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Does the Perigestational Exposure to Chlorpyrifos and/or High-Fat Diet Affect Respiratory Parameters and Diaphragmatic Muscle Contractility in Young Rats?

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1. Introduction

Maternal High-Fat Diet (HFD) is considered a reflection of the dietary habits in Western countries (Lucas, 1998; Symonds et al., 2007). Fetal development depends primarily on the maternal nutritional supply. Thus, any disruption in maternal metabolism could affect the intrauterine milieu, increasing the risk of offspring to further diseases in later life (Barker, 2004; Taylor and Poston, 2007) as described in the concept of Developmental Origin of Health and Diseases (DOHaD) (Kappil et al., 2016). Evidence from animal models revealed that perinatal HFD intake can increase the risk of behavioral disorders such as anxiety (Sullivan et al., 2010) and aggression in addition to altered locomotor activity in the offspring (Raygada et al., 1998). Studies in rats fed with HFD (Ramadan et al., 2007, 2006) have reported an association between metabolic dysfunctions and respiratory disorders as like increased sleep apnea (SA). On the other side, Hurst et al. (2019) have shown that HFD can reduce the contractile performance of the skeletal muscles in mice. Even though continuous HFD feeding including gestation and lactation periods resulted in an increased risk of obesity in the pups (Howie et al., 2009), 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, central respiratory failure associated with apnea is considered to be the major cause of death following organophosphorus (OP) pesticides poisonings (Lerman and Gutman, 1988; Yamashita et al., 1997). OP pesticides 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 junctions (Ballantyne and Marrs, 2017). Chlorpyrifos (CPF) is an OP

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; CNS, central nervous system; CPF, chlorpyrifos; CSA, cross-sectional area; DOHaD, developmental origin of health and diseases; F, respiratory frequency; GADPH, glyceraldehyde3-phosphate dehydrogenase; HFD, high-fat diet; L₀, maximum length; M, weight of the muscle; MHC, myosin heavy chain; OP, organophosphorus; qRT-PCR, quantitative real-time PCR; RyR1, ryanodine receptor; SA, sleep apnea; SERCA, sarcoplasmic reticulum Ca²⁺-transporting ATPase; SR, sarcoplasmic reticulum; T_E, expiratory time; T_I, inspiratory time; V_T, tidal volume; ρ, muscle density

pesticide suspected to affect the metabolic programming from the fetal period until adulthood (Slotkin, 2011; Slotkin et al., 2005). The metabolic influence of CPF over the adipogenic process contributes to its obesogenic effect (Blanco et al., 2020). The developmental exposure to CPF has been evaluated by different studies with a variety of exposure protocols. In this context, early-life CPF exposure during the preweaning developmental stage has been shown to alter the gut microbiota in preweaning mice (Guardia-Escote et al., 2020) and induce long-term cholinergic effects in rats during later life (Perez-Fernandez et al., 2020). We have recently shown that chronic pre- and postnatal exposure to CPF from the first day of gestation and through postnatal life until adulthood induced an increase in SA index and the contractility of the respiratory skeletal muscle in growing rats (Darwiche et al., 2018). However continuous perigestational exposure, before gestation till the end of lactation, at low dose of CPF without exposing the offspring to CPF until adulthood has not been examined.

Although some of the impacts of exposure to either CPF or HFD have been reported, the effects of combined exposure to both CPF and HFD have not been covered knowing that both CPF and HFD induce inhibition of AChE (Kondakala et al., 2017). In light of these premises, our protocol has been dedicated to determining the effects of alimentary risk factors including an unbalanced diet rich in fats and OP pesticide residues, with the view of assessing the impacts of widely used environmental contaminants (CPF) in a western diet model (HFD). Since pregnant females are being exposed to multiple toxic stimuli throughout life, then examining the effects of prolonged maternal exposure to such environmental and nutritional interaction during the intrauterine life in the progeny simulates the real-life exposures (Tsatsakis et al., 2019). In particular, 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

ventilatory parameters during sleep and 2) the contractile activity of the diaphragm as an essential respiratory skeletal muscle. In addition, we further aimed at determining the effect of this exposure on the gene expression of different mediators involved in the excitation-contraction coupling mechanism.

2. Materials and Methods

2.1. Chlorpyrifos Preparation

Chlorpyrifos (O, O-diethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) with a 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 at a concentration of 1 mg/mL and administered by gavage to the rats at 1mg/kg/day (the oral NOAEL for inhibition of brain cholinesterase activity in rats (Cochran et al., 1995)) for CPF groups. Controls received only vehicle. This dose was selected based on our previous study reported by Darwiche et al. (2018), where rats were exposed to CPF exposure at 1 or 5 mg/kg/day starting from the first day of gestation and during the development of the pups until the age of PND60. In this study (Darwiche et al., 2018); we have encountered a lot of problem with the dose 5 that is above the NOAEL for developmental toxicity. Many of dams died or killed their pups after delivery. Thus, we preferred to use in the recent study the dose 1 to avoid these problems mainly because our exposure is longer (around 5 months). This dose is chosen according to the findings of literature review available. Even though the few studies available on presence of CPF on food commodities, didn't report the concentration we tested, however the limitation of those studies is the lacking of a long-term monitoring plan for the evaluation of the evolution of CPF and its concentration in

food further to different seasons as well as the factors that affect its persistence. A study conducted by El Hawari et al. (2019) on 212 samples of apples to evaluate the presence of different pesticides including CPF showed that pesticide residues were found in 77% of the samples, among them 61% exceeded the maximum residue limits with the most frequently detected pesticide residues were CPF (n = 142). CPF ranged from 7.9 to 750 $\mu\text{g kg}^{-1}$, exceeding the MRL 10 $\mu\text{g kg}^{-1}$ (123 samples).

2.2. 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 \pm SEM) 225 \pm 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, containing 4.5% Crude fat with soybean oil as main component of fat) or high-fat diet HFD (Research Diets no. D12492, SSNIFF Spezialdiäten GmbH, 5.24 kcal/g). The fat in the HFD consists of 54.4 % lard and 5.6% soybean oil. Dams were force-fed daily with oil (as a vehicle) for the control group (oil, standard diet) and the HFD group (oil and HFD) or CPF (1 mg/kg/day)

for CPF group (CPF, standard diet) and HFD+CPF group (CPF and HFD) during 4 consecutive months. Regarding the HFD, we used the rat model developed by Lecoutre et al. (2016), where female rats were fed the same HFD (providing 5.24 kcal/g with 60% of total calories as fat) prior to and during gestation and lactation. This model has been shown to induce metabolic alterations including mild glucose intolerance, hyperinsulinemia and hypercorticosteronemia and program adiposity in the offspring at adulthood. Moreover, studies have shown that diet with a higher percentage of fats induces a greater metabolic response and more rapid weight gain with increasing fat content up to 60% fat (Hu et al., 2018; Speakman, 2019). Then, the females were mated with the male rats (Janvier Labs, two females per male). Male rats were housed in the same conditions as the female and fed the standard diet. The pregnancy was daily determined by the presence of spermatozoa checked with vaginal smears. The average size of the litter was 9 pups per litter. During gestation, the dams were subjected to the same treatment as before gestation until the end of the lactation period. The male pups represent 52% while the females represent 48% of the total obtained offspring from all groups. The effects of perigestational exposure to CPF and/or HFD were studied only in male offspring to avoid the interference of hormonal fluctuations in females during the estrous cycle (Lecoutre et al., 2016; Smith et al., 2015). 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, rats in all groups received only standard diet without CPF until PND60 (early adulthood). The body weight of the offspring was measured three times a week. At PND60 the respiratory parameters of all male offspring were measured via whole-body

