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High-fat diet promotes a pro-inflammatory environment in testis and inhibits antioxidant defenses in the progeny

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Abstract: The adoption of high-fat diets (HFD) is a major contributor to the increasing prevalence of obesity worldwide. Herein we study the impact of HFD from early age in testicular physiology and sperm parameters in two generations of mice, with a focus on testicular oxidative status. Mice of the diet-challenged generation (F0; n=36) were randomly fed after weaning with standard chow (CTRL) or high-fat diet (HFD) for 200 days or transient high-fat diet (HFDt) (60 days of HFD+140 days of standard chow). The offspring generation (F1; n=36) was obtained by mating with normoponderal females with 120 days post-weaning and fed with chow diet. Mice fed with HFD for a lifetime have impaired insulin tolerance, a trait inherited by their sons. The sons of mice fed HFD inherited decreased catalase activity, displayed lower activities of mitochondrial complexes I and IV. Similar to their progenitos, the sons of HFD mice had a higher prevalence of pinhead and bent neck defects, than the sons of CTRL. The adoption of HFD impairs testicular antioxidant defenses and mitochondrial function in the progeny, which is detrimental to sperm morphology.

Keywords: high-fat diet, intergenerational effects, pro-inflammatory state, antioxidant defenses, testis

1. Introduction

Overweight and obesity have achieved epidemic proportions worldwide, mostly due to lifestyle choices, such as low levels of physical activity and the adoption of a high-fat diet (HFD) [1,2]. These numbers raise concerns about the consequences of sexual health in men of reproductive age suffering from excess adiposity and common comorbidities, such as Type 2 Diabetes (T2D) [3,4]. These

concerns were aggravated by recent evidence of the intergenerational effects of acquired traits, *i.e.*, by the evidence that acquired traits can compromise the health of the offspring, especially in the male reproductive health [5,6].

We have previously described the effects of HFD, even if temporary, in sperm parameters and testicular composition of mice [7,8]. Hereby we investigate the effects of HFD on sperm parameters and testicular physiology of the offspring (sons), with a focus on testicular antioxidative status.

2. Experiments

2.1. Animal Model

This study was performed in 2 generations of *Mus musculus* C57BL6/J mice. The first generation (Generation F0), was originated from normoponderal progenitors (both male and female), fed with a standard chow (#F4031, BioServ, USA – Carbohydrate: 61.6%, Protein: 20.5%, Fat: 7.2% [16.3% Kcals]) and water *ad libidum*. After weaning (21-23 days), F0 mice (n=36) were randomly divided in three groups: control (CTRL) (n=12), HFD (n=12) and HFDt (n=12). Mice from the CTRL group were fed with a standard chow. Mice from the HFD group received a fat-enriched diet (#F3282, BioServ, USA – Carbohydrate: 35.7%, Protein: 20.5%, Fat: 36.0% [59.0% Kcals]). The mice from the HFDt group were fed with a fat-enriched diet for 60 days (#F3282, BioServ, New Jersey, USA), then switched to standard chow (#F4031, BioServ, New Jersey, USA). F0 mice were mated starting at 120 days of age with normoponderal, chow-fed, same-age randomly selected females to generate the F1 generation. Mating lasted for 8 days and consisted of placing a male and a female in the same cage for 6 hours each day, without water or food supply. After weaning, F1 mice were assigned to the same experimental group as their fathers: CTRL – Sons of CTRL (n=12); HFD – Sons of HFD (n=12); HFDt – Sons of HFDt (n=12). Litters were generated until the target number of mice per group (n = 12) was achieved. In this generation (F1), all mice were fed with standard chow. Food and water were supplied without restrictions. The mating of F1 mice was performed under the same conditions as their progenitors (Generation F0). Mice from both generations were killed by cervical dislocation 200 days after weaning, and tissues were collected for further analysis. Total body weight, water, and food intake were monitored weekly from weaning to sacrifice. The animal model is compliant with the ARRIVE guidelines and was licensed by the Portuguese Veterinarian and Food Department (0421/000/000/2016).

