Original Article

Physiological and Histological Effects of Some Nutritional Supplements and Doping in Male Rats

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ABSTRACT

Background: Nutritional supplements (NSs) are nutritional components, not foods such as protein, that are commercially available and consumed in addition to the diet.

Objectives: We aim to investigate the impact of NSs and testosterone hormone used in sports training centers on male reproductive hormones like spermatogenesis-stimulating hormone (SSH), interstitial cell-stimulating hormone (ICSH), testosterone hormone, and cortisol, as well as assessing histological changes in the testis of exercising male rats.

Methods: The study involved 80 rats divided into 8 groups. The first group was the control, while the second group was subjected to exercise. The third group received NS, the fourth group was injected with a testosterone blend, and the fifth group received NS and a testosterone blend. The sixth group was given NS with exercising, the seventh group was injected with a testosterone blend with exercising, and the eighth group was given NS and a testosterone blend with exercise.

Results: There was a significant increase in SSH, ICSH, cortisol, and testosterone hormone concentration in the group treated with NS and injected with testosterone blend compared to the rest of the groups. The results of histological examinations showed abnormal seminiferous tubules with an increase in diameter and thickness of the spermatogenic cell layer. Also, the seminiferous tubules were broadly separated from each other, and cell necrosis for rats treated with NS and testosterone blend compared to the rest of the groups revealed normal shape and arrangement of testicular cells. Exercise has positively reversed the negative effects of dietary supplementation and testosterone on male sex hormone levels and cortisol, in addition to reducing the negative effects on testicular tissue of male rats.

Conclusion: Treatment with NS and testosterone blend had adverse effects on the concentration of some hormones. There were also some histological changes in the testis.

Keywords: Cortisol, Rats, Sports training, Spermatogenesis-stimulating hormone (SSH), Testosterone

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Introduction

Nutritional supplements (NSs) have a long history, dating back to 776 BC when athletes in the ancient Greek Olympic Games first used them. Early forms of NSs included natural energy sources such as dried figs, cheese, and wheat, although no scientific evidence supported their effectiveness (Coleman-Jensen et al., 2022). Protein powders, such as whey and rice protein, are particularly popular for muscle growth and recovery, often enhanced with flavoring to improve their palatability (Al Nozha & Elshatarat, 2017).

NSs are now widely used across all age groups and levels of athletic participation. Their popularity is incredibly high among adolescents engaged in strength training and bodybuilding, where unsupervised usage, especially of supplements combined with steroids, is often driven by the desire for rapid body transformation (Guleria et al., 2018). One study shows that over half of the adult population in the United States uses at least one type of NS (Keisler-Starkey et al., 2020). Despite their popularity, there is limited scientific evidence confirming the efficacy and safety of these supplements, especially among recreational athletes (Heikkinen et al., 2011).

Unregulated use of NSs and anabolic androgenic steroids poses significant health risks. Prolonged use and high doses without medical guidance are associated with a range of adverse physiological effects, from hormonal imbalances to severe organ damage. Abuse of anabolic androgenic steroids, in particular, can lead to cardiovascular complications, reproductive issues, and liver toxicity (Albano et al., 2021). Side effects such as inhibited pituitary-gonadal function, sexual dysfunction, decreased libido, and testicular atrophy are common in steroid misuse, with a noted two-fold increase in infertility risk among male androgen users compared to non-users (Rahnema et al., 2014; Horwitz et al., 2019; Henriksen et al., 2023).

Knowledge gap

Although research acknowledges the health risks associated with excessive NS and steroid use, there remains a gap in understanding the specific physiological, histological, and biochemical impacts of these substances, especially in populations engaging in non-professional sports. This study aims to bridge this gap by examining the effects of NSs and testosterone-based substances on physiological and histological parameters in male New Zealand white rats, providing insights into potential health consequences relevant to recreational athletes.

Materials and Methods

Study area

The study was conducted in the animal house of the College of Science, Department of Biology, University of Mosul, Iraq.

