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# Postnatal overfeeding in rats leads to moderate overweight and to cardiometabolic and oxidative alterations in adulthood

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**Running title:** Postnatal overfeeding, heart, oxidative stress

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*Abbreviations* : Ach, acetylcholine; ANOVA, analysis of variance; AU, arbitrary units; BMI, body mass index; BK, bradykinin; +dP/dt, left ventricular maximal pressure development; -dP/dt, left ventricular minimal pressure development; HR, heart rate; IU, international units; L, Langendorff mode; LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; LVESP, left ventricular end-systolic pressure; MMP-2, matrix metallo-proteinase-2; NF, normal-fed; OF, overfeeding ; SOD, superoxide dismutase; RLU, relative light units; ROOH, hydroperoxides; SNP, sodium nitroprussiate; W, working mode

## Abstract

In contrast to the masses of data on obesity, few data are available concerning the cardiometabolic and oxidative consequences of moderate overweight. The model of postnatal-overfeeding (OF) induces an increase in body weight at weaning that remains during adult life. Litters of Wistar rats were either maintained at 12 (normal-fed group, NF), or reduced to 3 at birth in order to induce OF. At 6 months of age, metabolic parameters, circulating oxidative stress and aortic and coronary vasoreactivity were assessed. Cardiac susceptibility to ischemia-reperfusion injury was also evaluated *ex vivo* as were markers of cardiac remodeling. OF led to an increase in body weight at weaning (+50%); the increase in body weight persisted throughout adult life, but was less marked (+10%). Significant increases in plasma levels of fasting glucose, insulin and leptin were observed in OF. An increase in both plasma hydroperoxides and cardiac superoxide dismutase activity and a decrease in plasma ascorbate were observed in OF rats. Vasoreactivity was not modified, but *ex vivo*, after 30 minutes of ischemia, isolated hearts from OF rats showed lower recovery of coronary flow along with a greater release of LDH. Studies on heart tissues showed an increase in collagen content, and increased expression and activity of MMP-2.

Our findings show that moderate overweight in adult rats, induced by postnatal overfeeding, leads to both metabolic and oxidative disturbances as well as a higher susceptibility to cardiac injury after ischemia *ex vivo*, which may be explained, at least in part, by ventricular remodeling.

**1. Keywords:** Postnatal overfeeding, Oxidative stress, Heart, Ischemia, Rats

## **1. Introduction**

Metabolic syndrome, a cluster of several abnormalities including the build-up of abdominal fat, glucose imbalance, dyslipidemia and increased blood pressure, is a major risk factor for the development of cardiovascular diseases. We have already shown that the prevalence of metabolic syndrome in an unselected population of patients undergoing myocardial infarction was high, and that an elevated body mass index (BMI) and waist circumference were associated with a higher risk of heart failure and death at one year [1, 2]. Clinical and experimental evidence shows that the nutritional environment during the peri-natal period plays an important role in regulating both metabolic and hormonal tendencies in adulthood, and that nutrition in early life has an impact on the subsequent risk of overweight and hypertension [3]. In the past few years, certain authors showed that inducing early post-natal over-nutrition in rats, just by reducing litter sizes, led to profound changes in body weight at weaning (nearly 30% increase); the increase in body weight persisted with maturation, but was less marked (10-15% increase) [4-6]. Certain authors reported increases in fasting glucose, plasma insulin, triglycerides and leptin levels at weaning and later [7-9], while others observed a discrete increase in blood pressure [4, 10]. Thus, a cluster of symptoms characteristic of metabolic syndrome occurred in neonatally overfed rats. However, few in-depth explorations of the cardiovascular consequences of this moderate overweight have been reported.

While much work has been done on the cardiovascular consequences of high-fat diets or on genetically obese experimental animals, little research has been reported on the cardiovascular consequences of moderate overweight, and postnatal overfeeding might provide a good experimental model for studying classical risk factors for cardiovascular disease associated with early-induced permanent overweight. In addition, several experimental studies have

demonstrated that in overweight or obese individuals, an increase in energy metabolism increases the production of reactive oxygen species production [11]. As a consequence, this general increase in oxidative stress is able to initiate or reinforce various metabolic or functional disorders which in turn accentuate pathologies such as hypertension, atherosclerosis or heart failure [12].