plethysmography (n=7-10/group). These offspring 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 (due to technical problems, contractility parameters were measured for 8 of the 10 rats in the CPF group), while the remainder of the diaphragm was preserved at -80 °C for AChE activity measurement and gene expression evaluation. For gene expression, the diaphragm tissue samples were harvested and submerged in Invitrogen™ RNAlater™ Stabilization Solution (Courtaboeuf Cedex, France), a non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. The low number of male offspring in HFD+CPF group (7) refers to the fertility problems encountered in the dams of this group. In addition, we lost some of the pups (newborns) in HFD exposed groups (HFD and HFD+CPF).

2.3. 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), respiratory frequency (f_R), and minute ventilation (V_E) measurements were recorded for each animal according to the previously described protocol (Drorbaugh and Fenn, 1955) using data acquisition and analysis software (LabChart 7, ADInstruments, Oxford, UK). 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). This procedure has been previously validated in our

laboratory by several publications (Darwiche et al., 2018; Ramadan et al., 2006). Indeed, Shoham and Teitelbaum, (1982) and Van Twyver, (1969) have shown that the behavioral sleep reflects the physiological sleep as defined by electroencephalogram and electromyogram activity. However, in order to reduce the possible divergence that could appear between behavioral and electrophysiological evaluation, the measurements were taken only 5 mins after sleep entrance. Sleep Apnea, defined as two missed breaths, was scored according to a previously described procedure (Christon et al., 1996; Ramadan et al., 2006). This method of measuring the sleep apnea during behavioral sleep has been also previously used by Thomas et al. (1995). 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.

2.4. 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 (Darwiche et al., 2018; McGuire and MacDermott, 1998). The preparations were suspended vertically in a muscle bath containing oxygenated Krebs Henseleit solution maintained at 37 °C. The rib margin of the diaphragm was anchored to the base of the plexiglass bath while the central tendon was attached to an isometric force transducer (PIODEN CONTROLS LTD, UK, accuracy \pm 10mg) having a maximum response of 25 mg and controlled by data acquisition system (Digidata 1200, Axon Instruments) for further analysis.

Muscle strips were stimulated using square wave pulses at 2 ms duration and the voltage was set to give supramaximal stimulation at 12 V delivered via two platinum plate electrodes positioned on either side of the muscle strip. The length of the diaphragm strip

was stretched to reach the maximum length L_0 , defined as the optimal length at which maximal force was obtained. All the following measurements were made at L_0 . 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 ($\rho = 1.056 \text{ g.cm}^{-3}$) and L_0 ($\text{CSA} = M / \rho \times L_0$).

2.4.1. 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 N/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.

2.4.2. 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.

2.5. Acetylcholinesterase Activity

The Acetylcholinesterase (AChE) activity in the diaphragm was measured by applying a modified Ellman method (Ellman et al., 1961). The diaphragm samples were homogenized in mammalian cell lysis buffer 5X (ab179835; Abcam, Cambridge, UK) and then centrifuged. 1 μL of protease inhibitor (Abcam, Cambridge, UK) was then added to the obtained supernatant. The diluted supernatant (1:2) was incubated with 100 $\mu\text{mol/L}$ of the butyryl cholinesterase inhibitor, tetra isopropyl pyrophosphoramidate (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 diaphragm protein content was measured spectrophotometrically according to Bradford assay using bovine serum albumin (BSA) as a standard (Bio-Rad, Hercules, CA, USA). The AChE activity was normalized for protein content and expressed in $\mu\text{mol}/\text{min}/\text{mg}$ protein.

2.6. Myofibrillar Protein Extraction and Dosage

Frozen diaphragm muscles were homogenized with 5 volumes of washing buffer (20 mmol/L NaCl, 1 mmol/L EGTA (pH 6.4), and 5 mmol/L PO_4) and then centrifuged at 12000 rpm for 5 min. Then, the myofibrillar proteins were extracted with 3 volumes of extraction buffer (5 mmol/L EGTA, 1mmol/L di-thiothreitol (pH 8.5) and 100 mmol/L 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) (Bradford, 1976).

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 Cedex, 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 Cedex, France) and PCRs were run on a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Courtaboeuf Cedex, France).

Gene-specific primers were purchased from Thermo Fisher Scientific as TaqMan™ 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 (GAPDH) 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 Ca^{2+} - 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 $2^{-\Delta\Delta\text{Ct}}$ calculation compared to control samples ($\Delta\Delta\text{Ct} = \Delta\text{Ct exposed} - \text{mean } \Delta\text{Ct control}$). The expression level of the studied genes was 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

StatView software (version 5.0, Abacus Concepts Inc., Berkeley, CA, USA) was used to perform all our statistical analyses. Data were normally distributed according to a Kolmogorov-Smirnov test of normality. A two-way ANOVA was used to analyze the main effects of diet (HFD and HFD+CPF groups vs. control and CPF groups) or CPF (CPF and HFD+CPF groups vs. control and HFD groups) and the interaction between diet and CPF exposure. In the case of diet-CPF interaction, a post hoc analysis using unpaired t-test was used to compare between individual groups. In all analyses, the threshold for statistical significance was set to $p \leq 0.05$. Indicative results ($p < 0.1$) are shown when appropriate.

3. Results

3.1. Body Weight

To avoid possible early-life maternal separation stressor, the male pups were not weighed at the PND1. The mean body weight of male rat offspring was then assessed at 3 developmental stages: 3 days after birth (PND3), as juveniles (PND21), and as young adults (PND60) (Figure 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).

