin adenine dinucleotide |
| FRAP | = ferric reducing ability of plasma |
| GLP-1 | = glucagon-like peptide 1 |
| GDM | = gestational diabetes mellitus |
| GDIP | = glucose-dependent insulinotropic polypeptide |
| Glucose-6-P | = glucose 6 phosphate |
| GLUT 2 | = glucose transporter 2 |
| GLUT 4 | = glucose transporter 4 |
| GPX | = glutathion peroxidase |
| GSHPx | = GSH peroxidase |

| | |
|-------------------------------|--|
| GSHR _x | = GSH reductase |
| GSR | = glutathione reductase |
| GST | = glutathione transferase |
| H ₂ O ₂ | = hydrogen peroxide |
| HFD | = high fat diet |
| HIF1A | = hypoxia-inducible factor 1-alpha |
| HOMA | = homeostasis model assessment |
| IGF-I | = insulin-like growth factor I |
| IP 3 | = inositol triphosphate |
| IR | = insulin receptor |
| IRS | = insulin receptor substrate |
| IRS-1 | = insulin receptor substrate-1 |
| LC | = low calory |
| LDL | = low density lipoprotein |
| LHP | = lipid hydroperoxide |
| MAP | = mitogen activated protein |
| MAPK | = mitogen-activated protein kinase |
| MDA | = malondialdehyde |
| mtDNA | = mitochondrial DNA |
| NAD | = nicotinamide adenine dinucleotide |
| MVM | = microvillous plasma membrane |
| NFKB1 | = nuclear Factor Kappa B Subunit 1 |
| NRF-1 | = nuclear respiratory factor -1 |
| O ₂ ⁻ | = superoxide anion |
| OH [•] | = hydroxyl radical |
| OXPHOS | = oxidative phosphorylation |
| PACAP | = pituitary adenylate cyclase-activating polypeptide |

| | |
|----------------|--|
| PGC-1 α | = peroxisome proliferator guided-activated receptor co-activator -1 α |
| PI3K | = phosphatidylinositol 3-kinase |
| PIPD1 & 2 | = phosphatidylinositol dependent protein kinases 1 & 2 |
| PI3K | = phosphatidylinositol 3-kinase |
| PKB | = protein kinase B |
| PKC | = protein kinase C |
| PPAR | = peroxisome proliferator guided-activated receptor |
| PUFAs | = polyunsaturated fatty acids |
| ROS | = reactive oxygen species |
| SOD | = superoxide dismutase |
| SIRT3 | = sirtuin-3 |
| SH2 | = <i>src</i> homology-2 |
| SNAT | = sodium-coupled neutral amino acid transporter |
| TBARS | = thiobarbituric acid reactive substances |
| TFAM | = transcription factor A of mitochondrial |
| UCPs | = uncoupling proteins |
| VIP | = vasoactive intestinal peptide |

INTRODUCTION

The increased prevalence of metabolic diseases in our modern societies constitutes a major public health problem. Genetic factor, environment and lifestyle play a role in the susceptibility to several metabolic disorders. Based on epidemiological data, a link has been established between the environment during the first stages of life and diseases occurrence in adulthood leading to the concept of "Developmental Origins of Health and Diseases" (DOHaD). The intrauterine life environment is of importance for fetal growth and closely related to health status in later life (Gluckman 2005, 2008). Results show that the gestational environment can play an important role in influencing later susceptibility to chronic diseases such as diabetes in postnatal offspring (Gluckman, 2008). In addition, exposure to an inappropriate environment such as intrauterine oxidative stress during fetal and neonatal development may changes offspring susceptibility with permanent effects on the health of offspring (Zambrano *et.al* 2016).

On the other hand, pregnancy is a physiological state associated with a moderate increase of oxidative stress related to high metabolic turnover and elevated tissue oxygen requirements (Wisdom 1991, Morris 1998, Toescu 2002). Under normal condition, the increased reactive oxygen species (ROS) production will be compensated through increased production of endogenous antioxidants to maintain the equilibrium of redox status (Trachootham *et.al.* 2008). When the ROS production is high enough to exceed the capability of the body's antioxidant system, it will disrupt redox balance and causes oxidative stress. Intrauterine oxidative stress can alter mitochondrial function and may be one of a key underlying mechanism in fetal programming (Thompson *et.al.* 2012; Reusens *et.al.* 2011). During

pregnancy, unbalanced diet (Szostak-W 2014, Grieger *et.al.*2015), prolonged stress (Entringera *et.al.* 2011), alcohol consumption (Li *et.al.* 2004), smoking exposure (Genbacev *et.al.* 2003; See-Ling and Hamid 2014), physical inactivity (Laufs *et.al.* 2005) contribute to intrauterine oxidative stress, which may increase the probability of risk of diseases in childhood and adult life.

Intrauterine oxidative stress in pregnancy may be induced by maternal imbalanced diet, hypoxia, utero-placental insufficiency and/or a positive caloric balance. The impact of oxidative stress during pregnancy can promote pregnancy disorders such as placental growth disruption, preeclampsia, premature delivery and gestational diabetes mellitus (Kim 2014, Al Gubory 2010, and Agarwal 2012). Since oxidative stress in the pregnant female is closely linked to oxidative stress on the fetus (Argüelles 2006), it can disrupt the fetal development proces, fetal growth restriction, and low birth weight (Gavard 2008, Clapp 2002). Moreover, oxidative stress during pregnancy is also linked to the pathogenesis and progression of neonatal diseases (Harvey 2002, Biri 2007, Karowicz-Bilinska 2007, Jantasing 2008, and Ostlund 2003). Thus, it seems important to limit oxidative stress during pregnancy to reduce the risk of disease later in life of the offspring.

Modification of the environment around gestation by regular exercise is of importance. Level of physical activity is now a significant component of a good health (Paffenbarger 2001). Regular physical activity during pregnancy is already known to have many maternal benefits including weight and body composition control. Moreover, it improves cardiovascular health (Clapp 1995), reduces the risk of glucose intolerance and gestational diabetes. Finally, the benefits in offspring include a decrease in fat mass at birth and in childhood, a better stress tolerance and cognitive characteristics (Clapp 1996), an advance in neurobehavioural

maturation (Melzer 2010; Snapp 2008), an enhancement of insulin sensitivity in the adult offspring (Carter 2013), and a decrease in oxidative damage markers (Radak 2008). However, the mechanism by which exercise elicited such a metabolic adjustment remains unclear. We hypothesize that moderate-intensity endurance training before and during gestation could modify the pancreatic Islet β -cell and mitochondrial function of offsprings. In this way, endurance training has been known to have beneficial effects on redox balanced; it could be of potential interest on the redox state of the newborn.

In parallel, modification of the environment around gestation through unbalanced diet, such as high-fat/high-fructose diet may lead to intrauterine oxidative stress. On the other hand, gestation insulin sensitivity if modified during pregnancy, maternal high fructose diet may pose a challenge for the health of the mother and offspring. This is associated with both low and high birth weight of offspring, oxidative imbalance during fetal life, disruption of mitochondrial function and glucose metabolism of offspring, as well as gestational diabetes mellitus (Donovan 1990, Bernard 2005). Gestational diabetes mellitus (GDM) is marked by increased insulin resistance that associated with a rising level of oxidative stress (OS). In addition, excessive iron intake may also elevate insulin resistance and oxidative stress, which could aggravate the risk of GDM. The alterations in the blood glucose and redox balanced status in newborns suggest that fetuses are more sensitive than their mothers to the effect of an iron-enriched diet in the case of GDM.

Furthermore, streptozotocin may not be suitable to create an experimental model of GDM that represents insulin resistance, because it induces necrosis of the pancreatic β -cells leading to Type 1 diabetes (Damasceno 2014). On the other hand, a diet rich in fructose has been used to study insulin resistance in experimental research (Busserolles 2003, Kolderup 2015) but, so

far, not in pregnancy. The purpose of this study is to evaluate fructose diet as an alternative model of GDM on rats and the worst effect of a fructose iron-enriched diet on glucose tolerance and oxidative stress status during pregnancy.

In the present study, a mouse model was used to examine the effect of moderate intensity endurance training before and during gestation on the redox state (thiols level and glutathion peroxidase (GPX) activity) and on glucose metabolism (structure and function of pancreatic Islet β -cells, measuring of glucose tolerance, insulin sensitivity, insulin secretion, insulin signaling pathway (PKB phosphorylation) of offspring. In addition, this study proposed a novel experimental model for GDM, and gave an overview on the effects of a moderate iron consumption that increased the risk of disruption glucose and oxidative damage to rat's newborn.

This study was performed at the Laboratory of Bioenergetics and Fundamental Applied (LBFA) INSERM U1055 Grenoble-France and Laboratory INSERM UMR U870/INRA U123/INSA Lyon-France. This work was supported by INSERM, by Joseph-Fourier University and by the SFR-SEM from UFRAPS of Grenoble-France and a grant by Ministry of Education, Republic of Indonesia.

CHAPTER I

LITERATURE REVIEW

Introduction

Oxidative stress is related to mitochondria, which play an important role in cellular function and are very sensitive to environmental changes (Cui *et.al.* 2011). Intrauterine oxidative stress can alter mitochondrial function and may be one of a key underlying mechanism in fetal programming (Thompson *et.al.* 2012; Reusens *et.al.* 2011). This chapter will address recent reviews and current study identifying the influence of oxidative stress on the fetus.

The influence of low protein/high fat/high fructose diet of maternal and micronutrient supplementation of iron around gestation on the programming of fetal metabolism will be discussed in this chapter. Furthermore, their effects on oxidative stress status, mitochondrial function of brain and liver, and glucose metabolism of mother and offspring will be described in this chapter.

Exercise has been proposed to improved overall physiological, psychological and metabolic health (Melzer 2010). Thus, maternal exercise before and during pregnancy will be highlighted with respect to metabolic changes that can affect the early life environment. However, until now there are discrepancies in the results of studies on the impact of exercise on pregnancy both on pregnant women and their baby. This might be due to differences in the intensity, time, frequency, and duration of exercise. This chapter also describes common animal models used to study the fetal adaptive responses to maternal exercise through the alteration of mitochondrial and pancreatic Islet β -cell function in the early life.

I.1. MITOCHONDRIA and OXIDATIVE STRESS

1.1.1 Structure and Function of Mitochondria

Mitochondria have a major role in cellular function, not only in ATP production, but also play important roles in cellular processes such as ROS production, apoptosis signaling, Ca^{2+} homeostasis, metabolic cellular signaling, and regulation of energy expenditure (Cooper 2000, Farris *et.al.* 2005, Suen *et.al.* 2008, Cheng *et.al.* 2013, Tait *et.al.* 2012). It contains outer and inner membranes which are made up of phospholipids and also contain a number of enzymes and proteins involved in energy transduction. The space between outer and inner membrane is called the inter membrane space (Cooper 2000). (Fig.1)

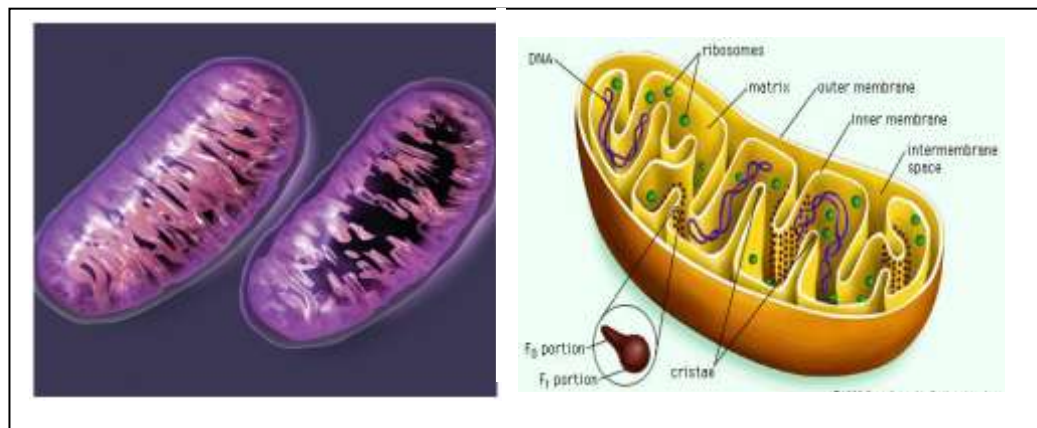


Figure 1: Structure of mitochondrion (Schmidt)

The outer membrane is composed of 50 % protein and 50 % lipid. It contains a special protein called porin which makes it permeable to molecules of about 10 kilodaltons or less, such as ions, nutrient molecules, ATP and ADP.

The structure of inner membrane is very complex, including the electron transport chain (ETC), ATP synthetase complex, and transport proteins. It is composed of about 80 % protein and 20 % lipid. The folds are organized into layers, called cristae. The cristae largely increases the total area of the inner membrane. The inner membrane has the respiratory chain and many specific transporters that ensure the passage of necessary elements for the production of ATP. This membrane is permeable to O₂, water, and CO₂ but is almost not permeable to protons, which allows the respiratory chain to build and sustain a gradient of protons required to drive the ATP synthesis.

The mitochondrial matrix contains mitochondrial DNA (mtDNA), enzymes of the Krebs cycle (citric acid cycle) and of the β -oxidation process (Fig.2). In the cytosol, the initial step of glucose metabolism (glycolysis) converts glucose in pyruvate. Then, in the mitochondrial matrix, fatty acids and pyruvate are imported from the cytosol and converted in acetyl CoA. Through the citric acid cycle, acetyl CoA is then oxidized to CO₂ (Fig.2). These pathways produce NADH + H⁺ and FADH₂ which provide electrons to the respiratory chain.

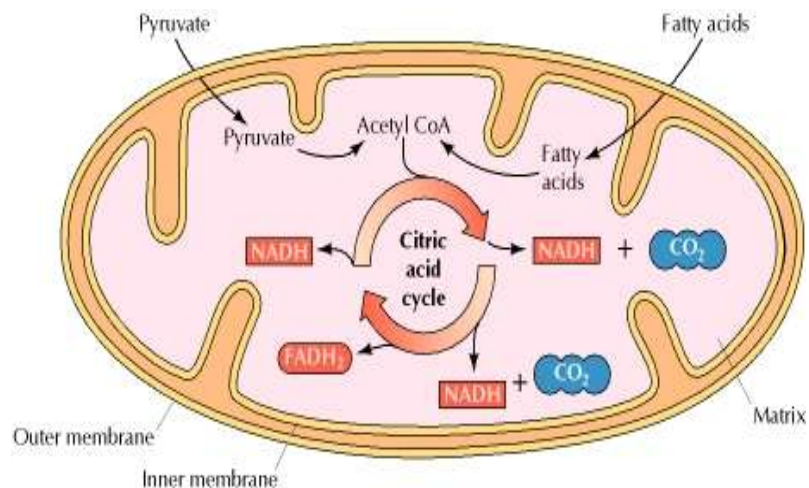


Figure 2: Metabolism of pyruvate and fatty acids in the matrix of mitochondria (Cooper)

I.1.2 Respiratory Chain

The respiratory chain contains four enzyme complexes, which are in turn acceptors and electron donors. The energy released during these redox reactions allows the vectorial transport of protons from matrix to the inter membrane space at complex I (NADH-ubiquinone reductase), III (ubiquinone-cytochrome c reductase), and IV (cytochrome oxidase) as shown in Fig 3 (Shaughnessy).

The production of mitochondrial ATP occurs through the electrons flow derived from the reduced equivalents: nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) (Fig.3). ATP production requires two main phases, namely the oxidation of $\text{NADH} + \text{H}^+$ or FADH_2 to NAD^+ or FAD and the phosphorylation of ADP to ATP (oxidative phosphorylation) (Yano 2002, Benard *et.al.* 2006).

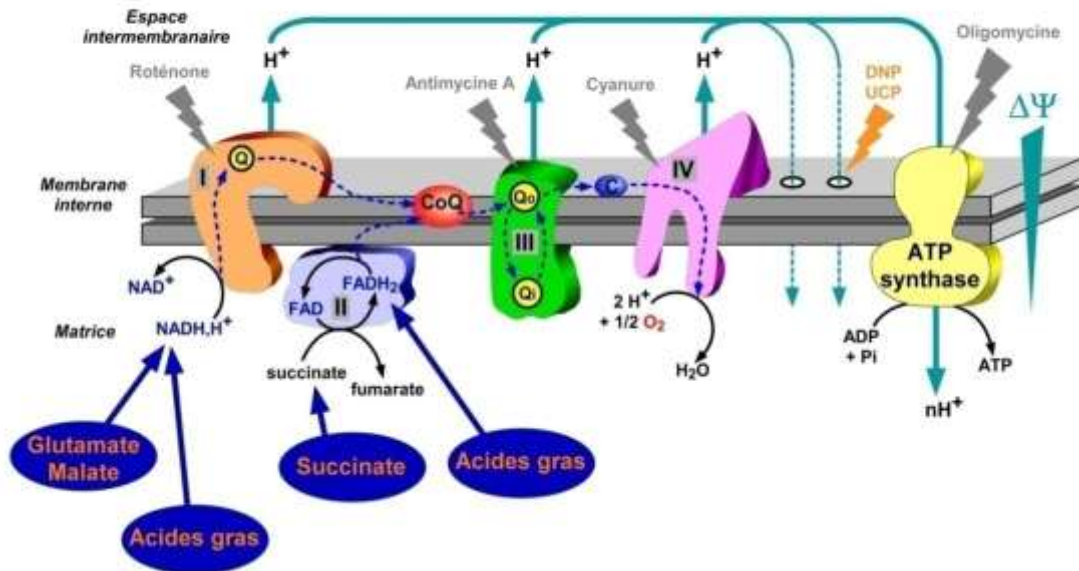


Figure 3: Schematic representation of the respiratory chain or electron transport chain, and ATP synthase.

Electrons pass through a series of complexes in the inner mitochondrial membrane known as the electron transport system (ETC). As a result of this electron transfer, protons (H^+) are transferred across the inner mitochondrial membrane producing a large mitochondrial membrane potential (Figure 3). The H^+ gradient created by the accumulation of proton outside the inner membrane activates a fifth complex enzyme called ATP synthase. When protons reenter the matrix through the F_0F_1 ATP synthase, they release an energy used by the enzyme to produce ATP from ADP and Pi present in the matrix. Moreover, when the electrons finally arrive at complex IV, they are accepted in the matrix by molecular oxygen to form H_2O in the matrix (Farris 2005). (Figure 3)

Complex I is the biggest component of the respiratory chain. In this complex, on-going oxidation of $NADH + H^+$ leads to several redox reaction that finally transfer electrons to ubiquinone (Yano 2002). Reduction of ubiquinone is coupled with the transfer of protons from the matrix to the inter membrane space. $FADH_2$ is oxidized at Complex II (succinate-ubiquinone oxidoreductase) which allows the transfer of electrons to ubiquinone that is not associated with the efflux of protons.

Mitochondrial respiration is activated by the reduced equivalents ($NADH+H^+$ and $FADH_2$) and by high concentrations of ADP. Extra-mitochondrial oxygen consumption can occur by enzymatic and non-enzymatic reactions, including NADPH oxidase, xanthine oxidase, uncoupled NO synthase, d-aminooxidase, cytochrome p450, and proline hydroxylation (Yano 2002).

I.1.3 Protein, DNA, and Biogenesis of Mitochondria

In human cells, mitochondrial proteins are encoded by genes derived from the nucleus and mitochondria. Mitochondrial genes encode 13 subunits proteins of oxidative phosphorylation complexes, twenty-two transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs), all of these proteins are important for electron transport and ATP production, and consequently for normal cellular physiology (Fariss 2005). The subunit expression levels of oxidative phosphorylation and the number and size of mitochondria determine the ability of mitochondrial oxidation (Fariss 2005).

mtDNA is highly sensitive to oxidative stress which can cause lethal injury through the loss of electron transport, mitochondrial membrane potential, and ATP generation. Furthermore, mitochondrial genome is more sensitive to mutagenic stressors because mitochondrial genes are less protected by histon while they are close to the source of ROS. Moreover, in mammalian cells, the repair system capacity of mitochondrial DNA is limited (Yakes 1997).

In addition to mitochondria's role in energy production and apoptosis, these organelles serve as sites of iron-sulfur cluster synthesis. Iron, like oxygen, is essential for life, but is also one of mitochondrial toxicants (e.g. alkylating agents, iron, hyperoxia, doxorubicin and rotenone) that stimulate ROS production (Liang 2004). High concentrations of iron rapidly reduce the transcription of a large set of genes that encode mitochondrial ribosomal proteins, as well as, reduce OXPHOS and the total components of electron transport chain. This disruption of electron transport can enhance H_2O_2 production of mitochondria. The combination of increased iron and physiological concentrations of H_2O_2 generated in the mitochondria during

OXPPOS might result in the generation of DNA damage (Yakes 1997). In addition, the results of two studies of Santos 2003, 2004 suggest that mitochondrial telomerase sensitizes cells to oxidative stress, which may result in apoptotic cell death, and imply a new function for telomerase in mitochondrial DNA. Finally, these results indicate that mtDNA damage following oxidative stress can lead to a vicious cycle of ROS propagation and mtDNA oxidation. The cascade initiated by oxidative mtDNA damage that leads to faulty gene expression, deficiency of important enzymes in the electron transport, subsequent ROS generation and ultimately cell death, is known as the mitochondrial catastrophe hypothesis. mtDNA damage, therefore, represents an important target for intervention.

I.1.4 Role of mitochondria in muscle

Mitochondria are essential for the function of skeletal muscle and they ensure the level of ATP required for contraction by the muscle sarcomere. The capacity of mitochondrial function directly affects the muscle function. Due to the main contribution to total body mass, muscle mitochondria have a very significant role in the metabolism of the body as a whole. This is supported by the discovery of an increase in skeletal muscle mitochondrial content of a person with hypermetabolism and not prone to weight gain (Luft syndrome).

I.1.5 Role of mitochondria in liver

The regulation of multiple metabolic functions is centralized in the liver. Ultrastructure and function of liver mitochondrial are different from in muscle. Areas of mitochondria in the liver are 44% lower than in the heart, with a smaller size, fewer cristae, and a lower density of

mitochondria. Moreover, the protein expression of several components of the oxidative phosphorylation and transcription factor A (TFAM) that regulates replication and transcription of the mitochondrial DNA, as well as citrate synthase activity is also lower in the liver (7% lower than the heart muscle). In function, the isolation of mitochondria has relative decreased oxidative phosphorylation proteins, cytochrome respiratory chain, and a maximum capacity of complex III and IV. In addition, fatty acid oxidation in the liver is known to be related with mitochondrial function. Disruptions in the number of mitochondria and/or function could impact multiple cellular processes in hepatocytes, both directly such as decreased ATP production, altered status of oxidative stress, reduced fatty acid oxidation, and indirectly through the influence on required energy processes, such as gluconeogenesis, synthesis of cholesterol, proteins, urea, bile acids, and detoxification. A study by Borengasser *et.al.* 2011 demonstrated a significant reduction in several markers of liver mitochondrial function in rat pups whose born to obese mothers, including SIRT3 mRNA as a marker of mitochondrial DNA copy number and biogenesis, mitochondrial protein content, decreased fatty acid oxidation, ETC complexes II, III, and ATPase, as well as expression of PGC-1 α mRNA as a regulator of mitochondrial biogenesis.

I.1.6 Reactive Oxygen Species (ROS)

Free radicals generated during normal cellular metabolism are chemical species, atoms or molecules that have unpaired electron on their molecular orbital electrons. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are the most important groups of free radicals produced by the body.

ROS are byproducts of mitochondrial ETC and also an inevitable byproduct of various cellular and extracellular redox reactions (Aljubory *et.al.* 2010, Gomez *et.al.* 2007). At low or moderate levels, ROS have role in physiological processes involved in the regulation of cell signaling, enzymes and gene expression that sensitive to redox, but higher concentration of ROS can cause oxidation of proteins, lipids and DNA (Gomez *et.al.* 2007).

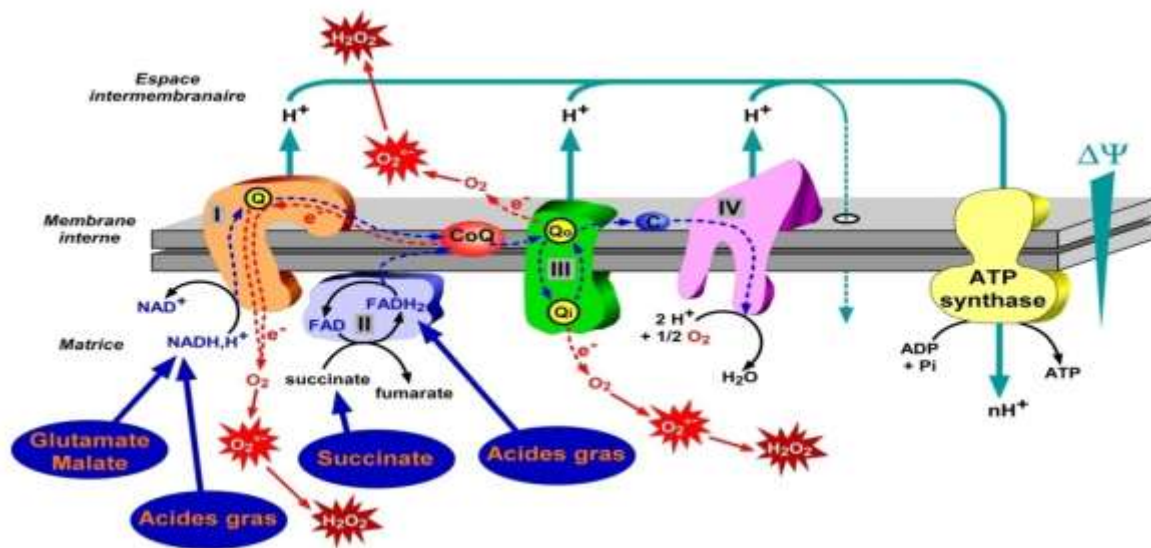


Figure 4. ROS production by electron transport chain in mitochondria

In normal aerobic metabolism, mitochondria are a major source of ROS production at complex I and III of the respiratory chains (Figure 4). Indeed, the electron transport chain is recognized as one of the main sites of superoxide anion generation (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^\bullet). 1-3% of the oxygen used by mitochondria is partially reduced to form ROS as a result of electron leakage (Turrens 2007, Fusch 2003).

During normal metabolism, many cellular components or enzymatic reactions produce ROS, especially superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Superoxide anion (O_2^-) has a very short half life, because it is immediately converted by mitochondrial superoxide

dismutase (Mn-SOD or SOD₂) to form H₂O₂ (Cadenas 1975). Then, catalase, which is an antioxidant enzyme, catalyzes the reaction converting H₂O₂ to H₂O and O₂. Under certain conditions, hydrogen peroxide (H₂O₂) can be catalyzed by copper and iron and form highly reactive hydroxyl radical (Fenton reaction). Hydroxyl radical (OH[•]) may cause the peroxidation of lipids, proteins, glucose, and DNA.

The highest ROS production occurs when the proton gradient is high, while oxygen consumption or ATP requirement is low. Excessive caloric intake and low energy expenditure will lead to high proton gradient and decreased ATP requirement, while exercise will increase the requirement for ATP, where electron transfer will be paired with ATP production and decrease proton gradient.

Moreover, ROS generation can occur through several different mechanisms at each level, such as ischemia-reperfusion, activation of neutrophils and macrophages (NADPH oxidase), the process of electron transport in the mitochondrial respiratory chain, Fenton chemical reaction, endothelial cell xanthine oxidase, metabolism of free fatty acids and prostaglandins, NO synthase from arginine, and hypoxia.

I.1.7 Antioxidant

Under normal/physiological conditions, antioxidants prevent excessive ROS generation to maintain in equilibrium through scavenger molecules or conversion of ROS into H₂O. In the body there are two groups of antioxidants, the enzymatic and non-enzymatic antioxidant.

I.1.7.1 Enzymatic antioxidant

Antioxidants in this group are also known as natural antioxidants which can neutralize excess ROS and prevent damage to the cell structure. The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Increased oxidative stress can induce the antioxidant enzymatic activity such as superoxyde dismutase. If SOD is found at the start of the pregnancy, the activity of this enzymatic antioxidant will protect the developing embryo from damage caused by free radicals (Wang *et.al.*2002). In line with the gradual increase of MDA levels in pregnant women in comparison with non-pregnant women, the levels of superoxyde dismutase, glutathione peroxidase and catalase decrease gradually. The lowest decline is in the third trimester (Patil *et.al.* 2007).

Superoxide anion (O_2^-) is produced by the reduction of molecular oxygen and will initiate a chain reaction of free radical formation. It was found that SOD can change the superoxide anions to hydrogen peroxide and the reaction was instrumental in antioxidant enzymatic reaction. In mammals, SOD has three isozyme: (1). SOD1 is encoded by the Cu, Zn-SOD containing Cu and Zn as the metal cofactor and is cytosolic isoform in cytoplasm; (2). SOD2 is encoded with Mn-SOD is a mitochondrial isoform containing metals Mn in mitochondria; (3). SOD3 encoded with ECSOD (extracellular form) that has a similar structure with CuZn-SOD and also contain Cu and Zn metal cofactors (Fukai 2011).

I.1.7.2 Non-enzymatic antioxidant

Other categories that are members of the non-enzymatic antioxidant defenses against the toxic effects of free radicals are represented by small molecules, vitamins and metals. Among them, vitamin C and E and beta carotene directly scavenge free radicals. Non-enzymatic antioxidants are also known as synthetic antioxidants or dietary supplementation. The antioxidant system is influenced by dietary intake of antioxidant vitamins or minerals such as vitamin C, vitamin E (tocopherol), selenium, zinc, taurine, hipotaurin, glutathione, beta carotene.

Antioxidants in this group play a role in the lipophilic environment. Vitamin A acts as an antioxidant breaker chain reaction, vitamin C acts as scavenging superoxide, free radicals and various lipid hydroperoxide. In addition, vitamin C can also stop the propagation of peroxidation process, and help cycle oxidation of vitamin E and glutathione. Lipid peroxidation was regarded as a marker of pro-oxidant. On the contrary, the reduced form of glutathione, vitamin E, vitamin C, and vitamin A can be seen as an antioxidant.

The power of antioxidant (total antioxidant) was assayed by determining of ferric reducing ability of plasma (FRAP) as a novel method by Benzi *et.al.*1996. The reduction of ferric to ferrous at low pH causes the formation of a colored complex of ferrous-tripyridyltriazine. The value of FRAP was obtained by comparing the absorbance of standard ferrous ion with the sample at 593 nm. The FRAP measurement is inexpensive and reagents are simple to prepare.

I.1.8 The Role of ROS in Normal Fetal Development

In physiological levels, ROS play important roles as second messengers in many intracellular signaling cascades (Burton 2011). They regulate various signaling transduction pathways from the beginning of reproductive processes such as folliculogenesis stage, oocyte maturation, corpus luteum and uterine function, embryogenesis, implantation and until the development of fetoplacenta (Agarwal *et.al.* 2008).

It has been known that pregnancy is a physiological state in which an increase in ROS production is associated with high metabolic activity of the mitochondria. Morris *et.al.* 1998 reported that in women with normal pregnancy there is a significant increase in markers of oxidative stress (lipid peroxidation [180%], malondialdehyde [34%], and the antioxidant vitamin E [84%]). Moreover, pregnancy is also associated with a decrease in the total antioxidant ability and uric acid in the first trimester but it is gradually increase during pregnancy, reaching normal values during the postpartum period. In addition, normal pregnancy is associated with significant changes in lipid metabolism marked by hypertriglyceridemia and shift to the dominant direction of LDL. Toescu *et.al.*2002 have shown that these changes correlated with increased levels of lipid peroxidation as the pregnancy progress.

The placenta has been identified as an important place of lipid peroxidation because of its high content of polyunsaturated fatty acids (PUFAs). Peroxidation marker levels, such as lipids and malondialdehyde (MDA) were higher in pregnant women than in non-pregnant women (Patil *et.al.* 2006). Lipid peroxidation increases during the second trimester, and then gradually decreases. The changes observed in the lipid hydroperoxide (LHP) were

significantly correlated with increased LDL profile. Moreover, the metabolism of maternal lipids is specifically altered during pregnancy. LDL particles become smaller and denser than in non-pregnant women, which make them more susceptible to oxidation. Thus, elevated triglycerides and the accumulation of small, dense LDL during pregnancy are thought to increase the risk of endothelial damage (Winkler *et.al.* 2000). Biochemical changes may be relevant for long-term cardiovascular health of women, especially with high parity or those who are at high risk for cardiovascular disease (for example, women with diabetes). The placenta is also a source of antioxidants that control enzymatic lipid peroxidation in pregnancy without complications. All the major antioxidant defense system, including SOD, catalase, GPX, glutathione, vitamin C and E are found in the placenta and may be sufficient to control lipid peroxidation in normal pregnancy.

Embryonic development occurs at a relatively low oxygen environment. This situation is very sensitive to injury caused by oxidant molecules due to low antioxidant capability (Toescu *et.al.* 2002). During the formation of the placenta, there is an increase in oxygen transfer which would increase the formation of reactive oxygen species (ROS) in the cell. This will encourage the conversion of the cellular redox status from reduction to oxidation, which will act as a driving force for cell differentiation. With the maturation of the placenta, there is an increase in the transfer of oxygen to the developing fetus, which is needed to sustain the increased speed metabolism during the phase of rapid growth of the fetus.

I.1.9 Oxidative Stress

Haliwell 1999 defines oxidative stress as the inability the body to defend against the attack of ROS or when the level of ROS exceeds the antioxidant capacity of the body. Oxidative stress (OS) is also defined as disequilibrium of the redox balance.

While ROS production plays an important role in cell signaling, a high level of ROS induces oxidative damage to protein, membrane lipid, and DNA. This leads to mitochondrial dysfunction (Cui *et.al.* 2011).

The generation of ROS has both physiologic and pathologic roles in the placenta and the fetus. Oxidative stress is also involved in the etiology of some diseases includes aging, cancer, diabetes, and cardiovascular diseases. ROS have extremely short half-lives, it is difficult to measure ROS directly. Instead, the evaluation of oxidative stress can be determined by measuring several products of the damage produced by oxidative stress, such as thiobarbituric acid reactive substances (TBARS) which is formed as a byproduct of lipid peroxidation (Janero 1990).

I.1.10 Oxidative Stress in Pregnancy

Oxidative stress in pregnancy is closely linked to oxidative stress in the fetus. The effects of oxidative stress intrauterine on offspring are influenced by the severity and duration as well as the gestational age of the fetus at the time of exposure to the stress. In addition, Gupta *et.al.*2007 indicates that oxidative stress has been suggested to play a role in recurrent abortion, preeclampsia, intra-uterine growth restriction and fetal death.

Arguelles *et.al.* 2006 showed that a high oxidative stress in pregnant women is closely related to oxidative stress in fetal and in newborns, leading to pathogenesis or progress of neonatal disease. Oxidative stress in children is even greater when compared to the mother, probably because of their antioxidant defense is still poorly developed. Excessive oxidative stress in pregnant women is also associated with other complications of pregnancy including preeclampsia and GDM. This pathology has a very bad effect on the health of the fetus and can even cause death (Rao 2007).

Emerging biological evidences suggest that oxidative stress can be induced among other things by unbalanced mother's diet (deficiency or excess of nutrients), hypoxia, placental insufficiency-utero, and/or positive caloric balance; a key that can be related to disease progression or permanent fetal programming.

I.2 GLUCOSE HOMEOSTASIS and PANCREAS

Maintenance of plasma glucose homeostasis, in response to nutrient conditions and hormonal signals, is important for the survival of mammalian organisms. Insulin decreases plasma glucose level by inhibiting glucose production in the liver and by increasing glucose consumption in muscle and adipose tissues. In contrast, glucagon and glucocorticoids hormones enhance glucose levels through the activation of gluconeogenesis, glycogenolysis in the liver and reduction of glucose utilization in skeletal muscle.

I.2.1 Glucose transporter proteins (GLUT)

Glucose transporter GLUT 1 is located in the red blood cells, placenta, micro vessels of blood brain barrier, kidney, colon, and other cells, while GLUT 2 in the liver cells, pancreatic β cells, and the basolateral surface of the small intestine. GLUT 3 is in neurons, placenta, and testis. GLUT 4 is in the fat cells, skeletal muscle, and heart and mediates glucose uptake stimulated by insulin. GLUT 5 as a transporter of fructose is located in the small intestine, testis, sperm, kidney, skeletal muscle, adipose tissue, and brain.

In order to maintain normal blood glucose levels, glucose metabolism is regulated by homeostatic mechanisms involving several hormones, liver and extra-hepatic tissues. Glucose enters the liver and the pancreatic β -cells freely through GLUT 2 and is phosphorylated by glucokinase. Concerning the function of β -cells, an increase in blood glucose increases the metabolism flow through glycolysis, the citric acid cycle, and ATP formation. Increased ATP inhibits K^+ pathway which is sensitive to ATP (ATP-sensitive K^+ channels) induces the cellular membrane depolarization, enhances the Ca_2^+ influx and stimulates insulin exocytosis. After a meal, skeletal muscle is the main site for glucose consumption. Insulin immediately decreases blood glucose by increasing glucose transport into muscle and fat tissue by mobilizing GLUT4 in the cell to the surface of cell membrane.

GLUT-4 is the primary glucose transporter in muscle cells and fat. GLUT-4 is different from other glucose transporter, mainly because insulin favors the translocation of GLUT-4 from the storage vesicles towards the plasma membrane (Murabayashi 2012) by a mechanism that involves the protein kinase B (PKB) pathway (Figure 5).

