

The Efficacy of Tribulus Terrestris Extract on Sperm Parameters and Sexual Function in Male Mice: A Potential Complementary Treatment for Male Infertility

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Abstract

Medicinal plants have gained increasing attention for their potential in treating infertility. Tribulus Terrestris, a widely used traditional medicinal plant, is reputed for its aphrodisiac and fertility-enhancing effects. This study aimed to evaluate the impact of Tribulus Terrestris extract on spermatogenesis and Androgen receptor (Ar) gene expression in male laboratory mice. Forty-two adult male BALB/c mice were randomly assigned to seven groups. Tribulus Terrestris extract was administered daily via gavage at doses of 5, 20, 40, 80, 160, and 320 mg/kg body weight for 55 days. Blood samples were analyzed for testosterone levels, sperm parameters were assessed using the CASA system, and Ar gene expression was measured in testis tissues through reverse transcription-real-time PCR. An increase in Tribulus Terrestris extract dose correlated with body weight gain. Notably, testosterone levels were significantly higher in the 80 mg/kg group compared to others ($p < 0.05$). Sperm motility, count, and viability were significantly improved in the 40 and 80 mg/kg groups ($p < 0.05$). Furthermore, Ar gene expression was significantly elevated in the 80 mg/kg group ($p \leq 0.05$). Tribulus Terrestris extract positively affects sperm parameters and sexual function in male mice. These findings support the potential of Tribulus Terrestris as a complementary treatment for male infertility.

Introduction

Male factor infertility accounts for 30–50% of infertility cases (1). Major contributors to male infertility include testicular disorders resulting in low sperm quality and count, which can be assessed through concentration, morphology, and motility(2). Male infertility can also arise from various factors, including reproductive system infections, chromosomal abnormalities, genitourinary cancers(3), hormonal imbalances(4) and unhealthy lifestyles(5). Various treatment options including medical/herbal, laboratory techniques, and surgical interventions exist to treat male infertility(6). Many couples prefer Complementary and Alternative Medicines, particularly herbal products, due to their perceived cost-effectiveness and accessibility. Herbal products, including Tribulus Terrestris, have been used in traditional medicine across different cultures, with studies highlighting its nutritional and biological benefits (7). The plant has had significant applications in traditional medicine in China, India, Iraq, Bulgaria, South Africa, as well as Iran. Tribulus Terrestris contains bioactive compounds like steroids, saponins, flavonoids, alkaloids, unsaturated fatty acids, vitamins, tannins, resins, potassium nitrates, aspartic acid, and glutamic acid(8), which are believed to stimulate spermatogenesis due to their effect on Sertoli cells(9). Additionally, Tribulus Terrestris extract is known for enhancing physical endurance, muscle mass, red blood cell count, blood circulation, and oxygen transfer(10). Studies have demonstrated its positive effect on sexual hormones, behavior, and ejaculation in men(10).

On the other hand, the *AR* gene plays a crucial role in male reproductive system development and spermatogenesis. Mutations such as A240S in the AR gene have been linked to azoospermia infertility(11). Hormonal regulation influences AR gene expression, affecting hormone secretion(12). Previous study have explored AR protein function and its impact on spermatogenesis using modified mouse models(13). A lot of studies have examined the significance of the lengths of trinucleotide

repeats GGN and CAG in the function of the AR protein. Trinucleotide repeat lengths, particularly GGN and CAG, in the AR gene have been associated with male infertility(14–16). Previous studies have shown that Tribulus Terrestris supplementation enhances sexual hormones, improves sexual behavior, and ejaculation in mice, suggesting a potential positive impact on spermatogenesis. Given the importance of androgens and the AR gene in spermatogenesis and male reproductive system function, our study aims to investigate the in vivo effects of Tribulus Terrestris extract on *Ar* gene expression and sperm parameters in testicular tissue of mice.

Materials and Methods

To prepare Tribulus Terrestris fruit extract

The Tribulus Terrestris plant was collected from the Botanical Garden of Iran with herbarium number 87328, growing at an altitude up to 1050 meters above sea level. The fruit of the plant was processed by a device, ground into a powder, and 100 grams of the plant powder was mixed with 100 ml of Merck ethanol and kept in the laboratory for one hour. The liquid and solid phases of this solution were separated by an ultrasonic standing waves device for 94 min. The separated liquid was put in a rotary evaporator (200g, 60 min, 20°C) to separate ethanol from the original extract. The extracts were subsequently stored in dark sterile glass at 4°C.

Quality control of Tribulus Terrestris extract

Examination of the quality of Tribulus Terrestris extract was conducted using Gas GC-MS (Agilent Technologies, USA, Model; 7890A-5975C). The maximum sample melting temperature for this analysis was approximately 400°C.

