Zinc alleviates high fat diet-induced spermatogenic dysfunction in Wistar rats: role of oxidative stress, HMGB1 and inflammasome

Eman Hamed Elmorsy¹, Rania Gaber Aly², Noha Mohamed Badae³, Mona Mohamed Aboghazala⁴, Salma Samir Omar¹, *

¹Department of Dermatology, Venereology & Andrology, Faculty of Medicine, Alexandria University, 21521 Alexandria, Egypt
²Department of Pathology, Faculty of Medicine, Alexandria University, 21521 Alexandria, Egypt
³Department of Medical Physiology, Faculty of Medicine, Alexandria University, 21521 Alexandria, Egypt
⁴Ministry of Health Hospitals, 21527 Alexandria, Egypt

*Correspondence
salma.samir@alexmed.edu.eg
(Salma Samir Omar)

Abstract
Whether chronic inflammation in the genital tract induced by obesity shares in spermatogenic dysfunction is not clearly known. We aimed to study the effect of high fat diet (HFD) on spermatogenesis, seminal oxidative stress (malondialdehyde (MDA)) and inflammatory markers (high mobility group box 1 (HMGB1), nucleotide-binding oligomerization domain, leucine rich repeat and pyrin-3 domain containing (NLRP3)) in the rat testes and the role of zinc on testicular dysfunction and chronic inflammation in high fat diet (HFD) fed rat testes. This parallel group comparative experimental study included 36 male wistar rats divided into 3 groups: group A (fed on normal control diet); group B (fed on high fat diet (HFD) only); and group C (fed on HFD with zinc supplementation 3.2 mg/kg/day orally). At the end of the 12th week, sperm count, viability and motility were assessed by computer-assisted semen analysis (CASA), seminal malondialdehyde measured by calorimetry and histopathological examination of testicular sections was done. Immunohistochemical staining was done for HMGB1 and NLRP3 evaluation. Sperm count was lowest in group B. Groups A and C showed statistically higher means sperm vitality, total and progressive motility scores ($p < 0.001$), while no difference was found between the groups A and C ($p > 0.05$). Seminal malondialdehyde level was significantly highest in group B. Tubular diameter, epithelial height and Johnsen score were significantly lowest in group B. Significantly higher HMGB1 and NLRP3 levels were demonstrated in group B ($p < 0.001$). Obesity is associated with testicular dysfunction, testicular oxidative stress and increased testicular HMGB1 and NLRP3. We suggest a beneficial effect of zinc on testicular function in HFD-rats.

Keywords
Obesity; Male infertility; Inflammasome; HMGB1; Oxidative stress; Zinc
El zinc alivia la disfunción espermatozaina inducida por una dieta rica en grasas en ratas Wistar: papel del estrés oxidativo, HMGB1 y el inflamasoma

Resumen

No se sabe claramente si la inflamación crónica en el tracto genital inducida por la obesidad participa en la disfunción espermatozaina. Nuestro objetivo fue estudiar el efecto de una dieta alta en grasas sobre la espermatozaina, el estrés oxidativo seminal y los marcadores inflamatorios en los testículos de ratas y el papel del zinc en la disfunción testicular y la inflamación crónica en una dieta alta en grasas testículos de ratas alimentadas. Este estudio experimental comparativo incluyó 36 ratas wistar macho divididas en 3 grupos: grupo A (alimentadas con una dieta de control); grupo B (alimentados únicamente con una dieta alta en grasas); y el grupo C (alimentado con dieta alta en grasas con suplementación de zinc). Se evaluó el recuento, la vitalidad y la motilidad de los espermatozoides mediante un sistema análisis de semen asistido por computadora, se midió el malondialdehído seminal mediante calorimetría y se realizó un examen histopatológico de las secciones testiculares. Se realizó tinción inmunohistoquímica para la evaluación de (grupo de cajas de movilidad alta 1, inflamasona 3). Los grupos A y C mostraron puntuaciones medias estadísticamente significativas más altas de vitalidad spermatográfica, motilidad total y progresiva, mientras que no se encontraron diferencias entre los grupos A y C. El nivel de malondialdehído seminal fue significativamente más alto en el grupo B. El diámetro tubular, la altura epitelial y la puntuación de Johnsen fueron significativamente más bajos en el grupo B. Se demostraron niveles significativamente más altos de HMGB1 y NLRP3 en el grupo B. Sugerimos un efecto beneficioso del zinc sobre la función testicular en ratas HFD.