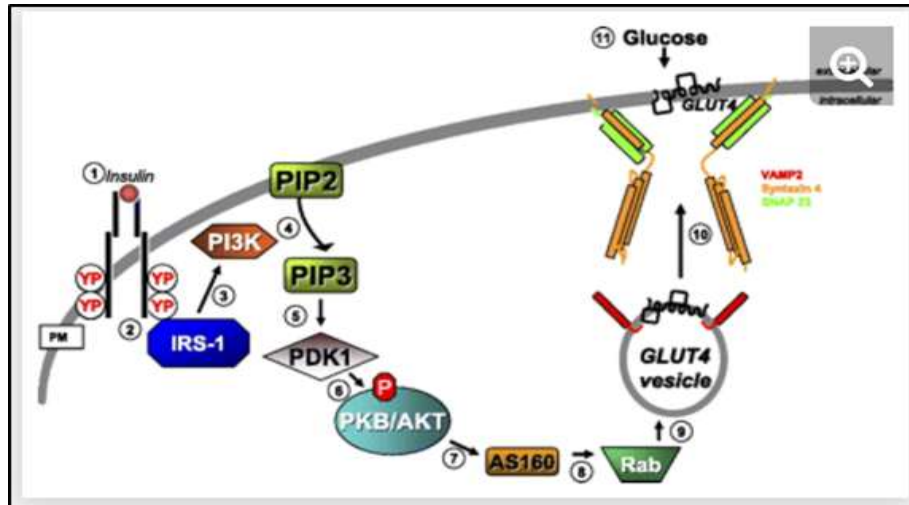


Figure 5. The role of protein kinase B (PKB) and GLUT 4 in glucose uptake (Jewell 2010).

I.2.2 Insulin

Insulin is highly important for glucose homeostasis but is also involved in cellular growth, cellular differentiation, synthesis and degradation of protein, transcription of gene, turnover of mRNA, and lipid metabolism (Kahn 1985, Rosen 1987). Synthesis and secretion of insulin is regulated by both nutrient and non-nutrient secretagogues in response to environmental stimuli in coordination with other hormones. Nutrient secretagogue such as glucose induces the secretion of insulin from β -cell by increasing intracellular ATP and closing of K^+ -ATP channels. Generation of cyclic AMP and other cellular energy intermediates are also augmented, further enhancing insulin release. Non-nutrient secretagogues that induce insulin secretion may act via neural stimuli such as cholinergic and adrenergic pathways, or through peptide hormones and cationic amino acids. Insulin has also the mitogenic and growth effect, which is mostly mediated by a cascade of Akt and activation of the Ras/MAPK pathway.

I.2.2.1 Insulin signaling pathways

Insulin mediates its actions through binding to insulin receptors. Insulin activates the insulin receptor tyrosine kinase (IR), which phosphorylates and recruits different substrates such as IRS family proteins. The insulin receptor is composed of a heterotetramer located on the cell membrane consisting of 2 α and 2 β glycoprotein subunits linked by disulphide bonds. Insulin binds to the extracellular insulin receptor (IR) α -subunit, resulting in conformational change enabling ATP to bind to the intracellular component of the IR β -subunit which contains an intrinsic tyrosine kinase (Kasuga *et.al.*1982).

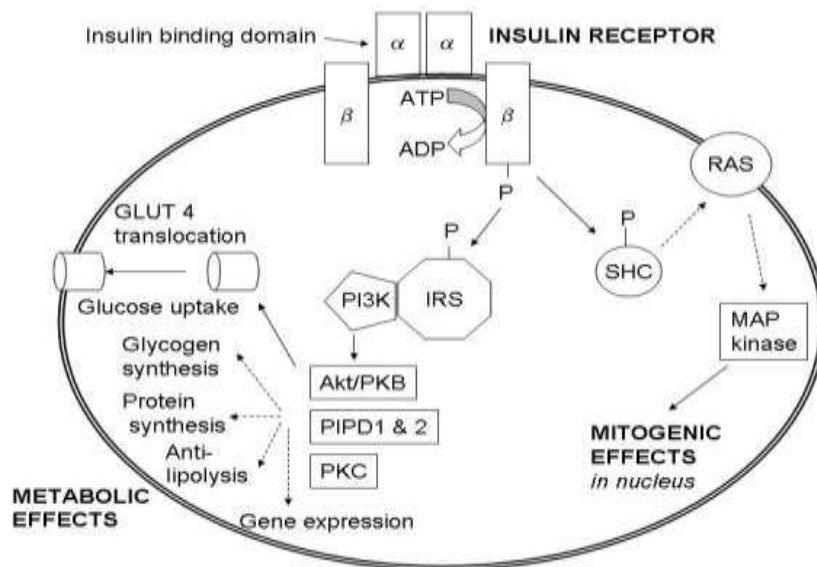


Figure 6. Insulin signaling pathway (Pessin 2000).

Subsequent steps in insulin signal transduction are mediated via the phosphorylation of specific intracellular proteins, including insulin receptor substrate-1 (IRS-1) (Sun et al. 1991). Tyrosine phosphorylated IRS-1 serves as a docking protein that binds to various signaling partners containing *src* homology-2 (SH2) domains such as phosphatidylinositol 3-kinase (PI3-kinase/PI3K) and phosphotyrosine phosphatase SHPTP2 (or Syp), and other SH2-

containing proteins that lack enzymatic activity but which connect IRS-1 and other intracellular signaling systems (White 1997). Among them, PI3K which has a major role in insulin function, mainly through activation of serine and threonine kinases such as Akt/protein kinase B (PKB), protein kinase C (PKC) and PI dependent proteine kinases 1 & 2 (PIPK 1-2). Akt/PKB activation induces glycogen synthesis through inhibition of GSK-3; protein synthesis through mTOR and downstream of the element; and cell survival through inhibition of several pro-apoptotic agents (Figure 6).

In addition, insulin signaling inhibits gluconeogenesis in the liver, through binding CREB/CBP/Torc2 disorders. Insulin signaling also promotes the synthesis of fatty acids through activation of SREBP-1C, USF1, and LXR. A negative feedback signal derived from the Akt/PKB, PKC ζ , p70 S6K, and MAPK cascades resulting in serine phosphorylation and inactivation of IRS signaling.

Exercise stimulates glucose uptake by an insulin-dependent pathway involving AMPK that plays a crucial role in arranging a glucose uptake during exercise and may be involved in improving insulin sensitivity as the effects of both acute and chronic exercise (O'Neill *et.al.* 2011). Moreover, AMPK protein expression and activity are increased by chronic exercise training (Lee-Yang 2009). The increasing of insulin sensitivity in muscle involves both insulin signaling downstream of Akt and GSK-3 and stimulation process directly involved in the activation of glycogen synthase and glucose transporter translocation (Huang 2007). Insulin (nutrient supply) stimulates GLUT 4 translocation through the PI 3-kinase pathway, while exercise (muscle contraction) stimulates it through both elevated AMP/ATP ratios and intracellular [Ca²⁺] leads to activation of protein kinases (Akt, aPKC λ/ζ , AMPK, CaMKII cPKC) that phosphorylate putative effectors (Figure 7) (Huang 2007).

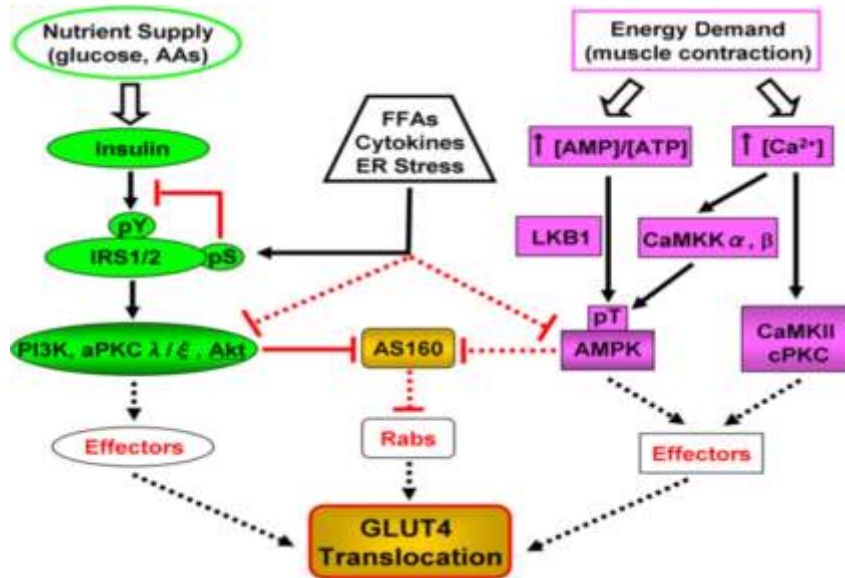


Figure 7. Signaling pathways stimulated by insulin and exercise leading to GLUT4 translocation (Huang 2007).

I.2.2.2 Insulin Secretion

Insulin secretion may be influenced by alterations in synthesis at the level of gene transcription, translation, and post-translational modification in the Golgi as well as by factors influencing insulin release from secretory granules. Longer-term modification may occur via influences on β -cell mass and differentiation.

Increased levels of glucose induce the “first phase” of glucose-mediated insulin secretion by releasing insulin from secretory granules in the β -cell. The entry of glucose into the pancreatic β -cell freely through GLUT 2 transporter increases ATP production as there is no possibility of glucose storage (glycogen synthesis) in β -cell. This results in the closure of K^+ -ATP-dependent channels leading to membrane depolarization and activation of voltage dependent calcium channels, which in turn leads to an increase in intracellular calcium concentration triggering pulsatile insulin secretion. Other mediators of insulin release include activation of

phospholipases and protein kinase C (e.g. by acetylcholine) and stimulation of adenylyl cyclase activity and activation of β -cell protein kinase A, which potentiates insulin secretion. This latter mechanism can be activated by hormones such as vasoactive intestinal peptide (VIP), PACAP, GLP-1, and GIP. These factors appear to play a significant role in the second phase of glucose mediated insulin secretion, after refilling of secretory granules translocate from reserve pools (Wilcox 2005).

I.2.2.3 The role of mitochondria in glucose homeostasis and insulin resistance

Insulin regulates homeostasis of nutrient and metabolic via the IRS–PI3K–AKT (PKB) signaling cascade that targets FOXO1 and mTOR (Cheng Z 2010). Mitochondria play the main role in metabolism, and metabolic diseases indicate that mitochondrial malfunction can cause insulin resistance. However, the proper molecular mechanism between insulin resistance and dysfunction of mitochondria remains unclear. In addition, mitochondria produce a moderate amount of ROS and improve sensitivity of insulin through the regulation of redox reactions at the protein tyrosine phosphatase and insulin receptors. However, chronic exposure to excessive level of ROS could induce the alteration of mitochondrial function leading to insulin resistance. Moreover, mitochondria play a role in cellular energy metabolism, including beta cells, regulation of insulin secretion and tissue sensitivity to the hormone insulin. Therefore, the mitochondrial dysfunctions induced by an increase in ROS production due to excess dietary factors seem to play an important role in insulin resistance.

Factors that affect mitochondrial function including oxidative stress, genetic factor, aging, and mitochondrial biogenesis may affect mitochondrial function leading to insulin resistance and a variety of pathological conditions. Excessive intake of nutrients (high calory or high fat diet)

increases ROS production, decreases mitochondrial biogenesis and causes mitochondrial dysfunction. Mitochondrial dysfunction will lower β -oxidation and ATP production, and increases the production of ROS.

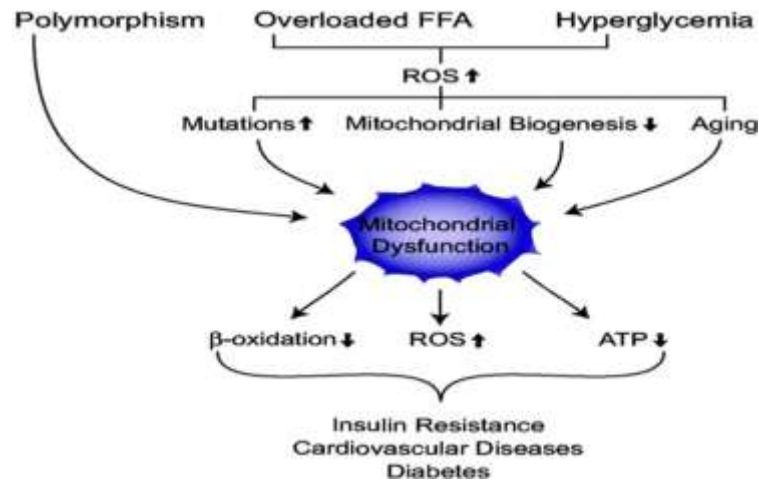


Figure 8. The mechanism of mitochondrial dysfunction (Kim *et.al.* 2008)

Decreased ability of oxidation would reduce the expression of mitochondrial proteins encoded by the mitochondrial genome (cytochrome c oxidase 1) and nuclear (succinate dehydrogenase and pyruvate dehydrogenase). The mitochondrial number and genes expression involved in biogenesis of mitochondria are significantly reduced in adipocytes from patients with type-2 diabetes or obesity. Mitochondrial gene mutation caused by aging or the conditions of stress cells may be one of the mechanisms underlying insulin resistance. PGC-1 α is a major regulator of mitochondrial biogenesis. However, its activity is not constant and can change in response to different metabolic conditions. Two metabolic sensors, AMPK and SIRT1 affect the activity of PGC-1 α through phosphorylation and deacetylation. The coordination between AMPK, SIRT1, and PGC1 α play an important role in the regulation of metabolic homeostasis. PGC-1 α is also known as a transcriptional regulator of uncoupling protein (UCP) that play a

role in thermogenesis in fat tissue. PGC-1 α is primarily expressed in high energy oxidative capacity tissues, such as skeletal muscle, heart, liver, brown adipose tissue and brain. Moreover, its activity is strongly induced in conditions that require energy, such as exercise, fasting and cold (Cantó 2013).

PGC-1 α is also a coactivator of nuclear transcription factors such as NRF-1, PPAR γ , and PPAR α . NRF-1 regulates the expression of many mitochondrial genes, including oxidative phosphorylation genes and transcription factor A (TFAM) of mitochondria (Patti 2003). TFAM is a direct regulator of replication and transcription of the mitochondrial DNA. PGC-1 α expression is reduced in insulin resistance and diabetes. Expression of NRF-1 also decreased in patients with DM (Patti 2003, Kim 2008). Thus, patients with insulin-resistance have fewer mitochondria in muscle that may be caused by a reduced expression of PGC-1 α and PGC-1 β (Wu 1999). Because PGC-1 expression is regulated by endothelial NO synthase (eNOS)/cGMP/PGC-1 axis, eNOS also plays an important role in mitochondrial biogenesis.

The evaluation of insulin resistance can be evaluated by simple and complex techniques. The important is their validity and reliability. Hyperinsulinaemic euglycaemic clamp is a highly sensitive technique for estimating insulin resistance, but it is a complex technique. However, there is a simple method assessed and validated, such as homeostasis model assessment (HOMA) (Borona 2000, Borai 2011).

I.2.3 Pancreas

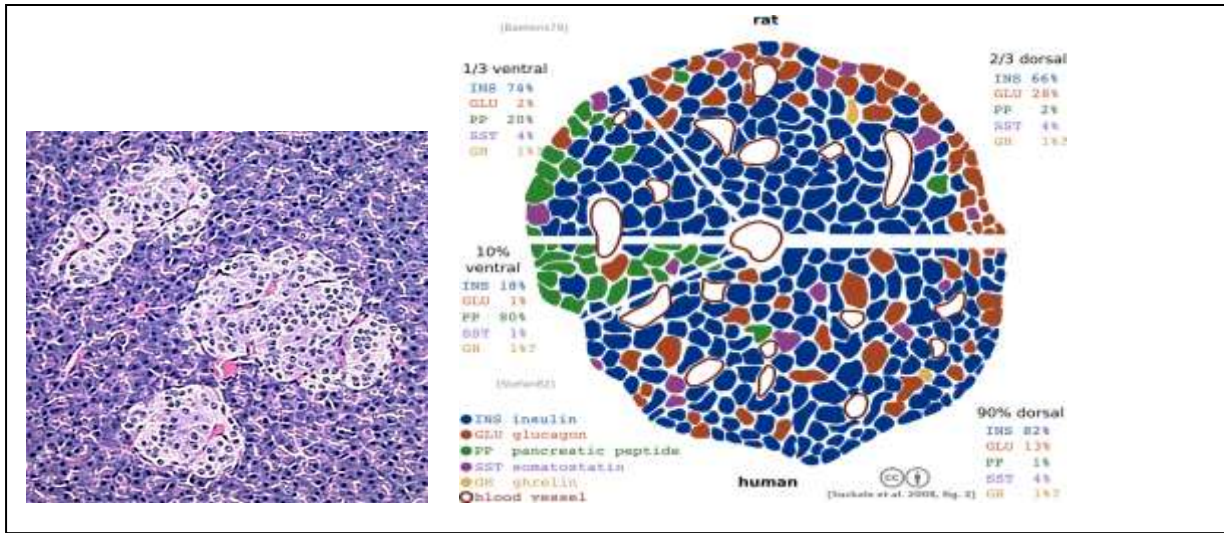


Figure 9. The differences structure between human and rat pancreas (islets of Langerhans)

Endocrine microorgans called islets of Langerhans occupy 1–4% of total pancreas volume (TPV), while exocrine and mesenchyme amounts to 96–99% of TPV (Dolenšek *et.al.*2015). Each lobe of pancreas consists of several lobules which their sizes are proportional to the size of the organism, but the islets of Langerhans are of fairly comparable size in humans and mice. In humans, the diameter of lobules are 1–10 mm, while they are 0,5-1,5 mm in mice (In't Veld 2010). Murakami *et.al.*1992 reported that islet of rat received streams of afferent vessels on the surface and issued through efferent veins on the inside, but there is no consistent rules concerning microcirculatory patterns in human islet. Cell composition and location of the islets of Langerhans within the pancreas are markedly different in the 2 species. The dstrubution of endocrine cells in humans are diffuse, while mantle-core pattern in mice (Dolenšek *et.al.*2015).

There are several types of polypeptide-hormone-secreting endocrine cells in the island of Langerhans, where most are beta cells that synthesize and secrete insulin. In human, Beta cells make up 50–70% of the total number of cells in islets; Alpha cells that secrete glucagon, contributing 20–40% in islets. Whereas in mice, 60–80% of cells are Beta cells and 10–20% of the total number of cells in islets are Alpha cells. Thus, the ratio between beta and alpha cells is higher in mice than in humans. The others types of endocrine cells are Delta cells, PP cells, and epsilon cells releasing somatostatin, pancreatic polypeptide, and ghrelin, respectively.

I.2.3.1 Programming of pancreatic β -cell

There is a critical period in the differentiation and maturation of tissues and cells that is involved in organogenesis during pregnancy and postnatal early life. Some studies on animal models revealed that stress in early life environment also triggers an adaptive response in growth of pancreatic β -cell in the fetus.

During the end phase of fetal life in the uterus and the early postnatal life, beta-cell mass is determined by the recruitment of undifferentiated precursors, as well as the level of replication and beta cell apoptosis. Obviously, disturbing in the intrauterine environment can affect the endocrine cells development and contribute to the growth of beta cells, corresponding to an adaptive metabolic response in the short term. However, this adaptive response may in turn be detrimental if maintained in the long term, because it can encourage beta-cell failure and diabetes in later life.

Additionally, Portha 2005 reported that both maternal undernutrition (i.e., low protein/low calorie diet) and overnutrition (i.e., high fat/high calorie/high fructose diet) stimulate an adaptive response of pancreatic β -cell in the fetus, impairing insulin secretion. Low-energy and low protein diet of pregnant women reduces β -cell mass development through different mechanisms. Low-calorie diet causes a reduction in β -cell neogenesis, while low protein diet reduces vascularization and proliferation without change- β cell differentiation (Portha 2005). Moreover, Cerf *et.al.*2005 showed that high fat diet during pregnancy significantly reduced the volume and the number of β -cells. The disturbance of β -cell growth causes the failure of β -cell function and impairs glucose tolerance, insulin secretion, and triggers the onset of insulin resistance leading to high blood glucose and overweight or obesity of offspring in adulthood (Wilcox *et.al.* 2005). This is supported by strong evidence suggesting that children, who are exposed to an unsuitable environment during the fetal life period associated with the nutritional status of maternal, are programmed for the development of a number of chronic diseases such as obesity and diabetes, which perpetuate the vicious circle of obesity and diabetes in an entire generation (Fernandez-Twinn *et.al.* 2006).

I.2.3.2 The role of mitochondria on pancreatic β -cell

In pancreatic beta-cells, mitochondria play a major role in coupling glucose metabolism through integrating and generating metabolic signals to stimulate insulin secretion (Maechler *et.al.*2010; Supale *et.al.*2012). This is supported by study of Soejima *et.al.*1996 which indicates that the removal of mitochondrial genes in β -cells impaired insulin secretion, whereas pancreatic β -cell function was recovered when β -cells were replenished with normal mitochondria.

Insulin secretion occurs in the pulsatile mode synchronous with influxes Ca^{2+} during both first and second phases. The rapid phase (first phase) and the longer phase (second phase) are highly dependent on glucose metabolism and mitochondrial oxidative capacity. Glucose oxidation by mitochondria will produce ATP and increase the ratio of ATP/ADP. The ratio of ATP/ADP is mainly regulated by mitochondrial function. The increased ratio of ATP/ADP contributes to the inhibition of potassium channels (K_{ATP}), which would cause membrane depolarization, opening of voltage-gated calcium channel, calcium influx, and insulin secretion (Kim *et.al.* 2008). Thus, disruptions in mitochondrial function induce β -cell dysfunction.

I.2.3.3 The correlation between decreased oxidative function of muscle mitochondria and insulin resistance

Over 80% of glucose disposal occurs in skeletal muscle. Therefore, insulin resistance in this tissue will greatly affect the whole body glucose homeostasis. In addition, multiple metabolic defects observed in insulin-resistant muscle will cause interference which includes: 1. Reduced insulin-stimulated glycogen synthesis; 2. Alteration in insulin signal transduction; 3. Increased lipid accumulation in muscle.

In humans, dysfunction of muscle mitochondria is associated with insulin resistance. Moreover, the oxidative activity of citrate synthase and acyl CoA dehydrogenase is strongly correlated with insulin sensitivity, even better than triglycerides or long-chain fatty acid acyl CoA. Simoneau and Kelley, 1997, who observed obese individuals, showed a decrease in citrate synthase, malate dehydrogenase, carnitine palmitoyltransferase 1 (CPT1) and cytochrome oxidase (COX) activity in fasting state, and in parallel an increase in activity of

glycolytic enzymes hexokinase and phosphofructokinase. Moreover, accumulation of lipids in skeletal muscle of sedentary people impaired insulin-stimulated glucose metabolism. A decrease in oxidative capability of tissues to adjust lipid oxidation to the lipid availability can cause the accumulation of fat tissue as triglycerides. This accumulation may induce lipotoxicity, which is reflected in the cellular accumulation of ceramides and diglycerides (Moro *et.al.* 2008). The lipid species can interfere with insulin signaling through different mechanisms. Both increased serine phosphorylation of insulin receptor and / or reduction of serine phosphorylation of PKB/Akt, which ultimately impair insulin sensitivity (Summers and Nelson, 2005). The decreased mitochondrial ability to regulate oxidative metabolism appropriately associated with decreased mitochondrial function are caused by: 1) abnormalities in mitochondrial density in vivo or 2) the intrinsic defects of lipid metabolism or other substrates. Ritov *et.al.* 2010 demonstrated that complex I activity of the respiratory chain, which is assessed through the rotenone sensitive NADH oxidoreductase activity, was reduced by 20% in biopsy samples of skeletal muscle of obese individuals and by 40% in type 2 DM. Boushel *et.a.l.*2013 also found a decrease in ADP and oxygen consumption stimulated by succinate (complex II) in the muscle fibers of obese people with type 2 DM.

I.3 THE EFFECT OF EXERCISE AROUND GESTATION ON OFFSPRING

According to the recommendation of the Centers for Disease Control and Prevention and the American College of Sports Medicine (CDC-ACSM), women with no obstetric complications in pregnancy may practice moderate physical exercise (60-90% of maximal heart rate or 50-85% of VO₂max) and light exercise (60-70% of maximal heart rate or 50-60% of VO₂max) for about 30 minutes or more a day, all days of the week for developing and maintaining

physical fitness. The CDC-ACSM also recognises that more intense exercise during pregnancy performed in 20–60 minute sessions on three to five days a week results in higher levels of physical fitness (Artal *et.al.* 2003).

Maternal exercise before and during pregnancy will be highlighted with respect to metabolic changes that can affect the early life environment. However, until now there are differences in the results of existing studies on the impact of exercise on pregnancy to pregnant women and their baby. Such differences are influenced by exercise intensity (mild 30-50% of VO_2max , moderate 65-75% of VO_2max , heavy 85-90% of VO_2max), time (the first/second/third of trimester), frequency, and duration during exercise. Moreover, there is substantial evidence suggesting that different patterns of exercise during pregnancy have diverse effects on the relationship between exercise and pregnancy outcome. A lower volume of regular exercise or a reduction in volume in the second half of pregnancy seems to stimulate fetal growth, leading to increased birth weight as compared with the offspring of control women who do not exercise during pregnancy. Increased birth size has been associated with balanced increase in fat and lean mass. In contrast, Clapp 1990 reported that maintaining a vigorous weight-bearing exercise program throughout pregnancy leads to reduced birth weight and neonatal fat mass (no change in percent lean mass). Moreover, Hopkins 2010 reported that regular moderate or vigorous exercise started in the second half of pregnancy consistently reduced the offspring birth weight without an alteration in fat percentage accompanied by decreasing levels of insulin-like growth factor I (IGF-I) and IGF-II hormones in offspring, suggesting that maternal exercise elicited adaptations in nutrient to the fetus, leading to reduced fetal growth stimulation by growth hormone.

In general, regular exercise during the early and mid-pregnancy stimulates the growth of the placenta, but if done at the end of pregnancy it can cause fetal growth retardation. Regular exercise with moderate intensity during pregnancy causes a decrease in oxygen delivery by 50% or more during exercise, but it can improve oxygen delivery at rest and when not exercising (Clapp 2006).

Radak *et.al.* 2013 gave an advice that training will be based on the theory of hormesis, which indicated that exercise had a beneficial effect when it done on a regular basis with moderate intensity. Additionally, exercise is usually accompanied by increased heart rate and changes in blood flow. Regular, moderate intensity exercise is advised for pregnant women (Practice *et.al.* 2002). Although in reality only a few women follow that advice (Petersen *et.al.* 2005). Altering maternal heart rate and blood flow affect fetal heart rate (Collings *et.al.* 1983) as well as oxygen and nutrients available to the growing fetus (Salvesen *et.al.* 2011).

Exercise will lead to the formation of ROS which in turn will induce the production of myokines in muscles that mediate the metabolic effects and increased the expression of genes involved in mitochondrial biogenesis. But, if it is performed regularly, exercise can reduce basal ROS production and oxidative damage, such as decreased susceptibility to lipid peroxidation, increase antioxidants and repair system against oxidative damage (e.g., increased SOD and catalase in the body). Others investigators reported an increase, decrease or no change in oxidative damage (Niess *et.al.* 2007).

The purpose of regular exercise is to avoid excessive increases in ROS, which has a double-edged sword effect. Excessive production will be harmful to organisms because they are responsible for the oxidative damage of membrane lipids and proteins and the level of DNA,

causing DNA mutations. Moreover, it has an effect on apoptosis and aging as well as oxidative damage associated with various diseases including metabolic syndrome. This condition was supported by the results of studies that showed the detrimental effects of free radicals in type 2 diabetes, because they play a role in insulin resistance.

In humans, maximal oxygen uptake is correlated with the expression of genes of oxidative phosphorylation. Decreased physical activity is associated with reduced oxidative phosphorylation genes in muscle. In contrast, oxidative phosphorylation gene expression increases with exercise training, a potent promoter of insulin sensitization.

I.3.1 The effects of exercise during gestation on glucose metabolism

Change in physical activity by regular exercise during pregnancy will influence the energy metabolism and pancreas function of mother and fetus. During exercise, skeletal muscle contraction stimulates glucose uptake and increases insulin sensitivity. In addition, exercise improves insulin activity and glucose tolerance in diabetic patient and animal models. Moreover, regular exercise prior to and during pregnancy attenuates weight gain during pregnancy (Kramer 2011), reduces by 50% the risk of glucose intolerance in pregnancy (Kramer 2011, Oken 2006,), increases insulin secretion and prevents gestational diabetes of mother (Clapp 2006, Leandro 2012). This condition is important for breaking the vicious circle between GDM and risk of obesity and type-2 diabetes mellitus of offspring.

Exercise and muscle contraction overcome insulin resistance by increasing the enzyme activity of adenosine 5-monophosphate kinase (AMPK) that enhanced the cellular glucose absorption. This occurred because AMPK mediates the translocation of glucose receptor

GLUT4 of skeletal muscle toward the plasma membrane. This is supported by the result study of Wisloff 2001 which showed an improvement of insulin action of rats trained by treadmill for one hour/day with a speed of 41.6 cm/sec, the slope of 10%, 75% VO₂ max.

Another study performed by Oken E *et.al.* 2006 reported that the strong activity (7-13 hours per week) prior to pregnancy and mild to moderate exercise (3-6 hours/week) during pregnancy may reduce weight gain during pregnancy, the risk of impaired glucose tolerance in pregnancy and gestational diabetes. In addition, James F Clapp III (2010) reported that regular exercise during pregnancy may reduce markers of insulin resistance and may decrease blood glucose levels during and immediately after exercise. In contrast, Hopkins *et.al.*2012 reported that 15 weeks of moderate-intensity cycling exercise that began mid-pregnancy had no impact on insulin sensitivity in late pregnancy. Reinforced by Stafne *et.al.*2012 and Ramirez-Velez 2012 who reported that there was no difference in the prevalence of insulin resistance in the group during the 12-week of training that begin in mid-pregnancy with a frequency of 3 days or more/week compared with control group.

However, study about the effect of maternal exercise during pregnancy and its underlying mechanism on offspring is minimal. This thesis will explore the influences of regular exercise before and during pregnancy on oxidative status of fetus by measuring oxidative stress marker of newborn plasma. The present study will also evaluate the effect of exercise on mitochondrial function of offspring by measuring O₂ consumption, ROS production, antioxidant capacity and activity of respiratory complex I, II, III, IV. Moreover, the effects of exercise before and during pregnancy on pancreatic Islet β -cell function will be studied by evaluating glucose tolerance, insulin sensitivity, insulin secretion, insulin signaling pathway (PKB phosphorylation), and structure of β -cell pancreas.

I.3.2. The effects of exercise during gestation on oxidative stress of offspring

Pregnancy is a physiological state associated with enhanced oxidative stress related to high metabolic turnover and elevated tissue oxygen requirements. Intrauterine stress induces increased risk of adult disease through fetal programming mechanisms. During pregnancy, the mitochondrial activity of the placenta produces reactive oxygen species (ROS), mainly superoxide anion. On the contrary, all the major antioxidative defense systems, including SOD, catalase, GPx, glutathione, vitamin C and E, are found in the placenta and may prevent lipid peroxidation in normal pregnancies (Myatt 2004). Excessive production of ROS may occur at certain windows in placental development and in pathologic pregnancies, such as those complicated by pre-eclampsia and/or IUGR, overpowering antioxidant defenses with deleterious outcomes (Myatt 2004).

ROS generated by a variety of intrauterine conditions may be one of the key downstream mediators that initiates epigenesis and programming of the offspring. ROS generation normally is balanced by the cell's antioxidant defense mechanisms, which maintains its redox state and is important in physiological regulation in both the embryo and fetus (Trachootham 2008).

In the first trimester, establishment of blood flow into the intervillous space is associated with a burst of oxidative stress (Myatt 2004). Levels of peroxidation markers, such as lipid hydroperoxide and malondialdehyde (MDA), are higher in pregnant than in non-pregnant women. Experimental investigations have well demonstrated the role of redox balance in modulating gene expression, and recent studies indicate that both the insulin functional axis and blood pressure could be sensitive targets to oxidative stress programming (Luo 2006).

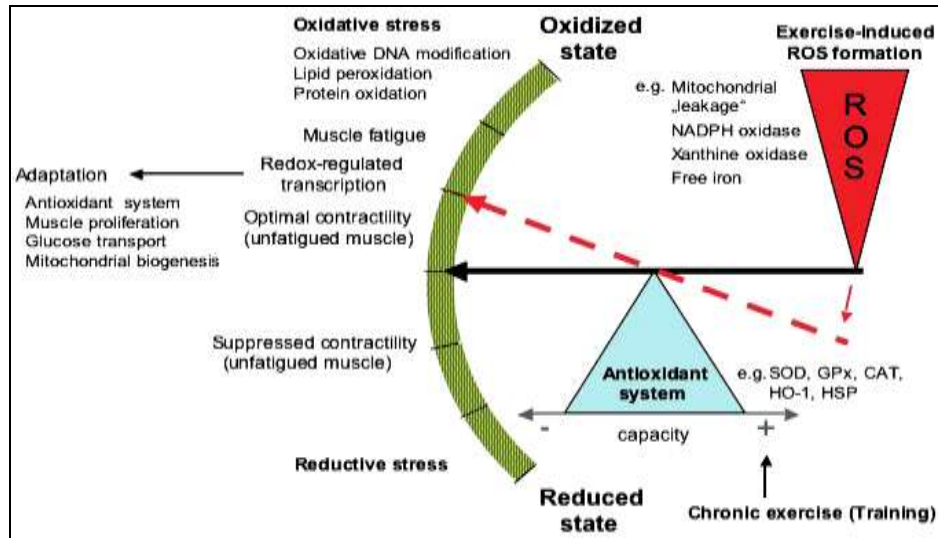


Figure 10. The consequences of changes in the redox balance in skeletal muscle (Niess 2007).

Exercise increases ROS generation and creates a pro-oxidant environment. This acidic environment during exercise induces oxygen release from hemoglobin and increases PO_2 in tissues, as well as the release of iron from transferrin. When exercise is repeated regularly, the body quickly adjusts so that oxidative stress is eliminated or reduced. The body's adaptations to a regular exercise seem to have an antioxidant effect (Neiss 2007) (Fig.10). In addition, exercise activates AMPK may through pathways: (1) phosphorylation of PGC-1 α leads to increase SIRT3 protein expression that modulate mitochondrial biogenesis; (2) enhances the ability of skeletal muscle to better handle ROS via deacetylation and activation of MnSOD, as a response to exercise training (Brandauer *et.al.* 2015).

In humans, regular exercise effects have been identified with an enhanced activity of antioxidative enzymes. Adaptation response to regular exercise stimulates women's bodies more resistant against production of pro-oxidants by increasing the number of mitochondria and decreasing the susceptibility to lipid peroxidation. Evidence suggests that women who are physically active are more resistant to the development of preeclampsia (Yeo 2008).

Naziroğlu 2004 have reported that moderate exercise training in pregnant rat improved vitamins A and E, increased MDA, glucose levels and white blood cell counts that decreased during pregnancy. A similar study in pregnant women, Wagey *et.al.*2011 demonstrated that exercise was performed during pregnancy from 20 weeks of gestation (30 minutes, twice a week) showed significantly higher decrease of (MDA) among treatment compared with control group without exercise. In addition, there were significantly higher increase of superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT) levels of exercise group compared with non-exercise group. Furthermore, Ramirez 2012 showed that a 12-weeks of aerobic exercise increased antioxidant status in nulliparous women.

I.4 THE EFFECT OF NUTRITION AROUND GESTATION ON OFFSPRING

The modification of nutrition and supplementation during gestation can influence oxidative balance during fetal life and function of mitochondrial as well as glucose metabolism of offspring (Gluckman *et.al.* 2005, 2008). The nutritional status during critical periods of early life is an important determinant for proper development of the organism and maturation of metabolic and endocrine systems. An aberration in the quality and/or quantity of maternal nutrition resulting in an altered intrauterine environment has been shown to be responsible for adverse consequences in the offspring. Fetal development in the hyperinsulinemic/obese HF maternal diet intrauterine environment results in fetal hyperinsulinemia without significant changes in their plasma glucose levels (Srinivasan 2006). Because insulin is a potent modulator of the central nervous system development, elevated concentrations during critical

periods of development can lead to malprogramming of central regulators of body weight and metabolism (Symonds *et.al.* 2007, Reusens *et.al.* 2006).

I.4.1 The effect of nutrition around gestation on glucose metabolism

I.4.1.1 High fat diet

Srinivasan *et.al.*2003 showed that prolonged consumption of a high fat diet (HFD) by maternal rats results in an adverse maternal intrauterine environment, predisposing the fetuses to metabolic malprogramming. These early fetal maladaptations eventually predispose them in their adult life to the metabolic syndrome-like phenotype (increased body adiposity, chronic hyperinsulinemia, glucose intolerance, and hyperlipidemia). Pancreatic adaptations, including increase in pancreatic insulin content and the amplified insulin secretory response of the HF fetal islets to various secretogogues, were most likely contributing factors for the observed fetal hyperinsulinemia (Srinivasan 2006). Other studies by Winzell *et.al.*2004, Woods *et.al.*2003 have demonstrated that a high-fat diet (HFD) to normal rats results in increased body weight, hyperinsulinemia, and insulin resistance in their adulthood.

In addition, maternal diet high in calories and high fat diet (HFD) cause similar long-term programming to the risk of offspring obesity (Catalano 2003). The HFD increased transplacental transport of glucose (5-fold) and neutral amino acids (10-fold) *in vivo*. In microvillous plasma membranes (MVMs) isolated from placentas of HFD animals, protein expression of GLUT1 was increased 5-fold, and protein expression of sodium-coupled neutral amino acid transporter (SNAT) 2 was elevated 9-fold. On the contrary, MVM protein expression of GLUT 3 or SNAT4 was unaltered. These data indicate that up-regulation of

specific isoforms of placental nutrient transporter is the mechanism that links maternal high-fat diet and obesity to fetal overgrowth (Jones *et.al.*2009).

Srinivasan *et.al.*2006 also demonstrated that on gestational day 21, plasma insulin levels and the insulin secretory response of islets to various secretagogues were significantly increased in high fat (HF) fetuses. The HF male offspring weaned onto low calory (LC) diet (HF/LC) demonstrated increases in body weight from postnatal day 60 onward. In adulthood, HF/LC male rats were significantly heavier than controls, had increased plasma levels of insulin, glucose, free fatty acids, and triglycerides, and demonstrated glucose intolerance. HF/LC male islets secreted higher amounts of insulin in response to low glucose concentrations, but their response to a high glucose concentration was similar to that of LC/LC islets. In another set of experiments, when the male offspring of HF maternal rats were weaned onto a high-sucrose diet (HF/HSu), their metabolic profile was further worsened. These results indicate that chronic consumption of a HF diet by mother rats malprograms the male offspring for glucose intolerance and development of increased body weight in adulthood (Srinivasan *et.al.* 2006).

