



Research Article

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## ***Repairing of the Distorted Hyperlipidaemic-Inflamed Testis and Gonads' Hormonal Axis of Rats Fed with a High-Fat Diet by Nigella Sativa***

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### ABSTRACT

*Objective: The goal of the current project was to identify the therapeutic benefits of Nigella sativa (NS) on enhancing the fertility of obese male rats. Methods: Rats were distributed into three groups: controls which were fed by a normal diet (ND); the group which was fed by a high-fat diet (HFD); besides, the group which was fed by HFD, then supplemented with NS. Gonadal Hormones plasma levels were investigated by ELISA. Inflammatory cytokines IL-6, IL-1 $\beta$  and IL10, were investigated by Multiplex Assays. The animals were sacrificed after eight weeks, and their testes were extracted for histology. Results: Testosterone levels in the HFD group were significantly lower than from the control group. Moreover, the HFD group treated with NS seeds showed significantly increasing levels of testosterone compared to the group treated with HFD only. The luteinizing hormone levels were unchanged amongst three groups. However, the follicle-stimulating hormone levels were the lowest in the HFD group compared to the other study groups. Cytokines were significantly raised in the HFD group compared with the controls. However, the rats treated with NS seeds showed lower cytokines than the group treated with HFD only. The gonads of HFD group showed disrupted seminiferous tubules, degenerated Leydig cells, and atypical spermatogenesis. Interestingly, the HFD group treated with NS restored the normal testicular structure, similar to the control group. Conclusion: NS could improve the reproductive efficiency in the abnormally distorted hyperlipidaemic-inflamed reproductive tissues in obese male rats.*

**Key words:** Obesity, Men Fertility, Hyperlipidaemia, Inflammation, spermatogenesis, Nigella Sativa.

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### INTRODUCTION

Hyperlipidemia refers to elevated levels of lipids and cholesterol in the blood, and it is a condition of excess fatty substances called lipids, largely cholesterol and triglycerides in blood [1], and obesity is life-threatening for most comorbid diseases, and is considered a critical cause of male infertility [2], it has been defined as excessive fat accumulation in adipose tissue leading to impaired health [3]. Obesity has been considered a worldwide concern over the past 20 years [4]. The pathogenesis of obesity is complicated by systemic inflammation and metabolic disarrays [5-7]. Taken together, their physiological disturbances lead to many organic dysfunctions, including testis.

Multiple studies have evidenced that the pro-inflammatory state is strongly associated with human obesity [8, 9]; parallel relations have been shown in animal models [10]. In testis, the pro-inflammatory cytokines, particularly TNF- $\alpha$  and IL-1, enter, from circulated blood, during systematic inflammation, and can directly damage the seminiferous epithelium, interrupt the critical regulation steps in spermatogenesis, and perturb the assembly of the junctional proteins of Sertoli cells [11]. In infertile men, the presence of high seminal reactive oxygen species (ROS) is associated with low sperm concentration, reduced sperm motility, dysmorphology and high DNA fragmentation [12].

The distraction of the negative-feedback cycle at the hypothalamic-pituitary-gonadal axis has been observed in obese men. The aromatase enzyme produced from their abundance of white adipose tissue converts androgen into oestradiol [13]. Subsequently, oestradiol inhibits gonadotrophin, follicle-stimulating hormones (FSH) and luteinizing hormones (LH) produced by the pituitary gland, and this results in further declines of testosterone production, particularly as an effect of LH inhibition [14]. The low FSH and testosterone levels in obese men might lead to impaired spermatogenesis and other fertility functions [14]. Therefore, sexual hormonal imbalances might remain a significant reason for male infertility fortified by obesity [13].

Both clinical and animal scientific reports have indicated that alterations in the fatty acid structure of sperm remain one of the pathways essential for decreased sperm quality of obese males. It has been shown that the substantial elevation in spermatozoa cholesterol composition in obese men is postulated toward sperm morphological anomalies, diminished motility and premature acrosomal-reaction [15].

Recently, there has been a particular interest in natural substances identified from herbal compounds [16]. *Nigella sativa*; *N. sativa* (NS) is a medicinal plant well-known, over the centuries, for its wide spectrum of pharmaceutical properties [17, 18]. Intensive research has been conducted to characterise its pharmacological aspects, concluding that it has the properties of an immunomodulatory, an anti-inflammatory, an antioxidant, an anti-dyslipidaemia, an analgesic, a bronchodilator, a diuretic, a spasmolytic and an antimicrobial agent [18]. Most of these properties allow this plant to have therapeutic potential in different diseases, including rheumatoid arthritis, hyperlipidaemia, asthma, hypertensive disease and gastrointestinal disorders [18].

