Impact of High Fat Diet on the Sterol Regulatory Element-Binding Protein 2 cholesterol pathway in the testicle.

Abi K. Funes [1], Layla Simón [1], Regina Colombo [1], María Virginia Avena [1], María Monclús [1, 2], Julieta Crescitelli [2], María E. Cabrillana [1, 2], María Inés Conte [1], Niubys Cayado [2, 3], Paola Boarelli [4], Miguel W. Fornés [1, 2, a], Tania E. Saez Lancellotti [1, 2, b]

[1] [a*] Laboratorio de Investigaciones Andrológicas de Mendoza (LIAM). [b*] Laboratorio de Biología Molecular del Metabolismo & Nutrición ([b]MeNu). Instituto de Histología y Embriología (IHEM), Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Centro Científico y Tecnológico (CCT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Mendoza, Argentina.

[2] Instituto de Investigaciones. Facultad de Ciencias Médicas. Universidad del Aconcagua. Mendoza, Argentina.

[3] Laboratorio de Oncología, Instituto de Medicina y Biología Experimental de Cuyo (IMBECU), CCT, CONICET, Mendoza, Argentina.

[4] Laboratorio de Enfermedades Metabólicas (LEM), Universidad Juan Agustín Maza, Mendoza, Argentina.

Short title: **Srebp2 activity in lipid stressed testicles**

Key words: cholesterol, sperm, SREBP, male infertility, testicle

Corresponding author e-mail: efsaez@fcm.uncu.edu.ar, Centro Universitario.

M5502JMA. Mendoza, Argentina

* These labs contributed equally to this work.

ABSTRACT Male fertility has been shown to be dependent on cholesterol homeostasis. This lipid is essential for testosterone synthesis and spermatogenesis, but its levels must be maintained in an optimal range for proper testicular function. In particular, sperm cells development is very sensitive to high cholesterol levels, noticeably during acrosomal formation. The aim of this work was to study whether the molecular pathway that regulates intracellular cholesterol, the Sterol Regulatory Element-Binding Protein (SREBP) pathway, is affected in the testicles of animals under a fat diet. To investigate this, we took advantage of the non-obese hypercholesterolemia (HC) model in New Zealand rabbits that displays poor sperm and seminal quality. The testicular expression of SREBP isoform 2 (SREBP2) and its target molecules 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) and low-density lipoprotein receptor (LDLR) were studied under acute (6 months) and chronic (more than 12 months) fat intake by RT-PCR, western blot and immunofluorescence. Our findings showed that fat consumption promoted down regulation of the SREBP2 pathway in the testicle at 6 months, but upregulation after a chronic period. This was consistent with load of testicular cholesterol, assessed by filipin staining. In conclusion, the intracellular pathway that regulates cholesterol levels in the testicle is sensitive to dietary fats, and behaves differently depending on the duration of consumption: it has a short-term protective effect, but became deregulated in the long term, ultimately leading to a detrimental situation. These results will contribute to the understanding of the basic mechanisms of the effect of fat consumption in humans with idiopathic infertility.

3

INTRODUCTION

Lipid metabolic disorders promoted by diet are constantly increasing in occidental societies and lead to the development of pathologies such as obesity, diabetes, and metabolic syndrome (Organization, 2020, Organization, 2016). Obesity has been recently associated with male infertility (Guh et al., 2009, Flegal et al., 2013, Hammoud et al., 2008, Carlsen et al., 1992, MacDonald et al., 2010, Teerds et al., 2011, Du Plessis et al., 2010), and hyperlipidemias (abnormally elevated levels of lipids or lipoproteins in the blood) may play an important role in semen quality, in addition to other environmental or lifestyle factors (Magnusdottir et al., 2005). The recent Longitudinal Investigation of Fertility and the Environment (LIFE) study indicated that serum lipid levels may affect semen parameters, highlighting the importance of cholesterol and lipid homeostasis for male fecundity (Buck Louis et al., 2014). Population studies showed that a higher BMI increased body adiposity and higher intakes of saturated fat are associated with deleterious effects on sperm concentration, motility, morphology, mitochondrial activity, acrosome integrity, and increased DNA fragmentation (Fariello et al., 2012, Jain, 2015).

Numerous animal studies have provided evidence of associations between cholesterol and male fertility. Cholesterol-fed mice, rats and rabbits showed decreased spermatid cell number, reduced seminiferous tubules' diameter, smaller Leydig cell nuclear dimensions, asthenozoospermia (low motility), oligozoospermia (low sperm count), teratozoospermia (abnormal morphology) and diminished sperm functions as capacitation and acrosomal reaction (Gwynne and Strauss, 1982).

4

Cholesterol is found in the sperm membrane and its levels within the membrane affects fluidity and helps determine motility, capacitation and acrosome reaction (Cross, 1998), all important processes required for successful fertilization (Yanagimachi, 1994). However, the negative impact of hypercholesterolemia on semen quality is still poorly understood at the molecular level.

The molecular regulation of intracellular cholesterol is tightly associated with circulating cholesterol and depends on a pathway with the Sterol Regulatory Element-Binding Protein 2 (SREBP2) as its lead molecule (Madison, 2016, Howe et al., 2016). This protein senses the amount of cholesterol available in the cell and activates a response according to whether the lipid is excessive or scarce. This pathway has not yet been thoroughly explored in seminiferous tubules, but recent proteomic studies found changes in the expression of SREBP2 in testes from mice fed with a high fat diet (Jarvis et al., 2020).

The aim of this work was to study the response of the intracellular machinery that regulates testicular cholesterol levels to lipid stress caused by a diet enriched in saturated fats in rabbits. We found that the fat diet in rabbits impacts at the testicular level, causing the deregulation of the pathway governed by SREBP2. As a result, there was an increase in membrane (and probably also intracellular) cholesterol, which would correspond to the sperm abnormalities previously observed.

5

MATERIALS AND METHODS

Ethics statement

Animal studies described here were reviewed and approved by the animal care and use committee of School of Medicine, National University of Cuyo (Institutional Committee for Use of Laboratory Animals, CICUAL http://fcm.uncuyo.edu.ar/paginas/index/cicual; 06_150702).

Reagents

Unless otherwise stated, all chemicals and solvents of the highest grade available were obtained from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany).

Euthanyle®: 40 g pentobarbital, 5 g diphenylhydantoin / 100 ml. SENASA - http://www.senasa.gov.ar, government animal health regulation (Brouwer laboratory S.A., Argentina).

Phosphate buffered saline (PBS): was prepared following the manufacturer's instructions (MP Biomedicals, California, USA).

First bovine juice: Juan Lopez y CIA. Argentina. Commercial preparation approved by Argentina Alimentary Code (http://www.anmat.gov.ar/resultados.asp?cx=018082787451070703178%3Arx-vbt5pdfu&cof=FORID%3A10&ie=UTF-8&q=CAPITULO_VII.pdf; article 543, resolution 2012, 19.10.84).

Cholesterol determination kit (GTlab kit): GT Lab Rosario (https://www.gtlab.com.ar; ISO 9001: 2015; Good Manufacturing Practices: ANMAT - 3623, 1997; CEE: 91/356; OMS/WHO Technical Report Series 908/2003).

6

Animals and diets

Twenty-four male rabbits (White New Zealand, 6 months old) from rabbit farm (Verde-Azul farm, Buenos Aires, Argentina) were caged and maintained with a photoperiod of 12 h light / day and a temperature ranging from 18 ± 25 °C. Rabbits were divided in two groups (**Figure 1**).

The control group, designated as normo-cholesterolemic rabbits (NCR; n = 12) was fed *ad libitum* with a standard rabbit diet (SD, GEPSA FEEDS, Buenos Aires - Argentina). The treatment group, designated as hypercholesterolemic rabbits (HCR; n = 12) was fed *ad libitum* with fat-enriched diet (high fat diet, HFD) following our previous animal model (Saez Lancellotti et al., 2010, Simon et al., 2017). Animals from both groups were sacrificed when the time of diet was between 3 and 6 months (corresponding to an acute effect and designated ≤ 6 M) or between 12 and 24 months (corresponding to a chronic effect and designated as ≥ 12 M). The control animals from ≤ 6 M and ≥ 12 M were grouped together and are shown as NCR because they showed no differences in the parameters evaluated. All rabbits were weighted fortnightly.

The HFD was prepared by heating (up to 60 °C) fat derived from cow "first bovine juice" (55% saturated fat), pouring the resulting oil over SD, and mixing mechanically. The resulting stock diet became enriched up to 0.05% cholesterol (chromatography analysis at National Institute of Industrial Technology, INTI - Argentina) and was stored in darkness under refrigeration until used to avoid peroxidation.

Serum cholesterol

7

Blood was obtained fortnightly from marginal ear vein with heparinized syringes from all animals. Blood was centrifuged at 1,100 *g* for 10 minutes and the supernatant was carefully aspirated for cholesterol determination (GTlab kit).