Nutritional complement

Bodybuilders and novice athletes centers use one typical NS in sports training (Table 1). Adult male rats were given this NS (mass attack), the official agent in Iraq (Mirias Group Company/Iraq Baghdad). It is a soluble powder in regular drinking water; the rats were orally given this NS (2.4 g/ rat/ day) using a gavage needle. The ingredients of NS used in the study are protein 70 g, carbohydrate 78 g, energy 646 kcal, BCAA (branched-chain amino acids) 12.6 g, L- glutamine 9.4, creatine 10 g, and tribulus terrestris Fenugreek, DAA 3 g.

Testosterone blend

The current study used one of the most common types of steroid hormones used by athletes and bodybuilders in sports training centers, known as Sustanon (testosterone blend), which is in the form of a multi-dose vial (250 mg/ mL). It is made by BOOY TECH company, Germany. Male rats were injected with testosterone blend into the thigh muscle at a dose of 1 mg/kg once every 10 days for three months (Frankenfeld et al., 2014; Jwad & Mohammed, 2017).

Study animals

In this study, 80 adult male Albino rats were used, with ages ranging from 2 to 3 months and their weights ranging from 250 to 400 g. They were obtained from the animal house of the College of Veterinary Medicine, Tikrit University. They were examined to be sure they were disease-free. They were raised in metal cages designated for rats under appropriate conditions, with a temperature of 25-28 ˚C and a lighting period of 14 hours a day with good ventilation. After transferring the rats, they were subjected to an introductory period of 10 days before they were included in the experimental groups. Before starting the experiment, the rats were given a balanced and standard diet in pellet feed. As for water, it was provided to the rats using appropriate bottles.

Table 1. The contents of the NS used in the study

Component	Content
Protein	70 g
Carbohydrate	78 g
Energy	646 KCAL
Branched-chain amino acids (BCAA)	12.6 g
L- glutamine	9.4 g
Creatine	10 g
Tribulus terrestris Fenugreek, DAA	3 g

Biochemical analyses

Blood samples were collected from male rats during the experiment, and at different intervals, EDTA (ethylenediaminetetraacetic acid) samples were taken using a capillary tube containing an anticoagulant and through the corner of the animal's eye. The blood samples were divided into two parts, and according to the type of examination, 2 mL of blood was placed in tubes. A plastic container with a tight lid containing EDTA, an anticoagulant to perform blood tests on, while 2 mL of blood was placed in gel tubes designated for separating the serum, free of anticoagulants. The tubes were left in the laboratory for 15 minutes until they coagulated, after which the samples were separated by a centrifuge at 4500 rpm for 6 minutes to obtain the serum. The serum was then divided into several parts by placing it in dry, sterile Eppendorf tubes to preserve the serum, and then frozen at a temperature of -20 ˚C until measuring the concentration of different parameters: Testosterone, spermatogenesis-stimulating hormone (SSH), interstitial cell-stimulating hormone (ICSH), and cortisol hormones, determined by ELISA method.

The study design

Rats were randomly divided into 8 equal groups. Each group consisted of 10 rats treated as follows:

Control group (G1): Given water and feed (balanced diet) only for three months from the date of the start of the experiment,

Exercise group (G2): Trained on a treadmill three days a week for three months from the start of the experiment, also giving them water and feed (a balanced diet),

NS group (G3): Administered the NS orally by gavage needle at a dose of 2.4 g/ rat /day for three months,

Testosterone group (G4): Injected with (testosterone blend), at a dose of 1 mg/kg once every 10 days for three months,

NS and testosterone group (G5): Administered orally by gavage needle with the NS at a dose of 2.4 g/rat and were injected with testosterone blend at a dose of 1 mg/kg once every 10 days for three months.

NS and exercise group (G6): Administered orally by gavage needle with the NS at a dose of 2.4 g/rat and were subjected to exercise on a treadmill at a rate of three days a week for three months,

Testosterone and exercise group (G7): Injected with (testosterone blend), at a dose of 1 mg/kg once every 10 days and were subjected to exercise on a treadmill at a rate of three days a week for three months,

NS, testosterone with exercise group (G8): Administered orally by gavage with the NS at a dose of 2.4 g/rat and injected with testosterone blend at a dose of 1 mg/kg once every 10 days and were subjected to exercise on a treadmill at a rate of three days a week for three months.

At the end of the treatment, when three months had passed since the experiment, the rats were anesthetized before being sacrificed after the last drawing of blood samples, using diethyl ether. Then, anatomical features were conducted on them to obtain the organs for histological study.