Kozak *et.al.* 2000, 2005 also demonstrated that a HF diet during gestation and lactation affected body weight regulation in the adult offspring via alterations in the functioning of neuropeptide Y. Exposure of fetal brain to excess insulin during development in the hyperinsulinemic HF maternal environment may result in an abnormal development of the energy homeostasis, predisposing to increased body weight gain in adulthood. The observed fetal hyperinsulinism in that study could be a contributing factor for the phenotype of the adult offspring of the HF female rats. Moreover, Cerf *et.al.*2005 demonstrated that feeding a HF diet to female rats throughout gestation resulted in significant decreases in β -cell volume and number and converse changes in α -cells, resulting in hyperglycemia in 1-day-old

newborn rat pups without changes in serum insulin concentrations. Furthermore, Taylor PD *et.al.* 2005 reported that a HF maternal diet during prenatal until suckling induced abnormal glucose homeostasis, reduced whole body insulin sensitivity, impaired-cell insulin secretion and changed the structure of pancreas (insulin secretory granule morphology) of offspring, which is preceded by reduced tissue mtDNA content and altered mitochondrial gene expression.

4.1.2 Low protein diet

Islets from fetuses of maternal fed a low-protein diet demonstrated a reduction of cell proliferation, islet size, islet vascularization, and an impairment of insulin secretory capacity (Dahri S 1991, 12, 36 from Sinivasan). Zambrano *et.al.* 2006 indicated that male but not female offspring of maternal with protein restriction during pregnancy showed evidence of insulin resistance, higher cholesterol, and also higher triglycerides (TG) compared with normal diet. Furthermore, Villar-Martini *et.al.* 2009 demonstrated that at day 10 (end of the nephrogenesis period), the number of renal corpuscles and mature corpuscles was lower in low protein offspring than in normal protein offspring of the same sex. In adulthood, low protein offspring had higher blood pressure, and showed thicker glomerular basement membrane (Villar-Martini).

4.1.3 High fructose diet

Fructose is a natural sugar and predominant monosaccharide found in fruit, honey, and some vegetables (Henry *et.al.* 1991). In the mid-19th century, fructose was used commercially and

widely as a sweetening substitute (fructose corn syrup) (Elliott *et.al.*2002, Tappy *et.al.*2010). Diet rich in fructose is associated with various adverse health consequences such as obesity and metabolic syndrome that risen dramatically (Johnson *et.al.* 2007).

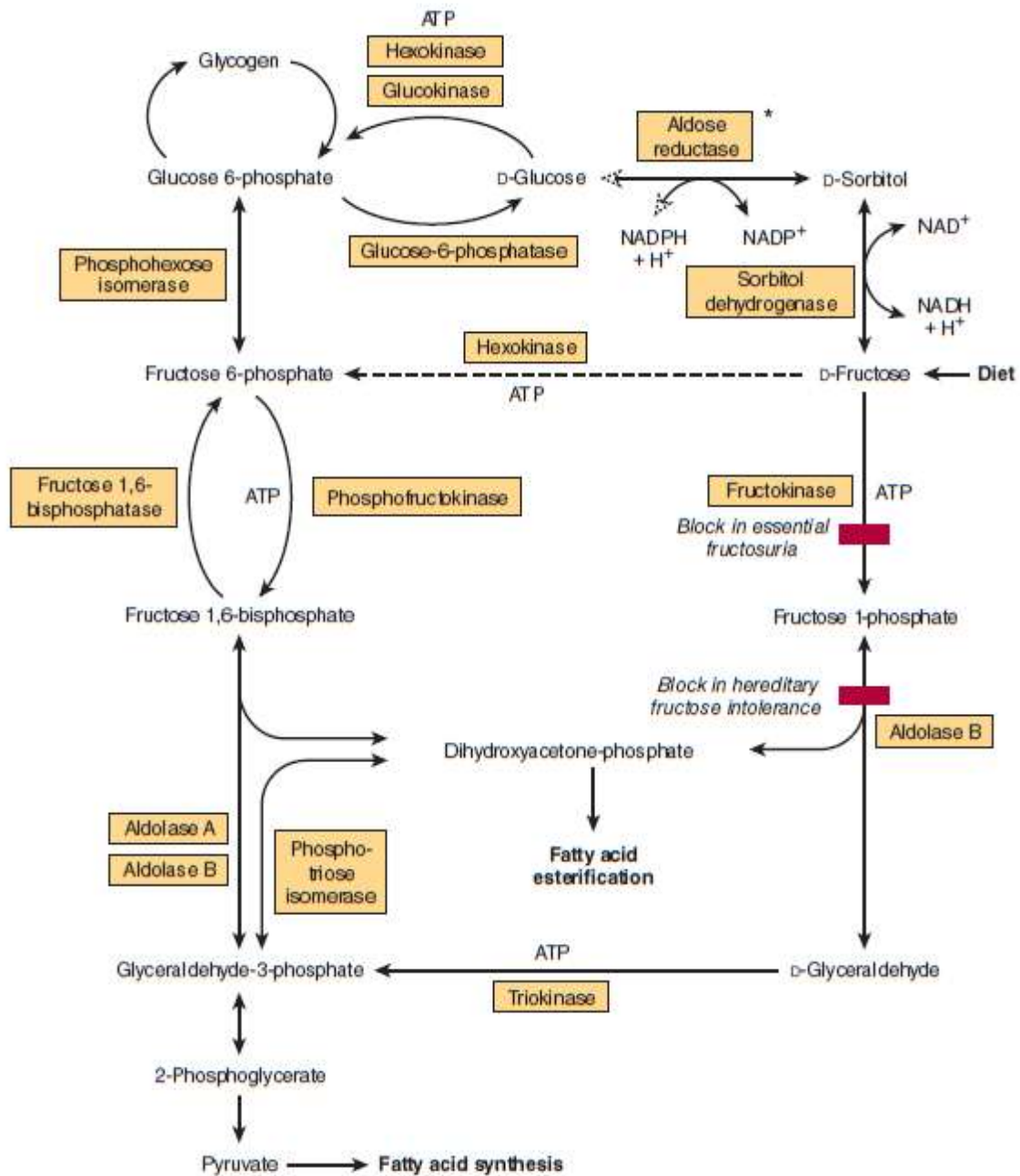


Figure 11. Metabolism of fructose (Harper)

It has been generally accepted that the absorption of fructose in humans intestinal occurs via facilitated diffusion alone, through the members of glucose transporter family, GLUT5 and GLUT2 (Riby JE *et.al.*1993). Metabolism of fructose is different to glucose pathway and undergoes a faster glycolysis in liver than does glucose, because it bypasses the regulatory step catalyzed by phosphofructokinase. Therefore, high fructose diet causes fructose to flood the pathway in liver increasing fatty acids and triacylglycerol synthesis, VLDL secretion, serum triglycerides, and finally LDL cholesterol level (Fig. 8). Some of fructose is converted to glucose. Sun *et.al.*2012 reported the mean of conversion rate from fructose to glucose in 3–6 hours after ingestion was $41\% \pm 10.5$.

Moreover, fructose stimulate the release of gastric leptin, which in turn leads to increased insertion of GLUT 2/GLUT 5 transporters into small intestine via apical membrane which increases fructose transport across the intestinal wall (Sakar 2009). It appears that excessive fructose consumption for long periods of time have potentially negative effect on metabolic function. Blakely *et.al.*1981, Tuovinen *et.al.*1975 reported that fructose diet altered several enzymes activity and metabolism of hepatic carbohydrate leading to hepatic insulin resistance, although the exact molecular mechanisms involved are unknown.

It is highly important; fructose consumption does not induce pancreatic insulin secretion due to an absence of GLUT5 transporters in the pancreas and it cannot stimulate gastric inhibitory peptide, which stimulates insulin secretion (Bray *et.al.* 2004). Bezera *et.al.* 2000 reported that a high fructose diet did not decrease the level of insulin receptor IR and the IRS-1 in the liver and muscle, but reduced tyrosine-phosphorylation of the insulin receptor after insulin stimulation (IRS-1 association with PI 3-kinase and IRS-1 association with SHP2) in the liver

of rats. These data indicate that fructose rich diet induced insulin resistance via the alterations in the early steps of insulin signal transduction.

On the other hand, literature review shows different results on glucose homeostasis by administration of high fructose diet (HFD). Some studies state that changes occurred (Kelley 2004, Girard 2006, Sharabi 2007), but others do not confirm (Jürgens *et.al.* 2005, Roglans *et.al.* 2007, Sanchez-Lozada *et.al.* 2007). Agrawal *et.al.* 2012 also demonstrated that high-fructose and low omega-3 fatty acid consumption induced a metabolic syndrome like state in the brain as well as impaired cognitive function, suggesting an interaction between consumption of fructose and the function of brain. In addition, a diet rich in fructose has been related to symptoms of metabolic syndrome, including hyperglycemia, dyslipidemia, and insulin resistance. However, few studies have examined the effect of high fructose diet during pregnancy on glucose metabolism and oxidative status of offspring.

In animal model, Douard 2008 reported that under normal conditions, in the prenatal and suckling period of rat development, intestinal GLUT5 (the fructose transporter at the apical cell membrane) mRNA levels and fructose transport rates are very low. Therefore, the administration of large amounts of fructose during fetal/neonatal life resulting in intolerance to the fructose, which may impairs body weight gain.

Ghezzi *et.al.* 2011 demonstrated that the glucose tolerance of the animals was not altered by the excessive intake of fructose (60%) in animal study. Ghezzi also reported no significant difference in glycogen synthesis, glucose uptake and glucose oxidation rates by the isolated soleus muscle between rats fed the HFD and those fed the control. However, insulin sensitivity decreased in the group fed the fructose-rich diet and maintained in proper litters

(8 pups/litter) during lactation. On the other hand, Ghezzi 2011 found that the fructose rich diet (60%) during pregnancy led to dyslipidemia and increased serum concentrations of total cholesterol and triglycerides. This study was supported by the results of research by Bezerra 2001 that found insulin resistance, but contrast with the study by Moura that showed that administration of HFD after weaning induced intolerance to glucose without changing insulin sensitivity. In addition, Rodríguez *et.al.*2013 reported that fructose administration (10% wt/vol) in drinking water throughout gestation resulted in hypertriglyceridemia of mothers, whereas fetuses showed a hypotriglyceridemia and higher hepatic triglyceride content than those from control fed mothers. Moreover, genes expression of lipogenesis was higher but genes expression of fatty acid catabolism was lower in fetuses from fructose-fed mothers.

In another study, Mortensen *et.al.*2014 showed that maternal fructose exposure did not affect the birth weight, litter size, and body fat percentage, but significantly lowered body weight of the offspring throughout life after weaning, Moreover, the isolation of mitochondrial brain showed a significantly increased of state 3 respiration (8%) with the combinations substrate of malate/pyruvate, malate/pyruvate/succinate, and malate/pyruvate/succinate/rotenone, as well as a significant decrease in P/O₂ ratio, compared to the control.

I.4.2 The effect of nutrition around gestation on oxidative stress

I.4.2.1. Iron supplementation

Iron is a major component of oxygen transporters in the body. Iron supplementation is usually recommended for pregnant women to fulfill the iron needs of both mother and fetus at dose 5-25 mg iron/100 g (Lin 1976). Although iron is an essential mineral, it can also be toxic in

certain conditions. As a redox-active transitional metal, iron is a strong prooxidant potentially hazardous when present in excess amounts. Through Fenton reaction, iron can produce reactive oxygen or nitrogen species and catalyzes several cellular reactions that result in the formation of hydroxyl radicals and induces oxidative stress (Andrews 1999, Rajpathak 2009). Moreover, iron supplementation can reduce the risk of preterm birth or low birth weight, but excessive iron intake may increase ROS production during pregnancy, which may cause pancreatic β -cell dysfunction (Goldstein 2005). Insulin resistance and gestational diabetes mellitus (GDM) have been associated with high plasma ferritin and biological evidence of oxidative stress (Casanueva 2003).

During pregnancy, a physiological increase of oxidative stress and defense mechanisms has been observed. In a large prospective cohort study, Bowers *et.al.* 2011 identified a significant and positive association between prepregnancy dietary heme iron intake and GDM risk. In an epidemiological study, Lachili 2001 studied the effect of a daily combined iron supplementation (100mg as fumarate) and vitamin C (500mg) during the third trimester. They found an increased thiobarbituric acid reacting substances (TBARs) plasma level. Another study (Rehema 2004) conducted in a small group of borderline anemic pregnant women reported that daily iron prophylactic doses (36 mg as aspartate) for 4 weeks did not deleteriously change the physiological pattern of oxidative stress parameters. Moreover, Devrim 2006 reported an increase in the oxidant malondialdehyde (MDA) and a decrease in antioxidant parameter glutathione peroxidase (GSH-Px) level in blood plasma at delivery in 27 pregnant women who took daily 100mg of iron supplements during pregnancy. On the contrary, Chan *et.al.* 2007 demonstrated that iron supplement in the prevalent dose (60 mg/daily) from early pregnancy did not increase the risk of GDM.

Moreover, mediated oxidative stress may contribute to B-cell dysfunction and insulin resistance even in the absence of significant iron overload (Swaminathan 2007, Rajpathak 2009). On the other hand, the pancreatic β -cell is particularly susceptible to oxidative stress because of a weak antioxidant defense. Therefore, high body iron levels can damage pancreatic β -cell function and impair glucose metabolism (Buchanan 2007).

Further, Yakes 1997 reported that high concentrations of iron rapidly reduce the transcription of a large set of genes that encode mitochondrial ribosomal proteins as well as reduce oxidative phosphorylation (OXPHOS) and the total complement of electron transport proteins. The dysregulation of electron transport can lead to increased mitochondrial production of H_2O_2 . The combination of increased iron and physiological concentrations of H_2O_2 generated in the mitochondria during OXPHOS might result in the damage of DNA. In mammalian cells, mtDNA is much more sensitive than nuclear DNA (nDNA) to H_2O_2 induced damage, probably due to the availability of iron in the mitochondria. Moreover, human cells have a limited capacity for repairing H_2O_2 -induced mtDNA damage (Yakes 1997).

4.2.2. High fat diet

Maternal consumption of a high fat diet (HF) was associated with changes in vascular function and oxidative balance in the offspring through increased ROS generation, reduced Nitric Oxid bioavailability, and led to increased oxidative stress of offspring (Torrens 2012). At energy balance, HF increases the flux of fatty acids through skeletal muscle for oxidation. HF down regulated PGC1 α and PGC1 β mRNA, as well as genes encoding proteins in complexes I, II, III, and IV of the electron transport chain (Sparks 2005).

Furthermore, Taylor *et.al.*2005 reported other consequences of a maternal HF during prenatal until suckling periode including abnormal glucose homeostasis, attenuated insulin sensitivity, impaired cellular insulin secretion and alteration of the structure of pancreas (insulin secretory granule morphology) of offspring, which is preceded by reduced tissue mtDNA content and altered mitochondrial gene expression.

In addition, Zhang *et.al.*2011 reported that maternal HF significantly increased plasma TG and hepatic TBARS concentrations and the size of hepatic lipid droplets in offspring rats. The expression of antioxidant defense genes, such as glutathione peroxidase-1 (GPx-1) and Cu/Zn superoxide dismutase (Sod1) were significantly lower in the liver of rat offspring. Other study by White *et.al.*2009 suggests that HF increases oxidative and inflammatory signaling in brain. Moreover, Watkins *et.al.*2011 showed that male offspring from dams fed low protein diet displayed significantly decreased expression of the mitochondrial uncoupling protein 1 (*Ucp1*) gene compared to normal diet offspring. Another study, Mortensen *et.al.*2014 showed that maternal fructose exposure caused a significantly lower body weight of the offspring throughout life after weaning, while birth weight, litter size, and body fat percentage were unaffected. Moreover, isolated brain mitochondria displayed a significantly increased state 3 respiration of 8%, with the substrate combinations malate/pyruvate, malate/pyruvate/succinate, and malate/pyruvate/succinate/rotenone, as well as a significant decrease in the P/O₂ ratio, compared with the control.

I.5 GESTATIONAL DIABETES MELLITUS

Gestational diabetes mellitus (GDM) is related to increased insulin resistance and an increased level of oxidative stress (OS) caused by both excess free radicals and/or a defect in antioxidant defenses (Lappas *et.al.*2011, Zein *et.al.*2014, Gelaleti *et.al.*2015, Shang *et.al.*2015). According to American Diabetes Association 2004, GDM is one of the most common complications of pregnancy. It was approximately 7% of all pregnancies and up to 14% of pregnancies in high-risk populations. Therefore, it is important to determine modifiable factors that may increase the risk of GDM. High iron consumption could increase insulin resistance and OS, which could aggravate GDM risk. GDM can increase the risks on both mothers (e.g., hypertension in pregnancy, preeclampsia, cesarean and diabetes later in life) and the fetus (macrosomia, neonatal hypoglycemia, shoulder dystocia).

However, epidemiological evidence suggests that the risk of GDM is increased along with maternal age (Marcinkevagea 2011), it could also predict the occurrence of diseases later in life for the newborn (Leiva 2011). Despite a better diagnosis of GDM and the identification of the detrimental consequences for mothers and babies in many countries, there is still no consensus regarding the origin of GDM (Harlev *et.al.* 2010). This increases a great interest in understanding the etiology and patho-physiological mechanisms of GDM.

CHAPTER II

PERSONAL WORK

II.1 SCIENTIFIC OBJECTIVES

The objective of the present study was to examine the effect of moderate intensity endurance training before and during gestation on the redox state in the plasma and on liver mitochondria of newborns from trained mother compared to those from untrained mothers. Moreover, the effect of maternal exercise on pancreatic islet β -cells function of offspring were evaluated by measuring of glucose tolerance, insulin sensitivity, insulin secretion, insulin signaling pathway (PKB phosphorylation) and evaluating the alteration of structure of pancreatic β -cells.

Furthermore, in this study, two studies were also performed to examine the effects of fructose diet and iron-enriched fructose diet during pregnancy on both maternal and offspring. The first preliminary study was conducted to show that the fructose diet could be used as a model of gestational diabetes. The second study demonstrated that iron supplementation could be deleterious in case of gestational diabetes” by evaluating the effects of iron-enriched fructose diet during pregnancy on glucose metabolism and oxidative status in brain and liver of rat offspring.

II.2 MATERIALS AND METHODS

II.2.1 THE INFLUENCE OF EXERCISE AROUND GESTATION ON OXIDATIVE STRESS AND GLUCOSE METABOLISM IN OFFSPRING

II.2.1.1 Animals and Experimental design

All study protocols were reviewed and approved by the Institutional Ethic Committee for Animal Care (Protocol 00174.02 accepted in March 2014). The rats were maintained and handled in agreement with the Guidelines for the Care and Use of Laboratory Animals published by the European Union (2010/63/UE) and the French Ministry of Research (2013-118).

A. FISRT PART: THE INFLUENCE OF EXCERCISE AROUND GESTATION ON OXIDATIVE STRESS IN OFFSPRING

In the first part of the core study, an experimental study was performed in female Wistar rats to examine the effect of moderate intensity endurance training 4 weeks before mating until the 18th day of gestation on the redox state in the plasma and on liver mitochondria of offspring at weaning (21 days old) from trained mother compared to those from untrained mothers. Moreover, assessment of oxygen consumption, ROS production, complex respiratory enzyme activities of the electron transport chain, and citrate synthase activity in liver and muscle mitochondria of offspring. Besides, the concentration of cytochromes, α -Tocopherol and Quinone in liver mitochondria, as well as fatty acids composition in liver, plasma, and liver mitochondria of offspring were measured.

In the core study (study 1) twenty four adult nulliparous female Wistar rats (12 weeks-old), bred in the animal facility, were housed three per cage for the first part of protocol (4 weeks before mating) in a controlled environment: 25°C, 50 ± 10% relative humidity and 12h:12h light: dark, during all the experiment (Study 1). All the rats have had free access to water and food (rat chow for growth SAFE A03, AUGIS, France). Females were randomly assigned to one of two age- and weight-matched groups (exercise MTr and control MSed). Exercise training consisted of continuous running on a motor-driven rodent treadmill (Bio lab, France) (Figure 12) 5 times/wk and the intensity was gradually increased. Rats progressively ran from 15 min/day at 15 m/min, 0% slope, up to 60 min/day at 25 m/min, 10% slope, for the last 4 weeks. Sedentary females were housed in identical cages without exercise training protocol. Half of the animals from the MSed and the MTr groups were used for the first part of study 1 (the influence of exercise around gestation on oxidative stress in offspring) and the remaining were used only to produce pups for the second part of study 1 (the influence of exercise around gestation on anthropometry and glucose metabolism) and were not included in the results on mothers.

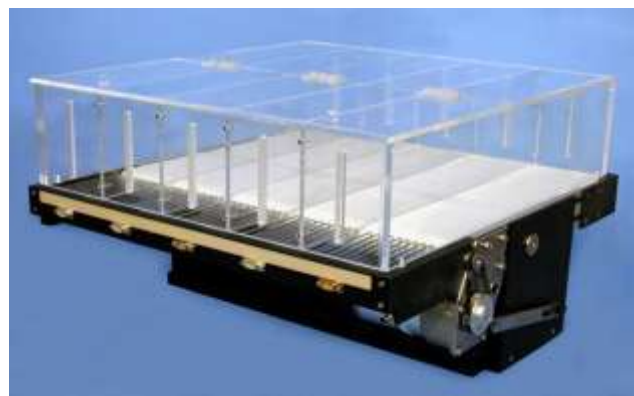


Figure 12. Motor-driven rodent treadmill (Bioseb, France)

In order to verify the efficacy of the exercise training protocol, we have done a pilot study on twelve females with the same moderate intensity of training 4 weeks before gestation until the 18th day of gestation. In order to measure citrate synthase (CS) activity, in this pilot study, mothers were sacrificed just after delivery. The morning of sacrifice, the mothers were weighted and decapitated without anesthesia. Retroperitoneal, urogenital and mesenteric adipose tissues were collected and weighed. Liver and plantaris muscle of the right leg were dissected out. Parts were frozen in liquid nitrogen and stored at -80°C. The activity of CS enzyme is recognized to be a good index of mitochondrial genesis and a good marker of muscle adaptation to exercise training. (Holloszy JO 1984).

Food intake and body weight were recorded once a week. For mating, two male rats were placed in contact with three females for one week. Pregnancy was diagnosed with examination of vaginal smears for the presence of spermatozoa (Figure 13). Pregnant female were single-housed.

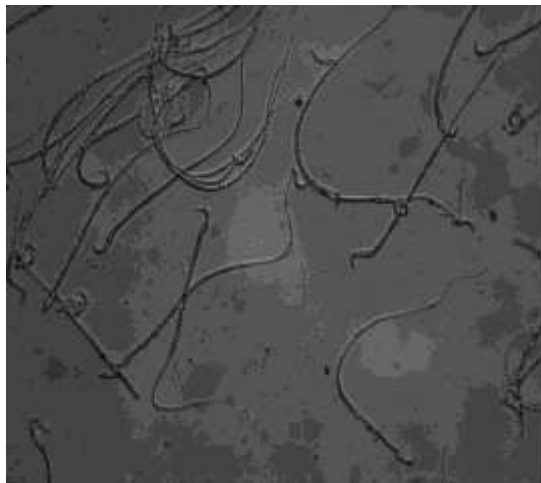


Figure 13. Spermatozoa on vaginal smears

On postnatal day 1, litter sizes were equalized to 8 pups. Pups were cross-fostered from other litters from the same group and the same age to maximize the number of males per litter. Offspring body weights were recorded at day 1, 7, 14, and 21. Although this study examined only babies at the weaning age (21 days old), but we want to continue in another study to examine in adult offspring. So that, only male babies were used as subject, because we want to minimize confounding factor such as hormonal factor in adult female offspring that may influence the result. To verify that no difference in mitochondrial function between both female and male babies, we have performed preliminary study showed that no difference in oxygen consumption and ROS production between male and female rat offspring.

Blood and tissue sampling

At weaning, 9 male babies from each group were weighted and decapitated without anesthesia. Blood samples were collected and centrifuged to separate plasma and packed red blood cells before storage at -80°C until further analysis. Retroperitoneal, epididymal and mesenteric adipose tissues were collected and weighed. Liver and muscles of both hind legs were dissected out. Parts were frozen in liquid nitrogen and stored at -80°C . Remaining parts were used to prepare isolated mitochondria as described below.

Mothers were also sacrificed after babies in the same conditions. Six females from each group were weighted and decapitated without anesthesia.

Adipose tissues (visceral fat) weight corresponds to the sum of the weight of mesenteric, retroperitoneal and urogenital adipose tissues were collected and weighed.

a. Isolation of muscle mitochondria

- Mitochondria were isolated from muscles from both hind legs. Muscles were chopped in a cold (4°C) isolation buffer (150 mM sucrose, 75 mM KCl, 1 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, 50 mM Tris-HCl, pH 7.4).
- In coldroom, muscles pieces were transferred into 30 mL of isolation buffer supplemented with 0.2 % fat-free bovine serum albumin and were then added with 0.2 mg/mL subtilisin for breaking down the wall of muscle cells to make the mitochondria to be more easily extracted.
- After one-minute incubation, the suspension was diluted twice with isolation buffer, transferred to a glass-teflon Potter, and homogenized using a motor-driven homogenizer at 500 rpm/minute, up and down \pm 25 times.
- Nuclei and cell debris were removed by centrifugation at 800 g for 10 min at 4°C. This step was done three times.
- Mitochondria were isolated by spinning twice the supernatant at 8000 g for 10 min at 4°C. The supernatant was discarded
- The mitochondrial pellet was resuspended in 300 μ L of isolation buffer and kept on ice.
- Mitochondrial protein contents were determined using the bicinchoninic acid assay (Pierce) using bovine serum albumin (BSA) as a standard. Samples were diluted 50 times and dispensed on a 96-well microplate and 200 μ L of the Pierce reagent was dispensed in each well.

- Samples were incubated 30 minutes at 37 °C and optical density were recorded at 462 nm and were analyzed using KcJunior software (Biotek) to express the protein concentrations in mg/ml.

b. Isolation of liver mitochondria

- Liver mitochondria were isolated using the same procedure than for muscle mitochondria except that there was no digestion with subtilisin.
- In coldroom, liver was cut into small pieces in 30 mL cold (4°C) isolation buffer (250 mM sucrose, 1 mM EGTA, 20 mM Tris-HCl, pH 7.4) added with BSA 15% to agglutinate fat traces.
- The suspension was transferred to a glass-teflon Potter, and homogenized using a motor-driven homogenizer at 500 rpm/minute, up and down \pm 20 times.
- Nuclei and cell debris were removed by centrifugation at 800 g for 10 min at 4°C. This step was done three times.
- Mitochondria were isolated by spinning twice the supernatant at 8000 g for 10 min at 4°C. The supernatant was discarded
- The mitochondrial pellet was resuspended in 300 μ L of isolation buffer and kept on ice.
- Mitochondrial protein contents were determined using the bicinchoninic acid assay (Pierce) using bovine serum albumin (BSA) as a standard. Samples were diluted 100 times and dispensed on a 96-well microplate and 200 μ L of the Pierce reagent was dispensed in each well.

Samples were incubated 30 minutes at 37 °C and optical densities was recorded at 462 nm and were analyzed using KcJunior software (Biotek) to express the protein concentrations in mg/m/l.

c. Oxygen consumption measurement

The rates of oxygen consumption of isolated muscle mitochondria were measured using a Clark-type O₂ electrode (Oxygraph, Hansatech Instruments). Mitochondria (0.2 mg/mL) were incubated at 30 °C in a respiration buffer containing 125 mM KCl, 5 mM Pi, 20 mM Tris-HCl, 0.1 mM EGTA, 0.1 % fat free BSA (pH 7.2). The suspension was constantly stirred with a built-in electromagnetic stirrer and bar flea. Measurements were carried out in the presence of either glutamate (5 mmol.L⁻¹)/malate (2.5 mmol.L⁻¹) and succinate (5 mmol.L⁻¹) as substrates, after the addition of 1 mmol.L⁻¹ ADP (state 3), followed by the addition of 0.25 mg.mL⁻¹ oligomycin (state 4) and addition of 2µl of dinitrophenol (DNP).

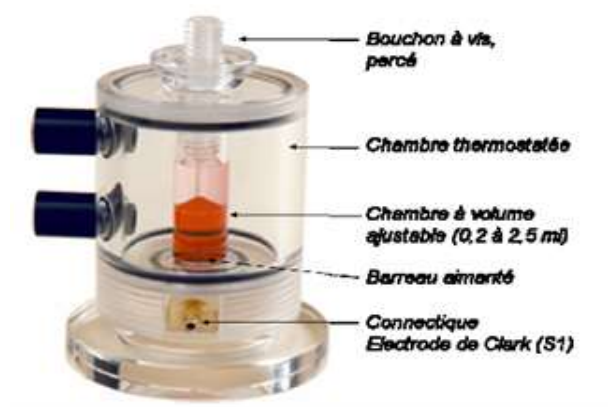


Figure 14. Oxygraphic chamber, Hansatech Instruments

Moreover, the assessment of oxygen consumption includes serial addition of various substrates, inhibitors, and uncouplers which allow a comprehensive assessment of mitochondrial function:

1. Basal respiration was measured by adding a suspension of mitochondria (0.2 mg/mL) in the absence of exogenous substrates.
2. Respiration state 2 (**complex I**) was measured by adding glutamate (5 mmol.L⁻¹) / malate (2.5 mmol.L⁻¹) as substrate. In the absence of adenylates, oxygen consumption with G / M reflects the state 2 respiration by the complex I.
3. Respiration state 2 (**complex I+II**) was measured by adding succinate (5 mmol.L⁻¹) to the respiration chamber after addition of glutamate/malate (G/M) to provide additional electrons through complex II.
4. Addition of 1 mmol.L⁻¹ ADP (state 3), followed by the addition of 0.25 mg.mL⁻¹ oligomycin (state 4).
5. Uncoupled respiration was measured after addition of 2µl of dinitrophenol (DNP).
6. The respiratory rate measured in each condition was determined from the linear portions of the curves.

d. Mitochondrial H₂O₂ release

Generation of mitochondrial H₂O₂ was determined by fluorimetry by measuring the linear increase in fluorescence due to enzymatic oxidation of amplex red (excitation at 560 nm, emission at 584 nm) by H₂O₂ in the presence of horseradish peroxidase (Batandier 2002,

Lacraz, 2008). Muscle mitochondria (0.2 mg/mL) were incubated at 30 °C in the same medium as for mitochondrial respiration added with 6 U/mL of horseradish peroxidase and 1 μM amplex red. The reaction was started by addition of the same combination of G/M/S as substrates as used for mitochondrial respiration. Mitochondrial H₂O₂ release was measured both in basal conditions (substrate alone) and after sequential addition of 2 mM rotenone and 2 mM antimycin A to determine the maximum rate of H₂O₂ production of complexes I and I+III by the respiratory chain, respectively.

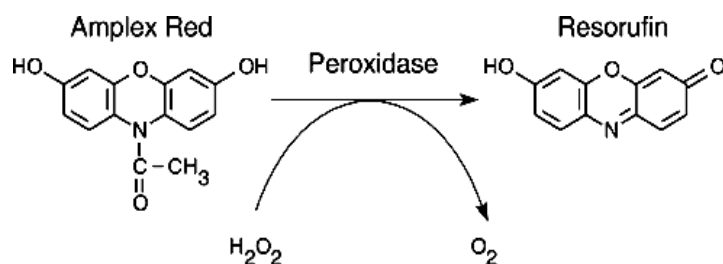


Figure 15. Equation oxidation of by H₂O₂ (Batandier C *et.al.* 2002)

e. **Determination of enzymatic activities:**

Enzymatic activities of individual complexes of the electron transport chain in liver and muscle mitochondria

The measurement of the specific activity of the respiratory chain complex I, II, and III was performed spectrophotometrically. Enzyme activity was expressed as μmoles of reduced or oxidized substrate per min and per mg of mitochondrial protein.

Rotenone-sensitive NADH-ubiquinone oxidoreductase (complex I) was assayed using 100 μmol.L⁻¹ decylubiquinone as electron acceptor and 200 μmol.L⁻¹ NADH as a donor, in a 10 mmol.L⁻¹ KH₂PO₄/K₂HPO₄ buffer, pH 7.5 containing 3.75 mg.mL⁻¹ BSA, 2 mmol.L⁻¹ KCN

7.5 $\mu\text{mol.L}^{-1}$ antimycin A. Oxidation of NADH was then measured at 340 nm, before and after the addition of 4 $\mu\text{mol.L}^{-1}$ rotenone to allow the calculation of the rotenone-sensitive specific activity which is characteristic of complex I.

Succinate-ubiquinone reductase (complex II) activity was quantified by measuring the decrease in absorbance due to the reduction of 100 $\mu\text{mol.L}^{-1}$ DCIP at 600 nm. The measurement was performed in 50 mmol.L^{-1} $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, pH 7.5 in the presence of 100 $\mu\text{mol.L}^{-1}$ decylubiquinone, 2 $\mu\text{mol.L}^{-1}$ rotenone and 2 mmol.L^{-1} KCN.

Ubiquinol cytochrome c reductase (complex III) activity was determined by measuring the reduction of cytochrome c when decylubiquinol was used as substrate and complexes I and IV were blocked by specific inhibitors. The change in absorbance at 550 nm was first evaluated at 30°C for 1 min in the presence of potassium phosphate buffer 90.7 mM, pH 7.4, EDTA 50 μM , bovine serum-albumin (1 mg/ml), KCN 1 mM, oxidized cytochrome c (100 μM), decylubiquinol 0.11 mM and the 200-times diluted sample in order to evaluate total activity. The non specific activity was then measured with antimycin A (5 $\mu\text{g/ml}$) for 2 min and complex III activity was then calculated by subtraction.

Cytochrome c oxidase (complex IV) activity was assayed on freshly isolated liver and muscle mitochondria by oxygraphy with TMPD/Ascorbate as electron donor/acceptor in the presence of DNP as uncoupling agent.

f. Citrate synthase activity

Citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle and is an exclusive marker of the mitochondrial matrix (Srere, 1969). Plantaris muscle or liver samples (50 mg)

were homogenized at 4°C in 450 µL of 100 mM KH₂PO₄, pH 7.4. The homogenates were centrifuged (1500 g, 5 min, 4°C), and the resulting supernatant was collected and stored at -80°C until determination of enzymatic assays. Activity of citrate synthase was assessed according to Srere (Srere, 1969). Results were expressed as µmol/min/mg of wet tissue.

g. Activity of glutathione peroxidase (GPx)

A portion of frozen liver (100 mg) was homogenized with a potter Elvehjem, at 4°C, in buffer containing KH₂PO₄ 100 mM, DTT 1 mM, and EDTA 2 mM, pH 7.4. After centrifugation (3000 g/min for 5 min), the supernatant was used for enzymatic assays. GPx activity in supernatant and in plasma was evaluated by the modified method of Günzler *et.al.* (1974) using tertbutyl hydroperoxide (Sigma Aldrich) as a substrate.

h. Biochemical assays:

Quantification of fatty acid esters was performed by gas chromatography coupled to mass spectroscopy. Samples from liver, liver mitochondria and plasma containing internal standards were saponified in 0.6 N ethanolic potassium hydroxide. Fatty acids were further extracted, derivatized and resuspended in 100 µL hexane, and 1 µL was used for fatty acid composition determination (Viens 1996). Cytochrome content of the respiratory chain (in liver mitochondria) was measured by comparison of the spectra of fully oxidized (potassium ferricyanide) compared with fully reduced (sodium dithionite) cytochromes, and absorbance values were used to calculate the amounts of cytochromes (Williams 1964). Quinones 9 (Q9) and 10 (Q10) were measured on powdered frozen tissues, liver mitochondria and plasma. After solubilization and extraction in 2-propanol, Q9 and Q10 were detected by reverse-phase HPLC with electrochemical detection on the same run (Galinier 2004).

Protein oxidation in the plasma and liver was evaluated by the disappearance of protein thiol groups (Faure, 1995). Standards and plasma samples (20 μ L) were measured spectrophotometrically at 415 nm in $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer 50 mM, EDTA 100 mM, pH 8 and bis-5,5'-dithio-bis(2-nitrobenzoic acid 10 mM).

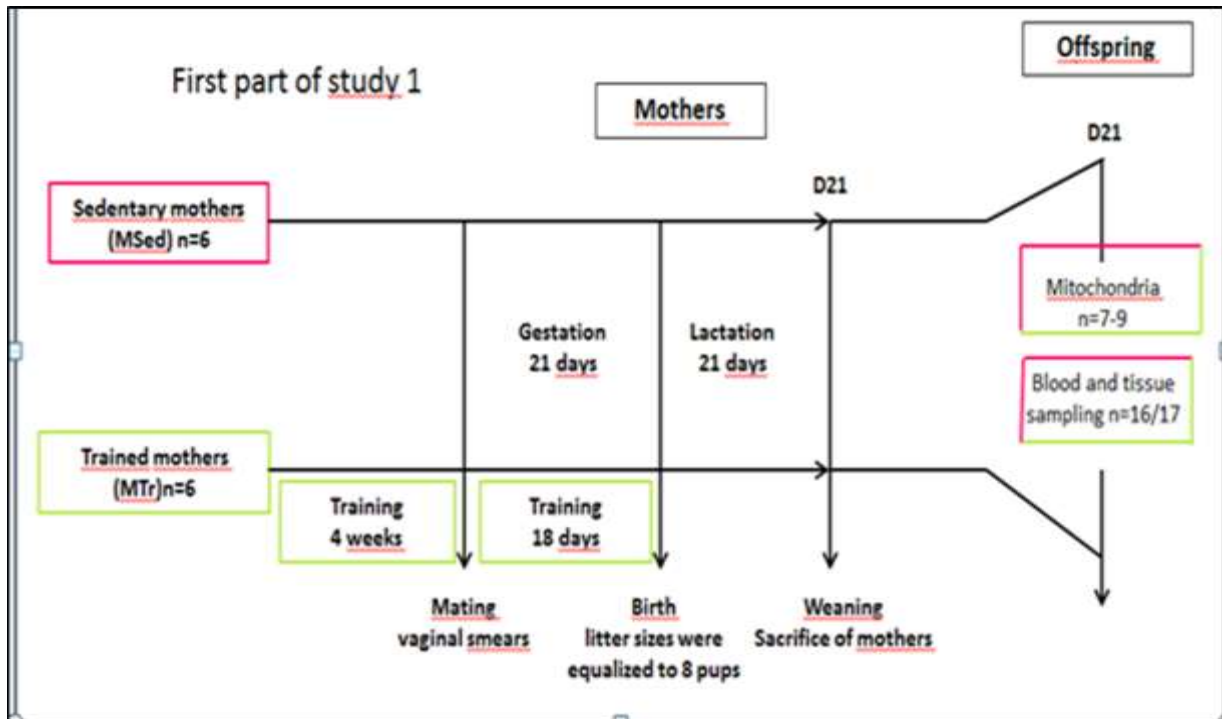


Figure 16. The scheme of first part of study the influence of exercise around gestation on oxidative stress of offspring

B. SECOND PART: THE INFLUENCE OF EXERCISE AROUND GESTATION ON GLUCOSE METABOLISM OF OFFSPRING

In the second part of the core study, effect of moderate intensity endurance training 4 weeks before mating until the 18th day of gestation on the pancreatic islet β -cells function of offspring were evaluated by measuring of glucose tolerance, insulin sensitivity, insulin secretion, insulin signaling pathway (PKB phosphorylation), insulin content, and evaluating the structure of pancreatic β -cells.

a. Glucose and insulin tolerance test

Intraperitoneal glucose tolerance test (IpGTT) and intraperitoneal insulin tolerance test (IpITT) were performed in male rat offspring.