Therefore, the aim of our work was to evaluate the metabolic, oxidative and cardiovascular consequences moderate overweight induced by post-natal over-nutrition in rats.

## **2. Materials and Methods**

### *2.1. Animals and diets*

Adult female Wistar rats (Charles River, L'Arbresle, France) were caged with male rats at a proportion of 2:1, then housed in individual cages and given water and standard pellet diet *ad libitum* (A03, SAFE Diets Augy, France), during pregnancy and lactation. To induce early postnatal overfeeding, the litter size was reduced. Litters were either maintained at 12 male-pups (normal-fed, NF group), or reduced to 3 male-pups in order to induce postnatal overfeeding (over-fed, OF group). After weaning (day 24), rats of both groups had free access to standard diet (A03, SAFE Diets Augy, France) and water. The composition of the standard diet was 69% of cereals, 20% of vegetable proteins (soya), 6% of animal proteins (fish) and 5% of vitamins and minerals mixture, corresponding to 52% of carbohydrates, 21% of protein, 5% fat, and 4% fibers as macronutrient intake. Throughout life, body weight and food intake were measured weekly then monthly. The local Ethics Committee approved the

experimental protocol (see paragraph 5), and the investigators complied with authorization 3408 of the French government. The investigation conforms to the Directive 2010/63/EU of the European Parliament.

## *2.2. Plasma and tissue sampling*

At 6-months old, and after 12-hours fasting, rats from NF and OF groups were anesthetized with sodium thiopental (60 mg/kg, i.p.), and heparin was intravenously injected (500 UI/kg). Blood was collected from the abdominal aorta, and immediately centrifuged at 4°C in order to separate the plasma, and samples were stored at -80°C until subsequent measurements. Thoracic aortas and hearts were quickly harvested to determine cardiovascular function and oxidative stress levels in tissues (see later).

## *2.3. Biochemical measurements*

Plasma glucose concentration was determined by a glucometer, and plasma insulin, leptin and adiponectin were determined by EIA using specific commercial kits (Gentaur, Paris, France). Cholesterol was assayed on a Victor 1420 Multilabel Counter (Wallac-Perkin Elmer Life Sciences, Courtaboeuf, France) using a commercially available kit (Diasys, Condom, France).

## 2.4. Oxidative stress measurements

### 2.4.1 Plasma oxidative stress level

Oxidative stress was determined in 10  $\mu$ L of plasma by the free oxygen radical test (F.O.R.T, FORM-PLUS-3000, Optimabio, Ollioules, France). The color intensity correlates directly with the amount of hydroperoxide and, consequently, with the oxidative stress level [13].

Vitamin C content was assessed in 100  $\mu$ L of plasma, stabilized by the addition of 200  $\mu$ L of metaphosphoric acid (5%) and measured by high-performance liquid chromatography coupled with fluorimetric detection ( $\lambda_{ex}$ =360 nm and  $\lambda_{em}$ =440 nm) [14].

### 2.4.2. Antioxidant enzyme activity in the heart

After an *ex vivo* ischemia/reperfusion protocol, the hearts were frozen in liquid nitrogen and stored at -80°C until the subsequent measurement of ventricular antioxidant enzyme activity.

Superoxide dismutase (SOD) general activity was measured in heart samples using a method described previously [15]. Catalase activity in the heart was measured using a modified method [16] derived from Aebi and Clairbone [17]. All cardiac enzyme activities were related to protein content determined by the Lowry method.

### 2.4.3. NADPH oxidase activity in the heart and aorta

The capacity of cardiac or vascular tissue to produce  $O_2^{\bullet-}$  in an NAD(P)H-dependent way was assessed using an LB9507 luminometer (Berthold Systems, Thoiry, France), by the measurement of superoxide-enhanced lucigenin (5  $\mu$ M) chemiluminescence, in the presence or absence of NAD(P)H (100  $\mu$ mol/L) as described previously [18]. The results are expressed

in relative light units (RLU) per mg of protein for the heart and in milligrams of dry tissue for the aorta.

