Original Article

Sustanon suppresses spermatogenesis and increases cell death

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Background: Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone. Sustanon, dissolved in peanut oil, is an AAS used by athletes to build muscle mass. This study aims to examine the effects of Sustanon on male reproductive health.

Methods: Adult male rats were divided into four groups under standard conditions. The control group received an intramuscular injection of the Sustanon solvent. The second, third, and fourth groups were treated with different doses (10, 20, and 30 mg/kg body weight) of Sustanon for 8 weeks. Blood samples, testes, and spermatozoa were collected for laboratory tests. Complementary DNA (cDNA) was synthesized from total RNA, and the expression of deleted in azoospermia like (*DAZL*) and B-cell lymphoma 2 (*BCL2*) genes was measured by real-time polymerase chain reaction (PCR). Histopathological analysis was performed on the testes.

Results: The *BCL2* gene had significantly lower expression in the treatment groups compared to the control group. There was no significant increase in the expression of *DAZL*. Significant reductions in testicular length, diameter, weight, sperm count, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) concentrations were observed in the treatment groups. Histological changes were evident in the testes of the treated groups.

Conclusions: Sustanon likely induces adverse effects on the male reproductive system, potentially decreasing fertility. The study provides critical insights into the negative impacts of Sustanon on spermatogenesis and cell survival.

Keywords: Sustanon; spermatogenesis; apoptosis; deleted in azoospermia like (*DAZL*); B-cell lymphoma 2 (*BCL2*)

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Introduction

Anabolic-androgenic steroids (AAS) represent a broad category of synthetic derivatives of the male sex hormone testosterone. These compounds are engineered to mimic the effects of natural testosterone, enhancing muscle growth and physical performance. Among these, Sustanon is a prominent example, characterized by its unique formulation of four distinct testosterone esters: testosterone propionate,

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testosterone phenylpropionate, testosterone isocaproate, and testosterone decanoate. This combination allows for a prolonged and steady release of testosterone into the bloodstream, stabilizing hormone levels for up to 3–4 weeks (1,2). Initially developed for the clinical management of conditions such as male hypogonadism and infertility, Sustanon has also found use in bodybuilding and athletic circles, often for its muscle-building and performance-enhancing properties (3,4).

The clinical applications of Sustanon are rooted in its ability to provide sustained testosterone replacement therapy, which is essential for individuals with low endogenous testosterone levels (5). Male hypogonadism, a condition characterized by insufficient testosterone production, can lead to a range of symptoms including reduced libido, fatigue, and diminished muscle mass. Sustanon's unique formulation addresses these issues by delivering a continuous supply of testosterone, thereby alleviating symptoms and improving overall well-being (6). Similarly, in the context of infertility, testosterone replacement can help restore normal spermatogenesis, the process by which sperm cells are produced in the testes (7).

The use of Sustanon extends beyond its therapeutic indications. In the athletic and bodybuilding communities, Sustanon is often used non-medically to enhance physical performance and muscle mass (8). Athletes and bodybuilders may misuse this steroid to gain a competitive edge, increase muscle hypertrophy, and improve strength (9). Despite its potential benefits, the non-medical use of Sustanon raises significant health concerns, particularly regarding its effects on male reproductive health (10). Since athletes, especially young people in Iran, frequently use Sustanon to increase muscle mass, this study focuses on its effects on fertility and reproductive health.

One of the major concerns with Sustanon use is its impact on spermatogenesis. Spermatogenesis is a highly regulated process that involves the production and maturation of sperm cells within the testes (11). This process relies on a delicate balance of hormonal signals, primarily testosterone, which is crucial for the progression of spermatogenesis through various stages, including stem cell proliferation, differentiation, chromatin condensation, and meiotic cell divisions. Elevated levels of exogenous testosterone from Sustanon can disrupt this balance, leading to a suppression of spermatogenesis and potential infertility (12).

The mechanisms underlying this suppression are complex. Testosterone plays a key role in the regulation of spermatogenesis by binding to androgen receptors in the testes (13). This interaction facilitates the progression through the meiotic cycle and the production of mature sperm cells. However, when testosterone levels are artificially elevated, the body may perceive this as a signal to downregulate its own production of testosterone. This feedback inhibition can result in decreased stimulation of the testes, leading to reduced sperm production and impaired fertility (14).