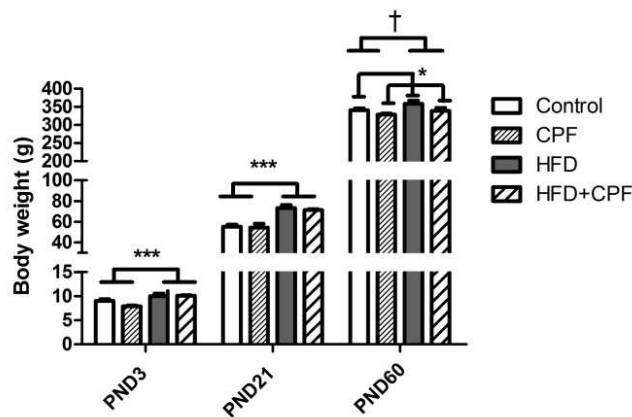


Figure 1. Effects of perigestational exposure to CPF and HFD on the body weight of male rat offspring 3 days after birth (PND3), as juveniles (PND21), and as young adults (PND60). Data are quoted as means \pm SEM ($n=7-10$ /group). Effect of diet (HFD and HFD+CPF vs. Control and CPF) ***: $p < 0.001$, †: $p < 0.1$. Effect of CPF (CPF and HFD+CPF vs. Control and HFD) *: $p < 0.05$

Parameter		CPF Exposure Effect (CPF and HFD+CPF vs. Control and HFD)	Diet Effect (HFD and HFD+CPF vs. Control and CPF)	Interaction (Diet x CPF exposure)
Body Weight	PND3	NS	↑↑↑	NS
	PND21	NS	↑↑↑	NS
	PND60	↓	‡	NS
Respiratory parameters	Frequency (fR)	↓	↓↓↓	***
	T _I	↓↓	↓↓↓	NS
	T _E	NS	NS	NS
	Tidal Volume (V _T)	↑	NS	*
	Minute Ventilation (V _E)	NS	NS	*
Sleep Apnea	Sleep Apnea Index	↑	↑	**
	Mean duration of an episode of apnea	NS	NS	*
Contractility parameters	Twitch Tension	NS	NS	*
	Time to peak tension	NS	NS	NS
	Half-relaxation time	NS	NS	*
	Fatigability Index	NS	NS	**
AChE activity		↓↓↓	NS	**
Myofibrillar protein content		↑↑↑	NS	NS
Genes expression	AChE	NS	↑↑	*
	MHCI	NS	↑↑	***
	MHCII	NS	↑	**
	RYR1	NS	↑↑↑	*
	Atp2a1	↓↓	↑	NS
	Atp2a2	↓↓↓	↑	NS

Table 1. Effects of CPF exposure, diet, and CPF-diet interaction on different parameters analyzed by two-way ANOVA. CPF or Diet effect: ↑ (increasing) or ↓ (decreasing):

p<0.05; ↑↑ or ↓↓: p<0.01; ↑↑↑ or ↓↓↓: p<0.001; †: p<0.1. CPF and Diet interaction: *: p<0.05; ** p<0.01; *** p<0.001, NS (Non-significant).

3.2. Respiratory Parameters

No significant diet-CPF exposure interaction was observed for T_I (Figure 2a) and T_E (Figure 2b). However, a significant diet-CPF exposure interaction was reported for fR (p = 0.0004) (Figure 2c) and for V_T (p = 0.0337) (Figure 2d). Compared to non-CPF groups, maternal CPF exposure was associated with a significant decrease for T_I (p = 0.005) (Figure 2a) and fR (p = 0.0403) (Figure 2c) in male offspring at PND60. Furthermore, comparing HFD groups to standard diet groups revealed a significant decrease for T_I (p = 0.0002) (Figure 2a) and for fR (p = 0.0008) (Figure 2c) (Table 1).

Particularly, a significant decrease in fR was observed in CPF group by 18% (p = 0.0101), HFD group by 23% (p = 0.0025), and in HFD+CPF group by 18% (p = 0.0194) compared to controls (Figure 2c). The V_T was significantly decreased in HFD group by 38% compared to controls (p = 0.0106) and by 47% compared to HFD+CPF group (p = 0.0083) (Figure 2d). The V_E was significantly decreased in HFD group by 46% compared to controls (p = 0.0227) and by 42% compared to HFD+CPF group (p = 0.016) (Figure 2e). Despite a non-significant interaction, the T_I was significantly lower by 17% in CPF group (p = 0.0016), by 23% in HFD group (p = 0.0007), and by 32% in HFD+CPF group (p = 0.0005) compared to controls (Figure 2a).

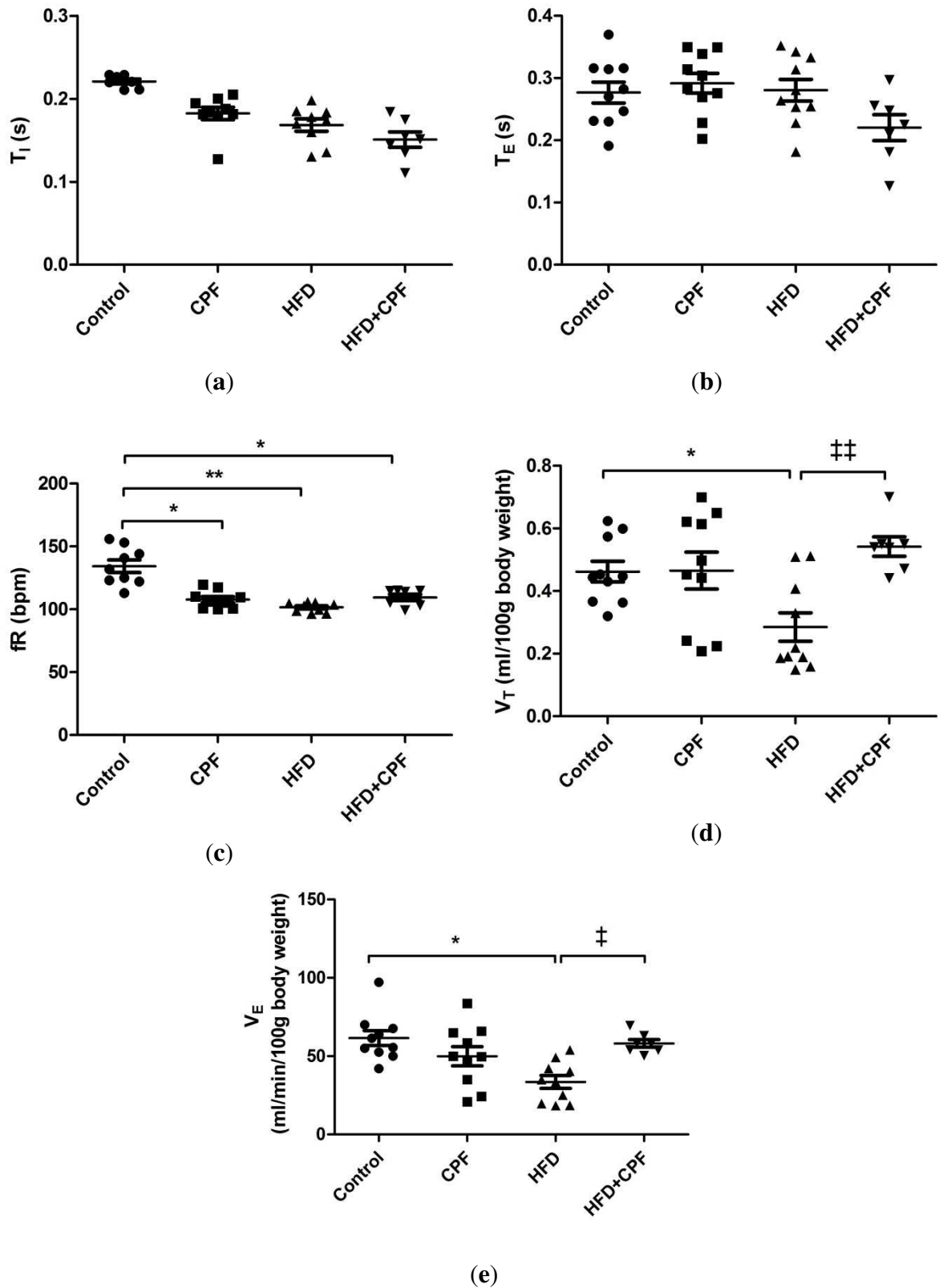
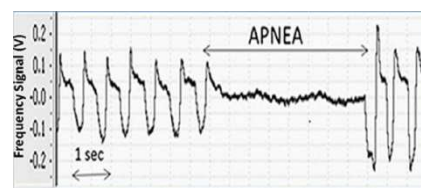


