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ORIGINAL ARTICLE

Metabolic Syndrome Induced by High Fat and Fructose Diet Affects the Semen Quality and Immunohistochemical Expression of AQP8 in Male Rats

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ABSTRACT

Background: The increasing prevalence of male subfertility and the possible association with the increasing rates of obesity is alarming. Fluid homeostasis in the testis is important for male fertility. This study aims to define the effect of diet induced metabolic syndrome on the testis and expression of the selective water channel AQP8. **Methods:** 12 male Sprague-Dawley albino rats were divided equally into control and high fat high fructose diet (HFFD) groups. After 12 weeks, the blood glucose, insulin, lipid profile and testosterone level were evaluated. Testicular sections were stained with H&E for histopathological examination and other testicular sections were immunohistochemically stained with anti-AQP8 antibody. The sperms of the cauda epididymis were analyzed.

Results: in comparison to control, blood glucose of HFFD rats was elevated and insulin level was decreased. Serum triglycerides, cholesterol and LDL levels were significantly elevated while testosterone level was decreased. There was a significant decrease in sperm number and motility with increase of abnormal forms. Testes of HFFD group showed distortion of many seminiferous tubules, depletion of germ cells with exfoliation of cells into the lumen. The diameter and thickness of the tubules were reduced. Also, the immunoexpression of AQP8 was significantly decreased in comparison to control especially in relations to spermatids.

Conclusions: AQP8 immunoexpression was decreased in the HFFD rats which might partially contribute to the reduced sperm quality. Other factors might also contribute to the changes in sperm and seminiferous tubules morphology like hormonal changes, hyperlipidemia and hypoinsulinemia.

Keywords: high fat fructose diet, metabolic syndrome, AQP8, immunohistochemistry, testis.

INTRODUCTION

Obesity is a global health problem with increasing prevalence worldwide. Lifestyle habits particularly of unhealthy fats intake act as a major contributor [1]. Obesity promotes metabolic syndrome (MetS) characterized by a cluster of metabolic dysregulations including insulin resistance, dyslipidemia, central obesity, non-alcoholic fatty liver (NAFLD) and hypertension [2].

The effect of obesity on human male fertility is controversial. Several studies and meta-analyses demonstrated a negative relation between body mass index (BMI) or metabolic syndrome (MetS) and semen quality: sperm count, sperm morphology, sperm motility and sperm viability [3,4,5,6,7,8]. While, several other studies reported that MetS does not negatively influence semen parameters and fertility [9,10,11].On the other

hand, animal models of obesity induced by high fat diet showed reduction of semen quality and reproductive performance [12,13,14,15].The effect of paternal obesity may even compromise offspring reproductive health and thus may subsequently amplify transgenerational subfertility [16].

Proper cell function and survival depends on appropriate concentrations of water and solutes [17]. In the mammalian testis, fluid homeostasis during spermatogenesis and sperm maturation is critical for male fertility [18].

Aquaporins are a family of highly conserved, integral transmembrane proteins that work as selective water channels [19]**.** They have different isoforms, AQP0–12, divided into groups according to permeability characteristics [20]**.** Aquaporin 8 (AQP8) belongs to the orthodox group of AQPs which are most selective for water transportand to the ammoniaporins group that allows transportation of ammonia. Additionally, AQP8 is a peroxiporin that transports hydrogen peroxide across cell membrane [21].

The description of AQP 8 localization in the testis is inconstant. AQP8 was described to be localized in Sertoli cells [22,23], primary and secondary spermatocytes [24] or spermatogenic cells [25,26]**.** Testicular localization of AQP8 led to the suggestion of having a role in the formation of testicular luminal fluid volume and reduction of spermatids during elongation [25].

Few studies investigated the effect of obesity or MetS on the expression of AQPs. Marchiani et al. [13] found in the rabbit testis that the mRNA expression of aquaporin 1 was reduced while that of aquaporin 9 was elevated after HFD. However, they did not study the effect on AQP 8 expression. The effect of HFD induced NAFLD and MetS on the cytoarchitecture of the testis and AQP8 expression still needs further investigation.

The present study aims to determine the immunohistochemical localization of AQP8 protein in rat testis and to evaluate the effect of high fat and fructose diet on its expression and its possible consequences on semen parameters.

METHODS

Animals used:

Twelve pathogen-free adult male Sprague-Dawley albino rats weighing 150-200 gmwere obtained from Medical Experimental Research Center (MERC) of Mansoura University.Rats were housed three per cage and maintained under stable conditions: humidity (60 ± 5%), temperature (21 ± 2 °C), light/dark (12/12 h) cycle, with free access to food and water for two weeks before the experiment for acclimatization and to ensure normal growth and behavior. The number of animals necessary to produce reliable scientific results were used to minimize animal suffering. This work was carried out in the Anatomy Department, Faculty of Medicine, Mansoura University and MERC over a period of one year.

All animal experiments comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals.

High fat and fructose diet (HFFD) model:

High fat diet consists of 4०% fat(40% animal fat + 5% fat present in basal diet) and fructose 20% (20 g of fructose was diluted in 100mL of drinking tap water) [27].

Experimental design:

The animals were divided randomly into two groups (6 rats each), control group fed on basal diet and water ad libitum for 12 weeks and high fat and fructose diet (HFFD) group fed on high fat diet and fructose for 12 weeks.

