



Assessment The Relationship Between High-Fat Diet Feeding and Male Infertility in Albino Rats

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THE advent of the high-tech devices has accompanied by low physical activity but high consumption of westernized style high fat diet leading to high prevalence of obesity worldwide particularly in young reproductive ages of both man and women creating a highly risk factor for fertility disorders. However, the molecular mechanism underlying the link between overweight and infertility is still unclear. Therefore, the current study planned to uncover the association between weight-gain from high fat diet and the consequent infertility in male rats. The study recruited 30 adult male rats divided equally into two groups: the 1st group was given normal diet and considered as a control group. The 2nd group was served high fat diet (HFD) 60% buffaloes' fat for 16 weeks to initiate similar to diet-related obesity. At the end of the 16th week, blood samples, testicular tissue and semen samples were obtained and underwent biochemical, histopathological, gene expression and microscopical investigation. Compared to control normal diet fed rats, the obtained data showed that there was an increase in blood cholesterol and triglycerides in addition to the unwanted increase in body weight. HFD group showed down regulation of steroidogenic genes *Star* and *CYP17A* with low levels of serum testosterone. Semen analysis taken from HFD males demonstrated low quality represented by low sperm count with reduced viability, motility and increased abnormalities. Testicular tissue sections of positive control group displayed degenerative changes with Caspase3 is crucial for the morphological alterations of cells as well as for the biochemical occurrences connected to the start and end of apoptotic processes and damaged cell indicator NLRP3. In contrast, the same sections displayed marked decrease of inhibitor of apoptosis, survivin. Accordingly, it can be concluded that HFD induced obesity negatively affect male fertility through several mechanisms including general cardiovascular health concern of hyper-lipidemia, hormonal imbalance, low quality semen resulted from degenerative changes of testicular functional tissue.

Key words: High fat diet, Male infertility, *Star*, *CYP17A*, Caspase3, NLRP3.

Introduction

The main cause of metabolic syndrome is defined as obesity, which is an inequality between high energy intake (food consumption) and expenditure (body activity) [1]. A combination of factors interact to predispose to the development of obesity such as high food consumption with low physical activity supported by environmental factors and genetic propensity, which is defined as sedentary lifestyles that enhance fat precipitation in the obese body [2]. Therefore, the regional distribution of body fat specifically the abdominal adiposity has

also been used to identify a person as obese [3] and currently waist circumference is considered the highest accurate measure of obesity [4]. Moreover, excessive fat storage and a high plasma level content are indicators of obesity [5]. By increasing the quantity and size of fat cells, the body's overall fat mass is increased [6]. Based on research supported potent association linking obesity to global health problems including cardiovascular diseases, hypertension, metabolic disorders like type 2 diabetes, liver diseases, renal diseases and

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subfertility in both women and men obesity is classified as one of the top concerns to global public health[7].

Obesity is unfortunately thought to alter several sperm parameters, including sperm concentrations, sperm cell counts, and mass, morphology and mobility [8]. Therefore, a direct link between male infertility and obesity is evident [9]. The adjustments that resulted in this outcome include hormonal levels as well as immediate modifications to the chemical structure of sperm functions and composition[10]. Typically, the poor semen quality and significantly reduced level in blood testosterone are connected to a higher body mass index (BMI) [11].

Experimentally, HFD induced obesity in rats lead to primary hypogonadism with low fertility hormones and increased biomarkers of testicular tissue oxidative stress [12]. Interestingly, obesity is inversely proportional to serum testosterone levels [13]. As a result, testosterone levels may be decreased by obesity, which could lead to the onset of male obesity in men [14]. However, low testosterone levels in rats fed HFD were restored to normal by changing to normal diet [15, 16].

Cytochrome *P450 17A1 (CYP17A1;* also, *P450c17* and *P450sccII*) is a gene encodes a member of the cytochrome P450 superfamily of enzymes. The result P450 proteins, is monooxygenases which catalyzes many reactions involved synthesis of all endogenous androgens [17]. *P450c17* is the single key enzyme mediating both 17 alpha-hydroxylase and 17,20 lyase

activities in the synthesis of steroid hormones [18]. The steroidogenic acute regulatory (*StAR*) protein mediates the transport of cholesterol from the outer to inner mitochondrial membrane, which is considered the rate-limiting step of steroid synthesis [19]

Accordingly, the current study targeted to investigate the adverse effect of HFD consumption on the male fertility compared with a control group that received a normal diet. This study included biochemical, histological and immunohistochemical investigations.