Figure 2. Effects of perigestational exposure to CPF and HFD on the respiratory parameters in male rats at PND60. (a) T_I (s), (b) T_E (s), (c) fR (bpm: breath/min), (d) V_T (mL/100g body weight), (e) V_E (ml/min/100g body weight). Data are quoted as

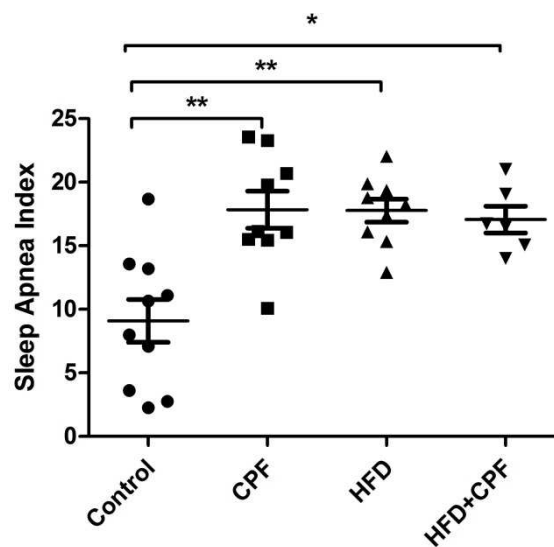
means \pm SEM (n=7-10/group). For a significant CPF exposure x diet interaction: *: $p < 0.05$ and **: $p < 0.01$ vs. control, ‡: $p < 0.05$ and ‡‡: $p < 0.01$ vs. HFD.

3.3. SA

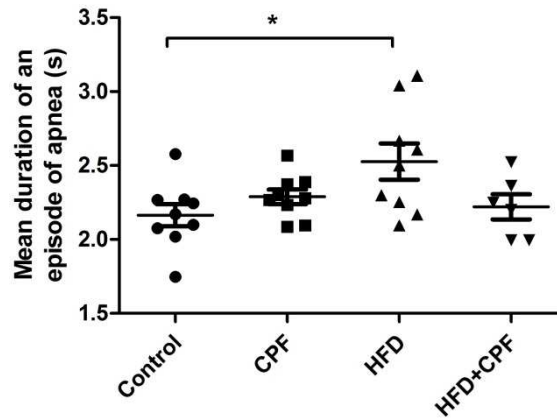
A significant interaction between diet and CPF exposure was observed for SA index ($p = 0.0087$) and the mean duration of an episode of apnea ($p = 0.0415$). The SA index was significantly increased with either maternal CPF exposure ($p = 0.0233$) or HFD feeding ($p = 0.0255$) (Table 1). The SA index was significantly higher by 96% in each of CPF group ($p = 0.0033$) and HFD group ($p = 0.0013$) and by 88% in HFD+CPF group compared to controls ($p = 0.0322$) (Figure 3b). A significant increase in the mean duration of an episode of apnea was observed in HFD group compared to controls ($p = 0.0297$) (Figure 3c).



(a)



(b)



(c)

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, significant differences between groups analyzed by unpaired t-test: *: $p < 0.05$ and **: $p < 0.01$ vs. control group.

3.4. Effects of CPF and HFD on the Contractility Parameters

There was a significant interaction between CPF exposure and diet for twitch tension ($p = 0.0115$), half-relaxation time ($p = 0.0492$), and fatigability index ($p = 0.0078$) (Table 1). The twitch tension was significantly increased in CPF group by 136% ($p = 0.0038$) and HFD group by 140% ($p = 0.0010$) compared to controls. This increase was less pronounced in HFD+CPF group ($p = 0.0532$) (Figure 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 ($p = 0.0199$) (Figure 4c). However, no changes were detected for time to peak tension (Figure 4b).

Regarding the diaphragm fatigability index, a significant increase by 31% was observed in CPF group compared to controls ($p = 0.0004$). However, the fatigability index was significantly decreased by 27% in HFD+CPF group compared to CPF group ($p = 0.0004$) (Figure 4d).