Assessment of body weight:

The body weight of rats was recorded at the beginning and the end of experimental period.

Assessment of blood glucose and insulin levels:

Fasting blood glucose of rats in each group was measured in blood from the tail vein at the beginning and end of experiment.The blood glucose level was estimated with the ACCU-CHEK glucose meter (Roche Diagnostic Co., Germany). Insulin level (Chemiluminenscence, Abbot, USA) was measured at the end of the experiment.

Biochemical study:

Blood samples were taken by direct puncture of the left ventricle at the end of the 12th week under diethylether anesthesia. Blood samples were received in EDTA (ethylenediamine tetra acetic acid) containing tubes and centrifuged at 1800xg for 10 minutes at 4^{o}C where plasma was separated and stored at -20^{o}C for assessment of lipid profile and serum testosterone level [28]**.**

Assessment of lipid profile: Serum levels of total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured using the commercially available kits; total cholesterol assay endpoint kit (MG, cat. No. MG230001), triglyceride assay endpoint kit (MG, cat. No. MG314001), and HDL cholesterol assay endpoint kit (MG, cat. No. G266001) respectively.Low-density lipoprotein (LDL) cholesterol was

determined by the formula: LDL cholesterol = Total cholesterol – HDL cholesterol – (Triglycerides/5)**.** ThenLDL/HDL, TG/HDL and TC/HDL ratios were estimated**.**

Testosterone level assessment: Measuring of serum testosterone was done for all groups by the Enzyme Linked Fluorescent Assay (ELFA) technique using an automated quantitative instrument of VIDAS family [29].

Collection of tissue samples:

After sacrification, the testis and epididymis were exposed by scrotal incision and removed. The testis was used for histological and immunohistochemical study and the epididymis for semen analysis.

Semen analysis:

Sperm counting was done as described by Dominic and Padmaja [30]. The right cauda epididymis was gently squeezed to discharge epididymal fluid on a slide. One drop of diluted semen was added to Neubauer's haemocytometer. The sperm count was done with the high-power (400×) after 5 minutes in humid chamber to settle down. The total numbers of sperms were counted in the 5 major squares of the haemocytometer and the sperm count per 1ml was calculated.To estimate the sperm concentration of the original cauda epididymal semen sample, the sperm count was calculated as: Sperm count/ml = (dilution factor) (count in five squares) ($0.05x\ 10^6$).

Sperm motility was determined microscopically in the same way as sperm count within 5 minutes of their isolation from epididymis. Percentage of motile sperms to the total sperm count was assessed [30].

Percentage of abnormal forms: seminal smears were spread on a glass slide, allowed to dry at room temperature, fixed with ethanol and stained by hematoxylin and eosin stain. The stains were washed after 5 minutes and allowed to dry at room temperature. All the slides were observed under light microscope at 400x magnification [31]**.** The slides were examined for percentage of abnormal sperms evaluated for head and/or flagellar defects in every 200 spermatozoa observed on each slide .

Processing of tissue specimens for light microscopic examination:

The two testes were fixed in Bouin's solution for 12 hours, then cut transversely and inserted back in the same fixative for four days. The testes were dehydrated in ascending grades of ethanol, cleared in xylene and then embedded in paraffin. Five µm thick sections were cut by using rotatory microtome. The sections were stained with H&E to evaluate the main histopathological findings among the two experimental groups.

Immunohistochemicalstain with Anti-AQP 8 antibody wasused to detect changes in testicular expression of AQP 8 among the two experimental groups. Tissue sections were dewaxed in xylene, washed in alcohol and rehydrated. Antigen retrieval was performed in a microwave oven in 0.02 M citrate buffer solution (pH 6.0) for 10 min. The sections were cooled at room temperature and washed in PBS. The sections were incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidase, then washed in PBS three times.The goat serum (dilution 1:50) was used for 30 min to avoid any non-specific reactions, followed by overnight incubation with anti-AQP8 (rabbit polyclonal, 1:200, Genetex, California, USA) antibody at 4°C. Goat anti-rabbit biotin conjugate (1:200) diluted in PBS was used as secondary antibody for 30 min at 37°C. Then the sections were subjected to diaminobenzidine chromogen, counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted. Negative controls were obtained by substituting the primary antibody with PBS.

Image Analysis:

Olympus CX41 light microscope and Olympus SC100 digital camera were used to observe and photograph the sections. Image analysis was performed using ImageJ 1.51j8 program. For H&E stained sections, two slides were used from each rat of the two groups and four randomly chosen images were captured from each slide at a magnification of x100 to measure the average diameter and thickness of tubules. The two longest perpendicular (e.g., horizontal and vertical) diameters (µm) of the seminiferous tubules were measured, and the average tubular diameter (µm) was defined. The thickness of the spermatogenic epithelium was also measured at different points of the seminiferous tubule circumference and the average thickness per tubule was calculated. Immunostained images without hematoxylin counter stain were used to analyze the area percentage of anti-AQP8 antibody immunoreactive cells. Two slides were used from each rat of the two groups and four randomly chosen images were captured from each slide at a magnification of x200. First, the colored image was converted by image J program into 8-bit image. Then, the brown color (indicating positive immunereaction) was highlighted using the command image/adjustment/threshold and then the

percentage of area was quantified using the analyze/measure command.