Additionally, the process of spermatogenesis is intertwined with programmed cell death, or apoptosis, which is essential for maintaining the health and quality of sperm cells (15). Apoptosis in the testes serves to eliminate defective germ cells and prevent the overproduction of sperm. This process is regulated through intrinsic and extrinsic pathways, with the intrinsic pathway involving mitochondrial function playing a particularly critical role in male fertility. Disruption of these apoptotic pathways can further contribute to the adverse effects of Sustanon on spermatogenesis (16).

Several studies have investigated the effects of Sustanon on male reproductive health, highlighting its potential to disrupt spermatogenesis and induce apoptosis. For instance, research by Bameri *et al.* (17) demonstrated that Sustanon

Highlight box

Key findings

- Significant reduction in B-cell lymphoma 2 gene expression in Sustanon-treated groups.
- No significant increase in deleted in azoospermia like gene expression.
- Significant reductions in testicular length, diameter, weight, sperm count, and hormone levels in treated groups.
- Histopathological changes observed in the testes of treated rats.

What is known and what is new?

- It is known that anabolic-androgenic steroids (AAS) can negatively affect male reproductive health.
- This manuscript adds new insights into the specific effects of Sustanon on gene expression, hormone levels, and testicular histology.

What is the implication, and what should change now?

- The findings imply that Sustanon use can lead to significant reproductive dysfunction and infertility.
- There is a need for increased awareness about the adverse effects of Sustanon among athletes and bodybuilders. Policies should be developed to regulate the use of AAS and promote safer alternatives for muscle building.

treatment resulted in significantly lower expression of the B-cell lymphoma 2 (*BCL2*) gene, a key regulator of the intrinsic apoptotic pathway, in treated groups compared to controls. This decrease in BCL2 expression indicates increased apoptosis and further underscores the negative impact of Sustanon on spermatogenesis. Additionally, the study observed reductions in testicular size, weight, sperm count, and concentrations of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), alongside notable histopathological changes in the testes.

This study also uniquely investigates the role of deleted in azoospermia like (*DAZL*), which is essential for normal spermatogenesis and germ cell development. While Bameri *et al*. focused on general reproductive impacts (17), we explore the gene-specific effects of Sustanon on *DAZL* expression, offering a more in-depth understanding of the molecular pathways affected by this steroid. By including a gene-level analysis, our research highlights how Sustanon can compromise fertility through both hormonal disruption and genetic regulation.

The misuse of Sustanon among athletes poses significant risks to male reproductive health, primarily through disruptions in spermatogenesis and induction of apoptosis. While Sustanon has therapeutic potential in treating certain andrological conditions, its non-medical use should be approached with caution due to its potential adverse effects. This study aims to address some of the existing gaps by investigating the molecular effects of Sustanon, specifically focusing on its impact on *BCL2* and *DAZL* gene expression. This offers a novel perspective on how Sustanon-induced apoptosis and disrupted spermatogenesis contribute to fertility issues, underscoring the need for further research to develop strategies to mitigate these negative effects. We present this article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-24-397/rc).

Methods

Study population

In this study, 24 adult male Wistar rats, each weighing between 200 and 250 grams at the start of the experiment, were bought from the Laboratory Animal Breeding Center, Ferdowsi University of Mashhad, Mashhad, Iran. The rats were housed in individual cages (six rats per cage) at the Animal House of Islamic Azad University, Mashhad Branch, Iran, where the entire experiment was conducted. Each ampoule contained 1 mL of Sustanon oil solution. Experiments were performed under a project license (No. IR.IAU.MSHD.REC.1398.214) granted by the Ethics Committee of Islamic Azad University, Mashhad Branch, in compliance with national guidelines for the care and use of laboratory animals. A protocol was prepared before the study without registration. The rats were maintained under standard laboratory conditions with a controlled environment of 24 ℃ and a 12-hour light/dark cycle. Food and water were provided ad libitum.