## 2.5. *Susceptibility to ex vivo Ischemia/Reperfusion (I/R) injury*

### 2.5.1. Preparation of isolated working hearts

After the rats had been anesthetized with sodium thiopental (60 mg/kg, i.p.) and heparin injection (see previously), the hearts were excised and placed in a cold (4°C) perfusion buffer bath until contractions ceased. The hearts were cannulated in the Langendorff mode, and perfused at 37°C with oxygenated (O<sub>2</sub>/CO<sub>2</sub>, 95%/5%), Krebs-Henseleit (pH 7.40) solution containing (in mmol/L) NaCl, 118; NaHCO<sub>3</sub>, 25; KCl, 4.7; MgSO<sub>4</sub>, 1.2; KH<sub>2</sub>PO<sub>4</sub>, 1.2; CaCl<sub>2</sub>, 3; glucose, 5.5; at a constant hydrostatic perfusion pressure (55 mmHg). A catheter was inserted into the left ventricle and connected to a pressure transducer to measure heart rate (HR) and left ventricular pressures: left ventricular end-diastolic pressure (LVEDP), left ventricular end-systolic pressure (LVESP), left ventricular developed pressure (LVDP=LVESP-LVEDP), and the first derivative of LVDP, left ventricular maximal pressure development (+dP/dt) and relaxation (−dP/dt). The above cardiac parameters were calculated using software (A.D.E., Technolog21, Dijon, France) developed in collaboration with computer and electronics scientists. Then, an atrial cannula was inserted through the orifice of the pulmonary veins in order to perfuse the right atrium with a constant preload pressure of 15 mmHg in order to switch to the working mode. In this condition, the fluid was ejected by left ventricular contraction through the aortic root against a stable post-load pressure of 55 mmHg. Throughout each experiment, the coronary flow, aortic and cardiac output (calculated as a total of coronary and aortic output) were recorded.

### 2.5.2. Ischemia/Reperfusion (I/R) protocol

The isolated hearts were perfused in the working (W) mode for 12 min, and then the perfusion was turned off in order to induce 30 min of total global normothermic ischemia, followed by reperfusion in the Langendorff (L) mode for 12 min and for 30 min in the W mode.

Samples of coronary effluents (50  $\mu$ L) were collected during reperfusion and stored at 4°C until further use (within 6 h). Lactate dehydrogenase (LDH) activity was measured using a kit (Promega, Charbonnieres-Les-Bains, France). The accumulated amount of LDH released was obtained by integrating the area under the time-course curve. Results are expressed in arbitrary units (AU) per g of tissue.

## 2.6. *Vascular reactivity*

### 2.6.1. Coronary vasoreactivity test

The isolated hearts were perfused for 12 min in the L mode under constant pressure (55 mmHg), then turned to 12 min of L mode in constant flow conditions, for which the flow rate was adjusted in order to obtain the same aortic pressure as in the L preparation (55 mmHg). During this constant flow mode, coronary vasoreactivity was tested by the perfusion of bradykinin (BK  $5 \cdot 10^{-7}$  mol/L) and sodium nitroprussiate (SNP  $10^{-6}$  mol/L). Coronary reactivity was evaluated through variations in aortic cannula pressure when the hearts were perfused in constant flow conditions. Coronary vascular resistance was calculated according to the formula: Coronary Resistance (CR) = Aortic Pressure (AP) / Coronary Flow (CF).

### 2.6.2. Vascular reactivity of the aorta.

Aortic reactivity was measured in freshly excised thoracic aortas as described previously [19]. The functional integrity of arterial rings was tested with KCl (60 mmol/L), and the presence or absence of functional endothelium was assessed by acetylcholine (ACh, 1  $\mu$ mol/L)-induced relaxation of aortic rings precontracted with phenylephrine (1  $\mu$ mol/L).