Statistical Analysis:

The computer program SPSS (Statistical package for social science) version 26 was used for tabulating, coding and analyzing data. Data were presented in the form of mean ± standard deviation (SD). Differences in means between groups for each parameter were analyzed using independent sample T test to compare between two groups of parametric data. Mann-Whitney test was used to compare between two groups of non-parametric data. P value ≤0.05 was considered statistically significant. All graphic representations of the data were performed with Microsoft® Excel® for windows®. (Microsoft Inc., USA).

RESULTS

Effect of HFFD on body weight:

At the start of the study, no significant difference in the body weight was detected between the two groups (p>0.05). Administration of high fat diet for 12 weeks resulted in a highly significant increase in the body weight in the HFFD group when compared to the control rats (p<0.001) (**Table 1**).

Biochemical results:

Blood glucose level: At the beginning of the study, there was insignificant difference in the blood glucose level between the studied groups (p> 0.05). Administration of HFFD for 12 weeks resulted in highly significant increase in the level of blood glucose in comparison to the control rats (p<0.001) (**Fig. 1A).**

Serum insulin level: showed highly significant decrease compared to the control rats (p<0.001) after 12 weeks of HFFD (**Fig. 1B).**

Lipid profile: consumption of HFFD for 12 weeks resulted in highly significant increase in the level ofserum triglycerides(p<0.001), serum cholesterol(p<0.001), low density lipoprotein (LDL)(p<0.001) in comparison to the control rats. While no significant increase in the level of high density lipoprotein (HDL) (p>0.05) was observed **(Fig. 2).**

The ratios of LDL/HDL, TC/HDL and TG/HDL exhibited highly significant increase in comparison to control (p<0.001) **(Table 2)**.

Serum Testosterone level (ng/ml): Administration of HFFD for 12 weeks resulted in highly significant decrease in the level of testosterone in comparison to the control rats (p<0.001) **(Fig. 3).**

Semen parameters: Administration of HFFD for 12 weeks resulted in significant decrease in the sperm count (p<0.001), significant decrease in the sperm motility (p<0.05) and significant increase in the abnormal sperm morphology (p<0.05) in comparison to the control rats (**Table 3**).

Histopathological and immunohistochemical results:

Hematoxylin and eosin stained sections of the testis:

The seminiferous tubules of testis in the control group appeared circular or slightly oval in cross section and lined by multiple layers of spermatogenic cells at different spermatogenic stages. Sertoli cells were columnar cells with oval or pyramidal nuclei located near the basement membrane. The tubules were separated by interstitial tissue containing polyhedral Leydig cells with a single eccentrically located ovoid nucleus **(Fig.4 A&B).**

Examined sections of rat testis in HFFD group revealed testicular damage with wide interstitial spaces. Some seminiferous tubules appeared normal while others appeared shrunken, smaller in diameter and irregular in shape. Their cells appeared degenerated with depletion of germinal epithelium and partially reduced sperms. The cytoplasmic processes of Sertoli cells were seen surrounding wide empty spaces that might indicate the sites of degenerated germ cells. There was exfoliation of degenerated spermatogenic cells toward lumen. In some tubules, spermatogenic cells were separated by wide spaces in between. The interstitial tissue showed decreased size of Leydig cells **(Fig.4 C&D).**

Morphometric study of hematoxylin and eosin stained sections:

Seminiferous tubular diameter: The mean diameter of the seminiferous tubules in the testis of the control group was 396.92 µm. The HFFD group showed a highly significant decrease in seminiferous tubular diameter in comparison to the control group (P<0.001) **(Fig.5 A).**

Germinal epithelial thickness: The mean germinal epithelial thickness of the seminiferous tubules in the testis of the control group was 135.51µm. The

HFFD group showed a highly significant decrease in the germinal epithelial thickness in comparison to the control group (P<0.001) **(Fig.5 B).**

Immunohistochemical stain with Anti-AQP 8 antibody sections:

In the seminiferous tubules of the control testis, positive immune reaction was detected as brown color in the cytoplasm of Sertoli cells and round spermatids. Strong positive immune reaction was present in elongated spermatids and residual bodies. The cytoplasm of the interstitial cells of Leydig showed also positive immune reaction (**Fig.6 A&B**). The HFFD showed decrease in the positive reaction in both the seminiferous tubules especially the elongated spermatids and interstitial cells (**Fig.6 C&D**).

Image analysis of immunohistochemically stained testis sections:

Testis sections stained with anti-AQP8 antibody without hematoxylin counter stain at a magnification of x200 **(Fig.7 A&B)** were submitted for image analysis to measure the area percentage occupied by positive reaction. The HFFD group showed significant low values of the area percentage of anti-AQP8 antibody expression in comparison to the control group (P<0.001) (**Fig. 7C**).

