

Impacts of a combined exposition to a pesticide, Chlorpyrifos (CPF), and a High-Fat Diet on the contractile properties of the gastrointestinal smooth muscle and striated diaphragm muscle

Hiba El Khayat El Sabbouri

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à l'Ecole doctorale des Sciences et Technologie **de l'Université Libanaise**

par

Hiba EL KHAYAT EL SABBOURI

pour obtenir le grade de Docteur de l'Université de Picardie Jules Verne

Impacts d'un pesticide, le chlorpyriphos (CPF), et d'un régime riche en graisses "High Fat Diet" sur l'activité contractile du muscle lisse digestif et strié diaphragmatique

Soutenue le 20 Décembre 2019, après avis des rapporteurs, devant le jury d'examen :

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- Impacts of prolonged chlorpyrifos exposure on locomotion and slow-and fasttwitch skeletal muscles contractility in rats: Nancy Hallal, Hiba El Khayat El Sabbouri, Ali Salami, Wiam Ramadan, Hassan Khachfe, Mohamed E. Moustafa, Mahmoud Khalil, and Wissam H. Joumaa. *Toxicology Reports* 6 (2019) 598–606. doi:10.1016/j.toxrep.2019.06.006

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List of abbreviations

5-HT: 5-hydroxytryptamine AC: Adenylyl cyclase ACh: Acetylcholine AChE: Acetylcholinesterase ADI: Acceptable daily intake ARfD: Acute reference dose ATP: Adenosine triphosphate BMI: Body mass index BuChE: Butyrylcholinesterase Ca²⁺: Calcium cAMP: Cyclic adenosine monophosphate CaE: Carboxylesterase **ChE:** Cholinesterase **CO: Carbon Monoxide** CO₂: Carbon dioxide COPD: Chronic Obstructive pulmonary disease CNS: Central nervous system **CPF:** Chlorpyrifos **CPO:** Chlorpyrifos-oxon CYP: Cytochrome P450 DAP: Dialkylphosphate DDT: Dichlorodiphenyltrichloroethane **DEP:** Diethylphosphate **DETP:** Diethylthiophosphate DGCCRF: French General Directorate for Consumer Affairs and Fraud Control **DIO: Diet-Induced Obesity** DOHaD: Developmental Origin of Health And Disease DTNB: 5,5'-dithio-bis-(2-nitrobenzoic acid) E%: percent of total energy edl: extensor digitorum longus **ECC: Excitation-Contraction Coupling** EFSA: European Food Safety Authority ELISA: Enzyme-Linked ImmunoSorbent Assay **ENS: Enteric Nervous System EPA: Environmental Protection Agency** EU: European Union EUCP: EU-coordinated control program f: Respiratory Frequency FAO: Food and Agriculture Organization FI: Fatigability Index FSH: Follicle stimulating hormone GABA: Gamma aminobutyric acid GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GD: Gestational Day **GDP: Gross Domestic Product GI**: Gastrointestinal **GH: Growth Hormone** HFD: High-Fat Diet **IBS: Irritable Bowel Syndrome** ICCs: Interstitial cells of Cajal ILK: Integrin-linked kinase JMPR: Joint FAO/WHO Meeting on Pesticide Residues LD₅₀: Lethal dose 50 LH: Luteinizing hormone LIBNOR: Lebanese Standards Institution LOO: limit of quantification LPS: Lipopolysaccharide mAChRs: muscarinic Acetylcholine Receptors MCFA: Medium-chain fatty acids MHC: Myosin heavy chain MLCK: Myosin light chain kinase MUFAs: Monounsaturated fatty acids **MRLs: Maximum Residue Limits** nAChR: nicotinic Acetylcholine receptors NGF: Nerve growth factor NKA: Neurokinin A NKB: Neurokinin B NMJ: Neuromuscular junction NO: Nitric oxide NPK: Neuropeptide K NP γ : Neuropeptide γ NOAEL: No Observable Adverse Effect Level **OPs: Organophosphates OSA:** Obstructive sleep Apnea CSA: Cross-Sectional Area **PAR : Predictive Adaptive Responses** PON1: Paraoxonase PCBs: polychlorinated biphenyls PIPs: Plant-Incorporated-Protectants **PND: Postnatal Day PNS:** Peripheral nervous system preBötC: pre-Botzinger complex PUFAs: polyunsaturated fatty acids **PPPs: Plant Protection Products** qRT-PCR : Quantitative Reverse Transcription Polymerase chain Reaction **REM:** Rapid-eye-movement RyR1: Ryanodine receptor1 SAS: Sleep apnea syndrome SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SFA: Saturated fatty acids SERCA: Sarcoplasmic Reticulum Ca²⁺ adenosine triphosphatase SP: Substance P SR: Sarcoplasmic Reticulum TCP: 3,5,6-trichloro-2-pyridinol T_E : Expiratory Time T₁: Inspiratory Time T-tubules: Transverse tubules VIP: Vasoactive intestinal polypeptide V_T: Tidal Volume WHO: World Health Organization ZIPK: Zipper-interacting protein kinase

RÉSUMÉ GÉNÉRALE

INTRODUCTION et HYPOTHESE

Les pesticides représentent un problème de santé publique majeur dans le monde actuel. Leur diversité d'action est élevée, principalement dans un pays qui a une histoire agricole extrêmement riche, tel que la France mais aussi le Liban. L'application intensive de pesticides a sans aucun doute contribué à améliorer la production alimentaire et à lutter contre les maladies (Berton et al. 2014). Ces pesticides sont largement utilisés dans les milieux professionnels et les zones résidentielles, en tant qu'outil économique, d'économie de travail et efficace pour la lutte contre les parasites et la production agricole.

Les résidus de pesticides constituent un réel problème de société de nos jours. Au cours des cinquante dernières années, ils ont été mis en évidence dans tous les compartiments de l'environnement, dans les rivières, les eaux souterraines et l'air. Ces résidus sont également présents dans les fruits, les légumes, les céréales et les produits d'origine animale. La population générale est régulièrement exposée aux résidus de pesticides en tant que contaminants présents dans les aliments et l'eau. Outre l'exposition directe des adultes à ces pesticides à l'âge adulte, les femmes enceintes peuvent être exposées aux pesticides au cours des phases critiques du développement, en affectant à leur tour le foetus et le lait maternel pendant la lactation.

Dans cette étude, le chlorpyriphos (CPF) a été sélectionné en raison de son utilisation élevée en Europe. Ses résidus ont été parmi les plus détectés dans les aliments en Europe et au Liban. Le CPF est un pesticide neurotoxique pour le développement. Il est classé parmi les substances les plus dangereuses à identifier comme étant une priorité dans la communauté européenne.

CPF est un insecticide organophosphoré largement utilisé qui agit en inhibant l'acétylcholinestérase au niveau des synapses cholinergiques. Le CPF est associé à des effets néfastes sur la santé au long cours, notamment des perturbations métaboliques, des perturbations endocriniennes, des effets gastro-intestinaux (GI), ou des maladies respiratoires. L'exposition au CPF est délétère pour les systèmes respiratoire et digestif pendant les périodes de développement *in utero* et postnatal (Darwiche et al. 2017; Darwiche et al. 2018). À l'âge adulte, l'effet de l'exposition alimentaire au CPF a été exploré sur les muscles squelettiques rapides ou à contraction lente (Hallal et al. 2019), mais pas sur la fonction du muscle squelettique respiratoire mixte.

D'autre part, l'obésité représente un risque majeur pour la santé du 21ème siècle. Le surpoids et l'obésité sont les principaux problèmes nutritionnels dans les sociétés développées, en particulier en France, où plus de 21 millions de personnes sont en surpoids ou obèses, en particulier en Champagne-Ardenne et en Picardie, où les taux d'obésité sont les plus élevés ("ObEpi-Roche 2012 : enquête nationale sur l'obésité et le surpoids,").

Ainsi, le moment et la durée d'exposition aux perturbations environementales ou alimentaires sont des facteurs déterminants pour le fœtus et sa croissance et maturation postnatales. La période périnatale est caractérisée par une grande plasticité des systèmes physiologiques avec une sensibilité et une vulnérabilité encore plus grande des individus aux facteurs environnementaux (programmation fœtale). Selon le concept de l'origine développementale des maladies (DOHaD), des perturbations pendant la vie prénatale et intra-utérine peuvent programmer les risques d'apparition de maladies plus tard dans la vie. Des études expérimentales et épidémiologiques ont montré que l'obésité maternelle constituait un environnement à risque qui programme le métabolisme du nourrisson et favorise l'apparition de l'obésité à l'âge adulte (Alfaradhi et al. 2016; Lecoutre et al. 2016). De nombreux facteurs entrent en jeu dans l'apparition de l'obésité: la consommation d'aliments déséquilibrés riches en graisses (junk food) chez les personnes prédisposées, mais également l'exposition répétée à des facteurs environnementaux tels que les pesticides, en particulier les résidus de pesticides organophosphorés.

Des études récentes sur des modèles animaux ont démontré qu'une alimentation riche en matières grasses de type « western diet » peut exacerber un certain nombre de perturbations métaboliques induites par l'exposition aux pesticides organophosphorés en début de vie (Slotkin 2011). D'autre part, des contaminants alimentaires tels que les pesticides organophosphorés (OPs) augmenteraient les troubles induits par un régime alimentaire riche en graisses (effets sur la digestion, les voies respiratoires et le métabolisme).

Cette étude est divisée en deux approches différentes:

1) Le premier objectif est d'examiner les effets de l'exposition répétée au CPF à l'âge adulte chez le rat sur la réponse contractile du diaphragme et les hormones susceptibles d'affecter les performances musculaires. Nous avons en outre cherché à étudier les mécanismes par lesquels le CPF induit l'altération de la contractilité musculaire en étudiant la composition de l'isoforme de la myosine, en tant que déterminant clé de la performance contractile dans les muscles squelettiques.

2) Le second objectif est de déterminer si une exposition périgestationnelle à long terme au CPF associée à un régime obésogène (HFD) pendant les périodes pré-gestationnelle, gestationnelle et d'allaitement pourrait affecter les performances des muscles squelettiques respiratoires et des muscles lisses digestifs de la descendance à l'âge adulte.

i. En fait, la respiration est un mécanisme rythmique continu essentiel tout au long de la vie. Des altérations du contrôle cholinergique sont par conséquent impliquées dans les perturbations des performances respiratoires. L'exposition à des pesticides ou au HFD est associée à une altération de la fonction respiratoire (Villa et al. 2007; Speretta et al. 2018) et affecte l'activité contractile d'un muscle respiratoire majeur; le diaphragme (Thiermann, Eyer, and Worek 2010; Tallis, James, and Seebacher 2018). L'exposition au CPF est délétère pour le système respiratoire pendant les périodes *in utero* et postnatale (Darwiche et al. 2018). Cependant, les effets cumulatifs de l'exposition périgestationnelle au CPF et/ou au HFD (de la gestation à la fin de la lactation) sur la performance respiratoire de rats à l'âge adulte, qui en conséquence n'ont pas été en contact direct avec le CPF et /ou le HFD n'ont jamais été explorés. Ainsi, l'un des objectifs principaux de cette étude est de déterminer les conséquences d'une exposition continue péri-gestationnelle à long terme des rates (mères) au CPF et/ou HFD sur les paramètres respiratoires, l'incidence de l'apnée du sommeil et les réponses contractiles du muscle squelettique respiratoire; le diaphragme.

ii. Le tractus gastro-intestinal est le premier compartiment en contact avec les aliments ingérés et les contaminants associés. La digestion et l'absorption des nutriments, qui sont essentiels à la survie, sont considérées comme des tâches critiques du système gastro-intestinal. Les autres fonctions comprennent l'excrétion et la protection. Il a été démontré que l'exposition aux OP ou la consommation de HFD altèrent la fonction gastrointestinale et affectent la contractilité du muscle lisse iléal. Il a été révélé que l'exposition prénatale et postnatale au CPF augmente la perméabilité intestinale (Joly Condette et al. 2014), provoque des anomalies métaboliques (Reygner et al. 2016) et diminue l'amplitude de la contraction du muscle lisse longitudinal iléal (Darwiche et al. 2017) au sein de la descendance à l'âge adulte. Dans cette étude, nous avons aussi examiné les effets à long terme de l'exposition maternelle à une faible dose de CPF associée à l'alimentation HFD avant et pendant la gestation et pendant la lactation sur l'activité contractile du muscle lisse iléal à l'âge adulte comme étant essentiel pour le maintien de l'homéostasie intestinale et la bonne absorption des nutriments.

ANIMAUX, MATERIEL et METHODES

Etude 1: exposition de rats adultes

1.1. Préparation du CPF et exposition des rats au CPF

Le CPF (O, O '- diéthyl-O-3,5,6-trichloro-2-pyridylphosphorothioate pur à 99,8%) a été dissous dans de l'huile de maïs (comme solvant) à deux concentrations de 1 et 5 mg/ kg.

30 rats Sprague Dawley mâles adultes (âgés de 6 semaines; poids corporel moyen 200 ± 10 g) ont été divisés de manière aléatoire en 3 groupes (10 rats/groupe): contrôle, CPF1 et CPF5. Les animaux des groupes CPF1 et CPF5 ont été nourris quotidiennement avec un régime alimentaire standard en granulés (20 g/jour/rat) mélangé à 1 ou 5 mg de CPF/kg de poids corporel/jour respectivement pendant 6 semaines consécutives. Les rats du groupe témoin ont été nourris avec le même régime en granulés contenant le solvant à 1

ml/kg/jour. Après la période d'exposition de 6 semaines, les rats ont été euthanasiés et le diaphragme a été disséqué.

1.2. Mesure des propriétés contractiles du muscle

Les bandelettes de diaphragme ont été suspendues verticalement dans une cuve en plexiglas contenant une solution de Ringer oxygénée thermostatée à 37 °C. Le bord des côtes était ancré à la base du bain en plexiglas et le tendon central du diaphragme était fixé à un capteur de force isométrique. Les muscles ont été stimulés par une stimulation supramaximale (12 V, 2 ms). La longueur de la bandelette de diaphragme a été étirée jusqu'à atteindre L₀, définie comme la longueur optimale à laquelle la force maximale a été atteinte (McGuire and MacDermott 1998).

Des secousses simples ont été obtenues après l'équilibration. Pour chaque contraction, la tension de contraction maximale a été normalisée par la surface de la section transversale et présentée en g.cm⁻². Le temps nécessaire pour atteindre la tension maximale (ms) et le temps de demi-relaxation (ms) ont été déterminés. La tension tétanique maximale a été produite par des trains de stimuli d'une durée de 1 s (20 Hz). La résistance à la fatigue a été examinée selon un protocole de stimuli de 2 Hz pendant 5 minutes. L'indice de fatigabilité a été calculé comme suit: FI = (Force initiale) - (Force finale) * 100 / (Force initiale).

1.3. L'extraction et le dosage des protéines myofibrillaires

Les muscles du diaphragme qui on été congelés, ont été homogénéisés avec 5 volumes de tampon de lavage. Ensuite, les protéines myofibrillaires ont été extraites avec 3 volumes de tampon d'extraction, et incubées une nuit à 4°C. Le surnageant contenant les protéines myofibrillaires a été utilisé pour déterminer les concentrations protéiques par spectrophotométrie à 595 nm selon le test de Bradford (Bio-Rad, Hercules, CA, USA) (Bradford 1976).

1.4. Séparation des isoformes de la chaîne lourde de la myosine par électrophorèse sur gel de polyacrylamide de sodium dodécylsulfate (SDS-PAGE)

La migration verticale simple de SDS-PAGE a été utilisée pour la séparation électrophorétique des isoformes de MHC (Joumaa and Léoty 2002). Les gels de séparation et d'empilement ont été préparés selon le protocole décrit précédemment (Talmadge and Roy 1993). 2 µg de protéines myofibrillaires ont été chargés dans chaque puits. Les gels ont été passés sous tension constante à 72 V pendant 28 h à 4-6 °C. Ensuite, les gels ont été colorés avec la solution de bleu de Coomassie. Après avoir numérisé les gels, l'expression relative des différentes isoformes de MHC a été déterminée par le logiciel Image J (National Institutes of Health, États-Unis).

1.5 Mesure de l'activité de l'acétylcholinestérase (AChE)

L'activité de l'AChE a été déterminée en appliquant la méthode d'Ellman modifiée (Ellman et al. 1961), en mesurant la quantité d'ACh hydrolysée par l'enzyme AChE chaque minute dans les échantillons analysés. Les tissus du diaphragme ont été homogénéisés dans un tampon de lyse pour cellules de mammifère (environ 179835; Abcam, UK). Après centrifugation, le surnageant a été utilisé pour la mesure de l'activité AChE à l'aide du kit de dosage colorimétrique AChE en une étape (ab138871; Abcam) conformément aux instructions du fabricant. L'activité de l'AChE a été normalisée par rapport à la teneur en protéines et présentée en µmol/min/mg de protéines.

1.6. Taux sériques de cortisol, de testostérone et d'hormone de croissance

Les taux d'hormones ont été déterminés par la technique ELISA (Enzyme-Linked ImmunoSorbent Assay). Le kit ELISA cortisol (K7430, BioVision, USA), le kit ELISA testostérone (K7418, BioVision) et le kit ELISA hormone de croissance (A05104, Bertin, France) ont été utilisés pour la quantification sérique du cortisol, de la testostérone et de l'hormone de croissance (GH), respectivement, selon les instructions des fournisseurs.

Etude 2: Exposition périgestationnelle au CPF et au HFD

2.1. Procédure expérimentale

16 rats Wistar femelles (âge à l'arrivée: 7 semaines) ont été répartis au hasard en 4 groupes (n = 4 / groupe) après une semaine d'acclimatation aux conditions d'hébergement. Les femelles ont été nourries soit de nourriture conventionnelle (Serlab3436, 3,1 kcal/g). ou un régime alimentaire riche en graisses HFD (5,24 kcal/g, 60% de kcal provenant des lipides), gavés quotidiennement avec de l'huile de colza biologique (en tant que solvant) pour les groupes témoins (huile, régime alimentaire normal) et les groupes HFD (huile, HFD) ou CPF (1 mg/kg/jour) pour CPF (CPF, alimentation standard) et HFD+CPF (CPF, HFD) groupes pendant 4 mois consécutifs. À la fin de ces 4 mois, les femelles ont été accouplées avec des rats mâles. Pendant la gestation, les femelles ont été soumises au même traitement qu'avant la gestation jusqu'à la fin de la période de lactation. Au sevrage; 21^{ème} jour postnatal, les ratons ont été séparés de leur mère et quatre groupes de rats mâles (n = 7-10 par groupe) ont été identifiés en fonction de leur exposition maternelle dans les groupes suivants: groupe témoin (régime standard et solvant), groupe HFD (groupe HFD et solvant), et le groupe CPF (régime standard et CPF) et le groupe HFD+CPF (HFD et CPF). Après le sevrage, les ratons ne recevaient qu'un régime standard sans CPF jusqu'à J60 (début de l'âge adulte).

2.2. Etude de la performance respiratoire chez le rat jeune adulte

i. Pléthysmographie corps entier: Mesures Respiratoires

Le temps inspiratoire et le temps expiratoire (respectivement T_I et T_E), le volume courant (V_T) (exprimé par 100 g de poids corporel) et la fréquence respiratoire (f) de tous les ratons mâles ont été mesurés par pléthysmographie corps entier (modèle PLY3213, Buxco-EMKA Technologies) à J60 (Drorbaugh and Fenn 1955). Chaque animal a été habitué à la chambre de pléthysmographie pendant deux jours consécutifs avant les mesures. Le protocole a duré 90 minutes, pendant lesquelles nous avons continuellement noté si le rat était réveillé ou endormi. L'apnée du sommeil, qui reflète deux respirations consécutives non réalisées, a été notée selon une procédure bien définie (Christon et al. 1996). L'indice d'apnée du sommeil correspond au nombre d'apnées par heure de sommeil comportemental. La durée moyenne d'un épisode d'apnée a également été calculée.

ii. Contractilité du diaphragme in vitro:

Les jeunes rats ont ensuite été euthanasiés, et les paramètres contractiles du diaphragme ont été étudiés grâce à la réalisation de bandelettes musculaires de diaphragme montées verticalement dans une cuve à organes isolés contenant une solution de Krebs-Henseleit oxygénée (Sigma Aldrich, Saint Quentin Fallavier, France) maintenue à 37 °C selon le protocole décrit ci-dessus.

iii. Évaluation biochimique

Dans des échantillons de diaphragme, l'activité de l'AChE a été étudiée en appliquant la méthode d'Ellman modifiée (Ellman et al. 1961) et le contenu myofibrillaire a été mesuré par spectrophotométrie (595 nm) selon la méthode Bradford (Bradford 1976) selon les protocoles décrits ci-dessus.

iv. Quantitative Reverse transcription polymerase chain reaction (qRT-PCR)

Les ARN totaux du diaphragme ont été isolés, quantifiés et transcrits de manière inverse en ADNc. Des amorces et le Master Mix Multiplex TaqMan $\[mathbb{M}$ (Thermo Fisher Scientific, Courtaboeuf, France) ont été utilisés pour la détection des transcrits par PCR quantitative en temps réel. Des amorces spécifiques aux gènes d'intérêt ont été utilisées comme tests d'expression génique TaqMan^m pour la β -actine et glycéraldéhyde 3-phosphate déshydrogénase (GADPH) (housekeeping genes), AChE, la chaîne lourde de la myosine (MHC) I, la MHCII, le récepteur de Ryanodine (RyR1), Atp2a1, Atp2a2.

2.3. Etude de l'activité de l'iléon

i. Contractilité in vitro des muscles lisses de l'iléon

Des lambeaux de muscle iléal longitudinal et circulaire (1 cm de long) ont été suspendus verticalement sous 1 g de tension dans un bain à organes isolés de 15 mL contenant une solution oxygénée de Krebs-Henseleit (pH=7,4) maintenue à 37°C. Après 30 minutes d'équilibration, chaque bandelette musculaire a été stimulée par champs électriques EFS

(100 V; 30 ms) créés par deux électrodes rectangulaires en platine, parallèles aux lambeaux musculaires (Darwiche et al. 2017). Les EFS induisent la libération d'ACh du plexus myentérique, ce qui entraîne la contraction des muscles (Takahashi, Ikemoto, and Ezaki 1999). Les amplitudes de contraction pour les muscles longitudinaux et circulaires ont été normalisées à la surface de la section transversale et rapportées en g/cm².

ii. Mesure de l'activité AChE

L'activité AChE iléale est étudiée selon la méthode d'Ellman modifiée.

iii. qRT-PCR

L'expression génique de différents médiateurs impliqués dans la contractilité de l'iléon, notamment GAPDH (gène de ménage ; étalon interne d'expression), les récepteurs M2 & M3 à l'ACh, la substance P (SP), les récepteurs NK1, ainsi que la calmoduline a été étudiée par PCR quantitative en temps réel, comme expliqué ci-dessus. La qRT-PCR a été réalisée à l'aide d'amorces spécifiques (Invitrogen Life Technologies, France) et de SYBR Green Master Mix (Applied Biosystems) conformément aux instructions du fournisseur.

iii. Analyse histologique

Des segments d'iléon ont été fixés avec du liquide de Carnoy pour préparer des coupes histologiques. Ensuite, les segments ont été déshydratés à l'éthanol, clarifiés au xylène, incorporés dans de la paraffine et découpés en sections de 5 µm d'épaisseur. Après déparaffinisation au xylène, les coupes ont été hydratées avec des bains successifs d'éthanol de concentration décroissante et colorées à l'hématoxyline/éosine de Mayer. Les épaisseurs des muscles lisses longitudinaux et circulaires ont été mesurées à l'aide du logiciel Image J (NIH, Bethesda, USA).

RÉSULTATS

Étude 1: Effet de l'exposition chronique au chlorpyrifos sur la contractilité du muscle diaphragmatique chez le rat adulte:

Après 2 semaines d'exposition, le poids corporel moyen des rats était significativement inférieur (p<0,05) mais uniquement dans le groupe CPF5 par rapport aux témoins.

L'amplitude des contractions a été significativement augmentée de 130% (p<0,01) et de 96% (p<0,05) dans les groupes CPF1 et CPF5 respectivement par rapport aux témoins. L'exposition au CPF induit un allongement du temps nécessaire pour atteindre le pic de tension maximale dans le groupe CPF1 (p<0,001) et dans le groupe CPF5 (p<0,05) par rapport aux témoins. Le temps de demi-relaxation était fortement augmenté respectivement de 203% et 134% (p<0,001) dans les groupes CPF1 et CPF5 (p<0,001) par rapport aux groupes témoins. La force tétanique était significativement augmentée dans le groupe CPF1 de 240% (p<0,01) et le groupe CPF5 de 186% (p<0,01). L'indice de fatigabilité était significativement accru dans le groupe CPF1 de 23% (p<0,01) et le groupe CPF5 de 21% (p<0.001) par rapport aux témoins. Ces changements étaient associés à une diminution de l'activité des AChE dans le diaphragme dans les groupes CPF1 et CPF5 de 33% et 36% respectivement par rapport aux témoins (p<0,05).

La teneur en protéines myofibrillaires était significativement augmentée dans le groupe CPF5 de 29% par rapport au groupe témoin (p<0,05) et de 52% par rapport au groupe CPF1 (p<0,05). L'exposition répétée au CPF1 a entraîné une diminution significative de 31% de la teneur musculaire en isoforme de type MHC I (p<0,05) et une augmentation significative de 14% de l'expression de l'isoforme de type MHC IIa (p<0,05) par rapport aux témoins.

Une altération de l'équilibre hormonal a été observée dans les groupes CPF. Le taux sérique de testostérone était significativement plus bas de 31% (p<0,05) et 23% (p<0,01) respectivement dans les groupes CPF1 et CPF5 par rapport aux témoins. Cependant, les taux sériques de corticostérone étaient significativement plus élevés de 183% dans les groupes CPF1 (p<0,05) et CPF5 (p<0,01) comme les taux de GH significativement plus élevés de 88% dans le groupe CPF1 et de 39% dans le groupe CPF5 (p<0,001) par rapport aux témoins.

Étude 2: Effets de l'exposition périgestationnelle au CPF et/ou au HFD sur la réponse contractile du muscle squelettique respiratoire et du muscle lisse gastro-intestinal

Le poids corporel moyen était significativement plus élevé dans les groupes nourris au HFD par rapport aux groupes nourris au régime standard à J3 et à J21 (p<0,001) et tend à devenir significatif à J60 (p=0,057). Comparée aux groupes non-CPF, l'exposition au CPF était associée à un poids corporel inférieur à J60 (p<0,05).

2.1. Impacts de l'exposition périgestationnelle au CPF et/ou au HFD sur la réponse contractile du muscle squelettique diaphragmatique et la performance respiratoire chez le rat mâle jeune adulte

À J60 (jeune adulte), une diminution significative de la fréquence respiratoire de 18% (p<0,05) était observée dans le groupe CPF, de 23% dans le groupe HFD (p<0,01) et de 18% (p<0,05) dans le groupe HFD+CPF par rapport aux témoins. La T_V était significativement réduite dans le groupe HFD de 38% par rapport aux témoins (p<0,05).

L'indice d'apnée du sommeil (AS) était significativement supérieur de 96% dans chacun des groupes CPF (p<0,01) et HFD (p<0,01) et de 88% dans le groupe HFD + CPF (p<0,05) par rapport aux témoins. La durée moyenne d'un épisode d'apnée était augmentée dans le groupe HFD par rapport aux témoins (p<0,05). La tension en secousse était significativement augmentée de 136% dans le groupe CPF (p<0,01) et de 140% dans le groupe HFD (p<0,01) par rapport aux témoins. Cette augmentation était moins prononcée dans le groupe CPF + HFD (p=0,053). Dans le groupe HFD, le temps de demi-relaxation était significativement inférieur de 10% (p<0,05) mais la force tétanique était significativement plus élevée (p<0,01) par rapport aux témoins. L'indice de fatigabilité était significativement augmenté de 31% dans le groupe CPF par rapport aux témoins (p<0,01).

Ces changements étaient associés à une réduction significative des niveaux d'activité de l'AChE de 53% dans le groupe CPF, de 29% dans le groupe HFD et de 40% dans le groupe HFD + CPF (p<0,05). L'expression de l'AChE du diaphragme était significativement plus élevée de 1,5 fois dans le groupe CPF (p<0,001), de 2,8 fois dans le groupe HFD (p<0,001) et de 1,6 fois dans le groupe HFD+CPF (p<0,01) par rapport aux témoins.

Malgré une interaction exposition-régime non significative avec le régime alimentaire, la teneur en protéines myofibrillaires était significativement plus élevée de 65% dans le groupe CPF (p<0,01) et de 82% dans le groupe HFD+CPF (p<0,05) par rapport aux témoins. L'expression de l'ARNm de l'isoforme MHCI était significativement plus élevée de 1,6 fois dans le groupe CPF (p<0,05), de 2,9 fois dans le groupe HFD (p<0,01) et de 1,5 fois dans le groupe HFD+CPF (p<0,01) et de 1,5 fois dans le groupe HFD+CPF (p<0,01) par rapport aux témoins. L'expression de l'ARNm de MHCII était 2,1 fois plus élevée dans le groupe CPF comparé aux témoins (p<0,01).

L'expression de l'ARNm de RyR1 au niveau du diaphragme était significativement plus élevée de 1,5 fois dans le groupe CPF (p<0,01), de 3,5 fois dans le groupe HFD (p<0,01) et de 2,4 fois dans le groupe HFD+CPF (p<0,01) par rapport aux témoins. Malgré une interaction exposition-régime non significative avec le régime alimentaire, l'expression de l'ARNm était significativement 1,5 et 1,9 fois plus élevée pour Atp2a1 (p<0,05) et Atp2a2 (p<0,05) respectivement dans le groupe HFD par rapport aux témoins.

2.2. Impacts de l'exposition périgestationnelle au CPF et/ou au HFD sur la réponse contractile de l'iléon chez le rat mâle jeune adulte

Malgré une interaction non significative entre le régime alimentaire et le CPF pour les deux muscles, les contractions maximales du muscle lisse iléal longitudinal étaient plus élevées de 123% dans le groupe CPF (p<0,05) et de 149% dans le groupe CPF+HFD (p<0,1) par rapport aux contrôles. L'amplitude de contraction maximale du muscle circulaire était significativement plus élevée dans le groupe CPF+HFD de 110% par rapport aux contrôles (p<0,01).

Les niveaux d'activité de l'AChE iléale étaient significativement plus bas dans le groupe HFD de 26% (p<0,05), dans le groupe CPF de 24% (p<0,01) et dans le groupe CPF+HFD de 29% par rapport aux témoins. Malgré une interaction non significative entre le régime alimentaire et le CPF pour les ARNm des récepteurs M2 et M3 de l'ACh, l'expression iléale de l'ARNm du récepteur cholinergique M2 était significativement plus élevée de 1,5 à 1,45

fois dans les groupes CPF (p<0,001) et CPF+HFD (p<0,01) par rapport aux contrôles. L'expression iléale de l'ARNm du récepteur cholinergique M3 était significativement plus élevée de 1,9 fois dans le groupe CPF par rapport aux témoins (p<0,05).

L'expression de l'ARNm de la SP était significativement augmentée tandis que celle de la calmoduline était significativement diminuée avec l'exposition maternelle au CPF par rapport aux groupes non-CPF (p<0,05). D'autre part, l'expression iléale de l'ARNm des récepteurs NK1 était significativement plus élevée de 1,5 fois dans le groupe HFD par rapport aux témoins (p<0,05).

L'exposition maternelle au CPF était associée à une augmentation significative de l'épaisseur du muscle iléal circulaire uniquement par rapport aux groupes non-CPF (p<0,05).

DISCUSSION

Les facteurs alimentaires et les contaminants associés peuvent induire des risques pour la santé des personnes à différentes étapes de la vie, de la période périnatale, une phase de développement extrêmement sensible, et jusqu'à la période postnatale, en plus de l'exposition directe à l'âge adulte. Le premier objectif de ce travail était de déterminer les effets d'une exposition continue au CPF à l'âge adulte sur la contractilité du diaphragme chez le rat.

Des études antérieures dans notre laboratoire amiénois ont montré que l'exposition *in utero* et postnatale au CPF modifie les fonctions respiratoire et digestive chez le rat au cours du développement (Darwiche et al. 2017; 2018). L'exposition était continue du premier jour de gestation et jusqu'à la fin de la lactation des nouveau-nés jusqu'au J60 (jeune adulte), illustrant les effets de l'exposition indirecte (gestation et allaitement) et directe (après le sevrage) au CPF.

Ensuite, le deuxième objectif était d'examiner les conséquences à long terme de l'exposition périgestationnelle de la mère au CPF, au HFD ou à l'exposition simultanée aux deux facteurs, à partir de 4 mois avant la gestation et jusqu'à la fin de la période de lactation.

Dans notre étude, la fonction respiratoire et la contractilité des muscles respiratoires et GI ont été analysés chez la descendance au début de l'âge adulte sans que ces jeunes rats soient soumis directement au CPF et/ou HFD après le sevrage, illustrant pour la première fois dans notre laboratoire, les effets de l'exposition indirecte au CPF et/ou au HFD chez les jeunes adultes.

1) Effet de l'exposition alimentaire au CPF chez le rat adulte:

L'exposition alimentaire au CPF correspond à l'exposition réelle et les doses choisies ont été utilisées dans l'évaluation de l'exposition alimentaire chronique au CPF (Hallal et al. 2019). Les effets de l'exposition au CPF ont été évalués dans le diaphragme, un muscle respiratoire hautement spécialisé qui assure une contraction rythmique continue tout au long de la vie (Polla et al. 2004).

Nous avons observé une diminution du poids corporel moyen uniquement chez les rats soumis au CPF5 par rapport aux témoins, ce qui indique très certainement un effet toxique. L'exposition au CPF peut augmenter la peroxydation lipidique et le stress oxydatif, ce qui pourrait entraîner une diminution du poids corporel dans le groupe CPF5 (Mansour and Mossa 2010; Kaur, Singla, and Dhawan 2019).

Nous avons montré que l'exposition au CPF aux deux doses augmentait l'amplitude de la tension de contraction, le temps nécessaire pour atteindre le pic de tension, le temps de demi-relaxation et la force tétanique. Ces changements ont entraîné une diminution de l'activité AChE du diaphragme, illustrant une altération cholinergique au niveau de la jonction neuro-musculaire. L'inhibition de l'AChE par un médicament anticholinestérase, l'écothiopate, a montré une augmentation similaire de la contraction due à l'action prolongée de l'ACh au niveau de la plaque motrice (Burd and Ferry 1987). Hallal et al. (2019) on montré que l'exposition au CPF peut altérer la contractilité des muscles squelettiques du soléus et de l'extenseur longus (edl). Une contraction accrue du diaphragme a été révélée par l'étude de Darwiche et al. (2018) chez des rats adultes exposés au CPF pendant les périodes pré- et postnatales associées à une activité AChE du diaphragme inférieure. Par conséquent, l'accumulation d'ACh au niveau des synapses

cholinergiques pourrait expliquer l'augmentation de la contractilité du diaphragme. L'augmentation de la fatigabilité du diaphragme peut être attribuée également à la stimulation cholinergique prolongée consécutive à l'inhibition de l'AChE.

Nous avons montré que l'exposition à 1 mg/kg/jour de CPF induisait une diminution significative de l'expression de l'isoforme de MHCI (lentes) associée à une augmentation de l'isoforme de MHC IIA, suggérant que la plasticité de la fibre musculaire respiratoire passerait au type rapide et plus fatigable (Isoforme MHC IIA). Ces données pourraient en particulier expliquer la fatigabilité accrue du diaphragme.

L'exposition au CPF a diminué les taux sériques de testostérone conformément aux études précédemment rapportées à des doses plus élevées de CPF (Joshi, Mathur, and Gulati 2007). La testostérone étant essentielle au développement et à la maintenance des muscles (Sinha-Hikim et al. 2006), les niveaux de testostérone plus faibles observés dans les groupes CPF pourraient expliquer l'augmentation de l'indice de fatigabilité du diaphragme. En outre, les taux sériques de corticostérone ont augmenté dans les groupes CPF, en accord avec Güven et al. (1999) qui ont montré des niveaux élevés de cortisol chez les patients présentant une intoxication par les OP. Ainsi, des sécrétions prolongées de cortisol peuvent entraîner une faiblesse musculaire (LaPier 1997).