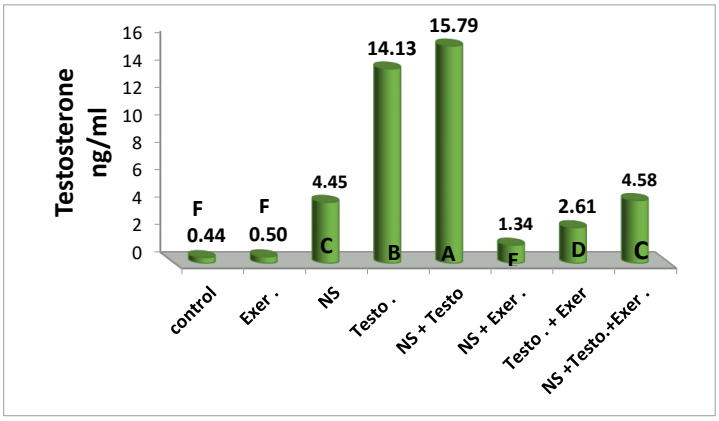

Figure 1. The effect of the NS and the testosterone blend on the concentration of the hormone testosterone (ng/mL) in the blood serum of male rats (P<0.05)

Histological study

The histological study included testicles. The process begins with collecting and preserving the sample in a fixative solution, usually formalin, to prevent degradation and maintain cell components. Next, water is gradually removed from the sample using a series of alcohol solutions of increasing concentrations, followed by replacing the alcohol with a waxy substance, often paraffin, to facilitate sectioning. Using a microtome, the tissue is sliced into very thin sections, typically between 4 to 10 µm. These sections are placed on glass slides and stained with specific dyes, such as hematoxylin and eosin (H&E), to highlight different cellular components and bring out details. These stained slides enable a clear examination of histological changes in the samples, whether for disease studies or to assess the effects of various factors on tissues.

Kits used in the study

The kits used in this study were as follows. Sustanon, or testosterone blend kit (BOOY TECH Co., Germany), measures testosterone levels in the blood, utilizing a unique blend of testosterone compounds for precise level determination. ICSH (interstitial cell-stimulating hormone) kit (Shanghai YL Biont Co., Shanghai, China) measures ICSH hormone levels in the body, using a sensitive immunoassay reaction for this hormone through the ELISA technique. SSH (sex steroid hormone) kit (Shanghai YL Biont Co., Shanghai, China) is designed to determine levels of sex steroid hormones in blood samples, providing quantitative analysis through ELISA for high accuracy. The cortisol hormone kit (Shanghai YL Biont Co., Shanghai, China) measures cortisol hormone levels in the blood, utilizing ELISA technology to detect precise cortisol concentrations via specialized antibodies.

ELISA measurement technique

The enzyme-linked immunosorbent assay (ELISA) technique is an analytical method used to detect specific protein or hormone concentrations in biological samples. It relies on the interaction between antibodies and antigens for accurate results. In this study, the ELISA Analyzer Cobas c311 was used. It is a fully automated device combining German and Japanese technology from Roche/HITACHI, ensuring precision and speed in results and simplifying routine hormone measurement processes.

Statistical analysis

Data were statistically analyzed using the SAS statistical program, version 9 (SAS, 2001) and in a completely randomized design. The differences are determined using Duncan's multiple range test at P≤0.01 (Hinton, 2014).

Results

Concentration of testosterone hormone

Figure 1 illustrates that the rats treated with the NS and testosterone blend showed significantly higher testosterone levels (P≤0.01) compared to the other groups, with a mean of 15.79±0.24 ng/mL.