Table 1: The mean body weight (gm)

	0 week		**12 weeks**	
Group Parameters	Control Mean±SD	HFFD Mean±SD	Control Mean±SD	HFFD Mean±SD
Body weight(gm)	190.67±7.23	189±8.0	306.33±7.17	345.83±7.27*

*P< 0.001

Table 2: LDL/HDL, TC/HDL and TG/HDL ratios

Group Parameters	**Control Mean± SD**	**HFFD Mean± SD**
LDL/HDL ratio	0.06 ± 0.004	1.63 ± 0.24*
TC/HDL ratio	1.39 ± 0.01	3.41 ± 0.32*
TG/HDL ratio	1.67 ± 0.02	3.86 ± 0.74*

*P< 0.001

1. **LDL: low density lipoprotein**
2. **HDL: high density lipoprotein**
3. **TC: Total cholesterol**
4. **TG: Triglycerides**

Table 3: Sperm count (10^6/ml), sperm motility (%) and abnormal sperm morphology (%)

Group Parameters	**Control Mean± SD**	**High fat diet Mean± SD**
Sperm count (10^6/ml)	125.83±4.84	73.23±11.41**
Sperm motility (%)	93.33±2.58	74.16±12.81*
Abnormal sperm morphology (%)	4.66±0.51	15.83±3.76*

*P< 0.05, **P< 0.001

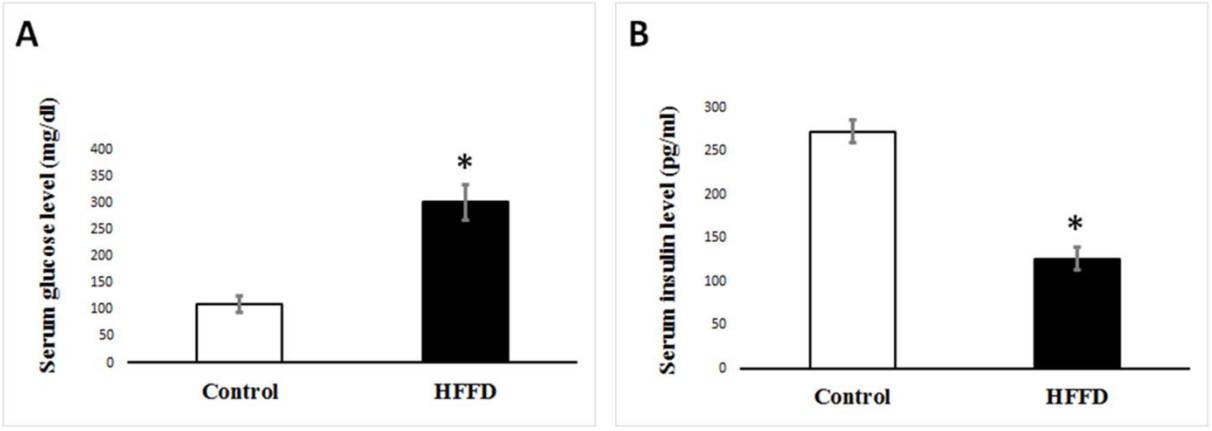

Figure 1: Effect of high fat fructose diet on (A) the blood glucose and (B) the insulin levels in the control and HFFD groups after 12weeks. Each bar represents the mean ± SD, n=6. (*) significant difference from control, $p < 0.001$.