2) Effets de l'exposition périgestationnelle au CPF et/ou au HFD

Nous avons observé un poids corporel moyen plus élevé à la naissance chez les rats exposés au HFD pendant la période périgestationnelle comparé aux animaux non exposés au HFD. Étant donné que la teneur élevée en lipides de l'alimentation peut modifier la composition du lait, nos rats sevrés de HFD étaient caractérisés par un poids corporel moyen plus élevé (Del Prado, Delgado, and Villalpando 1997). En accord avec Lecoutre et al. (2016), l'augmentation du poids corporel continue d'être observée au début de l'âge adulte (J60). Conformément à Darwiche et al. (2018), l'exposition au CPF était associée à un poids corporel moyen inférieur chez les rats à J60, suggérant un effet de programmation prolongé du CPF sur le poids corporel.
L'exposition périgestationnelle au CPF et/ou au HFD a révélé une diminution de la fréquence respiratoire, en accord avec les données rapportées sur les effets des OP en cas d'exposition aiguë (Villa et al. 2007) ou d'exposition chronique prénatale et postnatale (Darwiche et al. 2018). Cependant, dans notre étude, T_I et T_E n'étaient pas affectés. La diminution de la fréquence respiratoire était associée à un T_V inférieur, indiquant un volume total d'air inhalé et expiré réduit lors d'une respiration dans le groupe HFD. La T_V réduite pourrait être liée au poids corporel plus élevé des rats, à la suite d'éventuelles perturbations métaboliques et de l'état pro-inflammatoire associé, résultant de perturbations nutritionnelles en début de vie (Hancox et al. 2007).

Nos résultats ont montré que l'exposition périgestationnelle au CPF et/ou au HFD augmentait de manière significative l'indice d'apnée du sommeil chez les jeunes rats adultes. Deux mécanismes physiopathologiques pourraient expliquer l'apnée: le dysfonctionnement du SNC du contrôle ventilatoire des muscles respiratoires et l'obstruction périphérique des voies respiratoires supérieures (Kryger 2000). Bien que nos rats ne soient pas en contact direct avec le CPF, l'augmentation de l'indice d'apnée du sommeil était supérieure à celle observée dans Darwiche et al. (2018), dans lesquels des rats en développement ont été exposés au CPF pendant la gestation et les périodes postnatales, approuvant à leur tour le concept de la DOHaD. L'apnée observée après une exposition aiguë aux OP pourrait se rapporter à une inhibition de l'AChE dans les centres respiratoires (Carey, Dunn, and Gaspari 2013; Gaspari and Paydarfar 2011) ou à un effet périphérique de l'obstruction des voies respiratoires (Proskocil et al. 2010).

L'apnée du sommeil est liée aux perturbations métaboliques chez les personnes obèses. Cependant, l'indice d'apnée du sommeil plus élevé observé chez nos rats est lié à une légère augmentation du poids corporel, conformément à Ramadan et al. (2006; 2007) signalant des épisodes d'apnée augmentés chez des rats adultes nourris directement avec du HFD pendant cinq semaines. Nous avons observé que les ratons nés de mères exposées au HFD au cours de la période périgestationnelle présentaient un indice d'apnée du sommeil plus élevé et une durée moyenne plus longue de l'épisode d'apnée par rapport aux témoins bien qu'ils ne soient pas exposés au HFD après le sevrage. L'exposition combinée à la fois au CPF et au HFD a induit une augmentation similaire de l'indice d'apnée du sommeil par rapport au CPF ou au HFD seul, indiquant que l'effet n'est pas exagéré.

La tension de contraction du diaphragme et l'indice de fatigabilité ont été augmentés dans le groupe CPF, ce qui pourrait être attribué à l'activité plus faible de l'AChE et à l'hyperstimulation cholinergique qui en résulte. Bien que nos rats ne soient pas directement exposés au CPF, ces découvertes peuvent confirmer l'effet du CPF sur la contractilité du diaphragme, comme le montrent nos rats adultes exposés directement pendant 6 semaines au CPF (expliqué ci-dessus) et à Darwiche et al. (2018) où les rats ont été exposés au CPF à partir de la gestation et pendant la période postnatale. En effet, le CPF peut traverser la barrière placentaire et a été trouvé dans le lait maternel (Mattsson et al. 2000).

L'exposition maternelle au HFD a augmenté la tension de contraction du diaphragme et a réduit le temps de demi-relaxation dans le groupe HFD. Ces résultats étaient associés à une activité plus faible de l'AChE, suggérant ainsi une surstimulation cholinergique. La diminution de l'activité de l'AChE dans différentes régions du cerveau chez le rat qui consomme du HFD a été corrélée à une élévation des acides gras libres, ce qui affecte la fonction cholinergique (Kaizer et al. 2004; Morganstern et al. 2012). L'exposition maternelle à la fois au HFD et au CPF a été caractérisée par une augmentation de la tension de contraction associée à une réduction de l'AChE, similaire à l'effet observé dans chacun des groupes CPF et des groupes HFD seuls.

Ainsi, l'exposition périgestationnelle au CPF et/ou au HFD a diminué l'activité de l'AChE dans le diaphragme. En conséquence, l'expression de l'ARNm de l'AChE a été régulée positivement dans ces groupes en tant que réponse possible à l'activité réduite de l'AChE, comme décrit précédemment par Somnuek et al. (2009).

Le groupe CPF est caractérisé par une augmentation de la teneur en protéines myofibrillaires dans le diaphragme, associée à une expression accrue des isoformes de MHCI et de MHCII, ce qui pourrait expliquer une contraction accrue du diaphragme. L'augmentation plus importante de l'expression de l'isoforme MHC IIA a été corrélée à la fatigabilité accrue du diaphragme, ce qui est concordant avec nos résultats dans lesquels l'exposition au CPF à l'âge adulte pendant 6 semaines a augmenté l'expression de l'isoforme MHCII (décrite ci-dessus). De l'autre côté, seule l'expression de l'isoforme MHCI a été augmentée dans le groupe HFD, suggérant un passage à une fibre lente (type I). Ce résultat est corrélé à la tension de contraction accrue du diaphragme sans modification de la fatigabilité. L'exposition combinée au CPF et au HFD a montré une teneur plus élevée en protéines myofibrillaires associée à une expression accrue de l'isoforme MHCI, contribuant également à une contractilité accrue du diaphragme sans altération de l'indice de fatigabilité.

L'exposition périgestationnelle au CPF et/ou au HFD a augmenté l'expression du récepteur RyR1 de la ryanodine, suggérant une augmentation possible de la quantité de Ca²⁺ libérée par le réticulum sarcoplasmique, augmentant à son tour la quantité de Ca²⁺ myofibrillaire. Cet effet, en plus l'expression accrue des isoformes de MHC, aurait pu entraîner une augmentation de la contractilité du diaphragme. L'expression accrue de l'adénosine triphosphatase *Ca²⁺ du réticulum sarcoplasmique (SERCA) I et II dans le groupe HFD pourrait etre corrélée au temps de demi-relaxation inférieur (Gollnick et al. 1991).

En ce qui concerne l'iléon, seule l'exposition périgestationnelle au CPF a augmenté l'amplitude de la contraction des muscles lisses iléaux longitudinaux et circulaires chez les rats mâles à J60. Cela contraste avec l'étude de Darwiche et al. (2017) qui ont signalé une contraction plus faible du muscle longitudinal iléal chez de jeunes ratons adultes exposés pendant la gestation et la lactation jusqu'au début de l'âge adulte à une concentration de CPF de5 mg/kg/jour. Cette divergence pourrait être expliquée par le fait que nos rats n'ont pas reçu de CPF après le sevrage. Dans de telles conditions, le tube digestif du rat n'est donc pas en contact direct avec la molécule de CPF. Cependant, l'exposition périgestationnelle au HFD n'a pas modifié la contractilité du muscle iléal, en accord avec Patten et al. (2004). Cependant, l'épaisseur accrue du muscle circulaire iléal est corrélée à une contractilité accrue.

Conformément à Darwiche et al. (2017), l'activité de l'AChE iléale était significativement réduite dans le groupe CPF à J60. Cette inhibition peut entraîner une accumulation d'ACh au niveau des synapses cholinergiques, ce qui pourrait expliquer l'augmentation observée

des contractions iléales induites par l'EFS. La surstimulation des récepteurs muscariniques qui en résulte conduit à une régulation négative et à une désensibilisation des récepteurs (Michalek, Fortuna, and Pintor 1993; Abdollahi and Karami-Mohajeri 2012). Le métabolite actif du CPF (CPO) peut se lier directement au récepteur M2 de l'ACh et inhiber la liaison de l'agoniste dans le cœur (Bomser and Casida 2001) et dans le striatum (Huff et al. 1994). En conséquence, nous avons montré une augmentation significative de l'expression du récepteur M2 de l'ACh sans modification de l'expression du récepteur M3 dans l'iléon, de manière à compenser une éventuelle réduction de la fonction du récepteur M2 (Lein and Fryer 2005).

L'activité de l'AChE est diminué dans le groupe HFD sans altération de l'expression des récepteurs M2 et M3 de l'ACh indiquant une activation prolongée de la neurotransmission cholinergique. Bien que nos rats n'ont pas consommé directement de HFD, il a été démontré que la quantité accrue d'acides gras libres chez les rats qui en consommaient diminuait l'activité de l'AChE dans différentes parties du cerveau (Kaizer et al. 2004).

Nous avons montré que l'exposition au CPF augmentait de manière significative l'expression de la tachykinine, substance P (SP), sans affecter l'expression des récepteurs NK1. La SP régule la contraction non cholinergique des muscles lisses intestinaux en se liant au récepteur NK1 (Souquet et al. 1985). Une expression accrue de la SP peut entraîner une amplitude accrue de la contraction des muscles iléaux.

Dans le groupe HFD, l'expression des récepteurs NK1 a été augmentée sans altération de l'expression de SP. L'interaction entre SP et le récepteur NK1 joue un rôle essentiel dans le développement de la prise de poids en réponse à la HFD (Karagiannides et al. 2008). Par conséquent, l'augmentation de l'expression de récepteur NK1 pourrait affecter l'équilibre énergétique chez les rats, contribuant ainsi à l'augmentation du poids corporel observé chez les ratons à la suite de l'exposition maternelle au HFD.

En réponse à une augmentation du taux de Ca²⁺ intracellulaire, la calmoduline initie un cycle de pont croisé entraînant une contraction du muscle lisse (Webb 2003). Cependant, nous avons montré une expression plus faible de la calmoduline associée à l'exposition au

CPF. Cette découverte suggère une possible adaptation de l'iléon à une stimulation accrue aux niveaux cholinergique et non cholinergique.

CONCLUSION

Ce travail de thèse a mis en évidence les effets de l'exposition à des risques environnementaux et alimentaires critiques selon les thèmes associés aux deux laboratoires. En particulier, cette étude aavit 2 approches principales:

- Premièrement, nous avons identifié les effets d'une exposition directe à l'âge adulte à un insecticide OP, sur le muscle squelettique respiratoire. Ce travail a été effectué dans le laboratoire Rammal Hassan Rammal (Liban) dans le cadre de ses thèmes de recherche.

- Dans le cadre de l'une des recherches thématiques du laboratoire Peritox, nous avons également étudié les effets de l'exposition périgestationnelle des mères au CPF associée à un régime alimentaire riche en graisses, sur la fonction respiratoire et motrice intestinale chez les jeunes adultes. Dans ce nouveau protocole, le tube digestif des ratons n'est en contact direct ni avec la molécule de CPF ni avec le HFD, car les rats n'ont pas été soumis au CPF et / ou au HFD après le sevrage.

Par conséquent, cette étude a élucidé l'activité des muscles impliqués dans les fonctions vitales du corps à l'âge adulte après une exposition directe d'adultes ou une exposition périgestationnelle indirecte de la mère à des facteurs de risque alimentaires.

Compte tenu de nos résultats, plusieurs pistes de recherche pourraient être envisagées :

1) Nos résultats ont montré que l'exposition au CPF chez les adultes augmentait la contractilité du diaphragme, un muscle squelettique essentiel à la respiration. La fatigabilité du diaphragme a été augmentée, réduisant ainsi sa capacité à supporter la force de contraction. Ces modifications de la contractilité pourraient être liées à la modification cholinergique par inhibition de l'activité de l'AChE, passage à l'expression de l'isoforme MHC IIA de type plus rapide et plus fatigable et/ou perturbation de l'équilibre hormonal

anabolique-catabolique. D'autres modifications du mécanisme de couplage excitationcontraction pourraient entraîner une augmentation de la contractilité du diaphragme.

2) Par ailleurs, nos résultats sont conformes au concept de la DOHaD, qui souligne le lien entre les perturbations environnementales au début de la vie et le risque d'apparition de maladies chroniques à l'âge adulte dans le cadre de la programmation fœtale.

- Nous avons montré que l'exposition périgestationnelle au CPF et /ou au HFD diminuait la fréquence respiratoire et augmentait ensuite l'indice d'apnée du sommeil. Il serait intéressant d'examiner si cette exposition modifie la structure du sommeil chez le rat.

- L'exposition périgestationnelle au CPF et/ou au HFD a augmenté la contractilité du diaphragme associée à une plus grande fatigabilité. Les modifications contractiles peuvent être attribuées à une transmission cholinergique prolongée, à une altération de la fonction de libération / absorption de Ca²⁺ du réticulum sarcoplasmique et à une expression accrue des isoformes de MHC.

- Fait intéressant, selon les deux protocoles, nous avons montré que le CPF augmentait la contractilité du diaphragme chez des rats adultes exposés directement au CPF à l'âge adulte ou exposés indirectement par le biais d'une exposition maternelle continue au cours de la période périgestationnelle. Ces effets se sont traduits par le même mécanisme d'inhibition de l'activité de l'AChE et la régulation à la hausse de l'isoforme MHC IIA.

- L'exposition périgestationnelle au CPF a augmenté la contractilité des muscles iléaux longitudinaux et circulaires à l'âge jeune adulte, ce qui peut impliquer un mécanisme cholinergique et non cholinergique. L'analyse des modifications du temps de transit intestinal pourrait être pertinent car cette activité est dépendante de la contractilité musculaire de la paroi digestive.

 L'exposition périgestationnelle au HFD diminue l'activité de l'AChE iléale. D'autres études pourraient étudier les mécanismes par lesquels le régime HFD est susceptible d'affecter l'activité cholinergique dans un modèle impliquant une alimentation directe en HFD. - Malgré l'absence d'exposition directe, une exposition périgestationnelle au CPF et/ou au HFD peut programmer les risques de perturbations respiratoires et altérer l'homéostasie des muscles respiratoires et intestinaux, affectant ainsi les fonctions contractiles du diaphragme et de l'intestin à l'âge jeune adulte.

HYPOTHESIS

Pesticide residues constitute a real social problem nowadays. For nearly fifty years, they have been highlighted in all compartments of the environment, in the rivers, groundwater, and air. These residues are also found in fruits, vegetables, cereals, and animal products. The general population is regularly exposed to pesticide residues as contaminants in food and water. Chlorpyrifos (CPF) is a widely used organophosphorus insecticide which acts by inhibiting acetylcholinesterase at cholinergic synapses. Exposure to CPF is harmful for the respiratory and digestive systems during *in utero* and postnatal developmental periods (Darwiche et al. 2017; Darwiche et al. 2018).

The perinatal period is characterized by high plasticity of the physiological systems and even higher sensitivity and vulnerability of individuals to the environmental factors (fetal programming). Such developmental processes continue after birth, at least during the entire period of lactation and the first 24 months of life. Then any disruption in these physiological processes is described in the concept of DOHaD (Developmental Origin of Health and Diseases). Experimental and epidemiological studies have indicated that maternal obesity constitutes an environmental risk which programs infant metabolism and favors the occurrence of obesity or type 2 diabetes at adulthood (Alfaradhi et al. 2016; Dumortier et al. 2017; Lecoutre et al. 2016). Many factors are involved in the appearance of obesity: the consumption of unbalanced food rich in fats (junk food) in predisposed people but also the repeated exposure to environmental factors including pesticides, particularly organophosphate residues.

Recent studies on animal models have demonstrated that high-fat diet consumption as socalled "Western diet" can exacerbate many of the metabolic disruptions induced by earlylife organophosphates exposure (Slotkin 2011). On the other hand, food contaminants such as organophosphate pesticides would increase the high-fat diet-induced troubles (digestive, respiratory, metabolic effects).

This study is divided into two different approaches:

- The first aim is to examine the effects of repeated exposure to CPF in rats, during adulthood, on the contractile performance of the respiratory skeletal muscle.

- The second aim is to determine whether a long-term perigestational exposure to CPF combined with high-fat diet feeding during pre-gestational, gestational, and lactational periods could affect the performance of the respiratory skeletal muscles and digestive smooth muscles in the progeny at adulthood, by analyzing muscle contractile properties to electrical field stimulation. Further analysis is performed to identify different mediators of interest in the modification of the contractility function.

STATE OF THE ART

Chapter 1: Pesticides

The use of pesticides has been increasing regularly since the 1940s after the appearance of synthetic pesticides. Globally, around 4.6 million tons of pesticides are dispersed annually. This intensive application of pesticides and especially in industrialized countries has undoubtedly helped in improving food production and fighting diseases (Berton et al. 2014). These pesticides are extensively used in occupational settings and residential areas, as an economic, labor-saving, and efficient tool of pest control and agricultural production. Despite their intensive use, serious health concerns have been raised arising from the occupational exposure of farmers to pesticides or from residues in food and drinking water for the general population (Damalas and Eleftherohorinos 2011).

Pesticides can pose potential hazards to human health due to their biological reactivity. Worldwide, the acute pesticide poisoning, resulted in approximately 300,000 deaths per year (Hashemi-Domeneh et al. 2016). A summary of the adverse health risks includes asthma, chronic obstructive pulmonary disease (COPD), and lung cancer in addition to neurological, metabolic, and endocrine disruptions (Ye et al. 2013).

In this chapter, pesticides, and particularly chlorpyrifos, which is the pesticide used in our studies, will be discussed. The use of pesticides in France and Lebanon, as well as the exposure of the general population, will also be described in this chapter. The effects of chlorpyrifos on human health in general and the developmental exposure to chlorpyrifos through the perigestational period will be further detailed.

1.1. Definition

The term pesticide refers to any substance or mixture of substances used to prevent, destroy, repel or control harmful organism (pests) or protect plants during production, storage, and transport¹.

¹ <u>https://ec.europa.eu/food/plant/pesticides_en</u> [Accessed: 09-Mar-2019].

According to the Environmental Protection Agency (EPA)², pests are defined as living organisms that occur in undesired places or that cause damage to humans or crops or other animals. Examples include insects, mice and other animals, unsought plants (weeds), fungi, microorganisms such as bacteria and viruses, and prions...

In addition to agriculture, pesticides are used in public health to kill vectors of diseases. The definition of pesticides varied with times and countries depending on the regulations governing the market.

In Europe, a distinction is made between plant protection products and biocides.

According to the Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009, Plant Protection Products (PPPs) are defined as products containing at least one active substance presented to the user, intended for one of the following functions:

- protect plants or plant products from pests
- influence the life processes of the plants (such as substances influencing the growth, excluding nutrients)
- preserve the plant products
- prevent, check, or destroy undesired growth of plants or parts of plants

They may also contain other components such as synergists and safeners.

According to the Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012, biocidal products refers to any substance or mixture intended to destroy, deter, render harmless, prevent the action of, or control any harmful organism by any means excluding mere physical or mechanical action. The harmful organisms include pests and microorganisms. This definition excludes plant protection products.

1.2. Classification:

Pesticides can be categorized into different classes in several ways:

² <u>https://web.archive.org/web/20130411205520/http://www.epa.gov/pesticides/about/index.htm</u> [Accessed: 09-Mar-2019].

 The World Health Organization (WHO)(OMS 2009) classified pesticides into four categories based on the hazards of the compounds. The classification depends mainly on the acute oral and dermal toxicity to the rats through the estimation of the median lethal dose (LD50); which produces death in 50% of exposed animals. The classes are ranked in order from the lowest to highest toxic (I through IV), corresponding to extremely or highly toxic, moderately toxic, slightly toxic, and unlikely toxic respectively (table 1).

WHO class	Toxicity level	LD ₅₀ for the rat (mg/kg body weight)	
		Oral	Dermal
Class Ia	Extremely hazardous	<5	<50
Class Ib	Highly hazardous	5-50	50-200
Class II	Moderately hazardous	50-2000	200-2000
Class III	Slightly hazardous	>2000	>2000
Class IV	Unlikely to present acute hazard in normal use	≥ 5000	

Table 1: WHO Classification of Pesticides by Hazard

 Pesticides can be classified also according to the common source or production method into:

a) Chemical Pesticides:

These pesticides are classified in turn into four major families according to the chemical structure:

i. Organophosphate Pesticides: They affect the nervous system by inhibiting the enzyme that regulates the neurotransmitter acetylcholine. Most of them are insecticides. Although some are very poisonous (used in World War II as nerve agents), they are usually not persistent in the environment. Examples include Malathion, Chlorpyrifos and Diazinon.

- *ii. Carbamate Pesticides:* Also affect the nervous system by disrupting the enzyme that regulates acetylcholine degradation. The effects are usually reversible.
- iii. Organochlorine Insecticides: Many were removed from the market due to their harmful effect on health and environment in addition to their environmental persistence. Common examples of these pesticides include: DDT, lindane, endosulfan.
- *iv. Pyrethroid Pesticides:* These are the synthetic version of the pesticide pyrethrin which naturally occurs in the plant chrysanthemums. Some of them are toxic to the nervous system.

b) Biopesticides:

They are naturally present in animals, plants, bacteria, and certain minerals.

They are divided into three major classes:

- *i. Microbial pesticides:* Their active ingredient is a microorganism (e.g., a bacterium, fungus, virus or protozoan).
- *ii. Plant-Incorporated-Protectants (PIPs):* Plants produce these pesticides from genetic material that scientists add to those plants.
- *iii. Biochemical pesticides:* They are naturally occurring substances that control pests in a nontoxic way.
- c) Antimicrobials: They kill microorganisms such as bacteria.
- d) Pest Control Devices: These are instruments used to control pests³.
- 3) Pesticides are often referred according to the type of the target pest. The most widely used categories include:

³ "Types of Pesticides | Pesticides | US EPA," 28-Mar-2013. [Accessed: 12-Mar-2019]. https://web.archive.org/web/20130328231904/http://www.epa.gov/pesticides/about/types.htm#chemical

- a) Insecticides are used to kill insects such as organophosphates (Malathion, Chlorpyrifos...), organochlorines and carbamates. They also include insect repellents such as (DEET). Insecticides act through several mechanisms of action by targeting nerves and muscles, development and growth, and energy production.
- **b)** Fungicides are used to kill fungi (including mildews, molds, Azoxystrobin and rusts).
- c) Herbicides are used to kill weeds and other plants that grow in unwanted places.
- d) Bactericides are used to kill bacteria or acts against bacteria.
- e) Other pesticides include rodenticides (kill rodents), algaecides (kill algae), and miticides (kill moths)⁴...

1.3. Utilization:

According to the United Nations, Population Division estimations ("The World Population Prospects: 2015 Revision" 2015), the world population will reach 9.7 billion by 2050. This population growth will occur mostly in developing countries. In order to meet the needs, increasing food production depends mainly on the increase in yields and crop production as reported by the Food and Agriculture Organization of the United Nations (FAO), consequently increased demand for agrochemicals. Pesticides can reduce large crop losses and thus play a major role in agricultural production (WHO, 2018)⁵.

- World Pesticide Usage

Around one-third of the agricultural products depend on pesticides use (Liu, Men, and Liu 2002).

Worldwide around 1.8 billion people are engaged in agriculture and most use pesticides for food and commercial products (Alavanja 2009). At the production level, the annual total of pesticide usage scored around 6 billion pounds in both 2011 and 2012 with total expenditures of about \$56 billion in 2012. Between 2008 and 2012, the herbicides

⁴ <u>https://www.who.int/ceh/capacity/Pesticides.pdf</u>

⁵ "Pesticide residues in food."

https://www.who.int/news-room/fact-sheets/detail/pesticide-residues-in-food. [Accessed: 14-Mar-2019].

expenditures were the highest in all years, followed by the expenditures on insecticides, fungicides and other pesticides ("Pesticides Industry Sales and Usage 2008 - 2012 | US EPA,").

- Europe

The indicator of pesticide consumption in agriculture depends mainly on the data of pesticides sales. Among the 20 European Union (EU) Member States, Spain, France, Italy, and Germany accounts for the highest pesticide sales (79 %) in 2016. These countries collectively represent about one half (46 %) of the EU's total utilized agriculture area (Figure 1) (Eurostat, 2018a)⁶.



Figure 1: The quantities of Europe pesticides sales in 2016 (Source⁶)

As shown in Figure 2, despite a constancy of the total sales of pesticides in Europe, the average of the pesticide sales during (2014 till 2016) was increased by 5.6 % compared to the previous 3 years (2011-2013).

⁶ "EU Pesticide Sales by English team - Infogram."

https://infogram.com/eu-pesticide-sales-1h984w8555nd4p3. [Accessed: 14-Mar-2019].

In terms of the classes of pesticides sold, and according to the EU, the "Fungicides and bactericides" category represent the highest sales volumes (46%), followed by "Herbicides, haulm destructor and moss killers" category (29%) and "Insecticides and acaricides" (11%) category (Eurostat,2018b)⁷.



Figure 2: The total sales of pesticides categories (in tons of active ingredients) in the EU during 2011-2016 period (Eurostat, 2018c⁸).

- France

France is considered as the leading agricultural country in the EU, accounting for 23% of agricultural production in the fifteen member states. In particular, France is the primer European producer of poultry, beef, cereals, and wine, and the second for milk and sugar beet production. France is the leading EU exporter. Worldwide, it is among the largest agricultural exporters behind the United States of America⁸.

- French Agriculture

France contains around 27 million hectares of arable land, which account for almost half of the total surface area of the country⁹ (Figure 3). Metropolitan France includes around 500,000 farms and one million people involved in regular farms activities as reported in 2010. French agriculture depends on farms of all sizes. Although the number of farms was decreased between 2000 and 2010, the total volume of farm production was maintained.

⁸ France: the leading agricultural country in the EU –

http://www.terresdeurope.net/en/france-agriculture-leading-country-europe.asp (March 13, 2019). ⁹ <u>https://chambres-agriculture.fr/fileadmin/user_upload/National/002_inst-site-</u> chambres/pages/infos eco/FicheAgri Anglais.pdf Accessed March 15, 2019

⁷ <u>https://ec.europa.eu/eurostat/documents/4187653/8516161/Pesticide+sales+by+category+2016</u>

The agricultural accounts rose annually by 3.2% ("Ministère de l'agriculture et de l'alimentation - agreste - La statistique, l'évaluation et la prospective agricole - 2010).



Figure 3: The main farming regions in France (Source: Recensement Agricole 2010)¹⁰

The application of pesticides and fertilizers has multiplied the agricultural productivity in France. In the 1980s, the total amounts of active substances sales reported a major increase following the appearance of the synthetic fungicides. From 2000, the total quantities of sold active substances began to decrease due to the restrictions on certain products utilization and the replacements by innovating products with higher efficiency at low doses. Meanwhile, France remains among the leading European countries in pesticides consumption.

Around 90% of the 78,600 tons of active substances sold in 2008 were used in agriculture and the remaining 10% for other non-agricultural applications (maintenance of road and rail infrastructures, sidewalks, gardening...) (Pesticides et santé – Sénat 2009)¹⁰. The total

¹⁰ Rapport n° 421 (2009-2010) de MM. <u>Jean-Claude ETIENNE</u>, sénateur et Claude GATIGNOL, député, fait au nom de l'Office parlementaire d'évaluation des choix scientifiques et technologiques, déposé le 29 avril 2010

number of pesticides sold in 2016 was around 72 000 tons. Fungicides/Bactericides represent 44.30% of the total sales, followed by "Herbicides, haulm destructor and moss killers (41.70%), and Insecticides and acaricide (5.05%) categories (Eurostat 2016 - "Type of pesticides in Top 4 Countries by English team ").

- The Hauts-de-France region

The Hauts-de-France region is an excellent land for agriculture. Due to its diversified agriculture, unique training cycle in France, and developed agro-resources, Hauts-de-France is considered as the leading agricultural region in France. Around 67% of its regional territory is valued by agriculture. This region includes 2.1 million hectares of useful agricultural area and 27,400 farms providing 53,000 salaried jobs for the agri-food industry (Figure 4) ("Les Hauts-de-France, Première Région Agricole de France" 2015).

Hauts-de-France is the first producer of wheat, French potatoes, and industrial beets. Moreover, the region is the leader in the production of vegetable industry. The cereals and oilseeds account for 14% and 11% of French production respectively (Chambres d'agriculture France, 2019).





- Ecophyto Plan

Ecophyto is a plan set out to gradually reduce the use of plant protection products while maintaining economically efficient agricultural production.

An initiative was launched in 2008 following the Grenelle Environnement. The plan is led by the Ministry of Agriculture and Food; under the responsibility of the Ministry of Agriculture and Food, provided for a 50% reduction in the use of pesticides by 2018. However, the plan did not achieve its goal. On the contrary, the number of doses of pesticide units used in agriculture (Nodu) increased by 12% between 2014 and 2016 with a slight decrease in 2015 and stability in 2016 (Bénis 2018). In particular, the annual consumption of pesticides in the Hauts-de-France has just decreased by less than 1% between 2009 and 2016, thus remaining one of the leading sales groups in France ("Les Pesticides Ont Toujours La Cote Dans Les Hauts-de-France" n.d.).

"Ecophyto 2" plan was launched in the summer of 2017 with two strong figures: reducing the amount of pesticides used by 25% in 2020 and by 50% in 2025 compared to 2008.

- Lebanon

Lebanon's moderate climate, abundant water resources, and rich soil represent vital factors for agricultural production. Lebanon accounts for the highest proportion of agricultural areas in the Middle East with 64.3% of a total land area according to the latest report of the Food & Agriculture Organization (FAO). Most of the agricultural holdings are present in North Lebanon (31%) and Bekaa (20%) (Agriculture factsheet 2017).

In Lebanon, agriculture represents the third most vital economic sector after the services and the industry sectors¹¹. Although agriculture in Lebanon is still in general family-owned, it consists in total of around 4.500 small and medium enterprises SMEs with an average of 6 employees per unit (Kayrou and Atala, 2015). In 2010, the agricultural sector generates around 4.7% of the Gross Domestic Product (GDP), providing an income to approximately 10% of the Lebanese labor force. Vegetables are the leading agricultural outputs accounting for almost 62% of total agricultural production, followed by fruits (citrus, apples, grapes, and bananas...) and dairy products (Figure 5)("Agriculture fact book.pdf,").

¹¹ The Chemical pesticides use in the agriculture treatment is a must...?!, 2015. . GreenArea.me. URL <u>http://greenarea.me/en/65354/the-chemical-pesticides-use-in-the-agriculture-treatment-is-a-must/</u> (accessed 3.25.19).



Figure 5: Agricultural Production in Lebanon ("Agriculture fact book.")

The inappropriate use of pesticides is the most prominent conflict facing agriculture in Lebanon. In fact, the organization of pesticides utilization has started in 1982 by the scientific committee concerned in controlling pesticides utilization. The committee sets out authorizations for pesticides importation, sales, labeling, and usage. Even though the Lebanese Ministry of Agriculture controls pesticides registration systematically, banned pesticides can be illegally utilized by untrained public due to weak law enforcement (Salameh et al. 2004).

1.4. Exposure of Pesticides to the General Population:

i. Risk assessment:

The general population - involving people not present in the area of pesticides utilization is exposed mainly by the oral route through pesticide residues present in food at low doses, considered as chronic (or semi-chronic). By definition, "Pesticide residue" refers to the presence of any specified substances in agricultural commodities, food, or animal feed as a result of pesticide use. Other potential sources include drinking water contaminated with pesticides in addition to the personal environment such as the house dust, indoor and outdoor air and soil...The long term exposure can pose a health risk for the population.

The assessments of the risks from pesticide residues in food are carried out by international Joint FAO/WHO Meeting on Pesticide Residues (JMPR) based on several scientific and toxicity studies ("WHO | Joint FAO/WHO Meeting on Pesticide Residues

(JMPR),"). In order to ensure the safety levels of pesticides residues, the JMPR establishes limits for safe intake. The Long-term exposure studies on animal models allow determining the No Observed Adverse Effect Level (NOAEL) corresponding to the highest dose of the chemical that does not cause detectable adverse effects. This dose is used to define the acceptable daily intake (ADI) for humans, as the amount of pesticide that can be consumed daily over a lifetime without appreciable risk to the health of the consumer (expressed in mg of the chemical per Kg of body weight). International and national risk managers, such as the Codex Alimentarius Commission (International food standards-setting body) and the Lebanese Standards Institution¹² depend on these ADI in determining maximum residue limits (MRLs) for pesticides in food. MRL is defined as the maximum legally-allowed concentration of pesticides residues in food and agricultural commodities¹³. Codex standards are now identified for more than 100 different pesticides.

In the European Union (EU), the assessment of pesticide residues in food is established according to the Regulations (EC) No 396/2005¹⁴ and 1107/2009¹⁵. The control of the dietary intake of pesticides is accomplished by the national safety agencies and the European Food Safety Authority (EFSA).

According to the latest report published in 2018 and in the framework of the EUcoordinated control program (EUCP), 1.7% of the 12,168 samples analyzed in 2016 have exceeded the MRL corresponding to 203 samples in total. This exceedance rate was higher than that reported in 2013 (0.9%). The significant increase is attributed mainly to the exceedances of chlorpyrifos residues which have followed legal changes towards lower MRLs for many crops during 2016. Indeed, chlorpyrifos reported 59 exceedances out of the 10,212 analyzed samples. In total, 98.3% of the analyzed samples in 2016 were within the

¹³ Glossary | CODEXALIMENTARIUS FAO-WHO, n.d. URL

¹² LIBNOR Lebanese Standards Institution (2003). Pesticide Residues in Foods -Maximum Residue Limits for Pesticides. (Food Technology. NL 662: 2004. TC: NL TC IRI. ICS: 65.100.01)

http://www.fao.org/fao-who-codexalimentarius/codex-texts/dbs/pestres/glossary/en/ (accessed 3.20.19).

¹⁴ REGULATION (EC) NO 396/2005 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL

of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC

¹⁵ Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC

legal limits (11,965 samples) and 52.3% were free of quantifiable residues i.e. below the limit of quantification (LOQ) (European Food Safety Authority 2018).

According to the French General Directorate for Consumer Affairs and Fraud Control (DGCCRF), a recent study about pesticide residues in samples from 19 fruits and 33 vegetables in France was reported by the French NGO Générations Futures. Between 2012 and 2016, 41.1% of non-organic vegetables and 72.6% of non-organic fruit were contaminated by pesticides. 3.5% of vegetable samples and 2.7% of fruit samples have exceeded the maximum residue limit (MRL) (générations 2018).

A Lebanese study was performed on 1860 food samples to assess the dietary exposure of adults to 47 pesticide residues in two regions of Lebanon: Keserwan (semi-rural area) and Greater Beirut (urban region). Eighteen pesticide residues were either detected or quantified in the tested samples including, mostly, Chlorpyrifos, Procymidone, Primiphos methyl, Dimethoate, and Dieldrin (Nasreddine et al. 2016).

ii. Developmental Exposure to Pesticides

The exposure of pregnant women to the pesticides is particularly hazardous due to the sensitivity of the fetal nervous system which undergoes a rapid state of growth and development during the gestational process. The exposure to such environmental contaminants during the postnatal period is of great concern also, since the maturation of the organs function is still in process.

a. Exposure of pregnant women to pesticides (prenatal exposure):

The exposure of pregnant women to pesticides has been assessed in different global localities by measuring the metabolites of different pesticides which have been detected in urine and blood samples (Acosta-Maldonado et al. 2009). Between 2002 and 2006, the PELAGIE mother-child cohort included 3421 pregnant women from Brittany (France). Dialkylphosphate (DAP) metabolites of organophosphate pesticides were detected in most of the maternal urine samples (92%). In addition, organochlorine pollutants (such as DDE,

PCB...) were highly quantified in the cord blood samples (Debost-Legrand et al. 2016). Similarly, maternal hair has been analyzed for pesticides residues by other studies.

The assessment of fetal exposure to pesticides has been carried out by few studies through the analysis of the meconium; which is the first feces of the neonate (Ostrea et al. 2012; 2008; R. M. Whyatt and Barr 2001). Other studies have reported the analysis of the infant cord blood (Ostrea et al. 2008; Robin M. Whyatt et al. 2004), and infant's hair (Ostrea et al. 2008). The highest rates of pesticides and especially organophosphate metabolites were observed in the meconium, thus representing the most sensitive method in measuring fetal exposure to pesticides. Meconium is considered as a reflection of the cumulative exposure during the last 6 months of pregnancy.

