Research article

Adiponectin and the expression of BAX and caspase 3 in high-fructose - induced testicular injury in albino mice

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ABSTRACT

Introduction and Aim: The increase in the prevalence of obesity and metabolic syndrome in recent decades has been correlated with high consumption of high-fructose and high-fat diets and has been associated with increased rates of male infertility. The aim of this study was to investigate how high fructose diet exerts its effect upon testicular morphology in addition to examine the potential effects of adiponectin treatment in restoring the architecture of seminiferous tubules through the expression of immunohistochemical markers BAX and caspase-3.

Materials and Methods: Twenty-five adult albino mice were divided into three groups: In Group 1, mice fed with diet contained high concentration of fructose followed by adiponectin injection, Group 2, the mice fed with high concentration of fructose diet and received a saline placebo injection, and Group 3 (control) was nourished a regular food for 8 weeks. The parameters studied included changes in animal body weight, testicular spermatogenesis index, spermatogonia count, apoptotic index, exfoliative epithelium percentage and immunohistochemical scores for testicular BAX and caspase-3 expression.

Results: Animals on high fructose diet showed increase in body weight which was markedly reduced by adiponectin treatment. High fructose diet also resulted in reduced spermatogenesis index and spermatogonia count with increased apoptotic and epithelial exfoliation indices. High fructose diet was also associated with high-fructose induced obesity and significantly associated with increased BAX and caspase-3 expression alleviated by adiponectin treatment.

Conclusion: High-fructose intake induces obesity and obesity-related reduction in male fertility by reducing spermatogenesis and enhancing testicular cell apoptosis via different pathophysiological mechanisms. Such effects and mechanism can be reversed and corrected with adiponectin treatment.

Keywords: High-fructose diet; adiponectin; testicular tissue; infertility; BAX; caspase-3; apoptosis.

INTRODUCTION

Over the last decade, the consumption of high caloric soft drinks sweetened with sucrose and fructose has increased (1), contributing to an increase in obesity rates with high levels of serum cholesterol, free fatty acids and triglycerides (2), hypertension and metabolic syndromes (3). One of the main causes of metabolic syndrome is high fructose consumption as it increases lipogenesis, inflammatory reactions, endothelial dysfunction, and oxidative stress due to unregulated fructose metabolism (4).

Metabolic syndrome has been related to obesity and insulin resistance, contributing to testicular atrophy and hypogonadism (5). Replacement of testicular hormones has shown to improve metabolic syndrome by decreasing lipid indices, body weight, blood pressure and testicular atrophy (6). Additionally, obesity also affects the shape of the male testis and seminiferous tubules by lowering testosterone synthesis and sex hormone binding globulin. Further, fat deposition in testis up regulated the reactive oxygen species (ROS), considered as a material with high toxicity leading to DNA fragmentation and lipid peroxidation with subsequent damage to the spermatogenesis process (6,7).

Adiponectin, along with other adipokines such as free fatty acid, adipsin, leptin, resistin, and TNF (tumor necrotic factor), is a fat-derived hormone that is largely produced from white adipose tissue. Reduced adiponectin levels are accompanied by multiple obesity disorders like insulin resistance, type2 diabetes, atherosclerosis and hypogonadism (8). Adiponectin's exact physiological role is unknown, however it is thought to lower glucose, cholesterol, and free fatty acids, as well as have a part in metabolic syndrome etiology (9).

This study aimed to investigate the morphological and apoptotic changes induced in the seminiferous tubules on consuming high-fructose diet, as well as study the effect of adiponectin treatment on reversing these changes.