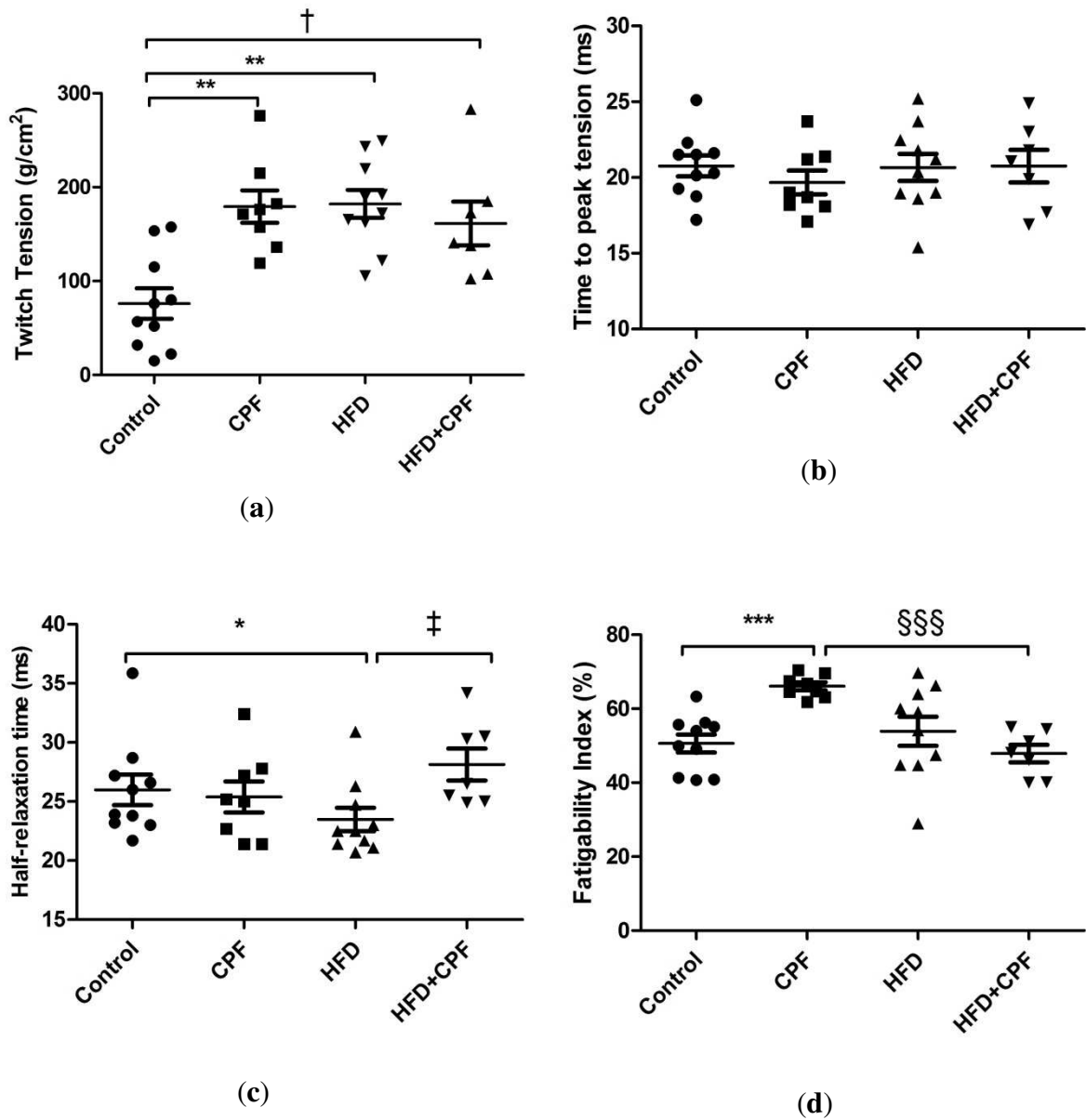


Figure 4. Effects of perigestational exposure to CPF and HFD on the contractility parameters in the diaphragm of male rats at PND60. (a) Twitch tension represented as a function of cross-sectional area (N/cm²), (b) time to peak tension

(ms), (c) half-relaxation time (ms) (d) 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<0.001, and †: p<0.1 vs. control, ‡: p<0.05 vs. HFD, §§§: p<0.001 vs. CPF.

3.5. 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). However, no significant diet effect was observed (Table 1). AChE activity levels were significantly reduced in CPF group (p = 0.0007) and HFD group (p = 0.0127) by 53% and by 29% respectively compared to controls. A significant decrease was reported in HFD+CPF group by 40% (p = 0.0376 vs. control) (Figure 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 (Figure 5b).

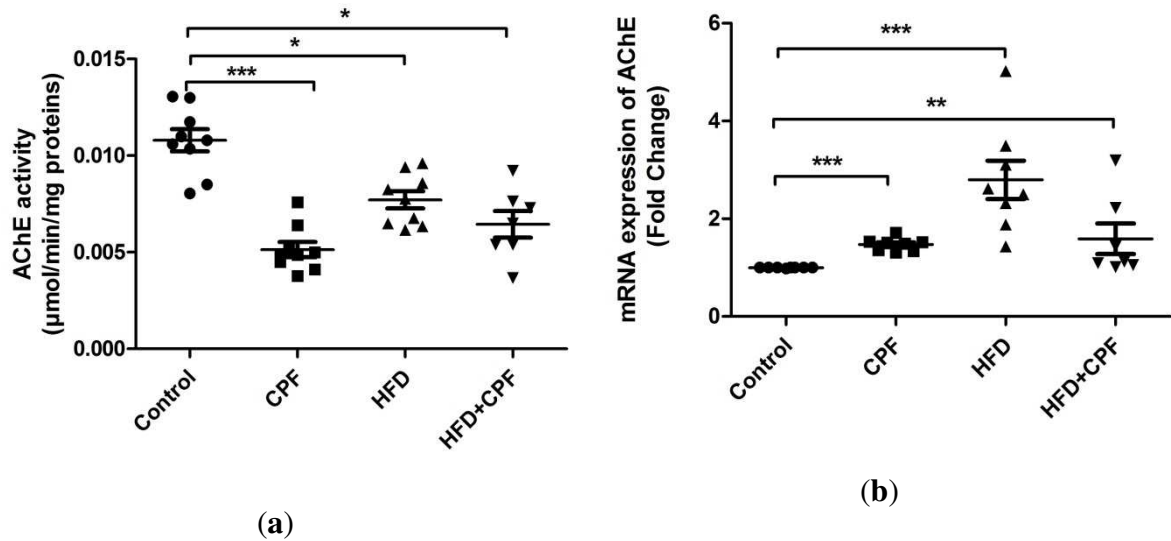


Figure 5. Effects of perigestational exposure to CPF and HFD on AChE in the diaphragm of male rats at PND60. (a) AChE activity quoted as the mean \pm SEM expressed as μmol of acetylthiocholine hydrolyzed per minute per mg of proteins, (b) expression of AChE mRNA quantified by real-time PCR depicted as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

3.6. 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 ($p = 0.0017$) and by 82% in HFD+CPF group ($p = 0.0021$) compared to controls (Figure 6).

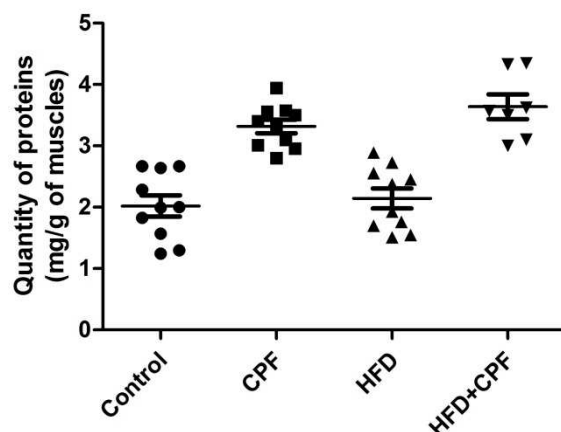


Figure 6. Effects of perigestational exposure to CPF and HFD on the myofibrillar protein content in the diaphragm of rats at PND60. Data are quoted as means \pm SEM expressed as mg of myofibrillar proteins per gram of muscle.

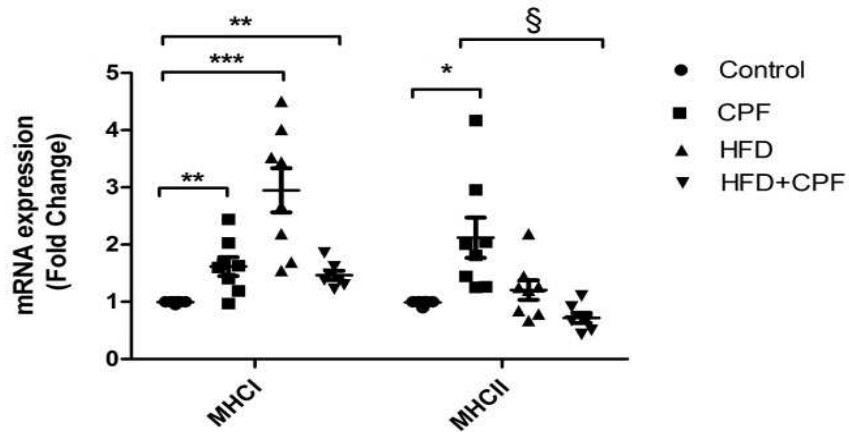
3.7. Gene Expression Analysis of MHC, RyR1 , and ATP2a1 and ATP2a2.