Material and Methods

Animals and experimental design

The present study recruited 30 adult male albino rats of 100±20 g average body weight. The animals were obtained from a private farm in Al-Gharbiya governorate. They were maintained in cages 5 rats each with soft-wood chips for bedding. These animals were kept under well-ventilated environmental conditions and light cycle 12/12h. During the 1st 2weeks, they were given a commercial basal diet and water *ad libitum* for acclimatization at the animal house of the faculty of Veterinary Medicine, Kafrelsheikh University. All the experiments in this present study were in agreement with ethical principles and guidelines for the care and use of laboratory animals adopted by Research Ethics Committee, Faculty of Veterinary Medicine, Kafrelsheikh University. After 2weeks, Rats were divided into two groups (15 rats per group). Group 1(control group) were kept on basal diet for 16 weeks while Group 2 (HFD group) received high-fat diet (HFD) (Table 1) for 16 weeks.

TABLE 1. Chemical composition of diet provided to experimental rat groups

Normal chow diet		High fat diet	
Component	Percentage (by weight)	Component	Percentage (by weight)
Water	9.7%	Normal chow diet	40.0%
Crude protein	20.5%	Buffalo tallow (from local Butcher shop)	60.0%
Crude fat	4.6%		
Ash content	6.2%		
Nitrogen free extract	52.5%		
Calcium	1.2%		
Phosphorous	0.9%		
Lysine	1.3%		
Methionine + cysteine	0.7%		

Sampling

At the end of the trial, blood was drawn from the medial canthus of the eyes using plain tubes. After blood was being drawn, serum was extracted from each separate tube and the sample is typically placed in cold storage at +2°C to +8°C for no longer than seven days. In order to analyze mRNA gene expression, one testis was promptly removed from the body and measured for weight, then

quickly frozen in liquid nitrogen and kept at -80°C until it was required. The other testis was retained for histological and immunohistochemical analyses in 10% buffered formalin solution.

Hormonal assays

In accordance with the manufacturer's recommendations, particular commercial ELISA kits were used to measure the serum levels of FSH,

LH, and testosterone (Cat. No. RH-251, DSI, Italy Inc., Cat. No. BC-1, 029, Bio Check, CA Inc.). The competitive inhibition enzyme immunoassay approach serves as the foundation for the assay. Three distinct microplates have each been pre-coated with a monoclonal antibody that is specific for Testosterone or LH or FSH, respectively. With the pre-coated antibody specific for rat Testosterone or LH or FSH, a competitive inhibitory reaction is started between biotin labelled rat Testosterone or LH or FSH and unlabeled rat Testosterone or LH or FSH (Calibrators or samples). Each microplate is washed of the unbound conjugate following incubation. After that, each microplate well is filled with avidin conjugated to horseradish peroxidase (HRP), which is then incubated. The ratio of the bound HRP conjugate to the levels of testosterone, LH, or FSH in the sample is inversely proportional. The amount of color created upon the addition of the substrate solution is inversely proportional to the amount of testosterone, LH, or FSH present in the sample.

Biochemical assays of serum lipids

HDL cholesterol was determined in accordance with Burstein and Legmann [20]; and LDL cholesterol was determined in accordance with

Wieland and Seidel [21]. **Gene expression assay by qRT-PCR.** Using the QIAzol reagent, total RNA was extracted from the testicular samples (QIAGEN Inc.). After that, RNA purity and concentration were measured spectrophotometrically at 260/280 nm. Following that, one μg of total RNA was used for reverse-transcription into cDNA synthesis using QuantiTect reverse transcription kit (Qiagen) in accordance with the manufacturer's instructions. The MacroGen Company created and manufactured the PCR primer sequences (Table 2) after using the primer 3 programmer (MacroGen Company, GAsa-dong, Geumcheon-gu, Korea). SYBR green qRT-PCR was utilized with the real-time PCR technique. The thermal cycling conditions were as follows: initial denaturation at 95°C for 10 min, 40–45 cycles of amplification of DNA denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 30 s. At the end of the last cycle, the temperature was increased from 63 to 95°C for melting curve analysis. Robotic data collection was done with a Rotor-Gene Q (Qiagen), which assessed the threshold cycle value (CT). The $2^{-\Delta\Delta\text{CT}}$ method was used to obtain the relative expression of *CYP17A1*, *StAR*.