The rats were randomly divided into four groups. The first group received an intramuscular injection of peanut oil as a control. Groups 2, 3, and 4 were administered Sustanon at doses of 10, 20, and 30 mg/kg body weight, respectively, via intramuscular injection once a week for 8 weeks. Sustanon was dissolved in peanut oil to prepare the respective doses: for the 10 mg/kg dose, 1 mL of Sustanon was diluted in 99 mL of peanut oil; for the 20 mg/kg dose, 1 mL of Sustanon was diluted in 49 mL of peanut oil; and for the 30 mg/kg dose, 1 mL of Sustanon was diluted in 32 mL of peanut oil.

Blood sampling

All the rats were sacrificed under anesthesia with diethyl ether at the end of the treatment course. Blood samples were collected and placed in complete blood count (CBC) tubes and clot tubes (18).

Sperm count, morphology, and DNA fragmentation analysis

After blood sampling, the testis and epididymis were removed. Sperm count, morphology, and DNA fragmentation were analyzed. Five millimeters of the epididymal cauda were incubated in 2 mL of Hams F10 medium at 37 ℃. Fifty microliters of sperm were added to 1 mL of Hams F10 (dilution ratio of 1:20). Ten microliters of this solution were placed on a Neubauer counting chamber for counting sperms in five squares under a light microscope. The mean count was multiplied by 10^6 (9,19). The Diff-Quik rapid sperm staining kit (Dayan Zist Azma Co., Tehran, Iran) was used for morphological analysis (20-22). DNA fragmentation index was examined according to the manufacturer's instructions (Dayan Zist Azma Co.), involving denaturation of sperm DNA and chromatin and observation of DNA strands as a halo around the sperm head (23).

Table 1 Primer sequences used in real-time PCR

Gene	Primer type	Sequence (5'-3')	Length (bp)
DAZL	Forward	CAGAAGATAGTAGAATCACAG	21
	Reverse	CGGATAAGGAGGATATGC	18
BCL2	Forward	CATGCGACCTCTGTTTGA	18
	Reverse	GTTTCATGGTCCATCCTTG	19
GAPDH	Forward	AGTGCCAGCCTCGTCTCATA	20
	Reverse	GATGGTGATGGGTTTCCCGT	19

PCR, polymerase chain reaction; *DAZL*, deleted in azoospermia like; *BCL2*, B-cell lymphoma 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Measurement of testis weight and diameter

The testis weights and diameters of the control and treated rats were measured at the end of the experiment using a scale.

Histological preparation

The left testis was extracted and fixed in Bouin's solution, then dehydrated in graded ethanol solutions with increasing concentrations (50%, 70%, 95%, and 100%). The testis was then cleared in xylene and infiltrated with paraffin wax, before being embedded in paraffin. For histological analysis, hematoxylin and eosin staining was used on 5-µm serial sections of the testis. The specimens were examined under a light microscope (Nikon, Tokyo, Japan) with a DinoCapture camera 2.0 (version 1.5) (18).

Isolation of total RNA and reverse transcription reaction

Total RNA was isolated using the Parstous kit according to the manufacturer's protocol at Azad University, Mashhad, Iran. complementary DNA (cDNA) synthesis was performed following the manufacturer's instructions (Parstous cDNA, Mashhad, Iran).

Real-time polymerase chain reaction (PCR)

Table 1 illustrates the primers designed with AlleleID software (version 6.0) (24). Synthesized cDNA was used in quantitative real-time PCR (QRT-PCR). The reaction mix included 1 µL cDNA, 0.5 µL microRNA (miRNA)-specific forward primer, 0.5 µL universal reverse primer, 6.5 µL miRNA quantitative PCR (QPCR) master mix, and 13 µL H_2O.

Statistical analysis

Data analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 28 (SPSS Inc., Armonk, NY, USA). For normally distributed variables, one-way analysis of variance (ANOVA) was performed. For continuous variables that did not meet the normality assumption, the Mann-Whitney *U* test was applied. The significance level was set at P=0.05.