Tissue collection

Rabbits were sacrificed by a lethal dose of pentobarbital (1 ml / 5 kg; Euthanyle) via pinna marginal vein. The testicles were cut into small cubes and some samples were fixed for light microscopy and the rest stored at -80 °C for RNA and protein extraction.

Light microscopy

Following standard procedures, histological analyses were performed on testicular tissues sections. Briefly, small pieces of testis were fixed (10% formol), dehydrated in ethanol-xylene series and embedded in paraffin. Sections of 5 μm thicknesses were stained with hematoxilin-eosin classics stain. The images were captured with a Nikon 80i microscope with objectives Plan Fluor 10X air, 0.3 NA (Numerical Aperture), 16 mm WD (Working Distance), Plan Fluor 40X air, 0.75 NA, 0.66 mm WD, DIC, Plan APO 60X oil, 1.4 NA, 0.13 mm WD, DIC (Nikon) and the image capture was carried out with a 5 Mp-Color DS-Fi2 CCD (charge-coupled divice) camera (Nikon) controlled with the NIS-Elements F3.2 software (Nikon).

Cholesterol staining / quantification

Semi-thin sections of the testicle were covered by 20 μl of Filipin III (0.15 mM in PBS) for 60 min at room temperature (RT) protected from light (stock solution:

8

7.6 mM filipin in dimethylsulfoxide 5 mg / ml). 20 µl of Propidium iodide (1 µg / ml, Sigma, P4864) was used to identify nuclear material. Finally, slides were washed with PBS and mounted with mowiol antifade. The images were captured with a FV 1000 confocal microscope with PLAPO40xWLSM and PLAPO60xWLSM objectives (Olympus) and the images were captured with the Olympus FluoView software with its latest 2018 update. Five microscopic fields of each section were chosen randomly for fluorescence intensity evaluation by ImageJ software (National Institute of Health Bethesda, Maryland; https://imagej.nih.gov/ij/) (Ma et al., 2018). The relative amount of cholesterol was based in the relative fluorescence intensity of filipin signal compared to NCR (fold changes). Signal at 480 nm - blue channel – was tabulated as mean (± SD), normalized to NCR, 5 times for all conditions.

Western-Blot analysis

Testicular proteins were extracted (Sheng et al., 1995) by homogenization with HEPES, KCl, EDTA, sucrose, spermine, spermidine, dithiothreitol (DTT) and protease inhibitors (Sigma, P8340). After centrifugation in (Beckman Optima TLX, 150000 g – 60 min), the supernatant was used to determine the protein concentration. Five percent of 2- mercaptoethanol was added to protein samples, boiled for 5 min and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8–10% mini-gels according to Laemmli (Laemmli, 1970). Forty µg of protein were separated by electrophoresis in acrylamide gels containing dual-prestained molecular weight standard (Bio Rad, Hercules, CA). Then, proteins were transferred to 0.45 mm nitrocellulose membranes (Bio Rad) and nonspecific reactivity was blocked by

9

incubation over night with 3% Teleostean fish gelatin dissolved in washing buffer (TBS, Towbin's buffer plus 0.1% Tween 20). Blots were incubated with primary antibodies 1:1000 in blocking buffer overnight at 4 °C (anti-SREBP2: H164, Santa Cruz Biotechnology (polyclonal), ab30682 ABCAM (polyclonal) and 22D5, Merck Millipore (monoclonal); anti- low density lipoprotein receptor (LDLR): BV-527, BioVision (polyclonal); anti- 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR): ABS229, Merck Millipore (polyclonal)). Biotin-conjugated anti-mouse/rabbit IgG (Sigma) was used as secondary antibody (1:1250) and horseradish peroxidase-conjugated ExtraVidine® (Sigma) was added at the end (1:750), both in blocking buffer at RT for 1 h each. Alpha tubulin was used as loading control (MP, 0869125). Detection was accomplished with an enhanced chemiluminescence system (ECL; Amersham Biosciences) and subsequent exposure to Blue Sensitive Cole-Parmer X-ray films (Cole-Parmer Instrument Company) for 5–30 s. The intensity of bands was obtained by ImageJ. Uncropped gels are shown in Supplementary figure S1.

Immunofluorescence staining

After decreasing autofluorescence with UV irradiation (Neumann and Gabel, 2002), tissue sections were dewaxed by 1 h at 60 °C, and two washes in xylol (15 min). Slide-mounted tissues were hydrated and subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6) containing 0.05 % (v/v) Tween-20 for 30 min. Non-specific binding was blocked with 10% (v/v) rabbit serum, 1% Triton X-100 and 0.2 % (w/v) gelatin in PBS, for 1 h at RT in a humidified chamber. Immuno-labelling was performed using primary antibody buffer containing anti-SREBP2 (1:100, ab30682 ABCAM, polyclonal), 2% rabbit

10

serum, 1% Triton X-100, and 0.2% gelatin in PBS, overnight at RT. Sections were rinsed in PBS and then incubated 2 h at RT with 1:200 secondary antibody (donkey) conjugated with Alexa Fluor 488 (Molecular Probes, A21206). DAPI (1:500, Sigma, D9542) was applied to identify nuclear material. Slices were rinsed and covered with Mowiol (81381, Sigma-Aldrich) and 24% glycerol in 0.1 M Tris–HCl buffer (pH 8.5)]. Positive and negative controls are shown in Supplementary figure S2. The images were captured with a FV 1000 confocal microscope with PLAPO40xWLSM and PLAPO60xWLSM objectives (Olympus) and the images were captured with the Olympus FluoView software with its latest 2018 update. Images were processed with *ImageJ* software. Evaluation of nuclear - cytoplasmic location of SREBP2 was made by a 3D reconstruction imaging (is shown in Supplementary figure S3 and Supplementary video). Briefly, a single cell was chosen and Z-stack imaging was performed. 3D reconstruction and display of cubic imaging were built up by ImageJ software.

Relative semi quantitative RT-PCR

Total RNA was extracted using TRIzol (Life Technologies) according to the manufacturer's instructions. RNA samples were solubilized in 25 µl UltraPure DNase / RNase-free distilled water (ThermoFisher Scientific) for reverse transcription/polymerase chain reaction (RT-PCR). Concentration and purity of the samples were spectroscopically determined at 260/280 nm. Two micrograms of total RNA were retrotranscribed with 200 units M-MLV enzyme reverse transcriptase (Invitrogen). Semi quantitative PCR of the products was performed using the following primers (http://simgene.com/primer3): *SREBP2*:

11

forward primer (Fw): CAGATTCCCTTGTTCTGACCACACTG; reverse primer (Rv): GCCAGCTTCAGCACCATGTTC; product: 442 bp; *LDLR*: Fw: CCACGAGTGTCATTGTCCCA; Rv: GACGTCCGTCCAGAACACTT; product: 865 bp; *HMGCR*: Fw: CCAGAAGGGTTCGCAGTGAT; Rv: TCGCCCCTCTATCCAGTTGA; product: 200 bp. *Beta actin* Fw: ACCAACTGGGACGACATGGAGAA; Rv: GTCAGGATCTTCATGAGGTAGTC; product: 353 bp was used as endogenous reference. The PCRs were carried out in a 25 µl final reaction volume containing 2 ml of cDNA, 20 pmoles of each primer, 200 mM deoxyribonucleotide triphosphate mix (dNTPs), 5 mM MgCl$_2$, 1.5 U of Taq DNA polymerase and 1 µl Taq DNA polymerase PCR buffer (Invitrogen). The cycling parameters were: 95 °C, 30 sec - 62 °C (*SREBP2),* 60 °C (*LDLR),* 57°C (*HMGCR)* and 54 °C (*beta actin)* 30 sec - 72 °C, 30 sec for 28 (*SREBP2* and *LDLR)* or *30* cycles (*HMGCR* and *beta actin).* Negative controls were included in each assay. Amplified products were run on an agarose gel (2% w/v) and dyed with SYBR-safe DNA Gel Stain (ThermoFisher Scientific). All PCR were performed in triplicate. The images were visualized and photographed on a Fuji LAS4000 imaging system. The mRNA abundance was determined by measuring the intensity of each band using ImageJ software.

Statistical analysis

Unless otherwise expressly noted, results are reported as means ± SD of at least three independent experiments. Differences between groups were evaluated by ANOVA test, followed by least significant difference (LSD) Fisher test, considering a *p* value of less than 0.05 as statistically significant.

RESULTS

Lipid profile

Animals under high fat diet (HCR groups) displayed significant hypercholesterolemia and hypertriglyceridemia as compared to chow fed control rabbits (Table I).

Seminal characteristics

We have previously seen that feeding cholesterol had a deleterious effect on semen parameters and sperm functionality (Saez Lancellotti et al., 2010, Simon et al., 2017). For this reason, data concerning semen volume, pH, sperm concentration, cell viability, motility and morphology were obtained at the end of experimental time to ensure the negative effect. Semen volume, sperm motility and abnormalities were significantly altered already at 6 months of diet and worsened time dependently in the case of semen volume and sperm motility (Table II).