In Picardy region of northern France, analytical study was conducted on 171 meconium samples for quantification of five major pesticide families (organophosphates, carbamates, phenylureas, pyrethroids, and phenoxy herbicides) and their metabolites. The most detected residues correspond to the metabolites of organophosphates (57.9%) and carbamates 22.8%, indicating fetal exposure to their corresponding pesticides (Berton et al. 2014).

b. Exposure of newborns to pesticides by breast milk:

Fetuses and young children can be more vulnerable to pesticides exposure due to the low levels of detoxifying enzymes that deactivate pesticides (Furlong et al. 2006). Since many toxic chemicals and xenobiotics are excreted in breast milk, lactational transfer is considered as a major route of exposure to infants which can pose a critical health risk (Pohl and Tylenda 2000). Several studies have reported the contamination of human milk with pesticides in France (Wolff 1983). Indeed 17 congeners of PCB and 13 organochlorine pesticides including HCB, aldrin, heptachlore-epoxide, dieldrin, endrin, were detected in milk samples of women in France (Bordet et al. 1993).

iii. Exposure of adults to Pesticides

Biomonitoring studies have been performed by several countries in Europe (Bouvier et al. 2005), Canada (Canada 2010), and the USA ("FourthReport_UpdatedTables_Feb2015.pdf,") in a way to measure individual's total exposure by assessing pesticides levels or their metabolites in human tissues (general population). In general, the mean levels have been found to be lower than, or sometimes similar to, occupationally exposed peoples. Since most of the pesticides have short half-lives (often less than 24 hours), then the population seems to be routinely exposed to pesticide residues. During the last years and according to different studies performed on urinary samples, adults have been shown to be exposed mostly to organophosphates (OPs) and by a lower degree to pyrethroids and carbamates. In an American NHANES III study on adult population, urinary levels of TCP which is a specific metabolite of chlorpyrifos-ethyl and chlorpyrifos-methyl, have been detected in 82% of the samples (Hill et al. 1995). Similar results were recorded in Europe (Aprea et al. 1999; H. M. Koch, Hardt, and Angerer 2001).

In the French National Nutrition and Health Survey, chemical pollutants or their metabolites have been measured in urine, blood and hair samples. Dialkyl phosphates (DAP), a metabolite of many organophosphorus insecticides, have been detected in around 90% of urine samples. In addition, other pesticides such as pyrethroids and polychlorinated biphenyls (PCBs) have been detected in the samples, even at higher levels compared to the United States and Germany ("ENNS: National Health Nutrition Study / Surveys and Studies / Nutrition and Health / Chronic Diseases and Injuries / Thematic files / Home,").

1.5. Chlorpyrifos

In this study, Chlorpyrifos was selected based on its high usage in Europe. Its residues are among the most detected in food in Europe and Lebanon. Chlorpyrifos is a developmental neurotoxic pesticide and it is classified among the most hazardous substances to be identified as a priority in the European community.

i. Overview

Chlorpyrifos (CPF) is a broad spectrum organophosphate insecticide, acaricide and miticide (Figure 6). Introduced by Dow AgroSciences into the marketplace in 1965, CPF has been commonly used to control crop pests in agricultural and non-agricultural areas. In 2001, the residential use of chlorpyrifos was eliminated in the United States by EPA. A global phase-out of nonagricultural uses of CPF has been started in 2006. Despite debate on CPF ban between 2016 and 2018, and according to EPA, CPF is still registered and its registration is under revision until 2022 (US EPA 2014).



Figure 6: chemical structure of chlorpyrifos

ii. Utilization

CPF is commonly used against a broad array of insects and mites. The diversity of arthropod pests controlled by CPF has made it one of the most widely used insecticides (Solomon et al. 2014). CPF is registered for use on a wide variety of crops but more than 60% of CPF use in the United States is based on 3 main crops, corn (39%), tree nuts (15%), and soybeans (9%). Also, the use of tree fruits accounts for 10% of total use. The nonagricultural uses of CPF account for less than 3% in total and are restricted to mosquito control for public uses and insect control on golf courses (Eaton et al. 2008).

According to the EU-coordinated program study for the control of food products in 2012, CPF was among the most detected on food products (469 detections) ("The 2012 European Union Report on Pesticide Residues in Food" 2014).

iii. Chemical and Physical Properties:

Chlorpyrifos or 0,0-diethyl-O-(3, 5, 6-trichloro-2-pyridinyl)-phosphorothioate is a chlorinated organophosphate chemical. Its chemical formula is C9H11Cl3NO3PS with a molecular weight of 350.6 g/mol. CPF is colorless to white crystalline solid, which has a mild mercaptan odor. Since CPF is a lipophilic chemical, it is soluble in most of the organic solvents however; it is slightly soluble in water (1.4 mg/ml at 25° C). CPF has an intermediate vapor pressure of 1.9×10^{-5} mm Hg at 25°C, thus it is capable of volatilization with the ability to drift from the site of application. CPF is relatively stable in acidic and neutral pH. Its stability decreases as pH increases. CPF can rapidly bind to particles on plants or in the soil, but in a very low extent to water sources (Eaton et al., 2008).

iv. Environmental Fate of CPF and Persistence

CPF binds to plants and soil after utilization, where slow to moderate degradation occurs (Eaton et al. 2008). CPF persistence in the environment is considered as short to moderate due to multiple dissipation pathways that might occur at the same time such as volatilization, abiotic hydrolysis, photolysis, and microbial degradation (Mackay, Giesy, and Solomon 2014). The persistence in soil depends on many environmental factors including moisture content, photolysis temperature, and the presence of microorganisms. Consequently, half-lives range from a few days to even 4 years (Gebremariam et al. 2012). CPF can undergo photodegradation, with a half-life of about 3 days. At water surfaces, CPF degradation depends on photosensitized oxidation or abiotic hydrolysis. CPF can persist for months at indoor environments due to the absence of water, sunlight or soil microorganisms that are present outdoors (Eaton et al. 2008).

The Atmo Federation performed a study on pesticide residues in the atmosphere in different environments of Picardy during 2012. CPF was detected in rural and urban sites with a concentration Maximum of 0.8 and 0.47 ng / m3, respectively (Sauvage 2013).

v. Exposure

The potential human exposures to Chlorpyrifos can occur through different pathways including the oral ingestion of residues in the diet, the inhalation of vapors, and the dermal absorption by skin contact. Dermal and inhalation exposures are the main routes for occupational exposure. Residential and occupational exposure occur through handling and applying CPF (Eaton et al. 2008). Agricultural workers can be exposed by post-application activities such as scouting and harvesting ("Revised Human Health Risk Assessment Chlorpyrifos | US EPA ARCHIVE DOCUMENT.).

Due to the restriction of indoor use of CPF for residential insect control in 2001 and the elimination of sales to the public (Carlton et al. 2004), the oral ingestion of CPF by food is considered to be the major source of exposure to the general population.

Chlorpyrifos is considered to be one of the 10 hazardous substances to be identified as a priority at Community level, according to the decision No 2455/2001/EC OF THE EUROPEAN PARLIAMENT (Union 2001).

CPF is one of the most detected pesticides in vegetables and fruits among Europe in 2015 ("Report_ED pesticides in EU food_PAN Europe.pdf, 2017). According to the 2015 European Union report on pesticide residues in food, CPF residues were detected at levels exceeding the toxicological reference value (ARfD) in bananas, peppers, table grapes, broccoli and aubergine ("The 2015 European Union Report on Pesticide Residues in Food" 2017).

vi. Toxicokinetics (ADME): Absorption, Distribution, Metabolism, and Excretion

In the view of pharmacokinetic studies, critical factors are taken into account such as the route, the dose, and the form of exposure. In this context, the pharmacokinetics parameters will be discussed in this part according to the oral route of exposure.

- Absorption

In vivo absorption of chlorpyrifos has been widely assessed by several studies by either measuring the levels of CPF metabolites such as TCP, DETP, or DEP in blood or urine or by determining acetylcholinesterase (AChE) effects depending on the administered dose.

The absorption of CPF after acute exposure has been examined in human and rodent studies. In humans, 70% of CPF was absorbed after 48 hours of a single oral dose administration assessed by measuring CPF metabolites in urine (Nolan et al. 1984). However, around 80% and 90% of CPF was absorbed in rats (Timchalk et al. 2002) and mice (Ahdaya and Guthrie 1981) respectively following CPF administration. Peak blood TCP was observed after 6 hours of dosing (Nolan et al. 1984). CPF is considered to be well absorbed from the small intestine through passive diffusion. According to the single-pass intestinal perfusion method, other mechanisms could affect ileal and probably the duodenal absorption such as the interaction with the efflux P-glycoprotein (Cook and Shenoy 2003).

The repeated administration of CPF (7 mg/kg/day) to pregnant rats through gestational days (GD) 14–18 resulted in similar levels of acetylcholinesterase (AChE) inhibition in the brain of dams and fetuses indicating the ability of CPF to pass into the fetus. The lower extent of AChE inhibition in the fetus is attributed to the ability of the fetus to synthesize new enzyme molecules (Lassiter et al. 1998).

Different studies have examined the CPF disposition across the placenta in pregnant rats. In a radiolabeled CPF distribution study, CPF residues were detected in the placenta and the amniotic fluid in addition to the fetal liver and brain (Akhtar, Srivastava, and Raizada 2006). According to the metabolite profile study, the peak levels of TCP occurs among the maximum levels of cholinesterase inhibition 5 hours following CPF administration to the fetus as reported by Hunter et al., (1999).

- Distribution

Following oral chlorpyrifos dosing in rats, CPF is distributed to body organs. Due to its lipophilicity, CPF accumulates in the fat and fatty tissues and is liberated in a slower manner with $t_{1/2}$ of around 62 hours compared to other tissues (kidney, liver and heart $t_{1/2}$ is about 10 to 16 hours) (Smith, Watson, and Fischer 1967). CPF can bind to plasma proteins such as albumin. According to a detailed study on CPF distribution in tissues, CPF is detected in brain, liver, kidney, and fat. CPF-oxon, a CPF metabolite being less lipophilic than CPF, has also been found in the organs at lower concentrations (Timchalk et al. 2002).

A distribution study of radiolabeled CPF was carried on pregnant rats. The maximum levels of radioactivity detected in the fetal plasma occurred after 15 min, representing around 1% of that found in the mother's plasma (Abdel-Rahman et al. 2002).

In humans, no specific studies regarding CPF distribution have been reported. Epidemiological studies confirm the passage of CPF across the placenta by detecting CPF in the umbilical cord blood (R.M. Whyatt et al. 2005).

- Metabolism

Biotransformation is one of the key factors which determine the toxicity of CPF. The parent molecule is a weak inhibitor of AChE, thus CPF is activated in the liver by cytochrome P450 (CYP) through a desulfuration reaction to form chlorpyrifos-oxon (CPO) (Figure 7), a potent AChE inhibitor (Tang et al. 2001).

CYP also promotes the detoxification of CPF by dearylation, producing diethylphosphate (DEP), and trichloro-2-pyridinol (TCP). Then the balance between both processes (desulfuration and dearylation) results in variable levels of AChE inhibition. B-esterases (B-est) such as AChE, butyrylcholinesterase (BuChE), and carboxylesterase (CaE) are inhibited by the CPO but A-esterases such as paraoxonase (PON1) are not inhibited (Poet et al. 2003).

The PON1 metabolism of CPO mediates the deactivation of the oxon to form TCP. Then both of the oxidative dearylation of CPF by CYP enzymes and hydrolysis of CPO by PON1 (or

other enzymes) lead to the formation of TCP, which is the major metabolite of CPF detected in urine (Eaton et al. 2008).

Even though the total detoxification of organophosphate insecticides is established by different enzymes (CYP450, B-est, PON1), the ability of PON1 enzyme to protect against OP toxicity has been examined in some studies. In rodents, the administration of PON1 has been shown to protect against acute OP poisoning (L. G. Costa et al. 1990; Li, Furlong, and Costa 1995; Li, Costa, and Furlong 1993). Another way of detoxification is mediated in human hepatocytes leading to GSH-derived conjugates in addition to O- and S-glucuronides through extensive phase II detoxification (Choi et al. 2006).

Comparative metabolic studies have been reported by (Timchalk, Poet, and Kousba 2006) on adult and juvenile rats of 5, 12, and 17 days-old. In 5 days-old young animals, the total detoxification capacity of CPF in the rats exceeds that of bioactivation. However, CPF blood levels in juveniles were higher than those in adults illustrating a lower ability of young rats to metabolize CPF. Indeed, the activity of brain oxidases is lower in the fetuses as compared to the mothers (Lassiter et al. 1998).



Figure 7: Major metabolic pathway of chlorpyrifos (Mink, Kimmel, and Li 2012)

- Excretion

Chlorpyrifos is excreted mainly by the kidneys in the urine. Detectable levels of CPF metabolites can be measured in the urine after 24 hours of CPF dosing in rats. The urinary metabolites are TCP (62%), DETP (40%), and DEP (4%). The maximum excretion in the urine occurs at 12 hours, but a significant excretion can be still detected at 72 hours (Timchalk et al. 2007). In humans, the elimination half-life for TCP after oral or dermal exposure is around 27 hours (Nolan et al. 1984).

CPF could not be found in the urine due to its lipophilicity, however, it could be found in breast milk. According to a study in rats, CPF was detected in the milk of lactating rats at 200 times higher than those found in blood during the perinatal period (Anderson and Wolff 2000).

vii. Mode of Action

The primary toxic mechanism of CPF is mediated by the ability of its oxon metabolite (CPO) to bind to and irreversibly inhibit acetylcholinesterase (AChE) in cholinergic synapses. The nervous system is the basic target because AChE hydrolyzes acetylcholine, a major neurotransmitter in the central and peripheral nervous systems thus terminating its synaptic function. Inhibition of AChE results in the accumulation of acetylcholine and subsequently the over-stimulation of the muscarinic and nicotinic cholinergic synapses (Eaton et al. 2008).

Other mechanisms include the disruption of cell signaling cascades that are critical to cell differentiation and homeostatic regulation. Among these is the pathway that generates cyclic adenosine monophosphate (cAMP), an essential second messenger that mediates several neurotransmitter and hormonal receptor signals, regulated by adenylyl cyclase (AC) (Meyer et al. 2003; Schuh et al. 2002).

viii. Toxicity of CPF

a) Acute Toxicity

According to WHO, CPF is classified as a Class II moderately hazardous substance (International Program on Chemical Safety, Inter-Organization Programme for the Sound Management of Chemicals, and World Health Organization 2010). In rats, values of acute oral lethal dose (LD50) ranges from 118–270 mg/kg for males and 82–174 mg/kg for females (B. McCollister et al. 1974). A summary of the LD50 values for CPF according to the species or the route of exposure is listed in table 2.

Route of Exposure	Species	LD50 (mg/kg)	Reference
Dermal	Sherman rats	202	(Gaines 1969)
Inhalation	Female Sprague-Dawley	78	(Berteau and Deen 1978)
	rats		
	Dow-Wistar Male rats	163	(B. McCollister et al. 1974)
Oral	Dow-Wistar Female rats	135	
	Sherman Male rats	118	(Gaines 1969)
	Sherman Female rats	155	
	Mice	60	(El-Sebae, Ahmed, and
			Soliman 1978)
	Guinea pigs, male	504	(B. McCollister et al. 1974)

Table 2: LD50 values for CPF in different species according to the routes of exposure

Like other OPs, the acute toxic effects of CPF are manifested mainly through the persistent phosphorylation, by its metabolite CPO, of AChE at cholinergic synapses within the peripheral (PNS) and central (CNS) nervous systems. This persistent phosphorylation results in inhibition of AChE leading to the accumulation of the neurotransmitter acetylcholine (ACh) in the synapses, and over-stimulation of the muscarinic and nicotinic cholinergic receptors. Since these receptors are present in most organs of the body, a "cholinergic syndrome" occurs, which includes increased sweating and salivation, intensive bronchial secretion, miosis, bronchoconstriction, increased gastrointestinal motility, tremors, muscular twitching, diarrhea, and other central nervous system effects (Lotti

2010). Death may occur due to respiratory failure as a result of the inhibition of respiratory centers in the brainstem, bronchoconstriction and increased bronchial secretion, and flaccid paralysis of respiratory muscles (Eaton et al. 2008). The occurrence of hypoxia as a result of peripheral acute cholinergic effects and central apnea contributes to the most of deaths of OPs poisoning (Hulse et al. 2014).

b) Chronic Toxicity

The chronic toxicity of CPF was evaluated in several species including rats, mice, and dogs. Inhibition of brain, plasma and RBC cholinesterase (ChE) occurred mainly at levels in the range of 0.03 to 3 mg/kg/day. In rats, the NOAEL was 0.1 mg/kg/day for plasma and RBC cholinesterase inhibition and 1.0 mg/kg/day for brain cholinesterase inhibition (table 3). The developmental NOAEL in rats is 1 mg/kg-day, based on developmental adverse effects such as neonatal survival, decreased pups body weight, and maturational delays (Hoberman, A. M. 1998).

The Acceptable Daily Intake (ADI) is 0.01 mg/kg body weight as set by the EU in 2005 using a safety factor of 100 ("Reconsideration of the Human Toxicological Reference Values (ARfD, ADI) for Chlorpyrifos - BfR Opinion No 026/2012, 1 June 2012," n.d.).

For a pregnant woman, ADI is less than 0.001 mg/kg based on a no adverse effect dose of 0.3 mg/kg/day (Maurissen et al. 2000).

Species	Duration	NOAEL	Adverse effect	Reference
		mg/kg/day	reported	
Rat	2 years	0.1	Plasma ChE	(Young, J.T 1988)
		0.1	RBC ChE	
		1	Brain ChE	
Human	9-28 days	0.03	Plasma ChE	(Coulston F et al.
		0.1	Brain ChE	1972)

Table 3: Adverse effects of CPF according to the species

ix. Health Impacts

i. Developmental toxicity

Epidemiological studies have examined the association between prenatal exposure to CPF and birth outcome by measuring CPF levels or its metabolites in maternal blood and umbilical cord (Perera et al. 2003; Robin M. Whyatt et al. 2004). Prenatal CPF exposure was associated with reduced birth length and weight. Indeed the prenatal exposure to CPF can mediate brain cell loss in addition to behavioral and neurochemical effects in the developing rats (Chanda and Pope 1996). CPF can affect cellular development by altering the adenylyl cyclase signaling pathways, which mediates cholinergic and adrenergic signals and cell differentiation, possibly through targeting M2 receptor (Meyer et al. 2003; Song et al. 1997).

- Neurodevelopmental toxicity

Several other studies have supported the developmental effects of CPF on the brain. These effects include altered synaptic development (Dam et al. 1999), alterations in DNA and protein synthesis (Campbell, Seidler, and Slotkin 1997), and interference with gene transcription (Crumpton, Seidler, and Slotkin 2000). Early prenatal exposure to CPF during the gestational period (1 or 5 mg/kg-day) have shown to induce serotoninergic, cholinergic, and behavioral changes in the offspring (Aldridge, Seidler, and Slotkin 2004; Icenogle 2004; Qiao et al. 2004).

The prenatal exposure to CPF during gestation caused an increase in locomotor activity on postnatal day (PND)25 (Ricceri et al. 2003), hyperactivity at PND70 (Ricceri et al. 2006), and increased agonistic response in male mice. CPF exposure during early postnatal life in rats (PND 1–6) induced decreased levels of mRNA for nerve growth factor (NGF), reelin which is an extracellular matrix protein essential for cerebral development, and muscarinic M1 receptors (Betancourt, Burgess, and Carr 2006).

Exposure to CPF (1 mg/kg/day) during gestation and lactation period has impaired the novelty-related activity and sensori-motor functions in the rats' progeny (Laporte et al. 2018).

- Metabolic Disruptions

The ability of CPF to induce developmental metabolic programming has been assessed in some studies. Meyer et al. have shown that subtoxic developmental exposures to CPF, during critical gestational and early postnatal life, induced alteration in the hepatic and cardiac cellular function in adulthood mediated by altered adenylyl cyclase function. The fact that many of the effects emerged at adulthood indicates the ability of CPF to alter the programming of the cell signaling (Meyer, Seidler, and Slotkin 2004). Consequently, an altered metabolic pattern of plasma lipids (hyperlipidemia) and insulin (hyperinsulinemia) in a way resembling the known major risk factors for atherosclerosis and type 2 diabetes mellitus in adulthood have been reported by Slotkin et al., (2005).

CPF exposure during gestation and lactation period has been shown to interfere with the regulation of metabolism characterized by metabolic syndrome signs in the progeny during their adult life (Reygner et al. 2016).

ii. Reproductive Toxicity and teratogenicity

Studies did not report teratogenic or abnormal reproduction effects with a daily oral dose of CPF up to 5 mg/kg-day (Eaton et al. 2008). At higher doses (12 and 15 mg/kg/day), prenatal growth retardation in addition to maternal signs of toxicity have been reported (Akhtar, Srivastava, and Raizada 2006). At very high doses (25 mg/kg/day), Farag et al. (2003) reported fetotoxicity and teratogenic effects of CPF administered through gestation.

iii. Endocrine Disruptions

Chlorpyrifos is suspected for endocrine disruptions. CPF can affect the adrenal gland and the thyroid homeostasis in human and animal models (De Angelis et al. 2009; Jeong et al.
2006). Other inhibitory effects were reported on the pituitary hormones secretions (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) in addition to reduced testosterone biosynthesis in the rats testes (Joshi, Mathur, and Gulati 2007). Peiris and Dhanushka (2017) have shown reduced serum testosterone, LH, and FSH hormone levels in rats following repeated exposure to CPF of 10 mg/kg/day.

iv. Cancers

CPF did not induce tumors in chronic oral studies in mice and rats. According to EPA, CPF is not listed as a carcinogen ("Chlorpyrifos Registration Review; Preliminary Human Health Risk Assessment; Extension of Comment Period" 2011). Epidemiological studies, however, suggest a possible relationship between CPF exposure and lung and rectal cancers in humans (Lee et al. 2007).

v. Gastrointestinal Effects

Limited gastrointestinal effects were reported in female Fisher 344 rats exposed to CPF of 5 mg/kg/day (Dow. 1993). Prenatal and postnatal CPF exposure was associated with increased gut permeability (Joly Condette et al. 2014) and altered gut motility with a weaker contraction of ileal longitudinal smooth muscle (Darwiche et al. 2017).

vi. Respiratory Diseases

The chronic prenatal and postnatal exposure to CPF was associated with increased sleep apnea index and perturbations in the respiratory parameters accompanied by altered diaphragm contractility in juvenile and adult rats (Darwiche et al. 2018). Such effects of OPs on the respiration are attributed to the hyperstimulation of the muscarinic receptors in the central nervous system (Houze et al. 2008).

- Therefore, the exposure to the insecticide, CPF, is associated with subsequent adverse impacts on health as described in this chapter. Consequently, another environmental factor, particularly high-fat diet consumption, which plays a major role in the occurrence of overweight and obesity in the developed world, will be discussed in the next chapter.

Chapter 2: Obesity and High-Fat Diet (HFD)

The second food risk that hovers over our society is the obesity representing a major health hazard of the 21st century. Especially in rich countries, junk food is easily accessible more than just a balanced diet. Overweight and obesity are the largest nutritionally based problems in the developed societies particularly in France where more than 21 million people are either overweight or obese.

In this chapter, obesity, overweight, and the effects of dietary fats on energy balance will be discussed. The effects of nutritional perturbations on fetal development and metabolic disruptions will be explained.

2.1. Definition of Overweight and Obesity

According to WHO, Overweight and obesity are defined as abnormal or excessive fat accumulation that poses a health risk. Clinically, the prevalence of obesity is measured by the body mass index (BMI), defined as the body weight in kilograms (kg) divided by the square of the height in meters (m) expressed in (kg/m²). BMI values are used to classify individuals as underweight (<18.50 (kg/m²), healthy (range lies between 18.50 and 24.99 kg/m²) overweight (≥ 25.00 kg/m²) or obese (BMI ≥ 30 kg/m²) according to table 4 ("WHO Obesity: Preventing and Managing the Global Epidemic" 2000).

Classification	BMI (kg/m2)	Risk of comorbidities	
Underweight	<18.50	Low (but risk of other clinical problems	
		increased)	
Normal range	18.50-24.99	Average	
Overweight:	≥ 25.00		
Pre-obese	25.00–29.99	Increased	
Obese class I	30.00–34.99	Moderate	
Obese Class II	35.00–39.99	Severe	
Obese Class III	≥ 40.00	Very Severe	

2.2. Prevalence of Overweight and Obesity

Worldwide, the prevalence of obesity has been almost tripled since 1975. In 2016, more than 1.9 billion adults were overweight, including more than 650 million obese adults. Indeed, 39% of adults aged 18 years and older were overweight representing 39% of men and 40% of women. In total, obesity accounts for almost 13% of the world's adult population. Around 41 million children under 5 years old and more than 340 million children and adolescents between 5 and 19 years old were reported as overweight or obese.

Obesity is considered as a high-income country problem. Globally, most of the populations are spread out through countries where obesity and overweight are more hazardous to people's life than underweight (WHO 2019).

In the WHO/European Region, more than 50% of people are either overweight or obese. According to 2016 data, 21.9% of men and 24.5% of women were obese¹⁶.

In France, as reported by the latest survey Obépi (2012), more than 21 million French are overweight or obese. The French adult population is 32.3% overweight ($25 \le BMI < 30 \text{ kg} / m^2$) and 15% obese (BMI $\ge 30 \text{ kg} / m^2$).

The prevalence of obesity has increased from 14.5% in 2009 to 15% in 2012, which was more observed in women than in men, especially among 18-25-years-old. It corresponds to a 3.4% relative increase in the world obesity.

The average BMI has increased from 24.3 kg / m^2 in 1997 to 25.4 kg / m^2 in 2012. An increase in the average weight of the French population by around 3.6 kg has been reported ("ObEpi-Roche 2012 : enquête nationale sur l'obésité et le surpoids,")(Figure 8).

¹⁶ "Chapter 2: Health Situation in the European Region." September 11, 2018. <u>http://www.euro.who.int/en/data-and-evidence/european-health-report/european-health-report-</u> <u>2018/european-health-report-2018.-more-than-numbers-evidence-for-all-2018/report-by-</u> <u>chapters/chapter-2-health-situation-in-the-european-region</u> (accessed 4.14.19).

Champagne-Ardenne and Picardie are the French regions with the highest rates of obesity with 20.9% and 20% respectively.



Figure 8: Evolution of BMI since 1997 according to gender in France

2.3. Factors contributing to Obesity

The tendency toward lifestyle changes has contributed to the increasing prevalence of obesity. The vulnerability to developing obesity is attributed to the imbalance between energy intake and expenditure. In modern societies, the reduced physical activities are accompanied by the consumption of High-calorie Diet or so-called "Western Diet" as being cheaper and thus more accessible than the healthy diet. The regulation of energy balance is a complex process involving the interaction of other factors such as genetic, psychosocial, hormonal and environmental influences (Hariharan, Vellanki, and Kramer 2015; Malterud and Tonstad 2009).

2.4. Western Diet

The Western diet or the meat-sweet diet is characterized by a high intake of red meat, animal fat, and high sugar- drinks and desserts, and a low intake of fruits and vegetables. The foods are usually highly processed and refined. They are rich in saturated fatty acids (SFA) and *trans* fats, fructose and sucrose, proteins of red meat, and sodium. However such a diet is low in monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), proteins from a plant source, and fiber content (Hariharan, Vellanki, and Kramer 2015; Odermatt 2011).

Ingested fats can affect energy balance through spontaneous energy intake. In fact, high fatdiet has a weaker satiating capacity compared to a low-fat diet resulting in the overconsumption of energy from fat-rich diets. Fats provide a greater flavor and palatability to foods, thus inducing a greater fat consumption (Willett 2003). Compared to protein and carbohydrate-rich foods, dietary fats are associated mainly with a greater energy density contributing to their fattening effects (Astrup 2005).

- Energy Balance

Several epidemiological studies suggest a positive correlation of dietary fats intake with a high incidence of obesity and in clinical studies between the levels of fats in diets and bodyweight gain (Saris et al. 2000; Tucker and Kano 1992; Lissner et al. 1987). Similar associations have been observed in animal models, in both rats (Ghibaudi et al. 2002) and mice (Takahashi, Ikemoto, and Ezaki 1999) with a positive relationship between dietary fat intake and body weight or fat gain. Thus, dietary fat content represents a key in energy balance. As energy uptake exceeds energy expenditure, obesity occurs. The energy stores in body fat are enlarged, especially in adipose tissues. Generally, diets with more than 30% of total energy from fats can lead to obesity development (Hariri and Thibault 2010). Studies have shown that SFA are more obesogenic than PUFA in humans (Kien, Bunn, and Ugrasbul 2005). Further studies on animal models have reported greater accumulation of body fats or increased body weight on feeding with diets moderate or rich in SFA (Yaqoob et al. 1995; Bourgeois, Alexiu, and Lemonnter 1983).

2.5. Maternal High-Fat Diet

The maternal high-fat diet (HFD) is considered a reflection of the dietary habits in western countries. Indeed, 15 to 20% of women in the reproductive age are obese (Seidell 2000) and around 42% of women gained more weight during pregnancy than recommended

(May 2007). Such increase in pregnancy weight gain and maternal obesity induce longterm consequences on the offspring during development leading to early puberty and increased risk for obesity in a continuous cycle of overweight mothers and obesity-prone children (Howie et al. 2009).

Maternal diet and metabolic status can directly affect the environment of the developing offspring. In this context, emergent evidence from epidemiological studies and animal models suggest a relationship between the intrauterine environment and the development of obesity and metabolic disorders (S. Muhlhausler and Y. Ong 2011; Yang and Huffman 2013). Since fetal development depends mainly on the maternal nutritional supply, then any perturbation in the intrauterine milieu could predispose offspring to further diseases in later life. This relationship is described by the developmental origin of health and disease concept (DOHaD) (Barker 2004).

i. The Developmental origins of adult disease (Barker hypothesis)

Adverse influence during early intrauterine life, at a critical period of developmental plasticity, results in permanent changes in metabolism and physiology in a way to adapt and survive the different environmental conditions during development (De Boo and Harding 2006). However, disparities between prenatal predictions and the actual postnatal environment, known as predictive adaptive responses (PAR) may result in metabolic disease at adulthood (Gluckman and Hanson 2004).

ii. Developmental Programming and Metabolic disturbances

Programming is the most common phenomenon proposed to underlie the developmental origins hypothesis. This process is attributed to the stimulus during a critical or sensitive period having irreversible long-term influences on development. Well-known mechanisms include altered fetal nutrition (De Boo and Harding 2006). Epidemiological studies have suggested a relationship between suboptimal early nutritional environment and the risk for cardiovascular disease, diabetes, and the metabolic disease in adult life including hypertension, insulin resistance, and obesity (Le Clair et al. 2009; Tamashiro and Moran 2010; McMillen and Robinson 2005). Studies on animal models have shown that

developmental exposure to maternal protein deprivation (Desai et al. 1997) or dietary restrictions (Woodall et al. 1996) is associated with hypertension and insulin resistance in adulthood when further fed a balanced diet.

Other studies on rodents have reported cardiovascular dysfunction, impaired glucose homeostasis and perturbations in liver lipid metabolism in adult progeny after maternal HFD consumption (Cerf et al. 2005; I. Y. Khan et al. 2005). A maternal diet rich in saturated fats was also associated with metabolic syndrome features at adulthood (Siemelink et al. 2002; Imran Y. Khan et al. 2003).

2.6. Co-exposition to HFD and pesticides

Studies have shown that the metabolic abnormalities caused by early-life organophosphate exposure can interact with other factors that are related to human obesity and diabetes. Since human dietary choices occupy crucial concern nowadays, the role of HFD was examined. In these studies, neonatal rats were exposed to low-dose of parathion then, at adulthood, half of the animals were switched to HFD with 58% of the calories came from fat, especially saturated fat. The HFD exacerbated many of the metabolic effects of early-life OP exposure including a much higher fat-induced weight gain compared to unexposed animals given the same diet, associated with prediabetic metabolic alterations. For instance, HFD has been shown to affect the AC pathway in the liver and the heart, indicating that OP exposure can produce changes in the same signaling events that were affected by fat consumption (Adigun et al. 2010; Slotkin 2011; Lassiter et al. 2008). Moreover, hepatic carboxylesterase and fatty acid amide hydrolase activities in 4 weeks HFD fed mice have been shown to be more potently inhibited than those in normal-diet fed mice following 4 weeks of CPF exposure. These results indicate that obesity could exacerbate some of the non-cholinergic effects of CPF exposure (Kondakala et al. 2017).

In contrast, HFD ameliorated some of the effects induced by neonatal parathion exposure by enhancing synaptic activities (Lassiter et al. 2010). It has been demonstrated that the dietary manipulations can evoke prominent changes in neurotransmitter function by affecting the membrane lipids composition in which receptors and cell signaling molecules are embedded (Gudbjarnason and Benediktsdóttir 1996), and thus can affect different brain regions and neurotransmitter systems. Parathion induced differential effects on the ACh measures in the various brain regions but the consumption of HFD in adulthood ameliorated many of the long-term ACh synaptic abnormalities evoked by such exposure. By this way, the exposure to developmental neurotoxicants could induce a later subconscious preference for HFD as a way of reversing the adverse effects, thus consuming an unhealthy diet and increasing the incidence of obesity and diabetes (Slotkin et al. 2009).

Chapter 3: Respiratory system

Breathing is the flow of air in and out of the mouth or nose. It serves the purpose of respiration defined as the exchange of oxygen and carbon dioxide. Respiration is an autonomic and continuous process during the whole life. The respiratory rhythmic activity is driven by the CNS. In this chapter, the cholinergic control of the respiratory system and associated disorders will be discussed. In addition, the contractility of respiratory skeletal muscles, particularly the diaphragm will be described.

3.1. Breathing and cholinergic control / central respiratory control

The initiation of respiratory rhythm and respiratory depth, collectively called central ventilatory drive, is mediated by multiple biochemical and biophysical processes in the CNS. These processes in the CNS are sensitive and responsive to any changes in oxygen or carbon dioxide tension and hydrogen ion concentration (Burton and Kazemi 2000).

Neurotransmitters can affect both the central ventilator drive and neural function. Acetylcholine is an essential neurotransmitter for the neural control of respiration. Cholinergic neurons located throughout the CNS and peripheral nervous system (PNS) are involved in several functional roles in the control of respiration. Cholinergic neurons of the medulla are involved in CO₂ chemosensitivity. Peripheral cholinergic receptors in the carotid bodies are sensitive to changes in oxygen levels. Peripheral muscle mechanoreceptors and pulmonary mechanoreceptors transmit the signal to the brainstem through cholinergic pathways (reviewed in Carey, Dunn, and Gaspari 2013). Disruptions in the central cholinergic system are associated with some neurological disorders related to the respiratory control such as sudden infant death syndrome (SIDS) and sleep apnea (Bellingham and Funk 2000; Kinney et al. 1995; Gilman et al. 2003).

3.2. Respiratory Parameters

Pulmonary ventilation is the act of breathing described by the process that causes air to enter (inspiration) and leave the lungs. A respiratory cycle is one series of inspiration and expiration. The diaphragm and the external intercostal muscles are the two major muscle groups involved during normal inspiration. The contraction of the diaphragm creates a larger thoracic cavity. Contraction of the external intercostal muscles expands the rib cage thus increasing the volume of the thoracic cavity. As a result, the decrease in intra-alveolar pressure creates a pressure gradient which drives air into the lungs. Normal expiration is a passive process. Due to its elasticity, the lungs recoil, and the diaphragm and intercostal muscles relax. The decrease in the volume of the thoracic cavity and lungs induce increased inter-pulmonary pressure which causes air to leave the lungs.

The respiratory center in the medulla oblongata of the brain can control the respiratory rate by responding to any changes of carbon dioxide (CO₂), oxygen and pH levels in the blood. Tidal volume is defined as the volume of air that normally enters the lungs during breathing ("22.3 The Process of Breathing – Anatomy and Physiology" 2013). Minute ventilation is the product between tidal volume and respiratory rate over one minute (Sharma and Goodwin 2006).

3.3. The sleep apnea

Sleep apnea and sleep-disordered breathing are common disorders among adults and are usually linked to other comorbid conditions (J. Lin and Suurna 2018). Sleep apnea syndrome (SAS) is manifested by repetitive episodes of cessation of breathing during sleep (sleep apnea), leading to sleep disruption and hypoxia (Banno and Kryger 2007). In humans, apnea is the cessation of airflow for at least 10 s (Malhotra and White 2002). SAS is associated with adverse consequences including neurocognitive dysfunction, daytime sleepiness, metabolic disturbances, and impaired quality of life (Banno and Kryger 2007).

There are three types of apneas:

 Obstructive sleep apnea (OSA) is characterized by complete (apnea) or partial (hypopnea) repetitive collapse or narrowing of the pharyngeal airway during sleep despite ongoing breathing efforts (Eckert and Malhotra 2008). The obstruction of the upper airway can be due to abnormal anatomy and/or abnormal control of the muscles that sustain the upper airway patency. Episodes of hypoxemia and increased arterial PCO_2 can occur as a result of reduced ventilation during sleep.