Palabras Clave

Obesidad; Infertilidad masculina; Inflamasoma; HMGB1; Estrés oxidativo; Zinc

1. Introduction

Obesity is known to induce a chronic inflammatory state. A chronic inflammatory state in the male genital tract secondary to obesity is proposed to participate in obesity-related impairment of spermatogenesis and male infertility [1]. It is suggested that oxidative stress could act as a bridge linking obesity and its complications [2]. Obesity-related factors as hyperglycaemia, hyperlipidemia and hyperinsulinemia contribute to increased oxidative stress which eventually accentuate the chronic inflammatory state in the body [3]. Obesity related elevated reactive oxygen species (ROS) and/or inflammatory mediators are therefore considered to be major causes of obesity complications such as infertility [2].

A growing body of evidence suggests that obesity is correlated with a local chronic inflammation in the male genital tract. High-mobility group box proteins are pro-inflammatory molecules belonging to a family of high mobility group family of proteins. There are four categories of HMG (1 to 4) with the high-mobility group box 1 (also known as HMGB-1, HMG1, HMG-1, HMG 1, amphoterin, p30) being the most represented. HMGB1 is considered a danger associated molecular pattern (DAMP) released into the surrounding tissues secondary to cellular damage or inflammation. Physiologically, HMGB1 acts to activate the immune response, however over expression of HMGB1 leads to damaging inflammation. HMGB1 is a “cytokine-type” RAGE ligand [4]. Geng et al. [5] demonstrated that in rats, HMGB1 activates the NLRP3 inflammasome through the receptor for advanced glycation end products (RAGE), accentuating liver inflammation and injury [5]. It has been reported that the inflammasome nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing-3 (NLRP3) could be associated with obesity and implicated in obesity-induced inflammation [4]. Activation of the inflammasome by damage or microbial or life style associated molecular patterns (DAMP, MAMP, LAMPs) or oxidative stress leads to caspase 1 activation, apoptosis, pyroptosis and release of inflammatory mediators [6, 7]. These were suggested to be associated with impairment of spermatogenesis [7, 8].

The results of a recent review showed that weight loss alone by bariatric surgery does not significantly improve seminal parameters in obese males [9]. The effect of weight reduction on fertility parameters in obese mice is controversial. Switching from a high fat diet to a normal diet in obese mice was shown to reduce body weight, decrease testicular oxidative stress, and improve testicular morphology and blood testes barrier integrity [10]. Nematollahi et al. [11], on the other hand showed that dietary intervention alone improved sperm motility only but did not improve testicular morphometry, sperm concentration or morphology. However, it is not clear if the multiple differences between HFD and normal diets, as higher carbohydrate concentration could account for observed changes [12].

Zinc was suggested to regulate RAGE expression and therefore reduce HMGB1 induced inflammation [4]. Data from literature support that that zinc could also serve as an anti-oxidative agent through inhibition of oxidation of DNA/ribonucleic acid (RNA) and other proteins thereby downregulating the inflammatory response and reducing further reactive oxygen species production [13].

We aimed to study the effect of high fat diet (HFD) on spermatogenesis, seminal oxidative stress (MDA) and inflammatory markers (HMGB1, NLRP3) in the rat testes and further evaluated the role of zinc supplementation on testicular dysfunction and chronic inflammation in high fat diet (HFD) fed rat testes.

2. Materials and methods
2.1 Sample size calculation

Based on a published research reporting that nano vitamin D3 treatment produced significant improvement of final testicular weight, testicular coefficient, epididymal sperm count, sperm motility and serum testosterone (greater than 30% compared to control group, \( p < 0.0001 \)) [14], the calculated minimal total hypothesized sample size was estimated to be 36 eligible rats (12 per group) to study the effect of zinc supplementation on testicular dysfunction in high fat diet fed rats. The study sample was calculated using Power Analysis and Sample Size Calculator (PASS 2020, NCSS, LLC. Kaysville, UT, USA) taking into consideration an alpha error of 5% and power of 80%.

2.2 Study design

This prospective, parallel group comparative experimental study was conducted on 36 male wistar albino rats (180–200 g, 8 weeks of age), obtained from the animal house of the medical physiology, Alexandria faculty of Medicine. All rats were maintained on standard conditions (natural dark/light cycle, controlled room temperature (25 ± 2 °C)). The ethical guidelines of Alexandria University for laboratory animals and the National Research Council’s Guide for the Care and Use of Laboratory Animals were adopted.