2.2. Glucose Homeostasis Assessment

One week before sacrifice, glucose homeostasis was evaluated by the intraperitoneal Glucose Tolerance Test (ipGTT) and intraperitoneal Insulin Resistance Test (ipITT), according to the previously described protocol [8]. Fasting Glucose was measured before sacrifice, as described [7,8]. Serum was separated from blood obtained by cardiac puncture at sacrifice, and insulin was measured via ELISA [7]. HOMA2 indexes were calculated based on the serum insulin and glucose at sacrifice, using the HOMA2 Calculator [9].

2.3. Enzymatic activity of antioxidant enzymes and mitochondrial complexes

The enzymatic activity of antioxidant enzymes and mitochondrial complexes were evaluated from testicular extracts. A phase separation protocol was used to obtain a mitochondria-rich fraction and cytosolic, mitochondria-free, fraction, according to a protocol previously described [7]. Lipid peroxidation was measured in the cytosolic fraction of the testicular extract by the TBARS assay [7]. Enzymatic activities of Glutathione Peroxidase (GPx), Glutathione S-Reductase (GSR) and Mitochondrial Complex I were measured by fluoroscopic methods in 96-well plates, as described [7]. Enzymatic activities of Superoxide Dismutase (SOD), Citrate Synthase, Mitochondrial Complex II and Mitochondrial Complex IV were measured by colorimetric methods in 96-well plates, as described [7]. All colorimetric and fluorometric readings were obtained using a Biotek Synergy H1

plate reader (Winooski, VT, USA). Catalase (CAT) activity was polarographically determined following oxygen production resulting from H_2O_2 decomposition using a Clark-type oxygen electrode (Hansatech, Norfolk, UK), as described [7].

2.4. Assessment of sperm parameters

Sperm was collected from the right epididymis of each mice after sacrifice. Sperm count and motility were immediately assessed as previously described [7,8]. Sperm viability and morphology were evaluated using specific staining techniques and optical microscopy as previously described [7,8].

2.5. Untargeted metabolomics and lipidomics

A combined extraction of polar and nonpolar metabolites from testicular tissue (50 mg) was performed as previously described [7,8]. Two fractions result from the method – an aqueous, polar fraction; and an organic, nonpolar fraction. ^{1}H-NMR was performed to analyse and quantify the metabolites in the polar fraction, according to our methods [7,8]. GC-MS was used to analyse and quantify the nonpolar metabolites soluble in the organic fraction [7].