The current study sought to determine the therapeutic benefits of NS on enhancing the fertility of obese male rats through regulating systemic inflammation, hormones and spermatogenesis functions.

## MATERIALS AND METHODS

### Animals

Fifteen adult albino Wister male rats, aged eight weeks, weighing 160-200gm, were kept in well-ventilated cages in an animal house, and maintained at a stable temperature of  $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$  per a 12h-bright/12h-dark cycle and  $55\% \pm 10\%$  humidity. This study was led according to the guidelines for dealing with experimental animals of the Biomedical Ethics Research Committee (Reference No 335 16).

### Rats Dietary Feeding and Weighing

The animals were randomly divided into the following three sets of five rats.

- 1) Group 1: the control, which was fed with a standard, normal diet (ND).
- 2) Group 2: fed with a high-saturated fat diet (HFD) at a dose of 6ml/day for eight weeks.
- 3) Group 3: fed with an HFD for four weeks, then supplemented with *Nigella sativa* (NS) seed powder at a dose of 300mg/kg/day for the last four weeks.

The HFD included: 20% saturated fat (butter) purchased from local markets in Jeddah, KSA, 2% cholesterol and 0.5% bile acid salts.

The black cumin seeds of *Nigella sativa* were purchased from the Nahdi Medical Company, Jeddah, KSA. The seed powder was dissolved in freshly prepared distilled water and given to the animals by intragastric intubation, as in previous research studies [19, 20].

The body weights of the rats were recorded on the first day of the experiment, after four weeks (before beginning the NS treatment), after five weeks, and after eight weeks on the day of sacrificing (Table 1).

**Table 1:** Weight changes

	G1=ND	G2=HFD	G3=HFD+NS	P value
Weight at Day 1(g)	181.6 $\pm$ 12.5	190 $\pm$ 12.9	174.8 $\pm$ 5.5	NS
Weight at Day 21 (g)	197.4 $\pm$ 3.3	244.4 $\pm$ 19	231 $\pm$ 21	G1 vs G2= 0.002 G1 vs G3= 0.02 G2 vs G3=0.44
Weight at Day 35 (g)	229 $\pm$ 15.4	283.6 $\pm$ 20.4	255.2 $\pm$ 18	G1 vs G2= 0.006 G1 vs G3= 0.3 G2 vs G3=0.03

**Abbreviations:** Group 1(G1): normal diet (ND) rats, Group 2(G2): high fat diet (HFD) rats, and Group3 (G3): high fat diet plus *Nigella sativa* (HFD+NS) rats. Values are expressed as mean  $\pm$  standard deviation. Statistical significance was determined by one-way ANOVA (n = 5).

### Biochemical analysis

Blood samples were collected from the rats one day before sacrificing to assess laboratory parameters related to dietary induction, including lipid profiles, glucose levels and insulin levels (Table 2).

**Table 2:** Laboratory parameters related to diet induction

Variables	G1=ND (n = 5)	G2=HFD (n = 5)	G3=HDF+NS (n = 5)	P-value
TG (mg/dl)	72 $\pm$ 11.3	107 $\pm$ 13.2	68.5 $\pm$ 12	G1 vs G2= * G1 vs G3= ns G2 vs G3=**
CHOL (mg/dl)	137 $\pm$ 2.6	212 $\pm$ 2.9	101.5 $\pm$ 2.1	G1 vs G2= * G1 vs G3= ns G2 vs G3=**
LDL	94 $\pm$ 14	151 $\pm$ 8.4	57.5 $\pm$ 9	G1 vs G2= * G1 vs G3= ns G2 vs G3=***
HDL	29 $\pm$ 7.6	40 $\pm$ 9.3	31 $\pm$ 8.4	G1 vs G2= * G1 vs G3= ns G2 vs G3=**
Insulin (IU/l)	3.67 $\pm$ 1	1.3 $\pm$ 0.6	3.42 $\pm$ 0.8	G1 vs G2= * G1 vs G3= ns G2 vs G3=**
Glucose (mg/dl)	89 $\pm$ 11	197 $\pm$ 14	123 $\pm$ 8	G1 vs G2= * G1 vs G3= ns G2 vs G3=*

**Abbreviations:** Group 1(G1): normal diet (ND) rats, Group 2(G2): high fat diet (HFD) rats, and Group3 (G3): high fat diet plus *Nigella sativa* (HFD+NS) rats. Values are expressed as mean  $\pm$  standard deviation. Statistical significance was determined by one-way ANOVA (n = 5); \*\*\* $p$  < 0.0001, \*\* $p$  < 0.001, \* $p$  < 0.01, ns, not significant.