TABLE 2. Primers sequences and Gene Bank accession numbers

Gene	Accession number	Direction	Primer sequence
<i>CYP17A1</i>	NM_012753.2	Sense	ACTGAGGGTATCGTGGATGC
		Antisense	TAGGTACCAGTTGTCATCAT
<i>StAR</i>	NM_031558.3	Sense	CTGCTAGACCAGCCCATGGAC
		Antisense	TGATTTTCCTTGACATTTGGGTTCC
<i>GAPDH</i>	NM_017008.4	Sense	TCAAGAAGGTGGTGAAGCAG
		Antisense	AGGTGGAAGAATGGGAGTTG

Analysis of sperm parameters

Cauda epididymides were crushed and added to 8 mL of phosphate-buffered glucose saline in a petri dish to produce semen, which was then incubated at 37 °C for 10 minutes in the humid incubator. Microscopically, the motility of epididymal sperm was assessed. Within 2-4 minutes after their isolation, a drop of the semen suspension was placed on a glass slide and analyzed. Data were presented as percentages of the investigated sperm population that were motile. The total number of sperm in the suspension was then estimated using the Neubauer chamber in accordance with [22]. To test the vitality of the sperm, 10 μl of sperm suspension was combined with 10 μl of 0.5% eosin stain on a microscope slide. Meanwhile, anomalous sperm rates were assessed using Eosin-Nirgosin[23]. **Histological analysis** Testes and epididymides were sliced, weighed and fixed by immersion in 4% formaldehyde in phosphate buffered saline or Bouin's solution for at least 24 h. Tissues were then

processed for paraffin embedding and sectioning by routine methods. Tissue sections were stained with Hematoxylin and Eosin using standardized procedures and examined by light microscopy (100 \times or 400 \times).

Immunohistochemical investigation

According to the study [24], the immunohistochemistry protocol was followed. After being removed from the wax, tissue pieces were cleaned in 0.05 M citrate buffer at pH 6.8. Then, 0.3% H₂O₂ and a protein fragment were applied to the sections to prevent non-specific binding. With the following rabbit polyclonal antibodies: anti-caspase 3, NLRP3 and survivin antibody. Segments were treated with goat anti-rabbit antibody (EnVision Framework Horseradish Peroxidase Named Polymer; Dako) for 30 min at room temperature after being washed in phosphate-buffered saline. Slides were counter-stained with Mayer's hematoxylin and photographed. The immunolabelling was determined through counting the positive cells within 1,000 cells per 8- (XHPF).

Statistical analysis

Data are presented as mean and standard error of the mean (SEM) from four separate experiments of each sample. Statistical examines were carried out by GraphPad Prism 10 software. Differences between mean values were analyzed by t-test. The differences were considered significant when $p < 0.05$.

Results

Effects of HFD on the body weight, BMI and serum lipids contents

Raising rats on HFD for 4 months, significantly ($p < 0.05$) increased body weight (Fig. 1A) which was accompanied with a significant increase ($p < 0.05$) in BMI (Fig. 1B) compared with the control group that received basal diet.