When data met the assumption of normality, parametric ANOVA was used to compare means between the control and Sustanon-treated groups. If the normality assumption was violated, the non-parametric Kruskal-Wallis test was applied. The results of ANOVA were considered reliable only when the assumption of homogeneity of variance (tested by Levene's test) was met. If Levene's test indicated unequal variances (P<0.05), the Welch's test was used as an alternative to the *F*-statistic.

Results

Testis weight, diameter, sperm count, and morphological analysis

Table 2 presents an overview of significant reductions in the testicular weight, length, and diameter of the treatment groups compared to the control group. Exposure to Sustanon altered sperm chromatin and reduced viability (P<0.05). There was a significantly lower sperm count in the treatment group compared to the control group. Additionally, the results of the optical micrographs, shown in *Table 3* and *Figure 1*, reveal various kinds of sperm deformities in the heads and tails of the rats in the exposed group. Furthermore, as shown in *Figure 2*, many sperms without a halo or with a small halo around the sperm head were observed in the treatment groups, indicating DNA fragmentation.

Histological analysis

Histological examination of the testes from the control group showed seminiferous tubules containing different types of germ cells, with tubules filled with spermatogonia, spermatocytes, spermatids, and spermatozoa along the basement membrane (*Figure 3*). The histological analysis of the testes treated with Sustanon demonstrated the presence

Table 2 Changes in rat testicular length, diameter, weight, and histological parameters in control and treated groups

Parameters	Control	10 mg/kg	20 mg/kg	30 mg/kg
Gonadosomatic index	0.5	0.27	0.26	0.27
SDFA	9.17	28.33	25.58	22.33
Mean of testicular length (mm)	19.6	16.04	16.02	15.49
Mean of testicular diameter (mm)	11.38	8.47	7.74	7.3
Mean of testicular weight (g)	1.482	0.732	0.672	0.675
Thickness of germinal epithelium (μm)	1.298	0.655	0.617	0.608
Diameter of seminiferous lumen (μm)	4.51	3.19	3.16	3.22
Spermatogonia	66	45.8	37.73	33.8
Primary spermatocytes	84.033	57.767	53.833	42.033
Sperm count	39.333	24.033	16.65	15.3
FSH (IU/L)	0.29	0.22	0.21	0.22
LH (IU/L)	0.26	0.2	0.23	0.21

SDFA, sperm DNA fragmentation assay; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Table 3 Percentages of morphologically normal and abnormal sperm in control and Sustanon-treated rats

Morphological parameters	Control	10 mg/kg	20 mg/kg	30 mg/kg
Normal	87%	37%	29%	22%
Without tail	7%	25%	26%	29%
Without head	5%	20%	25%	28%
Curved tail	1%	10%	8%	9%
Coiled tail	0	8%	10%	9%
Double head	0	0	2%	3%

of immature germ cells in the lumen and a reduction in the number of spermatogonia, spermatocytes, and spermatozoa (P<0.05). *Figure 4* reveals a significant reduction in the diameter of the seminiferous lumen and the thickness of the germinal epithelium (P<0.05). The control group exhibited a normal spermatogenesis cycle, with spermatogonia maturing into spermatozoa in all tubules. In contrast, spermatogenesis efficiency decreased in the treatment group, with differentiation not occurring at a dose of 30 mg/kg (*Table 2* and *Figure 4*).

Hormonal analysis

Table 2 shows a marked increase in the mean levels of LH and FSH in the control group compared to the other groups (P<0.05). However, there was no significant difference between the rats treated with different doses.

DAZL and BCL2 gene expression

Figures 5,*6* illustrate the relative expression levels of the *BCL2* and *DAZL* genes in the control and treatment groups. The results demonstrated significantly lower expression of the *BCL2* gene in the treatment groups compared to the control group (P<0.001). The expression of *DAZL* in the group that received 10 mg/kg of Sustanon was significantly higher than in the control group and the groups that received 30 and 20 mg/kg of Sustanon (P<0.05).

In summary, the results indicate that Sustanon administration has an adverse effect on testicular parameters, sperm morphology, and gene expression in male rats, with significant differences observed between the control and treatment groups.