The study found no significant difference in the mean testosterone levels between the rats treated with testosterone blend and exercise, and no significant difference between the rats treated with exercise and the control

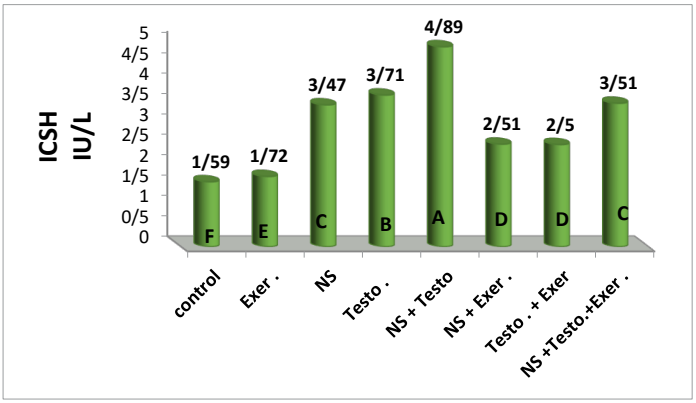

Figure 2. Effect of NS and testosterone blend compared on the concentration of ICSH (IU/L) in the blood serum of male rats ($P<0.05$)

group. Mean testosterone levels between the rats treated with testosterone blend and exercise, and no significant difference between the rats treated with exercise and the control group.

Concentration of ICSH

The concentration of ICSH in the rats treated with the NS and testosterone mix was significantly higher than in the remainder groups (Figure 2).

The study found no significant difference in the mean circulating levels of testosterone in rats treated with testosterone blend or exercise compared to those treated with the NS alone. The mean difference was similar across all groups, with the lowest mean in the control group being 1.59±0.03 IU/L.

Concentration of SSH

The study found a significant increase in serum testosterone levels in rats treated with a narcotic substance (NS) with a testosterone blend compared to the control group. The mean increase was 3.80±0.03 IU/L, followed by 2.94±0.02 IU/L for the NS and testosterone blend alone. The arithmetic mean of the NS and exercise group was 0.95±0.02 IU/L (Figure 3).

Concentration of cortisol hormone

Figure 4 shows a significant increase in cortisol levels in rats treated with NS with testosterone blend compared to other groups. The mean increase was 7.36±0.04 nmol/L, followed by 7.15±0.03 nmol/L and 4.37±0.04 nmol/L for the NS and exercise groups. No significant difference was found between the NS and testosterone blend groups.

Histological evaluations

Histological sections of the testis of the control group G1 and exercise group G2 showed a normal appearance of seminiferous tubules with the typical arrangement of spermatogonia, normal Sertoli cells resting on the basement membrane, the interstitial tissue containing normal blood vessels, and Leydig cells and the seminiferous tubules containing sperms (Figures 5 and 6).

The testis of the NS group G3 revealed the normal appearance of seminiferous tubules with the typical arrangement of spermatogonia. However, there are abnormal seminiferous tubules with the highest diameter and thickness of the spermatogenic cell layer; the seminiferous tubules are separated with a few Leydig cells and congestion of blood vessels (Figure 7). The histological section of the testis in the testosterone blend treated group G4 showed elongation of the seminiferous tubules with an increase in the diameter of these tubules to become oval-like, leading to a wide space between the tubules (Figure 8).

The histological section of the testis in the NS and testosterone blend treated group G5 revealed elongation of the seminiferous tubules with an increase in the diameter of these tubules to become an oval-like shape. Also, there was the thickness of the spermatogenic cell layer and the seminiferous tubules were broadly separated from each other (Figure 9). The histological section of the testis in the NS and exercise training group G6 demonstrated the normal appearance of seminiferous tubules with the typical arrangement of spermatogonia. However, there is a slight space between the seminiferous tubules (Figure 10). The testis in the testosterone blend with exercise training group G7 showed the normal shape of seminiferous tubules with the typical arrangement of primary spermatocytes, spermatids, and sperms, and there was

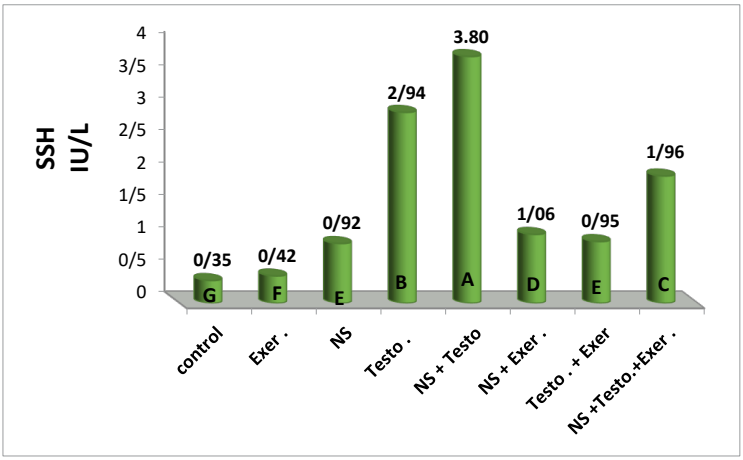

Figure 3. Effect of NS and testosterone blend on SSH concentration (IU/L) in the blood serum of male rats (P<0.05)