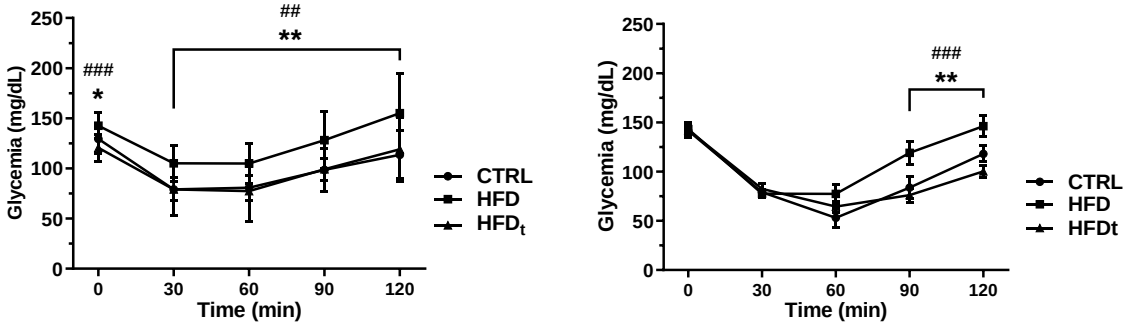
2.6. Statistics

Univariate parametric statistics were the preferred statistical methods. The assumptions of normality and homoscedasticity requested for parametric statistics were tested, for each variable, using the Kolmogorov-Smirnoff test with Lillefor's correction, and Levene's test, respectively. Univariate ANOVA was corrected for pairwise corrections by Tukey's Honest Significant Difference (HSD). ipGTT and ipITT data were tested using Repeated Mesures (RM) ANOVA corrected by Šidak's method for pairwise comparisons. Data was previously tested for sphericity using Bartlett's test. The distribution of sperm defects was tested using the χ^2 test, and column proportions were tested by z-test corrected for pairwise comparisons by Bonferroni's method. Significance cutoff was set when $p < 0.05$. All methods were performed using IBM SPSS Statistics v26 (Armonk, NY, USA).

3. Results

3.1. The offspring of HFD-fed mice display abnormal insulin tolerance

Glucose homeostasis was assessed by ipGTT, ipITT and HOMA2 indexes. The sons of the mice fed a lifelong HFD displayed higher serum glucose at 90 and 120 minutes of the ipITT than the sons of CTRL and HFDt (Figure 1). Regarding HOMA2 indexes, no differences were found between the sons of diet-challenged mice.



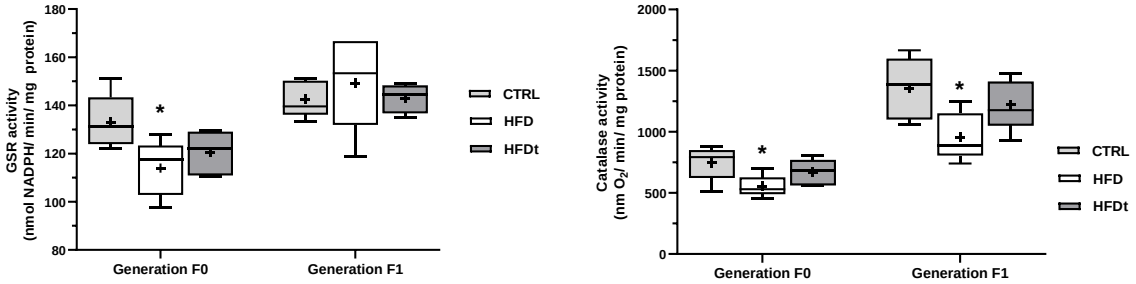
(**a**) (**b**)

Figure 1. Glycemia during the ipITT, in (**a**) mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFDt) (Generation F0); and (**b**) their progeny (Generation F1). Results are expressed as the mean (mg/dL) ± SD, in function of time

(minutes). Data was tested by two-way ANOVA corrected by Šidak's method. Significance was considered when $p < 0.05$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. * CTRL vs. HFD; # HFD vs. HFDt.

3.2. The adoption of HFD inhibits testicular antioxidant defences even in offspring

The enzymatic activity of antioxidant enzymes was measured in testes. Mice fed with HFD for a lifetime had decreased activity of GSR and Catalase (Figure 2). Interestingly, this phenotype was partially inherited by their sons, which had decreased testicular Catalase activity, compared to the sons of CTRL.

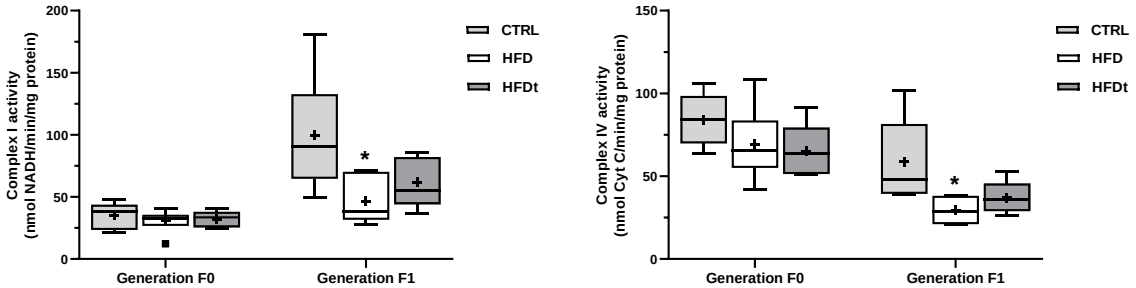


(**a**) (**b**)