Tissue changes

Effects of fat diet on testicular morphological structure

Morphological analysis of the testicles showed that HFD fed rabbits had anomalous seminiferous tubules compared to control animals (**Figure 2**). Some alterations are already observed at 6 months of fat diet, and increased with fat-feeding time. Detached cells from the epithelium were observed blocking the tubules lumen (**Figure 2 d to i**). Moreover, histological images of the seminiferous tubules of HCR showed an arrest in the progression from spermatogonia to spermatic cells **(Figure 2 d to i)** and between the cells round

13

spaces are depicted (**Figure 2 e, asterisks**). The interstitial tissue was enlarged with a noticeable increase in foamy cells, which accounts for lipid accumulation (**Figure 2 h, inset**).

Cholesterol detection and quantification

We had previously observed an increase in membrane cholesterol in sperm cells of rabbits on a fatty diet (Saez Lancellotti et al., 2010, Simon et al., 2017, Saez Lancellotti et al., 2013, Simon et al., 2018). Therefore, we examined whether the spermatozoa developed in a cholesterol-overloaded environment. Filipin staining showed that cholesterol was increased at all stages of the seminiferous epithelium from animals under short and long periods of HFD, compared to the control group **(Figure 3 A, a and b)**. The interstitium, where the testosterone-producing cells are located, was marked with filipin in all groups of rabbits, and foamy cells were also observed **(Figure 3 A**, **white squares).** When examined inside the tubules, the fluorescence (corresponding to cholesterol) showed similar distribution for all groups **(Figure 3 A, a,d and g)**, but the intensity increased towards the lumen in fattened animals **(Figure 3 A, d, white oval)**. In order to observe in more detail the distribution of cholesterol within the cells, a magnification was made using a confocal microscope zoom **(Figure 3 C)**. Cholesterol was detected around the nucleus in any condition during spermiogenesis in the seminiferous epithelium **(Figure 3 C, a, d and g)**, while maintaining the increase in both HCR groups as described above. The fluorescence intensity analyses show a significant increase of cholesterol in testicles of animals under fat diet in the short and long term compared to the control, and significantly higher at ≥ 12 M than ≤ 6 M. **(Figure 3 B and D)**.

14

Expression of lipid-metabolism related molecules.

To determine further effects of HFD on testicular lipid metabolism, we analyzed the expression of mRNA and proteins involved in intracellular cholesterol level regulation pathways, using RT-PCR and western blotting respectively. Six months of HFD significantly down-regulated the *SREBP2* mRNA expression (**Figure 4 A**) compared to the control group. However, after 12 months of HFD, *SREBP2 mRNA* was significantly up-regulated (**Figure 4 A**). In parallel, SREBP2 protein expression, as shown in **Figure 4 B**, followed the same pattern of as the mRNA: it decreased at 6 months and increased at 12 months of HFD. In addition, in the blot it can be observed both the 120 kDa membrane bound (mSREBP2) and the 68 kDa nuclear (nSREBP2) forms of the protein. The quantification was made from the active form of SREBP2, corresponding to the transcription factor **(Figure 4 C).**

Localization of SREBP2 was achieved by immunostaining (**Figure 5**). Control rabbits showed a high cytoplasmic expression of SREBP2 inside the cells of seminiferous tubules and interstitium, both in the nucleus and in the cytoplasm (**Figure 5, A, NCR).** Immunofluorescence examination of testis from animals under HFD showed that the number of cells that reacted to SREBP2 antibody decreased until 6 months of diet. Moreover, the remaining SREBP2 expression was located in the peritubular cells of the interstitium, and cells close to the luminal region of the tubules, at any stages **(Figure 5, A, HCR ≤ 6 M**). In contrast, the group HCR ≥ 12 M showed increased expression of the protein, observed in the nucleus of cells inside and outside the seminiferous tubules.

15

Moreover, a high expression was observed in the cells located along the basal membrane of the seminiferous tubules **(Figure 5, A, HCR ≥ 12 M**). Furthermore, SREBP2 immunofluorescence quantification showed the identical tendency as the expression of the protein evaluated by western blot (**Figure 5 B**), despite the fact that both the nuclear and membrane forms of the protein were detected by immunofluorescence and only the active form was analyzed by western blot.

Next, we investigated the expression of HMGCR and LDLR, both of which are downstream targets of SREBP2, and are involved in cholesterol synthesis and import. Between 3 and 6 months of HFD, a significant decrease in *HMGCR and LDLR* mRNA expression in the testicle was observed, compared with the levels in the control group (**Figure 6 A and B**). After 12 months or more of HFD, the *LDLR* mRNA expression did not change compared to ≤ 6 months, but the *HMGCR* mRNA increased, reaching levels similar to the control (**Figure 6, A and B**). However, both proteins followed the same pattern as SREBP2 expression when assessed by western blot (**Figure 6, C and D**). Thus, expression of the three proteins (SREBP2, HMGCR and LDLR) decreased at ≤ 6 M but increased at 12 ≥ M of HFD compared to control.

16

DISCUSSION

We described for the first time the differential effect of dietary fat consumption for 3 to 6 months (acute) or more than 12 months (chronic) on the cholesterol regulation pathway in rabbit testicles. Acute consumption caused downward regulation of SREBP2 and its target molecules, and chronic consumption upregulated them. This dysregulation ultimately causes lipid accumulation in the testicle of rabbit fed with fat diet. and probably others spermatogenic defects already described (Saez Lancellotti et al., 2010, Simon et al., 2017).

Cholesterol is an essential molecule for membrane structure, intracellular signaling as well as for the synthesis of hormones in the testicle. Therefore, the accurate cell cholesterol concentration is critical for the functional maintenance of this organ. In somatic cells, the synthesis and regulation of cholesterol and fatty acids are under the control of the transcription factors known as SREBPs (Horton et al., 2002, Persengiev et al., 1996). However, the role of these transcription factors in spermatogenic cells has not been sufficiently explored.

In the testicle, SREBP2 is present both in the Leydig cells (Shimizu-Albergine et al., 2016) and in the seminiferous tubules, both in Sertoli and spermatogenesis cells (Wang et al., 2002, Wang et al., 2004, Chen et al., 2019, Jarvis et al., 2020). One study suggested that the expression of genes for cholesterol biosynthesis during spermatogenesis is regulated by mechanisms independent of SREBPs (Rozman et al., 1999). In addition, an isoform called SREBPgc has been found in spermatocytes and round spermatids and acts as a constitutively active, soluble transcription factor (Wang et al., 2002, Fon Tacer et al., 2003, Wang et al., 2004). Recently, a proteomic study analyzed testes from mice fed a HFD, and found changes in the expression of proteins involved in lipid

17

homoeostasis, including SREBP2 (Jarvis et al., 2020). However, SREBP2's role in the testicle has not yet been clearly described, in particular with regard to the duration of the fat diet.

In previous works we had observed that rabbits fed with a HFD between 3 and 6 months already presented hypercholesterolemia and seminal alterations, and they were considered to test an acute effect of fat intake in the testicle. A minimum period of three months was chosen to observe the effects of the diet on the semen given the duration of spermatogenesis in the rabbits (Swierstra and Foote, 1965). Animals subjected to a period between 12 and 24 months of fat diet were grouped to analyze the chronic effect of the fat intake on the testicle. Other authors had seen an influence of time on the expression of SREBPs (Hernandez Vallejo et al., 2009, Jing Zhu 2018, Li et al., 2019, Fernandes-Lima et al., 2015). Nevertheless, the control animals did not present variations in relation to the time of experimentation, so they were clustered in a single group (NCR).

The HFD had a direct impact on the accumulation of cholesterol in the seminiferous tubules of rabbits, both in the membrane and inside the cells, even after a few months of a fatty diet. Other authors had previously seen a similar phenomenon in other animal models and for different periods of fat consumption (Campos-Silva et al., 2015, Morgan et al., 2014, Yu et al., 2019). As discussed below, acute cholesterol accumulation seems to be directly influenced by the diet while chronic accumulation seems to be related to the deregulation of the cholesterol pathway.

18

In the short term, the molecules of the cholesterol regulatory pathway (SREBP2 and targets) decreased their expression, in response to the high circulating cholesterol due to diet. In spite of this, the testicle is loaded with cholesterol and it is possible to observe some testicular and seminal abnormalities. In the long term, the regulation system fails to detect the high amount of circulating lipids and triggers all the machinery of synthesis and cholesterol incorporation into the cell, further worsening the situation.