To assess vasoconstrictive responses, the rings were contracted with cumulative additions of phenylephrine ( $10^{-9}$ - $3.10^{-5}$  mol/L) or U46619 (analog of the prostaglandin PGH<sub>2</sub>,  $10^{-10}$ - $10^{-6}$  mol/L). To assess vasorelaxing responses, arteries were pre-contracted with phenylephrine ( $10^{-6}$  mol/L), and cumulative concentration–response curves for ACh ( $10^{-9}$ - $3.10^{-5}$  mol/L) and SNP ( $10^{-10}$ - $10^{-5}$  mol/L) were constructed. Relaxation values were expressed as percentage decreases in phenylephrine-induced vasoconstriction.

## 2.7. *Ventricular remodeling*

### 2.7.1. Collagen content

Heart ventricles were fixed in 4% paraformaldehyde. Histological analysis was used to determine the development of cardiac fibrosis by staining of paraffin sections for total collagen with picrosirius red. This was quantified using computerized image analysis (Image-J-1.43 software, Wayne-Rasband NIH, MA, USA), which was manually set to a detection level threshold. Ten fields were randomly chosen from each section of left ventricle, analyzed by the same blinded experimenter, and the mean intensity was calculated. The percentage area was determined by calculating the positive stained area as a percentage of the total image area.

### 2.7.2. Matrix metallo-proteinase-2 (MMP-2) expression and activity

Total RNA was extracted from the ventricles with Trizol reagent (Invitrogen, Cergy Pontoise, France). mRNA (1 µg) was reverse transcribed with MMLV reverse transcriptase (Invitrogen, id.) using random hexamers (Invitrogen, id.) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-PCR) was performed with 2 µL of cDNA using the SYBR-Green PCR Master-Mix (Applied Biosystems) and both sense and antisense primers (5 µmol/L) in a final volume of 20 µL, in an 7500-Fast-Real-Time PCR system (Applied Biosystems). The primers used for the amplification of rat MMP-2 were: forward 5'-GAGAAAAGCGCAGCGGAGTGACG-3', and reverse 5'-TTCCCCCGCAAGCCCAAGTG-3'. Target gene expression was normalized against GAPDH gene expression.

Proteins were extracted from frozen heart tissue in lysis buffer containing 250 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, Nonidet-P40 1%. Protein concentrations were determined by the Lowry method. Samples were mixed with SDS buffer without a reducing agent and 30 µg of proteins were loaded onto a 10% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gels were incubated with 2.5% Triton X-100 for 2 times 30 min, and then incubated overnight at 37°C with buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 10 mmol/L CaCl<sub>2</sub> and 0.02% Triton X-100, pH 7.4). The gels were stained with 0.1% Coomassie-brilliant-blue, and gelatinolytic activity was revealed as clear bands against a blue-stained background. Digestion bands were quantitated by an image analyzer system (Image-J-1.43 software, Wayne-Rasband-NIH, MA, USA).

## 2.8. *Statistical analysis*

The results are expressed as mean values  $\pm$  SEM. The statistical significance of differences between two means was determined by a Student's *t*-test. For weight gain and food consumption, a repeat ANOVA was performed to investigate differences between the two groups.

## 3. Results

### 3.1. *Body weight and food consumption*

Weight gain was significantly greater in overfed pups during the suckling period (Figure 1A). At weaning (day 24), OF rats showed a 50% increase in body weight; the increase in body weight persisted with maturation, but was less marked (10%) (Figure 1B). This increase in body weight was associated with a significantly greater food intake (+15%) in OF than in NF rats (Figure 1C and D). Heart weight of 7-months old OF rats was also 10 % higher than that of NF ( $1.29 \pm 0.03$  vs.  $1.15 \pm 0.05$ ) but the heart weight/body weight ratio, a global marker of cardiac hypertrophy, was identical in the two groups.

### 3.2. *Biochemical parameters*

As shown in Table 1, we observed a 10% increase in fasting blood glucose in 6-month-old OF rats. No significant difference was observed between OF and NF rats concerning cholesterol and triglyceride levels. However, plasma levels of fasting insulin and leptin were significantly

greater in OF rats than in NF rats ( $p < 0.05$ ). In contrast, adiponectin levels were significantly lower in OF rats.