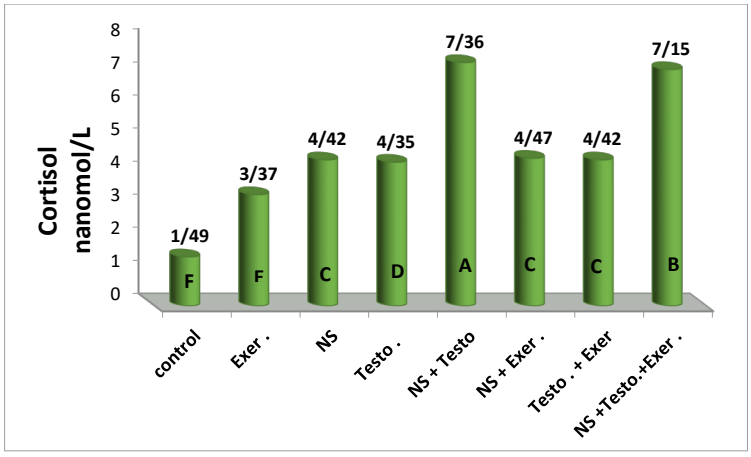

Figure 4. The effect of the NS and testosterone blend on cortisol (nmol/L) in the blood serum of male rats (P<0.05)

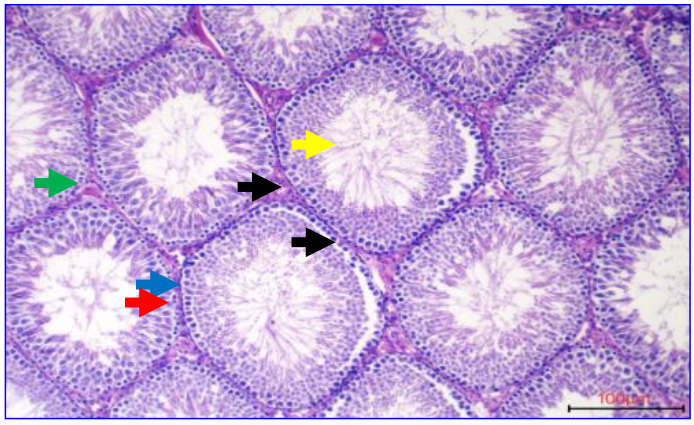

Figure 5. Histological section of testis of the normal rats (control)

Notes: It shows normal-appearing seminiferous tubules with the normal arrangement of spermatogonia (black arrows), normal Sertoli cells (blue arrow) resting on the basement membrane (red arrow), the interstitial tissue contains normal blood vessels and Leydig cells (green arrow), and the seminiferous tubules contain sperms (yellow arrow) (100 µm, H&E staining).

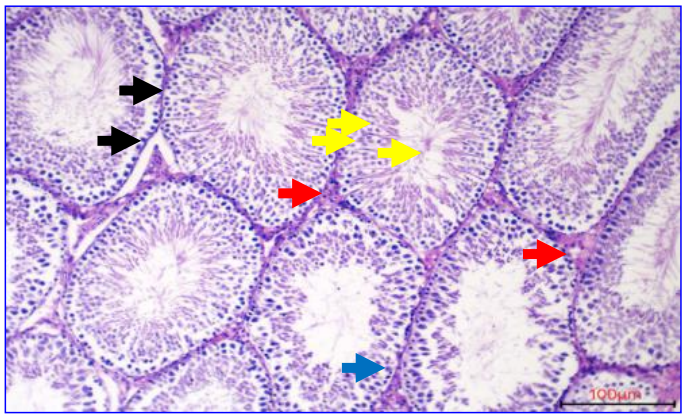

Figure 6. The testis of the exercise-trained rats' histological section

Notes: It displays normal-looking seminiferous tubules with a normal arrangement of spermatogonia (black arrows) and Sertoli cells (blue arrows), the interstitial tissue contains normal blood vessels and Leydig cells (red arrows), and the seminiferous tubules contain primary spermatocytes, spermatids, and sperms (yellow arrows) (100 µm, H&E stain).

a clear space between the seminiferous tubules (Figure 11). The histological section of the testis of the NS, testosterone injection with exercise group G8 revealed two types of seminiferous tubules: Some are normal in shape, and some are elongated tubules. The seminiferous tubules are separated (Figure 12).