The SREBP2 protein has a differential intracellular distribution depending on whether it is inactive (endoplasmic reticulum-membrane-bound) or active (nuclear). Control rabbits showed a high cytoplasmic expression of SREBP2 inside the seminiferous tubule and both in the nucleus and in the cytoplasm of the interstitial cells, corresponding to Leydig cells and in concordance with other authors (Jarvis et al., 2020). Inside the tubules it was present throughout the seminiferous epithelium, from the base to the apical region near the lumen, where the most mature sperm are found. This is consistent with the function of the protein in the testicle in a basal condition and as the main cholesterol regulating molecule in an organ that uses this lipid extensively. It should be noted that the antibody is also capable of detecting the isoform that other authors mention as being constitutively active (SREBP2gc). On the other hand, immunofluorescence examination of testis from animals under HFD showed that the number of cells that reacted to SREBP2 antibody decreased until 6 months of diet, with a slight interstitial expression remaining. A similar result was recently found in mice fed a HFD (Jarvis et al., 2020). In contrast, the long-term fat fed group (HCR ≥ 12 M) showed increased expression of the protein, especially in the nucleus of cells inside and outside the seminiferous tubules.

19

Moreover, a high expression was observed in the cells along the basal membrane of the seminiferous tubules.

Similar patterns of SREBP2 expression were observed between the control and the HCR groups with the three approaches used for their study (Immunofluorescence, RT-PCR and Western Blot). Furthermore, it is the first time that changes in the location of SREBP2 in testicle mediated by diet and (even more interesting) by diet duration are described. In the short term, the effect would be explained by the feedback mechanism of SREBP2 by which the active form inhibits the proteolytic processing of the native form, as described in liver (Espenshade and Hughes, 2007, Madison, 2016, Jiang et al., 2009, Desvergne et al., 2006). It is conceivable that positive fat imbalance chronically activates SREBP2, causing testicular lipotoxicity as reported for SREBP-1c in other tissues and organs (Yahagi et al., 2002, Takahashi et al., 2005). Lipophagy (lipid droplet mobilization by autophagia) is another cellular mechanism regulated by SREBP2 that could be altered and might account for the observed consequences (Jeon and Osborne, 2012). Cholesterol system deregulation has been also seen to occur with age (Morgan et al., 2014) and the same could be happening in testicles of hypercholesterolemic patients.

Regarding two well-known SREBP2 downstream targets involved in cholesterol import and metabolism in somatic cell cytoplasm (HMGCR and LDLR), we found that after 6 months of HFD both molecules behaved like SREBP2, decreasing the expression of mRNA and protein. However, over longer periods of HFD, HMGCR increased its expression (both mRNA and protein) as LDLR protein, following the behavior of SREBP2. However, *LDLR* mRNA was not modified.

20

Our results suggest a pathophysiological role of SREBP2 in testicles after a chronic fat intake, that would be related to the low seminal quality observed in rabbits. The present data reveal short and long term effects with a tendency to worsen over time. We had previously described sperm malformations due to high cellular cholesterol load, so in this work we report the probable cause of that cholesterol increase that connects some missing pieces. We consider that this is probably what applies to patients who consume this type of food for a long time and it is what other authors have observed in the liver (Wu et al., 2013, Caballero et al., 2009).

Data Availability

The data underlying this article are available in the article and in its online supplementary material.

Acknowledgements

The authors are grateful to Ituarte Leonor for her guidance in statistic. The authors would also like to acknowledge to Ibañez Jorge for his technical support.

Authors' roles

AF full experiment, LS biochemical determination, RC animal housing, MVA requested controls, MM animal housing and manuscript processing, JC sample managing, MEC biochemical determinations, MIC fluorescence images, NC mRNA running/primers, PB biochemical determinations, MWF direction and writing, TESL direction and writing.

21

Funding

Conflict of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

REFERENCES

BUCK LOUIS, G. M., SUNDARAM, R., SCHISTERMAN, E. F., SWEENEY, A., LYNCH, C. D., KIM, S., MAISOG, J. M., GORE-LANGTON, R., EISENBERG, M. L. & CHEN, Z. 2014. Semen quality and time to pregnancy: the Longitudinal Investigation of Fertility and the Environment Study. *Fertil Steril,* 101**,** 453-62.

CABALLERO, F., FERNANDEZ, A., DE LACY, A. M., FERNANDEZ-CHECA, J. C., CABALLERIA, J. & GARCIA-RUIZ, C. 2009. Enhanced free cholesterol, SREBP-2 and StAR expression in human NASH. *J Hepatol,* 50**,** 789-96.

CAMPOS-SILVA, P., FURRIEL, A., COSTA, W. S., SAMPAIO, F. J. & GREGORIO, B. M. 2015. Metabolic and testicular effects of the long-term administration of different high-fat diets in adult rats. *Int Braz J Urol,* 41**,** 569-75.

CARLSEN, E., GIWERCMAN, A., KEIDING, N. & SKAKKEBAEK, N. E. 1992. Evidence for decreasing quality of semen during past 50 years. *BMJ,* 305**,** 609-13.

CROSS, N. L. 1998. Role of cholesterol in sperm capacitation. *Biol Reprod,* 59**,** 7-11.

CHEN, L., MA, M. Y., SUN, M., JIANG, L. Y., ZHAO, X. T., FANG, X. X., MAN LAM, S., SHUI, G. H., LUO, J., SHI, X. J. & SONG, B. L. 2019. Endogenous sterol intermediates of the mevalonate pathway regulate HMGCR degradation and SREBP-2 processing. *J Lipid Res,* 60**,** 1765-1775.

DESVERGNE, B., MICHALIK, L. & WAHLI, W. 2006. Transcriptional regulation of metabolism. *Physiol Rev,* 86**,** 465-514.

DU PLESSIS, S. S., CABLER, S., MCALISTER, D. A., SABANEGH, E. & AGARWAL, A. 2010. The effect of obesity on sperm disorders and male infertility. *Nat Rev Urol,* 7**,** 153-61.

ESPENSHADE, P. J. & HUGHES, A. L. 2007. Regulation of sterol synthesis in eukaryotes. *Annu Rev Genet,* 41**,** 401-27.

FARIELLO, R. M., PARIZ, J. R., SPAINE, D. M., CEDENHO, A. P., BERTOLLA, R. P. & FRAIETTA, R. 2012. Association between obesity and alteration of sperm DNA integrity and mitochondrial activity. *BJU Int,* 110**,** 863-7.

FERNANDES-LIMA, F., MONTE, T. L., NASCIMENTO, F. A. & GREGORIO, B. M. 2015. Short Exposure to a High-Sucrose Diet and the First 'Hit' of Nonalcoholic Fatty Liver Disease in Mice. *Cells Tissues Organs,* 201**,** 464-472.

FLEGAL, K. M., KIT, B. K., ORPANA, H. & GRAUBARD, B. I. 2013. Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. *JAMA,* 309**,** 71-82.

FON TACER, K., KALANJ-BOGNAR, S., WATERMAN, M. R. & ROZMAN, D. 2003. Lanosterol metabolism and sterol regulatory element binding protein (SREBP) expression in male germ cell maturation. *J Steroid Biochem Mol Biol,* 85**,** 429-38.

GUH, D. P., ZHANG, W., BANSBACK, N., AMARSI, Z., BIRMINGHAM, C. L. & ANIS, A. H. 2009. The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. *BMC Public Health,* 9**,** 88.

GWYNNE, J. T. & STRAUSS, J. F., 3RD 1982. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr Rev,* 3**,** 299-329.

HAMMOUD, A. O., GIBSON, M., PETERSON, C. M., MEIKLE, A. W. & CARRELL, D. T. 2008. Impact of male obesity on infertility: a critical review of the current literature. *Fertil Steril,* 90**,** 897-904.

HERNANDEZ VALLEJO, S. J., ALQUB, M., LUQUET, S., CRUCIANI-GUGLIELMACCI, C., DELERIVE, P., LOBACCARO, J. M., KALOPISSIS, A. D., CHAMBAZ, J., ROUSSET, M. & LACORTE, J. M. 2009. Short-term adaptation of postprandial lipoprotein secretion and intestinal gene expression to a high-fat diet. *Am J Physiol Gastrointest Liver Physiol,* 296**,** G782-92.

HORTON, J. D., GOLDSTEIN, J. L. & BROWN, M. S. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest,* 109**,** 1125-31.

23

HOWE, V., SHARPE, L. J., ALEXOPOULOS, S. J., KUNZE, S. V., CHUA, N. K., LI, D. & BROWN, A. J. 2016. Cholesterol homeostasis: How do cells sense sterol excess? *Chem Phys Lipids,* 199**,** 170-178.

JAIN, A. P. A. G. C. 2015. Hyper-Lipidemia and Male Fertility: A Critical Review of Literature. *Andrology-Open Access* 4**,** 12.

JARVIS, S., GETHINGS, L. A., SAMANTA, L., PEDRONI, S. M. A., WITHERS, D. J., GRAY, N., PLUMB, R. S., WINSTON, R. M. L., WILLIAMSON, C. & BEVAN, C. L. 2020. High fat diet causes distinct aberrations in the testicular proteome. *Int J Obes (Lond).*

JEON, T. I. & OSBORNE, T. F. 2012. SREBPs: metabolic integrators in physiology and metabolism. *Trends Endocrinol Metab,* 23**,** 65-72.

JIANG, L., WANG, Q., YU, Y., ZHAO, F., HUANG, P., ZENG, R., QI, R. Z., LI, W. & LIU, Y. 2009. Leptin contributes to the adaptive responses of mice to high-fat diet intake through suppressing the lipogenic pathway. *PLoS One,* 4**,** e6884.