Discussion

AAS are frequently used by athletes, bodybuilders, and youths to enhance muscle mass and physical endurance. However, the high consumption of AAS can lead to serious side effects, including testicular dysfunction, which can result in infertility. The increasing use of Sustanon by

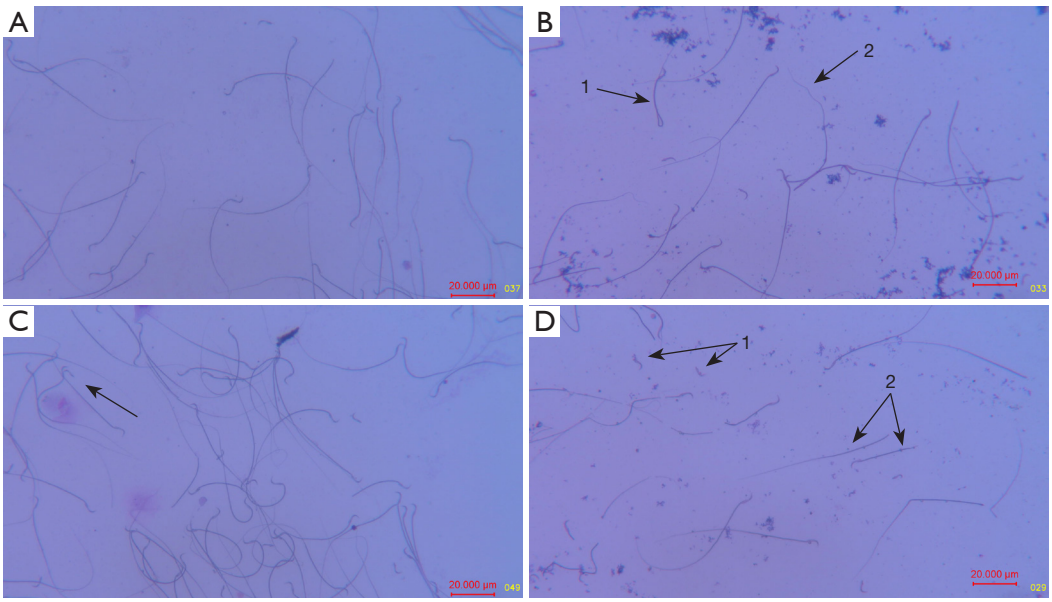

Figure 1 Normal and abnormal rat sperm morphology stained with Diff-Quik rapid sperm staining kit (magnification: ×200): (A) normal sperm; (B) arrow 1, coiled tail; arrow 2, curved tail double head; (C) double head (arrow); and (D) arrow 1, without tail; arrow 2, without head.