After fasting overnight, glucose was intraperitoneally injected at 1 g.kg⁻¹ body weight. Blood glucose readings were taken via tail pick before (T = 0 min) and 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, 120 min after glucose injection using an Accu-Chek glucometer (Roche Diabetes Care ®, Meylan, France). Areas under the curve were related to T = 0 min.

After fasting six hours, insulin was intraperitoneally injected at 0.15 g.kg⁻¹ body weight. Blood glucose readings were taken via tail pick before (T = 0 min) and 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, 120 min after insulin injection using an Accu-Chek glucometer (Roche Diabetes Care ®, Meylan, France). Areas under the curve were related to T = 0 min.

b. Isolation of rat Islet cells

Prior to isolation procedure, digestion solution and isolation buffer were prepared. Digestion solution consisted of 10 mg collagenase + 10 ml Hanks Balanced Salt Solution (HBSS) for young rat and 15 mg collagenase + 15 ml HBSS for an adult rat and was placed in an ice bag. Isolation buffer consisted of HBSS+SVFd and was kept at 4 °C.

1. After anaesthetization, pancreas was slowly injected with a small amount of digestion solution (2.5 ml for young and 5 ml for adult rat) via canal of bile duct. It was then dissected and placed in a 50-ml tube containing the digestion solution.
2. The pancreas was incubated for 12 minutes 45 seconds in a 37°C-waterbath.
3. Digested pancreas was filtered and suspension was transferred in two new 50-ml tubes.
4. 10 ml of solution HBSS+SVFd (without collagenase) were added in each tube and shaken well for 10 seconds.
5. 30 ml of solution HBSS+SVFd were then added in each tube that was centrifuged at 259 g at 4°C for 2 minutes.
6. The supernatant was slowly eliminated and the pellet was added with 10 ml of HBSS+SVFd, and was then slowly resuspended
7. Tubes were then filled with solution HBSS+SVFd and then centrifuged at 259 g at 4°C for 2 minutes. This step was repeated twice.
8. The pellet was kept and then added with 10 ml of HBSS+SVFd and was then slowly resuspended.
9. Tubes were then filled with solution HBSS+SVFd and then centrifuged at 259 g at 4°C for 2 minutes. This step was repeated twice.

10. The pellet was kept and added with 5 ml of Histopaque solution, slowly resuspended. 5 ml of Histopaque were then added.
11. The suspension composed of pellet+Histopaque and 10 ml HBSS (without SVFd) were layered very slowly in each falcon in order to make a three steps density gradient. Tubes were then centrifuged at 900 g at 20°C for 20 minutes.
12. The middle layer from the 2 tubes were taken carefully and placed in a new tube containing 10 ml of HBSS+SVFd that were centrifuged at 259 g at 4°C for 2 minutes.
13. The pellet was kept and slowly resuspended with 10 ml of HBSS+SVFd, and tubes were filled with additional HBSS+SVFd solution. Tubes were centrifuged at 259 g at 4°C for 2 minutes. This step was repeated twice.
14. The pellet was slowly resuspended in 700 µL of RPMI+antibiotics.
15. The suspended pellet was placed in a sterile petri dish and was then added with 700 µL RPMI used to rinse the tube.
16. Finally, 700 µL of RPMI were added into the petri dish.
17. The number of islet was counted under light microscope and islets were then stored overnight at -20°C for glucose stimulation insulin secretion (GSIS) tests.

c. **Glucose-stimulated insulin secretion (GSIS) test** (modified by Vial G, 2013)

Pancreatic islets isolation was performed for insulin secretion measurements using Serva® protocol with slight modifications by Guillaume Vial. A 35 mm Petri dish was coated using 50 µl of matrix and incubated for 2 h at 37 °C and then rinsed twice with sterile distilled water. Coated Petri dish was dried under the culture hood before use.

Preparation of Krebs:

KRBH solution

| | mM | MW | For 1L |
|---------------------------------|-----------|--|--------------------|
| NaCl | 125 | 58.44 | 7.305g |
| KCl | 4.74 | 74.55 | 0.353g |
| NaHCO ₃ | 5 | 84.01 | 0.42g |
| CaCl ₂ | 1 | 147.02 (2H ₂ O) | 0.147g |
| MgSO ₄ | 1.2 | 246.5 (7H ₂ O) | 0.2959g |
| K ₂ HPO ₄ | 1.2 | 174.18 (or 228.2 if 3H ₂ O) | 0.209g (or 0.274g) |
| HEPES | 25 | 238.3 | 5.957g |
| BSA | 0.1% w/v | | 1g |

pH 7.4

Filter through 0.22µM under hood.

Aliquote and store at -20 ° C.

Prepare Krebs solution with 2.8 mM glucose and another with 16.7 mM:

For 10 ml of 2.8 mM (0.5g / L): 50 µl of solution 100g / L (Sigma)

For 10 ml of 16.7 mM (3g / L): 300 µL of solution 100g / L (Sigma)

GSIS protocol:

1. The islets in Petri dish were taken and placed in two 2-ml sterile microtubes wetted with 1 drop of RPMI.
2. Incubation with glucose 0.5 g/L (2.8 mM): 1ml/each tube at 37 °C for 1 hour and then centrifugation at 1100 rpm/20°C/2 minutes
3. Elimination of the supernatant and addition of 1ml glucose 0.5 g/L to the pellet in each tube and incubation at 37 °C for 1 hour
4. Centrifugation at 1100 rpm/20°C/2 minutes
5. The supernatants were collected carefully and separated in two tubes (one for GSIS 0.5 g/L and one for GSIS Arg/0.5 g/L) that were stored at -80 °C until insulin secretion measurements.
6. The pellets were kept and incubated with 1ml/each tube of glucose 3 g/L (16.7 mM) at 37 °C for 1 hour and tube were then centrifuged at 1100 rpm/20°C/2 minutes
7. The supernatants were taken and separated in two tubes (one for GSIS 3 g/L and one for GSIS Arg/3 g/L) that were then stored at -80 °C until insulin secretion measurements.
8. The pellets were kept and incubated overnight in 1 ml of ethanol acid (EtOH 70% + 0.18N HCl) per tube at -20 °C.
9. The bottom was scraped with sterile tips and stored at -80°C until measurements of total insulin.

Composition of EtOH / HCl:

For 100 mL: 75 ml of absolute ethanol + 1.5 mL 12 N HCL + 23.5 mL H₂O.

d. Insulin load test and analysis of insulin signaling (PKB/Akt) in skeletal muscle and in liver

After a 6-hour fast, 3-week (others than those used for tolerance tests) old rats were intraperitoneally injected with NaCl (0.9%) or with insulin (10 mIU.g⁻¹ body weight). 15 min after injection, rats were killed and gastrocnemius muscle and liver were rapidly removed and frozen until their use to determine phosphokinase B (PKB) phosphorylation level by Western blotting as an indicator of insulin sensitivity.

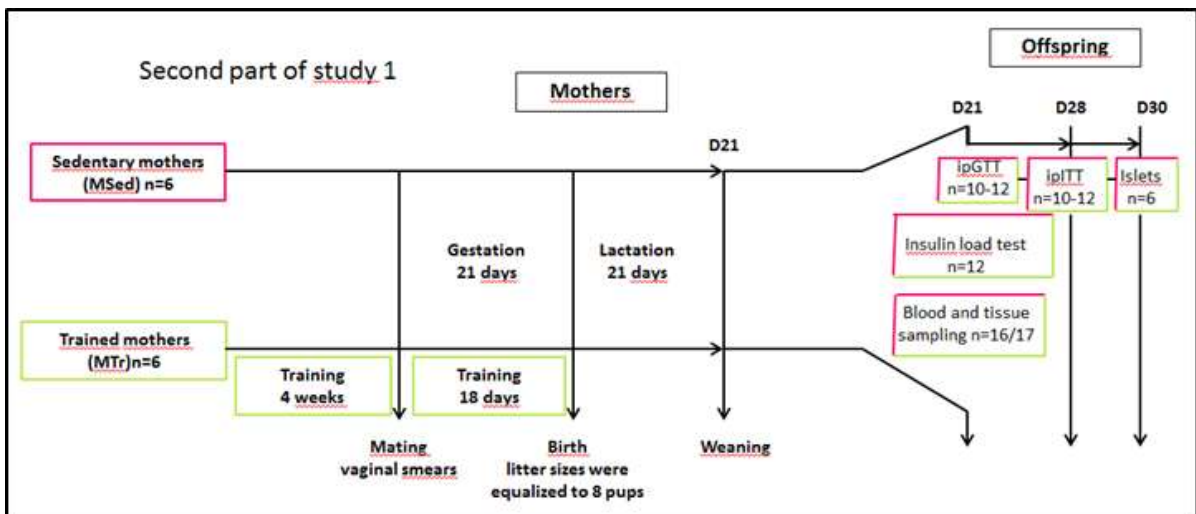


Figure 17. The scheme of second part of study the influence of exercise around gestation on glucose metabolism in offspring

II.2.1.1 Statistical analyses

All data are presented as mean \pm SE. One-way analysis of variance (ANOVA) was used to determine the global effect of training of the mothers on offspring. When appropriate, differences between groups were tested with a PLSD Fisher post-hoc test. Statistical significance was accepted at $P < 0.05$. Mann Whitney tests were applied when values were non-normally distributed.

II.2.2 THE INFLUENCE OF DIET AROUND GESTATION ON OXIDATIVE STRESS IN OFFSPRING

II.2.2.1 Animals care

All study protocols were reviewed and approved by the Institutional Ethic Committee for Animal Care (Protocol 00174.02 accepted in March 2014). The rats were maintained and handled in agreement with the Guidelines for the Care and Use of Laboratory Animals published by the European Union (2010/63/UE) and the French Ministry of Research (2013-118).

Eighteen female Wistar rats (12 weeks-old) from Charles River laboratory, L'Arbresle, France, were housed in individual cages in a controlled environment: 25°C, 50 ± 10% relative humidity and 12h:12h light:dark, during all the experiment with free access to food and water.

II.2.2.2 Diets

The diets (chow) were bought from SAFE, 89290 Augis, France. The 18 female Wistar rats were randomly divided in 3 groups (n=6 for each): control group (C), fructose group (F), and fructose iron-enriched group (FI). The control group received a standard diet (A04) as shown in Table 1. The FI group received the same fructose-rich diet but containing 22 mg of iron/100 g diet (Table 1). The diets were given 4 weeks before gestation and during the 3 weeks of gestation. The diet was given 4 weeks before gestation in order to get negative effect on metabolic function on mother before gestation. The iron content of the pellets for the Fructose and Fructose-Iron diet were assayed using a quadrupole ICP-MS Thermo X-series II completed with crash/reaction cell technology (CCT), concentric nebulizer and used Xt

interface. The collision reaction gas was a mixture of Helium (He) and H₂ (97/7). The samples were mineralized in nitric acid and were then diluted 100 times in water prior to analysis. Measuring of Fe²⁺ and Fe³⁺ used Ga as internal standard. Four calibration standards (0-200-1000, and 2000 nmol/l) were used to make a calibration curve. Method accuracy was assessed by analyzing NIST standardized reagent 1577b (bovine liver) and ARC/CL total diet standardized reagent at the beginning and at the end of the analytical run. The precision value was 3.40% and the bias was 1.67%.

Table 1: Composition of the diets (g/100g diet)

| Composition | Standard A04 (group C) | Fructose rich diet (group F) | Fructose-Iron diet (Group FI) |
|----------------------|---------------------------|---------------------------------|----------------------------------|
| Starch | 62 | 0 | 0 |
| Fructose | 0 | 65 | 65 |
| Casein | 22.7 | 20 | 20 |
| Vegetal oils | 4.5 | 5 | 5 |
| Mineral and vitamins | 6.25 | 6.25 | 6.25 |
| Iron mg/100 g diet | 10 | 12 | 22 |
| Cellulose | 4.50 | 5 | 5 |
| kCal/100 g diet | 379 | 385 | 385 |

II.2.2.3 Experimental procedures

All pregnant rats were weighed weekly and pups were weighed at delivery. One day after delivery and after an overnight fasting, the mothers were anaesthetized with sodium pentobarbital intraperitoneally. Some of the female rats were unfertilized, which is a common figure in animal facilities. Therefore, the final number of mothers with pups was for each group: C: n=4, F: n=5, FI: n=5. Blood samples were collected from mothers by heart

puncture in heparinized tubes protected from light and centrifuged at room temperature for 10 min at 3000 g. Plasma was immediately isolated, aliquoted and stored at -80 °C until analysis.

The morning of sacrifice males and females pups were weighed and decapitated without anesthesia. Blood glucose measurements were taken by a drop of blood using a glucometer as previously described. Immediately after blood collection, the rats were sacrificed and visceral fat mass was weighed. Pups liver and brain were removed, weighed, frozen in liquid nitrogen, and stored at -80 °C until analysis. Before analysis, tissue samples were homogenized (10% w/v) in a buffer composed of 10 mM Tris, 1 mM DPTA (diethylene triamine pentaacetic acid), 1 mM PMSF (phenylmethanesulfonylfluoride), pH=7.4 and centrifuged at 3000 g/4 °C/10 min.

II.2.2.4 Biological parameters

Enzymatic and colorimetric methods on Roche/Hitachi modular 912 (Roche diagnosis, Meylan, Fr) were used to determine the concentration of fasting glucose, triglycerides, and cholesterol in mothers. Insulin concentration in mothers was determined using a commercial radioimmunoassay kit (Merck Millipore Corp, Germany). Glycemia in pups was determined using an AccuChek[®] glucometer (Roche Diabetes Care, Meylan, France).

Insulin sensitivity was calculated using the Homeostasis Model Assessment-insulin resistance (HOMA-IR), first described by Matthews *et.al.* 1985. The value can be calculated from the fasting concentrations of insulin and glucose using the following formula:

$$\frac{\text{fasting glucose (mg/dL)} \times \text{fasting insulin } \mu\text{UI/mL}}{22.5}$$

Oxidative stress status was evaluated by measuring of thiobarbituric acid reactive substances (TBARS), plasma thiols (SH) groups, the reduced and oxidized form of glutathione (GSH/GSSG), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activity, and total plasma antioxidant (ferric reducing antioxidant power, FRAP).

TBARS level of plasma were assessed as described by Richard *et.al.* 1992. During measurement, lipoperoxidation process was inhibited by adding 20 g/L (10 μ L) of Butylated hydroxytoluene (BHT) in solution (Sigma Chemical Co., via Cogec, Paris, France) to each tube. Then, 100 μ L of polypropylene and 750 μ L of a buffer solution consisted in thiobarbituric acid and HClO_4 were then added to the sample. Tubes were mixed by vortex and incubated at 95 $^\circ\text{C}$ for 60 minutes. To stop the reaction, the samples were cooled in an ice bath. To each tube, 2 ml of butanol were added to extract the TBA-MDA complexes. The phases were then separated by centrifugation. The determination of TBARS was done at excitation wavelength of 532 nm and emission wavelength of 553 nm.

The power of antioxidant was assayed by measuring FRAP as a novel method. Reduction of Fe^{3+} to Fe^{2+} at low pH causes formation of a colored complex of ferrous-tripyridyltriazine. The value of FRAP was obtained by comparing the absorbance of standard ferrous ion with the samples at 593 nm. Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. Measured stoichiometric factors of Trolox, α -tocopherol, ascorbic acid, and uric acid are all 2.0 and that of bilirubin is 4.0. Activity of albumin is very low. Within- and between-run CVs are <1.0 and <3.0%, respectively, at 100–1000 $\mu\text{mol/l}$. The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The

FRAP assay offers a putative index of antioxidant, or reducing, potential of biological fluids within the technological reach of every laboratory and researcher interested in oxidative stress and its effects.

Plasma thiols (SH) groups were assayed as described by Faure and Lafond, 1995. The reduced and oxidized form of glutathione (GSH/GSSG) was determined by a kinetic method as prescribed by Akerboom and Sies, 1981. Glutathione peroxidase (GPx) activity was evaluated by the modified method of Gunzler *et.al.* 1974 using terbutyl hydroperoxide as a substrate instead of hydrogen peroxide. The glutathione-S-transferase (GST) activity was determined by the method of Habig *et.al.* 1974.

II.2.2.5. Statistical analyses

All data are presented as mean \pm SE. One-way analysis of variance (ANOVA) was used to determine the effect of diet in mother (high fructose diet and fructose iron-enriched diet) on offspring. When appropriate, differences between groups were tested with a PLSD Fisher post hoc test. Statistical significance was accepted at $p < 0.05$. Mann Whitney tests were applied when values were non-normally distributed.

CHAPTER III

RESULTS AND DISCUSSION OF

STUDY I:

THE INFLUENCE OF EXERCISE AROUND GESTATION

III.1. THE INFLUENCE OF EXERCISE AROUND GESTATION ON OXIDATIVE STRESS IN OFFSPRING

III.1.1. The influence of exercise around gestation on citrate synthase activity in the mother

Table 2. Citrate synthase activity in liver and muscle of mothers (Pilot study)

| | Sedentary | Trained |
|---|--------------------|----------------------|
| Citrate synthase activity in liver ($\mu\text{mol}/\text{min}/\text{g}$ of wet tissue) | 73.30 ± 2.70 | 82.72 ± 3.71 * |
| Citrate synthase activity in muscle ($\mu\text{mol}/\text{min}/\text{g}$ of wet tissue) | 240.40 ± 20.12 | 324.89 ± 15.55 * |

Values are means \pm SE of $n = 6$ rats/group.

*Significantly different from sedentary group ($p < 0.01$).

Since the major effects of training on Citrate synthase usually disappear after 3 weeks without exercise and as we have to use our offspring at weaning (21 days) to obtain sufficient tissue, we conducted a pilot study where mothers were trained using the same training protocol as for the central study. We sacrificed the mothers just after delivery to confirm that such training program was efficient and that mothers will be trained before and during gestation.

Maximal activity of citrate synthase (CS), a classic marker of skeletal muscle and liver mitochondrial adaptation to exercise training [Holloszy JO, Coyle EF (1984)], was significantly higher in the trained group compared to the sedentary group ($P < 0.05$).

III.1.2 The influence on anthropometry of mothers and offspring

Table 3. Characteristics of mothers and litters (central study).

| | Sedentary (C) | Trained (TG) |
|---|---------------|---------------|
| Body weight at the beginning | 260 ± 5 | 260 ± 4 |
| Body weight after 4 weeks training | 277 ± 22 | 276 ± 19 |
| Body weight after nursing | 297 ± 7 | 286 ± 4 |
| Weight gain during the last week of gestation (% of body weight) | 13.02 ± 3.62 | 9.49 ± 2.16 |
| Food intake during the last week of gestation (g/100g of body weight) | 53.44 ± 6.41 | 62.21 ± 16.18 |
| Adipose tissues weight (g/100g of body weight) | 54.3 ± 6.0 | 39.5 ± 2.7* |
| Muscle (g.kg PC ⁻¹) | 5,90±0,24 | 6,02±0,11 |
| Number of newborns | 10.7 ± 0.9 | 10.7 ± 1.5 |
| % of male | 63 | 45 |

Values are means ± SE of n = 6 rats/group.

*Significantly different from the control group ($p < 0.05$)

Adipose tissues weight corresponds to the sum of the weight of mesenteric, retroperitoneal and urogenital adipose tissues.

There was no difference in mother body weight between the two groups after the 4 first weeks of training (277 ± 22 g vs. 276 ± 19 g for Control and Trained groups, respectively) (Table 3). After nursing, body weight of trained mothers tended to be lower than that of Control mothers (297 ± 7 g vs. 286 ± 4 g for Control and Trained groups, respectively) (Table 3). Food intake was similar between both groups throughout the study from the beginning of the protocol to the end of lactation (Table 3). Relative muscle mass estimated as the sum of plantaris, externus longitudinalis, gastrocnemius, and quadriceps muscles measured at sacrifice was no

difference in mother between the two groups ($5,90 \pm 0,24$ vs $6,02 \pm 0,11$ for Control and Trained groups, respectively) (Table 3). However, relative fat mass estimated as the sum of retroperitoneal, urogenital and mesenteric fats depots, was significantly lower in trained mothers (TG) compared with control mothers (C) (39.5 ± 2.7 vs. 54.3 ± 6.0 g.kg⁻¹ body weight for trained and control groups, respectively. $P < 0.05$) (Table 3). Moreover, exercise training before and during gestation had no effect on the litter size or on sex ratio of the litter.

III.1.3. The influence of maternal exercise on citrate synthase activity in offspring

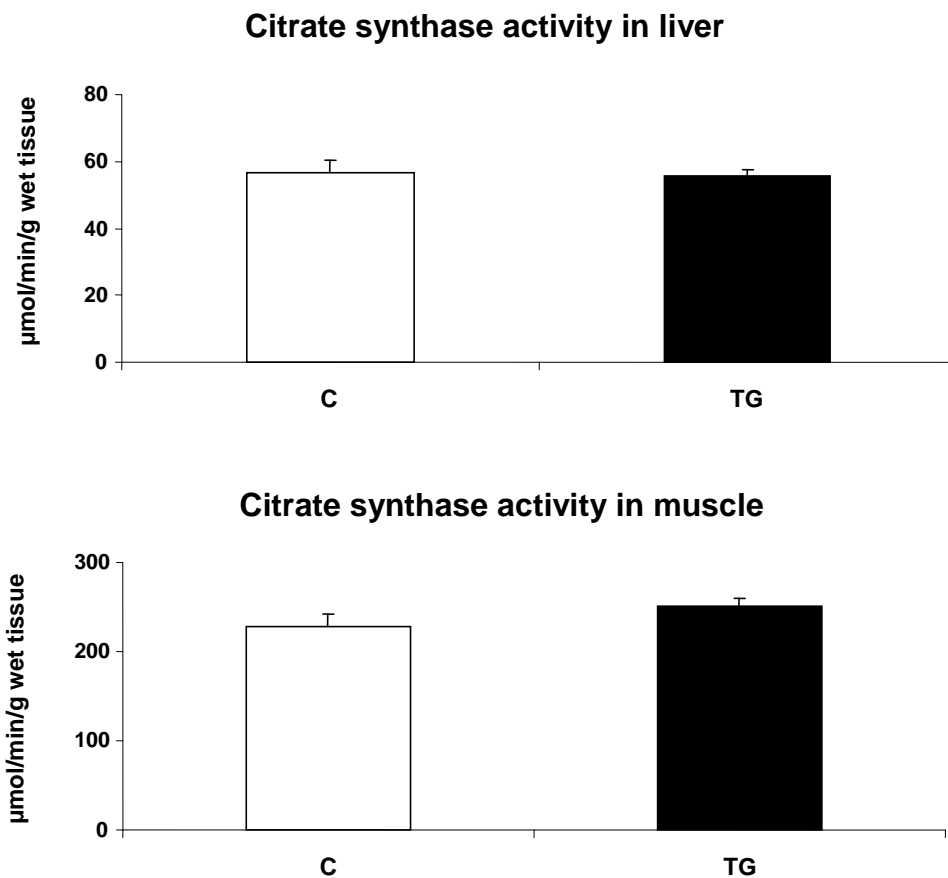


Figure 18. Citrate synthase activity in liver (upper panel) and in muscle (lower panel) in offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of $n = 9$ rats/group.

Endurance training of mother did not alter liver or muscle citrate synthase activity in offspring

III.1.4. The influence of maternal exercise on oxygen consumption of Offspring

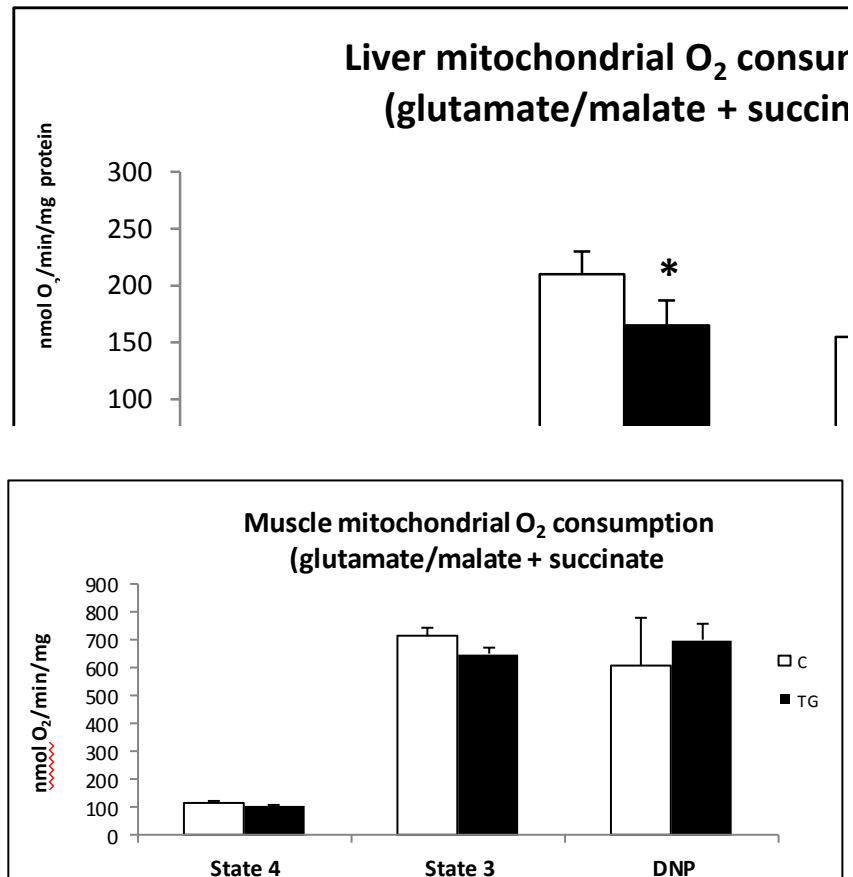


Figure 19. Oxygen consumption by isolated muscle mitochondria (upper panel) and by isolated liver mitochondria (lower panel) with glutamate/malate+succinate as substrates in offspring from C or TG mothers.

Values are means \pm SE of $n = 7-9$ rats/group

*Significantly different from control group ($p < 0.01$).

State 3 (ADP-stimulated) and state 4 oxygen consumptions (with glutamate/ malate + succinate as substrates) in liver mitochondria were lower in young rats from trained mothers compared to those from sedentary mothers. There is no significant difference in oxygen consumption by muscle mitochondria between groups and no differences as well in the uncoupled condition (+DNP).

III.1.5 The influence of maternal exercise on ROS production of offspring

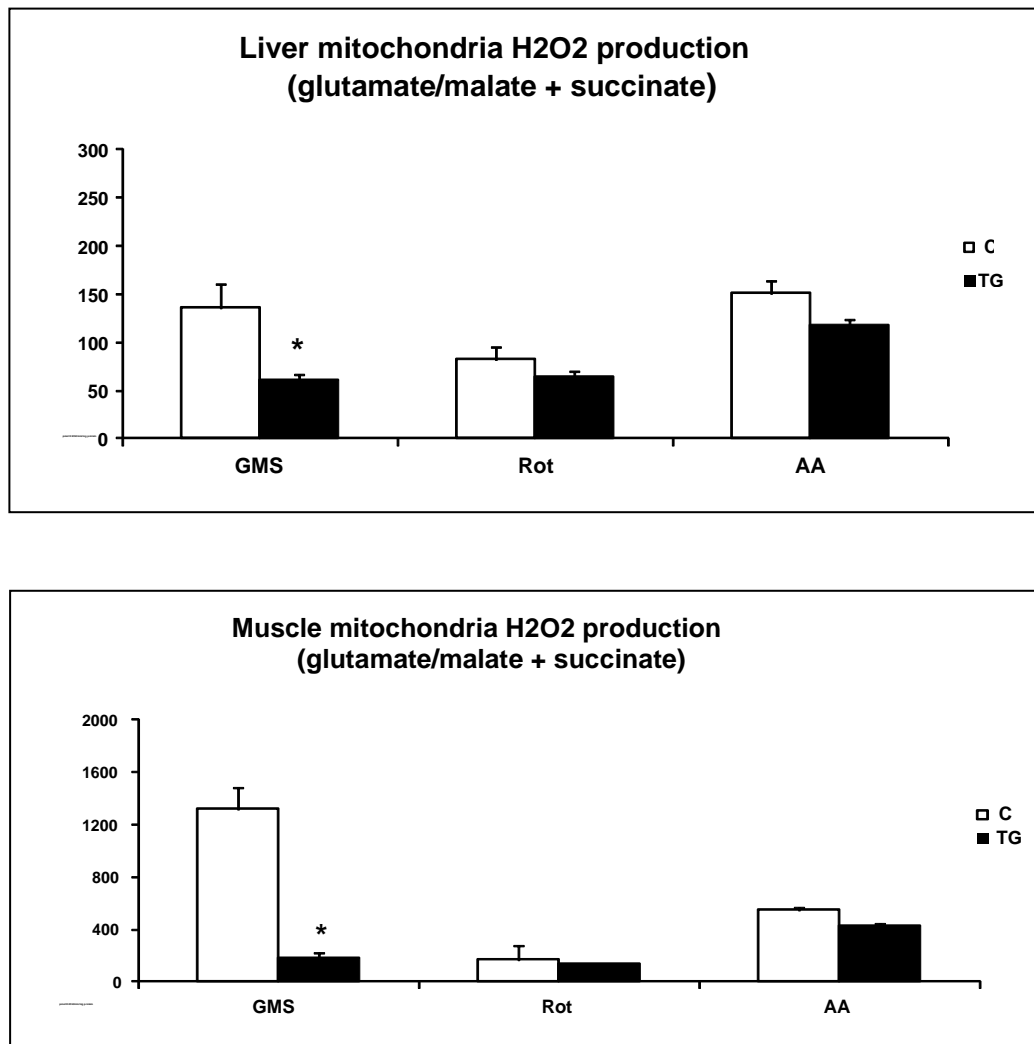


Figure 20. H₂O₂ production by isolated liver mitochondria (upper panel) and by isolated muscle mitochondria (lower panel) with glutamate/malate+succinate as substrates in offspring from C or TG mothers.

Values are means \pm SE of n = 7-9 rats/group.

*Significantly different from control group ($p < 0.01$).

There is a significant decrease in spontaneous H₂O₂ production with glutamate/malate + succinate (GMS) by liver and muscle mitochondria from young rats from trained mothers. This modification seems to be associated with a decrease in ROS production due to reverse electron flux through complex I since there is no more differences after addition of rotenone.

III.1.6 The influence of maternal exercise on GPX activity and thiols concentration in plasma of offspring

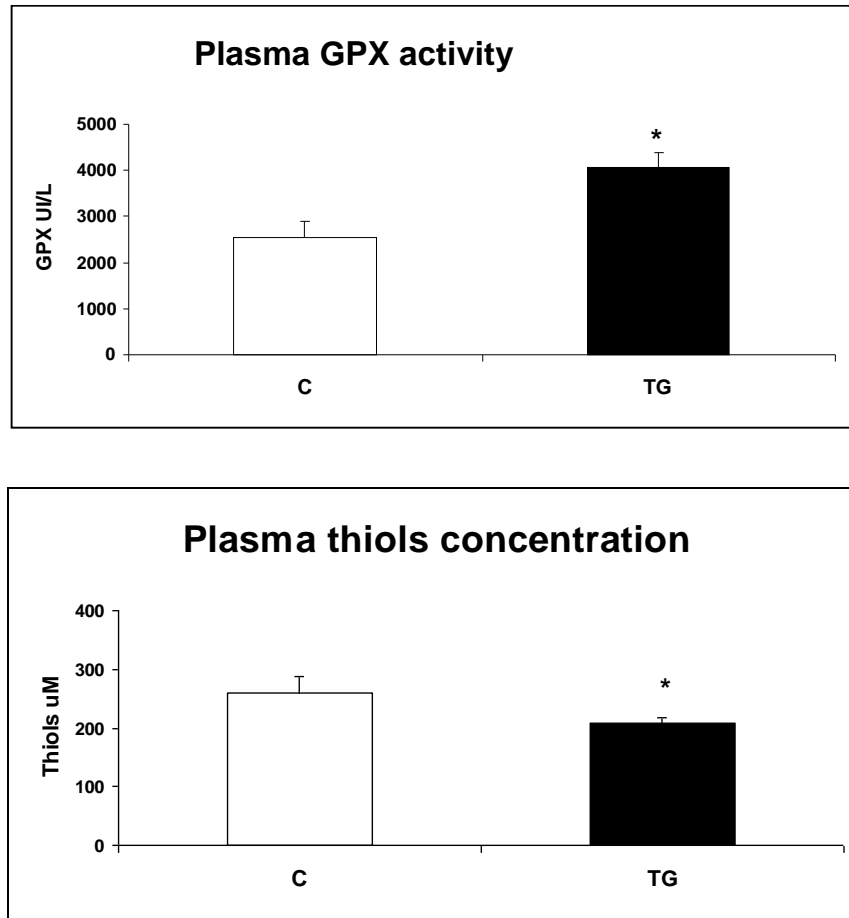


Figure 21. Plasma GPX activity (upper panel) and Thiols concentration (lower panel) in offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of n = 5-9 rats/group.

*Significantly different from control group ($p < 0.01$).

III.1.7 The influence of maternal exercise on GPX activity and thiols concentration in liver of offspring

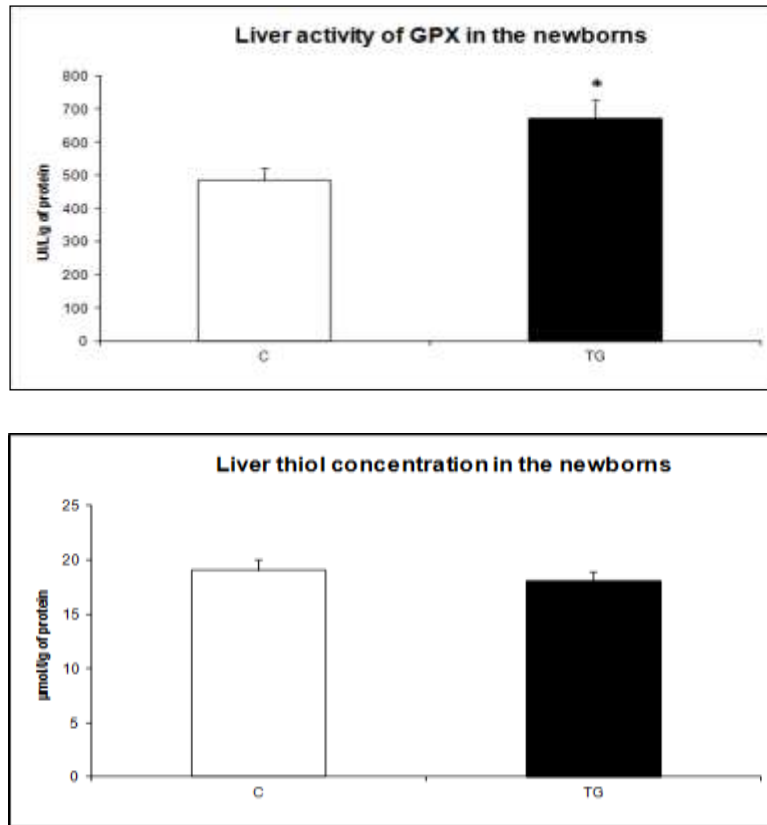


Figure 22. Activity of GPX activity (upper panel) and Thiols concentration (lower panel) in liver of offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of $n = 5-9$ rats/group.

*Significantly different from control group ($p < 0.01$).

Measurements of muscle GPX activity and muscle thiol concentrations could not be performed because rats were very young and there was not enough muscle tissue available for all analyzes.

The blood and liver redox status of young rats from trained mothers is altered: we observed an enhanced GPX activity associated with an increase in oxidative damages on proteins (as indicated by a reduced thiols concentration in blood).

III.1.8 The influence of maternal exercise on enzymatic activities of ETC in liver and muscle of offspring

Table 4. Enzymatic activities of individual complexes of the electron transport chain.

| Offspring | C | TG |
|--|---------------------|------------------------|
| C-I, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in liver | 28.47 \pm 3.31 | 28.5 \pm 2.83 |
| C-I, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in muscle | 54.34 \pm 5.68 | 50.76 \pm 5.39 |
| C-II, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in liver | 240.58 \pm 5.51 | 210.17 \pm 2.76 * |
| C-II, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in muscle | 548.56 \pm 47.87 | 339.66 \pm 25.08 * |
| C-III, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in liver | 484.08 \pm 17.32 | 483.78 \pm 24.19 |
| C-III, activity, $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ protein in muscle | 3045.94 \pm 95.51 | 2449.32 \pm 128.44 * |
| TMPD/ascorbate in liver nmolO₂/min/mg protein | 206.96 \pm 22 | 284.41 \pm 12.67 * |
| TMPD/ascorbate in muscle nmolO₂/min/mg protein | 707.93 \pm 47.36 | 869.42 \pm 26.93 * |

Values are means \pm SE of n = 7-9 rats/group.

*Significantly different from control group ($p < 0.01$).

Activities of CI, CII, CIII were expressed in nmoles of oxidized or reduced substrates by each complex.