Discussion

Muscle mass and strength enhancement are widely recognized to require intense physical training, necessitating adequate energy intake from NSss and anabolic agents, particularly testosterone. However, the administration of such substances should always be conducted under medical supervision to avoid adverse effects associated with unsupervised use (Guleria et al., 2018). In our study, we observed that treatment with a combination of NS and testosterone significantly elevated the levels of sex hormones, including luteinizing hormone (ICSH), thyroid stimulating hormone (SSH), testosterone, and cortisol.

Our findings contribute to the limited body of research in this area, marking one of the first investigations conducted in Mosul, Iraq. The study results merit careful interpretation, suggesting potential hormonal imbalances stemming from the supplementation. Specifically, elevated SSH, ICSH, and testosterone levels might indicate disruptions in thyroid hormone secretion, particularly thyroxine. NS contains essential amino acids such

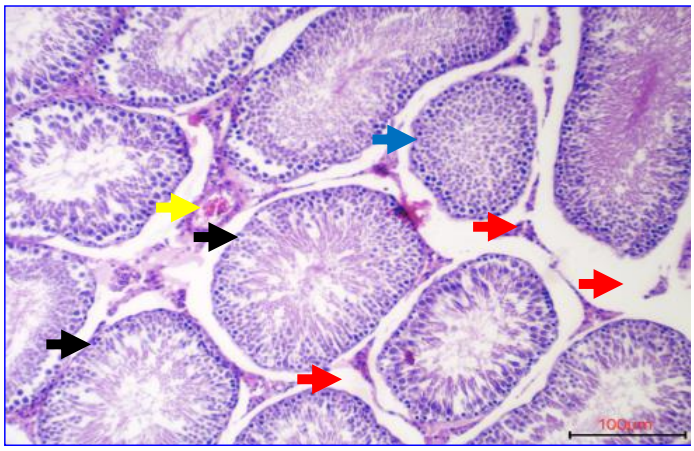

Figure 7. Histological section of testis of the NS-treated rats

Notes: It shows the normal appearance of seminiferous tubules with a normal arrangement of spermatogonia (black arrows). However, there are abnormal seminiferous tubules with the highest diameter and thickness of the spermatogenic cell layer (blue arrow), the seminiferous tubules are separated from each other with few numbers of Leydig cells (red arrows), and congestion of blood vessels (yellow arrow) (100 µm H&E stain).

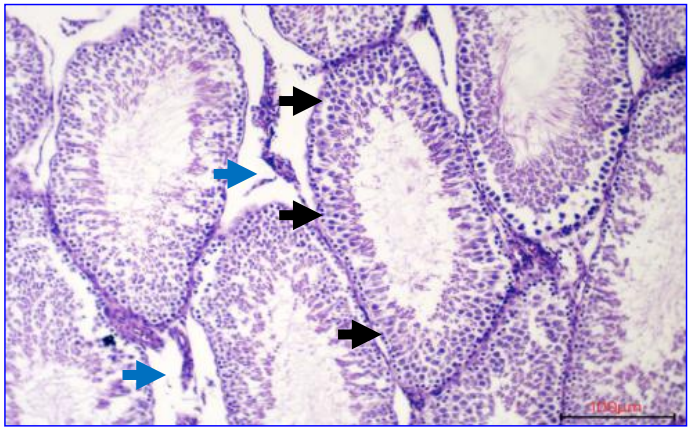

Figure 8. Histological section of testis in the testosterone blend treated rats

Notes: It shows elongation of the seminiferous tubules with an increase in the diameter of these tubules to become oval-like (black arrows), leading to an increase of a wide space between the tubules (blue arrows) (100 µm H&E stain).

as valine, leucine, and isoleucine, which are known to influence the levels of sex hormones (Guyton & Hall, 2023). An increase in these amino acids can stimulate the release of sex hormones, and there is a well-documented interplay between thyroid hormones and male sex hormones.