### 3.3. *Oxidative stress measurements in plasma and tissues*

We observed significantly higher plasma levels of hydroperoxide (Figure 2A) and lower levels of plasma vitamin C (Figure 2B) in the OF group than in the NF group.

In heart ventricular tissue, SOD total activity was significantly higher in the OF group than in the NF group (figure 2C). However, cardiac catalase activities in the OF ( $84.7 \pm 2.7$  IU/mg protein) and NF ( $84.5 \pm 2.3$  IU/mg protein) rats were not different.

NADPH oxidase activity in heart tissue homogenates (OF:  $95.2 \pm 15.6$ ; NF:  $115.6 \pm 8.2$  RLU/mg protein) and in thoracic aortas (OF:  $1778 \pm 193$ ; NF:  $1738 \pm 141$  RLU/mg dry weight) were not significantly different either.

### 3.4. *Heart's susceptibility to ischemia/reperfusion injury*

In baseline perfusion conditions, there was no difference between OF and NF rats in all of the cardiac parameters measured (Table 2). After 30 minutes of ischemia, reperfusion of the myocardium led to a progressive but incomplete recovery of the heart's circulating and contractile activity. After 12 min of reperfusion in the L-mode and 30 min in the W-mode, the recovery of coronary flow was approximately 65% of the pre-ischemic values, while recovery of LVDP,  $+dP/dt$  and  $-dP/dt$  were 38 %, 33% and 38% respectively. For these parameters, hearts from NF rats and OF rats did not differ significantly. However, the recovery of cardiac output was significantly lower (38% vs. 65%,  $p < 0.05$ ) in OF than in NF (Figure 3A). In

addition, the total amount of LDH, a marker of cell injury, released in the coronary effluent during the period of reperfusion, was found to be significantly higher in post-ischemic OF hearts than in NF hearts (Figure 3B).

### 3.5. *Vascular reactivity*

In isolated hearts perfused in constant flow conditions, the perfusion of BK or SNP in the coronary vasculature led to a drop in aortic perfusion pressure consecutive to a decrease in coronary vascular resistance (Figure 4). There was no difference between NF hearts and OF hearts for the coronary endothelium-dependent and independent response.

In isolated aortic tissue, the addition of increasing concentrations of phenylephrine or U46619, led to a gradual vasoconstrictive response, for which the aorta of NF or OF rats did not differ (Figure 5A and B). The addition of increasing concentrations of ACh or of SNP gradually relaxed precontracted arteries, showing the integrity of both endothelial and smooth muscle responses (Figure 5A and B). No differences between NF and OF groups were observed concerning their aortic vasoreactivity (Figure 5C and D).

### 3.6. *Ventricular remodeling*

Histological studies showed a higher collagen density (+54%) in the left and right ventricles of adult OF rat hearts (Figure 6A and 6B). The analysis of MMP-2 expression by quantitative RT-PCR and activity by gelatin zymography in whole ventricles (left and right) showed an increase in both MMP-2 expression and activity in OF rat heart ventricular tissue (Figure 6C and D).

## 4. Discussion

Earlier studies suggested that obesity and overweight were important factors in the development of diabetes and in the aggravation of cardiovascular risk in adulthood.

The experimental model of postnatal OF (overfeeding) induced by reducing litter size is a newly described model of early nutritional disturbances, due to the greater availability of breast milk in the immediate postnatal period. These disturbances can lead to the development of immediate and long-term modifications not only in body weight and food intake, but also in several biochemical and metabolic abnormalities [4, 20, 21]. In our study, we found that POF induced a tremendous increase in body weight at weaning (+50%); the increase in body weight persisted with maturation, though at a lower level (+10%), along with a permanent increase in food consumption (+15%). Additionally, we did not observe any changes in heart weight/body weight ratio in OF adult rats. These observations are consistent with earlier studies [22], with some differences that could be due to the number of pups per mother, strains and the composition of the standard diet. Some authors showed that the increase in body weight observed in OF rats was mainly due to an accumulation of visceral adipose tissue [5, 7, 23].