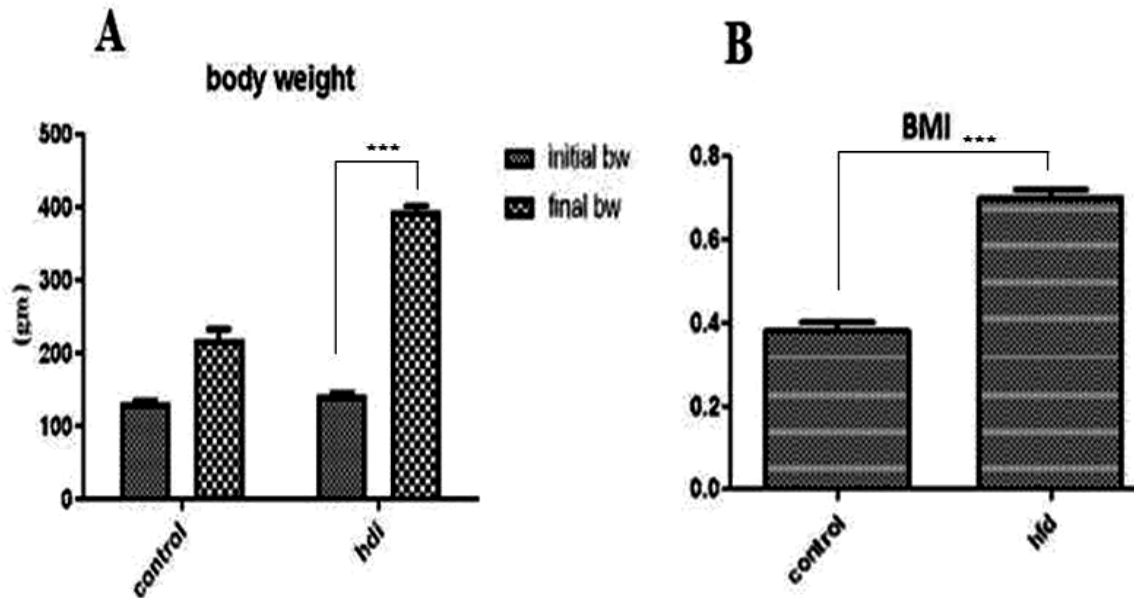


Fig. 1. The effect of HFD supplementation on initial and final body weight (A) and BMI (B)

Moreover, HFD induced significantly ($p < 0.05$) increased serum lipids content including triacylglycerol, total cholesterol and LDL-cholesterol. This was parallel

with a significant decrease in HDL- cholesterol compared with control group (Table 2).

Effects of obesity on serum hormonal levels

In group supplemented with HFD compared to the control group, Testosterone level was significantly reduced ($p < 0.05$). In contrast, both FSH and LH serum levels increased significantly ($p < 0.05$) (Table 3).

TABLE 3. Effect of HFD supplementation on serum lipids content. Data are represented as Mean \pm M.E.S. Different letters mean significant differences at ($p < 0.05$). Cholesterol, TAG, LDL-cholesterol and HDL cholesterol. The same letters indicate statistically insignificant differences while different letters mean significant differences at ($p > 0.05$).

Groups	Cholesterol mg/dl	TAG mg/dl	HDL mg/dl	LDL mg/dl
Control	50.37 \pm 4.6 ^a	76.00 \pm 3.1 ^a	27.5 \pm 1.47 ^b	15.8 \pm 2.3 ^a
HFD	96.14 \pm 8.6 ^b	150.78 \pm 17.2 ^b	^b 19.75 \pm 1.03 ^a	40.2 \pm 1.3 ^b

TABLE 4. Effect of HFD supplementation on serum hormones content

Groups	Testosterone	LH	FSH
Control	0.80 \pm .07 ^a	0.23 \pm .05 ^a	0.54 \pm .14 ^a
HFD	0.34 \pm .05 ^b	0.75 \pm .06 ^b	3.2 \pm .10 ^b

Data is represented as Mean \pm M.E.S. Different letters mean significant differences at ($p < 0.05$). Testosterone (ng/ml), LH (mIU/ml) and FSH (mIU/ml).

Effects of HFD on seminal parameters

The obtained results showed that sperm count (Figure 2A), viability (Figure 2B) and motility (Figure 2C) decreased significantly ($p < 0.05$) in the HFD group compared to control groups. Moreover,

sperm abnormalities % were increased significantly ($p < .05$) in HFD receive rats compared to control group (Fig. 2 D).