2.3 Experimental design

The adult male rats were randomly divided into 3 groups as follows: Group A (fed on normal control diet (CD); 12 adult male rats) and preserved under normal condition of diet and water; group B (fed on high fat diet (HFD) only; 12 adult male rats); and Group C (fed on HFD with zinc supplementation; 12 adult male rats).

The 36 rats were allocated to nine cages; four rats per cage. Oral gavage was started at 10 AM to about 12 AM daily.

The rats in control group were fed on standard diet prepared based on the composition of AIN-93G rodent diet containing 15% calorie of fat for 12 weeks. The rats in the other group were fed on high-fat diet (HFD) identical to standard diet, except for addition of 200 g lard fat/kg (45% calorie of fat) [15]. After a period of 8 weeks of obesity induction, obese rats (n = 24) were further randomly subdivided into two groups; (untreated HFD rats): (n = 12) maintained on the HFD only, and (HFD rats + zinc) group (n = 12) continued on HFD with zinc sulphate monohydrate supplementation (Sigma-Aldrich, St. Louis, MO, USA, 3.2 mg/kg/d) [16] for 4 weeks by oral gavage. The dose was re-adjusted every week based on the observed increase in body weight to maintain a constant dose per kg of rats over the entire study period.

2.4 Gonadal extraction

At the end of the 12th week, all rats were weighed and animal euthanization was performed under thiopental anaesthesia by cervical dislocation. The testes and epididymis were carefully dissected and removed. The weights of the testes were measured using an electronic balance. Then, the testicular coefficient was calculated according to the formula:

Testicular coefficient (gm/kg) = the weight of the testes (the mean of the weight of the two testes in gm)/final body weight (kg).

The caudal region of right epididymis in each rat was dissected out without fat tissue and cut into pieces in prewarmed (37 °C) 2 mL of Hank’s buffer salt solution (HBSS). Sperms were allowed to diffuse into the medium for 15 min at 37 °C, and then, sperm suspension was collected in a new Eppendorf tube. Finally, the sperm concentration, motility and progressive motility were assessed using a computer assisted semen analysis—Sperm Vision™ CASA System (MiniTüb, Tiefenbach, Germany) with Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan).

2.5 Seminal MDA evaluation

Seminal MDA levels were determined using the thiobarbituric acid method was measured colorimetrically at 534 nm according to Ohkawa et al. [17].

2.6 Histological examination

Testicular tissue was fixed in 10 % formaldehyde and embedded in paraffin blocks for histopathological examination. The testicular sections from all groups were stained with hematoxylin and eosin (H&E) and examined under the light microscope (DM300 binocular microscope, Leica, Wetzlar, Germany). Histological examination of all testicular sections for evaluation of the Johnsen’s score. A score from 1 to 10 was given based on the presence or absence of as spermatogonia, spermatids, spermatocytes, spermatogonia, germ cells and Sertoli cells. A higher Johnsen’s score indicates a better spermatogenesis, while a lower score denotes dysfunction. Score 1 signifies seminiferous tubules with complete inactivity while a score 10 means full epithelial maturation and tubules with maximum activity [18]. Following sacrifice, the liver and kidneys were excised and fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of these tissues were stained with hematoxylin and eosin for light microscopy for the histopathological examination to confirm the absence of toxicity.

2.7 Immunohistochemical (IHC) staining and interpretation

IHC of all sections was done using the Avidin-Biotin Peroxidase method. NLRP3 (A5652, polyclonal antibody, Abclonal, USA) and HMGB1 (A19529, polyclonal antibody, Abclonal, USA) using the fully automated immunostainer (Bond-MAX, Leica Biosystems, Durham, USA). In each IHC run, negative and positive controls were included in each run (according to the manufacturer’s datasheet). The quantification of the IHC in each slide in 10 high power fields (HPF), was done using ImageJ, v1.53 (Maryland, USA).