There was no difference in the complex I activity in both liver and muscle of offspring from trained mothers. However, complex II and complex III activities were lower in both liver and muscle from offspring from trained mothers while complex IV displayed a higher activity.

III.1.9 The influence of maternal exercise on cytochromes concentrations in liver mitochondria of offspring

Table 5. Cytochromes concentration (nmole/mg protein) measured by spectrophotometry in liver mitochondria isolated from offspring from sedentary (C) or trained (TG) mothers.

| Offspring | C | TG |
|---|----------------|------------------|
| Cytochromes a+a₃, pmol.mg prot⁻¹ | 131.27 ± 53.33 | 260.83 ± 46.14 * |
| Cytochrome b, pmol.mg prot⁻¹ | 190.47 ± 28.33 | 192.77 ± 16.71 |
| Cytochromes c+c₁, pmol.mg prot⁻¹ | 207.9 ± 63.59 | 306.89 ± 64.36 |
| Cytochrome c, pmol.mg prot⁻¹ | 126.39 ± 19.81 | 120.18 ± 29.9 |

Values are means ± SE of n = 7-9 rats/group.

*Significantly different from control group ($p < 0.01$).

While the major part of the content of respiratory chain cytochromes (b, c+c₁, c) was not affected by the training in the mothers, we observed an increased in a+a₃ cytochrome content in liver mitochondria from young rats from trained mothers compared to those from sedentary mothers.

III.1.10 The influence of maternal exercise on α -Tocopherol and Quinone concentrations in liver of offspring

Table 6. Electron transport chain composition in offspring is modified by training of the mother during gestation. Concentrations of α -Tocopherol reduced (red) and oxidized (ox) quinone pools (Q9 and Q10) were measured in liver tissue.

| | C | TG |
|--|------------------------------------|--------------------------------------|
| α -Tocopherol, nmol.g wet liver-1 | 8.26 \pm 0.78 | 13.42 \pm 0.65 * |
| redQ9, nmol.g wet liver-1 | 51.06 \pm 1.62 | 58.17 \pm 1.41 * |
| redQ10, nmol.g wet liver-1 | 8.84 \pm 0.54 | 11.39 \pm 0.61 * |
| oxQ9, nmol.g wet liver-1 | 12.16 \pm 1.14 | 12.30 \pm 1.07 |
| oxQ10, nmol.g wet liver-1 | ND | ND |
| redQ9/oxQ9 | 4.35 \pm 0.36 | 5.07 \pm 0.53 |
| Total Q9+Q10, nmol.g wet liver-1 | 72.07 \pm 2.51 | 82.56 \pm 0.86 * |

Values are means \pm SE of n = 7-9 rats/group. ND: not detected

Significantly different from control group ($p < 0.01$)

Training of the mothers was associated with a significant increase in reduced Q9 and Q10 (Table 6) in offspring. Q9 oxidized levels and redQ9-to-oxQ9 ratio were not significantly affected by mother training. Interestingly, the change in Q9 and Q10 reduced content in offspring from trained mother (TG) was associated with an increase in quinone pool (total Q9+Q10) and in alpha-tocopherol content.

III.1.11 The influence of maternal exercise on fatty acids in plasma, liver mitochondria, and liver of offspring

Table 7. Plasma, liver and isolated liver mitochondria fatty acids composition in young rats from trained (TG) and untrained (C) mothers.

| Plasma | | |
|---------------------------|------------------|--------------------|
| | C | TG |
| Fatty acids | | |
| Short chains | | |
| 12:0 (%) | 1.49 ± 0.50 | 5.01 ± 0.41 * |
| Long chains | | |
| 14:0-24:0 (%) | 98.48 ± 0.49 | 94.99 ± 0.41 * |
| Very Long chains | | |
| 26:0 (%) | 0.009 ± 0.001 | 0.005 ± 0.0004 * |
| Total MUFA (%) | 62.86 ± 1.22 | 54.85 ± 1.61 * |
| Total SFA (%) | 37.11 ± 1.23 | 45.15 ± 1.61 * |
| Total n-3-FA (%) | 4.76 ± 0.27 | 4.37 ± 1.15 |
| Total n-6-FA (%) | 42.01 ± 1.81 | 39.21 ± 1.33 |
| (n-3)/(n-6) | 0.116 ± 0.011 | 0.112 ± 0.003 |
| Grand Total (1) | 3056.47 ± 217.42 | 4215.56 ± 283.18 * |
| Liver Mitochondria | | |
| | C | TG |
| Fatty acids | | |
| Short chains | | |
| 12:0 (%) | 0.045 ± 0.004 | 0.14 ± 0.009 * |
| Long chains | | |
| 14:0-24:0 (%) | 99.95 ± 0.004 | 99.86 ± 0.009 |
| Very Long chains | | |
| 26:0 (%) | 0.001 ± 8.55E-5 | 0.001 ± 4.36E-5 |

| | | |
|-------------------------|---------------|-----------------|
| Total MUFA (%) | 55.84 ± 0.42 | 52.88 ± 0.45 * |
| Total SFA (%) | 44.16 ± 0.42 | 47.12 ± 0.45 * |
| Total n-3-FA (%) | 12.59 ± 0.39 | 14.11 ± 0.25 * |
| Total n-6-FA (%) | 36.09 ± 0.39 | 32.92 ± 0.41 * |
| (n-3)/(n-6) | 0.349 ± 0.013 | 0.427 ± 0.010 * |
| Grand Total (1) | 0.134 ± 0.006 | 0.140 ± 0.005 |

Liver

| | C | TG |
|-------------------------|-----------------|-----------------|
| Fatty acids | | |
| Short chains | | |
| 12:0 (%) | 0.450 ± 0.057 | 0.578 ± 0.046 * |
| Long chains | | |
| 14:0-24:0 (%) | 99.52 ± 0.05 | 99.41 ± 0.046 |
| Very Long chains | | |
| 26:0 (%) | 0.002 ± 3.07E-4 | 0.002 ± 6.65E-5 |
| Total MUFA (%) | 56.25 ± 0.56 | 55.77 ± 0.76 |
| Total SFA (%) | 43.72 ± 0.549 | 44.22 ± 0.766 |
| Total n-3-FA (%) | 9.52 ± 0.47 | 11.03 ± 0.27 * |
| Total n-6-FA (%) | 33.58 ± 0.34 | 32.69 ± 0.27 * |
| (n-3)/(n-6) | 0.284 ± 0.016 | 0.338 ± 0.01 * |
| Grand Total (1) | 26.94 ± 1.41 | 34.15 ± 1.03* |

(1) plasma: ng/μL ; liver: ng.μg wet liver-1 ; liver mitochondria: ng.g prot-1

*Significantly different from control group ($p < 0.01$).

Values are expressed as relative amounts from the total fatty acids content as means ± SE from n=7 in C group and n=9 in TG group. Grand Totals are expressed in ng/μL for plasma, in ng/mg protein for mitochondria and in ng/μg for liver.

Training in mother was responsible for a marked change in offspring body lipid content and composition. Total plasma and liver fatty acids were increased in offspring by mother's training and fatty acid composition was markedly altered (Table 7). Indeed there is an increased in fatty acids short chain in plasma, liver and liver mitochondria with a decreased in long chain (in plasma and liver mitochondria, not in liver) in offspring from training mother compared to offspring from control mother. Mono unsaturated fatty acids (MUFA) were lower (in plasma and liver mitochondria) TG group compared with C group while saturated fatty acids (SFA) were higher (in plasma, liver and just a tendency for liver mitochondria). Total n-3 fatty acids and n-3/n-6 ratios measured in liver and liver mitochondria were also higher in offspring from training mother compared to offspring from control mother. The changes found in liver and liver mitochondria were not similar to those found in the plasma where there is no difference in total n-3 fatty acids and n-3/n-6 ratio. The increasing of short chain fatty acids enhances the membrane fluidity (Puri 2006).

Discussion

Considering the potential negative consequences on the newborn of an excessive oxidative stress during pregnancy, the aim of this study was to investigate whether endurance training of the mother during pregnancy had effects on the redox status of the newborn.

For the first time, to our knowledge, we show that submaximal maternal exercise modifies the offspring mitochondrial function and structure and that it may be possible to act on the redox state in offspring by modifying maternal physical activity before and during pregnancy. Our purpose was to test the effect of daily chronic exercise during the gestation on fit females that were already active before gestation. We chose to train them for 4 weeks before mating; as

such duration is usually sufficient to get the first signs of adaptation to chronic endurance exercise (Wisloff U 2001). Studies using voluntary exercise in rodents with running wheels showed that the running distance decreased dramatically in the last week of gestation (Carter LG 2012, Eclarinal JD 2016). So we chose a compulsory treadmill exercise that allowed us to test the effects of exercise throughout all the gestational periods, with no change in training parameters and especially no reduction in exercise intensity and duration until day 18 of the gestation. To ensure that these adaptations were significant, we also chose a sufficiently high intensity. We used intensity parameters that are classically found in the literature (Bedford 1979, Lahaye 2010, Zguira 2013). Based on data collected in our laboratory, the speed and slope of the treadmill that we used would correspond to an intensity of about 55% of the maximal aerobic speed. That intensity matches the guidelines for exercise in pregnant women from a lot of countries around the world (Evenson 2014). However, the frequency and the duration are above most of the guidelines, except those from Denmark and the US that recommend at least 30 min of moderate-intensity exercise most of the days or daily (Evenson KR 2014).

We first established that endurance training imposed on rats during gestation was effective and did not disturb the gestation to the point of endangering the life of the mother and offspring. In order to, maximal activity of citrate synthase (CS) was performed in liver and in muscle of the control and training mother. CS activity is a classic marker of skeletal muscle and liver mitochondrial adaptation to exercise training (Holloszy 1984, Carter 2012, Vigelso *et.al.*2014). Its increase is a strong indication of adaptation to training. CS activity in liver and in muscle increased after mother training session ($P < 0.05$, Table 2) indicated that our training protocol was effective on training mothers and induced mitochondrial modifications.

Moreover, our result on litter was not difference with previously studies reported that voluntary and controlled low-intensity exercise during gestation has no negative consequences on dams and litter (Carter 2013, Platt 2013). Our training protocol appears to be safe for the mother and their pups since no differences were found in the number of pups per litters and the probability of gender their pups (Table 3), suggesting that moderate intensity endurance training before and during gestation has no negative effect on glucose metabolism of mother and offspring affect and maintaining the fulfillment of nutrients to the fetus (Hopkins 2010, Clapp 2006). Maternal exercise had no effect on maternal body weight, food intake or body composition.

On the contrary, no differences in liver or muscle citrate synthase activity in the offspring. This finding may caused by the exercise training was not performing to the offspring. In contrast to our finding, a study in mouse brain by Park *et.al.*2013 reported an increase in activity of citrate synthase of offspring born to trained mothers. Different to muscle and liver, the increased citrate synthase activity in the brain of offspring born to trained mothers suggests that brain is a highly oxygen and glucose dependent organ in order to maintain the $\text{Na}^+\text{-K}^+$ membrane potential required for transmission of the nerve impulses (Berg *et.al.*2002).

Our study presents novel data on modifying of offspring's oxygen consumption of mitochondria. Different to previous study in biochemical adaptation response to regular strenuous exercise by Holloszy 1967 that increased the oxygen consumption on mother, we found the mitochondrial oxygen consumption of offspring born to trained mothers was significantly decreased in state 3 and state 4 of the liver, but not significant in the muscle. This result may be as an adaptation to maternal exercise training before and during gestation. The decreasing of oxygen consumption can reduce the rate of ROS production, because 1-3%

of the oxygen used by mitochondria is partially reduced to form ROS as a result of electron leakage (Turrens 2007, Fusch 2003).

The reduction of ROS production is confirmed by our data in the current study (Figure 20) that showed a significant decrease in spontaneous H₂O₂ production with glutamate/malate +succinate (GMS) in the liver and muscle mitochondria of offspring born to trained mothers. This finding not differ with a study by Radak *et.al.*2005 reported a reduction in basal ROS production and in oxidative damage in rats subjected to moderate intensity exercise training. Moreover, the reducing of oxygen consumption that followed by the decreasing of enzymatic activity in complex II in the liver and muscle mitochondria and complex III in the muscle mitochondria (Table 3) might reduce the electrons flow in the electron transport chain in units of time. The decreasing of electron flow may reduce the electron leakage in the electron transport chain leads to reduce generation of ROS superoxide O₂⁻ (Park *et.al.* 2013). Moreover, the increased activity of Complex IV in liver and in muscle causes the electron have reached the complex III immediately transferred into the matrix of mitochondria to join with half of Oxygen (½ O₂) forms water (H₂O) which ultimately decreases ROS production (Harper *et.al.* 2004, Boveris *et.al.*1973; Kudin *et.al.* 2004). While the total amount of ROS production by Antimycin A (AA) were higher than those with GMS caused by a reverse-electron flux in complex I of the respiratory chain.

Since there is a link between mother and her fetus during intrauterine life (Argüelles 2006), we examined the redox state in the newborn as a representation of maternal environment. The current study showed an alteration in the redox state of newborn rats. Thiols level, as a marker of protein damages by oxidative stress, was significantly lower in the plasma of newborns

from trained mothers, not in the liver offspring. But the activity of GPx was significantly increased both in the plasma and liver tissue of newborn rats (Figures 21 and 22). This finding indicates that there is an increasing of protein damages by oxidative stress, but the rat offspring from trained mothers are able to adaptation by enhancing the activity of GPx in the plasma and liver tissue in order to maintain the balancing redox. No significant difference in thiols level of the liver tissue may be caused by a recycle of thiols synthesis in the liver offspring from trained mother. Regarding that the redox balance has a major role throughout embryonic, fetal, and postnatal development (Phyllis 2004) and that mitochondrial function plays an important role in cellular redox/oxidative balance control with consequences on the life and death of cells (Apostolova *et.al.* 2015), the balanced redox of rat offspring which is induced by moderate intensity exercise training can stimulate optimally programming of the cellular growth and fetal organogenesis, and also the structure, number and capability of offspring mitochondria during fetal life.

Moderate-intensity endurance training before and during gestation increased significantly the levels of reduced-Q9, reduced-Q10, oxidized-Q9, and quinone pools (total Q9+Q10) (Table 6) in the liver tissue of the offspring. These higher levels of Quinones, which are necessary to Complex I and Complex III, would increase the probability of catching the electrons within the electron transport chain and would prevent them from leaving the transport chain which is known to frequently occur at both Complexes I and III (Boveris and Chance, 1973; Kudin *et.al.* 2004). This would result in the reduction of ROS superoxide (O_2^-) production from escaped highly energized electrons. Cytochromes *a+a3*, transfer the electrons from cytochrome *c* to the molecular oxygen to form H_2O in the mitochondrial matrix and thus the increase in cytochrome *a+a3* that we found would contribute to inhibit the formation of ROS

superoxide (Yano T, 2002) by orienting electrons toward the last step of the electron transfer chain. A decrease in ROS production will increase producing of cytochromes a+a₃ with possible mechanism as follows: a decrease in ROS production leads to decreasing of ROS attack against proteins and mitochondrial DNA induces enhancing of the expression of mitochondrial proteins which is required to form cytochrome oxidase. Because the genome cytochromes a+a₃ derived from mitochondria (Fontanessi *et.al.* 2006), the increased expression of mitochondrial proteins causes increased production of cytochromes a+a₃. Moreover, the increasing of cytochrome oxidase contributes to an increase of energy storage in the formation of an electrochemical gradient that will be used by the oxidative phosphorylation system for the synthesis of ATP (Fontanessi *et.al.* 2006).

In addition to these findings, α -tocopherol, which is accepted as the most potent radical-scavenging lipophilic antioxidant (Lobo *et.al.* 2010) was increased in the offspring. In biological membranes, α -tocopherol is present in a low ratio of α -tocopherol: phospholipids, which are abundant and highly susceptible to oxidative damage. For every 1000–2000 molecules of phospholipid, it is predicted only 1 molecule of α -tocopherol is present for antioxidant defense (Sen *et.al.*2000). Hence, the increased level of α -tocopherol has beneficial effects either as an antioxidant by a peroxy radical scavenger (Traber 2007) and preventing the free radical chain reaction of lipid peroxidation (Apostolova *et.al.*2015) or non-antioxidant effect to modulate a number of cell functions (Azzi 2007) such as inhibition of monocyte reactive oxygen species, the changes in cellular membrane fluidity (Rimbach *et.al.* 2002, Van Aalst *et.al.* 2004), and also to maintain the integrity of long-chain polyunsaturated fatty acids (PUFA) in the cellular membrane to maintain their function (Traber 2007). These findings suggest that maternal exercise training reduced ROS production of offspring through

increased acceptor electron within respiratory chain and antioxidant in the mitochondria and tissue of the liver of the offspring.

We also present novel data on alteration in composition of fatty acids in plasma, liver, and liver mitochondria of offspring born to trained mothers. During pregnancy, growth of new tissue increases the requirement for essential fatty acids (EFA) and also raises mobilization of EFA from maternal tissue stores to the fetus for vital organ development (Ogburn 1991). However, Holman *et.al.*1991 reported significant lower essential fatty acids in pregnant women compared to non-pregnant women, suggesting lower mobilization EFA to the fetus. The deficiency must be minimized for the special needs of the fetus for brain and vital organ development. The present study showed an increase in total fatty acids in plasma, liver and liver mitochondria of offspring born to trained mothers. We also found an increase in levels of n-3 fatty acids, followed by decreasing levels of n-6 fatty acids and increasing n-3/n-6 ratio in the liver tissue and liver mitochondria of offspring from training mothers. The enhancing of omega-3 (n-3) which plays a role in the reduction of inflammation (Valentine *et.al.*2004) and increasing the ratio of n-3/n-6 stimulated by maternal exercise protects the liver cells and liver mitochondria of the inflammatory process of the offspring. Moreover, regarding to increasing n-3/n-6 fatty acid ratio could reduce the consequences of obesity-associated inflammation (Heerwagen *et.al.* 2013), it could be expected that the increasing of n-3/n-6 fatty acid ratio in the offspring of trained mother may reduce the risk of obesity-induced inflammation during fetal metabolic programming.

Our data also showed, in offsprings from trained mothers, that the composition of short chain fatty acids increased significantly, while the long chain fatty acids decreased significantly in the plasma, liver and liver mitochondria. The changes in this composition have a good effect,

because short chain fatty acids are more resistant to free radical attack or lipid peroxidation compared with long chain fatty acids (Hulbert 2008) leads to protection of lipids and protein membrane damage and improving the membrane function to maintain their bioactivity. In contrast, Argüelles 2006 showed the damage effect of the lipid peroxidation of the membrane caused the degeneration of membrane structure and loss of function of membrane proteins leads to changed membrane fluidity and increased membrane rigidity, and also increased membrane permeability due to the presence of oxidized lipids (Wong-ekkabut *et.al.* 2007).

CONCLUSIONS

Moderate intensity exercise training 4 weeks prior to and until the 18th day of gestation:

1. Modifies oxygen consumption and activity of complex respiratory chain II, III, IV in rat offspring
2. Reduces ROS production in rat offspring
3. Increases endogen antioxidant (GPx, Quinone), cytochrome a+a3, and α -tocopherol in rat offspring
4. Increases the level of short chain FA, n-3 FA, and ratio n-3/n-4 FA in plasma, liver, and liver mitochondria of offspring

III.2 THE INFLUENCE OF EXERCISE AROUND GESTATION ON ANTHROPOMETRY AND GLUCOSE METABOLISM

III.2.1 The influence on anthropometry in the rat offspring

Table 8. Body and relative organ weights, and area of the pancreatic islets of offspring at weaning

| | C | TG |
|-----------------------------------|------------|-------------|
| Body weight (BW) (g) | 51,6±3,4 | 47,5±1,2 |
| Organ weight (g/kg BW) | | |
| Liver | 31,81±0,75 | 30,97±0,19 |
| Kidney | 6,02±0,11 | 5,99±0,11 |
| Adipose tissue | 8,68±0,57 | 9,00±0,35 |
| Muscle | 4,58±0,13 | 4,54±0,07 |
| Pancreas | 4,54±0,14 | 3,96±0,18* |
| Area of Islets (µm ²) | 44669±6761 | 22822±4036* |

Data are the mean ± SE (n = 16 for C; n = 17 for TG; n = 11 to 13 for the area of islets).

*Significantly different from the control group ($p < 0.05$). Adipose tissues weight corresponds to the sum of mesenteric, retroperitoneal and epididymal weight.

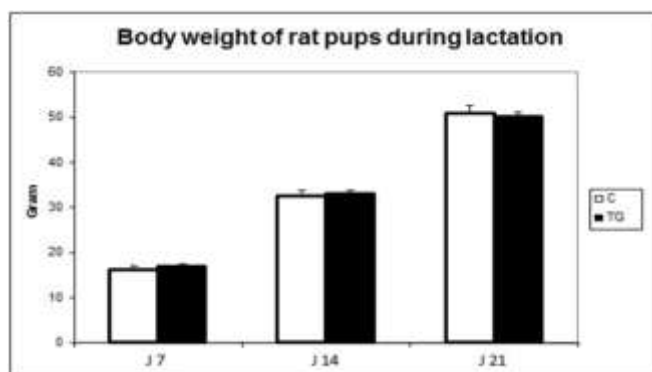


Figure 23. Body weight throughout the experiment in offspring from sedentary (C) or trained (TG) mothers

Values are means ± SE of n = 9 rats/group.

*Significantly different from the control group ($p < 0.05$).

At weaning, the body weight was not different between the two groups of pups (51.6 ± 3.4 vs. 47.5 ± 1.2 g, respectively, $p > 0.05$) (Table 8). Relative organ weights were not different between the two groups of pups for liver, kidney, muscles and adipose tissue but pancreas relative weight was significantly lower in TG group. In addition, the average area of pancreatic islets measured in 21-old days pups was significantly lower in the offspring of the TG group compared with those from the C group (Table 8).

III.2.2 The influence on the weight and structure of pancreas in offspring

The pancreas weight of offspring at weaning was shown in Table 8. The relative weight of the pancreas was significantly lower in the offspring of the TG group than in the C group (3.96 ± 0.18 vs. 4.53 ± 0.14 g.kg⁻¹, respectively, $p < 0.05$) (Table 8). In addition, the average area of pancreatic islets measured in 3-weeks old pups was significantly lower in the offspring of the TG group compared with those of C group (Table 8, Figure 24).

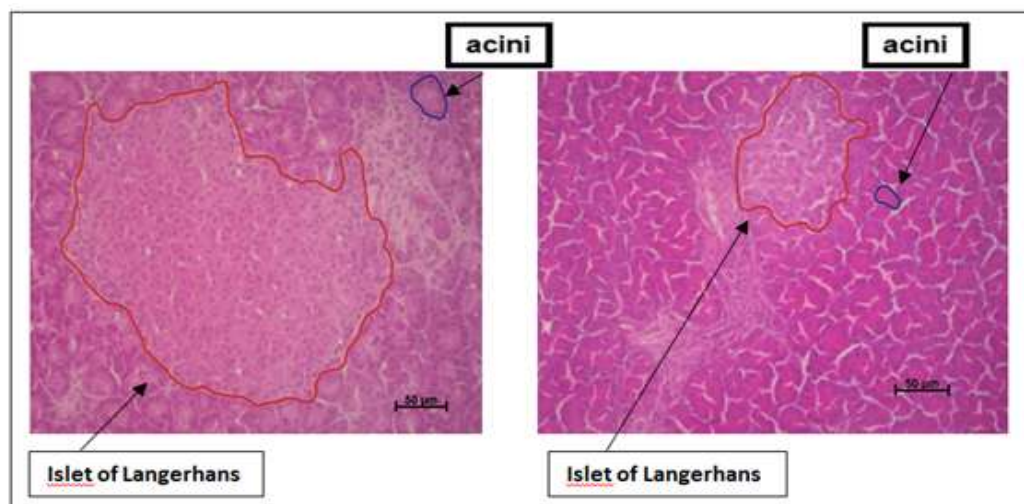


Figure 24. Histological structure of Langerhans islands and acini of pancreas of offspring from control (C, left) and trained (TG, right) mothers.

Values are means \pm SE of $n = 9$ rats for C group; $n = 12$ rats for TG group.

*Significantly different from control group, ($p < 0.05$)

Histological analysis of pancreas structure showed that the size islets of Langerhans of offspring from TG was significantly smaller ($22822 \pm 4036 \mu\text{m}^2$) than those from C mothers ($44669 \pm 6761 \mu\text{m}^2$) ($p < 0.05$) and that the acini of the rat from control mothers were larger than those from trained mothers (Figure 24).

III.2.3 The insulin content of the pancreas from the β -islets

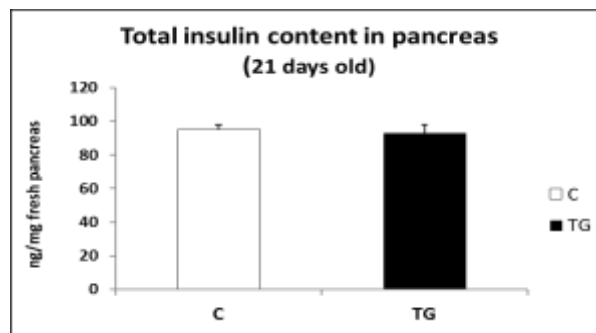


Figure 25. Insulin content of the pancreas from the β -islets of offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of $n = 6-8$ rats/group.

The pancreatic insulin content was not different between animals of TG and C mothers, at the age of 21 days old (Fig. 25).

III.2.4 The insulin secretion from the β -islets of offspring

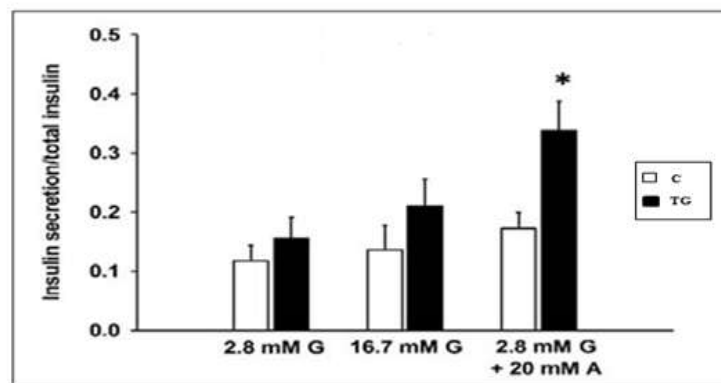


Figure 26. Insulin secretion from the β -islets of pups aged 30 days after incubation in low glucose (2.8 mM) and then high glucose (16.7 mM) or low glucose (2.8 mM) + Arginine (20mM) of pups from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of n = 6-8 rats/group.

*Significantly different from the other group ($p < 0.05$),

At weaning (21 days old), the secretion of insulin from the β -islets of pups of trained mothers was not different from those of sedentary mothers, whether provided by low glucose (2.8 mM) or high glucose (16.7 mM) (Figure 33). However, the secretion of insulin from the islets of pups of trained mothers was significantly higher than those β -islets pup sedentary mothers down glucose requirements (2.8 mM) + arginine (20 mM) (+ 96%, $p = 0.01$) (Figure 26).

III.2.5. Observation on basal/fasting blood glucose and insulin in 28 days old offspring

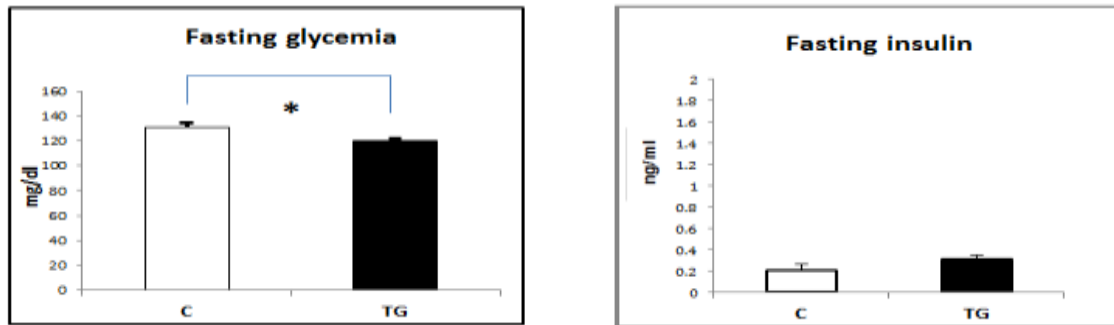


Figure 27. Basal/fasting blood glucose (left panel) and insulin (right panel) in offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of n=6 rats/group.

*Significantly different from the other group ($p < 0.05$)

After a 6h-fast, 28 day-old pups from trained mothers showed a lower glycemia compared with pups from control mothers (119.7 \pm 2.4 vs. 130.5 \pm 4.1 mg.dL⁻¹, respectively, $P < 0.05$) (Figure 26) while insulinemia was similar in both groups even if it tended to be higher in rats from trained mothers (0.31 \pm 0.04 vs. 0.21 \pm 0.05 ng.mL⁻¹ for trained and control mothers, respectively, $P = 0.158$) (Figure 26).

III.2.6. The influence on glucose tolerance (IpGTT) of offspring at 21 days old

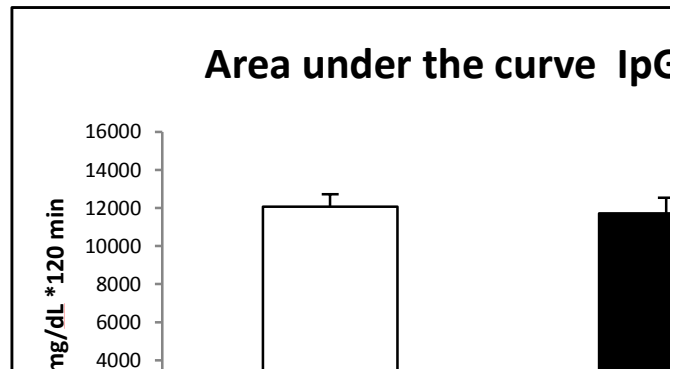


Figure 28. Area under the curve of glucose tolerance of offspring at 21 days old from control (C) and trained (TG) mothers.

Values are means \pm SE of n = 10-12 rats/group.

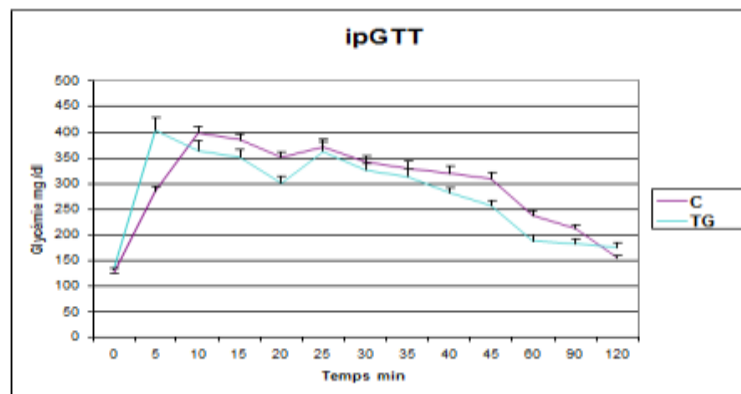


Figure 29. Glycemia of glucose tolerance of offspring at 21 days old from control (C) and trained (TG) mothers.

Values are means \pm SE of n = 10-12 rats/group.

At weaning, glucose tolerance was not different between the offspring of C and TG groups (108 ± 12.6 vs. 105 ± 11.3 mg/dL, respectively). Indeed, throughout the duration of ipGTT test, blood glucose was not different between the two groups (Figure 28). This is confirmed by the area under the curve, used as a measurement of the elimination of blood glucose, which is not significantly different between the two groups of pups (12068 ± 647 vs. 11717 ± 821 mg/dL. * 120 min for TG and C, respectively) (Figure 27).

III.2.7. The influence on insulin tolerance (IpITT) of offspring at 28 days old

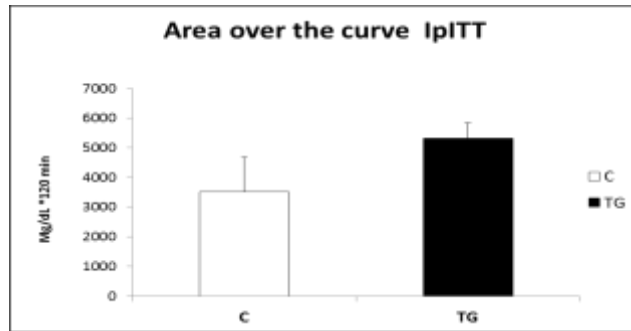


Figure 30. Area over the curve of insulin tolerance of offspring at 28 days old from control (C) and trained (TG) mothers.

Values are means \pm SE of $n = 10-12$ rats/group;

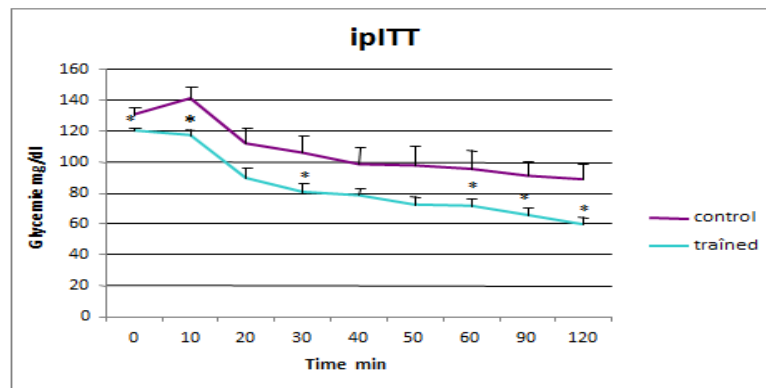


Figure 31. Glycemia of insulin tolerance of offspring at 28 days olds from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of $n = 10-12$ rats/group.

*Significantly different from the other group ($p < 0.05$),

At 28 days of age, pups from TG mothers had a lower blood glucose level than those from C mothers (118 ± 5.6 vs. 132 ± 4.3 mg/dL, respectively) for the duration of the test ipITT ($p < 0.05$) (Figure 30). However, the area over the curve is not significantly different between the two groups, though it tends to be higher in the offspring from the TG group (5318 ± 513 vs. 3507 ± 1178 mg.dL-1 * 120 min for TG and C, respectively) (Figure 29).

III.2.8 The influence on insulin signaling (PKB) of 21 days old offspring

III.2.8.1 Insulin signaling (PKB) in offspring at basal state

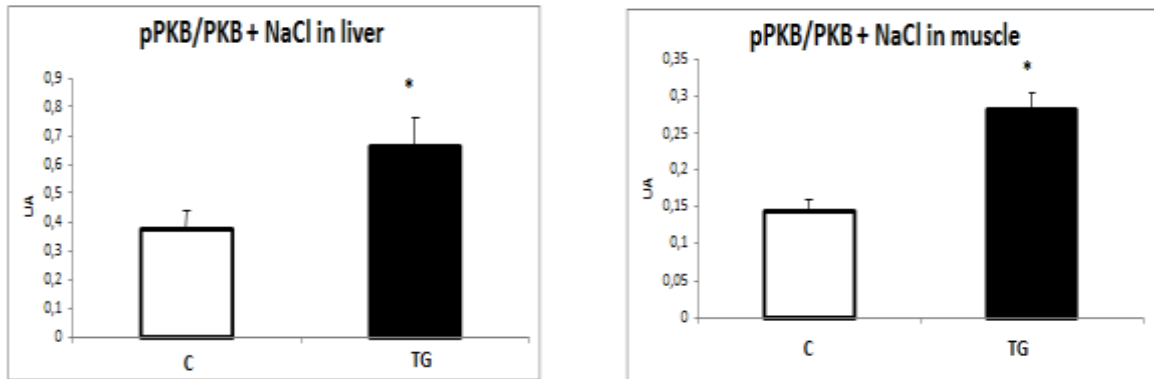


Figure 32. PKB phosphorylation (pPKB) in the basal state of the liver (upper panel) and muscle (lower panel) of offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of n=6 rats/group.

*Significantly different from the other group $p < 0.05$

III.2.8.2 Insulin signaling (PKB) activity stimulated by a high dose of insulin in offspring

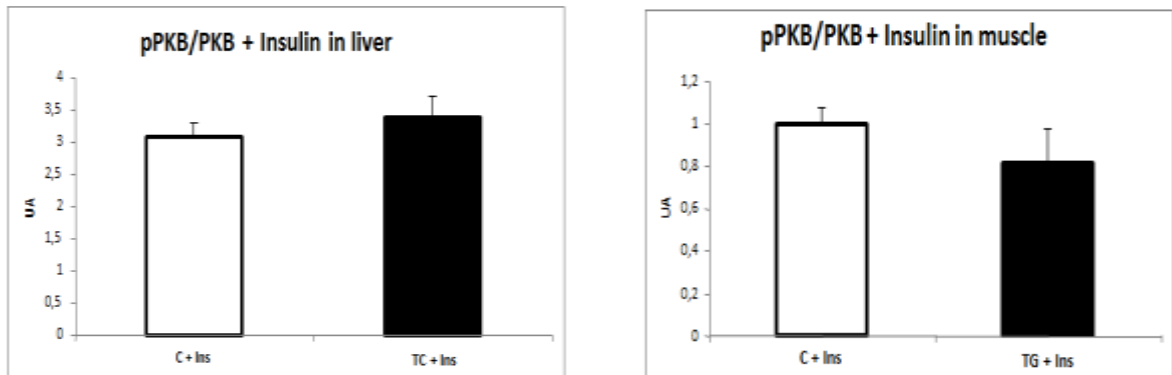


Figure 33. PKB phosphorylation (pPKB) under stimulation by a high dose of insulin in the liver (upper panel) and muscle (lower panel) offspring from sedentary (C) or trained (TG) mothers.

Values are means \pm SE of $n = 6$ rats/group.

Insulin signalling was studied by measuring the level of expression of PKB and its phosphorylated form (pPKB) both in liver and gastrocnemius muscle after either a NaCl or an insulin load. Figure 31, 32 shows the pPKB/PKB ratio obtained.

At 21 days of age, pups from trained group showed an increase in the pPKB/PKB ratio compared with pups from Control group in basal condition (respectively T- and C-) both in liver (+77%) and muscle (+97%) (Figure 31). However, these differences were not seen after an insulin load injection (T+ and C+) neither in liver nor in gastrocnemius muscle (Figure 32).