Animals of treatment

In this experimental study, a total of 42 adult male mice (Balb/C strain, 8–12 weeks old, 22–25 g)

were divided into seven groups of six randomly. They were kept at a temperature of 22°C to 27°C with 55 to 60% humidity and an 12:12-h light/dark cycles for 8 weeks at the department of laboratory animals of Royan Institute, according to the DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL. The animals had access to water and food. The prepared Tribulus Terrestris extract was administered to the mice in six doses of 5, 20, 40, 80, 160, and 320 mg/kg of body weight for each group consistently for 55 consecutive days in gavage form once daily. Additionally, control group received only distilled water. Measurement of the body weight of mice was recorded and compared for 8 weeks.

Testosterone measurement

After the experimental period, animals were anesthetized using a mixture of 10% ketamine and 2% xylazine (Alfasan, Netherlands, CAS Number: 61763-23-3) based on body weight. Then 2 ml of blood was directly taken from the live mouse's heart with a syringe. The samples were centrifuged, and the serum were collected and stored at -80 °C until hormone analysis. Testosterone hormone levels were measured using the Testosterone ELISA kit (IBL, Germany).

Collection and determination of sperm parameters

The mouse was dissected after blood sampling. The mice were sacrificed by cervical dislocation, the cauda epididymis was removed and placed in a sterile test tube containing 1 ml of fetal bovine serum (Sigma, USA. CAS Number: M 2520) and 10% Hams F10 culture medium (Biotest, Germany. CAS Number: B 05AA01) for sperm retrieval. Then the samples were incubated at 37 °C for 30 min to allow sperm to be released into the solution. Sperm parameters (sperm count and motility) were analyzed by a CASA (NikonTM, Eclipse E-200, Japan) as described by Krause (1995). Eosin-Y staining was used to evaluate sperm viability. In this method, 10 µl of sperm sample was mixed with 10 µl of dye (0.5% wt/vol; Merck Chemical Co., Darmstadt, Germany). In each slide, 200 sperm cells were counted. Live unstained sperm cells and dead red stained sperm cells were evaluated by a phase contrast microscopy (Nikon Co.).

RNA extraction and cDNA synthesis

Initially, a part of the testicular tissue was placed in liquid nitrogen for gene expression analysis until further experiments were conducted. Testicular tissue RNA was extracted using the TRIzol method following the protocol provided by Invitrogen. The tissue was rapidly homogenized using by scalper for 30–60 s on ice, and then 0.2 ml of chloroform per 1 ml of TRIzol reagent was performed. The samples were Centrifuge for 15 min at 12,000×g at 4 °C. The upper transparent aqueous phase (~ 500 µl) containing the RNA was removed and placed into a sterilized microcentrifuge tube. RNA samples were treated with DNase1 (Fermentase: Gran Island, NY, USA. CAS Number: EN0521) for 10 min at 75 °C and then RNA concentration (ng/µl) and purity at ratios A260/A280 were recorded using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was evaluated by agarose gel electrophoresis (Sigma, USA, Cat. CAS Number: A9539) to detect of RNA bands on Gel Doc (Gel DocTM XR + and ChemiDocTM XRS + Systems with Image LabTM Software). cDNA synthesis was performed using the cDNA synthesis kit (Fermentas: Gran Island, NY, USA. CAS Number: K1621) according to the manufacturer's instructions to convert RNA to cDNA.

Real-Time Quantitative PCR

Housekeeping gene, *Gapdh* (Glyceraldehyde 3-phosphate dehydrogenase), was used as a reference gene. The primers of *Gapdh* and *Ar* (Androgene receptor) were designed by the PerlPrimer software

(version 1.1.21). The chosen primer pairs were checked using BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the UCSC genome browser (<http://www.genome.ucsc.edu>).

qRT-PCR was carried out by utilizing the Power SYBR Green PCR Master (Applied Biosystems, Foster City, California). The thermal cycling program were followed by 40 cycles of denaturation step at 95°C for 4 min, annealing step at 60°C for 30 s, and extension step at 72°C for 30 s. The method was repeated 2 times independently for all samples. The threshold cycle values were normalized against threshold value of mice *Gapdh*. Relative gene expression was compared between the groups. The relative expression of the *Ar* gene to the *Gapdh* gene was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 16). After determining the normality of the data, one-way analysis of variance (ANOVA) and Duncan's multiple-range test comparison were used to compare the experimental groups. The results were reported as mean $\pm$ standard deviation. Differences were considered statistically significant at $p \leq 0.05$.

Results

Qualitative Examination of Tribulus Terrestris extract

The results of the qualitative analysis of the extract of Tribulus Terrestris by chromatograph are given in the figure 1.