In fact, postnatal overfeeding leads to the increased inhibition by neuropeptide Y (NPY) of hypothalamic ventro-median nucleus (VMN) neurons, which are involved in satiety regulation [24]. This could explain the hyperphagia observed in OF rats in our study. Hyperphagia in adulthood in our study was accompanied by an increase in plasma insulin and leptin, a result that confirms those of Plageman and others [4, 7, 9]. Leptin is an adipokine known to reduce food intake and increase energy expenditure, and circulating levels of leptin are directly proportional to whole body adipose mass. Though OF rats have increased circulating levels of leptin, recent studies suggest they have central leptin resistance, due to

lower type-b leptin receptor [9, 25], lower JAK2 and STAT3 and higher SOCS3 [26] expression in the hypothalamus. Previous studies have shown that malformations of hypothalamic nuclei are induced by hyperinsulinemia during the neonatal period [27]. In fact, hyperinsulinemia and hyperleptinemia start in the earliest stages of life in OF pups, and lead to permanent insulin resistance at both the hypothalamic [28] and peripheral [29] levels.

Alterations in certain biochemical parameters, such as increased fasting glucose and an impaired glucose/insulin ratio, were observed in OF rats. This elevation of fasting glucose by postnatal OF is sometimes observed at weaning [7, 26] and later in life [4, 7], but this change does not seem to be constantly present in overfed rats. On the contrary, a drop in the glucose/insulin ratio is frequently reported [7, 8, 23, 30], as is glucose intolerance shown by modifications in oral or intra-peritoneal glucose tolerance tests [4, 5, 7, 8, 23, 26], corresponding to a situation of pre-diabetes. Concerning plasma lipids, we found no modifications in fasting triglycerides and cholesterol levels in OF rats. Certain studies have shown not only an increase in plasma triglycerides and cholesterol in OF pups at weaning [31] and later [7], but also increases in circulating free fatty acid (FFA) [7, 8]. Some authors have shown that hyperinsulinemia may stimulate the storage and accumulation of FFA in adipose tissue [32, 33], but the experimental conditions of our study did not allow us to confirm this hypothesis.

Given that OF rats had increased adipose mass [5, 7, 23], we also assessed the plasma levels of two adipokines, leptin and adiponectin, at 180 days and found an increase in leptin and a decrease in adiponectin in OF rats. The increase in leptin levels in OF animals is constantly reported in the literature [6, 7, 9, 23, 26, 29, 34], and can be explained by the fact that leptin levels are related to the amount of fat mass. Concerning adiponectin, our results corroborate those obtained by Boullu-Ciocca and collaborators [8] at 150 days, but other studies showed no difference at 4 and 6 months between OF and NF groups [6, 26]. Adiponectin production

is inversely proportional to whole body adipose mass, and experimental studies suggest that it modulates the action of insulin and thus improves insulin sensitivity in peripheral tissues [35] and induces general cardioprotective effects [36].

Obesity and overweight are associated with increased oxidative stress not only in the bloodstream, but also in myocardial tissue [37, 38]. However, to date, no other study has explored the impact of OF on oxidative stress markers such as peroxidation products, tissue activity of enzymes such as NADPH oxidases or vitamin or enzyme-related antioxidant defenses. In our study, we evaluated circulating levels of hydroperoxides and vitamin C and observed an increase in hydroperoxides and a decrease in vitamin C levels indicating increased circulating oxidative stress in OF rats. Unlike humans, rats synthesize their own vitamin C in the liver, but they can also ingest it in their food. However, intestinal transporters of ascorbate or dehydroascorbate are not very abundant in rodents except in gulonolactone oxidase-deficient strains [39, 40] and the composition of the standard diet we have used in our study does not mention the presence of vitamin C. Therefore, the decrease in vitamin C could be related to a higher depletion of this hydro-soluble antioxidant vitamin due to increased circulating oxidative stress, evidenced by higher peroxidation markers. However, we cannot exclude the possibility of lower hepatic synthesis of vitamin C. This increase in oxidative stress cannot be explained by increased NADPH oxidase activity in vessels or tissues, since in our study it was not modified in OF aortas or hearts. In the context of overweight, other factors, such as a higher rate of mitochondrial oxidative activity, might explain an increase in oxidative stress. Paradoxically, we also observed a significant increase in SOD activity in heart tissue homogenates, which could reflect an adaptive response to oxidative stress. In fact, some studies have demonstrated an association between obesity and increased myocardial oxidative stress [38]. Concerning the adaptive response, we previously showed in rats with