### Enzyme-linked immunosorbent assay

Rat testosterone, LH and FSH plasma levels were investigated by ELISA kits from MyBioSource.com, with the catalogue numbers MBS9424769, MBS729873 and MBS2502190; respectively. The assays followed the instructions from the manufacturer's instruction sheets.

### Bead-Based Multiplex Assays

Inflammatory cytokines Interlukine-6 (IL-6), Interlukine-1 $\beta$  (IL-1 $\beta$ ) and Interlukine-10 (IL10), were investigated in rats' plasma by Bead-Based Multiplex Assays (Cat. No. RECYTMAG-65K) purchased from Millipore. The assays followed the manufacturer's instructions, and were subsequently analysed using the Luminex 200 System.

### Histological examination

The animals were sacrificed after eight weeks. The testis was extracted and soaked in a formalin fixative solution. Tissues were paraffin embedded, sectioned (4 $\mu$ m), and then stained by Hematoxylin and Eosin stain. All slides were visualised and captured through light microscope (EVOS cell imaging system from ThermoFisher) at X10, X20, X40 magnifications.

### Statistical analysis

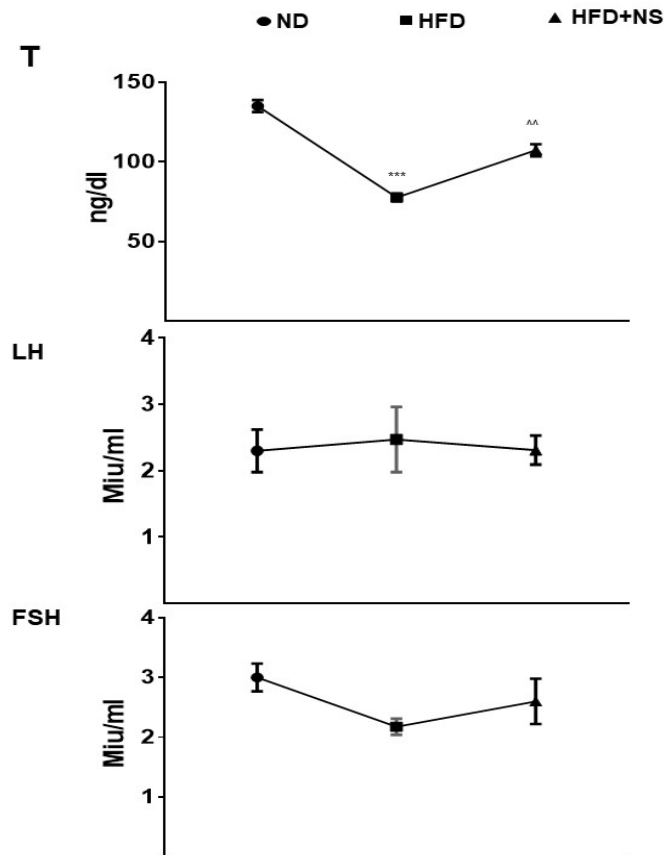
Graph-Pad Prism software version 7.0 was used to analyse the data of lipid profiles, hormones and cytokines. One-way analysis of variance was employed to compare the variances between the control group, the HFD group, and the rats treated with NS group.  $P$  values < 0.05 were considered significantly different.

## RESULTS

### NS improves hypotestosteronaemia

Testosterone levels were significantly lesser in the HFD rat group than in the group with the normal diet. Moreover, the HFD rat group treated with NS seeds showed significantly increasing levels of testosterone compared to the group with HFD only (Figure 1).

Alternatively, the LH levels were unchanged between the controls, the HFD group, and the group supplemented by NS seeds. However, the FSH levels were the lowest in the HFD group compared to the other study groups, but these data were not at statistically significant levels (Figure 1).