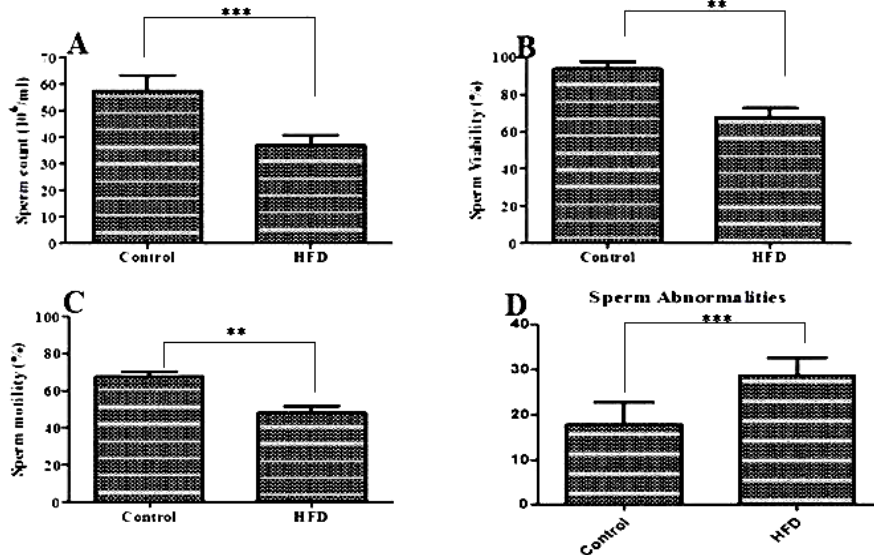


Fig. 2. Effect of HFD supplementation on Sperm count (A), sperm viability (B), Sperm Motility% (C) and sperm viability % (D). P values are indicated by *, ** and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively

Effect of HFD on *StAR* and *CYP17A1* mRNA gene expression

HFD induced highly significant ($p < 0.001$) down regulation the testicular *StAR* mRNA gene expression compared to control group (Fig. 3A).

Also, HFA induced significant decrease ($p < 0.001$) in *CYP17A1* mRNA gene expression compared to control group (Fig. 3B).

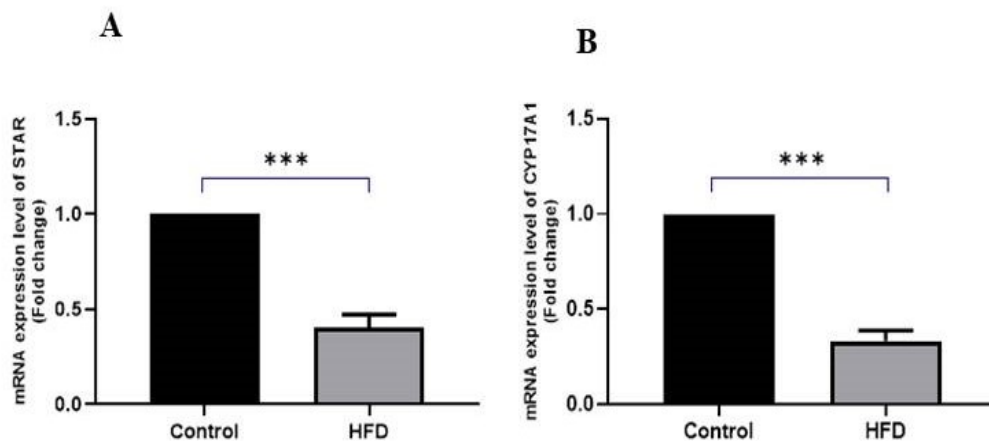


Fig. 3. Effect of HFD on testicular mRNA expressions of *StAR* gene (A), *CYP17A1* (B). P values are reported or indicated by *** for $P < 0.001$

Effects of HFD on histopathological investigations

When compared to Testis of HFD animals, which showed marked congestion of the blood vessels of the tunica albuginea (arrowhead) associated with interstitial oedema (arrow), noticeable degenerative lesions within the spermatogenic cells, and presence of proteinous

rich fluid within the seminiferous tubules (curved arrow), Testis of control animals showed normal seminiferous tubules lined with spermatogenic cell layers (arrow) with the presence of free sperm (Fig. 4).