Another consideration is the possibility of primary testicular failure induced by the combination of NS and testosterone, potentially resulting from pituitary gland dysfunction. This dysfunction could hinder the regulation of SSH and ICSH secretion, adversely affecting testicular function. SSH binds to receptors on Sertoli cells, which are crucial for sperm production and inhibin secretion, while ICSH stimulates testosterone production in Leydig cells, impacting Sertoli cell function and surrounding seminiferous tubules.

Furthermore, random use of NS may lead to an accumulation of free radicals and oxidative stress, impairing the endocrine function of hormone-secreting glands (Esencan et al., 2022). Exogenous testosterone administration has also been associated with suppression of the hypothalamic-pituitary-gonadal axis, leading to complications such as erectile dysfunction, reduced libido, and even testicular atrophy due to hypogonadism (Rahnema et al., 2014).

Regarding cortisol, its elevated levels—often termed the "stress hormone"—may be attributed to the stimulating effects of essential amino acids in conjunction with testosterone on the adrenal cortex, particularly the zona fasciculata (Guyton & Hall, 2023).

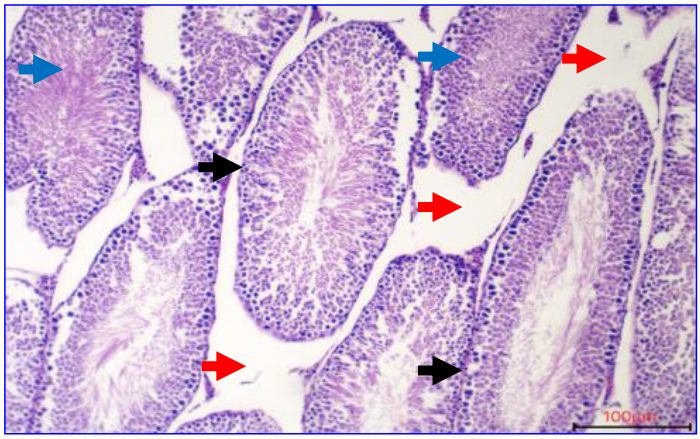

Figure 9. Histological section of testis in the NS and testosterone blend treated rats

Notes: It shows elongation of the seminiferous tubules with an increase in the diameter of these tubules to become oval-like shape (black arrows) with the thickness of spermatogenic cell layer (blue arrow), the seminiferous tubules are broadly separated from each other (red arrows). (100 µm H&E stain).

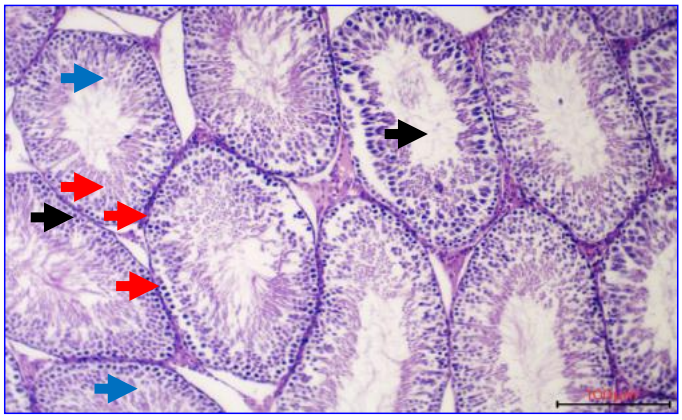

Figure 10. The histological section of the testis in the N-treated rats with exercise training

Notes: It shows the normal appearance of seminiferous tubules (black arrows) with normal spermatogonia (red arrows) arrangement. However, a slight space exists between the seminiferous tubules (blue arrows) (100 µm, H&E stain).