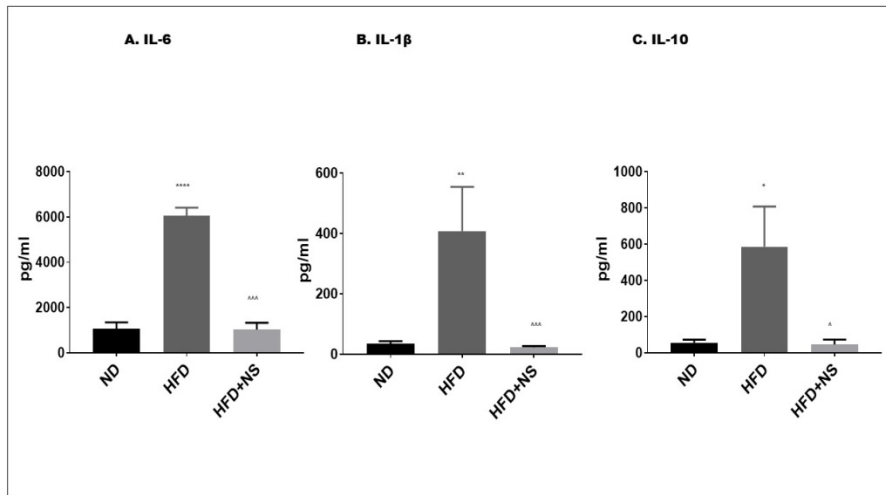


**Figure 1.** Testosterone (T), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) plasma levels in study groups.

T, LH, and FSH were compared between normal diet (ND) rats and high fat diet (HFD) rats;  $***p < 0.000$ . T, LH, and FSH were compared between HFD rats and high fat diet plus *Nigella sativa* seeds (HFD+NS) rats;  $^^p < 0.001$ . The values were expressed as mean  $\pm$  standard error of the mean. Statistical significance was determined by one-way ANOVA ( $n = 5$ ).

#### NS subsides inflammation

The plasma levels of inflammatory cytokines (IL-6, IL-1 $\beta$ , and IL-10) were significantly exaggerated from the HFD group compared by the controls. However, the rats treated with NS seeds showed reduced cytokines, similar to the controls and significantly lower than the HFD group (Figure 2).



**Figure 2.** Inflammatory cytokine—Interlukine-6 (IL-6), Interlukine-1 $\beta$  (IL-1 $\beta$ ) and Interlukine-10 (IL-10)—levels from the study groups