2.8 Statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 28.0 (IBM Corp, Armonk, NY, USA). The Shapiro-Wilk test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation, median
3. Results

3.1 Body weight and testicular co-efficient

The mean initial weight at the start of the study was 153.3 ± 11.44 g (128 to 168 gm) in group A, 158.8 ± 6.95 g (148–170 gm) in group B, and 161.4 ± 7.83 g (152–177 gm) in group C. There was no statistically significant difference among the three groups (p = 0.093). Mean body weight at the end of the study was 154.4 ± 11.07 g (135–170 gm) in group A, 295.6 ± 29.15 g (250–330 gm) in group B, and 283.6 ± 11.38 g (264–305 gm) in the group C. Comparing the initial weight to the final weight in each groups showed significantly higher weight in group B and C (p < 0.001). A statistically significant difference was found among the three groups regarding the weight at the end of the study (p < 0.001) (Table 1).

Concerning the testicular coefficient, group A showed the highest mean coefficient (22.43 ± 2.12), while group B showed the lowest mean coefficient (7.47 ± 0.91). Group C showed a mean testicular co-efficient of 10.13 ± 0.74 with a statistically significant difference between the three groups (p < 0.001).

3.2 Semen parameters

Mean sperm count was 30.40 ± 7.22, 13.68 ± 6.25 and 21.84 ± 9.46 in groups A, B and C respectively. Sperm count was significantly lower in group B compared to each of group A (p = 0.028) and C (p = 0.038). Regarding vitality, groups A and C showed significantly higher mean seminal plasma percentage (76.75 ± 12.92 and 70.42 ± 10.75, respectively) than the group B (39.76 ± 17.73) (p < 0.001), while no statistically significant difference was found between the groups A and C (p = 0.031).

Group B showed the lowest mean seminal motility (41.38 ± 16.50) compared to groups A (76.42 ± 16.06) and B (71.34 ± 12.50), while no statistically significant difference was found between group A and group C (p = 0.692). Likewise, groups A and C showed statistically significant higher mean values of progressive motility (52.33 ± 16.62 and 45.43 ± 15.45, respectively) than the group B (19.17 ± 12.22), with a p value < 0.001, and no statistically significant difference between group A and group C (p = 0.499). No significant difference was noted in the non-progressive motility (p = 0.534).

3.3 Seminal MDA levels

Group A showed the lowest mean seminal MDA levels (0.68 ± 0.32 nmol/mL), followed by group B (2.33 ± 0.36 nmol/mL), and highest in group C (1.09 ± 0.22 nmol/mL). This difference was statistically significant (p < 0.001) (Table 1).

3.4 Testicular histopathological parameters

Group A showed significantly greater mean tubular diameter (205.9 ± 8.51 µm) than group B (133.0 ± 9.92 µm) (p < 0.001), and group C (196.7 ± 6.83 µm) (p = 0.031). In addition, the mean tubular diameter in the group C was significantly higher than in group B (p < 0.001).

Group A showed significantly greater mean seminal plasma percentage (92.67 ± 4.74) compared to group B (40.0 ± 4.11) (p < 0.001), and group C (86.33 ± 5.05) (p = 0.006). Further, the mean epithelial height in group C was significantly higher than in the group B (p < 0.001).

The Johnsen score was significantly different among the three groups (p < 0.001), where group A showed significantly higher mean score (10 ± 0) than group B (5.0 ± 0.95) (p < 0.001) and group C (9.3 ± 0.65) (p = 0.049). The mean score in the group C was significantly higher than in group B (p < 0.001). (Table 1, Figs. 1,2,3)

3.5 Organ toxicity

Histopathologic examination of the kidneys and liver showed a preserved architecture in the three groups.

3.6 Testicular immunohistochemical parameters

There was significant elevation in the high mobility group box protein (HMGB1) in group B (106.92 ± 14.05) compared to group A (9.67 ± 1.56, p < 0.001), and group C (12.33 ± 3.82) (p < 0.001), while no significant difference was found between groups A and C (p = 0.722).

Similarly, nucleotide-binding oligomerization domain (NOD)-like receptor family, pyrin domain-containing 3 (NLRP3) was significantly elevated in the group B (111.92 ± 11.77) compared to the group A (11.75 ± 1.76) (p < 0.001), and group C (14.67 ± 3.89) (p < 0.001), while no significant difference was found between group A and the group C (p = 0.59) (Table 1, Figs. 1,2,3).

4. Discussion

Obesity is modern life’s global health problem linked to the sedentary lifestyles and the popular high caloric diets rich in simple sugars and fat [19]. Studies have shown that about 80% of men presenting to fertility clinics are classified as either overweight or obese [20]. It is believed that obesity is associated with chronic low-grade inflammatory state that leads to the generation of oxidative stress and accentuation of inflammation [21, 22]. Never the less, little is known about the relationship between these alterations and the factors that modulate or determine reproductive health in obese individuals.