Figure 2. Enzymatic activity of the antioxidant enzymes (**a**) GSR and (**b**) Catalase, in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFDt), and their progeny (Generation F1). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.3. Testicular mitochondrial defects are only detected in offspring of HFD-fed mice

The enzymatic activity of mitochondrial complexes was measured in testes. No changes were found between groups of diet-challenged mice (Generation F0) (Figure 3). The sons of lifelong HFD-fed mice showed reduced activity of mitochondrial Complex I and Complex IV.



(**a**) (**b**)

Figure 3. Enzymatic activity of the mitochondrial (**a**) Complex I and (**b**) Complex IV, in testes of mice fed standard chow (CTRL), life-long high-fat diet (HFD), and those subjected to diet correction after 60 days (HFDt), and their progeny (Generation F1). Results are expressed as mean ± standard deviation. Experimental groups were compared by one-way ANOVA with Tukey's HSD. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.4. Paternal HFD causes intergenerational sperm defects

Epididymal sperm parameters were evaluated after sacrifice. Sperm count and motility were assessed immediately after collection. Contrary to their progenitors, the sons of HFD and HFDt did not show differences in sperm motility and viability, when compared to sons of CTRL. Yet, regarding

sperm morphology, the sons of HFD mice have a greater prevalence of pinhead defects, comparing to sons of CTRL, and a greater prevalence of bent neck defects than the sons of HFDt (Figure 4).

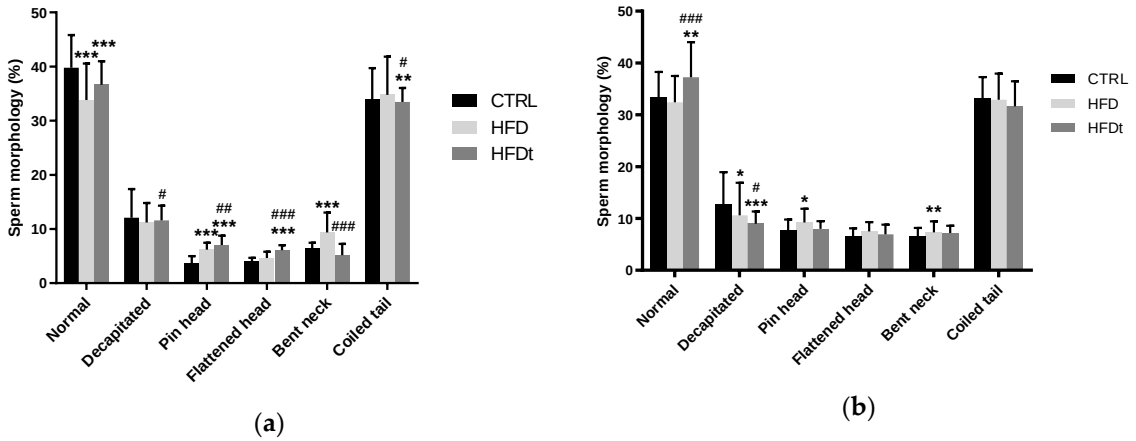


Figure 4. Distribution of sperm morphology, per experimental group in the (**a**) diet-challenged generation (F0), and their offspring (Generation F1). Data is presented as the mean (%) ± SD. Independence of experimental groups was tested by χ^2 test, and column proportions were tested with Z-test corrected for pairwise comparisons by Bonferroni's method. Significance was considered when $p < 0.05$. * vs. CTRL; # vs. HFD. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

3.5. Testicular metabolic and lipidomic signatures of HFD are not inherited by direct offspring

Testicular polar and nonpolar metabolites were extracted and quantified by either ^{1}H-NMR or GC-MS. However, no significant changes were found in the testicular content of the sons of the diet-challenged mice.