MATERIALS AND METHODS

Animals, diet and adiponectin treatment

Twenty-five adult albino mice (*Mus musculus*) aged 6-8 weeks and weighing of 23.7 grams in average was used in the study. The animals were bought from the Iraqi Center for Cancer and Medical Genetics Research and transported to the Department of Human Anatomy laboratory of Al-Mustansyiriah Medical College. Ethical approval was obtained from Al-Mustansiriyah Medical College Ethical committee. The weight of every mouse was obtained once at the starting and also obtained at the finishing of experiment using a digital scale (Diamond, China). The animals were maintained under controlled room temperature (22 ± 2C˚) and humidity (50% ± 10%) with 12:12 dark-light cycle, and fed with regular mouse feed (containing complex carbohydrates 39.5%, fat 4.4%, protein 25.2%, crude fiber 3.3% and ash 9.9%) and water *ad libitum*. High-fructose diet was prepared by dissolving 30g of fructose in 70 ml of distilled water and making up the volume to 100 ml (10). The animals were divided into three groups:

Group 1: HFD+A (High fructose diet + adiponectin) - ten mice were given regular chow and fructose water (30%; w/v) for 8 weeks. Following which they were injected daily with adiponectin (abcam® in form of histidine-tagged recombinant full length mature mouse adiponectin) intraperitoneally for a period of 7 days. A single daily intraperitoneal injection was given to each animal at a dose of 1.5 mg/kg (11)

Group 2: HFD (High fructose diet + placebo) - 10 mice were given regular chow and fructose water (30% w/v) for 8 weeks. This was followed by injecting each animal with 0.9% saline as placebo daily for one week. Group 2 served as control group to group 1(HFD+A)

Group 3: This was the control group wherein 5 mice were given regular chow and tap water for 8 weeks, followed by injection with intraperitoneal 0.9 % saline as placebo daily for a week.

Testicular histology and histomorphometry

At the end of 8th week of experiment the mice were anesthetized by injecting 50mg/kg sodium pentothal intraperitoneally, after which the abdominal cavity was opened and testicular tissue removed bilaterally. The testicular tissues fixed for one night in 10% neutral formalin and the dehydration was done in a series of graded ethanol after that the embedding was done with paraffin by using an autoprocessor (Leica®, Germany). After that sections of tissue of 4 μm thickness for H&E and immunohistochemistry staining by a microtome (Leica®, Germany), H&E staining slides were observed using a light microscope with digital camera connected to computer (Olympus, Tokyo, Japan). The stained slides were evaluated for overall seminiferous tubule histology.

Histo-morphometric evaluation was done using double blind method by two investigators. The spermatogenesis assessed by evaluating the cell layers number, cells types, and the existence of late spermatids in the seminiferous tubules. The spermatogenesis stage was graded as: 1-only spermatogonia present; 2 - spermatogonia and spermatocytes present; 3 - presence of spermatogonia, spermatocytes, and round (early) spermatids with ≤5 spermatid for one tubule, 4- presence of spermatogonia, spermatocytes, and round spermatids with ≤25 late spermatids for one tubule (12), the summation of grades over the number of seminiferous tubules examined (200 STs for each animal testis) was considered as spermatogenesis index. Exfoliation of cells, epithelium detachment from basal membrane of the tubule and the presence of vacuoles indicating disturbed spermatogenesis were also assessed.

Immunohistochemistry

For each sample two serial sections were selected, each with 4 micrometers thickness and placed on positive charge slides for staining with anti-BAX and anti-caspase-3 antibody (Santa Cruz® Biotechnology). The mouse specific primary antibody was detected using a HRP/DAB detection IHC kit (Abcam®, ab64261). After dewaxing using xylene and progressive rehydration, the retrieval of antigen was done by pressure cooking using citrate buffer for 20 minutes. The primary anti-BAX and anti-caspase-3 was diluted to a concentration of 1:200 using a reducing dilution buffer (Abcam, ab64211) and kept at room temperature for 30 minutes. Detection was achieved by means of labeled streptavidin biotin, followed by DAB and chromogen staining. The slides were briefly counterstained with hematoxylin and mounted by DPX (13). All tissues were evaluated blindly. Anti-BAX and caspase-3 staining showed distinct nuclear and cytoplasmic brown IHC staining. The positive and strong positive staining were additionally verified and confirmed by grading each slide from the low to high intensity and the spatiotemporal staining was also recorded for the markers. The stained slides were observed using a microscope at both low (10x) or high power (40x) to determine regions with highest staining. For each slide five fields were evaluated and scored semi quantitatively, the proportion of positive staining cells over the cells examined (%) (Total number of cells) were calculated and samples were classified according to the extent and intensity of staining (14). The score was calculated by multiplying the cells percentage with the intensity of staining. Indices were determined by counting the number of positive nuclei or cytoplasm among ≥200 STs in high-power fields and refer to be a percentages. The stained cells were