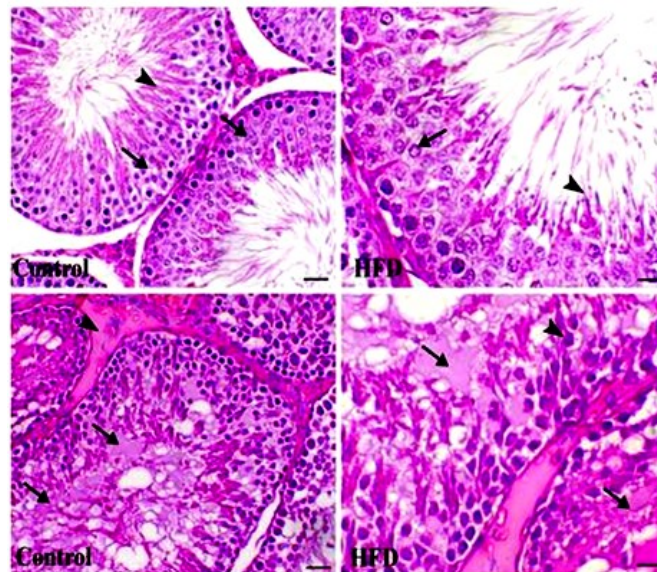


Fig. 4. Testis of control animal displaying typical spermatogenic cell layers lining the seminiferous tubules (arrow) and the presence of free sperms within their lumen (arrowhead), H&E, X200, bar=100 μ m. Comparing the testis of an HFD animal, the images show clearly constricted blood vessels in the tunica albuginea (arrowhead), interstitial oedema (arrow), and clearly degenerative changes in the spermatogenic cells, as well as the presence of protein-rich fluid in the seminiferous tubules (curved arrow). H&E, X200, bar= 100 μ m.

Effects of HFD on immunohistochemical findings

Figure 5 demonstrates a distinctly recognized and statistically highly significant ($p < 0.01$) immunoreactivity for caspase-3 protein was found in the HFD received group. In contrast caspase-3 protein displayed no immunoreactivity with testicular tissue from the control normal diet group. NLRP3 or survivin was strong and clearly detected in testicular sections prepared from normal diet group while it was significantly ($p < 0.01$) reduced in tissues of HFD fed group (Figure 5).

Discussion

One of the main public health concerns in the world is obesity [25]. Consuming an HFD is thought to be a significant risk factor for the development of obesity, despite the fact that it is a complex condition controlled by several environmental and genetic factors [26]. In clear agreement with that, our results revealed strong bond between HFD and obesity reflected by significant increase in body weight of HFD fed animals compared with normal fat diet fed rats. However, our study used new source of fat, buffalo's fat tallow. Up to published similar studies, it is the first experiment to explore the ability of buffalo's fat tallow in induction of obesity like condition. The use of buffalo's fat tallow is motivated by its high popularity in Egypt as a source of fat either in homemade food or in fast-food shops. In the present study, we looked

into how the HFD negatively affected male fertility and through disturbing in several ways including controlling genes, hormones, testicular functioning structure and rat sperm functioning.

This outcome is consistent with that of the earlier research [27]. The potential of HFD to create a state of a positive energy balance that results in an increase in visceral fat deposition and fits with our observation of increased visceral fat mass in the HFD group compared to control could be the cause of the development of obesity as a result of HFD feeding. Abdulwahab *et al.*, [28] revealed that the fertility of the HFD-received group was generally subnormal, as demonstrated by a marked reduction in serum testosterone joined with poor quantitative (low sperm count) and qualitative semen (sub normal viability, motility and high abnormality percentage). In line with Abdulwahab *et al* findings from 2021, our findings revealed a drop in serum testosterone as well as a decrease in sperm count, motility, and viability along with an increase in sperm abnormalities as compared to the basal diet-received group. Obesity affects testosterone production, sperm maturation, and development [10]. In addition to lowering testosterone levels, obesity can also cause oxidative stress. These modifications may alter how the testes function, and it has been suggested that obesity may be one of the etiological reasons causing male infertility [29].