A significant diet-CPF exposure interaction was observed for the expression of MHCI ($p = 0.0009$) and MHCII ($p = 0.0046$). A main effect of diet on MHCI ($p = 0.0035$) and MHCII ($p = 0.0318$) expression was observed (Table 1). MHCI mRNA expression was significantly higher by 1.6 fold in CPF group ($p = 0.0062$), 2.9 fold in HFD group ($p = 0.0004$) for HFD, and 1.5 fold in HFD+CPF group ($p = 0.0011$) compared to controls. The expression of diaphragm MHCII mRNA was 2.1 fold higher in CPF group than in controls ($p = 0.0103$). However, the combined exposure in HFD+CPF group induced a decrease in the expression of MHCII mRNA compared to CPF group ($p = 0.0223$) without significant difference compared to controls (Figure 7a).

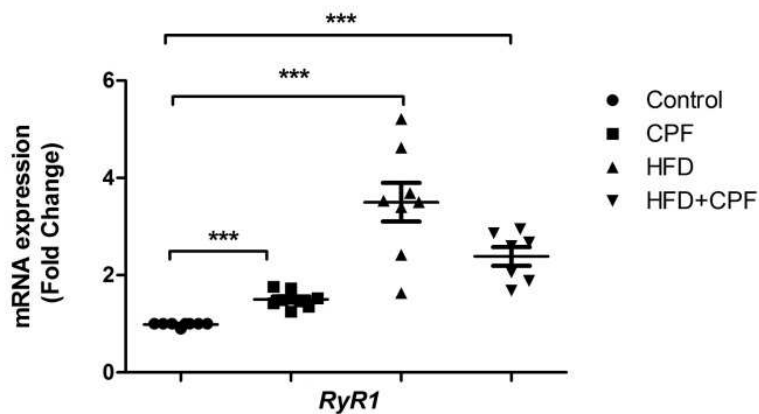
Then, the expression of different genes responsible for the Ca^{2+} signaling was studied in the diaphragm. A significant diet-CPF exposure interaction was observed for RyR1 mRNA expression ($p = 0.0101$). Compared to standard diet groups, HFD feeding showed an increase in RyR1 mRNA expression ($p < 0.0001$) (Table1). The diaphragm expression of RyR1 mRNA was significantly higher by 1.5 fold in CPF group ($p < 0.0001$), 3.5 fold in

HFD group ($p < 0.0001$), and 2.4 fold in HFD+CPF group ($p < 0.0001$) compared to controls (Figure 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 of 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* ($p = 0.0354$) and *Atp2a2* ($p = 0.0121$) respectively in HFD group compared to controls (Figure 7c).



(a)



(b)

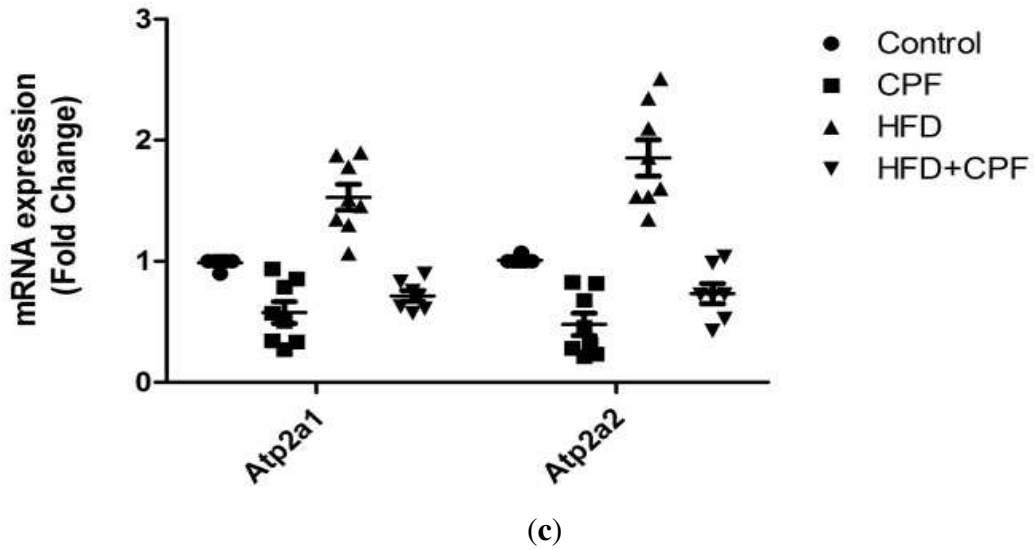


Figure 7. Gene expression analysis in the diaphragm of rats at PND60. (a) Expression of MHCI and MHCII, (b) expression of RyR1 mRNA coding for ryanodine receptor mRNA, (c) expression Atp2a1 and Atp2a2 mRNA coding for SERCAI and SERCAII pumps, quantified by real-time PCR. Data are depicted as the mean \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, significant differences between groups analyzed by unpaired t-test: *: p<0.05, **: p<0.01, and ***: p<0.001 vs. control group, §: p<0.05 vs. CPF.

4. Discussion

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 ventilatory parameters, SA incidence, respiratory muscle contractility, and

the expression of genes involved in the excitation-contraction coupling mechanism were studied during early adulthood in rats not directly exposed to risk factors (HFD or CPF).

- Effect of CPF and/or HFD on body weight

Our results revealed that the perigestational exposure to HFD, independent of CPF exposure, was associated with increased body weight in rats at birth. The increase in the body weight in the offspring continues to be observed at the end of the lactation period, and to a less extent, at early adulthood (PND60) as the offspring were raised on standard diet without CPF during the post-weaning period (from PND21 to PND60). Several studies have revealed a lower birth weight after maternal feeding with HFD (35.5% fat as lard, 20% protein, 36.3% carbohydrate [w/w]) (Hartil et al., 2009), HFD (24% fat, 24% protein, 41% carbohydrate [w/w]) (Howie et al., 2009), or even no effects with HFD (24% fat as partially hydrogenated vegetable oil, 20% protein, 30% carbohydrate [w/w]) (Guo and Jen, 1995) up to and/or throughout gestation and lactation. The fat contribution in these diets ranges from 45% to 60% kcal. However, our study showed higher body weight in HFD (35% fat, 26% protein, 26% carbohydrate [w/w]; 60% kcal from fat) 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 (42% kcal from fat) weanlings (Del Prado et al., 1997). The elevated body weight was still observed at PND60 in agreement with other studies where the increase in body weight persists till the age of 3 months and even more through adulthood as a result of developed hyperphagia (Kirk et al., 2009; Lecoutre et al., 2016).

On the other hand, in our study, a decrease in body weight was associated with CPF exposure only at early adulthood indicating 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 PND60 (Darwiche et al., 2018). The reduced body weight in CPF exposed rats reported in several studies (Kaur et al., 2019; Mansour and Mossa, 2010), was shown to be related to a possible increase in lipids and proteins degradation and the level of oxidative and cholinergic stress induced by CPF (Goel et al., 2000; Maurissen et al., 2000).