The present study showed that consumption of high fat diet
Several mechanisms were proposed to share in obesity-related tubular damage and a reduction in seminiferous epithelial height and function in the HFD fed rats. In agreement, Ibáñez et al. [23], Erdemir et al. [24], and Liu et al. [25] reported that HFD led to an increase in the number of abnormal seminiferous tubules and a reduction in seminiferous epithelial height and seminiferous tubular diameter in adult rats exposed to HFD. Several mechanisms were proposed to share in obesity-related induced significant alteration to testicular morphometric and histopathological parameters manifested as significantly lower testicular coefficient, tubular diameter, epithelial height and Johnsen score was shown in group B compared to the other two studied groups. Our findings denote the elicited cellular pathwayshavenotbeenfullyexplained inflammatory responses however, of them male reproductive tract, and the inflammation and oxidative stress inter-related pathophysiological phenomena. The unique inflammatory responses however, of the male reproductive tract, and the elicited cellular pathways have not been fully explained.

<table>
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<tr>
<th>Table 1. Biochemical, histopathologic and immunohistochemical characteristics of the three studied groups.</th>
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<td><strong>Group A</strong> (n = 12)</td>
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<td>Seminal MDA (nmol/mL)</td>
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<td>HMGB1 positive cells/HPF</td>
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<td>NLRP3 positive cells/HPF</td>
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Group A: rats on normal diet, Group B: rats on high fat diet only, Group C: rats on high fat diet with zinc supplementation.
SD: Standard deviation; HPF: high power field; MDA: malondialdehyde; HMGB: high mobility group box 1; NLRP: nucleotide-binding oligomerization domain, leucine rich repeat and pyrin-3 domain containing.

*: One way ANOVA test, Pairwise comparison bet. Each 2 groups was done using Post Hoc Test (Tukey).

p: p value for comparing between the studied groups.

p0: p value for comparing between Initial and Final in each group.

p1: p value for comparing between Group A and Group B.

p2: p value for comparing between Group A and Group C.

p3: p value for comparing between Group B and Group C.

*: Statistically significant at p ≤ 0.05, a: Significant with Group A, b: Significant with group B.

We demonstrated that group B HFD fed rats showed the highest seminal MDA levels. L-malondialdehyde is a byproduct of the oxidative degradation of lipids in cell membranes, and the change in MDA concentration is therefore used as an index of oxidative cell damage [27]. In agreement with our findings, El Kattawy et al. [14] demonstrated increased testicular MDA levels in HFD fed rats. This supports that obesity is associated with ROS accumulation and oxidative stress. It is well-known that inflammation and oxidative stress inter-related pathophysiological phenomena. The unique inflammatory responses however, of the male reproductive tract, and the elicited cellular pathways have not been fully explained.
FIGURE 1. **Control group showing.** (A) normal tubular diameter, epithelial height and Jhonson score. (B) with low HMGB-1 and NLRP3. Sg: spermatogonia, Se: Sertoli cell, Sc: spermatocyte, es: elongated spermatid, rs: round spermatid, s: spermatozoa.

FIGURE 2. **High fat diet fed group showing.** (A) decrease tubular diameter, epithelial height and low Jhonson score. (B) with increase interstitial space as well as increased HMGB-1 and NLRP3. Sg: spermatogonia, Sc: spermatocyte, s: spermatozoa.
High mobility group box protein 1 (HMGB1) is a nucleosomal proinflammatory signal unleashed by activated immune cells and necrotic cells, triggering robust inflammatory events including excessive production of pro-inflammatory cytokines [29]. The involvement of testicular pro-inflammatory HMGB1 in the pathology of autoimmune orchitis in rat models and in infertile males has been previously demonstrated [29]. However, its role in HFD-testicular injury has not been previously explored. It is known that oxidative stress is a central regulator of HMGB1’s translocation, release and activity in inflammation and cell death [30]. The present study showed significant elevation in testicular HMGB1 levels in group B compared to groups A and C suggesting that HMGB1 may play a role in HFD induced testicular injury.