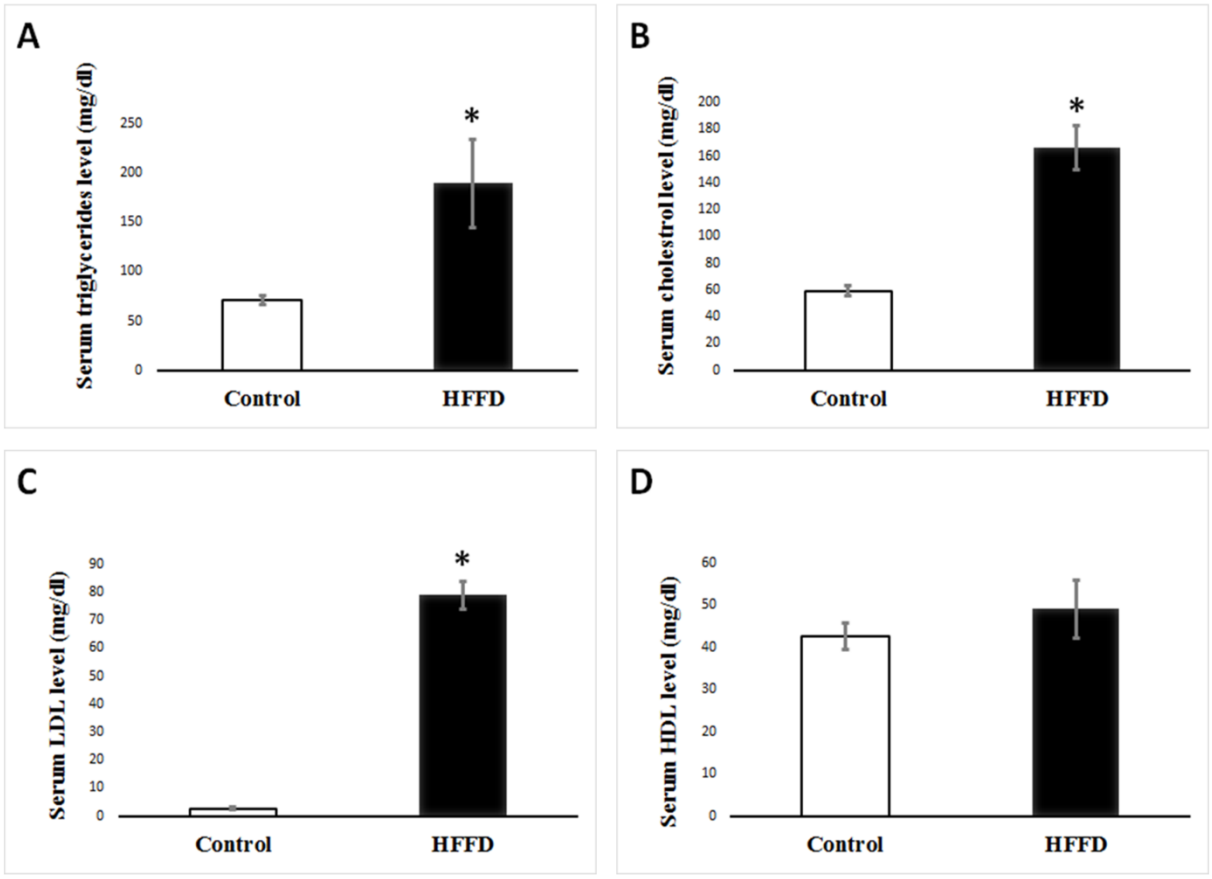

Figure 2: Effect of high fat fructose diet on (A) the triglycerides, (B) cholesterol, (C) LDL and (D) HDL serum levels in the control and HFFD groups after 12weeks. Each bar represents the mean ± SD, n=6. (*) significant difference from control, $p < 0.001$.

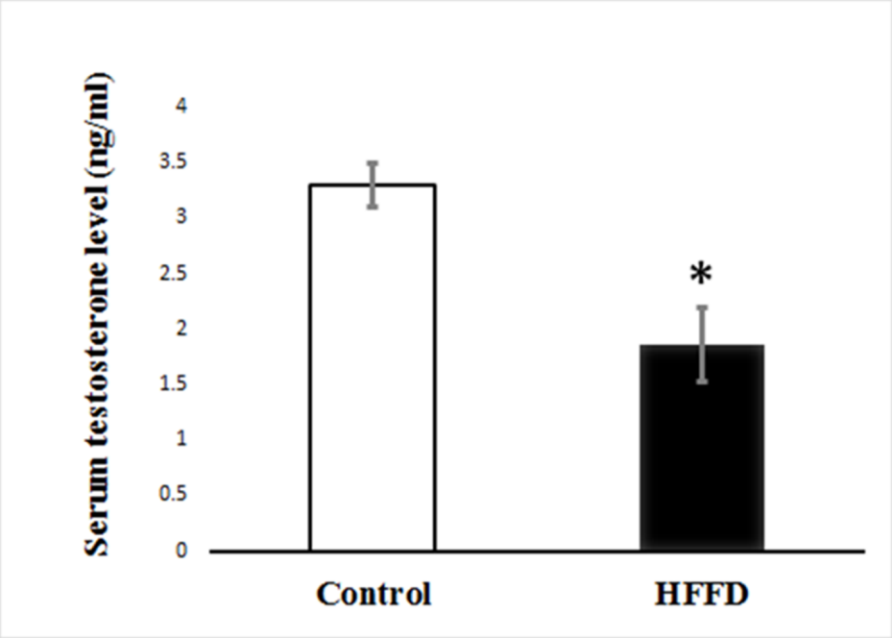

Figure 3: Effect of high fat fructose diet on the serum testosterone levelin the control and HFFD groups after 12weeks. Each bar represents the mean ± SD, n=6. (*) significant different from control, $p < 0.001$.