JING ZHU , J. J., JIEXIA DING , SIYING LI, PANPAN CEN, KEYI WANG, HAI WANG, JUNBO XIA,∗ 2018. Ganoderic Acid A improves high fat diet-induced obesity, lipid accumulation and insulin sensitivity through regulating SREBP pathway. *Chemico-Biological Interactions***,** 77-87.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-5.

LI, K. P., YUAN, M., HE, Z. R., WU, Q., ZHANG, C. M., LEI, Z. L., RONG, X. L., HUANG, Z., TURNBULL, J. E. & GUO, J. 2019. Omics Insights into Metabolic Stress and Resilience of Rats in Response to Short-term Fructose Overfeeding. *Mol Nutr Food Res,* 63**,** e1900773.

MA, K. L., GONG, T. K., HU, Z. B., ZHANG, Y., WANG, G. H., LIU, L., CHEN, P. P., LU, J., LU, C. C. & LIU, B. C. 2018. Lipoprotein(a) accelerated the progression of atherosclerosis in patients with end-stage renal disease. *BMC Nephrology,* 19**,** 192.

MACDONALD, A. A., HERBISON, G. P., SHOWELL, M. & FARQUHAR, C. M. 2010. The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Hum Reprod Update,* 16**,** 293-311.

MADISON, B. B. 2016. Srebp2: A master regulator of sterol and fatty acid synthesis. *J Lipid Res,* 57**,** 333-5.

MAGNUSDOTTIR, E. V., THORSTEINSSON, T., THORSTEINSDOTTIR, S., HEIMISDOTTIR, M. & OLAFSDOTTIR, K. 2005. Persistent organochlorines, sedentary occupation, obesity and human male subfertility. *Hum Reprod,* 20**,** 208-15.

MORGAN, D. H., GHRIBI, O., HUI, L., GEIGER, J. D. & CHEN, X. 2014. Cholesterol-enriched diet disrupts the blood-testis barrier in rabbits. *Am J Physiol Endocrinol Metab,* 307**,** E1125-30.

NEUMANN, M. & GABEL, D. 2002. Simple method for reduction of autofluorescence in fluorescence microscopy. *J Histochem Cytochem,* 50**,** 437-9.

ORGANIZATION, W. H. 2016. *Global Health Observatory (GHO) data* [Online]. Available: https://www.who.int/gho/ncd/risk_factors/overweight/en/.

ORGANIZATION, W. H. 2020. *Obesity and overweight* [Online]. Available: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.

PERSENGIEV, S. P., RAVAL, P. J., RABINOVITCH, S., MILLETTE, C. F. & KILPATRICK, D. L. 1996. Transcription factor Sp1 is expressed by three different developmentally regulated messenger ribonucleic acids in mouse spermatogenic cells. *Endocrinology,* 137**,** 638-46.

ROZMAN, D., FINK, M., FIMIA, G. M., SASSONE-CORSI, P. & WATERMAN, M. R. 1999. Cyclic adenosine 3',5'-monophosphate(cAMP)/cAMP-responsive element modulator (CREM)-dependent regulation of cholesterogenic lanosterol 14alpha-demethylase (CYP51) in spermatids. *Mol Endocrinol,* 13**,** 1951-62.

24

SAEZ LANCELLOTTI, T. E., BOARELLI, P. V., MONCLUS, M. A., CABRILLANA, M. E., CLEMENTI, M. A., ESPINOLA, L. S., CID BARRIA, J. L., VINCENTI, A. E., SANTI, A. G. & FORNES, M. W. 2010. Hypercholesterolemia impaired sperm functionality in rabbits. *PLoS One,* 5**,** e13457.

SAEZ LANCELLOTTI, T. E., BOARELLI, P. V., ROMERO, A. A., FUNES, A. K., CID-BARRIA, M., CABRILLANA, M. E., MONCLUS, M. A., SIMON, L., VICENTI, A. E. & FORNES, M. W. 2013. Semen quality and sperm function loss by hypercholesterolemic diet was recovered by addition of olive oil to diet in rabbit. *PLoS One,* 8**,** e52386.

SHENG, Z., OTANI, H., BROWN, M. S. & GOLDSTEIN, J. L. 1995. Independent regulation of sterol regulatory element-binding proteins 1 and 2 in hamster liver. *Proc Natl Acad Sci U S A,* 92**,** 935-8.

SHIMIZU-ALBERGINE, M., VAN YSERLOO, B., GOLKOWSKI, M. G., ONG, S. E., BEAVO, J. A. & BORNFELDT, K. E. 2016. SCAP/SREBP pathway is required for the full steroidogenic response to cyclic AMP. *Proc Natl Acad Sci U S A,* 113**,** E5685-93.

SIMON, L., FUNES, A. K., MONCLUS, M. A., COLOMBO, R., CABRILLANA, M. E., SAEZ LANCELLOTTI, T. E. & FORNES, M. W. 2018. Manchette-acrosome disorders and testicular efficiency decline observed in hypercholesterolemic rabbits are recovered with olive oil enriched diet. *PLoS One,* 13**,** e0202748.

SIMON, L., FUNES, A. K., YAPUR, M. A., CABRILLANA, M. E., MONCLUS, M. A., BOARELLI, P. V., VINCENTI, A. E., SAEZ LANCELLOTTI, T. E. & FORNES, M. W. 2017. Manchette-acrosome disorders during spermiogenesis and low efficiency of seminiferous tubules in hypercholesterolemic rabbit model. *PLoS One,* 12**,** e0172994.

SWIERSTRA, E. E. & FOOTE, R. H. 1965. Duration of Spermatogenesis and Spermatozoan Transport in the Rabbit Based on Cytological Changes, DNA Synthesis and Labeling with Tritiated Thymidine. *Am J Anat,* 116**,** 401-12.

TAKAHASHI, A., MOTOMURA, K., KATO, T., YOSHIKAWA, T., NAKAGAWA, Y., YAHAGI, N., SONE, H., SUZUKI, H., TOYOSHIMA, H., YAMADA, N. & SHIMANO, H. 2005. Transgenic mice overexpressing nuclear SREBP-1c in pancreatic beta-cells. *Diabetes,* 54**,** 492-9.

TEERDS, K. J., DE ROOIJ, D. G. & KEIJER, J. 2011. Functional relationship between obesity and male reproduction: from humans to animal models. *Hum Reprod Update,* 17**,** 667-83.

WANG, H., LIU, F., MILLETTE, C. F. & KILPATRICK, D. L. 2002. Expression of a novel, sterol-insensitive form of sterol regulatory element binding protein 2 (SREBP2) in male germ cells suggests important cell- and stage-specific functions for SREBP targets during spermatogenesis. *Mol Cell Biol,* 22**,** 8478-90.

WANG, H., SAN AGUSTIN, J. T., WITMAN, G. B. & KILPATRICK, D. L. 2004. Novel role for a sterol response element binding protein in directing spermatogenic cell-specific gene expression. *Mol Cell Biol,* 24**,** 10681-8.

WU, N., SARNA, L. K., HWANG, S. Y., ZHU, Q., WANG, P., SIOW, Y. L. & O, K. 2013. Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase during high fat diet feeding. *Biochim Biophys Acta,* 1832**,** 1560-8.

YAHAGI, N., SHIMANO, H., HASTY, A. H., MATSUZAKA, T., IDE, T., YOSHIKAWA, T., AMEMIYA-KUDO, M., TOMITA, S., OKAZAKI, H., TAMURA, Y., IIZUKA, Y., OHASHI, K., OSUGA, J., HARADA, K., GOTODA, T., NAGAI, R., ISHIBASHI, S. & YAMADA, N. 2002. Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. *J Biol Chem,* 277**,** 19353-7.

YANAGIMACHI, R. 1994. *"Mammalian Fertilization". The Physiology of Reproduction,* New York.

YU, C., JIANG, F., ZHANG, M., LUO, D., SHAO, S., ZHAO, J., GAO, L., ZUO, C. & GUAN, Q. 2019. HC diet inhibited testosterone synthesis by activating endoplasmic reticulum stress in testicular Leydig cells. *J Cell Mol Med,* 23**,** 3140-3150.

25

FIGURE LEGENDS

Figure 1. Schematic diagram of the experimental protocol. Twenty-four adult male rabbits were randomly divided in two: control (fed standard diet, SD) and treatment (fed high fat diet, HFD) groups. Animals from both groups were sacrificed between 3 and 6 months (acute effect) and between 12 and 24 months (chronic effect). The control animals from ≤ 6 M and ≥ 12 M were grouped together and are shown as NCR (normo cholesterolemic rabbits) because they showed no differences in the parameters evaluated. The treated animals became hypercholesterolemic rabbits (HCR) and were split in two groups: acute when the times of diet ≤ 6 months (≤ 6 M) or chronic when ≥ 12 months (≥ 12 M) of fat diet. n = number of experimental animals.