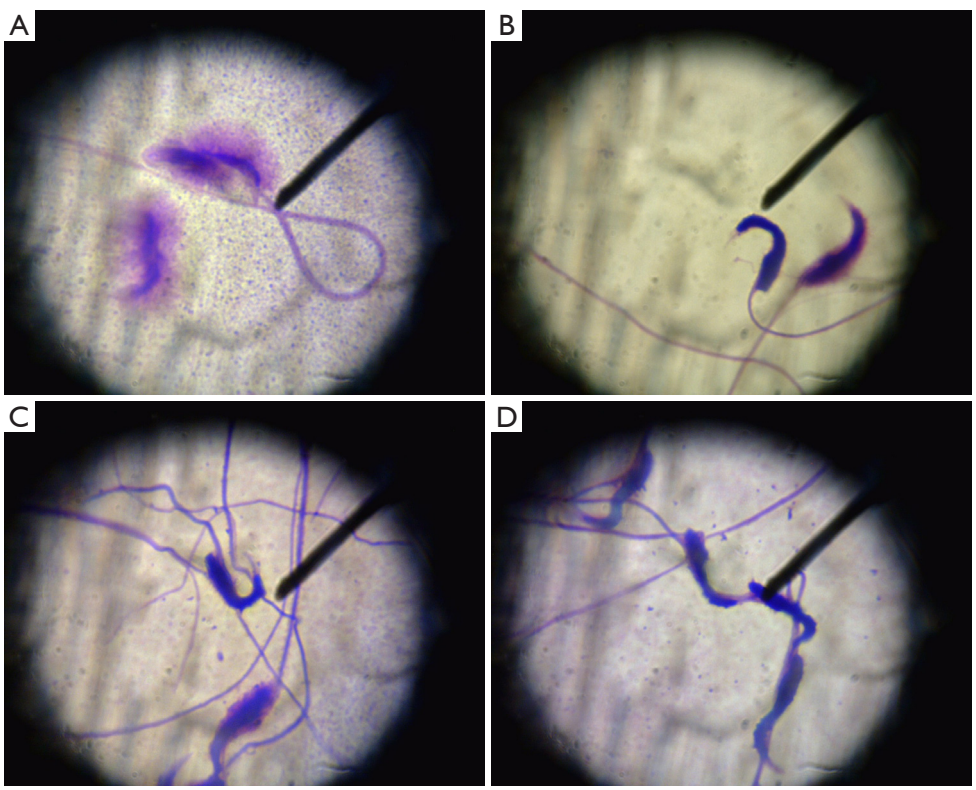

Figure 2 Light microscopy of rat spermatozoa (×40) using the sperm DNA fragmentation assay kit: (A) control group: many sperms with a halo around the sperm head; (B-D) treatment groups with doses of 10, 20, and 30 mg/kg, respectively: many sperms without a halo or with a small halo around the sperm head.

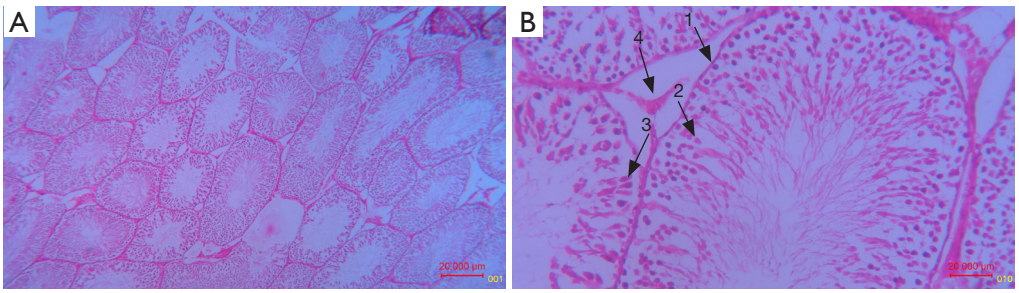

Figure 3 Photomicrograph of a section of rat testis in the control group stained with H&E (A: ×40; B: ×400): (A) seminiferous tubules showing natural appearance and active spermatogenesis; (B) spermatogonia with ovoid dark nuclei and largest cells (arrow 1), primary spermatocytes with large nuclei (arrow 2), Sertoli cells (arrow 3), Leydig cells (triangle; arrow 4). H&E, hematoxylin and eosin.