DISCUSSION

We have found that maternal moderate intensity endurance training 4 weeks before mating until 18th of gestational can improve long-term metabolic outcomes in offspring. Trained mothers have no effect on body weight at birth and during the lactation period. This finding was supported by Hopkins *et.al.* 2011 who demonstrated in humans that within normal, healthy pregnancy, maternal insulin sensitivity is regulated to achieve optimal fetal growth persistently and showed different response to the exercise training compared with non-pregnant individuals. Moreover, there was no significant difference in fat body mass between the two groups of offspring. Additionnaly, these results from Carter *et.al.*2013 and Clapp 2006 reported no difference in body weight and fat mass of offspring born to both trained and sedentary mothers consistently with our findings. These results suggest that moderate-intensity endurance training during 4 weeks before mating until 18th of gestational day did not affect the delivery of the nutrients to the fetus.

The effect of maternal exercise on mother weight gain during pregnancy. In the present study, we found that the food intake of trained mothers tended to increase ($p = 0.098$) during the last week of pregnancy, but the weight gain tended to be lower ($p = 0.136$) during the final weeks of pregnancy when compared to sedentary mothers as shown at Table 3. In contrast, the total mass of visceral fat was significantly lower ($p < 0.05$) in trained mothers than those in the sedentary mothers. The results suggest that a maternal moderate intensity endurance training 4 weeks before gestation until the 18th day of gestation induces increased of glucose uptake into cells and prevents fat accumulation and development of obesity during pregnancy. This similar to the findings of Haakstad *et.al.*2011 who reported that exercise before and

during pregnancy could prevent the excessive gestational weight gain and reduced post-natal weight retention. Another study by Tobias *et al.* 2011 showed that intensive exercise during pregnancy will reduce the risk of gestational diabetes mellitus (GDM) by 51%. In addition to this, Hopkins *et al.* 2011 showed that within healthy pregnant woman, maternal insulin sensitivity is regulated to achieve optimal fetal growth persistently and showed a different response to the exercise training compared with non-pregnant individuals. On the other hand, the prevention of obesity in pregnant woman has a positive impact in the prevention of mitochondrial dysfunction in the fetus. This was indicated by Borengasser *et al.* 2011 who reported a significant decrease in mitochondrial function of liver of offspring born from obese mice, decreases in SIRT3 mRNA, mitochondrial protein content, fatty acid oxidation, ETC complex I, II, and ATPase activity, as well as in the expression of PGC-1 α mRNA.

The effect of maternal exercise on the weight and structure of pancreas in offspring. The weight of pancreas of the rat from trained mothers was lower (-11%) than those of the control group at the age of 21 days ($p < 0.05$). This finding was supported by the histological structure of pancreas showed that the size of islets and acini of the rat from control mothers were larger than those from trained mothers. This is probably related to the programming of the pancreatic mass of the fetus in the uterine. The sufficient levels of fetal insulin induces the optimal proportion and development of pancreatic β -cells mass, while the lack of fetal insulin would induce cell proliferation, increase in vascularization and hyperplasia of β -cell pancreas during its development leads to larger islets of Langerhans. Due to larger size, central part of islets would be more susceptible to hypoxia leads to disruption of insulin synthesis in postnatal rat pups (Duvillié *et al.* 2002). In addition to this, the results of our study are in line with the results of Carter *et al.* 2012 who reported that smaller size of Langerhans islets was

more effective in insulin synthesis than larger islets of Langerhans due to the higher vulnerability to hypoxia in the center part. This will affect the synthesis of insulin of offspring. Regarding to this, the size of pancreas indicates that maternal moderate intensity endurance training during 4 weeks before gestation until the 18th day of gestation induces the development of optimal proportion of fetal pancreatic β -cells mass related to the normal function of pancreatic β -cells.

The effect of maternal exercise on the glucose and insulin tolerance of offspring. The impact of exercise training during pregnancy on glucose tolerance test of rats offspring at 21 days of age was not different between the two groups ($p > 0.05$). While, as response to ipITT, offspring from trained mothers had lower blood glucose levels than those from sedentary mothers during the whole ipITT. However, the area over the curve (AOC) was not significantly different between the two groups. These results suggest that moderate intensity endurance training during 4 weeks before mating until the 18th day of gestation does not affect the insulin sensitivity of offspring at weaning or during lactation period. This finding is supported by Carter 2012, 2013 who reported that differences in insulin sensitivity of rat were detected until offspring reached 10-months of age.

The effect of maternal exercise on insulin secretion of offspring.

We then looked at the insulin secretory capacity of these islets in various conditions. In low glucose condition, basal islet insulin secretion was not affected by maternal exercise at weaning. Under high glucose condition, the stimulated insulin secretion only tended to be higher in weaning rats from the trained mothers. This may be due to the fact that insulin release of β -cells islet in response to glucose stimulation is reduced in young rats (Bliss *et.al.*

1992). To clarify this problem, we then assessed the insulin secretion after a glucose + arginine incubation known to be efficient in fetal and young rats. In such a condition, we found that maternal exercise enhanced the islet insulin secretion in their offspring which may be due to smaller size of islets. This is consistent with the study by Carter *et.al.* 2012 and Huang *et.al.* 2011 who showed that small islets are more effective in insulin synthesis.

Moreover, the glucose enters through GLUT 2 into the pancreatic β -cells where it is detected by the glucokinase, which phosphorylates it into glucose-6-phosphate (G6P). Oxidation of glucose by mitochondria is used for ATP production and would increase the ratio of ATP/ADP. Increasing the ratio of ATP/ADP contributes to the inhibition of potassium channels (K_{ATP}), which causes membrane depolarization, opening of voltage-gated calcium channels, resulting in increased Ca^{2+} influx. Increased intracellular calcium stimulates the secretion (exocytosis) of insulin. Therefore, the function of the mitochondria may be correlated with β -cell function through the ratio of ATP/ADP (Fariss 2005). Improved mitochondrial function induced by a maternal regular exercise would increase the synthesis of ATP quicker and more efficient than in the sedentary group.

The effect of maternal exercise on insulin signaling (PKB phosphorylation) of offspring.

This tendency of elevated insulinemia without a hyperglycemia in pups from trained mothers could be an early marker of insulin resistance. To precisely assess such insulin resistance, we then performed an intra peritoneal glucose tolerance test (ipGTT) and an insulin tolerance test (ipITT) on our animals. Our results show that glucose disposal, as judged by the AUC during ipGTT, and overall insulin sensitivity reflected by the AOC during ipITT was not affected by maternal training. Taken together, these results suggest that, despite a trend of elevated insulinemia,

pups from trained mothers were not insulin resistant. This tendency of higher blood insulin could be also a sign for alterations in the insulin signalling. The main insulin sensitive tissues include skeletal muscle, liver and adipose tissue. Thus, we examined pPKB/PKB ratio in gastrocnemius muscle and in liver, both in basal conditions and after an insulin load. In basal conditions, the higher pPKB/PKB ratio measured in both tissues in 21 days old rats from trained mothers might be merely due to the trend in a higher plasma insulin level mentioned above. Results after the insulin load confirm that hypothesis since there is no difference in pPKB/PKB ratio between the two groups in that insulin stimulated condition. So, we can conclude that maternal exercise before and during gestation did not modify liver and muscle insulin pathway activation in offspring at weaning.

CONCLUSIONS

Based on the above results, it looks that moderate intensity exercise training during 4 weeks before mating to the 18th day of gestation tend to lower weight gain during pregnancy of trained mother, alters the microscopic structure of islets of Langerhans in the offspring, tend to increase basal insulin, decreases basal glycemia in the rat offspring, and increases insulin secretion and insulin signaling in rat pups.

**RESULTS AND DISCUSSION OF
STUDY II:
THE INFLUENCE OF DIET AROUND GESTATION**

III.3 HIGH FRUCTOSE DIET AS AN ALTERNATIVE EXPERIMENTAL MODEL OF GESTATIONAL DIABETES MELLITUS ON RATS

III.3.1 The influence of high fructose diet on anthropometric parameters of mothers and offspring

Table 9. Anthropometric parameters of mothers and rats pups after delivery

| | Control | Fructose | P value |
|---|-------------|---------------|-----------|
| Weight gain (g) | 17.2 ± 0.63 | 21.5 ± 1.95 | P > 0.05 |
| Total visceral fat (g/100 g body weight) | 3.41 ± 0.53 | 4.11 ± 0.35 | P > 0.05 |
| Number of pups/dam | 9.5 ± 2.18 | 10.0 ± 2.28 | P > 0.05 |
| Male pups/dam | 4.0 ± 0.58 | 3.6 ± 0.81 | P > 0.05 |
| Female pups/dam | 5.5 ± 1.66 | 6.4 ± 1.69 | P > 0.05 |
| Weight pups (g) all | 8.5 ± 0.9 | 9.5 ± 1.7* | P = 0.002 |
| Male pups | 8.94 ± 0.26 | 10.30 ± 0.45* | P = 0.03 |
| Female pups | 8.17 ± 0.2 | 9.13 ± 0.26* | P = 0.01 |
| Liver weight all (g/100 g body weight) | 3.54 ± 0.05 | 3.02 ± 0.04* | P < 0.001 |
| Liver male pups | 3.58 ± 0.09 | 2.94 ± 0.07* | P < 0.001 |
| Liver female pups | 3.51 ± 0.06 | 3.07 ± 0.05* | P < 0.001 |
| Brain weight all (g/100 g body weight) | 4.14 ± 0.06 | 4.12 ± 0.06 | P > 0.05 |
| Brain male pups | 3.98 ± 0.07 | 3.93 ± 0.1 | P > 0.05 |
| Brain female pups | 4.25 ± 0.07 | 4.21 ± 0.07 | P > 0.05 |

Values are expressed as mean ± SE. Number of mothers rats: n=4 for control fed group, n=5 for fructose fed group, and number of pups: n=38.

*Significantly different from control group (p < 0.05)

The results on the anthropometric parameters showed that the weight gain and the total fat mass in mothers fed high fructose were not significantly different compared to those from the control diet ($p > 0.05$). In contrast, maternal diet rich in fructose significantly increased the birth weight of their pups compared to those from mothers fed a control diet as shown in Table 8. The livers weight of pups from the F group was significantly lower than those from the control group, but the brain weight was unaffected by the maternal diet.

III.3.2 The influence of high fructose diet on biochemical metabolic parameters in mothers and offspring

Table 10. Biochemical metabolic parameters in mothers and rats pups after delivery

| | Control | Fructose | P value |
|------------------------------|-------------|----------------|-----------|
| Glycemia (mg/dL) | 143 ± 4.14 | 163 ± 3.60* | P = 0.008 |
| Insulin (ng/mL) | 0.58 ± 0.74 | 1.96 ± 0.80* | P = 0.016 |
| HOMA-IR(U) | 5.13 ± 0.75 | 19.46 ± 10.71* | P = 0.014 |
| Cholesterol (mg/dL) | 54 ± 0.01 | 66 ± 0.03* | P = 0.05 |
| Triglycerides (mg/dL) | 33 ± 0.05 | 88 ± 0.20* | P = 0.02 |
| Glycemia all pups (mg/dL) | 106 ± 2 | 78 ± 2 * | P < 0.001 |
| Glycemia-male pups | 112 ± 3 | 74 ± 3* | P < 0.001 |
| Glycemia-female pups | 100 ± 2 | 81 ± 2* | P < 0.001 |

Values were expressed as mean ± SE. Number of mothers rats: n=4 for control fed group, n=5 for fructose fed group, and the number of pups: n=38

The values of biochemical parameters of mother rats showed that a high fructose diet increased significantly the levels of glycemia, cholesterol, triglycerides, and also in insulin, leading to a significant increase in insulin resistance supported by the result of HOMA test. In contrast, the glycemia level of newborns was decreased with high fructose diet ($p < 0.05$).

Discussion

Modification of nutrition and supplementation during gestation can influence oxidative balance during fetal life and mitochondrial function as well as glucose metabolism of offspring. The nutritional status during critical periods of early life is an important determinant for proper development of the organism and maturation of metabolic and endocrine systems. Consumption of excessive fructose for long periods has potentially negative effects on metabolic function (Sakar 2009). Furthermore, gestation is marked by compromised insulin sensitivity; therefore, maternal high fructose diet may pose a challenge for the health of the mother and offspring. This is associated with weight gain of mother during pregnancy, high birth weight of offspring, impairing of glucose metabolism of offspring, as well as gestational diabetes mellitus (GDM) (Donovan 1990, Bernard 2005). On the other hand, there is no satisfactory model for studying GDM since streptozotocin, an agent usually used for experimental diabetes induction, might not be suitable to make experimental model of GDM as it induces pancreatic β -cells necrosis that leads to Type 1 diabetes (Damasceno 2014), while the features of GDM are more like Type 2 diabetes. Besides, a diet rich in fructose has been used to study insulin resistance in experimental research (Busserolles 2003, Kolderup 2015), but not in pregnancy. In addition, it has been shown that GDM is related with oxidative stress and increased insulin resistance. Thus, the purpose of this study was to evaluate diet rich in fructose as an alternative model of GDM by inducing insulin resistance on rats.

The levels of cholesterol and triglycerides rise as a response to high fructose diet. Fructose experience faster glycolysis in the liver than glucose, since it bypasses the regulatory pathway catalyzed by phosphofructokinase (Bender 2012). Therefore, diet rich in fructose

causes fructose to flood the pathway in liver. It increases fatty acids and triacylglycerol synthesis, VLDL secretion, serum triglycerides and finally increases LDL cholesterol level (Bender 2012). In addition to this, the increase in triglycerides level may also cause by an attenuation of the stimulation of insulin secretion after fructose administration that leads to reduce the lipoprotein lipase activity of adipose tissue and impair the triglycerides clearance (Chong *et.al*, 2007).

A significant elevation in the plasma levels of glucose and insulin was observed in mother's rats which were given a diet rich in fructose, suggesting that the ability of insulin to stimulate glucose disposal is clearly impaired in peripheral tissues associated with insulin resistance due to high fructose feeding. These results in the current study were supported by HOMA measurements that were significantly increased in the high fructose group. Similar results were obtained by Hininger-Favier *et.al*.2009 in adult male rats and Damasceno *et.al*.2002 with diabetes induced by streptozotocin. Insulin resistance in response to high fructose diet can be caused by several mechanisms: (1) high-fructose diet can induce intrauterine oxidative stress because some of the ingested fructose will be converted to glucose with a mean conversion rate of $41\% \pm 10.5$ from fructose to glucose in 3 to 6 hours after ingestion leading to hyperglycemia (Sun *et.al*. 2012). The hyperglycemia condition induces increase in ROS production and may cause pancreatic β -cell dysfunction (Goldstein 2005) that leads to impairing of insulin synthesis; (2) the elevated accumulation of ROS induces the activation serine / threonine kinase cascade which further phosphorylates several targets such as insulin receptor and insulin receptor substrate (IRS) proteins (Evans *et.al*. 2005). Enhanced serine phosphorylation of IRS will reduce its ability to phosphorylate tyrosine and accelerate the degradation of IRS-1 leading to disruption in glucose uptake by muscle, liver and adipose

tissues (Evans *et.al.*2005); (3) fructose diet alters several enzymes activity and metabolism of hepatic carbohydrate leading to hepatic insulin resistance, although the exact molecular mechanisms involved are unknown (Blakely *et.al.*1981, Tuovinen *et.al.* 1975); (4) high fructose diet does not decrease the level of insulin receptor IR and the IRS-1 in the liver and in the muscle, but reduces tyrosine phosphorylation of the insulin receptor after insulin stimulation (IRS-1 association with PI 3-kinase and IRS-1 association with SHP2) in the liver of rats. This indicates that fructose rich diet induces insulin resistance through the alterations in the early steps of insulin signal transduction that leads to an increased weight gain and to hyperinsulinemia in mothers (Bezera 2000) as were shown in Table 8, 9.

Moreover, maternal diet rich in fructose significantly increased the birth weight of newborns compared to birth weight of newborns from mothers with control diet. This finding was consistent with previous study which found that consumption of high fructose diets induces an increase in the risk of macrosomia (large birth weight) in the GDM women (KC *et.al.* 2015). The increase in birth weight may be caused by exposure to chronic maternal hyperglycemia during fetal life inducing hyperinsulinemia in the fetus and a higher deposition of fat leading to large birth weight (Capra *et.al.* 2013). It might also be caused by a higher expression of genes involved in lipogenesis but the gene expression of fatty acid catabolism is lower in fetuses of mothers fructose fed diet (Rodríguez *et.al.*2013). In addition, a study by Douard 2008 reported that under normal conditions, in the prenatal and suckling period of rat development, intestinal GLUT5 (the fructose transporter at the apical cell membrane) mRNA levels and fructose transport rates are very low. Therefore, administration of large amounts of fructose during fetal/neonatal life could result in intolerance to the substrate, which may impair body weight gain. Moreover, the increased body weight was followed by significantly

lower glycemia in both male and female offspring born to high fructose diet mothers. Hypoglycemia occurs as a response to prolonged fetal exposure to maternal hyperglycemia in intrauterine environment during fetal life leading to fetal hyperinsulinemia (Capra *et.al.* 2013). This finding was supported by the fact that the neonate of GDM mothers showed hypoglycemia associated with macrosomia as most common metabolic disorder (KC *et.al.* 2015). However, this is in contrast with the finding by Srinivasan 2006 who reported the fetal development in the hyperinsulinemic intrauterine environment due to high fructose maternal diet results in fetal hyperinsulinemia without significant changes in their plasma glucose levels

CONCLUSIONS

Maternal high fructose diet 4 weeks before and during gestation tend to increase the weight gain in mother rat, but it has no influence on number and gender of offspring. Moreover, it increases the glycemia, cholesterol, triglycerides, insulinemia and HOMA test leading to a significant increase in insulin resistance of mother rat. Our experimental GDM model induced by high fructose diet shows similarity in metabolic and anthropometric effects observed in women with GDM. It enables us to propose fructose diet as an experimental model of GDM. In contrast, high fructose diet during pregnancy increases the birth weight, decreases the glycemia level and liver weight of rat newborns.

III.4 THE INFLUENCE OF FRUCTOSE DIET (F) and FRUCTOSE-ENRICHED IRON DIET (FI) DURING PREGNANCY ON OXIDATIVE STRESS (OS), ANTHROPOMETRIC, AND BIOCHEMICAL PARAMETERS.

III.4.1 The influence of F and FI diet on anthropometric and biochemical metabolic parameters of mothers and offspring

Table 11. Anthropometric and biochemical parameters in mother rats fed with the F or the FI diet

| Parameters | F group | FI group | p value |
|-----------------------------|---------------|---------------|----------|
| Mother weight gain (g) | 21.5 ± 1.95 | 27.5 ± 1.6 | p > 0.05 |
| Mother glycemia (mg/dL) | 163.24 ± 3.6 | 161.44 ± 5.76 | p > 0.05 |
| Mother insulinemia (ng/mL) | 1.96 ± 0.78 | 1.05 ± 0.67 | p > 0.05 |
| HOMA-IR (U) | 19.46 ± 10.71 | 10.01 ± 6.15 | p > 0.05 |
| Mother cholesterol (mg/dL) | 66 ± 0.03 | 64 ± 0.66 | p > 0.05 |
| Mother triglyceride (mg/dL) | 88 ± 0.20 | 77 ± 0.17 | p > 0.05 |

Results were expressed as mean ± SE; n per group (F: n = 5 mothers and n = 50 pups) and (FI: n = 5 mothers and n = 38);

*Significantly different from F group (p < 0.05)

Table 12. Anthropometric and biochemical parameters in offspring of mother rats fed with the F or the FI diet

| Parameters | F group | FI group | p value |
|---------------------------|----------------|-----------------|----------------|
| Number of pups/dam | 10.0 ± 2.3 | 7.6 ± 2.2 | p > 0.05 |
| Male pups/dam | 3.6 ± 0.8 | 3.8 ± 1.7 | p > 0.05 |
| Female pups/dam | 6.4 ± 1.7 | 3.8 ± 0.9* | p < 0.05 |
| Pups glycemia (mg/dL) All | 78 ± 1.8 | 85 ± 1.8 * | p = 0.02 |
| Male pups | 74.4 ± 2.77 | 86.25 ± 2.52* | p = 0.003 |
| Female pups | 81.13 ± 2.26 | 83.18 ± 2.64 | p > 0.05 |
| Pup weight (g) all | 9.3 ± 0.10 | 11.0 ± 0.34* | P < 0.001 |
| Male pups | 10 ± 0.62 | 11.1 ± 0.16 | p = 0.06 |
| Female pups | 8.9 ± 0.37 | 10.9 ± 0.13* | p = 0.003 |

Results were expressed as mean ± SE; n per group (F: n = 5 mothers and n = 50 pups) and (FI: n = 5 mothers and n = 38);

*Significantly different from F group (p < 0.05)

Observation of anthropometric parameters showed fructose-enriched diet did not modify the levels of glycemia, insulinemia, cholesterol and triglycerides between the two groups of diet, except that the weight gain of the mothers fed FI diet tends to increase, but not significantly compared with F diet fed mothers (p = 0.0526) (Table 10). However, in the offspring born from the mothers fed the FI diet, the levels of glycemia and birth weight were higher and the number of females in the FI group was significantly lower than in F group.

III.4.2 The influence of F and FI diet on Oxidative Stress parameters in mothers and offspring

Table 13. Plasma OS parameters in mother fed with the F or the FI diet

| OS parameters of mothers | Group F | Group FI | <i>p</i> -value |
|---------------------------|----------------|-------------------|-----------------|
| TBARS ($\mu\text{M/L}$) | 4.6 ± 0.13 | $4.03 \pm 0.14^*$ | $p < 0.05$ |
| GPx activity (U/L) | 6349 ± 304 | 6464 ± 110 | 0.88 (NS) |
| FRAP ($\mu\text{M/L}$) | 273 ± 20 | 284 ± 25 | 0.36 (NS) |
| SH ($\mu\text{M/L}$) | 327 ± 38 | 295 ± 35 | 0.87 (NS) |

Results were expressed as mean \pm SE;

The number of mothers rats: n=4 for control fed group, n=5 for fructose fed group.

NS: not significant

The results of plasmatic OS parameters of mother rats are presented in Table 12. No significant difference was found on GPx activity and FRAP between mother rats fed with F and FI diet. However, surprisingly, iron decreases lipid peroxidation measured by TBARS in mother rats.

Table 14. OS parameters in the **liver** of offspring from mothers fed with the F or the FI diet

| OS parameters | F group | FI group | p value |
|----------------------------------|-------------------|-------------------|----------------|
| TBARS ($\mu\text{M/L}$) | 1.05 ± 0.2 | 0.99 ± 0.2 | $P > 0.05$ |
| Male | 1.12 ± 0.10 | 1.05 ± 0.10 | $P > 0.05$ |
| Female | 0.95 ± 0.13 | 0.92 ± 0.19 | $P > 0.05$ |
| GPx activity (U/gP) | 254 ± 7.0 | $225 \pm 6.6^*$ | $P = 0.03$ |
| Male | 253 ± 15 | 232 ± 7.9 | $P > 0.05$ |
| Female | 255 ± 10 | $217 \pm 11^*$ | $P = 0.005$ |
| GSH ($\mu\text{M/g P}$) | 636 ± 57 | 569 ± 32 | $P > 0.05$ |
| Male | 711 ± 73 | $522 \pm 33^*$ | $P = 0.03$ |
| Female | 562 ± 82 | 625 ± 52 | $P > 0.05$ |
| GSH/GSSG | 87.79 ± 6.34 | 88.87 ± 4.15 | $P > 0.05$ |
| Male | 83.82 ± 30.9 | 87.63 ± 13 | $P > 0.05$ |
| Female | 82.44 ± 21.36 | 91.79 ± 15.46 | $P > 0.05$ |
| FRAP ($\mu\text{M/g protein}$) | 210 ± 5.8 | 197 ± 4.9 | $P > 0.05$ |
| Male | 217 ± 7.8 | 201 ± 6.9 | $P > 0.05$ |
| Female | 202 ± 8.0 | 193 ± 7.0 | $P > 0.05$ |
| GST (nM/mg protein) | 232 ± 5.4 | $249 \pm 4.3^*$ | $P = 0.015$ |
| Male | 228 ± 8.5 | $257 \pm 6.9^*$ | $P = 0.02$ |
| Female | 236 ± 7.0 | 242 ± 4.0 | $P > 0.05$ |

Results were expressed as mean \pm SE; * $p \leq 0.05$,

The number of rats pups was in F Group (F): n = 50, 18 males; 32 females, and in FI Group: n = 38, 19 males;19 females.

Table 15. OS parameters in **brain** of offspring from mothers fed with the F or the FI diet

| OS parameters | F group | FI group | p value |
|----------------------|----------------|-----------------|----------------|
| GPx activity (U/gP) | 104,1 ± 3.8 | 92,8 ± 3.5* | P = 0.04 |
| Male | 113 ± 3.9 | 91 ± 2.4 * | P = 0.001 |
| Female | 95 ± 4.0 | 95 ± 8.0 | P > 0.05 |
| GSH (µM/g P) | 273 ± 6.4 | 256 ± 6.7 | P > 0.05 |
| Male | 259 ± 3.0 | 250 ± 6.0 | P > 0.05 |
| Female | 301 ± 6.0 | 266 ± 1.4 | P > 0.05 |
| GSH/GSSG | 76.08 ± 7.13 | 86.47 ± 11.16 | P > 0.05 |
| Male | 79.56 ± 19.54 | 88.08 ± 40 | P > 0.05 |
| Female | 70.55 ± 19.53 | 81.25 ± 24.2 | P > 0.05 |
| FRAP (µM/g protein) | 254 ± 4.0 | 253 ± 4.1 | P > 0.05 |
| Male | 258 ± 3.3 | 258 ± 6.5 | P > 0.05 |
| Female | 250 ± 7.3 | 248 ± 4.8 | P > 0.05 |
| GST (nM/mg protein) | 216 ± 8.3 | 228 ± 5.6 | P > 0.05 |
| Male | 213 ± 36 | 230 ± 17 | P > 0.05 |
| Female | 213 ± 27 | 226 ± 29 | P > 0.05 |

Results were expressed as mean ± SE; * p ≤ 0.05

The number of rats pups was in F Group (F): n = 50, 18 males; 32 females and in FI Group: n = 38, 19 males; 19 females.

The results represented in Tables 12 and 13 showed that GPX activity in the liver and brain was significantly decreased in offspring born from FI diet mothers. The gender analysis showed that in the liver, GPx activity was significantly lower in female offspring, while in the brain, it was significantly decreased in male offspring. The liver GSH level was significantly lower only in male pups of the FI group compared to those from F group. The activity of GST

was significantly increased in liver of the FI diet group, but only in male offspring. There was no significant difference in total antioxidant capacity measured by FRAP assay between the two groups.

Discussion

Iron supplements are usually advised for pregnant women to meet the iron needs of both mother and fetus at a dose 5-25 mg iron/100 g (Lin *et.al.*1976), but excessive iron intake increases ROS production during pregnancy probably through Fenton reaction. Furthermore, chronic consumption of high fructose diet contributes to excessive ROS production and thus to induction of oxidative stress, mediating insulin resistance (Houstis *et.al.*2006, Suwannaphet *et.al.*2010). In the current study, the dose of iron in the fructose-enriched iron (FI) diet was 22 mg/100 g diet, corresponding to a moderate dose, as iron is rarely consumed in an excessive dose during pregnancy, and because the purpose of this study was to examine the hypothesis that an increased iron intake would induce metabolic and oxidative disorders during GDM and may have sex-related differences effect in offspring.

The result showing that the number of female offspring from FI group was smaller than in F group might correlate with iron supplementation. This is supported by Vila` L *et.al.*2011; Rodríguez *et.al.* 2015 who showed a sex-dependent influence of the diet in the offspring born from mothers fed a fructose-enriched iron diet during pregnancy. To confirm this, further studies are necessary.

In mothers, the result showed that the fructose-enriched iron diet did not influence the oxidative status of the mothers between the two groups. In contrast, TBARS were reduced in

the FI group. Although this result seems surprising, clinical studies have also reported a decrease in TBARS in the case of GDM (Santra *et.al.*2003, Kharbs *et.al.*2008). This finding contrast with an epidemiological study in pregnant women by Lachili 2001 that showed the effect of a daily combination iron supplement (100 mg as fumarate) and vitamin C (500mg) for the third trimester that increased thiobarbituric acid reacting substances (TBARs) plasma level, but the the higher dose of iron given was 100 mg, while in our study only 22 mg/100 g diet iron were given.

Interestingly, although no difference in GPx activity and SH level between the two groups of mothers, but the activity of GPx and GSH level in livers and brains of newborns from FI diet was decreased. This suggests that fetuses were more sensitive to the effect of diet during pregnancy than their mothers (Table 11, Table 12). We found a sex-dependent effect on oxidative stress parameters in offspring. Male offspring born from FI diet mothers showed decreased GPx activity in the brain compared to those from F diet mothers ($p < 0.05$), but this result was unchanged in female offspring born from mothers in the FI diet group. Similarly, GPx activity in the liver offspring was decreased in both male and female offspring born from mothers in the FI diet group compared to those from F diet mothers, but the effect was only significant in the female offspring ($p < 0.05$). The inhibition of GPx activity has often been reported in cases of obesity and insulin resistance (Kobayashi 2009). Similar to our results, a difference by sex of liver GPx activity has been reported in pups born from diabetic mothers (Kruse MS 2014). The decrease in GPx is a common feature of the route toward Type 2 diabetes (Aouacheri *et.al.*2015.). In addition, the increase in catabolism of fructose causes decreasing of total glutathione concentrations and reducing of hepatic antioxidant enzyme activities such as CAT, SOD, and GPx (Suwannaphet *et.al.* 2010). The findings of this study

are in line with other studies that reported a significant increase in lipid peroxidation and a significant decrease of hepatic antioxidant enzyme activities in fructose-induced diabetic rats (Basciano *et.al.* 2016).

Moreover, there was no difference in total GSH level in the brain and liver between the two groups of diet, except for the GSH level in the liver of male offspring born from FI diet mothers that was significantly lower than in those from F diet mothers. In the liver, reduced GSH levels of males born from the FI diet-fed mothers served as an indicator of iron-induced OS, which can be attributed to the high glycemic values in this gender. In the liver of females, the level of GSH was not modified, which may be due to less oxidation caused by iron. In this study, the liver of male appeared to be more sensitive than liver of female to the effects of a diet rich in iron. A study by Kim *et.al.*2011 assessed the association of elevated serum ferritin concentration with insulin resistance and impaired glucose metabolism in Korean men and women, showing that iron overload was associated with insulin resistance in men, but not in women.

The activity of GST was increased in the brain and liver of offspring in FI diet group, but the effect was only significant in the male offspring. It could represent an adaptive response to address an increasing production of ROS caused by iron. Indeed, some evidence suggests that GST served to protect cells from oxidative stress, because GST is regulated by ROS such as H₂O₂. GST detoxification some oxidative metabolites produced by the OS (Hayes *et.al.*1995). GST is a phase 2 enzyme antioxidant prototype that is involved in the detoxification of various toxic and potentially carcinogenic compounds. In accordance with our results, an

increase in GST was associated with a decrease in GPx higher in iron-treated rats (Madra *et.al.*1996).

These alterations may be mediated by the pregnancy condition that displays an increased vulnerability to oxidative stress, because of mitochondria-rich placenta. Iron, one of the transition metals, is very abundant in the placenta and plays an important role in the production of free radicals. Plasma thiol levels (especially reduced glutathione) were found to be lower during pregnancy compared to non-pregnant women, suggesting an oxidative stress environment during pregnancy (Yamada *et.al.* 1991, Wisdom *et.al.* 1991). In parallel, fructose-induced hyperglycemia is one of the important factors that increase ROS production and causes the depletion of the antioxidant defense status in various tissues (Reddy *et al.*, 2009). Therefore, an environment rich in Fe³⁺ such as chronic maternal fructose-enriched iron diet induces an increase in oxidized thiols levels (especially reduced glutathione) (Wisdom *et.al.* 1991). In this reaction, iron can produce reactive oxygen or nitrogen species and catalyzes several cellular reactions that result in the formation of hydroxyl radicals and induces oxidative stress (Andrews 1999, Rajpathak 2009). Therefore, maternal high fructose-enriched iron diet may pose a worse impact on glucose tolerance and oxidative stress of mothers and offspring. Moreover, Yakes 1997 reported that high concentrations of iron rapidly reduce the transcription of a large set of genes that encode mitochondrial ribosomal proteins, oxidative phosphorylation proteins, and the total complement of electron transport proteins. The dysregulation of electron transport can lead to increased mitochondrial production of H₂O₂. The combination of increased iron and physiological concentrations of H₂O₂ generated in the mitochondria during OXPHOS might result in the DNA damage. In mammalian cells, mtDNA is much more sensitive than nuclear DNA (nDNA) to H₂O₂

induced damage, probably due to the availability of iron in the mitochondria. Moreover, human cells have a limited capacity for repairing H₂O₂-induced mtDNA damage (Yakes 1997).

CONCLUSIONS

Middle Iron enriched fructose diet 4 weeks before and during gestation reduces the female gender of rat pups, decreases the level of TBARS in the mothers rat. In male pups, this maternal diet increases glycemia, GST activity, and decreases GSH level. On the other hand in female pups, this diet increases birth weight and reduces GPx activity. These findings suggest that the influence of maternal moderate iron-enriched diet has been shown to be sex-dependent in the offspring, and the fetuses are more sensitive than their mother to the impact of a middle iron-enriched diet. Future studies are needed to clarify the relationship between the potential risk for male and female offspring and the characteristic of disorder.

CHAPTER IV

CONCLUSIONS

1. The modification of environment during gestation by moderate-intensity exercise 4 weeks before mating until and 18th day of gestation can modify mitochondrial function of offspring
2. The modification of environment during gestation by moderate-intensity exercise 4 weeks before mating until and 18th day of can modify glucose metabolism of offspring
3. High fructose diet during gestation can become a model of gestational diabetes mellitus on rat
4. High fructose enriched-iron diet during gestation can increase the risk of oxidative damage and glucose metabolism disorder in offspring.

CHAPTER V

THE LIMITATIONS OF THIS STUDY

1. The oxidative stress parameter, glycemia, and insulin on trained mother rats was not measured
2. The protocol training was performed only in one intensity until the eighteen day of gestation
3. The expression of proteins that regulated the mitochondrial function in mother and offspring were not measured in order to verify the modification of mitochondrial function in offspring was correlated with the characteristic of mitochondrial mother that given
4. The insulinemia of rat pups induced by high fructose and high fructose-iron should be measured

CHAPTER VI

PERSPECTIVE STUDY

To clarify the alteration of mitochondrial function of offspring born to trained mother is induced by maternal moderate-intensity exercise training, further studies are needed to assess:

- (1) Genes encode oxidative phosphorylation complex in mother and offspring
- (2) Protein expression of mitochondrial transcription factor A (TFAM) of pancreatic β -cell offspring
- (3) The proper number of pancreatic β -cells in the islets of Langerhans using propidium iodide (PI) staining which can distinguish β -cells from other endocrine cells
- (4) Continue this study in adult offspring (7 months old) in order to examine the alteration of mitochondrial function in 21 days old will persistent in adulthood
- (5) The benefit of these study will be recommended on pregnant woman

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Short-term and long-term effects of submaximal maternal exercise on offspring glucose homeostasis and pancreatic function

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Quiclet C, Siti F, Dubouchaud H, Vial G, Berthon P, Fontaine E, Batandier C, Couturier K. Short-term and long-term effects of submaximal maternal exercise on offspring glucose homeostasis and pancreatic function. *Am J Physiol Endocrinol Metab* 311: E508–E518, 2016. First published July 5, 2016; doi:10.1152/ajpendo.00126.2016.—Only a few studies have explored the effects of maternal exercise during gestation on adult offspring metabolism. We set out to test whether maternal controlled submaximal exercise maintained throughout all gestational periods induces persistent metabolic changes in the offspring. We used a model of 15-wk-old nulliparous female Wistar rats that exercised (trained group) before and during gestation at a submaximal intensity or remained sedentary (control group). At weaning, male offspring from trained dams showed reduced basal glycemia (119.7 ± 2.4 vs. 130.5 ± 4.1 mg/dl, $P < 0.05$), pancreas relative weight (3.96 ± 0.18 vs. 4.54 ± 0.14 g/kg body wt, $P < 0.05$), and islet mean area ($22,822 \pm 4,036$ vs. $44,669 \pm 6,761$ μm^2 , $P < 0.05$) compared with pups from control dams. Additionally, they had better insulin secretory capacity when stimulated by 2.8 mM glucose + 20 mM arginine compared with offspring from control dams (+96%, $P < 0.05$). At 7 mo of age, offspring from trained mothers displayed altered glucose tolerance ($\text{AUC} = 15,285 \pm 527$ vs. $11,898 \pm 988$ $\text{mg}\cdot\text{dl}^{-1}\cdot 120$ min, $P < 0.05$) and decreased muscle insulin sensitivity estimated by the phosphorylated PKB/total PKB ratio (–32%, $P < 0.05$) and tended to have a reduced islet insulin secretory capacity compared with rats from control dams. These results suggest that submaximal maternal exercise modifies short-term male offspring pancreatic function and appears to have rather negative long-term consequences on sedentary adult offspring glucose handling.

gestation; exercise; offspring; glucose homeostasis; pancreas

BASED ON EPIDEMIOLOGICAL DATA, Barker and Osmond (4), Hanson and Gluckman (36), and McMillen and Robinson (49) described the concept of the “Developmental Origins of Health and Diseases,” suggesting that the in utero environment and the

first stages of life environment may play a role in the occurrence of diseases in adulthood. Many epidemiological and experimental studies relate early nutritional environment to metabolic disorders such as diabetes, insulin resistance, and obesity as well as hypertension or cardiovascular diseases (3–5, 33, 45, 46, 48, 55, 56, 66, 73, 75, 82). Among them, type 2 diabetes mellitus (T2DM) is growing in modern societies at an alarming rate (79a). Environmental (i.e., food intake, obesity, and physical activity level, among others) and genetic factors are thought to play a role in the susceptibility to this disease (68).

Some studies have also established a link between increased glucose intolerance or T2DM prevalence in adulthood and a maternal nutrient intake during gestation (44, 49, 59, 60, 69, 73) or offspring low (3, 10, 35, 48, 81) or high (3, 8, 48, 81) birth weight. This suggests that an inadequate maternal nutritional environment during fetal life may influence fetal development.