4. Discussion

In this work, we evaluated the impact of an ancestral paternal exposure to HFD, either lifelong or up to early adulthood, on testicular metabolism and sperm parameters of the direct offspring (sons). To do so, we evaluate the oxidative status, the mitochondrial function and the metabolite content in testis of mice generated from mice fed with standard chow (CTRL), with a lifelong HFD (HFD) or with an HFD from weaning to early adulthood, then replaced with standard chow (HFDt).

We also evaluated the whole-body glucose homeostasis to search for cues of metabolic syndrome that may be inherited by the offspring. Indeed, the sons of HFD mice have shown signs of insulin intolerance, as for their performance in the ipITT (figure 1). We have previously described that the inhibition of testicular antioxidant enzymes catalase and GSR, and the increased testicular content of ω6-polyunsaturated fatty acids, elicit that a lifelong HFD promotes a pro-inflammatory environment in testis [7]. To balance this HFD-induced inflammation, mice fed with HFD have increased testicular glutathione (GSH) and taurine levels [8]. Similarly to their progenitors, the sons of HFD mice showed lower enzymatic activity of catalase in testes, suggesting an inheritable trait caused by the pro-inflammatory testicular environment of the progenitor. However, no differences in lipid peroxidation in either diet-challenged mice nor in their sons were found. Notwithstanding, no changes in testicular metabolome were found in the sons of HFD, compared to sons of CTRL and HFDt. Therefore, it is unclear whether the testicular oxidative balance is being balanced by other mechanisms. This is even more interesting considering the decrease in the mitochondrial activity of complex I and IV, found in the sons of HFD. Opposingly, the sons of HFD mice have the highest prevalence of sperm head defects (compared to sons of CTRL) and sperm neck defects (comparing to sons of HFDt). Indeed, oxidative damage in the testis has been linked to a higher prevalence of abnormal sperm [10]. Hence, the changes in antioxidant defenses and mitochondrial activity observed in the testis of sons of HFD mice might be associated with the increase of abnormal sperm.

Interestingly, the differences found in sons of diet-challenged mice, in all evaluated parameters, are restricted to the sons of HFD. These mice were the only group receiving HFD at the moment of conception. Several reports mention that sperm carries non-genomic factors, such as small non-coding RNA (sncRNA) and epigenetic modifications, that are sensitive to diet [11,12]. Therefore, the phenotypes observed in the sons of HFD mice are likely manifestations of intergenerational effects of HFD, *i.e.*, they are the result of direct exposure of the male gamete to the toxicant (HFD) [13].

5. Conclusions

The adoption of HFD by fathers causes intergenerational signatures in testis, notably in antioxidant defenses and mitochondrial activity. Those signatures, in turn, are associated with a higher prevalence of sperm head and neck defects. The negative impact of paternal HFD is more evident if it is continued at the moment of conception.

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Author Contributions: L.C., P.F.O., M.G.A. and R.L.B. contributed to study design, analysis and interpretation of data. L.R., I.J., K.S., T.M., R.A.V. and L.C. performed experimental work. L.C. edited the images and tables, performed the statistics and contributed to the analysis and interpretation of data. R.L.B., J.F.R. and R.A.V. critically reviewed the manuscript and suggested modifications. All the authors contributed to manuscript writing/editing and approved the final version.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

^{1}H-NMR: Proton Nuclear Magnetic Resonance
ANOVA: Analysis of Variance
CTRL: Standard diet (standard chow)
GC-MS: Gaseous Chromatography – Mass Spectroscopy
GPx: Glutathione Peroxidase
GSH: Glutathione
GSR: Glutathione S-Reductase
HFD: High-fat diet
HFDt: Transient High-fat diet
HSD: Honest Significant Difference
RM: Repeated Measures
SD: Standard Deviation
sncRNA: small non-coding RNA
SOD: Superoxide Dismutase

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