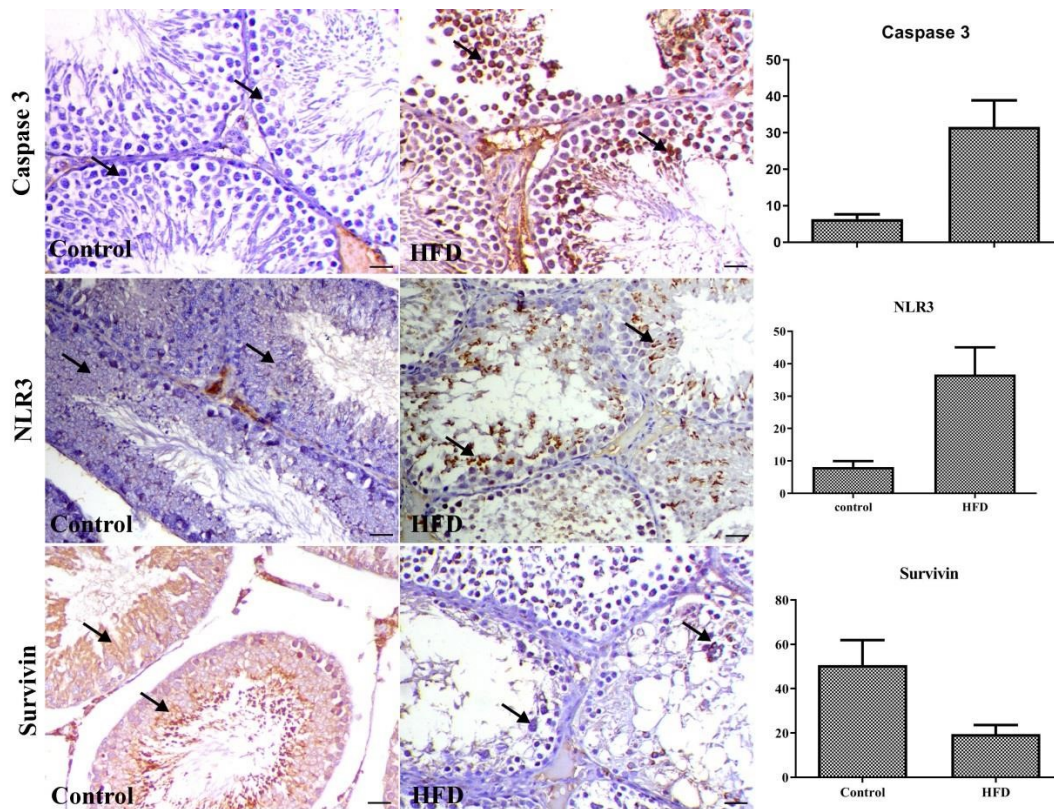


Fig. 5. The photo to the right of Caspase 3 IHC, X200, bar = 50 m. Image in top left depicts testis of control animal demonstrating low expression of caspase 3 antibody within the spermatogenic cells (arrows). The image in the middle right shows the testes of a control positive animal displaying prominent expression of the caspase 3 antibody within the spermatogenic cells (arrows indicate nuclear expression). The image in the middle left shows the testes of a control animal, which exhibit minimal cytoplasmic expression of NLRP3 within the spermatogenic cells (arrows). The image in the lower right shows the testes of a control animal with minimal cytoplasmic expression of NLRP3 within the spermatogenic cells (arrows).

The image in the lower left shows the testes of the control animal with prominent survivin immunostaining within the spermatogenic cells (arrows), Caspase 3 IHC, X200, and a bar of 50 m. The expression of survivin within the spermatogenic cells is significantly reduced in the testes of control positive animals (arrows); survivin IHC, X200, bar = 50 μ m.

It is well known that semen quality serves as a sign of fertility. Obesity undoubtedly causes physical changes and hormonal imbalances that have a negative impact on male fertility and key metrics [30]. Additionally, obesity negatively affects sperm morphology and motility [9]. Other studies focused on the negative effects of obesity on spermatozoa's activities [31].