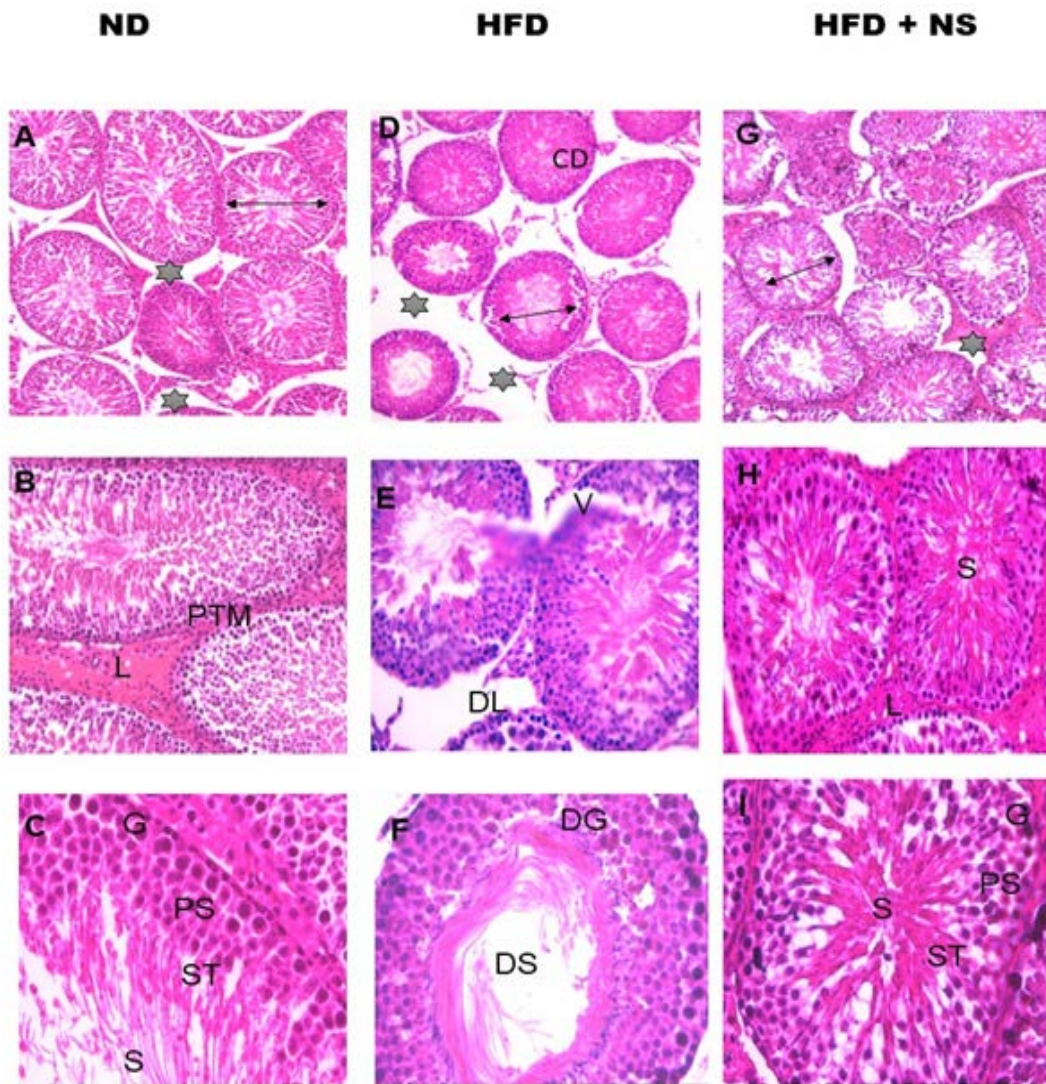
IL-6, IL-1 $\beta$  and IL-10 were compared between normal diet (ND) rats and high fat diet (HFD) rats;  $***p < 0.0001$ ,  $**p < 0.001$ ,  $*p < 0.01$ . IL-6, IL-1 $\beta$  and IL-10 were compared between HFD rats and high fat diet plus *Nigella sativa* seeds (HFD+NS) rats;  $^^^p < 0.0001$ ,  $^^p < 0.001$ ,  $^p < 0.01$ . The values were expressed as mean  $\pm$  standard error of the mean. Statistical significance was determined by one-way ANOVA ( $n = 5$ ).

#### **NS seeds enhanced the normal testicular structure**

As depicted in Figure 3 A-C, the control group showed a normal histology structure of testis, with ordinary seminiferous tubules, interstitial tissue composed of Leydig cells adjacent to blood vessels and spermatogenesis of different stages.

Unsurprisingly, the HFD group (Figure 3 D-F) showed disrupted seminiferous tubules, expansion of interstitial space with degenerated Leydig cells, vacuolation inside the germinal epithelium of seminiferous tubules and atypical spermatogenesis with disarrangement of spermatogonia and degeneration of germ cells.

Interestingly, the HFD group treated with NS seeds (Figure 3 G-I) exhibited restored normal testicular structure, similar to the control group, with an enhanced number of Leydig cells and spermatozoa. Additionally, the normal spermatogenesis stages were achieved.



**Figure 3.** Microscopic features of testis in normal diet (ND) rats, high fat diet (HFD) rats and high fat diet plus *Nigella sativa* seeds (HFD+NS) rats (H & E stain)

**A-C.** Photomicrographs represented normal testicular histology of ND. (A: the black-arrow indicates normal seminiferous tubules, and the star-shape indicates normal interstitial tissue space; B: Leydig cells (L) are seen in the intertubular space adjacent to blood vessels, and the peritubular-myoid cells (PTM) are observed encircling the seminiferous tubules; C: sequential stages of spermatogenesis (spermatogonia (G), primary spermatocytes (PS), spermatids (St) and spermatozoa (S)).

**D-F.** Photomicrographs represented HFD testicular histology. (D: the black-arrow indicates the dissolute seminiferous tubules, while the star-shape indicates the expansion of the interstitial space, and the cellular debris (CD) is seen in most of the seminiferous tubules; E: degeneration of Leydig cells (DL) is seen adjacent to the congested blood vessels, with vacuoles in the germinal epithelium of seminiferous tubules (V); F: abnormal spermatogenesis is observed with disarrangement of spermatogonia (DS) and degeneration of germ cells (DG).

**G-I.** Photomicrographs represented HFD+NS testicular histology. G: restoration of normal structure of seminiferous tubules and interstitial spaces; H: there is an increasing number of Leydig cells (L) and spermatozoa (S); I: normal spermatogenesis stages are achieved. Magnification of images A, D and G = X10; magnification of B, E and H = X20; and magnification of C, F and I = X40.