- Central sleep apnea is characterized by cessation of breathing caused by dysfunction of ventilatory control in the CNS. Central sleep apneas cause hypoxemia and frequent arousals and daytime sleepiness (reviewed in Banno and Kryger 2007).
- Mixed apnea consists of central pauses followed by obstructed respiratory efforts (Milner and Greenough 2004; Mathew, Roberts, and Thach 1982).

In rats, sleep apnea is defined as the cessation of respiratory effort for at least 2.5 s reflecting at least two "missed" breaths (Carley and Radulovacki 1999; Christon et al. 1996).

Obesity is the major epidemiological risk factor for OSA (Eckert and Malhotra 2008). Other factors include male gender and increasing age. Obesity can result in airway narrowing due to excess of fat tissue around the neck. Neck circumference corrected for height is considered as a predictor for OSA (Davies, Ali, and Stradling 1992). Several studies on obese populations have found an association between metabolic syndromes (including insulin resistance, glucose intolerance, and the associated diabetes) and sleep apnea (Strohl et al. 1994; Ip et al. 2000; Strohl and Redline 1996). Independent from obesity, OSA can cause insulin resistance which can result in turn in the development of type 2 diabetes mellitus (Vgontzas, Bixler, and Chrousos 2005).

Snoring is among the most prominent complaints of OSA. Other subjective symptoms include Daytime sleepiness and its adverse consequences on cognitive function, memory, and work performance. The reduced pharyngeal intraluminal pressure generated by diaphragmatic contraction is further decreased in patients with OSA as a result of increased respiratory efforts needed to sustain the flow due to collapsed pharyngeal passage (reviewed in Banno and Kryger 2007).

3.4. Respiratory Skeletal Muscle

Respiratory muscles are similar to other skeletal muscles in terms of function and structure. Respiratory muscles sustain breathing through gas exchange by supplying O2

and taking out CO2 from the bloodstream. Pump and upper airway muscles are two major types of respiratory muscles. Pump muscles move air into the lungs during ventilation including the diaphragm; the major muscle that is unique to mammals. Like other skeletal muscles, respiratory muscles are plastic and adapt to varying pathological and environmental conditions (Gransee, Mantilla, and Sieck 2012).

3.5. Skeletal Muscles: function and structure

Skeletal muscle represents around 40% of total body mass. It provides positional change, locomotion, and the exchange of respiratory gases. In order to perform such tasks, these muscles have a distinct structure, neural control, and fiber type composition.

Each skeletal muscle consists of multinucleated cells called fibers. The striated appearance is attributed to the highly organized thin and thick filaments within myofibrils that are arranged in turn within the muscle fiber. Myofibrils consist of polymerized protein molecules including myosin and actin that are responsible for muscle contraction. The functional unit of the myofibril is the sarcomere. Thick filaments (myosin) and thin filaments (actin and the regulatory proteins tropomyosin and troponin) within the sarcomere are anchored to the Z-disc. Each myofibril in the muscle fiber is surrounded by sarcoplasmic reticulum (SR), an intracellular membrane network that regulates Ca²⁺ concentration. Transverse tubular structures (T-tubules) are deep invaginations of the plasma membrane that mediate depolarization. The ability of a muscle fiber to generate force is primarily attributed to its myofibrillar protein composition, particularly myosin isoform composition (reviewed in Gransee, Mantilla, and Sieck 2012).

3.6. Excitation-Contraction coupling mechanism:

Synapses are the cellular determinants of neural circuitry, and thus essential to the function of the nervous system. The presence of postsynaptic neurotransmitter receptors represents a basic feature of the chemical synapse. So the assemblage of acetylcholine receptors (AChRs) at postsynaptic sites of the skeletal neuromuscular junction (NMJ) is the best studied in this respect (W. Lin et al. 2001).

Calcium (Ca^{2+}) is essential for the initiation of muscle contraction at the neuromuscular junction (Gehlert, Bloch, and Suhr 2015). As the action potential arrives to the presynaptic neuron terminals, voltage-dependent Ca²⁺ channels open and trigger the Ca²⁺ influx from the extracellular space into the presynaptic neural cytosol (Figure 9) (Zucker 1993). Then, ACh containing vesicles fuse with the presynaptic membrane and release ACh into the synaptic cleft (Sine 2012). ACh then binds to the nicotinic acetylcholine receptors (nAChRs), which serve as ion channels, located at the skeletal muscle cell membrane (sarcolemma) (Zucker 1993). The activated and opened ion channels lead to sodium (Na⁺) and potassium (K⁺) influx, causing a local depolarization of the sarcolemma. This depolarization can propagate across the sarcolemma reaching the T-tubules located in contact to the terminal cisternae of the SR (Capes, Loaiza, and Valdivia 2011), resulting in a voltage-dependent release of Ca²⁺ ions from the SR through the ryanodine receptors (RyRs) into the cytoplasm (Gehlert, Bloch, and Suhr 2015). Elevated Ca²⁺ levels induce a change in the positions of troponin and tropomyosin, and thus unmask the binding sites on actin molecules. As a result, myosin heads bind to actin and use energy stored in adenosine triphosphate (ATP) to create force for sarcomere shortening (Bertorini 2008), thus a crossbridge cycling initiates in myofibrillar compartments (Mense and Gerwin 2010). Relaxation is mediated by Ca²⁺ reuptake by the SR through the SR Ca²⁺ adenosine triphosphatase (SERCA) (Calderón, Bolaños, and Caputo 2014).



Figure 9: Architecture and Contraction of muscle fiber at the neuromuscular junction NMJ: (A and B) The NMJ is composed of three elements: pre-synaptic (motor nerve terminal), synaptic basal lamina, and post-synaptic component muscle fiber and muscle membrane (C) Calcium release from the sarcoplasmic reticulum allows cross-bridge cycling and force production (Gonzalez-Freire et al. 2014)

3.7. Neuromotor control of the skeletal muscle:

The central nervous system controls the skeletal muscle. The motor centers in the brain control the activity of motoneurons of the spinal cord which synapse with skeletal muscle fibers. A single motoneurons innervates each skeletal muscle fiber. The group of muscle fibers in the motor unit is activated synchronously by an action potential (Gransee, Mantilla, and Sieck 2012).

i. Motor unit classification:

Motor units are classified functionally depending on their mechanical and metabolic properties. The velocity of shortening is determined by the myosin heavy chain (MHC) isoform, in which motor units can be classified into slow-twitch and fast-twitch motor units. Slow-twitch (S) motor units are recruited during tasks with highly precise

movements with low power or force output. However, Fast-twitch motor units are recruited when high power output is required.

Fatigue resistance is identified in terms of muscle fibers ability to maintain energy balance in the form of ATP. Based on the mechanical and fatigue properties of the innervated muscle fibers, motor units are classified into 4 groups: slow-twitch, fatigue resistant (type S); fast-twitch, fatigue resistant (type FR); fast-twitch, fatigue intermediate (type FInt); and fast-twitch, fatigable (type FF) (Lucio G. Costa 2006; Gransee, Mantilla, and Sieck 2012).

ii. Classification of Muscle fibers:

Muscle fibers can be classified according to 3 different methods: for myosin ATPase histochemical staining, biochemical identification of metabolic enzymes, and MHC identification (W. Scott, Stevens, and Binder–Macleod 2001). Based on the myosin ATPase staining, muscle fibers are classified as type I (slow-twitch) or type II (fast-twitch) muscle fibers. By further analyzing the enzymes that reflect the metabolic pathways, 3 fiber types are classified: Slow-twitch oxidative (SO), Fast-twitch oxidative glycolytic (FOG), and Fast-twitch glycolytic (FG). Muscle fiber type is now classified based on myosin heavy chain (MyHC) isoform expression using antibodies against MyHC isoforms or by sodium dodecyl sulfate–polyacrylamide gel electrophoretic (SDS-PAGE) into 4 types: 1) type I (expressing MyHC I); 2) type IIa (expressing MyHC IIA); 3) type IIb (expressing MyHC IIB), 4) type IIx (expressing MyHC IIX) (reviewed in Gransee, Mantilla, and Sieck 2012) (Figure 10). Skeletal muscle is plastic which is able to adapt to varying physiological conditions and can have fibers with different myosin isoforms. Furthermore, MHC isoforms can change in response to different types of stimulations such as physical training, electrical stimulation, hormonal changes and environmental factors (Eddinger 1998).

MyHC type	Twitch duration	Shortening velocity	Cross-sectional area	Metabolism	Endurance	Energy efficiency
I.	Slow	Slow	Small	Oxidative	High	High
lla						
llx		-				
llb	Fast	Fast	Large	Glycolytic	Low	Low

Figure 10: Muscle fiber types properties in mammalian skeletal muscle (Gundersen 2011)

3.8. The Diaphragm:

The diaphragm is a thin, dome-shaped sheet of muscle that is inserted into the lower ribs and acts as a septum separating the thoracic (pleural) and abdominal (peritoneal) compartments of the body (Gransee, Mantilla, and Sieck 2012). The diaphragm contains two domains. The costal domain is a thin muscle containing a radial array of myofibers that extend from the ribs toward the central tendon. The crural domain is a posteriorly thicker muscle attached to the vertebrae (Pearce 2009). The left and right phrenic motor nerves originating from the cervical nerves, C3-C5, innervate the left and right halves of the diaphragm respectively (Stuelsatz et al. 2012; Allan and Greer 1997).

The diaphragm is a respiratory skeletal muscle involved in breathing under voluntary and neural control (Rochester 1985). In addition to its major role in respiration, the diaphragm, and especially the crural domain, play a fundamental role in emesis and swallowing (Pickering and Jones 2002). The muscle fiber composition of the diaphragm is well suited to its continuous rhythmic activity which does not allow any pause (Polla et al. 2004). Thus, diaphragm fibers should be very resistant to fatigue. Around 55% of the fibers in the adult human diaphragm are slow twitch oxidative (type I) and about 25% are of the fast twitch oxidative glycolytic (type IIa). 20% of the fibers are fast glycolytic (type IIx) (Faulkner et al. 1979). Moderate inspiratory breathing levels for a short period has been shown to increase the expression of slow MHC isoform mRNA in the diaphragm, suggesting a phenotypic adaptation of respiratory muscles (Gea et al. 2000).

3.9. Effects of pesticides on respiration:

i. Effects of pesticides on the respiratory parameters and the occurrence of sleep apnea:

Subcutaneous injections with the organophosphate pesticide, paraoxon, have been shown to alter the respiratory pattern in rats by decreasing the respiratory frequency and increasing the expiratory time and tidal volume without any effect on the inspiratory time (Villa et al. 2007). Atropine, a muscarinic antagonist, has been shown to reverse the low dose induced effects of paraoxon on respiration, thus confirming that these effects are related to a central cholinergic mechanism involving muscarinic receptors (mAChRs) in respiratory centers (Houze et al. 2008). Recently, Darwiche et al. (2018) have reported increased expiratory time and tidal volume in adult rats that are pre and postnatally exposed to CPF. The respiratory alteration is the result of overstimulated muscarinic receptors in the CNS. In addition, CPF exposure induced an increase in the sleep apnea index defined as the number of apnea episodes during an hour of behavioral sleep. On the other hand, acute OPs exposure is associated with the occurrence of central apnea episodes due to the inhibition of AChE in the respiratory centers (Carey, Dunn, and Gaspari 2013; Gaspari and Paydarfar 2011). A peripheral effect can induce apnea as a result of airway obstruction caused by OPs exposure (Proskocil et al. 2010).

ii. Effects of pesticides on the contractility of the diaphragm:

Like other skeletal muscles, force generation of the diaphragm depends mainly on the hydrolysis or elimination of the released ACh into the synaptic cleft. Thus the inhibition of AChE by OPs can induce effects on the contraction.

In vitro administration of ecothiopate, an anticholinesterase drug resulted in a prolonged contraction of the diaphragm following phrenic nerve stimulation. The contraction force, time-to-peak, and half-relaxation time were increased and indicated as enhancement of twitch as a result of prolonged action of ACh at the synaptic cleft (Burd and Ferry 1987). However, Thiermann, Eyer, and Worek (2010) have revealed a reduced force of contraction after *in vitro* exposure to paraoxon in a phrenic nerve-diaphragm model. Pre and postnatal

exposure to CPF induced an increase in the contractile force of the diaphragm in rats associated with increased fatigability as a result of decreased AChE activity (Darwiche et al. 2018).

3.10. Effects of obesity and HFD on respiration and diaphragm contractility

Overweight and obesity are considered as a prominent risk factor for many respiratory diseases, particularly obstructive sleep apnea. Appropriate pulmonary function necessitates harmonic processing of the structures that makes the respiratory system. In obese people, structural alterations of the thoracic-abdominal region result in limited diaphragm mobility and rib movement, which are fundamental for ventilatory mechanics. In addition, adipose tissues can produce a high amount of cytokines and bioactive mediators, generating a pro-inflammatory state which is in turn linked to hypodevelopment of the lungs, bronchial responsiveness, and increased risk of asthma (reviewed in Melo, Silva, and Calles 2014). Indeed, HFD feeding has been shown to exacerbate the increased respiratory frequency and reduce the expiratory time during chemoreflex activation by CO₂ in rats (Speretta et al. 2018).

The risk of OSA development increases at higher BMI (Lettieri, Eliasson, and Greenburg 2008). Other contributing factors include a large neck circumference and anatomically narrow upper airway (UA) (Isono 2009; Schäfer et al. 2002). The increased fat deposition around neck and tongue tissues results in the increase of the extra-luminal pressures in the pharynx which may lead to airway collapse (Patil et al. 2007). In this context, Ramadan et al. (2006, 2007) have reported an increase in sleep apnea index associated with metabolic disorders in rats fed with HFD despite the absence of obesity. Recently, Bove et al. (2018) have revealed that the severity of sleep apnea is correlated with the high intake of saturated fats in overweight patients.

The effects of HFD-induced obesity on diaphragm contractile performance have been established in several studies. In an isolated muscle work-loop (WL) model, high-calorie feeding for 16 weeks induced a decrease in the isometric stress and normalized WL power

of the diaphragm in mice, suggesting a reduction in muscle quality (Tallis et al. 2016). Hurst et al. (2019) have shown a reduction in the normalized isometric force of the diaphragm in rats fed a HFD for 8 or 12 weeks. The decline in contractility independent of feeding duration is attributed to the accumulation of body fat. Obesity can induce force and power output changes in muscles by affecting fiber type composition and altering calcium cycling (Funai et al. 2016; Seebacher et al. 2017).

- Thus, respiration is a continuous rhythm essential throughout life. Disruptions in the cholinergic control disruptions are consequently implicated in perturbed respiratory performance. As described in this chapter, the exposure to either pesticides or HFD is associated with altered respiratory function, increased the risk of sleep apnea and affected the contractile activity of a prominent respiratory muscle; the diaphragm. Accordingly, one of the primary aims of this study is to investigate the impacts of early-life disturbances mediated by the perigestational exposure to CPF and/or HFD on the respiratory performance and the contractile function of the diaphragm at early adulthood. It is also critical to determine the effects of repeated CPF exposure at adulthood on the contractile performance of the diaphragm.

Chapter 4: The digestive system

The digestive system consists of the gastrointestinal (GI) tract in addition to the liver, pancreas, and gallbladder. The digestion and absorption of the nutrients, which is essential for survival, is considered a critical task of the GI system. Other functions include excretion and protection. The GI tract is made up hollow organs which are the mouth, esophagus, stomach, small intestine, large intestine including the rectum and the anus. The ingested solids and liquids move from the esophagus into the stomach through peristaltic contractions. The peristaltic movements of the small intestine maximize the absorption of protein, fat, and carbohydrates.

The wall of GI organs can be divided into four overlapping layers from the lumen to the outside: 1) the mucosa surrounded by an absorptive and secretory epithelium; 2) the submucosa which contains mainly conjonctive tissue, immune cells, blood vessels and nerve fibers of the enteric nervous system (ENS) organized in the submucosa plexus of Meissner; 3) the longitudinal and circular muscle layers containing the other part of ENS with the Auerbach's myenteric plexus, and the interstitial cells of Cajal (ICCs) which control the contractility performance (K. L. Koch, Bitar, and Fortunato 2012); 4) the serosa which is the external layer of the intestine. In this chapter, the function and neural control of the small intestine will be described.

4.1. Neuromuscular function of the intestine

The gut is considered as a muscular tube for food ingestion, nutrients assimilation and waste elimination (K. M. Sanders 2008). The small intestine, a hollow neuromuscular tube, is made up of the duodenum, jejunum, and ileum (Jones and Bratten 2008). Chyme is broken down into the essential nutrients within the small intestine. Normal digestion and absorption of nutrients require normal intestinal motility in addition to pancreatic enzymes and bile secretions from the gallbladder. The ileum plays an important role in bile salts absorption (Dawson, Lan, and Rao 2009). The ileocecal valve mediates the emptying of ileal effluent to the cecum and prevents the backward reflux into the ileum. Normal small intestinal motility requires strong peristaltic contractions moving distally from the

duodenum reaching the ileum in a cycle manner every 90 minutes during fasting (S. M. Scott et al. 2006). Such contractions mediate the transport of indigestible fibers from the small intestine into the colon.

According to Bayliss and Starling (1899), the small intestine accomplishes two types of movements. Pendulum movements are rhythmic contractions affecting circular and longitudinal muscles simultaneously. Peristaltic contractions are coordinated downward unidirectional reflexes, initiated by the mechanical stimulation of the intestine and mediated by the local nervous mechanism (Auerbach's plexus) independent of the connections from the CNS. The production of the peristaltic wave is subjected to the law of the intestine: Local stimulation of the gut produces excitation above and inhibition below the exciting area. The intestinal activity of the muscles depends on the relative influence of the excitatory and inhibitory impulses.

Smooth muscles of the GI tract exhibit autonomous behavior and they also receive parallel regulatory inputs from multiple control systems, including ICCs, motor neurons, hormones, and inflammatory mediators resulting in highly integrated responses. ICCs are electrically coupled to the smooth muscle cells which provide spontaneous pacemaker activity in gastrointestinal muscles (K. M. Sanders 2008). Both circular and longitudinal muscle layers participate in the peristaltic movements. Contraction of the longitudinal muscles shortens the distance for chyme and stiffens the wall of the conduit. At the wave front the circular muscle contracts whereas the longitudinal muscle relax (Feher 2012) (Figure 11).



Figure 11: Factors involved in the regulation of gastrointestinal motility (Kenton M. Sanders et al. 2012)

4.2. Nervous System Control

In addition to the intrinsic neural plexuses of the ENS which provide a degree of autonomical function for the GI tract, the extrinsic neural inputs provided by the CNS, control and regulate these functions. The small and large intestines show of independent neural control ability and thus are able to function in without extrinsic neural inputs (Browning and Travagli 2014).

i. The CNS control: sympathetic and parasympathetic pathways:

The autonomic nervous system connects the GI tract to the CNS through the brain-gut axis (Figure 12). The CNS can regulate the motor activity through inputs mediated by the components of the extrinsic autonomic nervous system: the parasympathetic and the sympathetic nervous system (M. B. Hansen 2003). The vagus nerve and splanchnic nerves supply the stomach and upper intestine, while distal intestines are innervated by the pelvic nerves.

The parasympathetic and sympathetic extrinsic neurons can affect smooth muscles by acting on the myenteric plexus neurons. Thus, an electrical syncytium, innervated by multiple inhibitory and excitatory neurons, is formed in the smooth muscle cells (Kunze and Furness 1999; Olson 2008). The sympathetic nervous system exerts mainly an inhibitory effect on GI muscle and mucosal secretion and the blood flow in the GI tract. However, the parasympathetic nervous system can exert excitatory effects on the intestinal motility (Browning and Travagli 2014).

Many factors can affect the activity of the autonomic nervous system such as emotion, stress, and eating. After distension and inflammation, nociception is mediated by the release of different tachykinins and neurokinins (Gué and Buéno 1996).



Figure 12: Control of intestinal motor activity ("Chap-10_fondamentaux-Pathologie-Digestive_octobre-2014.Pdf")

ii. The enteric nervous system

The enteric nervous system is a large and complicated part of the PNS and autonomic nervous system (ANS) in vertebrates. It provides the GI tract with intrinsic innervation and contains ganglia, different types of neurons, glial cells, and an array of neuromodulators and neurotransmitters resembling those of the CNS. This interconnected network is distributed in two intramuscular plexuses; myenteric (Auerbach) plexus and the submucous (Meissner) plexus; involved in the control of smooth muscles contractile activity, intestine motility, flow of blood, and gut hormones secretions (Figure 13).

Despite several extrinsic sympathetic and parasympathetic innervation, the integrated neuronal circuits of the ENS can produce reflex contractile activity in the gut independent from the interventions of the CNS (Furness 2006; Sasselli, Pachnis, and Burns 2012).





The myenteric plexus and the submucous plexus:

The myenteric plexus is placed between the longitudinal and circular muscle layers throughout the GI tract, innervating these muscle layers. The submucous plexus is positioned in the submucosa between the layer of the circular muscle and the mucosa (Mark Berner Hansen 2003).

The neurons of the myenteric plexus are involved mainly in the regulation of muscle activity. In the stomach, the nerve cell circuits control the secretion of acids (gastric juice). The submucosal plexus is most prominent in the intestine. The submucosal nerves innervate mainly the mucosa and are involved in several mucosal functions including the regulation of ion secretion, the transport of transmucosal fluid, and supply blood to the mucosa. More than 30 neurotransmitters have been identified in the regulation of the neurotransmission along the ENS (Harrington, Hutson, and Southwell 2010).

- Neurotransmission of the enteric nervous system:

The muscle motor neurons are classified as either excitatory or inhibitory according to the released neurotransmitters which mediate contraction or relaxation of muscles.

a. Excitatory neurotransmitters:

- Cholinergic Neurotransmitters

Acetylcholine is the primary cholinergic neurotransmitter for excitatory motor neurons. It binds muscarinic receptors and thus depolarizes the muscles. Acetylcholine is synthesizedby choline acetyltransferase, acetylcholine synthesizing enzyme, in the enteric neurons. Extrinsic or intrinsic nerves stimulation at different frequencies resulted in contractions similar to those observed for acetylcholine application. These contractions are enhanced by AchE and abolished by muscarinic antagonists (McConalogue and Furness 1994).

- Non-cholinergic Neurotransmitters

Since the excitatory transmissions are only partially blocked by the muscarinic receptors, then a role of non-cholinergic transmission has been suggested (Ambache and Freeman 1968). Non-cholinergic transmissions are mediated mainly by tachykinins, a family of neuropeptides, including substance P, neurokinin A (NKA), and neurokinin B (NKB), neuropeptide γ (NP γ), and neuropeptide K (NPK). Substance P has been shown to promote the contractility of both longitudinal and circular muscles (Souquet et al. 1985) by inducing direct and indirect effects on muscles and excitatory neurons (Katayama, North, and Williams 1979). Other neurotransmitters include serotonin (5-hydroxytryptamine, 5-HT) and opioids.

b. Inhibitory neurotransmitters:

Accommodated inhibitory reflexes are mediated by several inhibitory transmitters including nitric oxide (NO), Adenosine triphosphate (ATP), vasoactive intestinal polypeptide (VIP), GABA, noradrenaline, and carbon monoxide (CO) (McConalogue and Furness 1994).

4.4. The interstitial cells of Cajal:

The interstitial cells of Cajal (ICC) are pacemaker regions of the slow waves placed at the submucous and myenteric borders of the circular muscles. ICC are muscle-like cells placed next to each other and in contact with other muscle cells and nerve fibers which initiate the rhythmic activity in the gastrointestinal muscles. Defects or loss in ICC is associated with GI motility disruptions (Huizinga, Robinson, and Thomsen 2000; Camilleri 2001).

4.4. Regulation of smooth muscle contraction:

The contraction of the smooth muscle cell is regulated mainly by receptor and mechanical activation of myosin and actin contractile proteins. Different agonists (such as hormones, neurotransmitters) bind to specific receptors and activate smooth muscle contraction (Webb 2003). The contraction is regulated by the phosphorylation of the myosin light chain subunits (MLC20) (Somlyo and Somlyo 2003), mediated either by Ca²⁺/ calmodulin-dependent actions of myosin light chain kinase (MLCK) or by the Ca²⁺-independent actions of different kinases, such as Rho-kinase, zipper-interacting protein kinase (ZIPK), and integrin-linked kinase (ILK) (K. M. Sanders 2008).

The initiation of contraction depends mainly on MLCK activation mediated by elevated Ca²⁺ concentration through the Ca²⁺ release from intracellular stores (SR) and from extracellular space by Ca²⁺ channels (receptor-operated Ca²⁺ channels) (Webb 2003).



Figure 14: Major pathways involved in excitation-contraction coupling in GI smooth muscles (Webb 2003)

4.5. Effects of Pesticides on the gastrointestinal tract

Following oral exposure to pesticides, which is the primary route of exposure to the general population, the gastrointestinal tract is considered the first compartment in contact with these contaminants. The activation of CPF to its oxon metabolite is mediated by CYP 3A4, 2B6 in humans and 2B1/2 in rats mainly within the liver (Poet et al. 2003). The enterocytes of the small intestine (duodenum and jejunum/ileum) are capable of CPF bioactivation but with lower efficiency than the hepatic metabolism (Leoni et al. 2012; C. Timchalk et al. 2002).

The exposure to sublethal doses of the organophosphate diazinon has shown to induce histological changes in the intestine in a dose-dependent manner. Submucosal hemorrhage, erosion in the epithelium of the small intestine, and lymphocytes aggregation have been observed especially in the duodenum (Rady 2009). Furthermore, Diazinon resulted in higher ileal contractions following electrical field stimulation in rats at the 14th day of exposure followed by a decrease in the ileal contractility on the 28th day. The tolerance to Diazinon administration is attributed to different adaptation mechanisms including downregulation of muscarinic and nicotinic receptors (Ivanović et al. 2016).

Several studies have examined the effects of CPF exposure on the intestinal function. CPF exposure induced a decrease in the contractility of the ileum and a strong decrease in the maximum effect of acetylcholine, as a result, reduced activity of the receptors (Cetinkaya and Baydan 2010). Recently Darwiche et al. (2017) have reported a decrease in the contractility of the longitudinal smooth muscle strips in the ileum in rats exposed pre- and postnatally to CPF through a nitrergic dependent mechanism involving increased inducible nitric oxide synthase expression. The reduced amplitude of contraction was associated with lower thickness of longitudinal ileal muscle. Moreover, altered epithelial barrier function associated with changes in the intestinal tight junctions' protein expression and localization has been also observed in rats exposed in utero and after birth to CPF. Consequently, the intestinal epithelial dysfunction was associated, in turn, with increased intestinal permeability and elevated bacterial translocation to the spleen (Joly Condette et al. 2014).

4.6. Effects of obesity and HFD on the gastrointestinal tract

Several studies on humans and animal models have shown that HFD consumption can promote endotoxemia (Cani et al. 2007; Erridge et al. 2007) characterized by increased lipopolysaccharide (LPS), the primary component of the Gram-negative bacterial cell wall, in blood (Ghanim et al. 2010) associated with metabolic disorders and low-grade systemic inflammation. Fat enriched meals induced an increase in bacterial LPS in mice (Cani et al. 2007) and humans (Erridge et al. 2007). HFD feeding in mice resulted in glucose imbalance, inflammation, and endotoxemia (Cani et al. 2008). Thus HFD contributes to the increase in blood LPS levels by several mechanisms including the formation of excessive chylomicrons needed for LPS transport and thus increased hepatic exposure to LPS in case of chylomicronaemia (Ghoshal et al. 2009) and by inducing changes in the composition of gut microbiota (Cani et al. 2007). High fat intake can affect the paracellular and transcellular intestinal permeability of the intestine through mast cells mediated by the secretion of inflammatory mediators (Keita and Söderholm 2010). In addition, hypercaloric diet consumption in rats was associated with hypertrophy of the longitudinal smooth muscle of the ileum and consequent reduction in the ileum contractility (de Souza et al. 2018). - Hence, OPs exposure or HFD intake has been shown to alter the gastrointestinal function and affect the ileal muscle contractility. Then, in this study, it is critical to examine the longterm effects of maternal exposure to a low dose of CPF associated with HFD feeding, before and throughout gestation and during lactation, on the contractile activity of the ileal smooth muscle as being essential for homeostasis maintenance and proper nutrients absorption.

MATERIALS AND METHODS

1. Study 1: Adult rats Exposure

1.1. Experimental Procedure

i. Chlorpyrifos preparation

Chlorpyrifos was used in powder form in 250 mg aliquots (99.8% pure; LGC Standards SARL, Molsheim, France) and dissolved in corn oil (as a vehicle) to prepare a stock solution of 10 mg/ml. CPF was administered at two doses: 1 mg/ml for CPF1 group or 5 mg/ml for CPF5 group. The oral dose was normalized to 1 ml/kg body weight of the rat. These chosen doses, 1 mg/kg/day and 5 mg/kg/day correspond to the NOAEL for the depression of cholinesterase activity in the brain and developmental the toxicity level in rats respectively (Maurissen et al. 2000). CPF solutions were kept at room temperature in dark.

ii. Exposure of rats to CPF and Animals Sacrifice

All animal experimentations in this study were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. 30 adult male Sprague-Dawley rats (6 weeks old; mean body weight 200 ± 10 g) were randomly divided into the 3 groups (10 rats/ group): control, CPF1, and CPF5.

Following one week of acclimation, each rat was individually housed in a plastic cage covered with a stainless steel grid, in ventilation standard conditions of light (12:12 hours alternating light: dark cycle) and temperature (23°C). The rats had access to fresh water ad libitum. Animals in CPF1 and CPF5 groups were fed daily a standard food pellet diet (20 g/day/rat) mixed with either 1 or 5 mg CPF/kg body weight/day respectively for 6 consecutive weeks. The CPF concentration in the diets was monitored weekly based on weekly body weight to maintain the targeted dose levels on an mg/kg-day basis. The rats in the control group were fed the same (pellet diet) containing the vehicle (corn oil) of 1 ml/kg/day (table 5).

	Control	CPF1	CPF5
Rats	n=10	n=10	n=10

After the 6-weeks exposure period, the rats were euthanized with an intraperitoneal overdose of sodium pentobarbital (1 ml/kg; 200 mg/ml solution). The diaphragm and adjacent rib cage were rapidly removed and placed in a petri dish containing an oxygenated (95% $O_2/5\%$ CO₂) pH= 7.4 Ringer solution (140 mM NaCl, 6 mM KCl, 5 mM HEPES, 3 mM CaCl₂) (Sigma Aldrich, Saint Quentin Fallavier, France). The contractile properties of the diaphragm were studied in rectangular strips of the costal diaphragm in which the rib margin and central tendon remained intact. Other parts were preserved at - 80°C for further protein quantification experiments.

1.2. Measurement of muscle contractile properties

The diaphragm strips were suspended vertically in a muscle bath containing oxygenated Ringer solution kept at 37°C by a stream of heated water passing through an external enclosure surrounding the bath. The rib margin was anchored to the base of the plexiglass bath and the central tendon of the diaphragm was attached to an isometric force transducer. The diaphragm muscle strip was positioned between two silver wire electrodes parallel to its axis. The force transducer output was fed to an isometric force sensor (PIODEN CONTROLS LTD, United Kingdom, accuracy ± 10mg) having a maximum response of 25 mg and controlled by data acquisition system (Digidata 1200, Axon Instruments) for further analysis (Figure 15).



Figure 15: Isolated organ system used to study the *in vitro* contractile properties of muscles

Muscles were stimulated directly using supra-maximal electric current pulses of 2 ms duration and the voltage was adjusted to induce supramaximal stimulation (12 V). Diaphragm strip length was stretched to reach the maximum length L₀, defined as the optimal length at which maximal force was reached. The subsequent measurements were made at L₀. The cross-sectional area (CSA) of each muscle strip was calculated according to the following formula (CSA=M/ ρ x L₀) where m is the muscle mass (in g) and ρ is the muscle density (ρ = 1.056 g.cm⁻³) (McGuire and MacDermott 1998).

i. Twitch measurements

Single twitches were elicited following equilibration. For each twitch, the peak twitch tension (measurement between baseline and peak force) was normalized by cross sectional area and presented (in g.cm⁻²), time to peak tension (time to peak force (in ms)) and half-relaxation time (time for the force to decrease to half of its peak value (in ms)) were determined. Then, the mean values for each strip were calculated.



Figure 16: Contractile parameters of the diaphragm muscle: peak twitch tension, time to peak tension, and half-relaxation time

ii. Tetanic contraction

The maximum tetanic tension was produced by 1 s duration trains of stimuli (20 Hz). The amplitude between the baseline and the steady part of the tetanic plateau was measured and the values were standardized for cross sectional area.



Figure 17: Tetanic contraction

iii. Fatigability Index

The fatigue resistance was examined by applying a protocol of 2 Hz train of supramaximal stimuli during 5 minutes. The fatigability index was calculated according to the following formula: FI = (Initial Force) - (Final Force) * 100 / (Initial Force)



Figure 18: Measurement of the fatigability Index of the diaphragm

1.3. Biochemical Assessment

The frozen diaphragm muscles were homogenized with 5 volumes of washing buffer containing 20 mM NaCl, 1 mM EGTA (pH 6.4), and 5 mM PO4 and then centrifuged (12000 rpm for 5 min). Next, myofibrillar proteins were extracted with 3 volumes of extraction buffer containing 5 mM EGTA, 1mM di-thiothreitol (pH 8.5) and 100 mM sodium pyrophosphate, incubated overnight at 4°C with gentle mixing, and then centrifuged

(12000 rpm, 15 min). The supernatant containing the myofibrillar proteins was collected for protein dosage. The myofibrillar protein content was determined spectrophotometrically at 595 nm according to Bradford assay using Bovine Serum Albumin (BSA) as standard (Bio-Rad, Hercules, CA, USA) (Bradford 1976).

1.4. Separation of Myosin Heavy chain isoforms by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Simple vertical migration of SDS-PAGE was used electrophoretic separation of MHC isoforms (Joumaa and Léoty 2002). The separating and stacking gels were prepared according to the previously described protocol (Talmadge and Roy 1993). The samples were diluted with a buffer containing Tris 125mM (pH=6.8), glycerol 20%, β -mercaptoethanol 5%, EDTA 1 mM, SDS 5% (Sigma Aldrich, Saint Quentin Fallavier, France) and then boiled for 5 min. 2 µg of myofibrillar proteins were loaded into each well. Gels were run at constant voltage at 72 V for 28 h at 4-6°C. Next, the gels were stained with Coomassie Blue R solution and the bands corresponding to slow MHC I and fast MHC IIa and MHC IIb isoforms were identified. After scanning the gels, the relative expression of the different MHC isoforms was determined by Image J software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

1.5. Measurement of Acetylcholinesterase (AChE) activity

The AChE activity was determined by applying modified Ellman's method (Ellman et al. 1961), through measuring the amount of hydrolyzed ACh by the AChE enzyme each minute in the analyzed samples. The diaphragm tissues were homogenized in mammalian cell lysis buffer 5X (ab179835; Abcam, Cambridge, UK). After centrifugation, the supernatant was used for AChE activity measurement by the colorimetric one-step AChE assay kit (ab138871; Abcam). The AChE activity was determined by the colorimetric quantification of the thiocholine produced after the hydrolysis of acetylthiocholine. The intensity of absorption of DTNB (5,5'-Dithio-bis- (2-nitrobenzoic acid)) was measured with a microplate reader (ELx808; Biotek,Winooski, VT, USA) at 412 nm for 15 minutes and used

to determine the amount of thiocholine formed, proportional to the AChE activity. Then, the AChE activity was standardized for protein content and the values were presented in μ mol/min/mg protein.

1.6. Serum Cortisol, Testosterone, Growth Hormone levels

Cortisol, serum testosterone, and growth hormone levels were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) technique. It relies on the principle of competitive binding. The assay depends on an antibody specific for the measured coated on a 96-well plate. The antibody is labeled with an enzyme whose substrate is a chromogen converted in a measurable product spectrophotometrically. Cortisol ELISA Kit (K7430, BioVision, USA), Testosterone ELISA Kit (K7418, BioVision), and Growth Hormone ELISA kit (A05104, Bertin, France) were utilized for serum quantification of cortisol, testosterone, and growth hormone respectively according to the suppliers' instructions.

2. Study 2: Perigestational exposure to CPF and HFD

2.1. Experimental Procedure

i. Chlorpyrifos preparation

Chlorpyrifos was prepared as described above. CPF was dissolved in rapeseed oil (MP Biomedicals, Illkirch, France) as vehicle and administered to the rats at a dose of 1 mg/kg/day (CPF group).

ii. Experimental Design

The experimental protocol was approved by the nationally accredited Regional Directorate for Health, Animal and Environment Protection (Amiens, France) and the French Ministry of Research (reference number APAFIS#8207-2016121322563594 v2). All animals were treated in accordance with the European Communities Council's guidelines (2010/63/EU).
In this study, outbred albino Wistar rats (Janvier Labs, Genest Saint Isle, France) were exposed to CPF, and/or high-fat diet HFD (Research Diets no. D12492, SSNIFF Spezialdiäten GmbH, Figure 19) during the perigestational period.