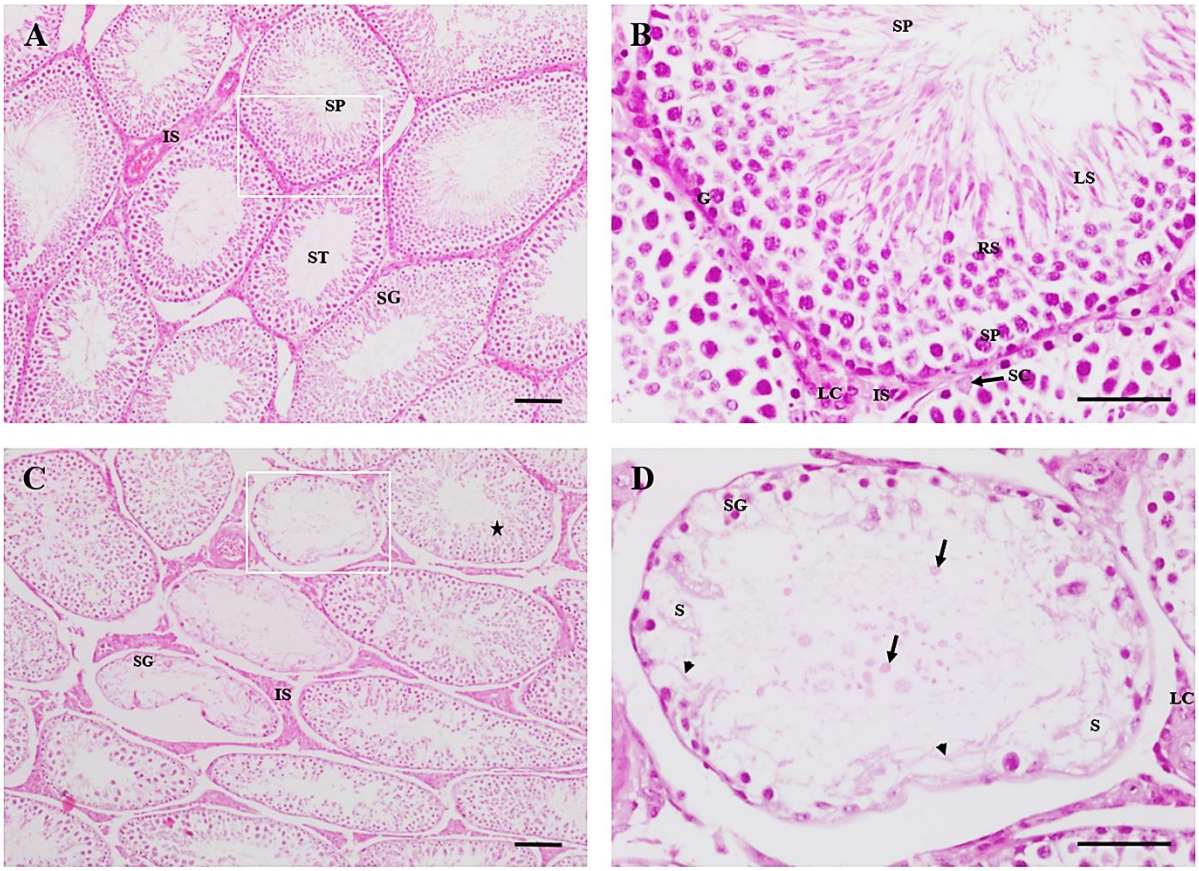

Figure 4: Photomicrographs of H&E-stained sections in the testes of **(A)** control adult albino rat, showing seminiferous tubules (ST) which appear circular or slightly oval in cross section and lined by multiple layers of spermatogenic cells (SG) and have lumen contains spermatozoa (SP). The tubules are separated by interstitial tissue (IS**). (B)** A higher magnification of (A) showing the basal layer of the seminiferous tubules is formed of

dark spermatogonia (G) and light Sertoli cells (SC). Large primary spermatocytes (SP) appear next to spermatogonia layer, then round or early spermatids (RS) and elongated or late (LS) spermatids and finally spermatozoa (SP) close to the lumen . The tubules are separated by interstitial tissue (IS) containing Leydig cells (LC); polyhedral cells with ovoid nucleus**. (C)** HFFD testis showing distortion of some seminiferous tubules with depletion of most germinal epithelium (SG) and wide empty lumen and an apparent increase in the interstitial tissue (IS). Some tubules appear normal (*). **(D)** A higher magnification of (C) showing one tubule with few spermatogenic cells (SG). Sloughed cells which appear without nuclei (black arrows) are seen in the wide lumen. There are wide empty spaces that may indicate degenerated germ cells (S) in between the cytoplasmic processes of Sertoli cells (arrow heads). Leydig cells are smaller in size (LC). (A&C x100, scale bar, 100µm, B&D x400, scale bar, 50µm).

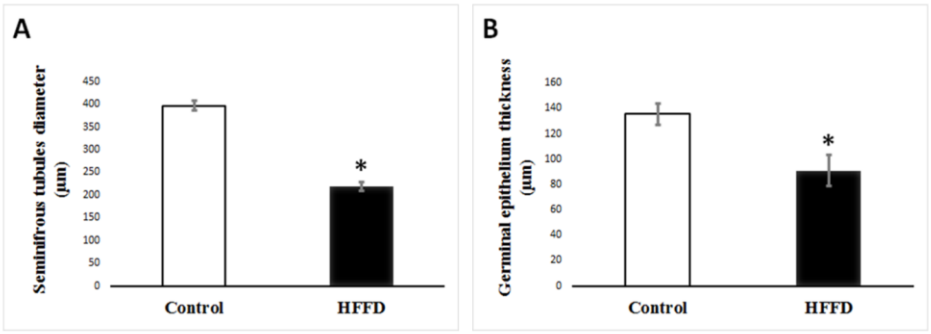

Figure 5: Effect of high fat fructose diet on (A) the seminiferous tubules diameter **(µm)** and (B) the germinal epithelial thickness **(µm)** in the control and HFFD groups after 12weeks. Each bar represents the mean ± SD, n=6. (*) significant difference from control, $p < 0.001$.