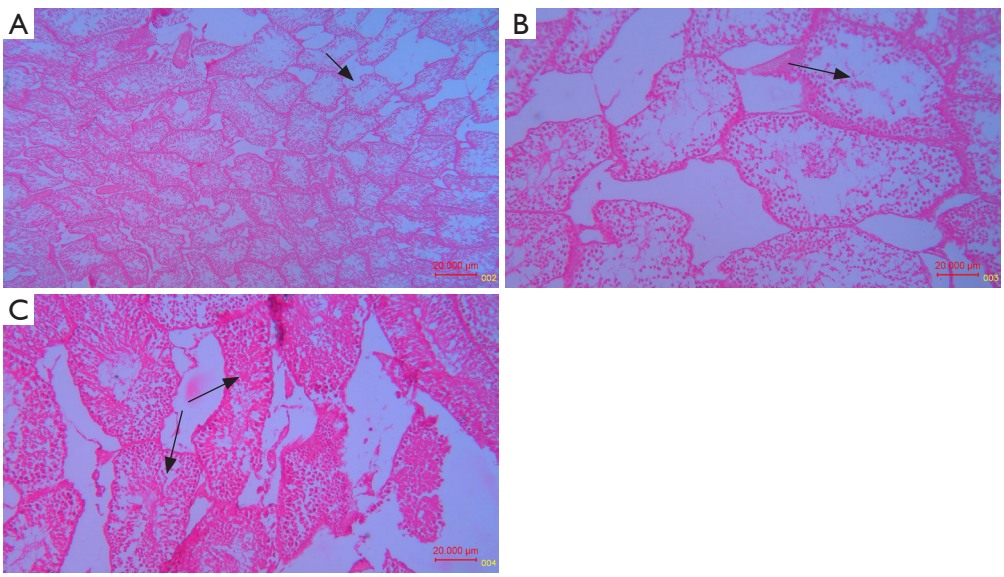

Figure 4 Photomicrograph of a section of rat testis from treated groups with Sustanon, stained with H&E (magnification: A: ×40; B,C: ×100): (A) treatment group with 10 mg/kg dose: destruction of seminiferous tubules (arrow); (B) treatment group with 20 mg/kg dose: reduced thickness of seminiferous tubules and germinal epithelium, with most sperm tubes emptied of sperm (arrow); (C) treatment group with 30 mg/kg dose: some seminiferous tubules without spermatogenesis (arrows). H&E, hematoxylin and eosin.

athletes to improve physical appearance prompted this study to investigate its effects on male reproductive health.

In this study, doses of 10, 20, and 30 mg/kg Sustanon were injected intramuscularly into rats in groups 1, 2, and 3, respectively. Our findings indicate that Sustanon administration induces adverse effects on the male reproductive system and decreases fertility.

Testicular weight and size

The study reported a significant reduction in the mean testicular weight and diameter in the treatment groups compared to the control group. These results agree with findings from other studies (25,26). For example, Mohd Mutalip *et al.* (26) reported a significant decrease in testicular weight in male Sprague Dawley rats following the administration of testosterone and anabolic steroids for 6 weeks. The weight of reproductive tissue is an indicator of the endocrine effects of AAS. The suppressive effects on testis weights observed in our study indicate the impact of AAS on fertility, consistent with previous research (20,27). Chang *et al.* (28) also documented a reduction in testicular

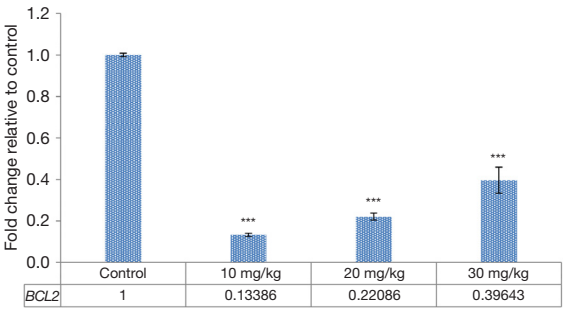

Figure 5 Expression of *BCL2* in rat testes in control and treatment groups with Sustanon doses of 10, 20, and 30 mg/kg. ***, significant difference at P<0.001. *BCL2*, B-cell lymphoma 2.

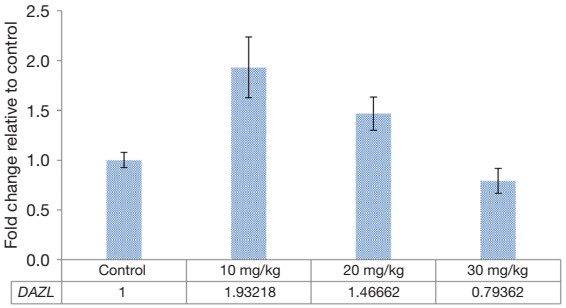

Figure 6 Expression of *DAZL* in rat testes in control and treatment groups with Sustanon doses of 10, 20, and 30 mg/kg. *DAZL*, deleted in azoospermia like.

dimensions following AAS exposure.

Hormonal impact and feedback mechanism

Exogenous testosterone, such as Sustanon, exerts a negative feedback effect on HPG axis, leading to the inhibition of endogenous testosterone production. The hypothalamic gonadotropin-releasing hormone (GnRH) regulates the synthesis and secretion of LH and FSH. FSH affects Sertoli cells, and its production in men is regulated by circulating testosterone and inhibin, both produced by the testes. FSH stimulates Sertoli cells to produce androgen-binding protein (ABP), which is crucial for spermatogenesis (29).