Formula

Product #D12492		gm%	kcal%
Protein Carbohydrate Fat		26.2 26.3 34.9	20 20 60
	Total kcal/gm	5.24	100

Figure 19: High-fat diet formula

After 1 week of acclimatization, 16 female Wistar rats (average body weight 225 ± 4.9 g) were assigned randomly to 4 groups (n=4/group) and housed two per cage. The females were fed either standard chow diet (Serlab3436, 3.1 kcal/g) or high-fat diet HFD (5.24 kcal/g, 60% kcal from fat) and gavaged daily with organic rapeseed oil (as a vehicle) for control (oil, standard chow diet) and HFD (oil, HFD) groups or CPF (1 mg/kg/day dissolved in organic rapeseed oil) for CPF (CPF, standard chow diet) and HFD+CPF (CPF,HFD) groups for 4 consecutive months. At the end of these 4 months, the females were mated with male rats of an equivalent age (2 females with 1 male). To determine whether mating took place, the presence of spermatozoa was checked with vaginal smears in the morning of the following day. The smear was stained by a drop of diluted purple crystal to visualize the presence of the spermatozoa (Figure 20).



Figure 20: Positive vaginal smear observed under a microscope

During the gestational period, the females were exposed to the same conditions as before pregnancy. At birth, the pups were weighed, sexed, and marked with veterinary ink. The dams continued to receive the same treatment until the end of the lactation period. At weaning (PND 21) pups were separated from their mothers and the male pups were categorized into the following groups:

1) Control group: Maternal exposure to standard diet with vehicle (rapeseed oil) (n=10)

2) HFD group: Maternal exposure to HFD with vehicle (rapeseed oil) (n=10)

3) CPF group: Maternal exposure to standard diet with CPF (n=10)

4) HFD+CPF group: Maternal exposure to both HFD and CPF (n=7)

Then, at the end of lactation (PND 21), the rats in all groups received the standard chow diet without CPF until the PND60 (early adulthood).



Figure 21: Different stages of animal experimental protocol including the pre-gestation, gestation, and lactation periods

2.2. Study of the respiratory performance at early adulthood

i. Whole-body plethysmography:

The respiratory parameters for all male pups were measured via whole-body plethysmography (Model PLY3213, Buxco-EMKA Technologies, Sharon, CT) at PND60±2 (n=7-10/group) during 1 hour. These parameters include the inspiratory and the expiratory time (T_I and T_E respectively), tidal volume (V_T) (expressed per 100g body weight), and the respiratory frequency (f) (Drorbaugh and Fenn 1955). Before each experimental run, the plethysmograph was calibrated to adjust the air volume by the software. The calibration was performed by injection via a syringe of 5ml for each plethysmograph chamber.

Before recording the respiratory measurements, two sessions of acclimatization to the plethysmograph chamber were carried out. A 60-minute adaptation period was performed the one-day before the measurements, and another 45-minute adaptation period preceded the measurements on the same day so that the rat got calm and sleepy when the recordings start. The protocol lasted 90 minutes, during which we continuously observe any change in the status of the rat as being awake (lying or standing with opened eyes) or asleep (lying down without movements and with closed eyes) (Figure 22).



Figure 22: Position of the rat in the plethysmograph chamber during recording

The sleep apnea, which reflects two missed breaths, was scored according to a well-defined procedure (Ramadan et al. 2006; Christon et al. 1996). Sleep apnea index corresponds to the number of apneas per hour. Also, the mean duration of an apnea episode was

calculated. All respiratory parameters were measured during sleep excluding apnea episodes. The measurements were integrated by data acquisition and analysis software LabChart 7 (ADInstruments, Oxford, UK) as shown in Figure 23.



Figure 23: LabChart recordings of two rats. The red curve and the green curves correspond to the plethysmograph flow for 2 different rats placed in two chambers. The blue and the pink curves represent the corresponding tidal volume.

ii. Invitro diaphragm contractility:

These pups were then euthanized with sodium pentobarbital (EXAGON; Axience, France) injection of 1 ml/kg (200 mg/ml solution). The contractile parameters of the diaphragm were studied through rectangular strips dissected from the midcostal region and mounted vertically in a muscle bath containing oxygenated Krebs Henseleit solution (Sigma Aldrich, Saint Quentin Fallavier, France) kept at 37 °C according to the protocol described above. The twitch tension (in g.cm⁻²), time to peak tension (ms), the half-relaxation time (ms), tetanic force (g.cm⁻²), and the fatigability index (%) of the diaphragm were measured using LabChart 7.0 (ADInstruments Oxford, UK).

iii. Measurement of AChE Activity

The AChE activity in the diaphragm samples was studied by applying modified Ellman method (Ellman et al. 1961) according to the protocol previously described above. Briefly, the diaphragm homogenates were incubated with 100 μ M of the butyryl cholinesterase inhibitor, tetra isopropyl pyrophosphoramide iso-OMPA (Sigma Aldrich, Saint Quentin Fallavier, France) for 15 minutes to measure only the AChE activity in the samples. Next, the AChE colorimetric

assay kit (ab138871; Abcam, Cambridge, UK) was used to measure the AChE activity using according to the manufacture's instructions.

iii. Biochemical Assessment

The myofibrillar content was measured spectrophotometrically (595 nm) according to Bradford assay (Bradford 1976) as described above.

iiii. Quantitative Reverse transcription polymerase chain reaction (qRT-PCR)

The total diaphragm RNA was isolated by FastGene® RNA Basic Kit (Nippon Genetics Europe GmbH, Germany), quantified by NanoDrop 1000 spectrophotometer (Thermo Scientific), and reverse transcribed into cDNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). Primers and TaqMan[™] Multiplex Master Mix (Thermo Fisher Scientific by Life Technologies SAS, Courtaboeuf, France) were used for transcript detection with quantitative real-time PCR. PCRs were run on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Gene-specific primers (Thermo Fisher Scientific) were used as TaqMan[™] Gene expression assays according to table 6.

Assay	Gene
Rn01412977_g1	β-actin (housekeeping gene)
Rn99999916_s1	Glyceraldehyde3-phosphate dehydrogenase (GADPH) (housekeeping gene)
Rn00596883_m1	AChE
Rn01751056_m1	Myosin heavy chain (MHC)I
Rn01470656_m1	MHCII
Rn01545085_m1	Ryanodine receptor (RyR1)
Rn00589545_m1	Atp2a1
Rn00568762_m1	Atp2a2

Table 6: TaqMan[™] Gene expression assays used in the quantitative real-time RT-PCR

PCR conditions were as follows: 50°C (2 min), 95°C (10 min) for hold stage followed by 40 cycles of denaturation at 95°C (15 seconds), and Annealing /Extension at 60°C (1 minute). The

experiments were done in duplicate. The results were expressed in arbitrary units, using a $2^{-\Delta\Delta Ct}$ calculation compared to control samples according to the following formula: $\Delta\Delta Ct = \Delta Ct$ exposed – mean ΔCt control.

2.3. Study of the ileum activity

i. In vitro contractility of the smooth muscles of the ileum

Longitudinal and circular ileum muscle strips (1 cm in length) were suspended vertically under 1 g of tension in a 15 mL organ bath containing Krebs Henseleit solution (pH=7.4) oxygenated with 95% O₂+5% CO₂ and maintained at 37 °C. The ileum was attached to two metal electrodes below and an isometric force sensor above through cotton yarn. After 30 min of equilibration, each muscle strip was stimulated by electric field stimulation EFS (100 V; 30 ms) created by two rectangular platinum electrodes separated by 2 cm, parallel to the muscle strips, (Darwiche et al. 2017). EFS induce the release of ACh from the myenteric plexus resulting in muscles contraction (Takahashi, Ikemoto, and Ezaki 1999).

The generated forces were recorded using a computerized data logger (MP100A-CE; Biopac Systems, Santa Barbara, CA, USA). The amplitudes of contraction for both longitudinal and circular muscles were measured with the peak analysis module in LabChart 7 software and the values were standardized to the cross-section area (CSA) as mentioned above (McGuire and MacDermott 1998). Data were reported in g.cm⁻².



Figure 24: Contraction of the ileum smooth muscle. A) Circular ileal muscle, B) Longitudinal ileal muscle

ii. Measurement of AChE Activity

The AChE activity in the ileum segments is studied according to modified Ellman method as cited above.

iii. Reverse transcription polymerase chain reaction

The gene expression of different mediators involved in the contractility of the ileum was studied by quantitative real-time PCR as explained above. The quantitative real-time PCR (qRT-PCR) was carried out in ABI Prism7900HT Real-time PCR System (Applied Biosystems[™]) using primers (Invitrogen Life Technologies, Saint Aubin, France), and SYBR[™] Green Master Mix (Applied Biosystems[™]) according to the supplier's instructions.

The housekeeping gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and were used as endogenous controls. The sequences of the primers are shown in table 7. The cycling conditions were as following: denaturation for at 95°C 1min, annealing at 60°C (56°C for calmodulin) and elongation at 72°C for 2 min. The results were expressed relative to GAPDH.

Table 7: The oligonucleotide sequences of primers used in quantitative real-time RT-PCR

Gene	Primer sequence	
GAPDH	Forward: 5'-GGTGCTGAGTATGTCGTGGAGT-3'	
	Reverse: 5'-ATTGCTGACAATCTTGAGGGAG-3'	
M2 mAChR	Forward: 5'-CACGAAACCTCTGACCTACCC-3'	
	Reverse: 5'-ACAGTCCTCACCCCTAGGATG-3'	
M3 mAChR	Forward: 5'-GCTCCATCCTCAACTCTACCA-3'	
	Reverse: 5'-TTCTCTCCACATCCAGAGTCC-3'	
Substance P (SP)	Forward: 5'-TGTTTGCAGAGGAAATCGGTG-3'	
	Reverse: 5'-GAACTGCTGAGGCTTGGGTC-3'	
rat NK1R	Forward: 5'-GGTACTACGGCCTCTTCTATTGC-3'	
	Reverse: 5'-CAGGAAGTAGATCAGTACAGTCACG-3'	
Calmodulin	Forward: 5'-GAATGGCACCATTGACTTCC-3'	
	Reverse : 5'-GTAGCCATTGCCATCCTTGT-3'	

iv. Histological analysis

Segments of ileum were removed and fixed with formol for preparing histological sections. The segments were further dehydrated with graded concentrations of ethanol, cleared in xylene, and then embedded in paraffin. These sections were cut into 5 µm thick-sections. After deparaffinization with xylene, the sections were hydrated with successive baths of ethanol and then stained with Mayer's hematoxylin/eosin. Digital images of the histological samples were obtained by light microscope attached with a camera using Zen software (Carl Zeiss AG, Oberkochen, Germany). Then, the thicknesses of both longitudinal and circular smooth muscles were measured using the Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA).

RESULTS

Study 1: Effect of chronic chlorpyrifos exposure on diaphragmatic muscle contractility in adult male rats

This study was the subject of an article submitted to the Toxicological & Environmental Chemistry which is currently under review. The study will be first summarized and then the detailed results will be presented in an article as below:

1. Summary:

Residues of CPF, an AChE-inhibiting insecticide, are commonly present in food chain representing the major risk of exposure to the general population. At the neuromuscular junction, reduced AChE activity in the diaphragm is associated with alteration in the contractile performance in developing rats exposed prenatally and postnatally to CPF (Darwiche et al. 2018). At adulthood, the effect of dietary exposure to CPF has been elucidated on either fast or slow-twitch skeletal muscles (Hallal et al. 2019), but not on the function of the mixed respiratory skeletal muscle.

This study aims demonstrated the impacts of repeated exposure to CPF during adulthood in rats on the contractile response of the diaphragm and the hormones that can affect muscle performance. We further sought to investigate the mechanisms through which CPF induces the alteration in the muscle contractility by studying myosin isoform composition, as the key determinant of the contractile performance in the skeletal muscles.

This study has been conducted according to the experimental protocol and techniques described in the materials and methods section. Male Sprague Dawley rats were fed daily a diet containing CPF at a low dose of (1 mg/kg/day) for CPF1 group or higher dose (5 mg/kg/day) vs. control (vehicle) for 6 weeks. Following the exposure period, the rats were euthanized and diaphragm strips were dissected and used for in vitro contractility assessment by isolated organ bath system and AChE activity measurement. The myofibrillar protein content and myosin heavy chain isoforms expression were studied in the diaphragm. Serum testosterone, cortisol, and Growth Hormone (GH) levels were also measured.

Our results showed that CPF exposure at both doses (1 and 5 mg/kg/day) altered the contractile properties of the diaphragm in adult rats. The twitch tension and the tetanic force of the diaphragm were increased. In addition, the time to peak tension, half-relaxation time, and the fatigability index of the diaphragm was also increased. These changes were associated with decreased AChE activity in the diaphragm at both doses (CPF1 and CPF5 groups) compared to controls. The exposure to CPF at 5 mg/kg/day dose showed an increase in the amount of myofibrillar proteins content. An increase in the expression of the fast-twitch myosin heavy chain (MHC) IIA isoform was observed in the diaphragm of rats exposed to the 1 mg/kg/day dose of CPF. Alteration in the hormonal balance was observed in CPF exposed groups. Indeed, the serum testosterone levels were reduced but cortisol and GH serum levels were increased.

In this study, we have shown that chronic dietary exposure to CPF for 6 weeks in adult rats induced an increase in the contractile performance in the diaphragm which is linked to alterations in cholinergic neurotransmission through inhibition of AChE activity. Consequently, the diaphragm fatigability was increased through perturbations in hormonal balance. The changes in the contractile responses of the diaphragm can be attributed to the modification in the expression of MHC isoforms and/ or other possible modifications in the excitation-contraction coupling mechanism.

2. Article:

Effect of chronic chlorpyrifos exposure on diaphragmatic muscle contractility in rats

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Effect of chronic chlorpyrifos exposure on diaphragmatic muscle contractility in rats

Abstract:

To investigate the impact of 6 weeks exposure to the pesticide, Chlorpyrifos, on the contractility of the diaphragm and hormonal regulation, male rats were exposed to chlorpyrifos through diet at 1 or 5 mg/kg/day. Chlorpyrifos exposure at both doses increased the twitch tension, time to peak tension, half-relaxation time, and fatigability index of the diaphragm with a lower AChE activity. The myofibrillar protein content was higher in 5 mg/kg/day dose. The expression of fast-twitch myosin heavy chain (MHC) IIA isoform was increased in 1 mg/kg/day dose. Serum testosterone levels were reduced; cortisol and GH levels were increased at both doses. These contractility modifications due to chronic CPF exposure at adulthood could be related to the increase in the expression of MHC IIA isoform, perturbations in hormonal balance, and/or modifications in the excitation-contraction coupling mechanism.

Keywords: Chlorpyrifos; diaphragm; contractility; fatigability; hormones

INTRODUCTION

Pesticides are biologically active compounds widely used to prevent pests and kill vectors of disease.¹ Many pesticides are neurotoxic to insect pests,⁴ and due to similar neurochemical processes, these chemicals are potentially hazardous to humans.⁵ Worldwide, about five billions pounds of pesticide are used annually,⁶ in which organophosphates represent up to one-half of the insecticides consumption.⁷

Organophosphorus (OP) pesticides poisoning accounts for major reported toxic exposure in the USA⁸ and across agricultural communities worldwide.⁹ Epidemiological studies have reported an association between chronic low level of OPs exposure and chronic peripheral neuropathy and neuropsychological effects in farm workers.^{10,11} Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] (CPF) is a broad spectrum OP insecticide, with a wide variety of crop protection and non-crop applications.¹² CPF has a moderate acute oral toxicity and is considered by U.S. Environmental Protection Agency (EPA) as a class II toxicant.¹³ Recent concerns regarding possible neurodevelopmental toxicity of CPF have resulted in the elimination of its most non-agricultural uses by the EPA.¹⁴ However, CPF remains one of the most commonly used insecticides in agriculture.¹⁵

Although the effects of acute CPF exposure have been widely reported,^{16,17} less studies have examined the impact of chronic exposure to traces of CPF in diet as a way to mimic the main route of exposure for the general population.¹² It was demonstrated that chronic long-term feeding with or without intermittent high dosages with CPF produces substantial several biochemical changes in rats including decreased cholinesterase (ChE) activity and muscarinic receptor density in the brain.¹⁸ Further studies on animal models have reported elevation in core temperature,¹⁹ long-lasting sensory and neuromotor effects,²⁰ and possible cognitive and emotional disorders during chronic dietary exposure to CPF.²¹

The main target of CPF is the enzyme acetylcholinesterase (AChE), which hydrolyzes the neurotransmitter acetylcholine (ACh) at cholinergic synapses. The excess of ACh at neuromuscular junctions can cause muscle twitching.¹² Severe acute OP poisoning is associated with prolonged neuromuscular weakness²² and death as a result of respiratory

failure.²³ We have recently shown that prolonged CPF exposure modified the contractility of soleus slow-twitch and *edl* fast-twitch skeletal muscles contractility in rats.²⁴

The diaphragm, a neither typical slow- twitch nor fast-twitch muscle,²⁵ is a highly specialized respiratory skeletal muscle unique in its continuous rhythmic activity.²⁶ The mammalian diaphragm has been assessed in animal models to study the effects of OP poisoning.^{27,28} In this context, we have previously reported increased diaphragm contractility associated with greater fatigability in growing rats at the age of 21 and 60 days following chronic feeding with CPF during critical pre- and postnatal developmental periods.²⁹ Although adults are extensively exposed to pesticides residues in food, to our knowledge, no studies have examined the impact of CPF exposure introduced by ingestion of contaminated food during adulthood on the contractile parameters of the diaphragm in rats.

Consequently, the present study was designed to investigate the effects of 6 weeks of chronic dietary exposure to two different doses of CPF on the contractility parameters of the diaphragm in adult rats. Since the myosin heavy chain (MHC) isoforms are the main determinants of the contractile performance of skeletal muscles,³⁰ we aimed to determine whether this exposure will induce any possible alteration in the composition of contractile proteins and MHC isoforms in adult male rats. Also, we further investigated if this exposure will induce alterations in some hormones that control muscle performance.³¹

Materials and Methods

Chlorpyrifos preparation

Chlorpyrifos (99.8% pure; LGC Standards SARL, Molsheim, France) was dissolved in corn oil (as vehicle) and a stock solution was prepared (10 mg/ml). CPF was administered at two doses: 1 mg/ml (CPF1 group) or 5 mg/ml (CPF5 group). The oral dose was standardized to 1 ml/kg body weight of the rat. The chosen doses, 1 mg/kg/day and 5 mg/kg/day correspond to the NOAEL for depression of cholinesterase activity in the brain and developmental toxicity level in rats respectively.³² These doses have been previously used by several studies.^{24,29} CPF solutions were maintained at room temperature in dark.

Experimental Design and Animals Sacrifice

All procedures in this study were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. 30 adult male Sprague-Dawley rats (6 weeks old; 200 ± 10 g) were randomly assigned to three groups: 10 rats for the control group and 10 rats for each of the two treated groups (CPF1, CPF5).

After one week of acclimation, each rat was individually housed in a transparent plastic cage covered with a stainless steel grid, in ventilation standard conditions of light (12-hour cycle) and temperature (23°C). These rats had free access to fresh water ad libitum. The rats in CPF1 and CPF5 groups were fed daily a standard food pellet diet (20 g/day/rat) mixed with either 1 or 5 mg CPF/kg body weight/day respectively during 6 weeks. The concentration of CPF in the diets was adjusted weekly based on weekly body weight to maintain the targeted dose levels on an mg/kg-day basis. The rats in the control group were fed the same pellet diet containing the vehicle (corn oil) of 1 ml/kg/day. The food intake for each rat was checked daily for any changes. Rats exposed to CPF have not shown any signs of cholinergic toxicity (such as tremors, salivation, and lacrimation) during the experimental protocol. Each week, the weight of the rats was assessed.

Following the exposure period, the rats were euthanized with an intraperitoneal overdose of sodium pentobarbital (1 ml kg⁻¹; 200 mg ml⁻¹ solution). The diaphragm and adjacent rib cage were rapidly removed and placed in Ringer solution (140 mM NaCl, 6 mM KCl, 5 mM HEPES, 3 mM CaCl₂; pH 7.4; 95% O2/ 5% CO2) (Sigma Aldrich, Saint Quentin Fallavier, France) Muscle contractile properties were studied in rectangular strips of the costal diaphragm in which the rib margin and central tendon remained intact. Several strips were suspended vertically in a muscle bath containing oxygenated Ringer solution maintained at 37°C while the remainder of the diaphragm was preserved at -80°C for protein quantification experiments.

Measurement of muscle contractile properties

The rib margin was anchored to the base of the bath and the central tendon of the diaphragm was attached to an isometric force transducer. Two silver wire electrodes were positioned on either side of the muscle strip, parallel to its long axis. The force transducer output was fed to an isometric force sensor (PIODEN CONTROLS LTD, United Kingdom, accuracy \pm 10mg) with a maximum response of 25 mg and recorded by data acquisition system (Digidata 1200, Axon Instruments) for subsequent analysis.

Muscles were stimulated directly using square wave pulses (2 ms duration) and the voltage was adjusted to give supramaximal stimulation (12 V). Diaphragm strip length was adjusted to obtain *L*o, defined as that length at which maximal force was reached. All subsequent measurements were made at L_0 . After completion of the tension measurements, the cross-sectional area of the strip was calculated by dividing the weight of the muscle by the product of muscle density (of 1.056 g.cm⁻³) and L_0 .

The experiments were performed at controlled room temperature (19–20°C).

Twitch measurements

After equilibration, single twitches were elicited. This stimulation mode is identical to that used by Darwiche et al.²⁹ For each twitch, the peak twitch tension (measurement between baseline and peak force) was normalized by cross sectional area and measured (in g.cm⁻²), time to peak tension (time to peak force (ms)) and half-relaxation time (time for the force to decrease to one-half of its peak value (ms)) were determined and the mean values for each strip were calculated.

Measurement of tetanic contraction

The maximum tetanic tension (P0) was determined using 1 s duration trains of stimuli (20 Hz). The amplitude between the baseline and the stable portion of the tetanic plateau was measured. Force values were normalized for cross sectional area and expressed in g.cm⁻².

Measurement of fatigue

The fatigue resistance was evaluated using a protocol consisting of a 2 Hz train of supramaximal stimuli for a 5 min period. The fatigue index was calculated as the percentage of initial force remaining after 5 min of muscle stimulation.

Proteins Extraction and dosage

The frozen muscles were homogenized with 5 volumes of washing buffer containing 20 mM NaCl, 1 mM EGTA (pH 6.4), and 5 mM PO4 and centrifuged at a speed of 12000 rpm for 5 min. Then myosin was extracted with 3 volumes of extraction buffer, containing 5 mM EGTA, 1mM di-thiothreitol (pH 8.5) and 100 mM sodium pyrophosphate, then incubated in cold for 24 hours with gentle mixing. The mixture was centrifuged at 12000 rpm for 15 min, and the supernatant containing myosin was collected. Small volumes were used to determine the myosin content by Bradford technique. The remaining part was diluted twice with glycerol and stored at -20 °C for electrophoresis.

The Bradford technique was used for the dosage of myofibrillar proteins (Bio-Rad, Hercules, CA, USA).³³ This assay was used to estimate spectrophotometrically the concentration of proteins obtained. A standard curve using Bovine Serum Albumin (BSA) of different concentrations of 0, 10, 15, 20, 30, 50 μ g/ml was constructed. 1000 μ l of Bradford reagent was added to 50 μ l of protein sample diluted 10 times in a protein dosage buffer prepared from 5 mM HEPES,5 mM Tris HCL, 400 mM Sucrose (Sigma Aldrich, Saint Quentin Fallavier, France). The absorbance of each sample was measured at 595 nm using a UV-visible spectrophotometer (Metertech SP-8001 UV/Visible Spectrophotometer, Taiwan).

SDS-PAGE electrophoretic separation of Myosin Heavy chain isoforms

The effect of chlorpyrifos exposure on the MHC isoforms was studied by simple vertical migration of SDS-PAGE electrophoretic separation, as described previously.³⁴ The separating gel was prepared from 99.5% glycerol, 30% acrylamide, 0.6% bis acrylamide, 1.5% M Tris (pH 8.8), 1M glycine, H2O, 10% SDS, 10% ammonium persulfate, and Temed. The staking gel was prepared from 99.5% glycerol, 30% acrylamide, 0.6% bis acrylamide, 0.5% M Tris (pH 6.8), 10% SDS, 0.1 M EDTA (pH 7), H2O, 10% ammonium persulfate, and Temed. Temed. Myosin samples were diluted with a buffer containing Tris 125mM (pH 6.8),

glycerol 20%, β -mercaptoethanol 5%, EDTA 1 mM, SDS 5% (Sigma Aldrich, Saint Quentin Fallavier, France). The mixture is then boiled for 5 min. 25 μ L of diluted myosin was loaded into each well. Gels were run at constant voltage (72 V) for 28 hrs at 4-6 °C and then stained with Coomassie Blue R solution that allowed the identification of the MHC bands corresponding to slow MHC I and fast MHC (IIa and IIb) isoforms. After staining, the gels were scanned and the relative expression of different MHC isoforms was quantified using Image J software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA).

Acetylcholinesterase (AChE) activity

AChE activity was measured according to a modified Ellman's method.³⁵ Briefly, diaphragm tissues were homogenized in lysis buffer (1:10 w/v; i.e., 1 mg of homogenized tissue per 10 μ L of buffer) (Abcam, Cambridge, UK). Next, the homogenate was centrifuged and the supernatant was diluted (1:10). The colorimetric AChE assay kit (ab138871; Abcam, Cambridge, UK) was used to measure the AChE activity according to the manufacturer's instructions. The activity was corrected for protein content and the data were expressed in μ mol/min/mg protein.

Serum Cortisol, Testosterone, Growth Hormone levels

Serum cortisol, testosterone, growth hormone, and levels were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) technique based on the principle of competitive binding. Cortisol ELISA Kit (K7430, BioVision, USA), Testosterone ELISA Kit (K7418, BioVision, USA), and Growth Hormone ELISA kit (A05104, Bertin, France) were used for serum quantification of cortisol, testosterone, and growth hormone respectively according to the manufacturers' instructions.

Statistical analysis

Statistical analyses were performed with StatView software (version 5.0, Abacus Concepts Inc., Berkeley, CA, USA). Data were analyzed using non-parametric Kruskal-Wallis and Mann-Whitney tests. Body weight was analyzed by repeated measures ANOVA. In all analyses, the threshold for statistical significance was set to $p \le 0.05$.

Results

Effect of CPF exposure on body weight

As shown in Figure 1, all animals gained weight during the 6 weeks of the experimental period. However, after 2 weeks of exposure, the mean body weight of the rats was significantly lower (p<0.05) only in CPF5 group compared to the control group without significant changes in the body weight in CPF1 group.

Effect of CPF on the contractile parameters of the diaphragm

Twitch tension

In control group and following electrical stimulation, the twitch tension was characterized by an amplitude of 90.32 ± 12 g/cm², a time to peak tension of 37.56 ± 2 ms, and a time constant of relaxation of 18.86 ± 1 ms (n=10 per group). As shown in Figure 2, 6 weeks of exposure to either dose of CPF (1 or 5 mg/kg) induced detectable changes in the contractile parameters of the diaphragm. For instance, the twitch amplitude was significantly increased in CPF1 group by 130% (207.6 ± 26 g/cm²; p<0.01 compared to control) and in CPF5 group by 96% (177 ± 41 g/cm²; p<0.05 compared to control) (Figure 2A). Furthermore, CPF exposure induced an increase in the time to peak tension in CPF1 group (59.89 ± 5 ms; p<0.001) and in CPF5 group (44.11 ± 2 ms; p<0.05) compared to control. This increase was significantly higher in CPF1 group compared to CPF5 group (p<0.05) (Figure 2B). The constant of relaxation was highly increased in both CPF1 and CPF5 groups by 203% (57.1 ± 1 ms; p<0.001), and 134% (44.2 ± 2 ms; p<0.001) respectively compared to the control group (Figure 2C).

Tetanic contraction

In the control group, the tetanic force of diaphragm muscle was 140.6 ± 26 g/ cm². CPF exposure was associated by a significant increase in the tetanic force in CPF1 group by 240% (477.6 g/cm² ± 56, p<0.01) and CPF5 group by 186% (402.5 g/cm² ± 75; p<0.01) as shown in Figure 3.

Fatigability Index

Diaphragm susceptibility to fatigue was assessed by repetitive stimulation of the diaphragm strips using the protocol described above. There was a significant increase in the fatigability index in CPF1 group by 23% (92.08 ± 2%; p<0.01) and CPF5 group by 21% (90.7 ±2%; p<0.001) compared to control group (74.88 ± 3.3%) (Figure 4).

Effect of CPF on the amount of the diaphragm myofibrillar proteins

In the control group, the amount of myofibrillar proteins was 8.301 ± 0.3 mg/g of the diaphragm muscle. As shown in Figure 5 the myofibrillar protein content was not affected in CPF1 group (7.5± 0.4 mg/g of muscle). However, the myofibrillar protein content was significantly increased in CPF5 group by 29% compared to control group (10.81 ± 0.5 mg/g of muscle; p <0.05) and by 52% compared to CPF1 group (p<0.05).

Effect of CPF exposure on MHC isoforms expression in diaphragm muscle

The separation and analysis of MHC isoforms expression by SDS-PAGE allowed the estimation of different bands corresponding to each of the MHC isoforms and the order of migration has been previously identified and shown to be MHC I > MHC IIb > MHC IIa (Figure 6).³⁶ Under normal conditions, the diaphragm expressed the different isoforms as $27.2 \pm 3.5 \%$ for MHC I, $0.27 \pm 0.11 \%$ for MHC IIb, and $72.6 \pm 3.5 \%$ for MHC IIa. As shown in Figure 7, repeated exposure to the CPF1 induced a significant decrease by 31% in MHC I isoform (p<0.05 vs control) and a significant increase by 14% in the expression of MHC IIa isoform (p<0.05 vs control) without significant modification in the expression of MHC IIb. However, the exposure to CPF5 didn't induce significant changes in MHC isoform expression compared to the control group. Despite that, there was a non-significant increase in the expression of MHC I isoform in CPF5 group compared to the control group.

Effect of CPF exposure on AChE activity

As shown in Figure 8, diaphragm AChE activity was significantly lower in both CPF1 and CPF5 groups by 33% and 36% respectively (p<0.05) compared to the control group (CPF1: 0.02 ± 0.0005 ; CPF5: 0.02 ± 0.002 ; control: $0.03\pm0.003 \mu$ moles/min/mg protein).

Effect of CPF exposure on Hormone Levels

The serum hormone levels of male rats exposed to vehicle or CPF (1 and 5 mg/kg/day) during 6 weeks are represented in Figure 9. Serum testosterone level was significantly lower in both CPF1 and CPF5 groups by 31% (p<0.05) and 23% (p<0.01) respectively compared to the control group (CPF1: 2.020 ± 0.3045 ; CPF5: 2.236 ± 0.08643 ; control: 2.912 ± 0.1324 ng/ml) (Figure 9A). However, exposure to CPF was associated with higher cortisol levels by 183% in the serum of both CPF1 group (180.0± 28.41 ng/ml; p<0.05) and CPF5 group (180.1± 21.96 ng/ml; p<0.01) (Figure 9B). Furthermore, GH level was significantly higher in CPF1 group by 88% (51.92 ± 2.663 ng/ml; p<0.001) and by 39% in CPF5 group (38.28± 1.967 ng/ml; p<0.001) compared to control group (27.56 ± 0.9778 ng/ml) (Figure 9C).

Discussion

The diaphragm is a primary skeletal muscle essential for breathing, especially the inspiratory phase. The exposure to organophosphates can interfere with the diaphragm morphology,³⁷ and induce biochemical alterations and electrophysiological disruptions.³⁸ The aim of this study was to assess the effect of exposure to CPF by the oral route through the ingestion of contaminated food since dietary exposure is the main route of intoxication for humans. We examined the effect of repeated CPF exposure on the contractile parameters and the proteins involved in contraction of the principal muscle of the respiration i.e., the diaphragm. Also, we measured the levels of some blood hormones that can have an effect on muscle function and morphology. CPF was administered at two different doses (1 and 5 mg/kg/day) which correspond to the NOAEL for depression of cholinesterase activity in the brain and developmental toxicity level in rats respectively.³²

No changes in food consumption were reported between CPF exposed groups and control group (data not shown here). Our results showed lower body weight in rats exposed only to the higher dose of CPF (CPF5 *vs.* control), indicating an index of toxicity. Several studies on rats have reported similar reductions in body weight at higher doses of CPF.^{32,39} The low body weight observed in the CPF group during the exposure period might be related to the oxidative and cholinergic stress exerted by organophosphates,^{32,40} as a result of increased lipid peroxidation.⁴¹ We then measured the serum levels of growth hormone (GH), a

protein hormone secreted by the pituitary gland which controls growth and metabolism. GH promotes lipolysis in adipose tissues and it is associated with reduced fat mass.⁴¹ Unexpectedly, we found a significant increase in the GH in the serum of CPF1 and CPF5 groups. In fact, GH is known to stimulate protein turnover, and it is considered a strong anabolic agent.⁴² It has been shown to induce intracellular amino acid accrual and increase the expression of myofibrillar proteins as reported by Fong et al⁴³ Our results are in agreement with the study of Mazza et al⁴⁴ who reported an increase in GH release after exposure to Pyridostigmine, an acetylcholinesterase inhibitor, illustrating a stimulatory role of acetylcholine and muscarinic M1 receptors of brain on GH secretions. Indeed, the central nervous system cholinergic pathways can positively affect the growth hormone secretion.

The functional characteristics of the diaphragm are mainly determined by its contractile properties. The main finding of the present study is the alteration of the contractile parameters of the diaphragm after the repeated exposure to two different doses of CPF along with a decrease in the AChE activity of the diaphragm. In fact, the exposure to CPF was associated with an increase in the twitch tension and tetanic force of the diaphragm. The time to peak tension and the constant of relaxation were also increased at both doses. Our findings are in line with previously reported effects of OPs on muscles contractility where CPF was shown to increase the contractility of slow-twitch soleus muscle at both doses and fast-twitch *edl* muscle only at the higher dose.²⁴ The increase in the maximum contractility of the diaphragm was reported by S.R. Ivanovic' et al²⁷ following exposure to Diazinon, an OP insecticide. Furthermore, the enhancement of the twitch characterized by the increase in the amplitude of contraction, the time-to-peak, and constant of relaxation following anticholinesterase exposure is the consequence of prolonged action of acetylcholine at the motor end-plate as described by Burd and Ferry.⁴⁵ Recently, Darwiche et al²⁹ have reported a similar increase in twitch tension in the diaphragm of adult rats exposed pre- and postnatally to 5 mg/kg/day of CPF associated with low level of AChE activity. Thus, the decrease in the AChE activity observed at the level of the diaphragm and the consequent accumulation of acetylcholine at the cholinergic synapses following CPF exposure could account for the increase in the contractility of the diaphragm. On the other

hand, the time to peak tension was increased in both CPF1 and CPF5 groups. This increase was significantly more pronounced in CPF1 group. A possible explanation could be the development of tolerance to organophosphates toxicity at high doses due to the down-regulation of muscarinic acetylcholine receptors.⁴⁶

Muscle Fatigue is considered as acute loss of contractile force after work.⁴⁷ Our results showed an increase in the fatigability index of the diaphragm in both groups exposed to CPF. This finding is in agreement with those of Darwiche et al²⁹ where the effect was only reported at the dose of 5 mg/kg/day. In fact, severe inhibition of AChE is the primary event responsible for muscle weakness that occurs in OP poisoning. This increase could be a consequence of the prolonged stimulation of the diaphragm after AChE inhibition. On the other hand, it is well established that increased muscle contractions involve a strong production of reactive oxygen species (ROS), unstable molecules primarily generated in the mitochondria of skeletal muscle. It has been shown that paraquat, an herbicide, induces damage to neurons by generating reactive oxygen species (ROS).⁴⁸ Thus, elevated ROS levels promote oxidation reactions with cell proteins resulting in muscle fatigue.⁴⁹

In the view of the present results, it seemed interesting to determine the mechanisms that may account for the finding of increased force and reduced muscle resistance. This increase in the contractility of the diaphragm could be related to several parameters such as modifications in Excitation-contraction coupling mechanism and alterations in myofibrillar protein content. In this view, our results showed a dose-dependent effect of CPF on the myofibrillar protein content. In theory, skeletal muscle contractile performance is determined by the properties of its constituent fibers, which depend, in turn, on the interactions of the myofibrillar proteins.⁵⁰ Consequently, the modifications in the protein composition of skeletal muscle, especially the myofibrillar proteins might contribute to the changes in the muscle function. Interestingly, the increase in the amount of myofibrillar protein content observed in CPF5 group can be correlated with the increase in the contractility of the diaphragm. However, in CPF1 group a relatively lower myofibrillar protein compared to CPF5 group was associated with stronger twitch tension. A decrease of myofibrillar content has been also shown to be correlated with the increase in twitch amplitude in soleus muscle following CPF exposure, suggesting an adaptation of muscle

fibers to the changes induced by CPF exposure mediated by structural and functional alterations as reported by Hallal et al.²⁴ This increase in the contractile force could be attributed to other alterations at the level of calcium homeostasis mechanism such as the modification in the expression of proteins involved in Ca²⁺ release (ryanodine receptor) and/or uptake (Ca²⁺ ATPase / SERCA pump) by the sarcoplasmic reticulum (SR)⁵¹, or to the modifications in muscle fiber types which is further assessed in this study.