To enhance the chances for a successful parturition and to improve both their health and that of their developing child, women are prone to adopt a healthy lifestyle when pregnant (29, 32). Many of them may be willing to eat a healthier diet and stay physically active during that period (32).

Regular moderate exercise has been shown to decrease susceptibility to T2DM in pregnant women (26, 27, 50, 63), by enhancing insulin sensitivity and fostering non-insulin-stimulated glucose uptake (61, 78). The effects of gestational exercise on the mother are now well known, including improved aerobic capacity and cardiovascular fitness or reduced gestational diabetes incidence (2, 24, 27, 53, 62, 63). However, potential outcomes on offspring are still to be studied. Recent studies show that voluntary exercise during pregnancy and nursing can improve glucose tolerance and insulin sensitivity in adult offspring (13, 14). It has been proven that a controlled mild pace of exercise during pregnancy is safe in mice and humans (18, 57, 67, 70, 79). However, the effects of a controlled submaximal yet intense exercise during gestation on offspring health are contradictory (41, 70, 71). The literature reports various effects of maternal exercise on offspring birth weight and body composition (20–23, 37, 38). However, most

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of them show decreased body and fat mass in offspring from exercised dams (20, 21, 37). Currently, one hypothesis to explain the differences observed in birth weight would be changes in placental growth and in glucose and oxygen delivery toward the fetus during maternal exercise (17). Even if the reduction in placental blood flow is blunted by gestational exercise training with increased oxygen and substrate delivery during resting conditions, placental oxygen and nutrient delivery remained lower during moderate- to high-intensity exercise sessions (16). However, the consequences of these changes in uterine blood flow during exercise on fetus well being are conflicting (64, 70).

Taken together, these data suggest that the antenatal period, with many factors, including the mother's physical activity level, that can influence the intrauterine development and offspring birth weight, could be of major importance in the development of chronic diseases later in life (11, 25, 31, 40, 65, 72).

Considering the known effects of voluntary maternal exercise on glucose tolerance and insulin sensitivity in adult rodent offspring (13, 14), we have developed a model of pregnant rats subjected to compulsory exercise to study, with a functional approach and physiological tools, the impact of this constraint on glucose handling in pups. We postulate that metabolic improvements (13, 14) can result from structural and metabolic changes of the pancreas in the offspring from exercised dams. The literature shows that the effects of exercise during pregnancy on offspring depend on the intensity and the duration of this exercise (19, 47, 79). We wanted to test whether the metabolic adaptations such as improved glucose tolerance and greater insulin sensitivity were also present if the exercise was compulsory, submaximal but yet intense (55% of maximal aerobic speed), and maintained until almost the end of pregnancy rather than voluntary.

RESEARCH DESIGN AND METHODS

Animals. Nulliparous 15-wk-old female Wistar rats (Charles River Laboratories, Saint Germain-Nuelles, France) were housed three per cage with access to food (A03; SAFE Diets, Augy, France) and water ad libitum. The animal facility was on a 12-h light-dark cycle and maintained at a temperature of $22 \pm 2^\circ\text{C}$. All experimental procedures were carried out in accordance with European Directive 2010/63/UE. They were reviewed by the Institutional Ethics Committee for Animal Care and Use and authorized by the French Ministry of Research (00174.01 accepted in March 2014). After a 1-wk acclimatization period, female rats were assigned to either a sedentary (control; $n = 9$) or trained ($n = 12$) group. Body weight and food consumption were monitored once/wk during breeding and pregnancy. Trained females were exercised using a motorized treadmill (Bioseb, Vitrolles, France) 5 days/wk during the 4 wk before gestation and during the first 18 days of gestation, whereas female rats from the control group were kept in their cages. The treadmill speed and the duration of the training session were gradually increased during the first 3 wk of training to reach a speed of 25 m/min for 60 min. After 4 wk of controlled exercise, females from each group were housed with male rats during 1 wk for mating. The male rats did not exercise during the study. Vaginal smears were performed each day until spermatozoa were found to determine the first day of

gestation. On postnatal day 2, litter sizes were equalized to eight pups. Pups were cross-fostered from other litters from the same group and the same age to maximize the number of males per litter. Mothers from the trained group did not exercise during nursing. Pups were weighted on postnatal days 7, 14, and 21. Only male pups were used in the study. Some pups were tested at weaning (3–4 wk of age), and others were housed two per cage until 7 mo of age without any controlled physical activity and fed with food (A04 rodent diet; SAFE Diets) and water ad libitum. Mothers were euthanized after nursing and offspring at weaning and at 7 mo of age. Selected fat depots, skeletal muscles, and organs were dissected and weighed to estimate the changes in body composition and/or collected and stored at -80°C for other measurements.

Intraperitoneal glucose tolerance test. Intraperitoneal glucose tolerance tests (IPGTT) were performed on pups at 3 wk and 7 mo of age after a 16-h overnight fast. Only a few pups ($n = 8$ –14) from each group were tested at 3 wk of age. Glucose was intraperitoneally injected at 1 g/kg body wt. Blood glucose readings were taken via tail pick before ($t = 0$ min) and 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, and 120 min after glucose injection using an Accu-Chek glucometer (Roche Diabetes Care, Meylan, France). Areas under the curve (AUC) were related to $t = 0$ min.

Intraperitoneal insulin tolerance test. Intraperitoneal insulin tolerance tests (IPITT) were performed on pups from each group used for IPGTT at 4 wk and 7 mo of age after a 6-h fast. Insulin was intraperitoneally injected at 1 mIU/g body wt. Blood glucose readings were taken via tail pick before ($t = 0$ min) and 10, 20, 30, 40, 50, 60, 90, and 120 min after insulin injection, as described above. Blood samples were collected before insulin injection to determine fasting plasma insulin by radioimmunoassay (RIA) kit (Merck Millipore). Areas over the curve (AOC) were related to $t = 0$ min.

Insulin load test and analysis of insulin signaling in skeletal muscle and liver. After a 6-h fast, 3-wk- (other than those used for tolerance tests) and 7-mo-old rats were intraperitoneally injected with NaCl (0.9%; control C– and trained T–) or with insulin (10 mIU/g body wt; control C+ and trained T+). Rats were euthanized 15 min after injection, and gastrocnemius muscle and liver were rapidly removed and frozen until their use to determine protein kinase B (PKB) phosphorylation level by Western blotting as an indicator of insulin sensitivity (76). Tissues were homogenized in PBS buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with 5 mM EDTA, 1 mM Na_3VO_4 , 20 mM NaF, 1 mM DTT, and protease inhibitor cocktail (P2714; Sigma). Proteins were subjected to SDS-10% PAGE. Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and incubated overnight with primary antibodies (total Akt/PKB, no. 9272, and phospho-Akt/PKB Ser⁴⁷³, no. 9271; Cell Signaling Technology). Primary antibodies were detected with a horse-radish peroxidase-conjugated secondary antibody (no. 172-10-19; Bio-Rad) and revealed with enhanced chemiluminescence system (Pierce).

Pancreas insulin content. Pancreases were dissected just after euthanasia and stored at -80°C . They were then homogenized at 4°C in 6 ml of acidified ethanol (0.18 M HCl in 70% ethanol) and placed overnight on a rotative wheel (20 rpm, 4°C). The day after, homogenates were sonicated twice for 10 s each time and then placed on a rotative wheel for 2 days (20

rpm, 4°C). Homogenates were then centrifuged (150 g, 5 min, 4°C). Supernatants were collected and centrifuged (3,200 g, 20 min, 4°C). The resulting supernatants were collected and stored at -80°C until insulin content assay by RIA kit (Merck Millipore).

Pancreas histology. In a subset of animals from each group, pancreases were dissected just after euthanasia, fixed in Tissue-Tek, and stored at -80°C. Cross-sections were done using a cryostat on glass slides. Sections were randomly selected and colored using hematoxylin-eosin staining. Islets were outlined and their areas expressed in square micrometers using ImageJ software.

Islet isolation and glucose-stimulated insulin secretion test. Pancreatic islet isolation was performed on the rats used for IPGTT and IPITT by collagenase digestion. Rats were anesthetized by an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt). The abdomen was opened and the pancreas exposed as much as possible. The pancreatic duct was clamped with a hemostat at its duodenal insertion with care to avoid pancreatic tissue injuries. The bile duct at the proximal end was isolated and cut with fine scissors at one-third of the way across. A 26G catheter was inserted and fixed in the bile duct. A collagenase digestion solution (10 ml of collagenase from *Clostridium histolyticum* type XI at 1 mg/ml; Sigma-Aldrich, Saint-Quentin Fallavier, France) in Hanks' balanced salt solution (HBSS) was infused slowly. The pancreas was then carefully removed and placed in 7.5 ml of HBSS at 4°C and incubated in a water bath preset at 37°C for 11 min. After incubation, the tubes were vigorously hand-shaken for 15 s before the addition of 25 ml of a "wash solution" (HBSS; 5% fetal bovine serum). The tubes were centrifuged (250 g, 2 min, 4°C) and the supernatants discarded. The pellets were filtered through a wire mesh. Twenty-five milliliters of wash solution was then added, the tube was centrifuged (250 g, 2 min, 4°C), and the supernatant was poured off. The washing procedure was repeated two more times. After the last centrifugation, 5 ml of the supernatant was kept with the pellet. A density gradient was then prepared by pouring 10 ml of Histopaque 1.119 (Sigma-Aldrich) in a 50-ml tube that was overlaid with 10 ml of Histopaque 1.077. Islets were then resuspended with 7.5 ml of Histopaque 1.119 + 3.5 ml of Histopaque 1.077, and the suspension was layered on the Histopaque 1.077 phase, followed by 10 ml of HBSS on top. The gradient was centrifuged (1,750 g, 20 min, 20°C) with slow acceleration and no braking. The islets were then collected from each of the interfaces. The islets were washed three times in 25 ml of wash solution (1 time at 350 g, 5 min, 4°C, and 2 times at 250 g, 2 min, 4°C). Islets were then cultured in RPMI, 10% FBS, 1% sodium pyruvate, and 1% antibiotic/antimycotic solution overnight at 37°C. The day after isolation, the islets were incubated with either glucose or glucose plus arginine to determine their insulin secretory capacity. After a 1-h preincubation in 2.8 mM low-glucose medium at 37°C, islets were then incubated for 1 h in 2.8 mM low-glucose medium at 37°C, and supernatants were collected. Islet insulin secretion was then stimulated by incubation with either 16.7 mM high glucose medium for 3-wk-old and 7-mo-old rats or 2.8 mM low glucose + 20 mM arginine medium for 3-wk-old rats only (1) for 1 h at 37°C, and supernatants were collected. Residual total insulin was extracted in 0.18 M HCl and 70% ethanol. Samples were stored

at -80°C until insulin assay by ELISA (3 wk of age; Alpcos) or RIA (7 mo of age; Merck Millipore) kits.

Statistical analysis. Data are expressed as means \pm SE. Data were analyzed using one-way or two-way ANOVA with Holm-Sidak post hoc test. Kruskal-Wallis tests were applied when values were not normally distributed (Sigma Plot). $P < 0.05$ was considered significant, and $P < 0.2$ was considered as tendency.

RESULTS

Exercise training before and during gestation reduces fat depots in the mothers without any alteration in food intake or other organs. Body weight of female Wistar rats was similar in the two groups at the beginning of the study and after the first 4 wk of training (Table 1). After nursing, body weight of trained mothers tended to be lower than that of control mothers (286 \pm 4 vs. 297 \pm 7 g for trained and control groups, respectively, $P = 0.136$; Table 1). Food intake was similar between both groups from the beginning of the protocol to the end of lactating period (Table 1). Exercise training did not affect the weight of organs such as liver, kidney, pancreas, or muscles (Table 1). However, relative mass of the sum of retroperitoneal, urogenital, and mesenteric fats depots was significantly lower in trained mothers compared with control mothers (39.5 \pm 2.7 vs. 54.3 \pm 6.0 g/kg body wt for trained and control groups, respectively, $P < 0.05$; Table 1).

Exercise training before and during gestation in mothers has no effect on the litter outcomes. The number of pups per litter as well as the sex ratio were similar in the trained and control groups (Table 2). Offspring body weights were not affected by mother exercise intervention at any time (Table 2).

Maternal exercise affects offspring pancreas weight and islets size at 3 wk of age. At weaning, body weight and organ relative weight were not different in offspring from control or trained mothers, except for the pancreas (Table 3). Indeed, pancreas relative weight was found to be lower in pups from trained mothers compared with that of pups from control mothers (3.96 \pm 0.18 vs. 4.53 \pm 0.14 g/kg body wt for trained and control groups, respectively, $P < 0.05$; Table 3). Moreover, mean islet area measured in 3-wk-old pups from trained dams is significantly smaller compared with that measured in pups from control dams (Table 3). At 7 mo of age, no

Table 1. Maternal BW, FI, and organ relative weight after nursing

| | Control | Trained |
|----------------------------|------------------|-------------------|
| BW before the study, g | 260 \pm 5 | 260 \pm 4 |
| BW before gestation, g | 277 \pm 22 | 276 \pm 19 |
| BW after nursing, g | 297 \pm 7 | 286 \pm 4 |
| FI before gestation, g/day | 18.4 \pm 0.5 | 18.4 \pm 0.4 |
| FI after gestation, g/day | 23.0 \pm 1.3 | 20.7 \pm 0.7 |
| FI after nursing, g/day | 70.3 \pm 1.5 | 73.0 \pm 1.4 |
| Pancreas, g/kg BW | 4.34 \pm 0.32 | 4.26 \pm 0.18 |
| Liver, g/kg BW | 26.01 \pm 0.98 | 30.34 \pm 1.94 |
| Kidney, g/kg BW | 2.89 \pm 0.13 | 3.17 \pm 0.08 |
| Fat, g/kg BW | 54.30 \pm 5.97 | 39.45 \pm 2.65* |
| Muscles, g/kg BW | 5.90 \pm 0.24 | 6.02 \pm 0.11 |

Data are means \pm SE ($n = 9$ for control and $n = 12$ for trained). BW, body weight; FI, food intake. Fat mass was calculated as the sum of retroperitoneal, urogenital, and mesenteric fat depots. Muscle mass was calculated as the sum of gastrocnemius, plantaris, and soleus muscles. * $P < 0.05$ vs. control.

Table 2. Litter outcomes

| | Control | Trained |
|----------------|------------|------------|
| Litter size | 12.1 ± 2.0 | 10.8 ± 3.8 |
| No. of males | 5.2 ± 1.8 | 5.2 ± 2.5 |
| No. of females | 6.9 ± 2.0 | 5.7 ± 2.6 |
| Day 7 BW, g | 16.1 ± 1.0 | 16.9 ± 0.6 |
| Day 14 BW, g | 32.5 ± 1.3 | 32.9 ± 0.9 |
| Day 21 BW, g | 50.9 ± 1.7 | 50.1 ± 1.0 |

Data are means ± SE ($n = 9$ for control and $n = 12$ for trained).

differences were found in body and organ relative weights, including pancreas (Table 3). However, we noticed that there is a tendency to a higher relative fat pad mass ($P = 0.153$) in rats from trained mothers compared with those from control mothers (Table 3).

Effect of maternal exercise on offspring blood glucose and insulin levels. After a 16-h fast, 21-day-old pups showed a similar glycemia in both groups (104.6 ± 4.6 vs. 108.0 ± 2.7 mg/dl for trained and control groups, respectively; Fig. 1A).

After a 6-h fast, 28-day-old pups from trained mothers showed a lower glycemia compared with pups from control mothers (119.7 ± 2.4 vs. 130.5 ± 4.1 mg/dl, respectively, $P < 0.05$; Fig. 1B), whereas insulinemia was similar in both groups even if it tended to be higher in rats from trained mothers (0.31 ± 0.04 vs. 0.21 ± 0.05 ng/ml for trained and control groups, respectively, $P = 0.158$; Fig. 1C).

In 7-mo-old rats, neither blood glucose nor insulin levels were different, whatever the duration of the fasting (Fig. 1, D–F).

Effect of maternal exercise on offspring glucose tolerance. At 21 days of age, there was no difference in blood glucose levels between the two groups at any point of the IPGTT (Fig. 2A), and area under the curve (AUC) as a measure of the overall glucose disposal was not significantly different between both groups (Fig. 2B).

However, in 7-mo-old rats, blood glucose levels were higher in animals from the trained group compared with those from the control group during the first 15 min of the IPGTT (Fig. 2E). Moreover, the AUC is significantly larger in the trained group compared with the control group ($15,258 \pm 924$ vs. $11,614 \pm 1,366$ mg·dl⁻¹·120 min for the trained and control groups, respectively, $P < 0.05$; Fig. 2F).

Effect of maternal exercise on offspring insulin sensitivity. At 28 days of age, offspring from trained dams displayed lower blood glucose levels during the IPITT compared with the pups

from control group (Fig. 2C) due to a significantly lower starting glycemia (as already shown in Fig. 1B). However, the overall insulin sensitivity estimated by the area over the curve (AOC) appeared to be similar in both groups ($5,318 \pm 513$ vs. $3,507 \pm 1,178$ mg·dl⁻¹·120 min for the trained and control groups, respectively; Fig. 2D).

At 7 mo of age, blood glucose levels were not different at any point of the IPITT between animals from the trained group compared with those from the control group (Fig. 2G). This is confirmed by the AOC, which was similar between both groups (Fig. 2H).

Effect of maternal exercise on offspring insulin signaling in liver and skeletal muscle. Insulin signaling was studied by measuring the level of expression of PKB and its phosphorylated form (pPKB) in both liver and gastrocnemius muscle after either a NaCl or an insulin load. Figure 3 shows the pPKB/PKB ratios obtained.

At 21 days of age, pups from the trained group showed an increase in the pPKB/PKB ratio compared with pups from the control group in basal the condition (T– and C–, respectively) in both liver (+77%, $P < 0.05$; Fig. 3A) and muscle (+97%, $P < 0.05$; Fig. 3C). However, these differences were not seen after an insulin load injection (T+ and C+) in either liver (Fig. 3A) or gastrocnemius muscle (Fig. 3C).

At 7 mo of age, pPKB/PKB ratio was similar in the liver of animals from the two groups whatever the condition (NaCl load or insulin load; Fig. 3E). In muscle, under insulin load, pPKB/PKB ratio was significantly lower in rats from the trained group (T+) when compared with rats from the control group (C+) (–32%, $P < 0.05$) (Fig. 3G), whereas it remained similar between groups in basal condition (T– vs. C–; Fig. 3G).

Effect of maternal exercise on offspring pancreas insulin content and islet insulin secretion. The total insulin content in pancreas was not different between trained and control groups whether at 3 wk of age (Fig. 4A) or at 7 mo of age (Fig. 4C).

At 28 days of age, the islet insulin secretion was not significantly different in pups from trained dams compared with those from control dams either after low glucose (2.8 mM) or high glucose (16.7 mM) incubation (Fig. 4B). However, islet insulin secretion after low glucose (2.8 mM) + arginine (20 mM) incubation was significantly higher in pups from trained dams compared with those from control dams (+96%, $P = 0.01$; Fig. 4B).

Table 3. Offspring body outcomes

| | Weaning | | 7 Mo of Age | |
|-----------------------------|----------------|-----------------|--------------|--------------|
| | Control | Trained | Control | Trained |
| Body weight, g | 51.61 ± 3.43 | 47.49 ± 1.16 | 506.9 ± 24.5 | 528.8 ± 16.6 |
| Organ weights, g/kg BW | | | | |
| Liver | 31.81 ± 0.75 | 30.97 ± 0.19 | 27.88 ± 0.76 | 28.36 ± 0.84 |
| Kidney | 6.02 ± 0.11 | 5.99 ± 0.11 | 2.26 ± 0.03 | 2.38 ± 0.05 |
| Fat | 8.68 ± 0.57 | 9.00 ± 0.35 | 45.97 ± 4.38 | 54.71 ± 3.04 |
| Muscles | 4.58 ± 0.13 | 4.54 ± 0.07 | 5.97 ± 0.21 | 5.53 ± 0.24 |
| Pancreas | 4.54 ± 0.14 | 3.96 ± 0.18* | 2.94 ± 0.17 | 3.03 ± 0.11 |
| Islet area, μm ² | 44,669 ± 6,761 | 22,822 ± 4,036* | | |

Data are means ± SE ($n = 16$ for control and $n = 17$ for trained at weaning; $n = 8$ for control and $n = 6$ for trained at 7 mo of age; $n = 11$ –13 for islet area). Fat mass was calculated as the sum of retroperitoneal, epididymal, and mesenteric fat depots. Muscle mass was the sum of gastrocnemius, plantaris, and soleus muscles. * $P < 0.05$ vs. control.

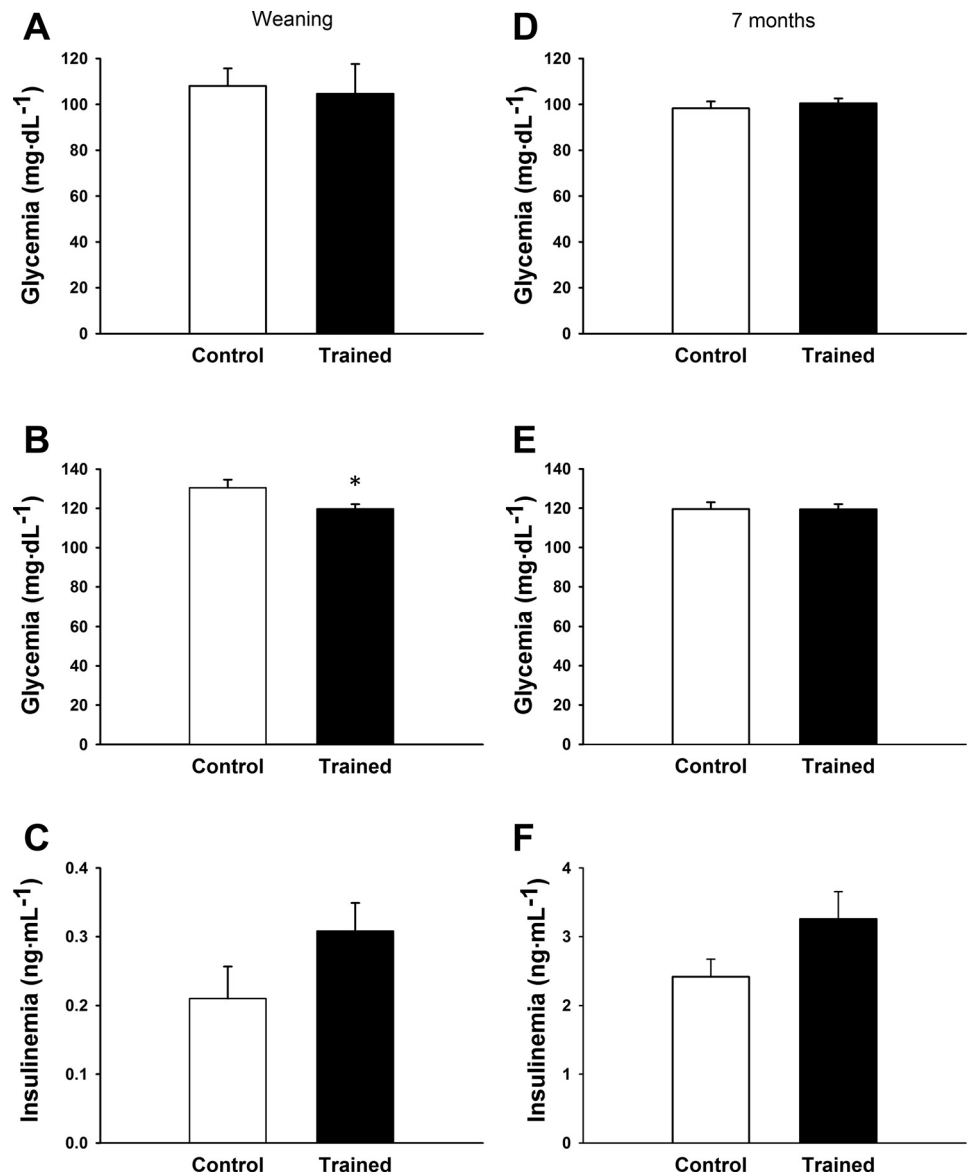


Fig. 1. Maternal exercise reduces 6-h-fasting glycemia and tends to increase 6-h-fasting insulinemia in 28-day-old offspring. Rat offspring fasting glycemia was measured after a 16-h fast (A and D) and a 6-h fast (B and E) at 21 days (A), 28 days (B), and 7 mo of age (D and E). Fasting insulinemia was determined after a 6-h fast (C and F) at 28 days (C) and 7 mo of age (F). Data are means \pm SE; $n = 4$ for control and $n = 6$ for trained groups in C; $n = 8-14$ in A, B, D, E and F. * $P < 0.05$ vs. control.

At 7 mo of age, islet insulin secretion was higher after high glucose (16.7 mM) incubation compared with the low-glucose (2.8 mM) incubation condition ($P < 0.05$) (Fig. 4D). However, the islet insulin secretion tended to be lower in rats from trained group compared with rats from control group whether in the low-glucose (2.8 mM) or high-glucose (16.7 mM) condition ($P = 0.102$ and $P = 0.255$, respectively; Fig. 4D).

DISCUSSION

For the first time, to our knowledge, we show that submaximal maternal exercise alters the offspring pancreatic function. Especially, we observed 1) a decline in insulin secretion with age in animals from the exercised mothers along with 2) a glucose intolerance and 3) a skeletal muscle insulin resistance that are not present in offspring from sedentary dams.

Our purpose was to test the effect of daily chronic exercise during the gestation on fit females that were already active before gestation. We chose to train them for 4 wk before mating, as such a duration is usually sufficient to get the first

signs of adaptation to chronic endurance exercise (77). Studies using voluntary exercise in rodents with running wheels showed that the running distance decreased dramatically in the last week of gestation (13, 28). So we chose a compulsory treadmill exercise that allowed us to test the effects of exercise throughout all the gestational periods, with no change in training parameters and especially no reduction in exercise intensity and duration until day 18 of the gestation. To ensure that these adaptations were significant, we also chose a sufficiently high intensity. We used intensity parameters that are classically found in the literature (6, 43, 84). Based on data collected in our laboratory, the speed and slope of the treadmill that we used would correspond to an intensity of about 55% of the maximal aerobic speed. That intensity matches the guidelines for exercise in pregnant women from a lot of countries around the world (30). However, the frequency and the duration are above most of the guidelines, except those from Denmark and the US that recommend at least 30 min of moderate-intensity exercise most of the days or daily (30).

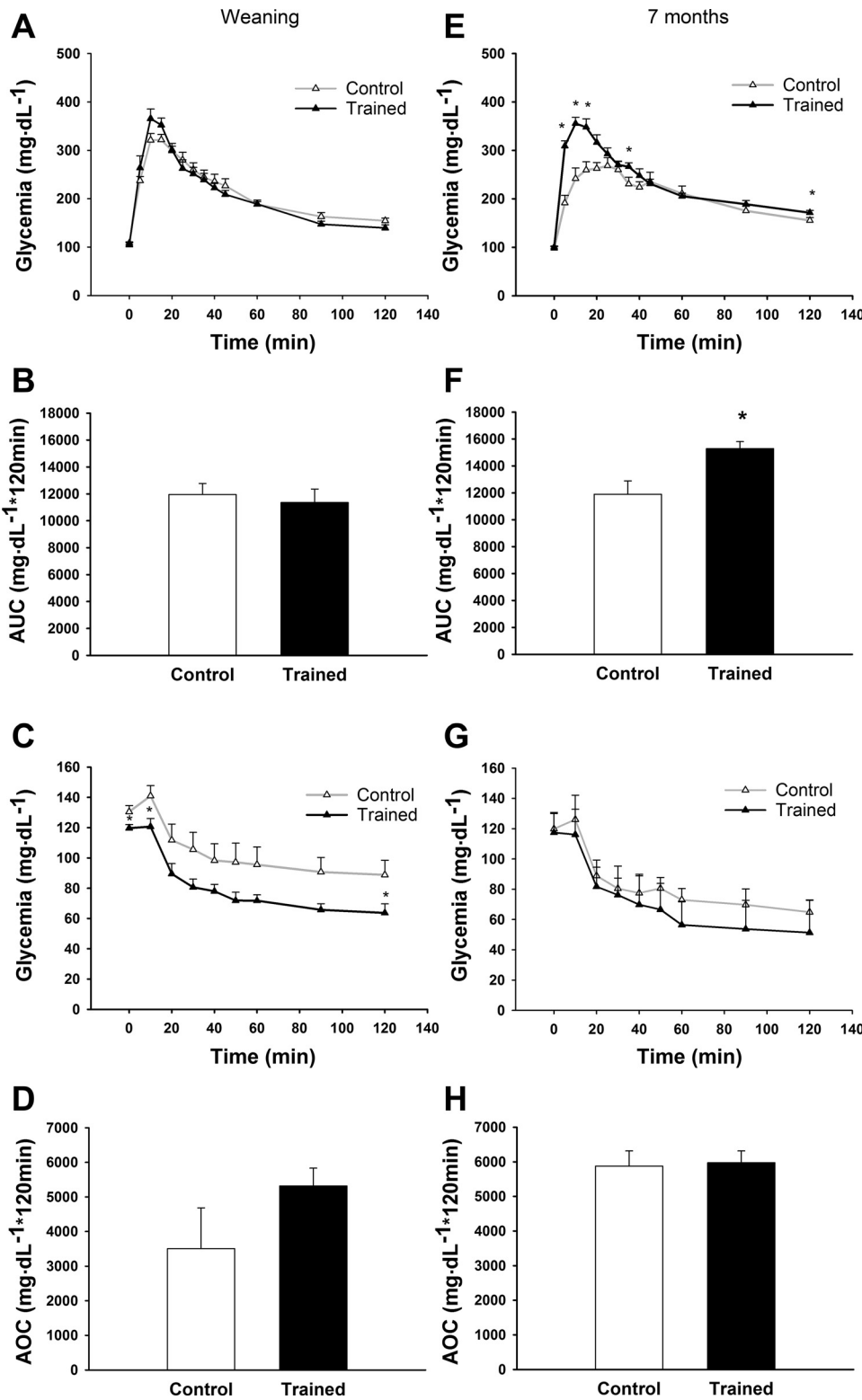
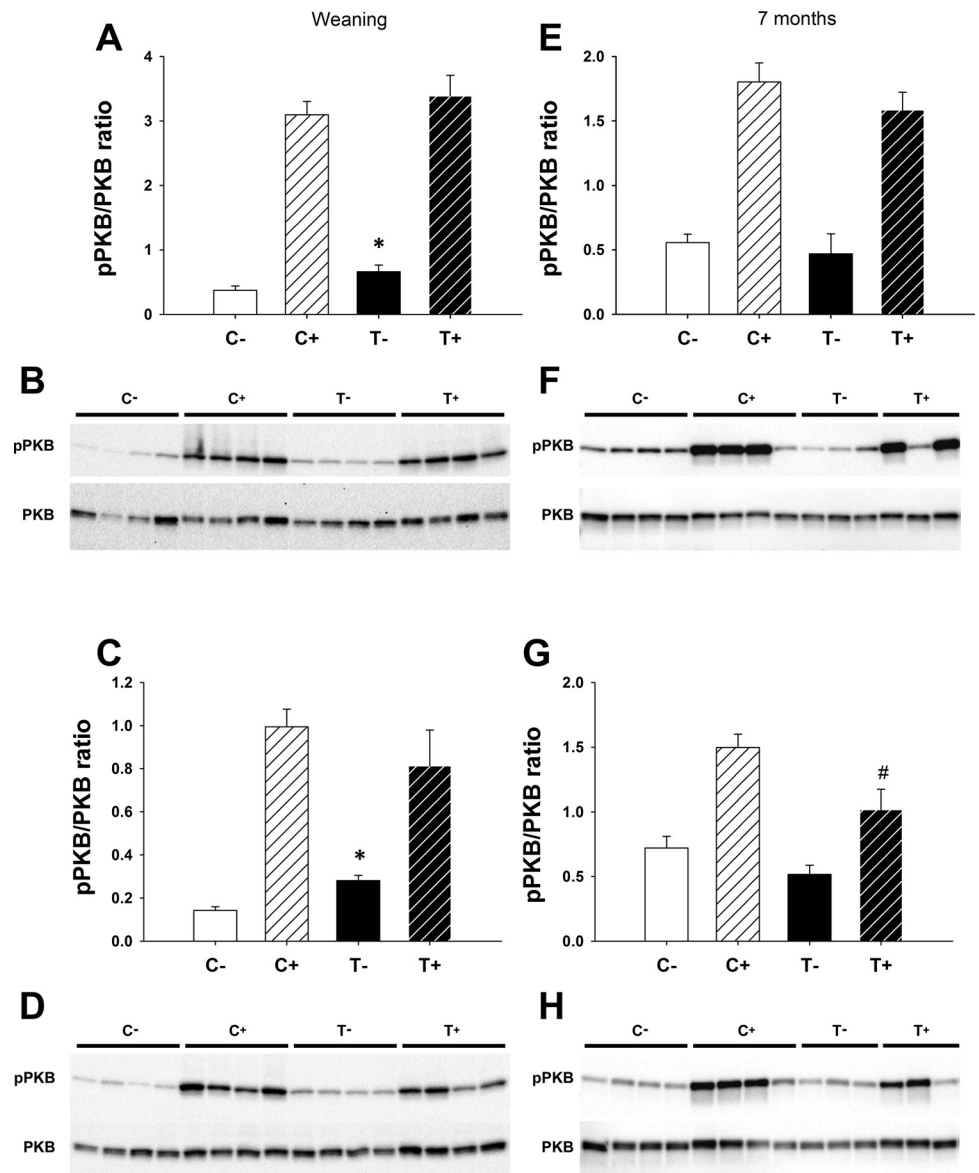


Fig. 2. Maternal exercise alters 7-mo-old offspring glucose tolerance. Rat offspring underwent an intraperitoneal glucose tolerance test (IPGTT) at 3 wk (A and B) and at 7 mo of age (E and F) to assess whole body glucose tolerance. Following a 16-h fast, glucose was injected intraperitoneally at 1 g/kg body wt. Blood glucose levels were monitored before injection (0) and 5, 10, 15, 20, 25, 30, 35, 40, 45, 60, 90, and 120 min after glucose injection (A and E). Areas under the curve (AUC) of IPGTT (C and G) were related to the value at $t = 0$ min. Rat offspring also underwent an intraperitoneal insulin tolerance test (IPITT) at 4 wk (C and D) and at 7 mo of age (G and H) to assess whole body insulin sensitivity. Following a 6-h fast, insulin was injected intraperitoneally at 1 mIU/g body wt. Blood glucose levels were monitored before injection (0) and 10, 20, 30, 40, 50, 60, 90, and 120 min post-insulin injection (C and G). Areas over the curve (AOC) of IPITT (D and H) were related to the value at $t = 0$ min. Data are means \pm SE; $n = 8-14$. * $P < 0.05$ vs. control.