Previous studies have suggested that weekly administration of nandrolone decanoate (10 mg/kg) can suppress the secretion of LH and FSH in treated animals (18,30). Our study supports these findings, demonstrating that Sustanon disrupts hormonal regulation, leading to decreased spermatogenesis and testicular atrophy.

Sperm count, morphology, and DNA fragmentation

Sustanon treatment resulted in a significant reduction in sperm count and altered sperm morphology. These findings are consistent with previous studies showing that AAS adversely affect sperm parameters. Testosterone is essential for continuous sperm production, and its reduction, along with oxidative damage, contributes to decreased sperm count and quality (21,31). Increased formation of free radicals and subsequent apoptosis of germ cells due to Sustanon administration likely explain the observed reduction in sperm count (32).

AAS can increase germ cell apoptosis, with apoptosis in germ cells being associated with the Fas-signaling system activated by exogenous toxicants (33). Our study demonstrated that Sustanon increased apoptotic changes in the testes of treated rats, with histological changes including severe degradation of seminiferous tubules and increased germ cell apoptosis observed in the seminiferous epithelium (34). Nolan and Levy (35) reported that high doses of Sustanon induced early apoptotic activity in gonadectomized animals. These findings are in agreement with our results, highlighting the pro-apoptotic effects of Sustanon on germ cells (31).

Gene expression: DAZL and BCL2

This study evaluated the expression of *DAZL* and *BCL2* genes, both of which play critical roles in spermatogenesis and apoptosis. Our findings revealed a significant decrease in *BCL2* expression in the treatment groups compared to the control group (P<0.001), indicating increased apoptotic activity in the testes of Sustanon-treated rats. The *BCL2* gene functions as a critical regulator of apoptosis, and its reduced expression suggests that Sustanon administration induces apoptosis, consistent with previous studies on AAS-induced apoptosis in testicular cells (25).

Although the effects of Sustanon on *BCL2* have been reported previously, our study sought to confirm these findings in the specific context of our experimental design, which also included sperm morphology and testicular histology. While other apoptosis markers such as *Bax* and the *BAX/BCL2* ratio were not assessed in this study due to resource constraints, their inclusion in future research would further clarify the molecular mechanisms underlying Sustanon-induced apoptosis. These markers, when studied together, provide a more comprehensive view of the pro-apoptotic pathways activated by AAS, as suggested by other research.

In contrast, *DAZL*, a gene essential for germ cell development and sexual differentiation (30,36), exhibited significantly higher expression in the group receiving 10 mg/kg of Sustanon compared to both the control and higher-dose groups ($P<0.05$). This finding is consistent with the work of Zhang *et al.* (37), which demonstrated that lower doses of AAS can enhance *DAZL* expression. *DAZL* is primarily active during the pachytene stage of spermatogenesis and is not dependent on gonadotropin regulation, which aligns with our observations that there was no significant increase in DAZL expression at the 20 and 30 mg/kg doses (38).

Conclusions

In conclusion, this study confirms that Sustanon significantly adversely affects male reproductive health. The observed reductions in testicular weight, sperm count, and alterations in sperm morphology and DNA integrity highlight the detrimental impact of AAS on fertility. Disruptions in hormonal regulation and increased apoptosis further underscore the potential risks associated with Sustanon use. These findings are consistent with the theory underpinning our hypotheses and contribute to the existing body of knowledge by providing detailed insights into the specific effects of Sustanon on male reproductive parameters. These results emphasize the need for caution regarding AAS use, especially among athletes and individuals seeking to enhance physical performance.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-24-397/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-397/dss

Peer Review File: Available at https://tau.amegroups.com/article/view/10.21037/tau-24-397/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-24-397/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. IR.IAU.MSHD.REC.1398.214) granted by the Ethics Committee of Islamic Azad University, Mashhad Branch, in compliance with national guidelines for the care and use of laboratory animals.

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