Myosin proteins make up about 45% of the myofibrillar protein in skeletal muscle which is composed of different fiber types. Each fiber type is identified by myosin heavy chain isoform (MHC) which is expressed as slow I, fast IIA, fast IIX, and fast IIB. The diaphragm contains about 55% of slow twitch fibers and 45% of fast twitch fibers with different fatigability characteristics.⁴⁷ Then any change in MHC isoforms expression could induce a change in fatigability of the diaphragm. The exposure to 1 mg/kg/day of CPF induced a significant decrease in the expression of the slow MHCI isoform accompanied by an increase in MHC IIA isoform. Such modifications could refer to the plasticity of respiratory muscle fibers, which is the ability of a fiber to switch from one type to another one in response to several physiological conditions such as training, nutrition, hypoxia, and aging.⁵² In general, the fast-type genes seem to be expressed "by default", whereas the slow-type genes would be expressed as a response to changes in activity.⁵³ Since slow fibers are resistant to fatigue whereas fast IIX and IIB fibers are easily fatigable and fast IIA fibers exhibit intermediate fatigue resistance,⁵² then the decrease in MHCI isoform and the subsequent increase in MHC IIA isoform expression could explain the increase in fatigability observed in the diaphragm in CPF1 group. It has been demonstrated that the contractile properties of the muscle fibers correspond with the expression of MHC isoforms.⁵⁴ Thus the increase observed in the relative composition of the fast isoform (MHC IIA) might explain the increase in the contractility of the diaphragm in CPF1 group. In this context, a similar slow-to-fast type transition has been reported in the soleus skeletal muscle in a study performed on AChE knockout mouse.⁵⁵ For instance, AChE deficiency could induce the transformation of the slow pattern of motor unit discharges into a fast one. Hence, the prolonged inactivation of AChE induced by CPF and the consequent hyperstimulation of the cholinergic receptors could have induced critical perturbations in

both signal neurotransmission and intrinsic muscle properties resulting in increased muscle contractility. However, no changes were observed at the level of MHC isoform expression following the exposure to the higher dose of CPF. This finding is in agreement with previous work reporting no changes in MHC isoform following the exposure to 5 mg/kg/day of CPF in slow (soleus) and fast (*edl*) twitch muscles. In fact, the accumulation of Ach at cholinergic synapses due to prolonged inhibition of AChE is thought to induce a protective neurochemical compensation for the inhibited enzyme.⁵⁶ Therefore, the lack of response at the MHC level (despite clear AChE inhibition in CPF5 group) could be attributed to the development of tolerance to the repeated exposure to CPF at relatively high doses.²⁰

In addition to the nervous system which initiates muscle contractions, muscles morphology and function are controlled by the endocrine system.³¹ Then, the perturbations at the levels of hormones could subsequently affect muscle performance. Accordingly, we sought to determine the influence of repeated CPF exposure on two critical hormone levels in the serum of rats. These hormones include testosterone, which is the primary male sex hormone, and cortisol, a representative marker of stress. Although OPs are not considered as endocrine disruptors, several studies have shown that CPF can induce antiandrogenic⁵⁷ and oestrogenic effects.⁵⁸ In fact, CPF can decrease the biosynthesis of testosterone in the testes of rats by inhibiting the secretions of the pituitary hormones (luteinizing hormone (LH) and follicle stimulating hormone (FSH)). The reduced testosterone levels observed in our study are similar to previous reports showing a decrease in testosterone level after the exposure to higher doses of CPF.^{59,60} It is well known that testosterone is an essential ingredient in the development and maintenance of muscle strength,⁶¹ then the decrease in testosterone levels observed following CPF exposure could also explain the decrease in the fatigability resistance of the diaphragm. In addition, we found a significant increase in serum cortisol levels after CPF exposure. Cortisol is a major glucocorticoid hormone produced by the adrenal cortex.⁶² The activation of the pituitary-adrenal axis is an important neuroendocrine response to stress. Cholinesterase inhibitors have been shown to modify the pituitary-adrenal axis, inducing increased cortisol levels as reported in patients with OP poisoning.⁶³ This effect could be attributed to the accumulation of acetylcholine and/or the oxidative stress induced by CPF exposure.⁶⁴ Since cortisol is known as catabolic, or muscle-breaking hormone, then the prolonged secretion of cortisol causes muscle weakness and fatigue.⁶⁵

In conclusion, the present study has highlighted the effects of exposure to CPF for 6 weeks in adult rats on the contractile performance and the endocrine regulation of the diaphragm. Repeated dietary exposure to CPF was associated with increased diaphragm contractility which could be related to the decrease in AChE activity. The myofibrillar protein content was increased and the muscle fibers shifted toward fast and more fatigable type (expressing MHC IIA isoform). The increase in the fatigability of the diaphragm could be attributed to the perturbation in anabolic-catabolic hormone balance. CPF induced a decrease in serum testosterone levels but an increase in cortisol and growth hormone levels. Finally, further studies are needed to investigate the possible effects of CPF exposure on the calcium homeostasis as well as the expression of different proteins involved excitation-contraction coupling mechanism.

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Declaration of Interest

No potential conflict of interest was reported by the authors.

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Figure 1. In vivo effects of repeated exposure to CPF during 6 weeks on the body weight of young adult rats. Data are quoted as the mean \pm SEM for control, CPF1, and CPF5 groups (n=10 /group); *: p<0.05 vs. control



Figure 2. Effect of CPF exposure on the contractile parameters of the diaphragm: (A) twitch tension expressed as a function of cross-sectional area (g/cm^2) , (B) time to peak tension

(ms), (C) half-relaxation time (ms). Values are quoted as means ± SEM (n=10/group); *: p<0.05; **: p<0.01; ***: p<0.001 vs. controls; #: p<0.05 CPF5 vs. CPF1



Figure 3. Effect of CPF exposure on the tetanic force of diaphragm muscle in rats: Values are quoted as means ± SEM (n=10/group); **: p<0.01;***: p<0.001 vs. controls



Figure 4. The fatigability index of the diaphragm expressed as the % force remaining after 5 min of electrical stimulation in rats exposed to CPF versus control: values are expressed as means ± SEM (n=8 /group); **: p<0.01; ***: p<0.001


Figure 5. Effect of CPF exposure on the myofibrillar protein content in the diaphragm muscle of male rats: Data are represented as means \pm SEM expressed as mg of myofibrillar proteins per gram of muscle (n=10/group); ***: p<0.001 vs. control, # # #: p<0.001 CPF5 vs. CPF1



Figure 6. Representative SDS-PAGE gel illustrating the electrophoretically separated Myosin Heavy Chain isoforms in the diaphragm of the control and CPF exposed rats. This figure shows the bands corresponding to each of the MHC isoforms (MHC I, IIa/x, IIb)



Figure 7. Effect of CPF on the expression of different MHC isoforms in the diaphragm muscle. Values are expressed as percentages of total MHC (n=10/group) *p<0.05 vs. control



Figure 8. Effect of CPF exposure on AChE activity in the diaphragm of male rats. Values are expressed as the mean \pm SEM expressed as μ mol of acetylthiocholine hydrolyzed per minute per mg protein for the control (open bars), CPF1 (gray bars) and CPF5 groups of rats (black bars). *P<0.05 vs. control (n=10/group)



Figure 9. Effect of CPF exposure on hormone levels in rats. (A) Serum testosterone level, (B) serum Cortisol level, (C) Growth Hormone level. Values are quoted as means ± SEM for

the control (open bars), CPF1 (gray bars) and CPF5 groups of rats (black bars). *P<0.05; **: p<0.01; ***: p<0.001 vs. control (n=10/group)

Study 2: Effects of perigestational exposure to CPF and/ or HFD on the contractile response of the respiratory skeletal muscle and gastrointestinal smooth muscle

According to the Developmental Origin of Health and Disease concept (DOHaD), perturbations during prenatal and intrauterine life can program the risks for further diseases later in life. The maternal obesity on one side and the early life exposure to organophosphates residues on the other side constitute an environmental risk favoring the occurrence of obesity in adulthood.

This study has been divided into two aims. For each aim, the obtained results will be first summarized and then the detailed results will be presented in a separate article.

2.1. Impacts of perigestational exposure to CPF and/ or HFD on the contractile response of the respiratory performance and the contractile respiratory skeletal in male rats at early adulthood

This study was a subject for an article submitted to Scientific Reports journal and is currently under review.

2.1.1. Summary:

Exposure to CPF is harmful to respiratory system during in utero and postnatal period, however, the cumulative effects of perigestational exposure to CPF and/or HFD (before gestation till the end of lactation) on the respiratory performance of rats at adulthood that are not in direct contact with either CPF and/or HFD has not been covered.

The objective of this study was to determine the consequences of continuous long term peri-gestational exposure of mother rats to a CPF and/ or HFD on the respiratory parameters, sleep apnea incidence, and the contractile responses of the major respiratory skeletal; the diaphragm muscle.

As discussed in the materials and methods section, female Wistar rats were exposed to either CPF (1mg/kg/day vs vehicle) and/or HFD during the perigestational period, starting from 4 months before gestation till the end of lactation period at PND21. Then all rats were maintained on normal diet without CPF. At early adulthood (PND60), *in vivo* measurements of sleep apnea and respiratory parameters were performed by whole-body plethysmography. Following euthanasia, diaphragm strips were used for *in vitro* contractility assessment by isolated organ bath system. Other diaphragm segments were used for proteins quantification, AChE activity measurements, and gene expression analysis by real-time PCR.

Our results showed alteration in the respiratory function and increase in the sleep apnea index in the male pups from dams in contact with a CPF and/or HFD during the perigestational period. Consequently, the diaphragm contractile force and the fatigability index of the diaphragm were increased. These changes were associated with decreased AChE activity and subsequent increase in the expression of AChE mRNA. As the skeletal muscle contraction is mediated by a Ca²⁺-dependent process, alterations in the expression of proteins involved for Ca²⁺ release/reuptake by the SR were observed in the diaphragm. The expression of MHC isoforms in the diaphragm was increased also increased.

Early-life exposure of dams to either pesticides residues and/ or HFD during perigestational period can alter the respiratory performance and the diaphragm contractile function in the offspring at early adulthood that are not directly exposed to either CPF or HFD.

2.1.2. Article:

Does the perigestational co-exposure to chlorpyrifos and/or high-fat diet affect respiratory performance and diaphragmatic muscle contractility at adulthood in rats?

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Abstract: The perinatal period is characterized by developmental stages with high sensitivity to environmental factors. Among the risk factors, maternal obesity and early-life pesticide exposure can program infant metabolism and promote obesity at adulthood. We established the effects of perigestational exposure to Chlorpyrifos (CPF) and/or High-Fat Diet (HFD) on respiratory parameters, sleep apnea and diaphragm contractility in adult rats. Four groups of female rats were exposed starting from 4 months before gestation till the end of lactation period to CPF (1 mg/kg/day vs vehicle) with or without HFD. Sleep apnea and respiratory parameters were measured by whole-body plethysmography in male pups at postnatal day 60. Then diaphragm strips were dissected for the measurement of contractility, acetylcholinesterase (AChE) activity, and gene expression. The perigestational exposure to CPF and/or HFD induced an increase in the sleep apnea index and respiratory frequency. The twitch tension and the fatigability index were also increased, associated with reduced AChE activity and elevated mRNA expression of AChE, ryanodine receptor, and myosin heavy chain isoforms. Therefore, the perigestational exposure to either CPF and/or HFD could program the risks for altered respiratory performance and diaphragm contractility in young adult offspring despite the lack of direct contact to CPF and/or HFD.

Key words: Chlorpyrifos, high-fat diet, sleep apnea, diaphragm, respiration, Excitation-Contraction mechanisms

1. Introduction

The perinatal period is characterized by high plasticity of the physiological systems, exposing the individual to higher vulnerability to his environmental factors. Any disruption in these physiological processes during development is described in the concept of Developmental Origin of Health and Diseases (DOHaD)¹. Studies have shown that maternal obesity constitutes an environmental risk which programs infant metabolism, favoring the occurrence of obesity or type 2 diabetes at adulthood²⁻⁴. Obesity can be induced by High-Fat Diet (HFD) associated to other factors including the exposure to environmental contaminants as organophosphorus (OP) pesticides, suspected to alter fetal programming metabolism⁵.

Maternal (HFD) is considered a reflection of the dietary habits in western countries^{6,7}. In a cohort of obese pregnant women, extreme obesity was associated with obstructive Sleep Apnea (SA) in mid-pregnancy⁸. Other studies in obese populations^{9,10} and in rats fed with HFD^{11,12} have reported an association between metabolic dysfunctions and SA. Since fetal development depends primarily on the maternal nutritional supply, then any disruption in maternal metabolism could affect the intrauterine milieu, increasing the risk of offspring to further diseases in later life^{13,14}. Evidence from animal models revealed that perinatal HFD intake can increase the risk of behavioral disorders such as anxiety¹⁵ and aggression in addition to altered locomotor activity in the offspring¹⁶. Even though a continuous HFD feeding including gestation and lactation periods resulted in an increased risk of obesity in the pups¹⁷, to our knowledge, no studies have examined the impact of the perigestational exposure to HFD on the respiratory muscle performance and SA incidence in the progeny at adulthood.

Alternatively, OP insecticides are commonly used for a variety of agricultural, industrial, and household applications¹⁸ and are detected in food and drinking water¹⁹. They are potent acetylcholinesterase (AChE) inhibitors resulting in the accumulation of acetylcholine (ACh) at cholinergic synapses and consequent overstimulation of the Central Nervous System (CNS) and neuromuscular junctions²⁰. Central respiratory failure associated with apnea is considered to be the major cause of death following OP poisonings^{21,22} resulting from the depression of the respiratory center in the brainstem and paralysis of the respiratory muscles²³. Chlorpyrifos (CPF) is an OP insecticide suspected to affect the metabolic programming from the fetal period until adulthood^{5,24}. We have recently shown that the exposure to CPF from the first day of gestation and through postnatal life until adulthood induced an increase in SA index and alter respiratory muscle contractility during development²⁵. However continuous perigestational exposure, before gestation till the end of lactation, at low dose of CPF without exposing the pups to CPF until adulthood has not been examined.

Since pregnant females are being exposed to multiple toxic stimuli throughout life, then examining the post-effects interaction of prolonged exposure combinations during the intrauterine life in the progeny simulates the real-life exposures²⁶. In this context, the present study is designed to investigate the consequences of long term perigestational exposure to CPF and/or HFD, 4 months before gestation and throughout gestational and lactational periods, in young adult male offspring raised on postweaning standard diet without CPF on 1) the respiratory performance during sleep and 2) the contractile activity of the diaphragm as an essential respiratory skeletal muscle. In addition, we further aimed at determining the mechanisms underlying these effects by studying the gene expression of different mediators involved in excitation-contraction coupling mechanism.

2. Results

2.1. Body weight

The mean body weight of male rat pups was assessed at 3 developmental stages: 3 days after birth (PND3), as juveniles (PND21), and as young adults (PND60) (Fig. 1). The mean body weight was significantly higher in HFD fed groups compared to standard diet fed groups at PND3 (p = 0.0005) and PND21 (p < 0.0001) and tends toward significance at PND60 (p = 0.0577). Compared to non-CPF groups, CPF exposure was associated with lower body weight at PND60 (p = 0.0367) (Table 1).

2.2. Respiratory Parameters

The effects of perigestational exposure to CPF and/or HFD on the respiratory pattern were analyzed in male rats at PND60. A significant diet-CPF exposure interaction was reported for f (p = 0.0004) and for V_T (p = 0.0337). However no significant diet-CPF exposure interaction was observed for T_I (p = 0.2977) and T_E (p = 0.0772). Compared to non-CPF groups, maternal CPF exposure was associated with significant decrease for T_I (p = 0.005) and f (p = 0.0403), and a significant increase for V_T (p = 0.0277) in male pups at PND60. Furthermore, comparing HFD groups to standard diet groups revealed a significant decrease for T_I (p = 0.0002) and for f (p = 0.0008) (Table 1).

Particularly, a significant decrease in f was observed in CPF group by 18% (p = 0.0118), HFD group by 23% (p = 0.0045), and in HFD+CPF group by 18% (p = 0.0472) compared to controls (Fig. 2c). The V_T was significantly decreased in HFD group by 38% compared to controls (p = 0.0263) and by 47% compared to HFD+CPF group (p = 0.0237) (Fig. 2d).

2.3. SA

A significant interaction between diet and CPF exposure was observed for SA index (p = 0.0087) and the mean duration of an episode of apnea (p = 0.0415). The SA index was significantly increased with either maternal CPF exposure (p = 0.0233) or HFD feeding (p = 0.0255) (Table 1). The SA index was significantly higher by 96% in each of CPF group (p = 0.0071) and HFD group (p = 0.0053) and by 88% in HFD+CPF group compared to controls (p = 0.0283) (Fig. 3b). A significant increase in the mean duration of an episode of apnea was observed in HFD group compared to controls (p = 0.0464) (Fig. 3c).

2.4. Effects of CPF and HFD on the contractility parameters

The effects of perigestational exposure of HFD and/or CPF exposure on the contractility of the diaphragm were studied in rats at PND60. There was a significant interaction between CPF exposure and diet for twitch tension (p = 0.0115), half-relaxation time (p = 0.0492), tetanic force (p = 0.0351), and also for the fatigability index (p = 0.0078). A main effect of diet was observed for tetanic force (p = 0.0445) (Table 1). The twitch tension was significantly increased in CPF group by 136% (p = 0.0095) and HFD group by 140% (p = 0.0095) compared to controls. This increase was less pronounced in HFD+CPF group (p = 0.0095)

0.0532) (Fig. 4a). The half-relaxation time was significantly lower in HFD group by 10% compared to controls (p = 0.0260) and by 20% compared to HFD+CPF group (p = 0.0199) (Fig. 4c). However, no changes were detected for time to peak tension ((Fig. 4d). The increase in tetanic force was highly significant in HFD group compared to controls (p = 0.0071) (Fig. 4e).

Regarding diaphragm fatigability index, a significant increase by 31% was observed in CPF group compared to controls (p = 0.0024). However, the fatigability index was significantly decreased by 27% in HFD+CPF group compared to CPF group (p = 0.0105) (Fig. 4e).

2.5. Acetylcholinesterase Activity and expression

The perigestational exposure to HFD and CPF was associated with significant interaction for AChE activity (p = 0.0093). A main effect of CPF was also shown (p = 0.0003) (Table 1). AChE activity levels were significantly reduced in CPF group (p = 0.0105) and HFD group (p = 0.0415) by 53% and by 29% respectively compared to controls. A significant decrease was reported in HFD+CPF group by 40% (p = 0.0433 vs control) (Fig. 5a). Consequently, the expression of AChE mRNA was quantified by real-time PCR.

A significant diet-CPF exposure interaction was observed for AChE mRNA expression (p = 0.0136). A main effect of diet was also observed. When compared to standard diet groups, HFD feeding was associated with a significant increase in the expression of AChE mRNA (p = 0.0054) (Table 1). The diaphragm AChE expression was significantly higher by 1.5 fold in CPF group (p = 0.0008), 2.8 fold in HFD group (p = 0.0009), and 1.6 fold (p = 0.0024) in HFD+CPF group compared to controls (Fig. 5b).

2.6. Myofibrillar protein content in the diaphragm

The diet-CPF exposure interaction was not significant. A main effect of CPF was observed for the myofibrillar protein content in the diaphragm. Compared to non-CPF groups, CPF exposure was associated with a significant increase in the myofibrillar protein content (p<0.0001) (Table 1). Despite a non-significant interaction, the myofibrillar protein content was significantly higher by 65% in CPF group (p = 0.0043) and by 82% in HFD+CPF group (p = 0.0159) compared to controls (Fig. 6).

2.7. Gene Expression analysis of MHC isoforms, Ryanodine receptor, and SERCA pump

The effects of HFD and CPF exposure on diaphragm genes expression were quantified by real-time PCR. A significant diet-CPF exposure interaction was observed for the expression of MHCI isoform (p = 0.0009) and MHCII isoform (p = 0.0046). A main effect of diet on MHCI (p = 0.0035) and MHCII (p = 0.0318) expression was observed (Table 1). MHCI isoform mRNA expression was significantly higher by 1.6 fold in CPF group (p = 0.0167), 2.9 fold in HFD group (p = 0.0010 for HFD), and 1.5 fold in HFD+CPF group (p = 0.0054) compared to controls. The expression of diaphragm MHCII mRNA was 2.1 fold higher in CPF group than in controls (p = 0.0010). However, the combined exposure in HFD+CPF group induced a decrease in the expression of MHCII mRNA compared to CPF group (p = 0.0040) without significant difference compared to controls (Fig. 7a).

Then, the expression of different genes responsible for the Ca²⁺ signaling RyR1, Atp2a1, and Atp2a2 coding for RyR1, SERCAI, and SERCAII proteins, respectively, were studied in the diaphragm. A significant diet-CPF exposure interaction was observed for RyR1 mRNA expression (p = 0.0101). Compared to standard diet groups, HFD feeding showed an increase in RyR1 mRNA expression (p<0.0001) (Table1). The diaphragm expression of RyR1 mRNA was significantly higher by 1.5 fold in CPF group (p = 0.0019), 3.5 fold in HFD group (p = 0.0012), and 2.4 fold in HFD+CPF group compared to controls (Fig. 7b).

Although no significant diet-CPF exposure interaction was observed, a main effect of diet was observed for Atp2a1 and Atp2a2 expression in the diaphragm. HFD feeding was associated with a significant increase in mRNA expression of Atp2a1 (p = 0.0457) and Atp2a2 (p = 0.0105) compared to standard diet groups. A main effect for CPF was observed for Atp2a1 and Atp2a2. Compared to non-CPF groups, CPF exposure was showed a significant decrease in mRNA expression of Atp2a1 (p = 0.0023) and Atp2a2 (p = 0.0007) (Table 1). Despite a non-significant interaction, the mRNA expression was significantly 1.5 and 1.9-fold higher for Atp2a1 (p = 0.0265) and Atp2a2 (p = 0.0262) respectively in HFD group compared to controls (Fig. 7c).

3. Discussion

The environment experienced by the developing offspring during the perinatal period is influenced by maternal health and dietary supply. Consequently, intrauterine life disturbances are recognized to program the risks for adverse health effects at adulthood²⁷. Evidence suggests that suboptimal maternal nutrition can induce obesity and metabolic disorder features during adulthood^{17,28}. Although the maternal HFD has been characterized in developing offspring in several studies^{27,29}, the interaction of maternal combined exposure to nutritional and environmental stimuli on the offspring at early adulthood has not been elucidated. Accordingly, the present study was designed to examine, to our knowledge for the first time, the perigestational effects of continuous exposure of female rats to CPF (1 mg/kg/day) and HFD (60% kcal from fat) 4 months before gestation, during pregnancy till the end of lactation period and weaning of the pups (PND21). Therefore, the long-term consequences of continuous maternal exposure to two alimentary risk factors, junk food and OP pesticide residues on the respiratory performance, SA incidence, respiratory muscle contractility, and the expression of genes involved in the excitation-contraction coupling mechanism were studied during early adulthood.

Our results revealed that the perigestational exposure to HFD, independent of CPF exposure, was associated with increased body weight in rats at birth. This increase continue to be observed at the end of lactation period, and to a less extent, at early adulthood (PND60) as the pups were raised on standard diet without CPF during the postweaning period (from PND21 to PND60). Several studies have revealed a lower birth weight^{17,30} or even no effects³¹ after maternal HFD feeding up to and/or throughout gestation and lactation. However, our study showed higher body weight in HFD groups at the PND3. These discrepancies could be ascribed to the duration of maternal HFD feeding, the content of dietary lipids, and even the fatty acid composition of the high caloric diet. HFD is transformed into the milk, increasing the lipid content of milk and contributing to a higher body weight of HFD weanlings³². The elevated body weight was still observed at PND60 in agreement with other studies where the increase in body weight persist till the age of 3 months and even more through adulthood as a result of developed hyperphagia^{3,33}. On the other hand, in our study, a decrease in body weight was associated with CPF exposure only at early adulthood indicating a long-term programming of body weight due to CPF toxicity. Recently, we have shown a similar decrease in body weight in rat exposed

continuously during pre- and postnatal periods, and throughout development, to a higher dose of CPF at PND60²⁵.

We have recently studied the effect of perinatal exposure to CPF on the respiratory parameters at young adulthood. Other risk factors can affect the respiratory pattern such as obesity³⁴. Consequently, the impacts of perigestational exposure to CPF and/or HFD are studied. Our results showed lower respiratory frequency as expressed in breath per minute during sleep associated with increased SA index without significant changes in T_I and T_E . Our findings are consistent with previously reported effects of either acute OPs exposure³⁵ or even repeated prenatal and postnatal CPF exposure on the respiratory frequency²⁵. The decrease in the respiratory frequency in the HFD group came along a decrease in the tidal volume (V_T). Overweight and obesity are associated with a higher risk of respiratory symptoms³⁶. Moreover, adipose tissues produce different cytokines and mediators, generating a pro-inflammatory state which could contribute, in turn, to reduced lung functions³⁷. Although normal T_I and T_E were observed in the HFD group, the V_T was significantly decreased compared to controls. The lung volume and capacity have been shown to be reduced in obese individuals³⁸. In our study, the lower volume of air moved in and outside the lungs during each respiratory cycle could be related to the increased body weight of the rats; despite they are not obese, as a result of possible metabolic disruptions and associated pro-inflammatory state induced by early-life nutritional perturbations.

ACh is an essential neurotransmitter for the neural control of respiration at the central and peripheral nervous system³⁹. Any modification at the cholinergic level in the neuromuscular junction could induce several central and peripheral symptoms, leading to the centrally mediated respiratory dysfunction such as SA.

The current study revealed a significant increase in the SA index in the young adult rats whose mothers were exposed to CPF and/or HFD. SA syndrome is demonstrated by repetitive episodes of breathing cessation during sleep resulting in adverse consequences including neurocognitive dysfunction, and metabolic disturbances⁴⁰. The dysfunction of the ventilatory control from the CNS to the respiratory muscles and the peripheral obstruction of the upper airway are two pathophysiological mechanisms that account for apnea incidence⁴¹. Accordingly, the decrease in the frequency of respiration, without any changes in the respiratory pattern of each breath as manifested by unchanged T_I and T_E could be

correlated to increased SA index observed in these groups. A 65% increase in SA index was reported by Darwiche et al.²⁵ in rats exposed to CPF (1 mg/kg/day) through gestation and postnatal periods. In our study, the increase in the SA was higher (around 100%) in CPF exposed rats. This incremental increase can approve the concept of DOHaD where maternal exposure to environmental toxicants can affect the life of the offspring even if they are not exposed to the same toxicants as shown in our study. Appeal episodes observed following acute OPs exposure are attributed to either central effects caused by the AChE inhibition in the respiratory centers^{39,42} or to peripheral effect through airway obstruction⁴³. It has been documented that the risk of SA increases with a higher body mass index resulting from fat deposition at the upper airways and subsequent peripheral obstruction⁴⁴. Even though SA is associated to metabolic disturbances in obese populations, in our study, maternal HFD feeding showed an increase in SA incidence associated with slight increase in the body weight. Our findings are similar to previous studies where HFD given directly to adult rats for five weeks induced an increase in SA index in rats despite the absence of obesity. This increase was attributed to the metabolic disruptions, mainly insulin resistance, resulting in ventilatory dysregulation^{11,12}. Moreover, evidence suggests a correlation between SA and the increased intake of saturated fatty acids in overweight patients⁴⁵. Remarkably, in our study, we showed that the mothers consuming HFD before and during pregnancy and throughout lactation expose their pups to a higher risk of apnea even though they are not exposed anymore to HFD after lactation. Not only the number of respiratory cessations episodes is increased, but also, the duration of apnea episode is also affected. The longer the duration of apnea, the higher the amount of CO₂ will be accumulated in the body. Then, the combined effects of hypercapnia and hypoxia and the perturbations in sleep can increase the degree of sympathetic activation which could lead, in turn, to elevated blood pressure⁴⁶. Interestingly, our results showed that the combined maternal exposure to both CPF and HFD resulted in an increase in the SA index as compared to controls. This increase was similar to both CPF and HFD groups speculating that the combination of both CPF and HFD will not exaggerate the effects observed in each group apart.

Otherwise, respiration is a distinctly coordinated process that necessitates the integration of motor function from different musculature, particularly the diaphragm⁴⁷. We aimed to figure out the observed *in vivo* respiratory alterations by measuring, *in vitro*, the contractile

performance of the diaphragm. Our results showed an increase in the twitch tension in CPF group without significant alterations in the time to peak tension and half-relaxation time. Consistent with our previous findings in rats exposed in utero and postnatally to CPF ²⁵, the increase in the twitch tension of the diaphragm was attributed to lower AChE activity as revealed in our study although our rats were not in direct contact with CPF. CPF, widely known to cross the foeto-placental barrier⁴⁸, exerts its toxicity through the inhibition of AChE enzyme inducing elevation of ACh at the post-junctional receptors. The resulting overstimulation of the motor-end plates could explain the increase in the diaphragm contractility⁴⁹.

Furthermore, the diaphragm contractility was increased in the rats at PND60 accompanied by reduction in half-relaxation time following maternal HFD exposure. The impacts of HFDinduced obesity on the contractile performance of the diaphragm have been assessed in different studies. By contract to our results, HFD was shown to induce a decrease in normalized work-loop power⁵⁰, and in the normalized isometric force of the diaphragm which could be related to the accumulation of body fats⁵¹. These contradictory results can be elucidated by the differences in the HFD exposure protocol. In our study, the rats are not directly exposed to HFD in addition to the fact that the pups assessed at PND60 didn't show obesity. This increase in the contractile force could be attributed to the reduced AChE activity resulting in the prolonged action of the cholinergic transmission. In our study, we cannot figure out the impact of direct HFD consumption on the activity of AChE in the diaphragm since our rats were not directly exposed to HFD. A decrease in the AChE activity has been previously reported in different regions of the brain in rats fed directly HFD for seven days or six months as a result of elevated free fatty acids. These findings suggest that HFD can induce cholinergic alterations mainly by inhibiting ACh degradation^{52,53}. To confirm our results, the amount of free fatty acids could be further quantified in the diaphragm and the AChE activity could be also assessed in the diaphragm of rats exposed directly to HFD.

Alternatively, our results showed that the combined exposure to HFD and CPF induced an increase in the twitch tension compared to controls accompanied by lower AChE activity, and this increase is similar to that observed after the exposure to either CPF or HFD. Despite no changes in the tetanic force, the exposure to CPF was associated with increased

fatigability of the diaphragm, thus reducing its ability to sustain the force of contraction. The increased fatigability could be attributed to ACh accumulation at cholinergic synapses due to lower AChE activity in the diaphragm, similar to Darwiche et al.²⁵. However, elevated tetanic contractions were observed in the HFD group without any changes in the fatigability index. Indeed, the AChE gene expression is the basis for cholinergic neural transmission in the central and peripheral nervous system⁵⁴. As the perigestational exposure to CPF and/or HFD was associated with lower AChE activity, consequently the inhibition of AChE activity could further induce the expression of AChE in a way to compensate the reduced enzymatic activity, as shown in our study by greater mRNA expression level of AChE observed in the diaphragm⁵⁵.

It is worth mentioning that a greater level of diaphragm contraction might be related to the lower respiratory frequency observed in the rats. Thus the diaphragm contractile force could be increased in a way to overcome the elevated apnea episodes resulting from upper airway obstruction²⁵, and to compensate for the subsequent lower respiratory rate. Increased respiratory efforts could be made against occluded airway and diaphragm fatigue has been shown to be a possible consequence of the chronic overload made on the diaphragm in obstructive SA^{56,57}.

The modifications observed in the contractile force of the diaphragm might reflect changes in fiber type composition. Muscle fibers are generally classified into type I (slow-twitch) and type II (fast-twitch) fiber types with different fatigability characteristics⁵⁸. Due to the specialized physiological demands placed, the diaphragm, a neither typical fast-twitch nor slow-twitch muscle, is characterized by plasticity; ability to regulate myofibrillar gene expression in response to various environmental cues⁵⁹. Consequently, the expression of MHC isoforms, representing the major protein in skeletal muscle, was examined. In CPF group, the increase in the twitch tension was associated with increased myofibrillar protein content. This increase in the myofibrillar proteins could be correlated with an increase in the expression of both MHC isoforms, MHCI and MHCII, thus contributing to increased force of contraction. As type I fibers are resistant to fatigue and type II fibers are easily fatigable⁵⁹, then the greater increase in the expression of MHCII could contribute to the increased diaphragm fatigability. On the other side, in HFD group, the expression of MHCI isoform was significantly increased without significant changes in MHCII isoform indicating a shift toward slow fiber type, which also could be correlated to the increase in the twitch tension reported in the diaphragm. A similar fast-to slow shift in MHC phenotype associated with obesity has been reported in the diaphragm indicating an increase in oxidative capacity⁶⁰. Such changes could contribute to the delay of fatigue during periods of prolonged contractile activity. The increase in the twitch tension revealed in our study without increasing the fatigability index could confirm the switching. In HFD+CPF group, the increase in the myofibrillar protein contents was associated with an increase in the expression of MHCI without modifications in the expression of MHCII. This increase could be correlated with a slight increase in the twitch tension without any change in the fatigability index.

Since optimal contractile function necessitates the maintenance of Ca²⁺ homeostasis and as the contractile function of the diaphragm muscle is altered in our study, then the expression of proteins involved for Ca²⁺ release-reuptake by the sarcoplasmic reticulum (SR) has been examined. A significant increase in the expression of RyR1 was observed with the perigestational exposure to either CPF and/or HFD compared to controls. Then the increased expression of RyR1 receptor will probably induce, subsequently, the release of greater amounts of Ca²⁺ ions from the SR, thus increasing the myofibrillar Ca²⁺ concentrations. Such greater Ca²⁺ concentrations could contribute in turn to the increase in the force generation. An increase in RyR1 expression was observed in atrophied soleus muscles only after 4 weeks of hindlimb unloading associated with the transformation of the SR from a slow to a fast phenotype⁶¹. As the myosin heavy chain expression has been shown to be increased in each of CPF and HFD groups, such alterations would, collectively, have induced an increase in the contractile force as reported in the diaphragm.

Likewise, the Ca2+ release from the SR depends on the Ca2+ stores uptaken from the cytoplasm through SERCA; consequently, the contractile function of the diaphragm could be affected by the expression levels of SERCA pump isoforms and by the Ca2+ uptake function. Since SERCAI is predominantly expressed in fast-twitch muscles, and SERCAII is expressed in slow-twitch skeletal muscles, then the total expression levels of SERCA are manifested by the assessment of both isoforms levels in the adult diaphragm⁶². A significant increase in the expression of SERCAI and SERCAII was reported in HFD group.

Since muscle relaxation depends on Ca²⁺ reuptake to SR⁶³, which consequently depends on the transport activity of the SERCA pump, then the decrease in the half-relaxation time observed in the HFD group could be possibly related to the increase in the expression of SERCA pump.

By contrast, previous studies have shown an association between the impaired diaphragm contractility observed in septic rats and reduced RyR1 expression. Besides, the slowed relaxation of the diaphragm was associated with the reduced expression of SERCAI and SERCAII isoforms⁶⁴ and reduced Ca²⁺ uptake function⁶². Decreased SERCAI expression could depress the maximal rates of SR Ca²⁺ uptake and by consequence its release⁶⁵. This could contribute to the reduction of maximal rates of both force development and output as a result of decreased Ca²⁺ release from the SR as shown in mice with targeted disruption of the ATP2A1 gene⁶⁶ exacerbating, in turn, diaphragm contractility impairment⁶⁴. The fact that the effect of HFD was the opposite indicates that elevated expression of RyR1 and SERCA isoforms and the consequent possible altered SR Ca²⁺ release/uptake function might contribute to the increased contractile force of the diaphragm.