Concerning the obtained results for immunohistochemical investigations, testicular tissue from the control group lacked any caspase-3 antibody. However, the HFD group had a significant immunoreactivity for caspase-3. In parallel to the results of Caspase-3, NLR-3 showed the same patterns. One of the best-characterized receptors that activate the inflammasome is NLRP3. Furthermore, a growing body of evidence suggests that the NLRP3 inflammasome contributes to a number of inflammatory illnesses. The release of interleukin (IL)-1 and IL-18 as a result of NLRP3 activation [32].

Growing evidence points to the NLRP3 inflammasome as a metabolic sensor of metabolic dysregulation, including Type II diabetes mellitus. Thioredoxin-interacting protein (TXNIP) associates with NLRP3 upon inflammasome activation in a ROS-dependent way, resulting in the subsequent production of IL-1 and insulin resistance [33].

Inflammatory activation brought on by obesity is prevented in mice with Nlrp3 ablation, and insulin signaling is improved. These findings demonstrate that the NLRP3 inflammasome perceives danger signals associated with obesity and contributes to obesity-induced inflammation and insulin resistance. Furthermore, eliminating NLRP3 in obese mice lowers IL-18 and adipose tissue interferon- (IFN-) expression [34].

However, as an inhibitor of the apoptosis protein, Survivin affects apoptosis during cell division [35]. It regulates apoptosis, the cell cycle, and cell division. Consequently, it is yet another

candidate gene that may be involved in the regulation of the development of germ cells [36]. Survivin has been shown to be highly expressed during human spermatogenesis, and its downregulation is frequently linked to spermatogenic failure [37]. The findings of this study showed that the testes of obese rats had significantly lower levels of survivin and higher levels of testicular caspase-3 expression.

The results of the hormone assay prompted researchers to look into higher levels of gene regulation. *StAR*, a prototype cholesterol controller, transports cholesterol into mitochondria, where cytochrome P450 11A1 initiates the synthesis of steroids in diverse endocrine cell types. Intriguingly, testicular tissue from HFD animals has lower levels of *StAR* gene expression than that from normal rats. These results appear to be in agreement with lower testosterone levels in the same group.

Conclusion

Collectively, from gene expression through hormone synthesis to spermatogenesis, there is a direct correlation between obesity and decreased steroid production as well as infertility in males.

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Conflict of interest

The authors declare no conflict of interest.

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This research received no external funding. *Author's contribution: all authors contributed equally. All authors have read and agreed to the published version of the manuscript.*

Ethical approve

The study was approved by Research Ethics Committee, Kafrelsheikh University.

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دراسة كيميائية حيوية لتقييم العلاقة بين تغذية النظام الغذائي العالي الدهون والخصوبة في ذكور الفئران

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تطور استخدام الاجهزة التكنولوجية أصبح مصحوباً بقله النشاط الحيوي الفيزيائي وأتباع نظام غذائي غير صحي وتناول الوجبات السريعة التي تحتوي على نسبة عالية من الدهون والتي تؤدي بدورها الي اصابه الانسان بالسمنة المفرطة في جميع انحاء العالم وخاصة في الشباب رجالاً ونساء مما يؤثر بطريقة غير مباشرة على الخصوبة. وعلي الرغم من تأثير السمنة علي الخصوبة ولكن العلاقة بينهم تظل غير معروفه الي حد ما لذلك سنتناول في هذه الدراسة بعض الاسباب الجينية وبعد الانزيمات والعمليات ت الحيوية والخلل الهرموني الناتج عن السمنة المفرطة ومدى تؤثر الخصوبة.

وذلك عن طريق اجراء تجرية على ٣٠ ذكور الفئران وقسمت الي مجموعتين المجموعة الاولى تتناول اكل صحي يوميا والمجموعة الثانية تتناول اكل يحتوي على نسبة عالية من الدهون (٦٠%) على الغذاء واستخدام دهن الجاموس كمصدر للدهون لمدة تصل الي ١٦ اسبوعاً ثم القيام بقياس بعض التحاليل الكيميائية ونسب بعض الانزيمات والهرمونات وكفاءة وحركه الحيوانات المنوية في المجموعتين لبيان مدى تؤثر ذكور الفئران بالغذاء المحتوي على الدهن.

الكلمات الافتتاحية : *StAR*, *CYP17A*, *Caspase3*, *NLRP3*; اكل عالي الدهن ، العقم عند الرجال.