assessed for their intensity of immune-reactivity on basis of 0 to 3+ scales. The staining intensity and percentage was scored according to an earlier method (13) and the intensity scored based on a 4-point system: 0 for no staining, 1+ for weak, 2+ for moderate, 3+ for strong immune-reactivity. The staining extent percentage was scored as: 0 for no stain, 1= < 10% of cells stained, 2 = 10-50% cells stained, 3= >50% cells stained. The total IHS score was calculated by multiplying the scores obtained for intensity and percentage. Manually obtained scores for intensity and percentage was also crosschecked using computerized (Immunohistochemical Profiler Plugin and Macro) Image J method.

Statistical analysis

Data were plotted and analysed using Statistical Package for the Social Sciences version 24 and Microsoft office Excel 2016. Statistical significance was calculated at a confidence interval of 95% with a $p < 0.05$ being of statistical significance. Numerical data were presented as means ± standard deviation (SD) for hypothesis testing (t-test) while categorical data were presented as frequency and percentage for Chi-square testing.

RESULTS

The changes in body weights of mice over the experimental period are summarized in Table 1. As seen, the initial and final body weights of mice differed significantly among the three groups. A significant increase in weight was seen in mice belonging to Group2 as compared to that of Group1 and Group3.

Table 1: Body weight changes in animals over the experiment period

Body weight (g)	Group 1 HF+A	Group 2 HF	Group 3 Control	P value
Initial	20.6±1.5	23.4±1.3	26.3±0.7	0.001
Final	24.1±1.9	31.4±0.9	30.3±0.7	0.001
Change %	23.1±1.7	30.4±1.1	29.3±0.7	0.001

Data presented as mean ± standard deviation

Histo-morphometric parameters such as spermatogenesis index spermatogonia number, apoptotic index and exfoliative epithelium percentage evaluated for mice testicular tissue in the three groups is presented in Table 2. Regarding parameters of cellular division, the Group2 animals had the least spermatogenesis and spermatogonia index values. On the other hand, the mean apoptotic index and exfoliative epithelium percentage values were significantly higher in the HF group (Table 2). The histological changes are shown in Fig. 1.

A statistically significant difference in immunohistochemical marker staining scores was observed among the three groups (Fig. 2). Both BAX and caspase-3 scores were highest in the HF group and lowest in the control animals (Fig. 3).

Table 2: Histomorphometric evaluation of mice testicular tissue

Parameter	Group 1 HF+A	Group 2 HF	Group 3 Control	P value
Spermatogenesis index	3.2+-0.2	3.1+-0.05	3.8+ -0.21	0.001
Spermatogonia number	65+-4.2	35+-3.2	83+ -5.4	0.001
Apoptotic index	0.58+-0.079	0.6+-0.147	0.21+ -0.075	0.001
Exfoliative epithelium %	2.5±0.4	23±2.2	1 ± 0.06	0.001

(Data presented as mean ± standard deviation)