Figure 2. Histological characteristics of rabbit testis after acute and chronic consumption of fat diet. Seminiferous tubules consisted in seminiferous epithelium around the lumen (L) and it was possible to observe the stages of spermatogenesis at any time in control rabbits (a, b and c) as well as a preserved interstitial space with numerous Leydig cells and blood vessels. HCR showed some tubules with a blocked lumen (d to i). Some tubules also showed empty areas inside the epithelium (*), and enlarged interstitium (h, inset). Magnification: (a), (d) and (g), 200 X. (b), (e) and (h), 400 X. (c), (f) and (i), 600 X. NCR: normal cholesterolemic rabbits; HCR: hypercholesterolemic rabbits; HCR ≤ 6 M: less or equal to 6 months of fat diet; HCR ≥ 12 M: greater or equal to 12 months of fat diet

Figure 3. Cholesterol content in testicles. A. Representative fluorescent images of filipin-stained testicle sections obtained from control rabbits and rabbits fed a HFD for short and long periods of time. Cholesterol content inside (white ovals) and outside (white squares, interstitial cells) the seminiferous tubules was compared among NCR (upper row), acute (middle row, ≤ 6 months of treatment) and chronic (low row, ≥ 12 months) HCR. Notice that cholesterol distribution was similar between diets and different periods of time (600 X). Cholesterol= blue, nucleus= red. B. Densitometric Filipin III fluorescence image analysis. Filipin-positive areas of seminiferous tubules are shown as mean ± SD, normalized to NCR (1), n = 5. C. Representative fluorescent images of NCR and HCR testicle slices at higher magnification - confocal view / digital zoom. The cholesterol content inside the seminiferous tubules detected by the filipin III probe shows more signaling around the nucleus during spermiogenesis (clearly defined in merge images) (Magnification 600 X and zoom 49). D. Densitometric filipin III fluorescence image analysis of cholesterol content inside seminiferous tubules of NCR and HCR rabbits. Filipin positive areas of seminiferous tubules are shown as mean ± SD, normalized to NCR (1), n = 5. NCR: normal cholesterolemic rabbits; HCR: hypercholesterolemic rabbits; HCR ≤ 6 M: less or equal to 6 months of fat diet; HCR ≥ 12 M: greater or equal to 12 months of fat diet. Bars, white = NCR, grey = HCR ≤ 6 M and dark grey = ≥ 12 M). Asterisks denote significantly different, *= $p < 0.05$; **= $p < 0.01$ and *** = $p ≤ 0.001$.

Figure 4. SREBP2 expression in testicles. A. Sterol Regulatory Element-Binding Protein isoform 2 (SREBP2) mRNA expression was determined by semi-quantitative RT-PCR (using β actin as internal control) of the total RNA extracted from the testes of the different groups. SREBP2 mRNA levels were plotted as bars and are expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SEM of 5 different determinations (n=5). B. Expression of SREBP2 protein in testicular extracts from both control and HFD-fed rabbits evaluated by western blot using α tubulin as load control. C. SREBP2 protein levels were plotted as bars and

26

are expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SD of four different experiments (n=4). NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (≤ 6 M), less or equal to 6 months of fat diet; (≥ 12 M), greater or equal to 12 months of fat diet. mSREBP2, membrane bound SREBP2 (120 kDa) and nSREBP2, nuclear form of SREBP2 (68 kDa). *, ** and *** denotes significantly different according to ANOVA test (p ≤ 0.05, 0.01 and 0.001 respectively).