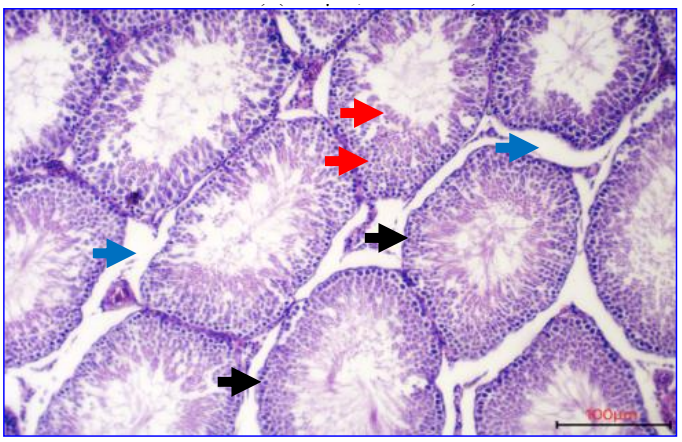

Figure 11. Testicular histology in rats given testosterone blend after exercise training

Notes: It revealed a normal seminiferous tubule shape (black arrow), normal primary spermatocyte, spermatid, and sperm arrangement (red arrows), and a distinct gap between the seminiferous tubules (blue arrows) (100 µm H&E stain).

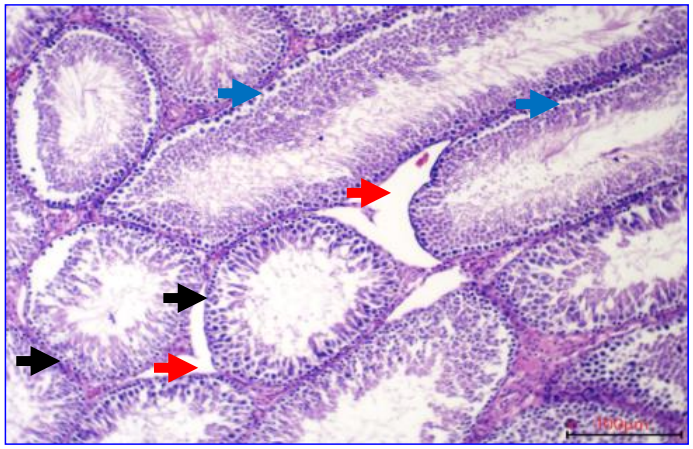

Figure 12. The histological section of the testis in the NS and testosterone blend treated rats with exercise training

Notes: It shows two types of seminiferous tubules: some are normal seminiferous tubules (black arrows), and some are elongated tubules (blue arrows); the seminiferous tubules are separated from each other (red arrows) (100 µm, H&E stain)

Histological examination of the testes in our study revealed significant pathological changes, including vascular congestion and elongation of seminiferous tubules, with an increased diameter that resembled a thickened layer of spermatogenic cells. These alterations were notably pronounced in rats receiving the NS and testosterone blend, in contrast to the control group. Such findings align with prior research indicating that testosterone administration can lead to atrophic seminiferous tubules characterized by large intertubular gaps and vacuolation of germinal epithelium (Amer & Selim, 2011).

Additional studies support these observations. For instance, a study by Seif et al. (2021) demonstrated that testosterone administration in male rats led to similar histological alterations, including degeneration of germ cells and disruption of normal spermatogenesis. Another comparative study by Wang et al. (2023) further corroborated these findings, revealing that long-term testosterone supplementation adversely affected testicular morphology and function, potentially due to oxidative stress.

In summary, while the use of NS and testosterone may offer short-term benefits in muscle mass and strength enhancement, the potential for significant hormonal imbalances and testicular pathology necessitates caution. Further research is essential to elucidate these treatments' long-term effects and establish safer protocols for their use.

Conclusion

This study concluded that the NS and testosterone blend had adverse effects on SSH, ICSH, and testosterone hormones, as well as the concentrations of SSH, ICSH, and testosterone. Increase in the case of treating male rats with the NS and injecting them with testosterone blend. The concentration of the hormone cortisol also increased if this NS and the hormone testosterone were used compared to the control group.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Institutional Animal Care and Use Committee, College of Veterinary Medicine, University of Mosul, Mosul, Iraq (Code: UM. VET. 2023.071; Dated: August 17, 2023).

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Authors' contributions

All authors contributed equally to the conception and design of the study, data collection and analysis, interception of the results and drafting of the manuscript. Each author approved the final version of the mnuscript for submission.

Conflict of interest

The authors declared no conflict of interest.

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