- Effect of CPF and/or HFD on AChE

Our results confirm the toxic effects of CPF and/or HFD on the AChE activity observed in the diaphragm of the rats. In fact, CPF, widely known to cross the foeto-placental barrier (Akhtar et al., 2006), exerts its toxicity through the inhibition of AChE enzyme, inducing elevation of ACh at the post-junctional receptors. 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 suggesting that HFD can induce cholinergic alterations (Kaizer et al., 2004; Morganstern et al., 2012). A possible mechanism could be related to the increased free fatty acids (FFA) metabolites in the HFD fed rats (Buettner et al., 2000), which have been shown to decrease in vitro the AChE activity (Tel et al., 2010; Vajreswari et al., 2002). 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. It is critical to note that the decrease in the AChE activity observed following the co-exposure to both CPF and HFD was similar to CPF or HFD exposure alone indicating that the effect was not exacerbated in HFD+CPF group. In fact, in our study, there was an indirect contact of the progeny after weaning to HFD, CPF or both. This could explain why there were no differences in the AChE activity between groups. Thus, it could be convenient to check directly in the dams if the exposures to CPF alone, HFD alone or to their interactions affect differently AChE activity. Very few studies have examined the

effects of both CPF and HFD in animal models. Kondakala et al. (2017), showed that although the AChE activity was not affected in the brain and skeletal muscles, a similar decrease in the plasma ChE activity was observed in HFD fed animals compared to controls and in CPF exposed animals (in either normal diet or HFD fed animals) compared to controls. 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 (Somnuek et al., 2009). This has been shown in our study by the greater mRNA expression level of AChE observed in the diaphragm.

- Effect of CPF and/or HFD on respiratory parameters

Our results showed lower respiratory frequency as expressed in breath per minute during sleep associated with increased SA index. Despite a non-significant diet-CPF interaction, the T_I was significantly lower in CPF, HFD and HFD+CPF groups. Thus, the decrease observed in the respiratory frequency is related to decrease in T_I with unchanged T_E . Our findings are consistent with previously reported effects of either acute OPs (paraoxon) exposure (Villa et al., 2007) or even repeated prenatal and postnatal CPF exposure on the respiratory frequency (Darwiche et al., 2018). Overweight and obesity are associated with a higher risk of respiratory symptoms (Zammit et al., 2010). Moreover, adipose tissues produce different cytokines and mediators, generating a pro-inflammatory state which could contribute, in turn, to reduced lung functions (Hancox et al., 2007). In fact, organophosphate-induced acute respiratory failure has been thought to result from a direct depressant effect on the respiratory center in the brainstem, constriction of and increased secretion by the airways, and paralysis of the respiratory musculature. Villa et al. (2007), suggested that the prolongation of the T_E reported in their study is related to the peripheral airway obstruction and flow restriction. In our study, T_E was not changed in rats exposed to CPF, thus we can exclude airways obstruction that could be induced by indirect CPF and/or

HFD exposure. In addition, the decrease in respiratory frequency and T_I with no significant effects on T_E or V_T failed to support the hypothesis of a major depressant effect of CPF, HFD or their combination on the control of breathing. On the other hand, respiratory failure is related to alteration of arterial blood gases (Speretta et al., 2018). Thus, the ventilator alterations observed in our study could be induced to arterial blood gases changes that need to be checked in order to confirm any respiratory distress. A decrease in T_I and V_T with normal T_E were observed in the HFD group compared to control one. These disruptions in respiratory parameters could be explained by a weaker adaptation of ventilation, which may result from impairment in the respiratory control responsible for regulating the basic respiratory rhythm. This could be checked for example by testing the response of the central respiratory system to a hypercapnia challenge which could be explained, in turn, by alterations of cholinergic and catecholaminergic mechanisms involved in signal transmission within the central and peripheral chemosensitive areas (Tantucci et al., 1997; Wahba and Soliman, 1988). In fact, the ventilatory responses to hypoxia and hypercapnia are associated with increased inspiratory and expiratory motor activities (Lemes and Zoccal, 2014) that are coordinated by neurons located in the brainstem (Feldman et al., 2003; Guyenet et al., 2005). The inspiratory motor activity is crucial to enhance respiratory frequency and tidal volume (Powell et al., 1998) and the peripheral and central chemoreceptor stimulation also activates expiration, promoting contractions in the abdominal expiratory muscles (Abdala et al., 2009; Moraes et al., 2012). The lung volume and capacity have been shown to be reduced in obese individuals (Melo et al., 2014). In our study, the lower volume of air moved in and outside the lungs during each respiratory cycle could be related to possible metabolic disruptions and associated pro-inflammatory state induced by early-life nutritional perturbations. Our finding suggests developmental impacts that could have affected the development/maturation of lungs in HFD group. Indeed, cytokines and bioactive mediators generated by the adipose tissues could induce a pro-

inflammatory state that is associated with hypodevelopment of the lungs. Rasmussen et al. (2009) have shown that higher levels of C-reactive protein (CRP) are associated with reduced lung volumes in young adults suggesting that low-grade systemic inflammation early in adult life may lead to a decline in lung function. However, the mechanisms for this association are still poorly understood (Hancox et al., 2016). In fact, metabolic parameters in the dams at the end of the exposure period (PND21), showed elevated blood glucose levels only in HFD dams compared to controls. CRP levels were also increased, thus indicating an inflammatory state. In addition, our results showed alterations in lipid profile (significant increase in triglycerides, indicative increase in LDL, and a significant decrease in HDL) in CPF dams. However, the co-exposure of dams to CPF and HFD induced a significant increase in the triglycerides levels and an indicative increase in the blood glucose levels compared to controls without alteration in CRP levels (data not shown).

ACh is an essential neurotransmitter for the neural control of respiration at the central and peripheral nervous systems (Carey et al., 2013). Any modification at the cholinergic level 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. 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 incidence (Kryger, 2000). Accordingly, the alterations observed in the frequency of respiration and T_I associated with unchanged 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. (2018) 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. Apnea episodes observed following acute OPs exposure are attributed to either central effects caused by the AChE inhibition in the respiratory centers (Carey et al., 2013; Gaspari and Paydarfar, 2011) or to peripheral effect through airway obstruction (Proskocil et al., 2010). 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 a slight increase in 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 (Ramadan et al., 2007, 2006). It has been proposed that obstructive sleep apnea (OSA) is an indicator of metabolic syndrome; indeed, both conditions share common intermediate pathogenic pathways including alterations in the regulation of the autonomic nervous system, increased inflammatory activity, alterations in adipokine levels and endothelial dysfunction (Lurie, 2011). These mechanisms may be involved in the interplay between these 2 conditions. Overall, this complexity makes it especially difficult to reveal and understand the links between sleep apnea and metabolic disorders. Moreover, evidence suggests a correlation between SA and the increased intake of saturated fatty acids in overweight patients (Bove et al., 2018). Remarkably, in our study, we showed that the mothers consuming HFD before and during pregnancy and throughout lactation expose their offspring 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. This increase of the duration of apnea episodes could induce an increase of the PaCO₂ that was not measured in our study. Interestingly, our results showed that the increase in SA index in the CPF+HFD group was similar to both CPF and HFD groups. This speculates that the combination of both CPF and HFD will not exaggerate the effects observed in each group apart. This could be related to

the fact that offspring in our study are not exposed directly to CPF or HFD as explained before for AChE activity.