Figure 5. Immunostaining of SREBP2 on testicular sections. A. Left column (green signal) corresponds to detection of SREBP2, middle column (blue signal) to nucleus detection by DAPI, and right column shows the merge. Magnification: a to i, 600 X; c', f' and i' are two-fold enlarged images from c, f and i, respectively. B. The histogram shows densitometry analysis data of SREBP2 immunostaining expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SD of four different experiments (n=4). NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (≤ 6 M), less or equal to 6 months of fat diet; (≥ 12 M), greater or equal to 12 months of fat diet. *** denotes significantly different (p ≤ 0.01).

Figure 6. Expression patterns of downstream molecules of SREBP-2 in testicles. A and B. The expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) (A) and low-density lipoprotein receptor (LDLR) (B) mRNAs were determined by semi-quantitative PCR (using β actin as internal control) of total RNA from testicles. mRNA levels are expressed as percentage of control (NCR). Each bar corresponds to mean ± SEM of 5 determinations (n=5). C and D. HMGCR (C) and LDLR (D) protein expression in testicular extracts after fat diet assessed by Western blot, compared to control (NCR) and using α tubulin as loading control. The histograms represent densitometric analysis data expressed as percentage of levels in control (NCR), mean ± SD of four different experiments. NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (HCR ≤ 6 M), less or equal to 6 months of fat diet; (HCR ≥ 12 M), greater or equal to 12 months of fat diet. HMGCR, HMG CoA reductase; LDLR, LDL receptor. Different letters denote significance according to ANOVA test (p ≤ 0.05).
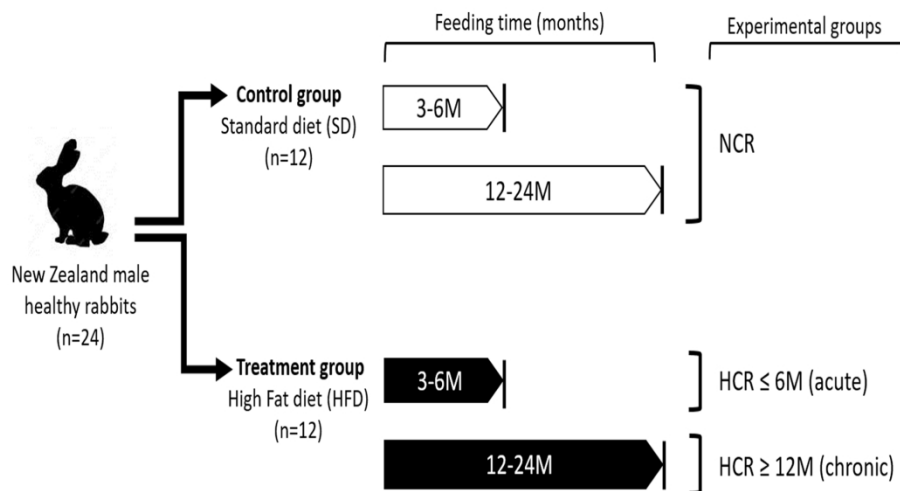
Figure 1. Schematic diagram of the experimental protocol. Twenty-four adult male rabbits were randomly divided in two: control (fed standard diet, SD) and treatment (fed high fat diet, HFD) groups. Animals from both groups were sacrificed between 3 and 6 months (acute effect) and between 12 and 24 months (chronic effect). The control animals from ≤ 6 M and ≥ 12 M were grouped together and are shown as NCR (normo cholesterolemic rabbits) because they showed no differences in the parameters evaluated. The treated animals became hypercholesterolemic rabbits (HCR) and were split in two groups: acute when the times of diet ≤ 6 months (≤ 6 M) or chronic when ≥ 12 months (≥ 12 M) of fat diet. n = number of experimental animals.
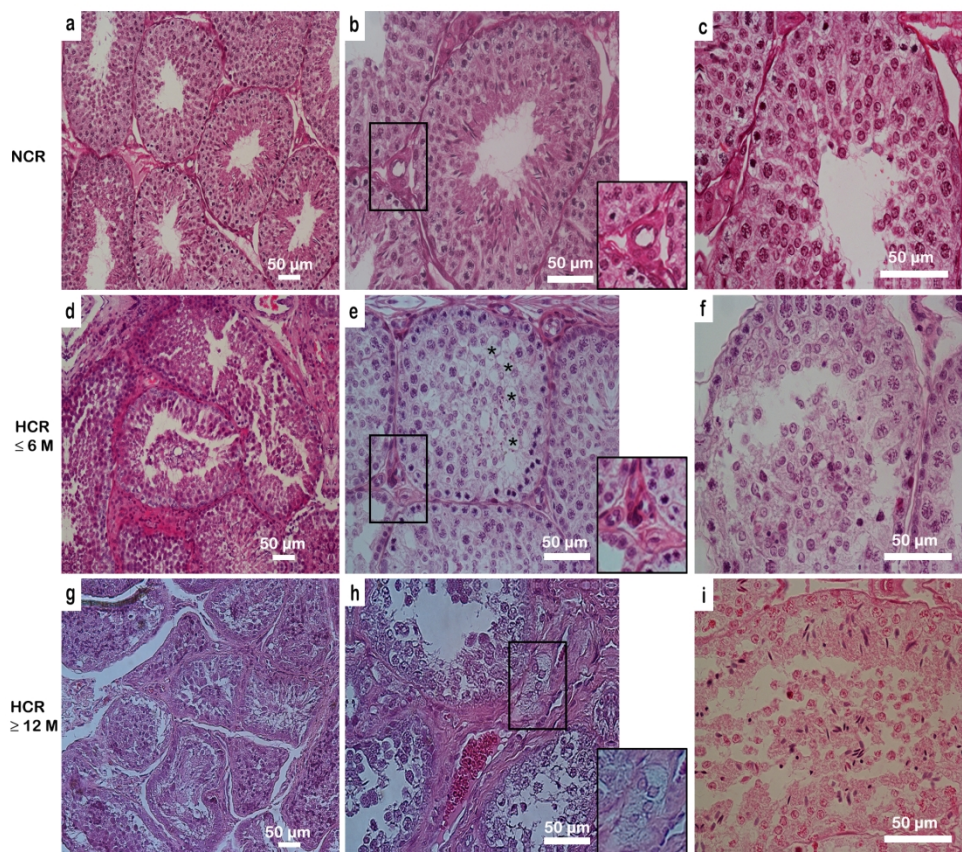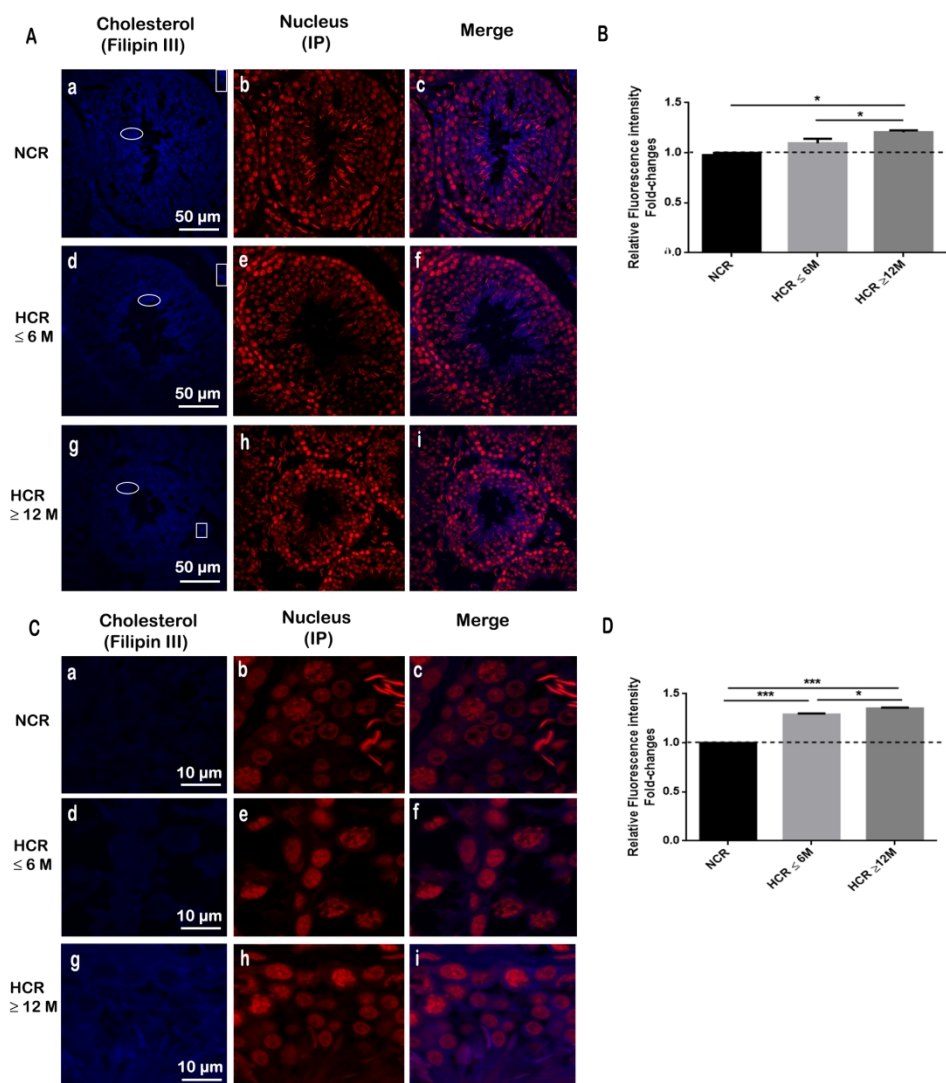
Figure 2. Histological features by light microscopy in NCR and HCR rabbits after acute (HCR ≤ 6 M) and chronic (HCR ≥ 12) consumption of fat diet. Seminiferous tubules consisted in seminiferous epithelium around the lumen (L) and it was possible to observe the stages of spermatogenesis at any time in control rabbits (a, b and c) as well as a preserved interstitial space with numerous Leydig cells and blood vessels. HCR showed some tubules with a blocked lumen (d to i). Some tubules also showed empty areas inside the epithelium (*), and enlarged interstitium (h, inset). Magnification: (a), (d) and (g), 200 X. (b), (e) and (h), 400 X. (c), (f) and (i), 600 X.