## DISCUSSION

Obesity is involved in men's subfertility in various ways, including defects of gonads' hormonal axis, increased scrotal temperature, local testicular dysfunctions and abnormal spermatogenesis [2, 13]. Previous research on

animal models has shown that HFD has adverse effects on testicular functions [21-23], and similar results were shown in this study. Despite other research showing that NS causes significant improvements in obesity indices [24], the current study demonstrated the improvements NS may have on dysfunctional spermatogenesis resulting from HFD.

In normal statuses, FSH and LH are released through the anterior part of the pituitary gland as a response to gonadotropin-releasing hormones produced by the hypothalamus—a relationship called the hypothalamic-pituitary-gonadal (HPG) axis [14]. The LH then turns on the Leydig cells from testis for releasing of testosterone, while FSH regulates the Sertoli cells for facilitating spermatogenesis [14]. Obese men have shown reduced testosterone and FSH levels, while LH may be either normal or reduced. The overall result of these hormonal changes was reduced fertility [25, 27]. This might possibly be explained by high oestradiol levels, owing to the aromatisation of testosterone from adiposity in the obese men, which has had a negative feedback suppression on the HPG axis [27]. It has also been suggested that the leptin produced by white adipose tissues correlates with low testosterone levels, and amplified adiposity results in leptin resistance [28]. Previous research studies have shown that cultured Leydig cells demonstrate high leptin levels associated with suppressed testosterone release [29], mechanisms which might occur in obese men. The results of the current study showed that HFD rats had a low level of testosterone and FSH with unchanged LH, which might be improved after supplementing their diets with NS.

Bashandy (2007), who studied lipid profiles, sperm parameters and testosterone levels to evaluate the fertility index of obese rats, demonstrated that NS improves the fertility index of hyperlipidaemic rats [30]. The obese rats in Bashandy's work represented abnormal lipid profiles, including high cholesterol, high LDL and low HDL, which were similar to the results of the present study that were reversed after NS supplementation. Both studies showed the improvement of the reproductive potentials of obese rats, after the administration of NS, by distinct aspects. While Bashandy studied sperm parameters, this study screened the HPG axis and examined testicular histology. It has been demonstrated that sperm count, sperm motility and decreased numbers of abnormal sperm were enhanced from hyperlipidaemic rats treated with NS [30]. Additionally, the current study demonstrated that, in obese rats, NS can restore normal testicular structure, enhance the number of Leydig cells, and achieve normal spermatogenesis stages similar to those of the control rats. Other studies have also approved that testosterone concentrations were returned to normal values after obese rats were treated with NS. Moreover, the current study showed the ability of NS to improve FSH values, and suggested that high doses of the alcoholic extract from NS seeds surged testosterone levels in non-obese rats [31]. In humans, the abnormal semen index from infertile men also improved by the daily consumption of NS for two months [32]. However, the study measuring this in humans did not relate infertility to obesity.

Inflammation is another area in which NS may affect the reproductive efficiency of obese rats. NS oil and its constituents have anti-inflammatory potential, and the potential for antioxidative stress, as shown by a series of in-vitro and in-vivo studies [33, 34]. Systemic and testicular inflammation in obese subjects is indicated by increased circulatory cytokine levels, high leptin levels, oxidative stress and apoptotic biomarkers [5, 11, 35]. These inflammatory mechanisms impair the testicular microenvironment, including testicular cells and the epididymis responsible for sperm formation and maturation. Altogether, obesity-induced inflammation would cause an obese person to be subjected to subfertility, represented by abnormal sperms and/or worsened epigenetic inheritance represented by DNA fragmentation/methylation [12, 36]. Therefore, this study suggests that NS has an anti-inflammatory effect in obese male rats, indicated by dropping systemic cytokine levels, and, thus, the implementation of NS treatment in obese subjects might increase the reproductive effectiveness.

## **CONCLUSIONS**

In conclusion, NS could improve the reproductive efficiency in the abnormally distorted hyperlipidaemic-inflamed reproductive tissues in obese male rats. The specific signalling pathways that contributed in the inflammation process should be identified and seen if the NS can act as anti-inflammation substance for the promotion of reproductivity. Therefore, for further studies in humans, it would be worthwhile to add NS to the regimen for obese, subfertile men.

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