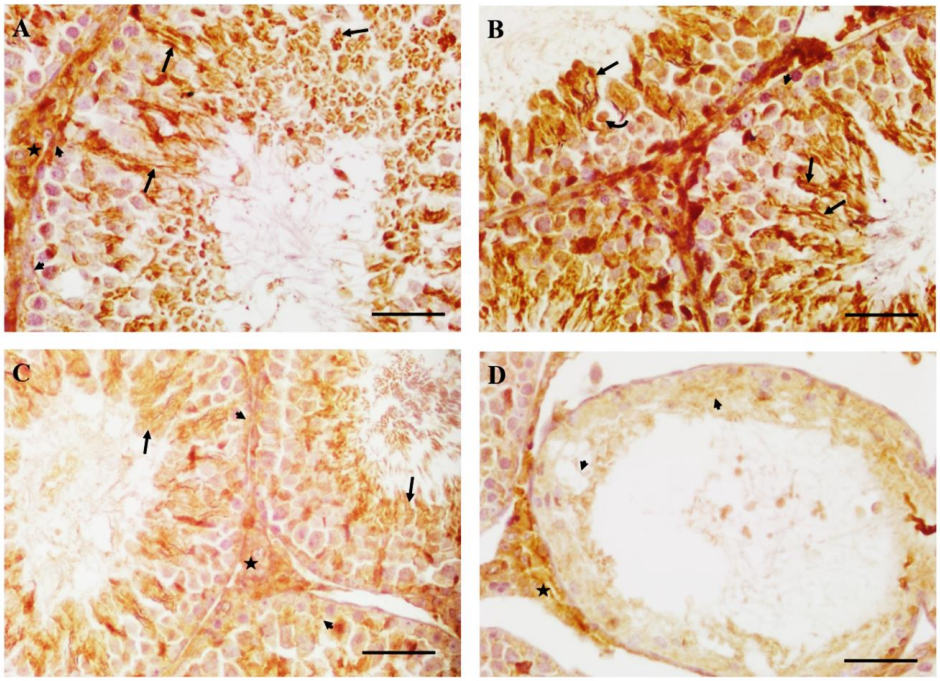

Figure 6: Immunohistochemical localization of aquaporin 8 (AQP8) in the rat testis. Representative photomicrographs of rat testes of the (A&B) control group showing strong positive anti-AQP8 antibody expression in the elongated spermatids and residual bodies (long arrows), some round spermatids (curved arrow) and cytoplasmic processes of Sertoli cells (short arrow) in between germ cells. Leydig cells (*) show also immune reaction. (C&D) HFFD group show moderately positive anti-AQP8 antibody expression mainly in spermatids (long arrows) and faint positive reaction in Sertoli cells (short arrows). Leydig cells (*) show also less intense positive immune stain (x400, scale bar, 50µm).

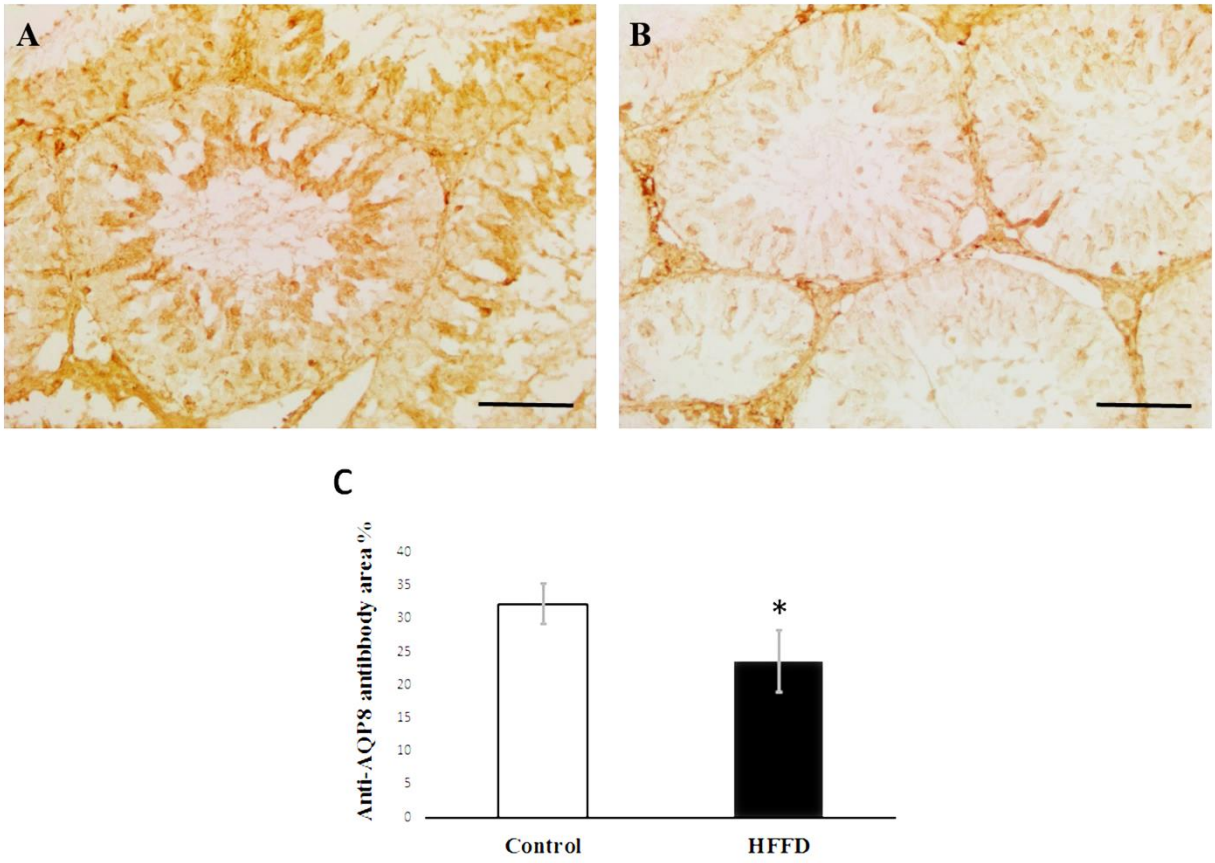

Figure 7: (A), (B) Representative photomicrographs of rat testes immunohistochemically stained with anti-AQP8 antibody without hematoxylin counter stain (x200, scale bar, 100µm) that were subjected to image analysis to measure area percentage of the immune positive reaction of (A) control group and (B) HFFD group. (C) Area percentage of the anti-AQP8 antibody immunohistochemical expression in the testes of the two groups. Each bar represents the mean ± SD, n=6. (*) significant difference from control, $p < 0.001$.