In the context of inflammatory events, inflammasomes were also suggested to play a central role in the pathophysiology of testicular damage [31]. By responding to several cell stressors such as damage signals as HMGB1 and reactive oxygen species (ROS), the NLRP3 inflammasome can be assembled and activated, resulting in activation of the pro-inflammatory cytokines [31, 32]. In this work, we found that NLRP3 level was significantly elevated in the group B compared to both groups A and C, while no significant difference was found between group A and the group C. Mu et al. [33] similarly demonstrated an increased expression of testicular NLRP3 in obese mice. They suggested that increased NLRP3 promoted obesity-related impaired spermatogenesis, testicular histopathological changes and decreased sperm count and motility which were improved by NLRP3 depletion. This suggests that HFD-induced could participate in the upregulation of NLRP3.

Zinc (Zn) is an important co-factor for over 300 enzymes as RNA polymerases, alcohol dehydrogenase, carbonic anhydrase and alkaline phosphatase. It has been previously demonstrated that zinc is important to maintain sperm membrane integrity. The sperm nucleus and chromatin contain high concentrations of zinc. Zinc is important for cellular DNA synthesis, cell proliferation and immunocompetence [34]. Obese subjects were suggested to have lower serum zinc levels which was related to contribute to systemic oxidative stress and inflammation [35]. Zinc is also known to be an important anti-inflammatory factor and plays an important role in the sperm’s oxidative metabolism [36]. The role of zinc supplementation in improving fertility in obesity-relates testicular dysfunction hasn’t been sufficiently studied.

Group C HFD + zinc fed rats showed significantly better testicular morphometric and histopathologic parameters (testicular coefficient, Johnsen score, epithelial height and tubular diameter). Similarly, sperm count and motility were significantly better in group C rats than HFD only fed rats. These suggest a potential beneficial role of zinc in HFD-related testicular dysfunction. Ma et al. [16] similarly reported that zinc sulphate supplementation improved testicular structure and spermatogenesis abnormalities [16]. Aziz et al. [37] showed that zinc supplementation alongside metformin provided additional benefits over metformin in improving testicular histopathologic changes, improving sperm quality, reduc-
ing testicular MDA and reducing testicular inflammation in diabetic rats.

The significantly better MDA levels in group C emphasizes the antioxidant activity of zinc [34]. Furthermore, the significantly lower HMGB1 levels in group C suggests an anti-inflammatory effect of zinc supplementation. The potent anti-inflammatory properties of zinc compounds has been proposed as the mechanisms of action of underlying such therapies effects of zinc [38, 39]. In harmony with our study, the study of Ooi et al. [40] reported that pretreatment with zinc compounds can inhibit the secretion of HMGB1 protein. Furthermore, the study of Siddiqui et al. [41], observed that HMGB1 cannot mediate proinflammatory activity at physiological pH and zinc concentrations. The authors attributed this to the zinc-dependent association of HMGB1 with sialoglycoproteins, thereby preventing its binding with proinflammatory receptors [42]. The anti-inflammatory effect of zinc was further suggested by the significant reduction of NLRP3 levels in group C rats that were fed on high fat diet and zinc. This agrees with the study of Summerrgill et al. [43], who reported that zinc deficiency was associated with NLRP3 inflammasome activation and the production of interleukin-1β (IL-1β) in macrophages. Also, Fan et al. [44], found that zinc treatment inhibited NLRP3 inflammasome activation by attenuating oxidative stress.

5. Conclusions

Our results are preliminary findings pointing to an association of obesity with testicular dysfunction, testicular oxidative stress and increased testicular HMGB1 and NLRP3. Our findings also suggest a beneficial therapeutic effect of zinc supplementation on testicular function in HFD-rats. Zinc sulphate supplementation is associated with decreased testicular oxidative stress and HMGB1 and NLRP3 inflammasome expression. Further studies are needed however to confirm the role of HMGB1 and NLRP3 in HFD-related testicular dysfunction. Zinc sulphate supplementation can be considered an effective and safe option to improve spermatogenesis and thereby fertility in obesity-related subfertility.

AVAILABILITY OF DATA AND MATERIALS

Data is available upon reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

EHE—supervised the work and participated in writing the manuscript. RGA—performed the histopathological assessment. MMA—supervised the animal house and experiment. SSO—carried out the experiment and writing the manuscript. MMA—supervised the work and analyzed the results.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of the institutional ethical committee was obtained (IRB NO: 0007555-FWA NO: 00018699, approval #0107067).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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