Moreover, some authors have reported that voluntary and controlled low-intensity exercises during gestation have no negative consequences on dams and litter (14, 57). Our training protocol appears to be safe for the mother and the litter since no differences were found in sex ratio, number, or birth weight of pups. Maternal exercise had no effect on maternal body

weight but seemed to change body composition since the weight of the sum of several selected fat pads was lower in trained mothers. Such reduced relative fat mass after endurance training has been described previously in male and female rats (34, 83). It may be explained by a higher energy expenditure in trained mothers, whereas food consumption is the same as for

Fig. 3. Muscle insulin sensitivity is reduced in 7-mo-old trained group rats. Rat offspring underwent an insulin load test at 3 wk (A and C) and 7 mo of age (E and G) to assess liver and muscle insulin pathway activation as an indicator of insulin sensitivity. Insulin bolus was injected intraperitoneally at 10 mIU/g body wt to half of the rats in each group (C+ and T+), and the others were injected with NaCl (C- and T-). Liver (A and E) and gastrocnemius muscle (C and G) samples were collected 15 min after injection to determine phosphorylated (p)PKB/PKB ratio (Ser⁴⁷³) by Western blotting. Representative Western blots show phosphorylated and total (pPKB and PKB, respectively) PKB content in liver and gastrocnemius muscle of 3-wk- (B and D, respectively) and 7-mo-old (F and H, respectively) offspring from control (C) and trained mothers (T) after NaCl (C- and T-) or insulin (C+ and T+) injection. Quantitation of the signals was expressed in arbitrary units. Data are means \pm SE; $n = 8$ for C-, C+, T-, and T+ (A and C); $n = 4$ for C- and C+, and $n = 3$ for T- and T+ (C and G). * $P < 0.05$ vs. C-; # $P < 0.05$ vs. C+.



controls. In the offspring, the maternal training had no consequence on body weight or body composition as suggested by the muscle mass and the fat mass measured. This surprising result could be explained by the duration of exercise sessions (1 h/day), which is shorter than voluntary exercise using running wheels and the running distance (1.5 km/day in our study vs. 1.5–8 km/day at early gestational age to ~1 km/day at late gestational age on running wheels) (12, 14, 28, 58). Reduced caloric intake in trained mothers appears to have no negative consequences on offspring growth, as judged by a similar body weight in both groups. This could be due to the fact that maternal exercise training is thought to increase resting placental bed blood flow (16) and then nutrient delivery at the maternal-placental interface. During pregnancy, postprandial maternal blood glucose levels would vary in accordance with the type of carbohydrates ingested. In our study, diet was composed mainly of low-glycemic carbohydrates that are associated with no change in blood glucose levels throughout pregnancy and with normal-size offspring contrary to

high-glycemic carbohydrate ingestion (16). However, the training program was associated with a lower relative pancreas weight in weanling rats that was not associated with changes in pancreas total insulin content. Moreover, histological cuts revealed that islet area is reduced in pups from trained mothers. We then looked at the insulin secretory capacity of these islets in various conditions. In low-glucose conditions, basal islet insulin secretion was not affected by maternal exercise at weaning. Under high-glucose condition, the stimulated insulin secretion tended to be higher only in weanling rats from the trained mothers. This may be due to the fact that islet insulin release in response to glucose stimulation is reduced in young rats (7, 9, 42, 51). To alleviate this problem, we then measured the insulin secretion after a glucose plus arginine incubation known to be efficient in fetal and young rats (1). In such a condition, we found that maternal exercise enhanced the islet insulin secretion, which is consistent with the literature. Indeed, a recent study showed that small islets have a higher insulin secretion (39). These changes in islet insulin secretion

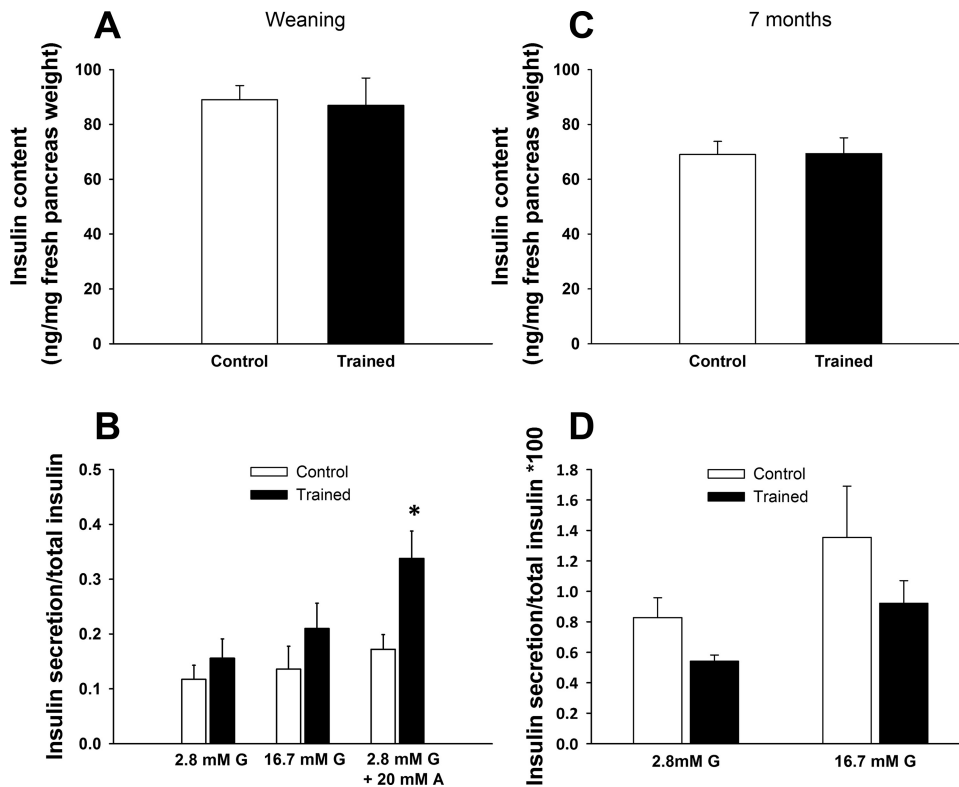


Fig. 4. Maternal exercise does not modify offspring pancreas insulin content but enhances short-term islet insulin secretory capacity and tends to reduce it with aging. Offspring pancreas was collected at 3 wk (A) and at 7 mo of age (C) to determine total insulin content. There were no differences in pancreas insulin content between both groups at 3 wk of age (A) or at 7 mo of age (C). Offspring pancreatic islets were isolated at 4 wk (B) and at 7 mo of age (D) to assess their insulin secretory capacity. The day after isolation, islets were incubated with low glucose (2.8 mM G) and then with high glucose (16.7 mM G) (B and D) or low glucose + arginine (2.8 mM G + 20 mM A) (B). Supernatants were collected and islet insulin secretion determined by ELISA (B) or RIA (D). Data are means \pm SE; $n = 5-9$. * $P < 0.05$ vs. control.

could be responsible for the trend of higher insulinemia found in pups from trained mothers after a 6-h fast.

This tendency of elevated insulinemia without hyperglycemia in pups from trained mothers could be an early marker of insulin resistance. To measure such insulin resistance precisely, we then performed glucose tolerance (IPGTT) and insulin tolerance tests (IPITT) on our animals. Our results show that glucose disposal, as judged by the AUC during IPGTT, and overall insulin sensitivity reflected by the AOC during IPITT were not affected by maternal training. Taken together, these results suggest that, despite a trend of elevated insulinemia, pups from trained mothers were not insulin resistant. This tendency of higher blood insulin could be also a sign for alterations in the insulin signaling. The main insulin-sensitive tissues include skeletal muscle, liver, and adipose tissue (52). Serine/threonine protein kinase Akt/PKB (15) and the ratio between phosphorylated PKB and total PKB content (pPKB/PKB) is classically considered as a good marker of insulin pathway activation (76, 80). Thus, we examined pPKB/PKB ratio in gastrocnemius muscle and in liver both in basal conditions and after an insulin load. In basal conditions, the higher pPKB/PKB ratio measured in both tissues in 3-wk-old rats from trained mothers might be due merely to the trend in a higher plasma insulin level mentioned above. Results after the insulin load confirm that hypothesis since there is no difference in pPKB/PKB ratio between the two groups in that insulin-stimulated condition. So, we can conclude that maternal exercise before and during gestation did not modify liver and muscle insulin pathway activation in offspring.

These effects of maternal exercise on the offspring are age dependent, since results on 7-mo-old rats are different from

those obtained on 3-wk-old rats. Indeed, at 7 mo of age, organ weights were not different between both groups, suggesting that maternal exercise effects would have no significant impact on body composition, including the pancreas. However, relative fat mass tended to be higher ($P = 0.153$) and relative muscle mass lower ($P = 0.183$) in offspring from trained dams. Together, these two results suggest that offspring of trained rats tended to have more fat and may have a significantly higher percentage of body fat. In another set of similar experiments, food consumption was monitored during the first 10 wk after weaning (i.e., 3 mo old), and we did not find any difference between rats from either group (unpublished data). Unfortunately, we do not have any data for the 3- to 7-mo period, but we cannot exclude that food intake becomes different; meanwhile, that could explain the tendency of increased fat mass in rats from the trained group at 7 mo of age. It can also be a consequence of the impossibility to perform their spontaneous activity, resulting in a positive energy balance. Indeed, a study showed that offspring from females that had access to running wheels during gestation were more active in adulthood (28). This would suggest that the lifelong propensity of offspring to exercise depends on maternal physical activity level during gestation (28). In our study, offspring from active mothers could not exert their potential higher spontaneous physical activity while being fed ad libitum. Taken together, these conditions would ultimately lead to an increase in adiposity. Moreover, there were no differences in 6-h or 16-h fasting glycemia, but insulinemia tended to be increased in rats from trained dams after a 6-h fast. However, AUC during IPGTT was significantly larger in 7-mo-old rats born from Trained mothers. This suggests that submaximal maternal exercise would be associated with a worse glucose tolerance in

adult offspring. Here, we show that maternal exercise may have various effects on prospective offspring glucose tolerance, depending on the training session intensity and offspring sex and age. Indeed, our submaximal compulsory exercise program led to a decrease in glucose tolerance in adult offspring, whereas some studies showed that low-intensity voluntary maternal exercise improved it (13, 14). These discrepancies could be explained by several factors. First, these studies were conducted on mice (13) or female Sprague-Dawley rats (14) aged ≤ 16 mo. Moreover, the females had access to running wheels before and throughout gestation and during lactation. In our study, we used 7-mo-old Wistar rats. Moreover, only male offspring were included, and maternal treadmill exercise was compulsory and conducted only before and during gestation, with no change in exercise parameters. In animals from trained mothers, the lower glucose tolerance observed may be due to either exhaustion in islet insulin secretion or a higher insulin resistance (74). Even if pancreas total insulin content was similar between both groups, the tendency to have a lower islet insulin secretion both in basal and in glucose-stimulated conditions may play a role in the glucose intolerance observed in 7-mo-old rats from trained dams. It is unlikely that liver could contribute to that glucose intolerance since the phosphorylation levels of PKB in liver are similar in both groups in basal and insulin-stimulated conditions. In skeletal muscle, the lower pKB/PKB ratio under insulin stimulation in the rats from trained mothers suggests that long-term insulin signaling in muscle could be reduced by maternal exercise done before and during gestation. These results suggest that even if the muscle insulin pathway activation observed with maternal exercise is altered at 7 mo of age, this has no consequences on the overall insulin response at the whole body level, as shown by the lack of difference in AOC during IPITT.

The smaller relative pancreas weight and the smaller islet size could be related to a suboptimal nutritional state of both the mother and the fetus. Indeed, despite the increase of energy expenditure due to training sessions, trained dams had the same food intake as the control dams. This supports the idea that offspring from trained dams may have encountered reduced substrate availability during maternal exercise. It has already been shown that during exercise sessions there could be a transient fetal hypoglycemia (17). These two factors (reduced energy balance and hypoglycemia) could impact the islet insulin secretory capacity under stimulation that could contribute to the trend of increased insulinemia that we observed in pups from trained dams.

Our study shows that submaximal maternal exercise leads to an inappropriate insulin secretion relative to ambient glucose in rodent offspring. Associated with the reduced muscle insulin pathway activation and glucose intolerance that we observed in 7-mo-old offspring, such maternal exercise could increase the susceptibility to T2DM that could be amplified when exposed to bad nutrition such as high-fat diet or to physical inactivity during adulthood. This suggests that daily exercise during the late period of the gestation would be detrimental to offspring, but further studies are needed to determine whether the intensity of such exercise programs may play a role as well.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.Q., F.S., H.D., C.B., and K.C. conception and design of research; C.Q., F.S., G.V., and K.C. performed experiments; C.Q., H.D., C.B., and K.C. analyzed data; C.Q., H.D., P.B., C.B., and K.C. interpreted results of experiments; C.Q. and H.D. prepared figures; C.Q., C.B., and K.C. drafted manuscript; C.Q., H.D., G.V., E.F., C.B., and K.C. edited and revised manuscript; C.Q., F.S., H.D., G.V., P.B., E.F., C.B., and K.C. approved final version of manuscript.

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Middle Iron-Enriched Fructose Diet on Gestational Diabetes Risk and on Oxidative Stress in Offspring Rats

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Abstract Gestational diabetes mellitus (GDM) is associated with increased insulin resistance and a heightened level of oxidative stress (OS). Additionally, high iron consumption could also increase insulin resistance and OS, which could aggravate GDM risk. The aim of this study is to evaluate a high fructose diet (F) as an alternative experimental model of GDM on rats. We also have evaluated the worst effect of a fructose iron-enriched diet (FI) on glucose tolerance and OS status during pregnancy. Anthropometric parameters, plasma glucose levels, insulin, and lipid profile were assessed after delivery in rats fed an F diet. The effects observed in mothers (hyperglycemia, and hyperlipidemia) and on pups (macrosomia and hypoglycemia) are similar to those observed in women with GDM. Therefore, the fructose diet could be proposed as an experimental model of GDM. In this way, we can compare the effect of an iron-enriched diet on the metabolic and redox status of mother rats and their pups. The mothers' glycemic was similar in the F and FI groups, whereas the glycemic was significantly different in the newborn. In rat pups born to mothers fed on an FI diet, the activities of the antioxidant enzyme glutathione peroxidase (GPx) and

glutathione-S-transferase in livers and GPx in brains were altered and the gender analysis showed significant differences. Thus, alterations in the glycemic and redox status in newborns suggest that fetuses are more sensitive than their mothers to the effect of an iron-enriched diet in the case of GDM pregnancy. This study proposed a novel experimental model for GDM and provided insights on the effect of a moderate iron intake in adding to the risk of glucose disorder and oxidative damage on newborns.

Keywords Gestational diabetes · Oxidative stress · High fructose diet · Iron

Introduction

In the field of obstetrics, gestational diabetes mellitus (GDM) is one of most common complications of pregnancy affecting up to 14 % of all pregnancies, depending on the population studied and the diagnostic tests employed [1]. Since GDM is a cause of concern due to increased risks on both mother (e.g., hypertension, preeclampsia, cesarean delivery, and diabetes later in life) and fetus (macrosomia, neonatal hypoglycemia, shoulder dystocia), there is great interest in understanding the etiology and pathophysiological mechanisms of GDM. As the majority of cases return to normal glycemic levels postpartum, GDM has been considered a “transient condition.” However, evidence suggests that GDM should be viewed more as a marker for chronic disease as mothers age [2], but it could also predict occurrence of diseases later in life for the newborn [3]. Despite the better diagnosis of GDM and recognition of its adverse consequences for mother and baby in many countries, there is still no consensus regarding the origin of GDM [4]. It is well known that this risk increases with advancing maternal

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age, racial/ethnic disparities, and obesity, but other factors might also be involved [4, 5].

It is well documented that GDM is associated with oxidative stress (OS), owing to both overproduction of free radicals and/or a defect in the antioxidant defenses [6–9]. Multiple biochemical pathways and mechanisms of action for glucose toxicity have been suggested [10]; all these pathways have in common the formation of reactive oxygen species (ROS), and they relate to insulin resistance [11].

So far, streptozotocin, an agent of choice for experimental diabetes induction, leading to specific necrosis of the pancreatic β -cells [12], has been extensively [13, 14] used to clarify or to prevent [15] the biochemical mechanisms of GDM. However, this experimental model leads to type 1 diabetes, while the features of GDM are more like a type 2 diabetes (DT2). Therefore, streptozotocin is probably not a good model for GDM [13, 14]. A fructose-rich diet has been used as an experimental model for the study of insulin resistance [16, 17] but, so far, not in pregnancy.

Recent studies suggest that iron overload may impair the regulation of body glucose metabolism [18]. A meta-analysis concluded that high iron intake is significantly associated with a greater risk of type 2 diabetes [19]. It is still not clear whether iron leads to the development of GDM, and despite the association between iron intake and GDM risk being examined in several studies [20–24], so far no consensus has been reached [25]. Iron is an essential trace element required for crucial functions of the body, such as oxygen transport and energy production. However, a high iron level increases ROS production, which may cause pancreatic β -cell dysfunction [26], and insulin resistance and gestational diabetes have been associated with high plasma ferritin and biological evidence of oxidative stress [27].

This study had a dual purpose: to propose the fructose diet as an experimental model for studying GDM and to determine the effects of a moderate iron-enriched fructose diet on the metabolic and OS status of newborns.

Material and Methods

Animal Care

All experimental procedure was reviewed and approved by the Joseph Fourier University Institutional Ethic Committee for Animal Experiment. The rats were maintained and handled in agreement with the Guide for the Care and Use of Laboratory Animals. The female Wistar rats (Charles River, L'Arbresle, France), 12 weeks old, were housed in wire-bottomed cages in a temperature-controlled room (22 °C), 50 ± 10 % relative humidity, and a 12-h light/12-h dark cycle.

Diets

The diets were purchased from SAFE, 89290 Augis, France. The control group (C, $n = 6$) was fed by a standard Purina chow. The fructose group received the fructose-rich diet (F, $n = 6$) containing 65 % of fructose and 12 mg iron/100 g as indicated in Table 1. The fructose iron-enriched diet received the same fructose-rich diet but containing 22 mg iron/100 g diet (FI, $n = 6$) (Table 1). All rats were fed for 4 weeks before mating and during gestation for 3 weeks. The analytical measurement of the iron content of the pellets for the F and FI diet was determined using a quadrupole ICP-MS Thermo X-Series II equipped with collision/reaction cell technology (CCT), quartz impact bead spray chamber, and concentric nebulizer. The Xt interface option was used. The collision reaction gas was a mixture of He and H₂ (97/7). The samples were mineralized in nitric acid and then diluted 100-fold in water prior to analysis. Fe 56 and Fe 54 were measured and Ga 71 was used as internal standard. An external standard calibration curve was generated using four calibration standards (0–200–1000 and 2000 nmol/l). Method accuracy was assessed by analyzing NIST standard reference material 1577b (bovine liver) and ARC/CL total diet reference material at the beginning and end of the analytical run. The between-run precision was 3.40 % and the bias was -1.67 %.

Table 1 Composition of the diets (g/100 g diet)

| Composition | Purina chow (group C) | Fructose-rich diet (group F) | Fructose-iron diet (group FI) |
|----------------------|-----------------------|------------------------------|-------------------------------|
| Starch | 62 | 0 | 0 |
| Fructose | 0 | 65 | 65 |
| Casein | 22.7 | 20 | 20 |
| Vegetal oils | 4.5 | 5 | 5 |
| Mineral and vitamins | 6,25 | 6,25 | 6,25 |
| Iron mg/100 g diet | 10 | 12 | 22 |
| Cellulose | 4,50 | 5 | 5 |
| kCal/100 g diet | 379 | 385 | 385 |

Experimental Procedures

Eighteen rats are weighed weekly and pups were weighed at delivery. One day after delivery and after overnight fasting, the mothers were anesthetized with sodium pentobarbital intraperitoneally. Some of the mothers were unfertilized which is common figure in animal facilities; therefore, the number of mother with pups was for each group: C ($n = 4$), F($n = 5$), FI ($n = 5$). Blood from mothers was collected by heart puncture in heparinized tubes protected from light and centrifuged at room temperature for 10 min at 3000g. Plasma was immediately isolated, aliquoted, and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. The morning of sacrifice, males and females pups were weighed and decapitated without anesthesia. Blood glucose readings are taken via a drop of blood using a glucometer. Immediately after blood collection, the rats were sacrificed and visceral masses were weighed. Pups' livers and brains were removed, weighed, frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$ until analysis. Before analysis, tissue samples were homogenized (10 %) in buffer (10 mM Tris-base, 1 mM diethylene triamine pentaacetic acid (DPTA)), 1 mM phenylmethanesulfonyl fluoride (PMSF), pH = 7.4) and centrifuged at 3000g and $4\text{ }^{\circ}\text{C}$ for 10 min.

Biological Parameters

Fasting glucose, cholesterol, and triglyceride levels were evaluated by enzymatic and colorimetric methods on Roche/Hitachi modular 912 (Roche diagnosis, Meylan, France). Glycemia from pups were assessed using an Accu-Chek[®] glucometer (Roche Diabetes Care, Meylan, France). Insulin levels were assessed using commercial radioimmunoassay kit (Merck Millipore Corporation, Germany). Insulin sensitivity was calculated using the homeostatic model assessment-insulin resistance (HOMA-IR) (formula: fasting glucose (mg/dL) \times fasting insulin $\mu\text{UI}/\text{mL}/405$). Plasma thiobarbituric acid reactive substance (TBARS) concentrations were assessed as described by Richard et al. [28]. Total plasma antioxidant status was estimated using ferric reducing antioxidant power (FRAP) assay as described by Benzie et al. [29]. Plasma thiol (SH) groups were assayed as described by Faure and Lafond [30]. The reduced (GSH) and oxidized (GSSG) form of glutathione was determined by a kinetic method as prescribed by Akerboom and Sies [31]. Glutathione peroxidase (GPx) activity was evaluated by the modified method of Gunzler et al. [32], using tertbutyl hydroperoxide as a substrate instead of hydrogen peroxide. The glutathione-S-transferase (GST) activity was determined by the method of Habig et al. [33].

Statistical Analysis

Data statistical analyses were performed using the statistical software package (Statistica Program, Statistical Software, Paris, France). Values were expressed as mean \pm standard error of the mean (SEM). Statistical analyses of the data were performed by analysis of variance, using the *t* test for comparison of the means. Statistical significance was set at $p < 0.05$.

Results

Evaluation of a Fructose Diet as a Model of Gestational Diabetes

The results of anthropometric and biochemical parameters of mother rats and their offspring are represented in Table 2. A high-fructose diet increased significantly the weight of their pups compared to the control fed group. The weight gain and the visceral fat mass were enhanced but not significantly different between the two groups of mothers. The livers of pups from the F group were significantly lower than those of the control group, but the brain weight was unaffected by the maternal diet. The results showed that a high-fructose diet increases significantly glycemia, insulin, cholesterol, and triglyceride of mothers, leading to a significant increase in insulin resistance followed by the HOMA test. On the contrary, glycemia of newborns were decreased.

Evaluation of Anthropometric and Oxidative Stress Parameters in Pups and Mothers Fed a Fructose Diet (F) or a Fructose Enriched with an Iron Diet (FI) during Pregnancy

Although a trend to an increase, the FI diet did not modify significantly the body weight gain during pregnancy, compared to the F fed group (Table 3). In addition, the FI diet had no effect on mothers' glycemia, insulinemia, cholesterol, and triglycerides. However, glycemia and body weight were increased in the pups born of the mothers fed the FI diet and the number of females in the FI group was significantly lower. There are gender differences since the glycemia was significantly increased only in male pups ($p < 0.03$) and the difference of body weight was significant for the female ($p < 0.003$) but almost significant ($p = 0.06$) in male pups.

The results of plasmatic OS parameters of mother rats are represented in Table 4. No significant difference was found on GPx activity, TBARS, and FRAP between rats fed with F and FI diet. The oxidative status was also assayed in livers and brains of offspring from the two groups, and results are

Table 2 Anthropometric and biochemical parameters of mother rats and pups after delivery

| Parameters | Control | Fructose | <i>P</i> value |
|--|-------------|----------------|-----------------|
| Weight gain (g) | 17.2 ± 0.63 | 21.5 ± 1.95 | <i>P</i> > 0.05 |
| Total visceral fat (g/100 g body weight) | 3.41 ± 0.53 | 4.11 ± 0.35 | <i>P</i> > 0.05 |
| Glycemia (mg/dL) | 143 ± 4.14 | 163 ± 3.60* | 0.008 |
| Insulin (ng/mL) | 0.58 ± 0.74 | 1.96 ± 0.80* | 0.016 |
| HOMA-IR(U) | 5.13 ± 0.75 | 19.46 ± 10.71* | 0.014 |
| Cholesterol (mg/dL) | 54 ± 0.01 | 66 ± 0.03* | 0.05 |
| Triglyceride (mg/dL) | 33 ± 0.05 | 88 ± 0.20* | 0.02 |
| Number of pups/dam | 9.5 ± 2.18 | 10.0 ± 2.28 | <i>P</i> > 0.05 |
| Male pups/dam | 4.0 ± 0.58 | 3.6 ± 0.81 | <i>P</i> > 0.05 |
| Female pups/dam | 5.5 ± 1.66 | 6.4 ± 1.69 | <i>P</i> > 0.05 |
| Weight (g) all | 8.5 ± 0.9 | 9.5 ± 1.7* | 0.002 |
| Male pups | 8.94 ± 0.26 | 10.30 ± 0.45* | 0.03 |
| Female pups | 8.17 ± 0.2 | 9.13 ± 0.26* | 0.01 |
| Liver weight all (g/100 g body weight) | 3.54 ± 0.05 | 3.02 ± 0.04* | <0.001 |
| Male pups | 3.58 ± 0.09 | 2.94 ± 0.07* | <0.001 |
| Female pups | 3.51 ± 0.06 | 3.07 ± 0.05* | <0.001 |
| Brain weight all (g/100 g body weight) | 4.14 ± 0.06 | 4.12 ± 0.06 | <i>P</i> > 0.05 |
| Male pups | 3.98 ± 0.07 | 3.93 ± 0.1 | <i>P</i> > 0.05 |
| Female pups | 4.25 ± 0.07 | 4.21 ± 0.07 | <i>P</i> > 0.05 |
| Glycemia (mg/dL) all | 106 ± 2 | 78 ± 2 * | <0.001 |
| Male pups | 112 ± 3 | 74 ± 3* | < 0.001 |
| Female pups | 100 ± 2 | 81 ± 2* | < 0.001 |

Results were expressed as mean ± SEM; the number of rats was in the control fed group (*n* = 4 mothers and *n* = 38 pups) and in the fructose-fed group (*n* = 5 mothers and *n* = 50 pups). **P* < 0.05

represented in Tables 5 and 6, respectively. In pups born to mothers in the FI diet group, brain and liver GPx activity was significantly decreased. The gender analysis showed that in the brain, GPx activity was related to a significant decrease in

males, while the FI diet has no effect in females. Similarly, we found a gender effect on hepatic GPx, which is decreased in both groups, but the effect was also significant only in females.

Table 3 Anthropometric parameters of mothers and pups fed with an F diet or an FI diet

| Parameters | F | FI | <i>P</i> value |
|------------------------------|---------------|---------------|-----------------|
| Mothers weight gain (g) | 21.5 ± 1.95 | 27.5 ± 1.6 | <i>P</i> > 0.05 |
| Mothers glycemia (mg/dL) | 163.24 ± 3.6 | 161.44 ± 5.76 | <i>P</i> > 0.05 |
| Mothers insulinemia (ng/mL) | 1.96 ± 0.78 | 1.05 ± 0.67 | <i>P</i> > 0.05 |
| HOMA-IR(U) | 19.46 ± 10.71 | 10.01 ± 6.15 | <i>P</i> > 0.05 |
| Mothers cholesterol (mg/dL) | 66 ± 0.03 | 64 ± 0.66 | <i>P</i> > 0.05 |
| Mothers triglyceride (mg/dL) | 88 ± 0.20 | 77 ± 0.17 | <i>P</i> > 0.05 |
| Number of pups/dam | 10,0 ± 2,3 | 7,6 ± 2,2 | <i>P</i> > 0.05 |
| Male pups/dam | 3.6 ± 0.8 | 3.8 ± 1.7 | <i>P</i> > 0.05 |
| Female pups/dam | 6.4 ± 1.7 | 3.8 ± 0.9* | 0.05 |
| Pups glycemia (mg/dL) All | 78 ± 1.8 | 85 ± 1.8 * | 0.02 |
| Male pups | 74.4 ± 2.77 | 86.25 ± 2.52* | 0.003 |
| Female pups | 81.13 ± 2.26 | 83.18 ± 2.64 | <i>P</i> > 0.05 |
| Pup weight (g) all | 9.3 ± 0.10 | 11.0 ± 0.34* | <0.001 |
| Male pups | 10 ± 0.62 | 11.1 ± 0.16 | 0.06 |
| Female pups | 8.9 ± 0.37 | 10.9 ± 0.13* | 0.003 |

Results were expressed as mean ± SEM; the number of rats was in fructose group (F: *n* = mothers and *n* = 50 pups) and in fructose-iron (FI: *n* = 5 mothers and *n* = 38); **P* < 0.05

Table 4 Plasmatic OS parameters in mother rats fed with the F or the FI diet

| OS parameters of mothers | F (<i>n</i> = 5) | FI (<i>n</i> = 5) |
|---------------------------|-------------------|--------------------|
| TBARS ($\mu\text{M/L}$) | 4.6 \pm 0.13 | 4.03 \pm 0.14 |
| GPx activity (U/L) | 6349 \pm 304 | 6464 \pm 110 |
| FRAP ($\mu\text{M/L}$) | 273 \pm 20 | 284 \pm 25 |
| SH ($\mu\text{M/L}$) | 327 \pm 38 | 295 \pm 35 |

Results were expressed as mean \pm SEM

Even though there was no difference in total hepatic GSH between the two groups, the hepatic GSH concentration was significantly lower in male pups of the FI group but no difference was observed for female pups. The activity of GST was significantly increased in livers of the FI diet group, and the gender analysis showed a significant increase only in males' livers. In the brain, no difference for GSH and GST was observed between groups. There was no significant difference in total antioxidant capacity measured by FRAP assay between the two groups.

Discussion

Diabetes in pregnant women is associated with increased risk of maternal, fetal, and neonatal complications, which makes GDM a significant public health challenge. Aside from non-modulating factors of GDM as the mother ages, inadequate

nutritional habits, such as high consumption of fructose and iron, might induce oxidative stress and insulin resistance. Therefore, we aimed to assess the fructose diet as a model of diet-induced GDM and then the effect of an iron-enriched fructose diet in inducing metabolic disorder and oxidative stress.

The Fructose Diet as a Model of GDM

So far, streptozotocin diabetes is the first choice for studying experimental GDM [12] and, to our knowledge, the only model. However, streptozotocin induces specific necrosis of the pancreatic β -cells, such as type 1 diabetes, rather than insulin-resistant diabetes, and it is still challenging for inducing GDM with streptozotocin [13, 34]. Thus, we evaluated a fructose-rich diet to feed pregnant rats as an experimental animal model for GDM by inducing insulin resistance. Here, the effect of an F diet to induce insulin resistance assessed with the HOMA score during pregnancy was consistent with that obtained with the same F diet in adult rats [35] and with diabetes induced by streptozotocin [36]. Furthermore, in this experimental model, the weight of newborns was significantly larger in the group of mothers fed with the F diet, and this effect is in agreement with the well-documented increased risk of macrosomia in the GDM women [37]. According to the recent guidelines of the Institutes of Medicine, weight gain associated with GDM increases the risk of miscarriages and other adverse outcomes [38], leading to a need for dietary counseling. While the pups were bigger, their blood glucose

Table 5 Liver OS parameters in newborn rats born to rats fed with F and with FI diet

| OS parameters | Group F (<i>n</i> = 50) (18 males; 32 females) | Group FI (<i>n</i> = 38) (19 males; 19 females) | <i>P</i> value |
|----------------------------------|--|---|-----------------|
| TBARS ($\mu\text{M/L}$) | 1.05 \pm 0.2 | 0.99 \pm 0.2 | <i>P</i> > 0.05 |
| Male | 1.12 \pm 0.10 | 1.05 \pm 0.10 | <i>P</i> > 0.05 |
| Female | 0.95 \pm 0.13 | 0.92 \pm 0.19 | <i>P</i> > 0.05 |
| GPx activity (U/gP) | 254 \pm 7.0 | 225 \pm 6.6* | 0.03 |
| Male | 253 \pm 15 | 232 \pm 7.9 | <i>P</i> > 0.05 |
| Female | 255 \pm 10 | 217 \pm 11* | 0.005 |
| GSH ($\mu\text{M/g P}$) | 636 \pm 57 | 569 \pm 32 | <i>P</i> > 0.05 |
| Male | 711 \pm 73 | 522 \pm 33* | 0.03 |
| Female | 562 \pm 82 | 625 \pm 52 | <i>P</i> > 0.05 |
| GSH/GSSG | 87.79 \pm 6.34 | 88.87 \pm 4.15 | <i>P</i> > 0.05 |
| Male | 83.82 \pm 30.9 | 87.63 \pm 13 | <i>P</i> > 0.05 |
| Female | 82.44 \pm 21.36 | 91.79 \pm 15.46 | <i>P</i> > 0.05 |
| FRAP ($\mu\text{M/g protein}$) | 210 \pm 5.8 | 197 \pm 4.9 | <i>P</i> > 0.05 |
| Male | 217 \pm 7.8 | 201 \pm 6.9 | <i>P</i> > 0.05 |
| Female | 202 \pm 8.0 | 193 \pm 7.0 | <i>P</i> > 0.05 |
| GST (nM/mg protein) | 232 \pm 5.4 | 249 \pm 4.3* | 0.015 |
| Male | 228 \pm 8.5 | 257 \pm 6.9* | 0.02 |
| Female | 236 \pm 7.0 | 242 \pm 4.0 | <i>P</i> > 0.05 |

Results were expressed as mean \pm SEM; **P* \leq 0.05

Table 6 Brain OS parameters in newborn rats born to rats fed with F and with FI diet

| OS parameters | F (<i>n</i> = 50) (18 males; 32 females) | FI (<i>n</i> = 38) (19 males; 19 females) | <i>P</i> value |
|----------------------------|--|---|-----------------|
| GPx activity (U/g protein) | 104,1 ± 3.8 | 92,8 ± 3.5* | 0.04 |
| Male | 113 ± 3.9 | 91 ± 2.4 * | 0.001 |
| Female | 95 ± 4.0 | 95 ± 8.0 | <i>P</i> > 0.05 |
| GSH (μM/g protein) | 273 ± 6.4 | 256 ± 6.7 | <i>P</i> > 0.05 |
| Male | 259 ± 3.0 | 250 ± 6.0 | <i>P</i> > 0.05 |
| Female | 301 ± 6.0 | 266 ± 1.4 | <i>P</i> > 0.05 |
| GSH/GSSG | 76.08 ± 7.13 | 86.47 ± 11.16 | <i>P</i> > 0.05 |
| Male | 79.56 ± 19.54 | 88.08 ± 40 | <i>P</i> > 0.05 |
| Female | 70.55 ± 19.53 | 81.25 ± 24.2 | <i>P</i> > 0.05 |
| FRAP (μM/g protein) | 254 ± 4.0 | 253 ± 4.1 | <i>P</i> > 0.05 |
| Male | 258 ± 3.3 | 258 ± 6.5 | <i>P</i> > 0.05 |
| Female | 250 ± 7.3 | 248 ± 4.8 | <i>P</i> > 0.05 |
| GST (nM/mg protein) | 216 ± 8.3 | 228 ± 5.6 | <i>P</i> > 0.05 |
| Male | 213 ± 36 | 230 ± 17 | <i>P</i> > 0.05 |
| Female | 213 ± 27 | 226 ± 29 | <i>P</i> > 0.05 |

Results were expressed as mean ± SEM; **P* ≤ 0.05

level was significantly lower than in the control group. This result is also consistent with the fact that the hypoglycemia associated with macrosomia is one of the most common metabolic disorders of the neonate of a GDM mother [37]. It occurs due to the hyperinsulinemia of the fetus in response to the maternal hyperglycemia in utero. The lower liver weight, observed both in male and female pups, could be explained by a decrease in glycogen storage level in response to a state of hepatic insulin resistance [39]. Taken as a whole, our results suggest that the fructose diet could be an alternative successful experimental model for studying GDM.

The Fructose Diet as a Model of GDM

In the second part of our study, we tested the hypothesis that an increased iron intake would induce metabolic and oxidative disorders during GDM. Iron requirements to meet the fetal pups' needs were estimated at 5–25 mg iron/100 g diet [40]. In our study, the dose of iron in the FI diet (22 mg/100 g diet) corresponded to a moderate, but not too heavy dose, because our goal was to assess the consequences of a high, but not an excessive iron intake, which is rarely the case during pregnancy. Furthermore, our goal was not to confirm the benefit of iron in cases of anemia, but we aimed to evaluate the effect of an iron-rich diet to rats of normal iron status in case of GDM, to increase insulin resistance and OS. Therefore, in the fructose diet, the level of iron was normal. Despite that the FI diet did not induce significant metabolic changes in mothers, the body weights of the newborns were increased and their glycemia was significantly increased. The increase in body weight induced by iron during GDM was more important in

female. The number of female progeniture was also smaller in the group of pups born from mothers with a higher iron intake. In a previous study, iron supplementation to pregnant rats was associated to an increased number of placentas without a developing fetus, which could take in part to our result [41]. Since diet gender effects have been reported in offspring born from mothers fed with fructose liquid intake during pregnancy [42, 43], we can hypothesize that the FI diet could play a part in the small number of females in the FI diet group. Further experiments are necessary to confirm that this effect is caused by the FI diet.

The FI diet did not affect the oxidative status of the mothers. A previous study [44] in an experimental model of GDM induced by streptozotocin reported an increase in OS induced by intra-peritoneal iron. However, in this study, both the method and the high dose of iron, in contrast to ours, might explain the discrepancies in oxidative stress.

Interestingly, despite the apparently similar OS level between the mothers fed with the F or the FI diet, the redox status of the livers and brains of newborns was altered. This suggests that fetuses were more sensitive to the effect of iron during pregnancy than their mothers.

The GPx activity was decreased both in the liver and the brain of pups born from the FI-fed mothers. The inhibition of GPx activity has often been reported in cases of obesity and insulin resistance [45]. In line of our results, a difference by sex of hepatic GPx activity has also been reported in pups born to diabetic mothers [46]. The decrease in GPx is a common feature of the route toward DT2 [47] and aging [48] and in neurodegenerative diseases [49]. Interestingly, it has been shown that iron supplementation in neonate mice increases the

risk of Huntington's disease associated with an increased oxidative stress [50] while GPx was neuroprotective in a model of this neurodegenerative disease [51]. Therefore, the GPx decline observed in the brains of the FI males could have important implications for cognitive function. In future studies, it would be interesting to evaluate the consequences of this decrease on their behavior in adulthood.

In our study, males' livers seemed to be more sensitive than those of female to the effect of iron-rich diet. Indeed, the reduced GSH levels of males born to the FI diet-fed mothers served as an iron-induced OS indicator, while in the liver of females, the GSH levels were not modified. In line with this effect, Kim et al. [52] found that iron overload is associated with insulin resistance in men, but not in women. In addition, the GST activity was increased in the liver of pups from the FI group. This would appear to represent an adaptive response to cope with an increased ROS production induced by iron. Indeed, several lines of evidence suggest that GST plays a role in protecting cells from the consequences of such stress, since GST is regulated by ROS such as H₂O₂. The induction of GST by ROS has been described as an adaptive response to detoxify some oxidative metabolites produced by OS [53]. GST is a prototypical phase 2 antioxidant enzyme, which has a part in the detoxification of a broad range of toxic and potentially carcinogenic compounds [53]. In accordance with our results, an enhancement of GST was associated with a higher decrease in GPx in iron-treated mice [54]. Also in agreement with our data, suggesting that male might be more sensitive to female to the diet of mothers during pregnancy, a gender effect has been reported with a 10 % (w/v) fructose in drinking water during pregnancy, which increased oxidative stress in the liver of male progenitor but not in female [43]. In this study, in relation with an increased oxidative stress in livers of male offspring, a feature of a metabolic syndrome was observed in male while the females were more resistant. However, it is worth noting that the model of fructose in drinking water is not a model of GDM since it is not associated with glucose impairment [55] during pregnancy. Furthermore, in this study the analyses were performed later after delivery (90 days) while at delivery in our study. Therefore, we cannot exclude that postnatal nutrition could modify metabolic abnormalities induced by iron-fed fetal programming associated with GDM. This deserves further investigation to evaluate later in life the consequences of an oxidative stress in the liver of pups born from mothers with a GDM and with high iron intake.

In conclusion, the similarity in the metabolic and anthropometric effects observed in GDM in women and those of our experimental model allows us to propose the fructose diet as an experimental model of GDM. Clearly, these results indicate that a moderately high iron intake, in the case of GDM, induces adverse effects in the pups (macrosomia and impaired redox status both on the liver and the brain) without significant effects on their mothers. Future studies are warranted to clarify

the link between these potential characteristics of a metabolic and oxidative altered state and a possible increased risk of chronic diseases later in life. Proper management of GDM, regarding iron intake in particular, would be of benefit for the newborn's health and the prevention of diseases in adulthood.

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Compliance with Ethical Standards All experimental procedure was reviewed and approved by the Joseph Fourier University Institutional Ethic Committee for Animal Experiment. The rats were maintained and handled in agreement with the Guide for the Care and Use of Laboratory Animals.

Conflict of Interest The authors declare that they have no conflict of interest.

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