A Cholesterol (Filipin III) Nucleus (IP) Merge **B**

NCR
a b c
50 µm

HCR ≤ 6 M
d e f
50 µm

HCR ≥ 12 M
g h i
50 µm

C Cholesterol (Filipin III) Nucleus (IP) Merge **D**

NCR
a b c
10 µm

HCR ≤ 6 M
d e f
10 µm

HCR ≥ 12 M
g h i
10 µm

Caption : Figure 3. Cholesterol content in testicles. A. Representative fluorescent images of filipin-stained testicle sections obtained from control rabbits and rabbits fed a HFD for short and long periods of time. Cholesterol content inside (white ovals) and outside (white squares, interstitial cells) the seminiferous tubules was compared among NCR (upper row), acute (middle row, ≤ 6 months of treatment) and chronic (low row, ≥ 12 months) HCR. Notice that cholesterol distribution was similar between diets and different periods of time (600 X). B. Densitometric Filipin III fluorescence image analysis. Filipin positive areas of seminiferous tubules are shown as mean ± SD, normalized to NCR (1), n = 5. C. Representative fluorescent images of NCR and HCR testicle slices at higher magnification - confocal view / digital zoom. The cholesterol content insidethe seminiferous tubules detected by the filipin III probe shows more signaling around the nucleus during spermiogenesis (clearly defined in merge images) (Magnification 600 X and zoom 49). D. Densitometric filipin III fluorecensce image analysis of cholesterol content inside seminiferous tubules of NCR and HCR rabbits. Filipin positive areas of seminiferous tubules are shown as mean ± SD, normalized to NCR (1), n = 5. Bars, white = NCR grey = HCR ≤ 6 M and dark grey = ≥ 12 M). Asterisks denote significantly different, *= $p < 0.05$; **= $p < 0.01$ and *** = $p ≤ 0.001$.
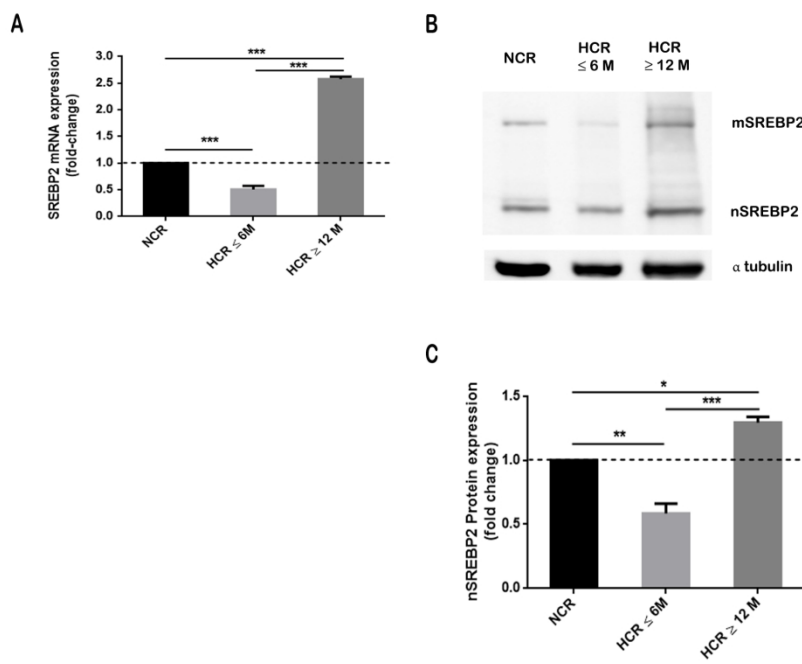
Figure 4. SREBP2 expression in testicles. A. SREBP2 mRNA expression was determined by semi-quantitative RT-PCR (using β actin as internal control) of the total RNA extracted from the testes of the different groups. SREBP2 mRNA levels were plotted as bars and are expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SEM of 5 different determinations (n=5). B. Expression of SREBP2 protein in testicular extracts from both control and HFD-fed rabbits evaluated by western blot using α tubulin as load control. C. SREBP2 protein levels were plotted as bars and are expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SD of four different experiments (n=4). NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (≤ 6 M), less or equal to 6 months of fat diet; (≥ 12 M), greater or equal to 12 months of fat diet. mSREBP2, membrane bound SREBP2 (120 kDa) and nSREBP2, nuclear form of SREBP2 (68 kDa). *, ** and *** denotes significantly different according to ANOVA test (p ≤ 0.05, 0.01 and 0.001 respectively).
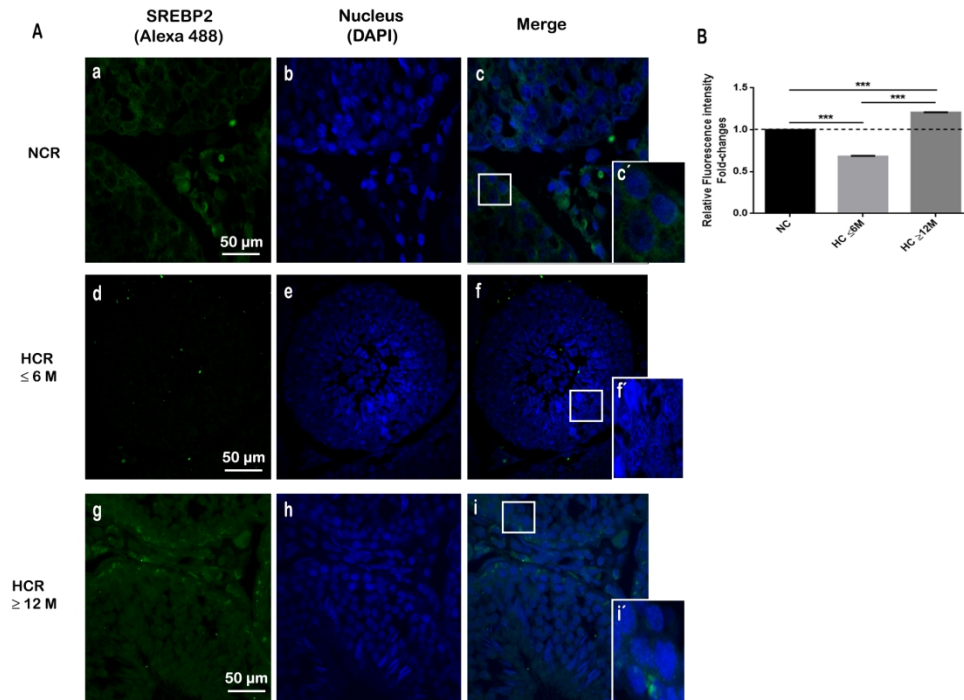
Figure 5. Immunostaining of SREBP2 on testicular sections. A. Left column (green signal) corresponds to detection of SREBP2, middle column (blue signal) to nucleus detection by DAPI, and right column shows the merge. Magnification: a to i, 600 X; c', f' and i' are two-fold enlarged images from c, f and i, respectively. B. The histogram shows densitometry analysis data of SREBP2 immunostaining expressed relative to NCR, which is considered as 1. Each bar corresponds to mean ± SD of four different experiments (n=4). NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (≤ 6 M), less or equal to 6 months of fat diet; (≥ 12 M), greater or equal to 12 months of fat diet. *** denotes significantly different (p ≤ 0.01).
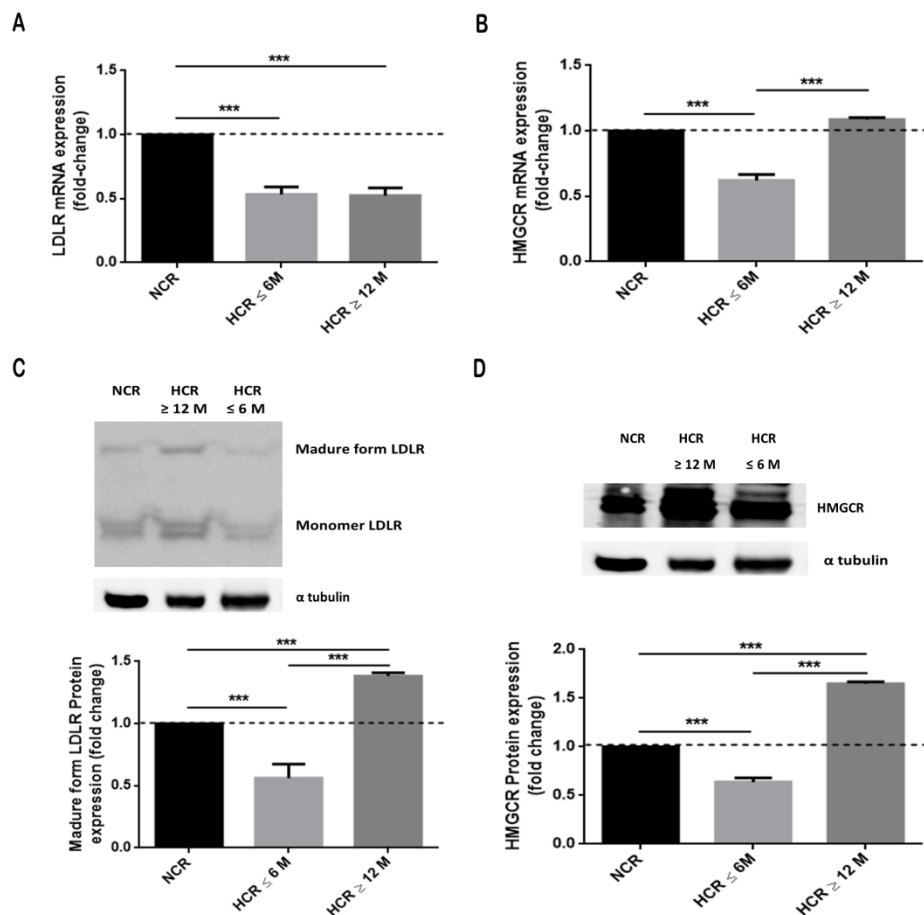
Figure 6. Expression patterns of downstream molecules of SREBP-2 in testicles. A and B. The expression of HMGCR (A) and LDLR (B) mRNAs were determined by semi-quantitative PCR (using β actin as internal control) of total RNA from testicles. mRNA levels are expressed as percentage of control (NCR). Each bar corresponds to mean ± SEM of 5 determinations (n=5). C and D. HMGCR (C) and LDLR (D) protein expression in testicular extracts after fat diet assessed by Western blot, compared to control (NCR) and using α tubulin as loading control. The histograms represent densitometric analysis data expressed as percentage of levels in control (NCR), mean ± SD of four different experiments. NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; (HCR ≤ 6 M), less or equal to 6 months of fat diet; (HCR ≥ 12 M), greater or equal to 12 months of fat diet. HMGCR, HMG CoA reductase; LDLR, LDL receptor. Different letters denote significance according to ANOVA test (p ≤ 0.05).

Table I

	NCR	HCR ≤ 6 M	HCR ≥ 12 M
Body weight (kg)	3.48 ± 0.34	3.39 ± 0.2	3.72 ± 0.21
Total Cholesterol (mg/dL)	30.1 ± 2.21	109.3 ± 40.9 [*]	110.5 ± 30 [*]
HDL-c (mg/dL)	16.15 ± 1.8	12.8 ± 0.2	14.4 ± 2.47
TG (mg/dL)	105.9 ± 12.6	144.21 ± 9.1 [*]	166.01 ± 16.85 [*]

Table I. Body weight and serum lipids. NCR, normal cholesterolemic rabbits; HCR, hypercholesterolemic rabbits; ≤ 6 M and ≥ 12 M correspond to months of fat diet; HDL-c, high density lipoprotein-cholesterol; LDL-c, low density lipoprotein-cholesterol; TG, triglycerides. Data are shown as mean values ± SEM. Sample size (n): NCR, n = 12, HCR ≤ 6, n = 6 and HCR ≥ 12 M, n = 6. * indicates $p<0.05$.

1

Table II

	NCR	HCR ≤ 6 M	HCR ≥ 12 M
Volume (µl)	759.8 ± 164.8	499.2 ± 0.29 *	432.2 ± 0.05 *
pH (mean ± SD)	7.5 ± 0.12	7.5 ± 0.24	7.5 ± 0.6
Sperm viability after eosin staining (%)	88.8 ± 3.07	84.2 ± 3.34	85.8 ± 2.76
Sperm concentration (x 10^5/ml)	629.2 ± 217.4	552 ± 215.3	521 ± 284.16
Total Motility (% A+B+C)	76.8 ± 7.92	60.1 ± 7.68 *	54.6 ± 9.84 *
Total sperm abnormalities (%)	21.1 ± 5.76	35.7 ± 9.36 *	33.6 ± 8.4 *

Table II. Seminal parameters. Data are shown as mean values ± SEM except for pH. For total motility, the sum of the different degrees of motility of the sperm samples is considered (A: good to rapid unidirectional forward progression; B: poor to moderate, erratic, poor progression and C: weak, twitching, no progression). Sample size (n): NCR, n = 12, HCR ≤ 6, n = 6 and HCR ≥ 12 M, n = 6. * indicates $p < 0.05$.