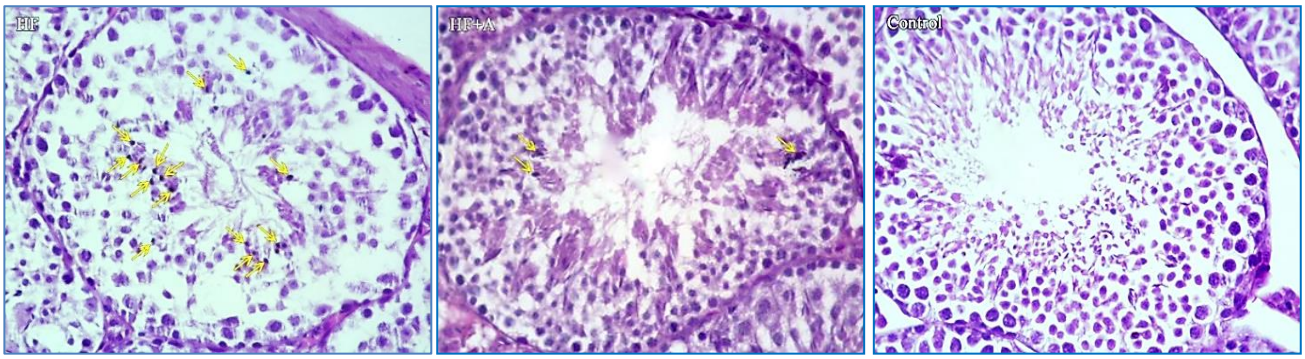

Fig. 1: Light photomicrographs of mice seminiferous tubules showing morphological changes induced by **a.** high fructose diet (HF): much epithelial exfoliation (tubule center) and numerous apoptotic cells (yellow arrows). **b**. High fructose fed animals that received adiponectin (HF+A) show less epithelial exfoliation and less apoptotic figures and are fairly comparable to **c**. control animals. (H&E 40 X).

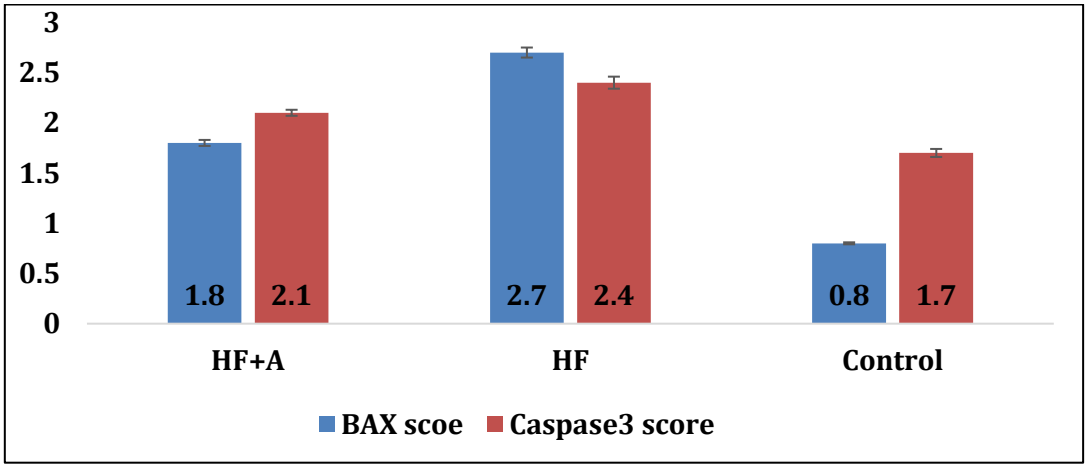

Fig. 2: Difference in testicular BAX and Caspase3 immunohistochemical scores for staining of testicular tissue in animals receiving high fructose diet (HF) with and without adiponectin (+A) and control animals. (Bars = mean, error bars= standard deviation.