- Effect of CPF and/or HFD on muscle contractility

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. These results are consistent with our previous findings (Darwiche et al., 2018).

Furthermore, the diaphragm contractility was increased in the rats at PND60 accompanied by reduction in half-relaxation time following maternal HFD exposure. By contrast to our results, HFD was shown to induce a decrease in normalized work-loop power (Tallis et al., 2016), and in the normalized isometric force of the diaphragm which could be related to the accumulation of body fats (Hurst et al., 2019). These contradictory results can be elucidated by the differences in the HFD exposure protocol and animal models. In our study, the rats are not directly exposed to HFD in addition to the fact that the offspring assessed at PND60 didn't show obesity.

Alternatively, our results showed that the combined exposure to HFD and CPF induced an increase in the twitch tension compared to controls, and this increase is similar to that observed after the exposure to either CPF or HFD. The exposure to CPF was associated with increased fatigability of the diaphragm, thus reducing its ability to sustain its contraction for a long time as shown by Darwiche et al. (2018). However, no changes in the fatigability index were observed in the HFD group.

It is worth mentioning that a greater level of diaphragm contraction might be related to the changes observed in the respiratory parameters mainly the increase in SA. Thus the diaphragm contractile force could be increased in a way to overcome the elevated apnea episodes and could 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 (El-Kabir et al., 2003; MacIntyre, 2006).

- Effect of CPF and/or HFD on Gene Expression Analysis of MHC, RyR1, *ATP2a1* and *ATP2a2*

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 characteristics (DeNardi et al., 1993). The diaphragm, a neither typical fast-twitch nor slow-twitch muscle, is characterized by plasticity (Pickering and Jones, 2002). 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. Our results are consistent with Hallal et al. (2019a) showing that the increased twitch tension observed in each of *edl* (fast-twitch skeletal muscle) and soleus (slow-twitch skeletal muscle) in adult rats following direct CPF exposure at a higher dose, correlates with increased myofibrillar protein content in these skeletal muscles. 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 fatigable (Pickering and Jones, 2002), then the greater increase in the expression of MHCII could contribute to the increased diaphragm fatigability.

On the other side, in HFD group despite there is no effect on myofibrillar protein content, the expression of MHCI isoform was significantly increased without significant changes in MHCII isoform indicating a shift toward slow fiber type. A similar fast-to slow shift in MHC phenotype associated with obesity has been reported in the diaphragm indicating an increase in oxidative capacity (Powers et al., 1996). Such changes could contribute to the delay of fatigue during periods of prolonged contractile activity as shown in our HFD rats.

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. Despite this increase in myofibrillar protein content, the twitch tension was slightly increased. This could be related to the increased expression of the slow MHCI isoform that might be linked to the maintained resistance to fatigue.

Surprisingly, the switch in MHC isoforms does not correlate with any changes in the contraction time and in the half-relaxation time. These results are consistent with Vignaud et al. (2008) reporting no changes in the twitch kinetics of the soleus skeletal muscle despite the transition from slow to fast MHC expression in AChE knockout (KO) mice.

On the other hand, 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 Ca^{2+} ions from the SR, thus increasing the myofibrillar Ca^{2+} concentrations. Such greater Ca^{2+} 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 phenotype (Bastide et al., 2000). However, a significant decrease in mRNA expression level of RyR1 was observed in Soleus after 6 weeks exposure to CPF (Hallal et al., 2019b). 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 Ca^{2+} release from the SR depends on the Ca^{2+} stores uptaken from the cytoplasm through SERCA. 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 diaphragm (Zhang et al., 2017). A significant increase in the expression of

SERCAI and SERCAII was reported in HFD group. Since muscle relaxation depends on Ca^{2+} reuptake to SR (Gollnick et al., 1991), 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 isoforms (Wu et al., 2016) and reduced Ca^{2+} uptake function (Zhang et al., 2017). Decreased SERCAI expression could depress the maximal rates of SR Ca^{2+} uptake and by consequence its release (Green et al., 2003). This could contribute to the reduction of maximal rates of both force development and output as a result of decreased Ca^{2+} release from the SR as shown in mice with targeted disruption of the ATP2a1 gene (Pan et al., 2003) exacerbating, in turn, diaphragm contractility impairment (Wu et al., 2016). 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 Ca^{2+} release/uptake function might contribute to the increased contractile force of the diaphragm. In order to confirm these results, the protein expression of SERCA and RyR1 should be further studied.

Finally, recent approaches have integrated the exposure to multiple stimuli combinations in a way to simulate the real-life exposure scenarios (Kostoff et al., 2018; Tsatsakis et al., 2019). These interactions could result in either a weaker or a stronger combined effect. Surprisingly, Slotkin et al. (2011) have showed that many of the central synaptic defects induced by organophosphate exposure have been ameliorated by HFD consumption. In our study, the Tidal Volume was reduced in HFD group compared to controls. However, the perigestational co-exposure to CPF and HFD showed that CPF exposure could ameliorate

the HFD-induced effects on the Tidal Volume as observed by unchanged Tidal Volume in the HFD+CPF group compared to controls. In addition, an increase in the fatigability index was observed in CPF group. This increase could be related to the higher increase in the expression of MHCII isoform mRNA compared to controls. In contrast, in the HFD+CPF group, the fatigability index was not changed compared to controls suggesting that the interaction between CPF and HFD could also ameliorate the effects induced by CPF exposure on the fatigability index of the diaphragm. Nevertheless, the SA indexes were increased similarly in CPF, HFD and HFD+CPF groups showing that the early life consumption of HFD has not exacerbate the effects of early-life OP exposure. In humans or rats with mechanically stable upper airways, central apneas or hypopneas may be provoked by the dysregulation of the patterning of the respiratory motor output (Carley and Radulovacki, 1999). Christon et al. (1996) suggested that central apnea in the rat might result directly from an inadequate respiratory input from chemoreceptors but may arise from other processes such as transient inhibition of the respiratory rhythm as well. Thus, in our study, the increase in 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.

5. Conclusions

This study aimed to understand the consequences of HFD and/or CPF exposure in endorsing changes in the breathing functions. In fact, we have shown that the maternal exposure of rats to CPF and/or HFD during the perigestational period could program the

risks for altered ventilatory parameters 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. It is indispensable to advance the interventional strategies to avoid and treat common disorders associated with the maternal exposure to CPF and /or HFD such as sleep apnea and respiratory distress. More studies are necessary to inspect the mechanisms involved in these responses.

Author contributions:

Hiba El Khayat El Sabbouri: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Validation, Writing – original draft, Writing – review & editing. Jérôme Gay-Quéheillard: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. Wissam H. Joumaa: Conceptualization, Funding acquisition, Validation, Visualization, Writing – review & editing. Stephane Delanaud: Methodology, Resources. Marion Guibourdenche: Methodology. Walaa Darwiche: Methodology, Writing – review & editing. Narimane Djekkoun: Methodology. Véronique Bach: Conceptualization, Funding acquisition, Resources. Wiam Ramadan: Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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