In conclusion, the maternal exposure of rats to CPF and/or HFD during the perigestational period could program the risks for altered respiratory performance and contractility characteristics of the respiratory diaphragm muscle in their offspring at early adulthood although they are not in direct contact to either CPF and/or HFD. The increased SA incidence and diaphragm contractile activity during early adulthood could be related to prolonged cholinergic activity, altered SR calcium release/uptake, and modifications in the expression of myosin heavy chain isoforms.

4. Methods

4.1. Chlorpyrifos preparation

Chlorpyrifos (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) with purity of 99.8% was supplied by LGC Standards (Molsheim, France). Chlorpyrifos was dissolved in organic rapeseed oil (MP Biomedicals, Illkirch, France) as a vehicle and administered by gavage to the rats at 1mg/kg/day (the oral NOAEL for inhibition of brain cholinesterase activity in rats⁶⁷) for CPF groups. Controls received only vehicle.

4.2. Experimental Design

The experiments were performed according to the protocol approved by the nationally accredited Regional Directorate for Health, Animal and Environment Protection (Amiens, France) and the French Ministry of Research (reference number APAFIS#8207-2016121322563594 v2). All animals were treated according to the European Communities Council's guidelines (2010/63/EU).

Sixteen female Wistar rats were obtained from Janvier Labs (Le Genest Saint Isle, France, age on arrival in the facility: 7 weeks). The animals were housed in NexGen Max cage system mounted on EcoFlow rack system (Allentown Inc, Bussy Saint Georges, France) under constant conditions in a controlled-air- temperature room (23°C), with a 12 hours light/dark cycle. After 1-week of acclimatization, female rats (body weight (mean ± SEM) 225 ± 4.9 g) were assigned randomly to 4 groups (n=4/group), and housed two per cage. The females were fed either standard chow diet (Serlab3436, 3.1 kcal/g) or high-fat diet HFD (Research Diets no. D12492, SSNIFF Spezialdiäten GmbH, 5.24 kcal/g, 60% kcal from fat) and force-fed daily with organic rapeseed oil (as a vehicle) for control (oil, standard diet) and HFD (oil, HFD) groups or CPF (1 mg/kg/day in organic rapeseed oil) for CPF (CPF, standard diet) and HFD+CPF (CPF,HFD) groups during 4 consecutive months. Then, the females were mated with the male rats (Janvier Labs, two females per male). The pregnancy was daily determined by the presence of spermatozoa checked with vaginal smears. During gestation, the dams were subjected to the same treatment as before gestation until the end of the lactation period. At weaning time (postnatal day (PND) 21), pups were separated from their mothers and 4 groups of male rats are formed as below:

- 1) Control group: Maternal exposure to standard diet with vehicle (oil) (n=10)
- 2) HFD group: Maternal exposure to HF diet with vehicle (oil) (n=10)
- 3) CPF group: Maternal exposure to standard diet with CPF (n=10)
- 4) HFD+CPF group: Maternal exposure to both HF diet and CPF (n=7)

Then after, rats in all groups received only standard diet without CPF until PND60 (early adulthood). At PND60 the respiratory parameters of all male pups were measured via whole-body plethysmography (n=7-10/group). These pups were then euthanized with sodium pentobarbital (EXAGON; Axience, France) injection (1 ml/kg; 200 mg/ml solution). The diaphragm and the adjacent rib cage were rapidly removed and directly placed in Krebs Henseleit solution (Sigma Aldrich, Saint Quentin Fallavier, France). Rectangular strips

were dissected for *in vitro* contractility assessment, while the remainder of the diaphragm was preserved at -80°C for AChE activity measurement, and genes expression evaluation.

4.3. Respiratory measurements

Whole-body plethysmography (Model PLY3213, Buxco-EMKA Technologies, Sharon, CT), was used to record the respiratory parameters of the rats at PND60. The Inspiratory time (T_I), expiratory time (T_E), tidal volume (V_T) (expressed per 100g body weight), and respiratory frequency (f) measurements were recorded for each animal according to the previously described protocol⁶⁸ using data acquisition and analysis software (LabChart 7, ADInstruments). The rats were allowed to acclimatize in the plethysmograph chamber for 60 min one day before the experimental day. On the next day, further acclimatization for 45 min preceded the measurements. During the 90 min measurement period, we continuously noted whether the rat was awake (i.e., lying or standing with opened eyes) or was asleep (i.e., lying down without movements with closed eyes). Sleep Apnea, defined as two missed breaths, was scored according to a previously described procedure^{12,69}. Sleep apnea index was calculated as the number of apnea episodes during one hour of behavioral sleep. The mean duration of an apnea episode was also measured. Respiratory measurements were calculated during sleep, excluding apnea episodes.

4.4. Diaphragm contractility

The muscle contractile properties were studied in rectangular strips of the costal diaphragm in which the rib margin and central tendon remained intact as previously described^{25,70}. The preparations were suspended vertically in a muscle bath containing oxygenated Krebs Henseleit solution maintained at 37 °C.

Muscle strips were stimulated directly using square wave pulses at 2 ms duration and the voltage was set to give supramaximal stimulation at 12 V. The length of the diaphragm strip was stretched to reach the maximum length L₀, defined as the optimal length at which maximal force was obtained. All the following measurements were made at L₀. The cross-sectional area (CSA) of each muscle strip was calculated by dividing the weight of the muscle (M) by the product of muscle density (ρ = 1.056 g.cm⁻³) and L₀ (CSA=M/ ρ x L₀).

4.4.1. Twitch measurements

Single twitches were elicited after equilibration. For each twitch, the peak twitch tension (measurement between baseline and peak force) was normalized by cross-sectional area and expressed in g.cm⁻².The time to peak tension (time to peak force (ms)) and the half-relaxation time (time for the force to decrease to one-half of its peak value (ms)) were also determined. The mean values for each strip were then calculated.

4.4.2. Measurement of tetanic force

The maximum tetanic tension was determined using 1s duration trains of electrical stimuli at 50 Hz. The amplitude between the baseline and the stable part of the tetanic plateau was measured and the values were expressed in g.cm⁻².

4.4.3. Measurement of fatigue

The diaphragm fatigue resistance was studied by applying a protocol of 2 Hz train of supramaximal stimuli for a 5 min period. The fatigability index was determined as the percentage of the difference between the initial force and final force obtained after 5 min of continuous electrical stimulation.

4.5. Acetylcholinesterase activity

The Acetylcholinesterase (AChE) activity in the diaphragm was measured by applying modified Ellman method⁷¹. The diaphragm samples were homogenized in mammalian cell lysis buffer 5X (ab179835; Abcam, Cambridge, UK) and then centrifuged. 1 μ L of protease inhibitor (Abcam) was then added to the obtained supernatant. The diluted supernatant (1:2) was incubated with 100 μ M of the butyryl cholinesterase inhibitor, tetra isopropyl pyrophosphoramide (Sigma Aldrich, Saint Quentin Fallavier, France) for 15 minutes before measuring the AChE activity using the AChE colorimetric assay kit (ab138871; Abcam, Cambridge, UK) according to the supplier's instructions. The AChE activity was corrected for protein content and expressed in μ mol/min/mg protein.

4.6. Myofibrillar Proteins Extraction and dosage

Frozen diaphragm muscles were homogenized with 5 volumes of washing buffer (20 mM NaCl, 1 mM EGTA (pH 6.4), and 5 mM PO4) and then centrifuged at 12000 rpm for 5 min. Then, the myofibrillar proteins were extracted with 3 volumes of extraction buffer (5 mM EGTA, 1mM di-thiothreitol (pH 8.5) and 100 mM sodium pyrophosphate), incubated overnight at 4°C with gentle mixing, and then centrifuged at 12000 rpm for 15 min. The supernatant was collected for the dosage of myofibrillar proteins content spectrophotometrically at 595 nm according to Bradford assay and using Bovine Serum Albumin as standard (Bio-Rad, Hercules, CA, USA)⁷².

2.7. Reverse transcription polymerase chain reaction

Total RNA were isolated from diaphragm samples using FastGene® RNA Basic Kit (Nippon Genetics Europe GmbH, Germany) according to the manufacturer's instructions. The total RNA concentration was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific) and 1 µg of RNA was used for cDNA synthesis with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). For transcript detection with quantitative real-time PCR (qRT-PCR), primers, TaqMan[™] Multiplex Master Mix was used according to the manufacturer's instructions (Thermo Fisher Scientific by Life Technologies SAS, Courtaboeuf, France) and PCRs were run on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems).

Gene-specific primers were purchased from Thermo Fisher Scientific as TaqMan^M Gene expression assays. Assays Rn01412977_g1 and Rn99999916_s1 were used for mRNA analysis of the housekeeping genes coding for β -actin and glyceraldehyde3-phosphate dehydrogenase (GADPH) and were used as endogenous controls. Assay Rn00596883_m1 was used for mRNA analysis of AChE, assays Rn01751056_m1 and Rn01470656_m1 for myosin heavy chain (MHC)I and MHCII isoforms respectively, Rn01545085_m1 for ryanodine receptor (RyR1 gene), Rn00589545_m1 for sarcoplasmic reticulum Ca²⁺ - transporting ATPase (SERCA)I (Atp2a1 gene), and finally assay Rn00568762_m1 for mRNA analysis of SERCAII (Atp2a2 gene).

PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min for hold stage followed by 40 cycles of denaturation for 15 sec at 95°C, and Annealing /Extension at 60°C for 1 min. All PCR reactions were done in duplicate. Results were presented in arbitrary units, using a

 $2^{-\Delta\Delta Ct}$ calculation compared to control samples ($\Delta\Delta Ct = \Delta Ct$ exposed – mean ΔCt control). The expression level of the studied genes were the same relative to β -actin and GAPDH mRNA, and only in the final analysis, all results were expressed relative to β -actin.

2.8. Statistical analysis

Statistical analysis was performed with StatView software (version 5.0, Abacus Concepts Inc., Berkeley, CA, USA). A two-way analysis of variance (ANOVA) was used to analyze the main effects of diet (control and CPF groups vs. HFD and HFD+CPF groups) or CPF (control and HFD groups vs. CPF and HFD+CPF groups) and the interaction between diet and CPF exposure. If the diet-CPF exposure interaction was significant, a non-parametric Mann-Whitney U test was performed. In all analyses, the threshold for statistical significance was set to $p \leq 0.05$. Indicative results (p<0.1) are shown when appropriate.

Data Availability

Raw data were generated at PERITOX UMR-I-01 University of Picardy Jules Verne, 80025, Amiens, France. Derived data supporting the findings of this study are available from the corresponding author, Jérôme Gay-Quéheillard, on request.

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Author contributions

All authors contributed to the study conception and design. H.E.K.E.S performed the experiments. H.E.K.E.S., W.D., and S.D prepared the materials. H.E.K.E.S., J.G.Q., W.H.J., W.D., and W.R analyzed and interpreted the data. H.E.K.E.S. wrote the main manuscript and all authors reviewed the manuscript.

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Additional Information

Competing Interests:

The authors declare no competing interests.

Tables

Table 1: Effects of CPF exposure, diet, and CPF-diet interaction on different parameters analyzed by two-way ANOVA. CPF or Diet effect: \uparrow (increasing) or \downarrow (decreasing): p<0.05; $\uparrow\uparrow$ or $\downarrow\downarrow$: p<0.01; $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow:$ p<0.01; $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow:$ p<0.01; $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow:$ p<0.01; $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.01; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.01; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.001; $\uparrow\uparrow$ or $\downarrow\downarrow$: p<0.01; $\uparrow\uparrow$ or $\downarrow\downarrow\downarrow$: p<0.01; $\uparrow\uparrow$ or $\downarrow\downarrow$: p<0.01; $\uparrow\downarrow$: p<0.01; $\downarrow\downarrow$: p<0.01; $\downarrow\downarrow$: p<0.01; $\uparrow\downarrow$: p<0.01; $\uparrow\downarrow$: p<0.01; $\downarrow\downarrow$: p<0.01;

Parameter		CPF Exposure Effect	Diet Effect	Interaction
Body Weight	PND3	NS	$\uparrow \uparrow \uparrow$	NS
	PND21	NS	$\uparrow \uparrow \uparrow$	NS
	PND60	\rightarrow	1	NS
Respiratory	Frequency (f)	\rightarrow	$\downarrow\downarrow\downarrow\downarrow$	***
parameters				
	T _I	$\downarrow \downarrow$	$\downarrow\downarrow\downarrow\downarrow$	NS
	T _E	NS	NS	NS
	Tidal Volume	\uparrow	NS	*
	(V_T)	•	•	
Sleep Apnea	Sleep Apnea Index	\uparrow	\uparrow	**
	Mean duration	NS	NS	*
	of an episode of			
	apnea			
Contractility	Twitch Tension	NS	NS	*
parameters		210	210	
	Time to peak	NS	NS	NS
	tension	NO	NO	<u>ب</u>
	time	NS	NS	^
	Tetanic Force	NS	\uparrow	*
	Fatigue Index	NS	NS	**
AChE activity		$\downarrow \downarrow \downarrow$	NS	**
Myofibrillar		$\uparrow \uparrow \uparrow$	NS	NS
protein content				
Genes	AChE	NS	$\uparrow \uparrow$	*
expression				
	MHCI	NS	$\uparrow\uparrow$	***
	MHCII	NS	\uparrow	**
	RYR	NS	$\uparrow \uparrow \uparrow$	*
	SERCAI	$\downarrow\downarrow$	\uparrow	NS
	SERCAII	$\downarrow\downarrow\downarrow\downarrow$	\uparrow	NS

Figures



Figure 1. Effects of perigestational exposure to CPF and HFD on the body weight of male rat pups 3 days after birth (PND3), as juveniles (PND21), and as young adults (PND60). Data are quoted as means \pm SEM (n=7-10/group). Effect of diet ***: p<0.001, †: p<0.1



Figure 2. Effects of perigestational exposure to CPF and HFD on the respiratory parameters in male rats at PND60. (a) T_I (s), (b) T_E (s), (c) f (bpm), (d) V_T (ml/100g body weight). Data are quoted as means \pm SEM (n=7-10/group). For a significant CPF exposure x diet interaction: *: p<0.05; **: p<0.01 vs. control, \ddagger : P<0.05 vs HFD



b



Figure 3. Effects of perigestational exposure to CPF and HFD on the sleep apnea in male rats at PND60. (a) Representative Plethysmograph signal showing an apnea episode. (b) Sleep Apnea Index expressed as the mean number of apnea episodes per hour of behavioral sleep. (c) Mean duration of an episode of apnea. Data are quoted as means \pm SEM (n=7-10/group). For a significant CPF exposure x diet interaction: *: p<0.05; **: p<0.01 vs. control



Figure 4. Effects of perigestational exposure to CPF and HFD on the contractility parameters in the diaphragm of male rats at PND60. (a) Twitch tension represented as a function of cross-sectional area (g/cm²), (b) time to peak tension (ms), (c) half-relaxation time (ms) (d) tetanic force, (e) fatigability index expressed as the percentage of the difference of force obtained after 5 min of continuous electrical stimulation. Data are quoted as means \pm SEM (n=7-10/group). For a significant CPF exposure x diet interaction: *: p<0.05; **: p<0.01; †: p<0.1 vs. control, ‡: p < 0.05 vs. HFD; §: p < 0.05 vs. CPF



Figure 5. Effects of perigestational exposure to CPF and HFD on AChE in the diaphragm of male rats at PND60. (a) AChE activity quoted as the mean \pm SEM expressed as µmol of acetylthiocholine hydrolyzed per minute per mg of proteins, (b) expression of AChE mRNA quantified by real-time PCR depicted as the mean \pm SEM expressed relative to the gene expression in the control according to $2^{-\Delta\Delta CT}$ method. For a significant CPF exposure x diet interaction: *: p<0.05; **: p<0.01; ***: p<0.001 vs. control. (n=7-10/group)


Figure 6. Effects of perigestational exposure to CPF and HFD on the myofibrillar protein content in the diaphragm of rats at PND60. Data are quoted as means \pm SEM expressed as mg of myofibrillar proteins per gram of muscle (n=7-10/group)



Figure 7. Gene expression analysis in the diaphragm of rats at PND60. (a) Expression of MHCI and MHCII, (b) expression of RyR1 mRNA coding for ryanodine receptor mRNA, (c) expression Atp2a1 and Atp2a2 mRNA coding for SERCAI and SERCAII pumps, quantified by real-time PCR. Data are depicted as the mean \pm SEM expressed relative to the gene expression according to $2^{-\Delta\Delta CT}$ method (n=7-10/group). For a significant CPF exposure x diet interaction: *: p<0.05; **: p<0.01 vs. control, §§: p<0.01 vs. CPF

2.2. Impacts of perigestational exposure to CPF and/ or HFD on the contractile response of the ileum in male rats at early adulthood

This study is being prepared for submission.

2.2.1. Summary:

The GI tract is the first compartment in contact with the ingested food and associated contaminants. This study investigated the effects of long term exposure of dams to either CPF and/ or HFD during critical and sensitive phases of the offspring development on the contractile response of the ileal smooth muscle at early adulthood.

This study was conducted according to the experimental protocol detailed in the materials and methods section. Female Wistar rats were exposed to CPF (1 mg/kg bw/day vs. vehicle) and fed with either HFD standard diet during the perigestational period starting from 4 months before gestation till the end of lactation period and the weaning of the rats at PND21. Then, the male pups were raised on standard diet without CPF. At PND60, ileal segments were used for *in vitro* contractile properties assessment by isolated organ bath system. Other segments were used for histological analysis, AChE activity measurement, and gene expression analysis by real-time PCR.

Our results showed that the exposure of dams to CPF during the perigestational period was associated with higher amplitude of contraction for both the longitudinal and the circular ileal smooth muscle in the male pups at PND60. The thickness of the circular ileal muscle was also increased. These changes were related to the alterations in the cholinergic and non-cholinergic transmission mediated by increased expression of the muscarinic m2 acetylcholine receptor mRNA and tachykinin (substance P) mRNA, respectively, and to the decrease in the expression of calmodulin in the ileum. Ileal contractility was not affected in the rats of HFD-fed mothers, however, the expression of tachykinin NK1 receptor mRNA was greater in the HFD group compared to controls. Furthermore, the exposure to either CPF and/or HFD induced a decrease in AChE activity in the ileum.

Therefore, the perigestational exposure to CPF, 4 months before gestation and during gestation till the end of lactation, affects the contractile function of the ileum. Ileal contractility was not affected in the rats of HFD-fed mothers despite elevated NK1 receptor expression and reduced AchE activity levels. Integrating this study helps in preventing chronic gut diseases in adults.

2.2.2. Article

Impacts of perigestational exposure to Chlorpyrifos (CPF) and High -Fat Diet

(HFD) on ileum contractility in male rats at early adulthood

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Abstract

Background: The perinatal period is characterized by strong plasticity and higher individual sensitivity to environmental factors through fetal programming. An association of junk food and early exposure to pesticide residues seems to be involved in the increase of pathologies such as obesity. This study established the effects of perigestational exposure to CPF and high-fat diet (HFD) on the contractility of smooth muscle in adult rats.

Methods: 4 groups of 4 female rats were exposed during 4 months before and later during gestation and lactation periods to CPF (1 mg/kg bw/day *vs.* vehicle) with or without HFD. After being sacrificed at the age of 60 postnatal days (PND60), ileal smooth muscle strips were used for *in vitro* contractility measurements in an isolated organ bath system. Other ileal segments were used for AChE activity assessment, genes expression evaluation, and histological analysis.

Results: At PND60, CPF exposure induced an increase in the ileal longitudinal and circular muscles contractility. The circular muscle thickness was also increased. CPF exposure was associated with greater expression of the tachykinin (substance P) and the muscarinic m2 acetylcholine receptor mRNA but lower expression of calmodulin mRNA in the ileum. Furthermore, the expression of tachykinin NK1 receptor mRNA was increased in HFD group compared to controls. The exposure to either CPF and/or HFD induced a decrease in AChE activity in the ileum.

Conclusion: Despite the lack of direct contact, early life exposure to CPF and /or HFD programs the ileal homeostasis and increase the risk of altered gut contractile function at early adulthood.

Keywords: Chlorpyrifos; high-fat diet; ileum; contractility

Introduction

The intrauterine life environment plays a pivotal role in programing the risks of adverse health outcomes at adulthood. According to the Developmental Origin of Health and Disease concept (DOHaD), well-established mechanisms include nutritional environment perturbations during prenatal and intrauterine life, as a critical candidate for fetal programming promoting irreversible long-term effects on development (Kappil, Wright, and Sanders 2016). Experimental and epidemiological studies have shown that on one hand junk food and other hand exposure pesticides residues are associated with altered fetal metabolism and increased risk in the development of obesity in adult life (Lecoutre et al. 2016; Howie et al. 2009).

The components of ingested food, including the natural ingredients, additives, and contaminants represent the major sources of chemical agents in contact with gastrointestinal (GI) tract. Emergent evidence from animal and human studies suggest the ability of western-style diet characterized by high intake of saturated fats in the contribution of endotoxemia through changes in GI function associated with low-grade systemic inflammation (Cani et al. 2007; Erridge et al. 2007). The consumption of hypercaloric diet in rats has been shown to reduce the rat intestinal contractility due to modified architecture of the intestinal smooth muscle (de Souza et al. 2018). As high-fat (HFD) intake is common among the pregnant women in western countries, recent concerns have been raised about obesity during pregnancy due to the well-recognized risk factors on the mothers and consequently on their infants. In this context, maternal HFD during gestation and lactation has been shown to inhibit intestinal development and disrupt the intestinal mucosal barrier (Xie et al. 2018). However, the impact of perigestational HFD intake on the contractile activity of the ileum in the offspring at adulthood has not been examined.

The gut represents the main point for the uptake of orally delivered chemicals, through internalization and subsequent interaction with other body organs and systems (Groh, Geueke, and Muncke 2017). Among the well-known environmental contaminants, Chlorpyrifos (CPF) is one of the most common used organophosphates insecticides. Its residues are frequently detected in food and drinking water (Arain et al. 2018). The primary toxic mechanism of CPF is

inhibiting acetylcholinesterase (AChE) at the central and peripheral nervous system and neuromuscular junctions leading to overstimulation of cholinergic synapses (Eaton et al. 2008; Timchalk et al. 2002). CPF exposure is associated with altered programming of fetal metabolism and long-term consequences on health (Slotkin, Brown, and Seidler 2005; Slotkin 2011). Preand postnatal exposure to CPF has been shown to increase the intestinal permeability (Joly Condette et al. 2014) and metabolic effects (Reygner et al. 2016) in the offspring at adulthood. Although the exposure to CPF from gestation and throughout lactation till early adulthood showed a weaker *invitro* contractility of the longitudinal ileal smooth muscle, the impact of long term perigestational exposure to CPF at low levels, prior and throughout pregnancy till the end of lactation, in the progeny at early adulthood has not been elucidated (Darwiche et al. 2017).

Since humans are exposed to multiple adverse stimuli simultaneously, then the combined exposure of pregnant women to different dietary risks mimic real-life exposure (Tsatsakis et al. 2019). Therefore, the present study investigated the consequences of continuous perigestational exposure to CPF and/or HFD, 4 months before gestation and during gestation till the end lactation periods on the contractile response of the ileal smooth muscle in young adult offspring maintained on a standard diet without CPF after weaning. We further sought to identify several mediators involved in the alteration of the ileum function.

Material and Methods

Chlorpyrifos preparation

Chlorpyrifos (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate; 99.8% pure) was purchased from LGC Standards (Molsheim, France). Chlorpyrifos was dissolved in rapeseed oil (MP Biomedicals, Illkirch, France) as vehicle at a dose 1 mg/ml and administered by gavage at 1 mg/kg body weight/day to the rats in the CPF group. The chosen dose corresponds to the oral NOAEL for inhibition of brain cholinesterase activity in rats (Cochran et al. 1995).

Experimental Design

The study protocol was approved by the nationally accredited Regional Directorate for Health, Animal and Environment Protection (Amiens, France) and the French Ministry of Research (reference number APAFIS#8207-2016121322563594 v2). All animals were treated according to the European Communities Council's guidelines (2010/63/EU).

16 female Wistar rats (Janvier Labs, Le Genest Saint Isle, France,) aged 7 weeks on arrival were housed in cages under constant conditions in a controlled-air- temperature room (23°C), with a 12 hours light/dark cycle. The rats were housed in NexGen Max cage system with 81 in $^{2}/523$ cm² floor area mounted on EcoFlow rack system (Allentown Inc, Bussy Saint Georges, France). After 1 week of acclimation period, the female rats, with average body weight 225 ± 4.9 g, were assigned randomly to 4 groups and housed 2 per cage (n=4/group). The females were fed either standard chow diet (Serlab3436, 3.1 kcal/g) or high-fat diet HFD (Research Diets no. D12492, SSNIFF Spezialdiäten GmbH, 5.24 kcal/g, 60% kcal from fat) and gavaged daily with organic rapeseed oil (as a vehicle) for control (oil, standard diet) and HFD (oil, HFD) groups or CPF (1 mg/kg/day in organic rapeseed oil) for CPF (CPF, standard diet) and CPF+HFD (CPF,HFD) groups during 4 consecutive months. At the end of these 4 months, the females were mated with the male rats (Janvier Labs, two females per male). Once the pregnancy was recognized (smear and presence of spermatozoa), the females were isolated in individual cages. During gestation, the dams were kept under the same conditions as before gestation until the end of the lactation period. At postnatal day (PND) 21, the weanlings were separated from their mothers and the male rats were categorized into 4 groups as below:

- 1) Control group: Maternal exposure to standard diet with vehicle (oil)
- 2) HFD group: Maternal exposure to HF diet with vehicle (oil)
- 3) CPF group: Maternal exposure to standard diet with CPF
- 4) CPF+HFD group: Maternal exposure to both HF diet and CPF

Then after, rats in all groups received only standard diet without CPF until the age of 2 months postnatal (n=7-10/group). At PND60, these pups were then euthanized with sodium pentobarbital EXAGON (Axience, France) injection (1 ml.kg⁻¹; 200 mg.ml⁻¹ solution). The ileum was rapidly dissected and directly placed in Krebs Henseleit solution (Sigma Aldrich, Saint Quentin Fallavier, France). The abdomen was then opened and segments of ileum of around 1

cm in length that are 5 cm proximal to the ileocecal valve were dissected and used for *in vitro* contractility assessment. Other parts were used for AChE activity measurement, gene expression and histological analysis.

Measurement of isolated ileum contractility

Longitudinal and circular ileum muscle strips were suspended under 1 g of tension in a 15 mL organ bath containing Krebs Henseleit solution of pH 7.4 (Sigma Aldrich, Saint Quentin Fallavier, France) oxygenated with 95% O_2 +5% CO_2 and maintained at 37 °C. The strips were then left to equilibrate in the bath for 30 min and the Krebs Henseleit solution was replaced every 15 min. The electric field stimulation EFS (100 V; 30 ms) was performed by an electric stimulator (model 2100, A-M Systems; Phymep, Paris, France) using two rectangular platinum electrodes placed 2 cm apart, parallel to the muscle strips, as previously described by (Darwiche et al. 2017). EFS induce ACh release from the myenteric plexus, promoting in turn muscle contraction (Takahashi, Ikemoto, and Ezaki 1999). The ileal strips were attached to a force transducer (model UF1; Pioden Controls Ltd, Newport, Isle of Wight, UK; precision ±10 mg), connected in turn to an isometric amplifier (HAZAX20208-01, Bionic Instruments; Phymep). A computerized data logger (MP100A-CE; Biopac Systems, Santa Barbara, CA, USA) was used to record the generated forces. The amplitude of contraction was analyzed with the peak analysis module in LabChart 7 software (AD Instruments, Oxford, UK) and normalized against the cross section area (CSA, in cm²) according to the following formula: CSA = $M/(L \times \rho)$, where m is the mass (g), L is the length (cm) and σ is the muscle density ($\rho = 1.056 \text{ g.cm}^{-3}$). Values were reported in g/cm^2 .

Acetylcholinesterase activity

The Acetylcholinesterase activity in the ileum was measured according to modified Ellman method (Ellman et al. 1961). The ileum samples were homogenized using a hand-held homogenizer in mammalian cell lysis buffer 5X (ab179835; Abcam, Cambridge, UK). Next, the ileum homogenates were centrifuged and 1 μ L of protease inhibitor (Abcam) was then added to the supernatant. Before measuring the AChE activity, the supernatant was then diluted (1:10)

and incubated with 10^{-5} M of butyryl cholinesterase inhibitor, tetra isopropyl pyrophosphoramide iso-OMPA (Sigma Aldrich, Saint Quentin Fallavier, France) for 15 minutes in order to obtain only AChE activity. The colorimetric assay kit (ab138871; Abcam) was used to measure the AChE activity according to the manufacturer's instructions. Briefly,50 µl of the reaction mixture containing assay buffer, 20X 5,5'-dithio-bis-(2-nitrobenzoic acid): (DTNB), and acetylthiocholine were added to 96-well plate containing 50 µl of the supernatant and incubated for 10 min. Then, the absorbance was measured with a microplate reader (ELx808; Biotek,Winooski, VT, USA) at 412 nm during 15 minutes. The AChE activity was normalized for the protein content, and the data were presented in µmol/min/mg protein.

Reverse transcription polymerase chain reaction

Total RNA was extracted from the ileum samples using FastGene[®] RNA Basic Kit (Nippon Genetics Europe GmbH, Germany) according to the supplier's instructions. After quantification of the total RNA concentration using NanoDrop 1000 spectrophotometer (Thermo Scientific), 1 µg of RNA was used for cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Courtaboeuf, France). For gene expression measurement with quantitative real-time PCR (qRT-PCR), primers were purchased from Invitrogen (Life Technologies, Saint Aubin, France), and SYBR™ Green Master Mix (Applied Biosystems[™]) was used according to the manufacturer's instructions and PCRs were run on an ABI Prism7900HT Real-time PCR System (Applied Biosystems[™]). The housekeeping gene coding for GAPDH was used as endogenous control. The oligonucleotide sequences of the primers are presented in Table 1.

PCR was performed under the following conditions: denaturation for 1min at 95°C, annealing temperature was 56°C for calmodulin and 60°C for other primers, and elongation for 2 min at 72°C. All PCR reactions were carried out in duplicate. The results were reported in arbitrary units using a $2^{-\Delta\Delta ddCt}$ calculation, relative to the controls according to the following equation ($\Delta\Delta$ Ct= Δ Ct exposed–mean Δ Ct control). The expression level of each gene was expressed relative to GAPDH.

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Histological analysis

Segments of ileum were fixed with formol, embedded in paraffin and cut to 5 μ m thicksections. Next, the sections were deparaffinized with xylene and further hydrated with successive baths of alcohol. The fixed sections were then stained with Mayer's hematoxylin/ eosin for histomorphometric analysis. The histological sections were observed under a light microscope attached with a camera and the digital images were acquired using Zen software (Carl Zeiss AG, Oberkochen, Germany). Then the Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to measure the thicknesses of both longitudinal and circular smooth muscles.

Statistical analysis

Statistical analysis was analyzed with GraphPad Prism[™] software (version 5.0; GraphPad Software Inc., San Diego, CA, USA). A two-way analysis of variance (ANOVA) was used to study the main effects of CPF (control and HFD groups vs. CPF and HFD+CPF groups) or diet (control and CPF groups vs. HFD and HFD+CPF groups) and the interaction between CPF and diet exposure. In case of significant CPF-diet interaction, a non-parametric Mann-Whitney U test was then applied. The threshold for statistical significance was set to p ≤0.05. Indicative results (p<0.1) are represented when needed.

Results

Body weight

The mean body weight of the male pups was studied at different developmental stages. At PND3 (3 days after birth) and PND21 (weaning), the mean body weight was significantly higher in HFD fed groups compared to standard diet fed groups (p<0.01). The increase in the body weight was still observed at early adulthood (PND60) (p = 0.0577). In addition, the mean body weight was lower in CPF groups as compared to the other groups not exposed to CPF (p<0.05) (Figure 1).

Effects of CPF and HFD on the contractility of the ileum in male rats at PND60

The effects of perigestational exposure to CPF and/or HFD and their interaction on the contractility of the ileum induced by electrical field stimulation were analyzed at PND60 (Figure 2). A main effect of CPF was observed. Compared to non-CPF groups, maternal CPF exposure was associated with a significant increase in the amplitude of contraction in the longitudinal muscle (p = 0.0018) and circular muscle (p = 0.0223) compared to controls. Despite a non-significant diet-CPF interaction for both muscles, the peak contractions of ileal longitudinal smooth muscle were higher in CPF group (p<0.05) and in CPF+HFD group (p<0.1) by 123% and by 149% respectively compared to the control group. For the circular muscle, the peak contraction was significantly higher in CPF+HFD group by 110% compared to the control group (p<0.01).

The activity of Acetylcholinesterase enzyme in the ileum

The perigestational exposure to HFD and CPF was associated with significant interaction for AChE activity in the ileum (p = 0.05). A main effect of CPF (p = 0.0198) and HFD (p = 0.0080) was also shown. AChE activity levels were significantly lower in HFD group (p<0.05) and CPF group (p<0.01) and by 26% and by 24% respectively compared to controls. A further decrease by 29% was also observed in CPF+HFD group (p<0.01 vs control) (Figure 3).

Gene Expression analysis of muscarinic AChR isoforms in the ileum

The effects of HFD and CPF exposure on the expression of muscarinic AChR isoforms were quantified by real-time PCR (Figure 4). A main effect of CPF was shown for m2 AChR mRNA. Indeed CPF exposure was associated with a significant increase in the m2 AChR mRNA compared to non-CPF groups (p = 0.0004). Even though the diet-CPF interaction for both m2 & m3 AChR mRNA was not significant, the ileal expression of m2 AChR mRNA was significantly 1.5- and 1.45- fold higher in CPF and CPF+HFD groups, respectively, compared to controls (p<0.001 for CPF and p<0.01 for CPF+HFD). The ileal expression of m3 AChR mRNA was significantly higher by 1.9 fold in CPF group compared to controls (p<0.05).

Gene Expression analysis of tachykinin (substance P) and neurokinin 1 receptor (NK1R) in the ileum

The effects of CPF and HFD and exposure on the expression of SP and its preferred receptor (NK1R) were quantified by real-time PCR (Figure 5). Despite a non-significant CPF-diet interaction for the expression of SP, a main effect for CPF exposure was observed. SP mRNA levels were significantly increased with maternal CPF exposure compared to non-CPF groups (p<0.05) (Figure 5A).

However, the interaction between CPF and diet tends toward significance for NK1R mRNA expression (p = 0.0953). The ileal expression of NK1R mRNA was significantly higher by 1.5 fold in HFD compared to controls (p<0.05) (Figure 5B).

Gene Expression analysis of calmodulin in the ileum

The ileal expression of calmodulin was studied by real-time PCR (Figure 6). Although a non-significant CPF-diet interaction for the expression of calmodulin, a main effect for CPF exposure was reported. Calmodulin mRNA levels were significantly decreased with maternal CPF exposure compared to non-CPF groups (p<0.05) (Figure 6).

Thickness of longitudinal and circular muscle layers in the ileum

The effects of HFD and CPF exposure on the thickness of longitudinal and circular muscles in the ileum are shown in Figure 7. Despite a non-significant CPF-diet interaction for both muscle layers, maternal CPF exposure was associated with a significant increase in the thickness of the circular ileal muscle by compared to non-CPF groups (p = 0.0476). However, no significant changes were observed for the longitudinal muscle layer.

Discussion

Neural development during the perinatal and early postnatal environment is highly affected by the maternal nutrient provision, a prominent candidate of fetal programming. Knowing that the susceptibility of pregnant women to the environmental factors is distinctly increased, in this study; we investigated the consequences of simultaneous maternal exposure to two major alimentary factors: OPs residues and HFD. Although recent studies on animal models have demonstrated the effects of HFD consumption on the metabolic disruptions induced by early-life OPs exposure, (Adigun et al. 2010; Lassiter et al. 2008; Slotkin 2011), no studies have examined the impacts of continuous long-term

maternal CPF and/ or HFD exposure starting from 4 months before gestation till the end of lactation on the GI smooth muscle function in the progeny at early adulthood.

In our study, only rats exposed during the perigestational period to HFD with or without CPF showed higher body weight at birth. Maternal HFD exposure assessed in animal models have reported contrasting results with some showing decreased body weight (Howie et al. 2009; Hartil et al. 2009; Cerf et al. 2005) while others reported unchanged body weight at birth (Guo and Jen 1995; Umekawa et al. 2015). These contradictory results could be attributed to the duration of maternal HFD consumption and the fatty acid composition of the HFD used among these studies. Since HFD can be transformed into the milk (Del Prado, Delgado, and Villalpando 1997), our rats reported an increase in the body weight at the end of lactation (PND21). Consistent with (Lecoutre et al. 2016) the elevated body weight was still observed at early adulthood (PND 60). On the other side, CPF exposure was associated with a decrease in body weight at adulthood (PND60) in agreement with (Darwiche et al. 2017). Since the effect was only observed at early adulthood suggest a prolonged effect of CPF toxicity through fetal programming.