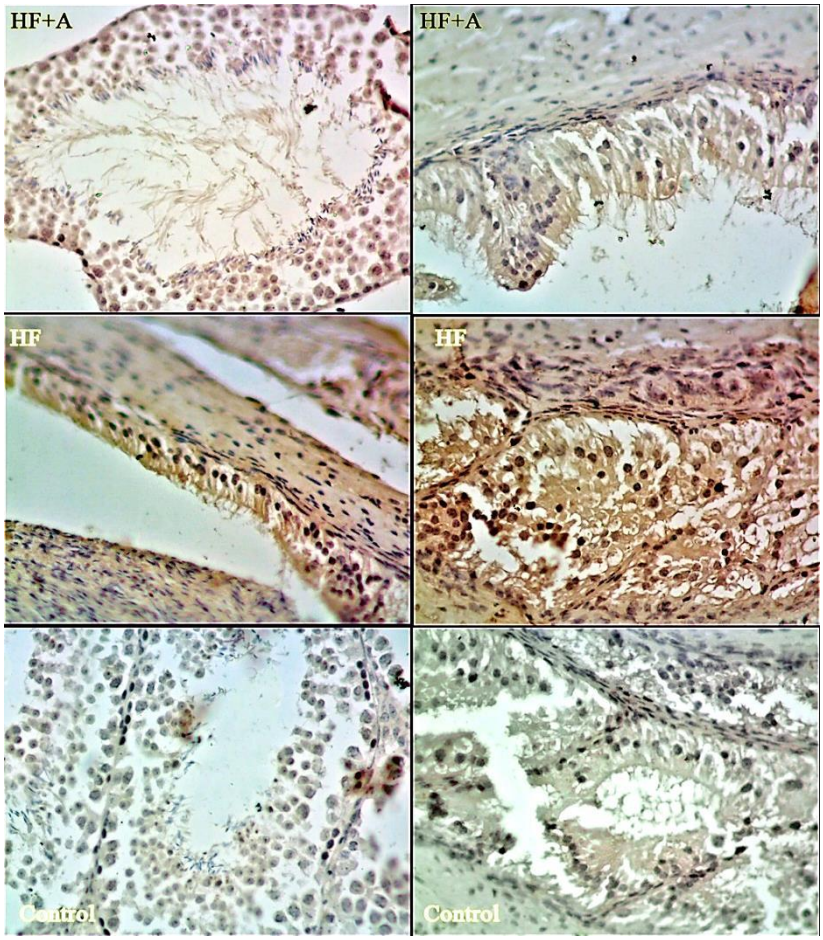

Fig. 3: Light photomicrographs of albino mice testicular tissue stained with BAX (left panel) and Caspase-3 (right panel) immunohistochemical markers in animals receiving high fructose diet (HF) with and without adiponectin (+A) and control animals (40x magnification).

DISCUSSION

High fructose consumption is directly linked to obesity and metabolic syndrome through different pathophysiological mechanisms that involve oxidative stress and inflammatory body response in addition to metabolic disturbances in brain and gut regulation of metabolism (15). The current study showed that adiponectin treatment was successful in limiting these adverse effects possibly by its insulin-sensitizing and anti-inflammatory effects (16). The increased prevalence of obesity in the past few decades has been associated with a proportional decline in male fertility (17). The current study showed that high fructose induced weight gain is associated with reduced spermatogenesis index and spermatogonia number with increased apoptotic and epithelial exfoliation indices. These effects may be related to the obesity-

induced production of reactive oxygen species (ROS) with subsequent spermatic DNA fragmentation and cellular death (18) and/or increased autophagy-activated apoptosis which leads to oligozoospermia and infertility (19). Previous *in vitro* studies showed that testicular treatment with adiponectin stimulated proliferation and survival of cells, and inhibited apoptosis of cells by augmenting transport of energetic substrates like lactate and glucose to keep cells from suffering apoptosis (20). Adiponectin-induced increase in intra-testicular concentrations of glucose and lactate enhances serum testosterone levels and improves the expression of testicular steroidogenic markers proteins (21). Apoptotic cell death is an essential cell process that employs the cell death receptor family signaling system with BAX and Caspase-3 being two important pro-apoptotic markers of this system (22). The current study showed that high-fructose induced obesity was associated with significantly increased expression of these two markers and that this increased expression was alleviated by adiponectin treatment. This is in agreement with several previous studies, wherein, an increased expression has been shown to be mainly related to increased oxidative stress with concomitant reduction in expression of anti-apoptotic marker Bcl-2 (23). Adiponectin has been shown to have both anti-apoptotic and pro-apoptotic effects in different issue and under different metabolic environments (24). High-glucose-induced apoptosis is reduced by adiponectin treatment, adiponectin also reduces inflammation in several cell types through AdipoR1 and R2 signaling mechanisms that modulate cellular responses to oxidative stress regardless of its controversial and tissue specific influence on BAX and caspase-3 expression (25).

CONCLUSION

High-fructose intake induces obesity and obesity-related reduction in male fertility by reducing spermatogenesis and enhancing testicular cell apoptosis via different pathophysiological mechanisms. Such effects and mechanism can be reversed and corrected with adiponectin treatment.

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CONFLICT OF INTEREST

There is no conflict of interest related to this article.

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