major vitamin C deficiency, an up-regulation of several antioxidant and protective pathways such as CuZn and MnSOD [39].

Given the metabolic alterations and increased oxidative stress induced by postnatal overfeeding, we addressed the consequences of overfeeding on cardiovascular parameters. We first examined the recovery of pre-ischemic developed pressure, contractility and relaxation after I/R injury, and then aortic and coronary vasoreactivity. We found that postnatal-OF induced higher susceptibility of the heart to ischemia-induced injury, as shown by a lower recovery of pre-ischemic cardiac output and an increase in cardiac LDH release and activity, indicating more severe cell damage. However in our study, we found no significant differences in basal vascular parameters such as aortic and coronary vasoreactivity, or in levels of systemic arterial pressure (data not shown). Nevertheless, some studies have shown that a high-fat diet induces dysfunctions in endothelium-dependent vasorelaxation [41] which can be restored by dietary antioxidants [41, 42]. However, since rodents are known to have low plasma LDL and are not prone to developing atherosclerosis, wild-type rats may not be the ideal model to study the vascular consequences of moderate overweight, and future research should be conducted in genetically-modified models to overcome this problem. Therefore, two conclusions may be drawn from these contrasting results. In our model, the OF-induced metabolic and hormonal alterations were not severe enough to induce modifications of vascular reactivity, despite the increase in circulating markers of oxidative stress. However, in a stressful condition such as I/R injury, which generates a massive release of reactive oxygen species [43, 44], the metabolic alteration induced by OF renders the myocardium more susceptible to injury. In addition, it is unlikely that this higher susceptibility is due to the restricted recovery of coronary perfusion (not modified in basal and post-ischemic conditions), and is therefore more probably related to impaired responses

from the cardiomyocytes themselves. In keeping with this hypothesis, previous studies suggested that overfeeding in the neonatal period can induce left ventricular hypertrophy at 21 days [31] and increased cardiac fibrosis in 16-week-old adult hearts [34].

We therefore explored the possibility of ventricular remodeling and found both an increase in collagen content and an increase in MMP-2 expression and activity in the ventricles of OF rats, showing an alteration of the architectural organization of hearts from moderately overweight 6-months-old OF rats. Several environmental factors such as oxidative stress or increased leptin levels might explain the occurrence of this remodeling phenomenon. Indeed, studies have highlighted the role of leptin and insulin in the elongation of cardiac myocytes [45], ventricular hypertrophy [46, 47], and heart failure [48]. The accumulation of fibrous tissue, mainly type I and III collagen, is a major contributing factor in heart failure, and certain authors showed, in an angiotensin II model of non-hypertrophic remodeling [49], that rat hearts with accumulations of collagen similar to those observed in our study were more prone to ischemia-reperfusion injury. In fact, collagen accumulation could lead to increased stiffening of the myocardium, thus impairing diastolic filling of the ventricle (although we found no changes in ventricular function using *in vivo* echocardiography), which may hamper the recovery of cardiac output after ischemia.

## **5. Conclusion**

To conclude, our study highlights OF-induced increases in circulating markers of oxidative stress, while showing for the first time that the myocardium of moderately-overweight adult OF rats is more prone to ischemia-reperfusion injury, which may be explained, at least in part, by ventricular remodeling. Indeed, our study supports the well-known hypothesis that

nutritional changes occurring in the neonatal period may induce epigenetic modifications that affect gene expression and thus alter metabolic features in adulthood [50]. From a clinical point of view, this genetic imprinting encountered in either low birth weight [51] or, on the contrary, postnatal overweight infants in a context of maternal diabetes, is now recognized as risk factor for cardiovascular disease in overweight individuals [52, 53]. These results suggest that the nutritional state in the immediate postnatal period should be taken into account since it may have an impact on cardiovascular risk in adulthood, even in moderately overweight individuals.