DISCUSSION

Previous studies indicated that MetS would be induced successfully by the high-fat and fructose diet (HFFD) model because the combination use of both gradients would have a synergic effect [32]. This is demonstrated by increased abdominal fat deposition, impaired glucose tolerance, dyslipidemia, and increased systolic blood pressure in rats [33], and thus mimetics what is called the American Lifestyle-Induced Obesity Syndrome [34]**.** This was evident in this study, HFFD for 12 weeks resulted in progressive increase in body weight, hyperglycemia, hypoinsulinemia and hyperlipidemia and fatty liver. Hyperlipidemia is signposted by high levels of serum TC, TG, and LDL and a low level of HDL. Ratios of TG/HDL, LDL/HDL and TC/HDL were significantly increased in HFFD group relative to the control group. The ratio of TG/HDL-C is an indicator of insulin resistance and is a predictor of type 2 diabetes and NAFLD [35].

Tooke & Hannemann [36] said that MetS is initially associated with hyperinsulinemia to maintain blood glucose level, later compensation eventually fails, leading to a decrease in insulin levelswhich was observed in this work.

Metabolic syndrome has been linked with male sexual dysfunction, hypogonadotropichypogonadism but relation to infertility is not clear [10]. Most animal models of obesity induced by high fat diet showed reduction of semen quality and reproductive performance [12-15].

In the current study, there was a significant decrease in testosterone level, sperm count and motility and a significant increase in abnormal

sperm morphology in HFFD group. The decreased testosterone level and sperm count in high fat diet may be attributed to increased production of oxygen free radicals and oxidative stress that inhibit steroidogenesis by Leydig cell [37].

Histological examination of the testis of HFFD group confirmed histopathological changes in many seminiferous tubules which appeared distorted with depletion of germinal epithelium and partially reduced sperms and exfoliation of degenerated spermatogenic cells in many tubules. The diameter of seminiferous tubules and germinal epithelial thickness were decreased in the testis of HFFD rats. In agreement with these findings, Jarvis et al.[38] demonstrated a reduction in Sertoli cells and germ cell numbers with effects on meiotic indexin the chronic HFD mice. They attributed these effects to blood-testis barrier (BTB) disruption caused by elevated plasma levels of cholesterol [39] and reduced testosterone levels. The latter would affect androgen receptor and tight junction proteins [14].

The development of male and female gametes is a sum of multiple complex processes in which water regulation is essential [40]. Water homeostasis is pivotal for spermatogenesis [41] and sperm motility [42]. Many aquaporins (AQPs) have been reported to play roles in testicular water homeostasis. In the seminiferous tubules, the expression of AQP7, AQP8 and AQP9 has been established [23,43].

In this study, AQP8 immunostain was observed in the cytoplasm of Sertoli cells and spermatids (round and elongated). Previous studies showed that AQP8 immunostain in the rat was localized in the ramified cytoplasm of Sertoli cells (SCs) between spermatogenic cells [22] or along the adluminal segment of SCs plasmatic membrane [23]. However, other studies described immune expression in the spermatocytes [24] or throughout spermatogenesis [25]. Aquaporins (AQPs7&8) present in SCs might create water movement crucial for spermatogenesis and liberation of mature sperm cells into the seminiferous tubules [22]. Aquaporin8 expression in rat elongated spermatids may highlight the participation of AQP8 in the transformation of round spermatids into spermatozoathrough cytoplasmic condensation [25].

It is well known that MetS is associated with increased reactive oxygen species production [44]. Medraño-Fernandez et al. [45] found that H2O2 has opposite roles in cells, magnifying growth factor signaling in physiological conditions, but excess can induce cell damage. The peroxiporin AQP8 can be considered as a compensatory protein for H2O2 detoxification. This was proved in theAQP8 knock down β-cells which displayed higher sensitivity to ROS as a result of elevated H2O2 concentrationin mitochondrialeading to toxicity[46]. Noteworthy, oxidative stress conditions inhibit AQP8-mediated H2O2 scavenging, and that, in a vicious circle, contribute to worsening the condition [47]. Similarly, we can suggest that the reduction of AQP8 protein may result from increased oxidative stress in MetS induced by HFFD. The reduction of AQP8 reduces the protective effect on Sertoli cells and germ cells especially the developing spermatids and partially contribute to the reduced sperm quality as observed in this study. This needs further investigation as AQP8-null mice were fertile with normal sperm count and morphology[41].Probably, AQP7 might functionally compensate for AQP8 in these transgenic animals [48].

CONCLUSIONS

In the present study, a decreased AQP8 immunostain expression was established in the high fat and fructose diet rats which might partially contribute to the reduced sperm quality. Other factors might also contribute to the changes in sperm and seminiferous tubules morphology like hormonal changes, hyperlipidemia and hypoinsulinemia observed in this study.

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