The contractile activity of the longitudinal and circular muscle layers play a functional role in propelling the luminal contents of the intestine (Hill-Eubanks et al. 2011). As the GI tract is the primary compartment in contact with ingested food and associated alimentary contaminates, in this study, we examined the effects of prolonged early-life exposure to CPF and HFD on the contractile function of the ileal muscles. Our results showed that the perigestational exposure to CPF was associated with higher amplitude of contraction for both longitudinal and circular muscles of the ileum. By contrast, Darwiche et al. (2017) have recently shown a weaker contraction of ileal longitudinal smooth in rat pups exposed pre- and postnatally, throughout development, to a higher dose of CPF (5 mg/kg/day) at PND60. Noting that in Darwiche et al. (2017), the rats continue to be exposed to CPF after weaning until PND60. However, our rats stop to receive either CPF or HFD after weaning. This could explain the difference between the results presented in both studies. On the other side, the amplitude of contraction for both muscles was not affected by HFD exposure. These findings are in agreement with (Patten et al. 2004) reporting no changes in the maximal contraction of the ileum with increasing levels of dietary saturated fats supplementation.

It has been admitted that the architectural changes of the intestinal smooth muscle could affect the contractile response of the ileum (de Souza et al. 2018; Bertoni et al. 2008). In our study, the increase in the amplitude of contraction in the ileum observed with CPF exposure could be attributed to the increased thickness of the circular ileal smooth muscle. Indeed, the hypertrophic growth of the circular musculature leads to increased hyperresponsiveness to contractile mediators and thus increasing the efficiency of contraction (Bertoni et al. 2008). In agreement with Darwiche et al. (2017), ileal AChE activity was significantly decreased in the CPF group compared to controls despite that our rats are not directly exposed to CPF. In fact, CPF, which is known cross the placental barrier (Akhtar, Srivastava, and Raizada 2006), elicits its toxicity through inhibition of AChE enzyme at cholinergic synapses (Timchalk et al. 2002). Then, the accumulation of acetylcholine can overstimulate the postsynaptic cholinergic receptors (Eaton et al. 2008); accounting at least, in part, to the increase in the contractility of the ileum. Indeed, the muscarinic acetylcholine receptors (mAChR) within the muscle myenteric circuits play an essential role in controlling intestinal mediated muscles contraction through the direct effect of ACh on muscle reflexes (Harrington, Hutson, and Southwell 2010). M2 AChR activation mediates adenylate cyclase inhibition, whereas m3 AChR lead to phosphoinositide hydrolysis, resulting in the mobilization of calcium (Ca²⁺) and consequent muscle contraction (Griffin and Ehlert 1992). It has been demonstrated that cholinergic hyperstimulation can result in the desensitization of the muscarinic receptors in the ileum and brain (Michalek, Fortuna, and Pintor 1993). Our results showed that CPF exposure is associated with a significant increase in the expression of m2 AChR mRNA without significant changes in m3 AChR expression. Several studies have previously shown that Chlorpyrifos oxon (CPO), the active metabolite of CPF can bind directly to muscarinic receptors, in particular the m2 AChR, inhibiting the muscarinic agonist binding to cardiac (Howard and Pope 2002) and striatum (Bomser and Casida 2001) muscarinic receptors. Then the increase in the expression of m2 AChR observed in our study could represent an adaptive response to compensate for the possible decreased function of m2 AChRs (Lein and Fryer 2005).

On the other side, the perigestational exposure to HFD was associated with a significant decrease in the AChE activity in the pups at PND60 suggesting prolonged cholinergic

activation through inhibiting ACh degradation without modifications in the expression of m2 AChR. Although our rats did not directly ingest HFD, direct HFD feeding in rats has been shown to reduce the AChE activity in several brain areas. Such cholinergic alterations have been attributed to the increased levels of circulating energy molecules, such as free fatty acids (Kaizer et al. 2004; Morganstern et al. 2012).

Non-cholinergic excitatory neurotransmissions are mediated by tachykinins located in the central and peripheral nervous system. The tachykinin Substance P (SP) acts directly on smooth muscles contraction of both the circular and the longitudinal muscle layers via the binding to the neurokinin 1 receptor (NK1R) (Souquet et al. 1985). Thus, the expression of SP and NK1R has been studied in our rats. Besides the effects on cholinergic transmission, perigestational CPF exposure induced a significant increase in the expression of SP without modifications in the expression of the NK1R. Since the stimulation of the myenteric neurons induces the release of SP which can activate, in turn, other neurons and promote non-cholinergic intestinal smooth muscle contraction (Barthó and Holzer 1985), then the increase in the amplitude of contraction observed in our study could be related to the increase in the expression of SP in the ileum. However, the expression of NK1R was significantly elevated in the HFD group compared to controls without significant changes in the expression of SP. A growing body of evidence suggests the significance of SP and NK1R interactions in the response of adipose tissue to HFD and weight gain. Indeed, NK-1R antagonist has been shown to reduce weight gain and prevent fat accumulation in mice fed HFD for two weeks and promote weight loss in Diet-Induced Obesity (DIO) mice model (Karagiannides et al. 2008). In addition, less body weight has been shown to accumulate in mice genetically deficient in NK1R, suggesting the role of NK1R in the development of obesity (Karagiannides et al. 2011). Then, the increase in the expression of NK1R could alter the energy balance and induce the increase in body weight observed in our rats after the perigestational exposure to HFD.

The role of Ca²⁺ has been widely demonstrated as a critical regulator for smooth muscle contraction (Walsh 1994). In this context, the expression of calmodulin, a Ca²⁺ binding protein, has been studied in ileum samples. CPF exposure induced a decrease in the expression of calmodulin compared to groups that were not exposed throughout the perigestational period to CPF. In response to contractile stimuli and subsequent increased

cytoplasmic Ca²⁺, calmodulin activates the cross bridge cycling for the generation of contractile force in the smooth muscles (Webb 2003). The decreased calmodulin expression could be an adaptive response to the increased cholinergic and non-cholinergic stimulation of the ileum.

Therefore, in accordance with the DOHaD concept, the perigestational exposure to CPF is associated with increased ileal muscle contractility at adulthood via cholinergic and non-cholinergic mechanisms. Rats of HFD-fed mothers elevated NK1 receptor expression and reduced AChE activity levels. Despite the lack of direct contact, early life exposure to CPF and /or HFD programs the ileal homeostasis and increase the risk of altered gut contractile function at early adulthood.

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Tables

Table 1: The sequences of primers used in real-time (RT-qPCR)

Gene		Primer sequence
GAPDH	Forward	5'-GGTGCTGAGTATGTCGTGGAGT-3'
	Reverse	5'-ATTGCTGACAATCTTGAGGGAG-3'
M2 AChR	Forward	5'-CACGAAACCTCTGACCTACCC-3'
	Reverse	5'-ACAGTCCTCACCCCTAGGATG-3'
Substance P (SP)	Forward	5'-TGTTTGCAGAGGAAATCGGTG-3'
	Reverse	5'- GAACTGCTGAGGCTTGGGTC -3'
rat NK1R	Forward	5'- GGTACTACGGCCTCTTCTATTGC -3'
	Reverse	5'- CAGGAAGTAGATCAGTACAGTCACG-3'
Calmodulin	Forward	5'- GAATGGCACCATTGACTTCC-3'
	Reverse	5'- GTAGCCATTGCCATCCTTGT-3'





Figure 1: Effects of perigestational exposure to CPF and HFD on the body weight of male rat pups at birth (PND3), as juveniles (PND21), and as young adults (PND60). Data are quoted as means ± SEM (n=7-10/group). Effect of diet ***: p<0.01, £: p<0.1. Effect of CPF *: p<0.05



Figure 2: Effects of perigestational exposure to CPF and HFD on the in vitro peak contraction of ileal longitudinal and circular smooth muscles in male adult rats expressed as a function of cross-sectional area (g/cm2). Data are depicted as means ± SEM (n=7-10/group). Effect of CPF exposure *: p<0.05; **: p<0.01



Figure 3: Effects of perigestational exposure to CPF and HFD on AChE activity in the ileum of male rats at PND60. Data are expressed as the mean ± SEM (n=7-10/group). Effect of CPF *: p<0.05. For a significant CPF x diet interaction: #: p<0.05; # #: p<0.01 vs. control



Figure 4: The expression of muscarinic AChR isoforms quantified by real-time PCR in the ileum of rats at PND60. (A) m2 AChR mRNA levels, (B) m3 AChR mRNA levels. Data are quoted as the mean \pm SEM expressed relative to the control gene expression according to $2-\Delta\Delta$ CT method (n=7-10/group). Effect of CPF ***: p<0.001



Figure 5: The expression of SP mRNA coding for tachykinin (substance P) (A), NK1R mRNA coding for neurokinin 1 receptor (B) quantified by real-time PCR in the ileum of rats at PND60. Data are quoted as the mean \pm SEM expressed relative to the control gene expression according to $2^{-\Delta\Delta CT}$ method (n=7-10/group). Effect of CPF *: p<0.05. For a significant CPF x diet interaction: #: p<0.05 vs. control



Figure 6: The expression of calmodulin quantified by real-time PCR in the ileum of rats at PND60. Data are quoted as the mean \pm SEM expressed relative to the control gene expression according to $2^{-\Delta\Delta CT}$ method (n=7-10/group). Effect of CPF *: p<0.05



Figure 7: Effects of perigestational exposure to CPF and HFD on the on the thickness of the longitudinal and circular ileal smooth muscle layers in male rats at PND60. Data are expressed as the mean ± SEM (n=7-10/group). Effect of CPF *: p<0.05

General Discussion

The contraction of muscles is initiated by signals from the nervous system. These muscles, in turn, mediate the steadiness of the inner body compartments and the coordination of body movements. Such vital functions are controlled by the CNS and PNS (sympathetic, parasympathetic, and enteric).

ACh is a major excitatory neurotransmitter which intervenes in the control of respiratory and digestive functions, inducing the contraction of the striated diaphragm muscle and gastrointestinal smooth muscles.

Several physiological and environmental factors can affect the contractile performance of the muscles including ageing (Miller, Callahan, and Toth 2014), unbalanced nutrition rich in fat (de Souza et al. 2018; Hurst et al. 2019), and the exposure to electromagnetic waves (Martynyuk, Melnyk, and Artemenko 2016) or to residues of environmental pollutants such as pesticides (Darwiche et al. 2017; Thiermann, Eyer, and Worek 2010; Darwiche et al. 2018). Indeed, the alimentary factors and associated contaminants represent critical cues that pose health risks for individuals during different stages throughout their life starting from the perinatal period, which represent highly sensitive phases of development and throughout the postnatal period. In addition, the direct exposure is also critical through adulthood.

In this context, the first objective of this study was to determine whether continuous exposure during adulthood to CPF, an AChE inhibitor organophosphorus insecticide, affects the contractile function of the diaphragm in rats.

Previous studies in our laboratory have shown that the continuous *in utero* and postnatal exposure to CPF alter the respiratory and digestive function in rats during development (Darwiche et al. 2017; Darwiche et al. 2018). The exposure was continuous starting from the first day of gestation and through lactation and development until the age of PND60 (early adulthood), illustrating the effects of indirect (pregnancy and lactation) and direct exposure (post-weaning) to CPF.

Then the second objective was to further investigate the long-term consequences of maternal perigestational exposure to CPF, HFD, or the simultaneous exposure to both

factors (CPF and HFD), starting from 4 months before gestation and throughout gestation until the end of lactation period. In our study, the respiratory function and the contractility of respiratory and GI muscles were analyzed in the offspring at early adulthood that were not subjected to CPF and/ or HFD after weaning. According to DOHaD, such environmental perturbations during early life can result in long-term consequences during development through fetal programming, predisposing offspring to further diseases in adulthood (Gluckman and Hanson 2004). Studies have shown that adverse early life nutrition is associated with the development of obesity and metabolic disturbances at adulthood (Howie et al. 2009; Alfaradhi et al. 2016). Our protocol is different from the previously conducted work in our laboratory as it illustrates the effects of indirect exposure to CPF as well as HFD and the combined exposure to both factors in the young adult offspring for the first time.

Effect of dietary CPF exposure in adult rats:

The oral route is considered the primary route for non-occupational exposures through the residues of these pesticides present in food and drinking water at low doses. In particular, CPF has been widely detected in the food chain. The long-term exposure to CPF residues poses several health risks to the general population. In this study, the dietary exposure to CPF closely mimics the real-life exposure and the chosen doses have been used in the assessment of potential chronic dietary exposure to CPF (Hallal et al. 2019).

Although CPF neurotoxicity has been widely investigated in the CNS and PNS, fewer insights have been drawn at the level of the neuromuscular junction. Then in our study, the effects of CPF exposure have been assessed in the diaphragm, a highly specialized respiratory muscle essential for breathing. The diaphragm contraction is specialized for its continuous rhythmic fashion throughout life without any period of rest (Polla et al. 2004).

We observed a decrease in the mean body weight in rats exposed to CPF only at 5 mg/kg/day compared to controls without significant modifications of rats exposed to CPF at 1 mg/kg/day. This decrease in the body weight persists until the end of the exposure period, suggesting an index of toxic effects. CPF exposure can increase the lipid

peroxidation and the oxidative stress which could lead to the lower body weight in CPF5 group (Mansour and Mossa 2010; Kaur, Singla, and Dhawan 2019). However, our rats showed increased serum levels of GH which is known as a strong metabolic agent. Mazza et al. (1994) have reported a similar increase in GH levels following exposure to pyridostigmine, an AChE inhibitor, suggesting the stimulatory role of ACh on GH secretions. This increase could also be attributed to the endocrine disruptor effect of CPF.

Our results showed that CPF exposure at both doses altered the contractile force of the diaphragm associated with lower AChE activity. Indeed, the amplitude of the twitch tension was increased associated with increased time to peak tension and half-relaxation time. The tetanic force was also elevated. These changes came along a decrease in the AChE activity of the diaphragm, illustrating cholinergic alteration at the neuromuscular junction. Several studies have reported the effects of OPs on the muscles contractility. Ivanović et al., (2016) showed a similar increase in the diaphragm contractility after the exposure to Diazinon, an OP insecticide. The inhibition of AChE by an anticholinesterase drug, ecothiopate, showed a similar enhancement of the twitch, as observed in our study, due to prolonged action of ACh at the motor-end plate (Burd and Ferry 1987). CPF exposure has been shown to alter the contractility of different skeletal muscles. CPF increased the contractility of soleus muscle at both doses (1 and 5 mg/kg/day). However, the contractility of extensor digitorum longus (edl) was decreased at the lower dose and increased at the higher dose of CPF (Hallal et al. 2019). A similar increase in the diaphragm amplitude of contraction has been recently shown by Darwiche et al. (2018) in adult rats exposed to 5 mg/kg/day of CPF during gestation and postnatal period. This increase was associated with decreased AChE activity in the diaphragm. The consequent accumulation of ACh at the cholinergic synapses could explain the increased diaphragm contractility.

In agreement with Darwiche et al. (2018), we observed an increase in the diaphragm fatigability index defined as a decline in contractile function after work in the CPF groups. It could be attributed to the prolonged cholinergic stimulation of the diaphragm following the inhibition of AChE.

The alteration in the contractile properties could be related to different parameters including the changes in Excitation-contraction coupling mechanism (ECC) and changes in the myofibrillar protein content. We have shown that the increase in the myofibrillar proteins content in the diaphragm of CPF5 group correlates with the increase in the contraction. However a decrease in the amount of myofibrillar proteins was observed in CPF1 group compared to CPF5 group. A similar decrease in the myofibrillar protein content has been shown to correlate with higher contraction of soleus muscle indicating an adaptation mechanism in the muscle fibers following the exposure to CPF (Hallal et al. 2019). The increased force of contraction could be related to other changes at the level of Ca^{2+} homeostasis mechanism including altered expression of proteins involved in Ca^{2+} release (ryanodine receptor) and/or uptake (Ca^{2+} ATPase / SERCA pump) by the SR.

We have shown that CPF exposure at 1 mg/kg/day induced a significant decrease in the expression of the slow MHC I isoform associated to an increase in the MHC IIA isoform, suggesting the plasticity of respiratory muscle fiber to shift toward fast and more fatigable type (MHC IIA isoform). This could refer in turn to the increase in diaphragm fatigability.

Regarding the endocrine regulation of muscle function, CPF exposure induced a significant decrease in the serum testosterone levels in agreement with previously reported studies at higher CPF doses (Joshi, Mathur, and Gulati 2007; Peiris and Dhanushka 2017). In fact, CPF can inhibit the LH and FSH secretions from the pituitary gland, decreasing in turn testosterone biosynthesis in the testis. Since testosterone is essential for muscles development and maintenance (Sinha-Hikim et al. 2006), then the lower testosterone levels observed in CPF groups could also account for the increased fatigability index of the diaphragm. Besides, the serum cortisol levels were increased in CPF groups in agreement with Güven et al. (1999) who showed elevated cortisol levels in patients with OPs poisoning. Anticholinesterases can modify the pituitary-adrenal axis inducing increased cortisol levels. Prolonged cortisol secretions can lead to muscle weakness (LaPier 1997).

Effects of perigestational exposure to CPF and/or HFD

The sensibility of pregnant women to different environmental factors is highly elevated during pregnancy. In turn, the developing offspring is substantially affected by maternal health and the composition of the diet. The exposure to primary alimentary factors and related pollutants, HFD and CPF residues, has been elucidated in this study.

We observed a higher mean body weight in rats exposed during the perigestational period to HFD at birth as compared to non-HFD exposed animals. Animal models showed contradictory results on the effects of maternal exposure to HFD on the body weight of pups at birth. While lower birth weight was reported by some studies (Hartil et al. 2009; Cerf et al. 2005), others showed no changes in the body weight at birth (Guo and Jen 1995; Umekawa et al. 2015). These discrepancies among the studies could be referred to several factors including the duration of the maternal exposure to HFD, the content of lipids in the diet, and the composition of the fatty acid in the HFD.

Since the high-fat content can be transformed into the milk increasing in turn the lipid concentration and composition in the milk, our rats showed elevated body weight in HFD weanlings (Del Prado, Delgado, and Villalpando 1997). In agreement with Lecoutre et al. (2016) the increase in the body weight continue to be observed at early adulthood (PND60) where the rats were not subjected to any treatment after weaning. It could be related to their programmed metabolism during *in utero* and lactation periods.

CPF exposure was associated with lower mean body weight in rats at early adulthood. The fact that the effect was only observed at PND60 indicates a prolonged programming effect of CPF on body weight. A similar decrease in the body weight in rat pups exposed during prenatal and postnatal periods, and throughout development, to a higher dose of CPF at PND60 (Darwiche et al. 2018). The effect of CPF on body weight was also observed in several studies (Mansour and Mossa 2010; Kaur, Singla, and Dhawan 2019), who reported that the reduced body weight in CPF exposed rats might be related to increased lipids and proteins degradation and the level of oxidative and cholinergic stress induced by CPF (Goel, Chauhan, and Dhawan 2000; Maurissen et al. 2000).

The perigestational exposure to CPF and/or HFD showed a decrease in the respiratory frequency in terms of breath per minute. These results are consistent with the reported data on the effects of OPs by acute exposure (Villa et al. 2007) or chronic prenatal and postnatal exposures (Darwiche et al. 2018). However, in our study, T_I and T_E were not affected. On the other side, the decreased respiratory frequency was associated with a decrease in the V_T indicating a lower total volume of inhaled and exhaled air during a breath. Overweight and obesity can increase the risk of respiratory symptoms (Zammit et al. 2010). Indeed, adipose tissues produce different mediators which generate a proinflammatory state contributing in turn to reduced lung function (Hancox et al. 2007). The lung volume and capacity have been shown to be decreased in obese individuals associated with restrictive respiratory pattern (Melo, da Silva, and Calles 2014). The reduced V_T, in our study, could be linked to the elevated body weight of the rats, although not obese, as a consequence of possible metabolic perturbations and the related proinflammatory state as a result of early-life nutritional perturbations. This finding suggests developmental impacts that could have affected the development/maturation of lungs. It might be also related to the central control of respiratory mechanisms that modulate the volume of breathing. Ramadan et al. (2006) have reported impaired ventilation in rats fed HFD suggesting impaired respiratory control which regulates the respiratory rhythm.

Since ACh is the major primary neurotransmitter for the control of respiration at both CNS and PNS (Carey, Dunn, and Gaspari 2013), then the cholinergic perturbations can result in respiratory disruption such as sleep apnea. Our results showed that perigestational exposure to CPF and/or HFD significantly increased the sleep apnea index in young adult rats. Two pathophysiological mechanisms could account for apnea: CNS dysfunction of the ventilatory control to the respiratory muscles and peripheral obstruction of the upper airways (Kryger 2000). In our study, the reduced respiratory frequency observed in these groups, without any alteration in the respiratory pattern of each breath (unchanged T_I and T_E), can be correlated to the increased sleep apnea index.

Although our rats are not in direct contact with CPF, the increase in sleep apnea index was higher than that observed in Darwiche et al. (2018), in which developing rats were exposed

to CPF through gestation and postnatal periods, approving in turn the DOHaD concept. The apnea observed after acute exposure to OPs could refer to either inhibition of AChE in the respiratory centers (Carey, Dunn, and Gaspari 2013; Gaspari and Paydarfar 2011) or a peripheral effect of airways obstruction (Proskocil et al. 2010).

On the other hand, the risk of sleep apnea development increases at a higher body mass index. Indeed, increased fat deposition can promote upper airways obstruction (Lettieri, Eliasson, and Greenburg 2008). It is widely admitted that sleep apnea is linked to metabolic perturbations in obese people. However, the higher sleep apnea index observed in our pups is linked to a slight increase in body weight. This finding is consistent with Ramadan et al. (2006; 2007) reporting a similar increase in apnea episodes in adult rats directly fed HFD for five weeks. We have shown that pups from dams exposed during perigestational period to HFD presented higher sleep apnea index even though they are not exposed to HFD after weaning. Moreover, the mean duration of apnea episode was also longer in the HFD group compared to controls. The combined exposure to both CPF and HFD induced a similar increase in sleep apnea index as CPF or HFD alone, indicating that the effect is not exaggerated.

The diaphragm contractile properties were studied *in vitro* in order to figure out the observed *in vivo* respiratory findings. We observed a significant increase in the twitch tension and diaphragm fatigability in CPF group which could be ascribed to the lower AChE activity and consequent cholinergic hyperstimulation. Although our rats are not directly exposed to CPF, these findings can confirm the effect of CPF on diaphragm contractility as shown in our adult rats that were directly exposed during 6 weeks to CPF (explained above) and to Darwiche et al. (2018) where the rats were exposed to CPF starting from gestation and throughout postnatal period. CPF and its metabolites are found in biological fluids such as amniotic fluid indicating the ability to cross the placental barrier (Akhtar, Srivastava, and Raizada 2006) and has been shown to be found in breast milk (Mattsson et al. 2000).

The maternal exposure to HFD increased the twitch tension of the diaphragm and reduced the half-relaxation time in HFD group. These findings were associated with lower AChE activity, thus suggesting cholinergic overstimulation. Although our rats are not in direct contact to HFD, direct HFD consumption resulted in lower AChE activity in different brain regions as previously reported in rats (Kaizer et al. 2004; Morganstern et al. 2012). Decreased AChE activity has been correlated to elevated free fatty acids in rats consuming HFD, affecting in turn the cholinergic function. In our study, the combined maternal exposure to HFD and CPF showed increased twitch tension associated with reduced AChE, similar to the effect observed in each of the CPF group and HFD group alone.

Thus, the perigestational exposure to CPF and/ or HFD decreased the AChE activity in the diaphragm. Consequently, the expression of AChE mRNA was upregulated in these groups as a possible feedback response to the reduced AChE activity as previously described by Somnuek et al. (2009).

The increased contractile force of the diaphragm observed in our study is correlated with the reduced respiratory frequency in the rats. In order to overcome the apnea episodes and the consequent decrease in the respiratory frequency, the diaphragm can contract with a higher force (Darwiche et al. 2018). The higher inspiratory efforts against obstructed airways could lead to diaphragm overload and fatigue in OSA (El-Kabir et al. 2003).

These modifications in the diaphragm contractility are reflected by alterations in the muscle fibers composition that are identified by MHC isoforms. CPF group showed an increased myofibrillar proteins content in the diaphragm associated with increased expression of both MHCI and MHCII isoforms which could lead in turn to the higher force of diaphragm contraction. Knowing that type II fibers are easily fatigable while type I fibers are more resistant to fatigue (Pickering and Jones 2002), the higher increase in the expression of MHC IIA isoform is correlated to the increased diaphragm fatigability, consistent with our results where CPF exposure at adulthood during 6 weeks increased the expression of MHCII isoform (discussed above). On the other side, only MHCI isoform expression was increased in the HFD group suggesting a shift toward slow fiber (type I). It is correlated to the increased twitch tension of the diaphragm without any changes in the fatigability. The combined exposure to CPF and HFD showed higher myofibrillar protein
content associated with increased expression of MHCI isoform, also contributing to the increased diaphragm contractility without alterations in the fatigability index.

Since proper diaphragm contraction relies on adequate Ca^{2+} homeostasis, we then studied the expression of proteins involved in Ca^{2+} release (RyR1) and reuptake (SERCA pump) by the SR. The perigestational exposure to CPF and/or HFD increased the expression of RyR1 receptor suggesting a possible increase in the amount of Ca^{2+} released from the SR, increasing, in turn, the amount of myofibrillar Ca^{2+} . This effect, in addition to the upregulation of MHC isoforms expression, could have resulted in the increased diaphragm contractility. The increased expression of both SERCAI and SERCAII in HFD is correlated to the decrease in the half-relaxation time since relaxation relies on the Ca^{2+} reuptake by the SR (Gollnick et al. 1991). In contrast to our results, Green et al. (2003) showed that downregulation of SERCAI could reduce the maximal rates of Ca^{2+} uptake by the SR and subsequently decrease Ca^{2+} release, thus exacerbating the impairment of the diaphragm. Therefore, the increased expression of RyR1 and SERCAI isoforms could contribute to the higher diaphragm contractility.

Regarding ileum, only perigestational exposure to CPF increased the amplitude of contraction of longitudinal and circular ileal smooth muscles in the male pups at PND60. The contractile activity of these muscles is essential for propelling the intraluminal contents. This contrasts Darwiche et al. (2017) who reported lower contraction of the ileal longitudinal muscle in young adult rat pups exposed during gestation and throughout lactation till early adulthood to CPF at a higher dose (5 mg/kg/day). This discrepancy could be elucidated by the fact that our rats did not receive CPF after weaning. The digestive tract of the rat is therefore not in direct contact with the CPF molecule. However, the perigestational exposure to HFD did not alter the ileal muscle contractility similar to Patten et al. (2004). The increased thickness of the ileal circular muscle is correlated to the increased contractility. Bertoni et al. (2008) have shown that circular muscle hypertrophy is associated with increased motor response to contractile mediators resulting in increased contractile efficacy.

Consistent with Darwiche et al. (2017), ileal AChE activity was significantly reduced in the CPF group at PND60, although our rats are only exposed to CPF till weaning. This inhibition can result in the accumulation of ACh at the cholinergic synapses which could account for the observed increase in the ileal EFS-induced contractions. The consequent overstimulation of muscarinic receptors leads to receptor downregulation and desensitization (Michalek, Fortuna, and Pintor 1993; Abdollahi and Karami-Mohajeri 2012). The active metabolite of CPF (CPO) can directly bind to m2 AChR and inhibits agonist binding in the heart (Bomser and Casida 2001) and striatum (Huff et al. 1994). Consequently, we showed a significant increase in the expression of m2 AChR without modifications in the expression of m3 AChR in the ileum, in a way to compensate for a possible reduction in m2 AChR function (Lein and Fryer 2005).

The AChE activity was also decreased in the HFD group without alterations in the expression of m2 and m3 AChRs indicating prolonged activation of cholinergic neurotransmission. Despite that our rats didn't directly consume HFD, the increased amount of free fatty acids in rats consuming HFD has been shown to decrease the AChE activity in different parts of the brain (Kaizer et al. 2004; Morganstern et al. 2012).

In addition to the effect of CPF on the cholinergic signal transmission, we have shown that CPF exposure significantly increased the expression of the tachykinin, Substance P (SP), without affecting the expression of NK1R. SP mediates the non-cholinergic contraction of the intestinal smooth muscles by binding to its preferred receptor, NK1R, but also to a lesser extent to NK2R and NK3R (Souquet et al. 1985). Increased expression of SP can lead in turn to the increased amplitude of contraction of ileal muscles.

We have shown increased expression of NK1R in the HFD group without alterations in the expression of SP. The interaction between SP and NK1R has been shown to have an essential role in the development of weight gain in response to HFD. The administration of NK1R antagonist reduced weight gain in rats and prevented the accumulation of fats in mice after HFD feeding for 2 weeks and resulted in the loss of body weight in mice with Diet-Induced Obesity (DIO) model (Karagiannides et al. 2008). Consequently, the increased

expression of NK1R could affect the energy balance in the rats contributing to the higher body weight observed in the pups following the maternal exposure to HFD.

Since Ca²⁺ plays a fundamental role in regulating smooth muscle contractility, then the altered amplitude of contraction in the ileum could be related to changes in calmodulin. As a response to elevated intracellular Ca²⁺, calmodulin initiates cross-bridge cycling resulting in smooth muscle contraction (Webb 2003). However, we have shown a lower expression of calmodulin associated with CPF exposure. This finding suggests a possible adaptation of the ileum to its increased stimulation at the cholinergic and non-cholinergic levels.

CONCLUSION AND PERSPECTIVES

This thesis work has highlighted the effects of the exposure to critical environmental and alimentary risks according to the themes associated to both laboratories. In particular, this study had 2 main approaches:

- First, we identified the effects of direct exposure to the widely used OP insecticide, CPF, at adulthood on respiratory skeletal muscle. This work is carried out in Rammal Hassan Rammal Laboratory (Lebanon) as a part of its research themes.

- Within the framework of one of its thematic research, Peritox laboratory focused the combined effects of two alimentary risks on the pregnant woman and the progeny. Thus, we further investigated the perigestational exposure of mothers to CPF associated to high-fat diet, another major alimentary factor, on the respiratory and intestinal motor function in the offspring at early adulthood through indirect exposure. In this new protocol, the digestive tract of the pups is almost not in direct contact with neither CPF molecule nor the HFD as the rats were not subjected to CPF and/or HFD after weaning.

Therefore, this study has elucidated the activity of muscles involved in vital body functions at adulthood after either a direct exposure of adults or an indirect maternal perigestational exposure, to alimentary risk factors.

Considering our findings, several insights on research could be considered:

1) Our results showed that CPF exposure to adults increased the contractility of the diaphragm, an essential skeletal muscle in breathing. The diaphragm fatigability was also increased, indicating the decreased capacity of the respiratory muscle to generate force. These contractility modifications due to chronic CPF exposure at adulthood could be related to cholinergic modification by inhibition of AChE activity, shift toward the expression of fast and more fatigable type MHC IIA isoform, and/or perturbation in anabolic/catabolic hormone balance. Other modifications in the excitation-contraction coupling mechanism could also result in increased diaphragm contractility. It would be interesting to further investigate the effects of CPF exposure during adulthood on the profile of breathing, by measuring the respiratory parameters in the rats.

2) On the other hand, our findings are in line with the DOHaD concept explaining the link between the environmental perturbations during early life, and the risk of occurrence of chronic diseases in adulthood through fetal programming.

- We have shown that the perigestational exposure to CPF and/or HFD decreased the respiratory frequency and subsequently increased the sleep apnea index. As sleep apnea syndrome disrupts the sleep, it would be interesting to examine whether this exposure modifies the sleep structure in rats. This part is currently under investigation by a researcher in our laboratory.

- The perigestational exposure to CPF and/or HFD increased the diaphragm contractility associated with a higher fatigability. We have shown that these contractile changes can be attributed to prolonged cholinergic transmission, altered SR Ca²⁺ release/uptake function, and increased expression of MHC isoforms.

- Interestingly, according to both protocols, we have shown that CPF increased the diaphragm contractility in adult rats exposed directly to CPF during adulthood or when exposed indirectly through continuous maternal exposure during perigestational exposure. These effects have been manifested by the same mechanism through inhibition of AChE activity and upregulation of MHC IIA isoform.

- The perigestational exposure to CPF increased the contractility of both longitudinal and circular ileum muscles in early adulthood which can be referred to cholinergic and non-cholinergic mechanisms. The analysis of changes in intestinal transit time might also be critical as it can be affected by muscular contractility. The transit time is a key determinant of a healthy digestive system. Changes in the transit time can affect the absorption rate of nutrients.

- We have shown that the perigestational exposure to HFD decreases the ileal AChE activity. Further studies could investigate the mechanisms in which HFD can affect cholinergic activity in a model involving direct HFD consumption.

- Despite the lack of direct exposure, perigestational contact with CPF and/or HFD can program the risks of respiratory disruptions and alters the homeostasis of respiratory and

intestinal muscles, affecting the diaphragm and gut contractile functions at early adulthood. Further studies are needed to determine the mechanisms underlying respiratory and digestive perturbations during development due to early life disturbances. It could be interesting to measure pro-inflammatory markers in the blood of the rats and study the lung and gut inflammation. Abnormal gut motility pattern can lead to irritable bowel syndrome (IBS) symptoms including abdominal pain, distention, diarrhea, and constipation. Irregular motor activity of the small intestine can be associated with pain; then it could be promising to study the perception of pain in the gut of the animals.

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Résumé

Le chlorpyriphos (CPF) est un pesticide inhibiteur de l'acétylcholinestérase (AChE). L'exposition au CPF est délétère pendant la période périnatale et à l'âge adulte. L'obésité maternelle et l'exposition aux pesticides peuvent programmer le métabolisme des enfants et favoriser l'obésité. Le premier modèle a évalué les effets de l'exposition au CPF pendant 6 semaines chez le rat adulte sur la fonction du diaphragme. Le deuxième a examiné les effets de l'exposition périgestationnelle des mères au CPF et / ou à un régime riche en graisses (HFD) pendant 4 mois avant la gestation jusqu'à la fin de la lactation. Les fonctions respiratoires et intestinales ont été étudiées chez des ratons de la descendance à I60. L'exposition des adultes au CPF augmentait la contractilité du diaphragme et sa fatigabilité, associée à une activité réduite de l'AChE, ainsi qu'à une perturbation de la régulation hormonale et de la composition en isoformes de la chaîne lourde de la myosine (MHC). L'exposition périgestationnelle augmentait l'indice d'apnée du sommeil, ainsi que la contractilité du diaphragme. Ceci peut être attribué à une transmission cholinergique prolongée, à une altération de libération/absorption du calcium du réticulum sarcoplasmique et à une expression accrue de l'ARNm des isoformes de la MHC. Cette exposition au CPF a augmenté la contractilité des muscles iléaux. Malgré l'absence d'exposition directe, l'exposition périgestationnelle au CPF et/ou au HFD programme les risques d'altération de la fonction respiratoire et intestinale chez les jeunes adultes. En outre, l'exposition au CPF chez l'adulte affecte la fonction physiologique du diaphragme. Mots-clés: Chlorpyriphos, Régime riche en graisses, Diaphragme, Iléon, Respiration, Apnée,

Exposition périgestationnelle, Exposition adulte

Abstract

Chlorpyrifos (CPF) is an acetylcholinesterase (AChE)-inhibiting organophosphorus insecticide. CPF exposure is harmful during the perinatal period and adulthood. Such developmental perturbations are linked to disorders in adults. Maternal obesity associated to pesticides exposure can program children's metabolism and promote obesity onset. The first model assessed the effects of CPF exposure for 6 weeks in adult rats on diaphragm function. The second examined the impacts of perigestational exposure of dams to CPF and/or high-fat diet (HFD) during 4 months before gestation till the end of the lactation. Respiratory and intestinal functions were studied in young adult pups at postnatal day 60. We showed that CPF exposure to adults increased diaphragm contractility and fatigability associated with lower AChE activity and altered hormonal regulation and myosin heavy chain (MHC) isoforms composition. Perigestational exposure to CPF and/or HFD increased the sleep apnea index and diaphragm contractility. These changes can be ascribed to cholinergic transmission, altered sarcoplasmic prolonged reticulum calcium release/uptake function, and elevated expression of MHC isoforms mRNA. Perigestational CPF exposure increased ileal muscles contractility through cholinergic and non-cholinergic mechanisms involving muscarinic m2 acetylcholine receptor and substance P. Exposure to CPF and/or HFD reduced ileal AChE activity. Despite the lack of direct exposure, perigestational exposure to CPF and/or HFD programs the risks for altered respiratory and intestinal functions in young adult offspring. Besides, CPF exposure in adults affects diaphragm physiological function.

Keywords: Chlorpyrifos, High-Fat Diet, Diaphragm, Ileum, Respiration, Apnea, Perigestational Exposure, Adult Exposure