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## **Legends to figures:**

### **Figure 1: Increase in body weight and food intake during life in NF and OF rats.**

Graph B shows the percentage of body weight increase in OF rats as compared to that in NF rats. Number of animals per group = 10.

\*,  $P < 0.05$  was considered significant (one-way ANOVA).

### **Figure 2: Oxidative stress indexes in plasma and heart tissue.**

A: plasma hydroperoxides. B: plasma vitamin C concentration. C: cardiac SOD activity.

D: cardiac catalase activity

### **Figure 3: Myocardial contractility parameters and tissue injury after ischemia. A:**

recovery of pre-ischemic cardiac output, B: LDH activity in coronary effluent, a marker of the extent of necrosis. Hearts were subjected to 30 min of ischemia (37°C) and 45 min of reperfusion.

### **Figure 4: Coronary vasoreactivity as measured by variations in coronary resistance in**

**NF and OF isolated hearts:** perfused in the presence of endothelium-dependent ( $BK\ 5.10^{-7}$  mol/L) and endothelium-independent ( $SNP\ 10^{-6}$  mol/L) vasodilators. Variations in coronary resistance are expressed in percentages.

### **Figure 5: Aortic reactivity of isolated thoracic aortas of NF or OF rats:**

subjected to different concentrations of vasoconstrictors and vasodilators. A: Phenylephrine, B: U46619 (analog of the prostaglandin  $PGH_2$ ), C: Acetylcholine, D: Sodium nitroprussiate (SNP).

### **Figure 6: Ventricular remodeling of hearts from NF or OF rats:**

A: representative picrosirius collagen staining of ventricular tissue. B: collagen density (% of area), C: MMP-2 mRNA expression (quantitative RT-PCR) and D: activity (gelatin zymography).

**Table 1:** Metabolic parameters of NF and OF rats groups. Fasting blood glucose was assayed by glucometry; cholesterol and triglycerides were assayed using colorimetric methods and plasma insulin, leptin and adiponectin were estimated using ELISA. P was calculated by Student's *t*-test.

	NF	OF
Fasting glucose (mmol/L)	7.49 ± 0.32	8.30 ± 0.37 *
Cholesterol (g/L)	0.80 ± 0.03	0.88 ± 0.10
Triglycerides (g/L)	0.75 ± 0.08	0.90 ± 0.18
Insulin (mIU/L)	40.2 ± 8.8	67.0 ± 7.9 *
Leptin (ng/dL)	4.03 ± 0.48	5.84 ± 0.48 *
Adiponectin (µg/mL)	7.07 ± 0.55	5.12 ± 0.57 *

\*,  $P < 0.05$ , NF vs. OF group.

**Table 2:** Pre-ischemic isolated heart parameters of normally fed (NF) or postnatal overfed (OF) 7-months' old rats. Parameters were recorded after 12 min in the Langendorff mode, (L 12) and after 12 min in the working mode (W12). P was calculated by Student's *t*-test.

	NF		OF	
	L 12	W 12	L 12	W 12
Coronary output (ml/min/g)	13.0 ± 0.5	12.1 ± 1.0	13.2 ± 0.7	15.3 ± 2.2
Cardiac output (ml/min/g)	13.0 ± 0.5	14.57 ± 1.94	13.24 ± 0.67	16.77 ± 2.52
LV developed pressure (mmHg)	66.9 ± 4.1	76.8 ± 6.2	66.8 ± 2.3	81.5 ± 6.3
+dP/dt (mmHg)	1796 ± 97	1886 ± 169	1785 ± 35	1988 ± 150
-dP/dt (mmHg)	-1163 ± 77	-1252 ± 142	-1152 ± 42	-1366 ± 139