Body Weight Changes in Mice

As shown in the figure 2, the descriptive research findings indicate a significant increase in the body weight of mice over time, correlating with the dosage of Tribulus Terrestris.

Testosterone Level

Figure 3 illustrates a significant difference in the average testosterone levels among the investigated groups. Duncan's test revealed that the 80 mg group exhibited notably higher testosterone levels compared to other groups. However, testosterone levels increased significantly in the 160 mg, 40 mg, 320 mg, 20 mg, 5 mg, and control groups, respectively (Table 1).

Cauda Epididymis Sperm Quality

The results from Duncan's test, as outlined in table 2 and figure 4, highlight a significant increase in sperm parameters (including average count, progressive motility, percentage of normal morphology and viability) in 80 mg and 40 mg groups compared to the other groups ($p \leq 0.05$).

Gene Expression of *Ar* in Mice Testicular Tissue

Real-time PCR analysis of *Ar* mRNA expression levels in testicular tissue revealed a significant upregulation in the 80 mg group compared to other groups ($p \leq 0.05$). Additionally, *Ar* mRNA expression was upregulated in the 40 mg, 160 mg, and 320 mg groups compared to the control groups 5 mg and 20 mg ($p \leq 0.05$) (Figure 5).

Discussion

In recent years, there has been extensive global research into the potential use of medicinal plants for addressing the sexual dysfunctions. In the current study, a direct correlation between the passage of time, the escalating dosage of *Tribulus Terrestris*, and a significant increase in mice weight. was observed. A study conducted by El-Tantawy, Temraz, and El-Gindi (2007) revealed that the alcoholic extract of certain parts of the *Tribulus Terrestris* plant, administered at a dose of 50mg/kg, led to a significant elevation in the serum-free testosterone production in mice(17). Similarly, Gauthaman & Ganesan (2008), demonstrated that *Tribulus Terrestris* supplementation increased blood testosterone levels in castrated mice(18). In our study, the aqueous extract of the *Tribulus Terrestris* significantly increased the testosterone levels in 80 mg group compared to the others. Recently, it has been observed that an alcoholic extract of certain parts of the *Tribulus Terrestris*, at a of 50mg/kg dose, significantly enhanced sperm motility and count (19). Likewise, the results of the present study revealed a significant increase in the average number of spermatozoa cells, sperm motility, morphology and viability in 40 mg and 80 mg groups compared to the control group.

Tesarik, Guido, Mendoza, and Greco (1998) found that follicle-stimulating and testosterone hormones alone play an enhancing role in spermatogenesis and Sertoli cell survival. (20). *Tribulus Terrestris* contains various estradiol glycosides such as saponin, which stimulate the increases in testosterone level. The natural estradiol, in turn, may facilitate the conversion of estradiol to androgen as an intermediate, therefore, leading to an elevation in the testosterone hormone levels. Also, this hormone promotes the process of spermatogenesis. Moreover, testosterone hormone performs its biological function by binding to the androgen receptor. Wu, Yang, and Wang (2017) noted that *Tribulus Terrestris* extract administration to rats undergoing high exercise training resulted in increased testosterone levels, enhanced AR protein expression, and muscle weight gain. (21). The findings of the present study showed that the treatment with *Tribulus Terrestris* extract significantly increased the expression of the *Ar* gene. Notably, the average expression of this gene in Groups 40 mg and 80 mg was significantly higher than in other groups, providing support for the hypothesis that *Tribulus Terrestris* extract elevates testosterone levels. This is the first study to document that *Tribulus Terrestris* extract not only enhances testosterone levels but also increases *Ar* gene expression in mouse testes.

Conclusion

In conclusion, our findings suggest that *Tribulus Terrestris* extract administration effectively improves sperm parameters and sexual function in male mice. Further investigations are recommended to determine the appropriate dosage for human trials.

Declarations

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgments

The authors would like to dedicate this paper to the memory of Dr. Saeid Kazemi Ashtiani, the late founder of Royan Institute.

Author Contributions

A. Mohseni Meybodi was involved in the conception and design. M. Sabbaghian and A. Mohseni Meybodi provided overall supervision. F. Fazeli and P. Mokhtari participated in all experimental work, data analysis, statistical analysis, and interpretation. P. Mokhtari drafted the manuscript, which underwent revisions by A. Mohseni Meybodi and M. Sabbaghian. All authors reviewed and approved the final manuscript.

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Ethical Approval

This experimental study approved by the Ethics Committee of Royan Institute (Approval ID: Ec/92/1084), Tehran, Iran.

Competing Interests

The authors declare that they have no conflicts of interest in the research.

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Tables

Tables 1 and 2 are available in the Supplementary Files section.

Figures

Figure 1

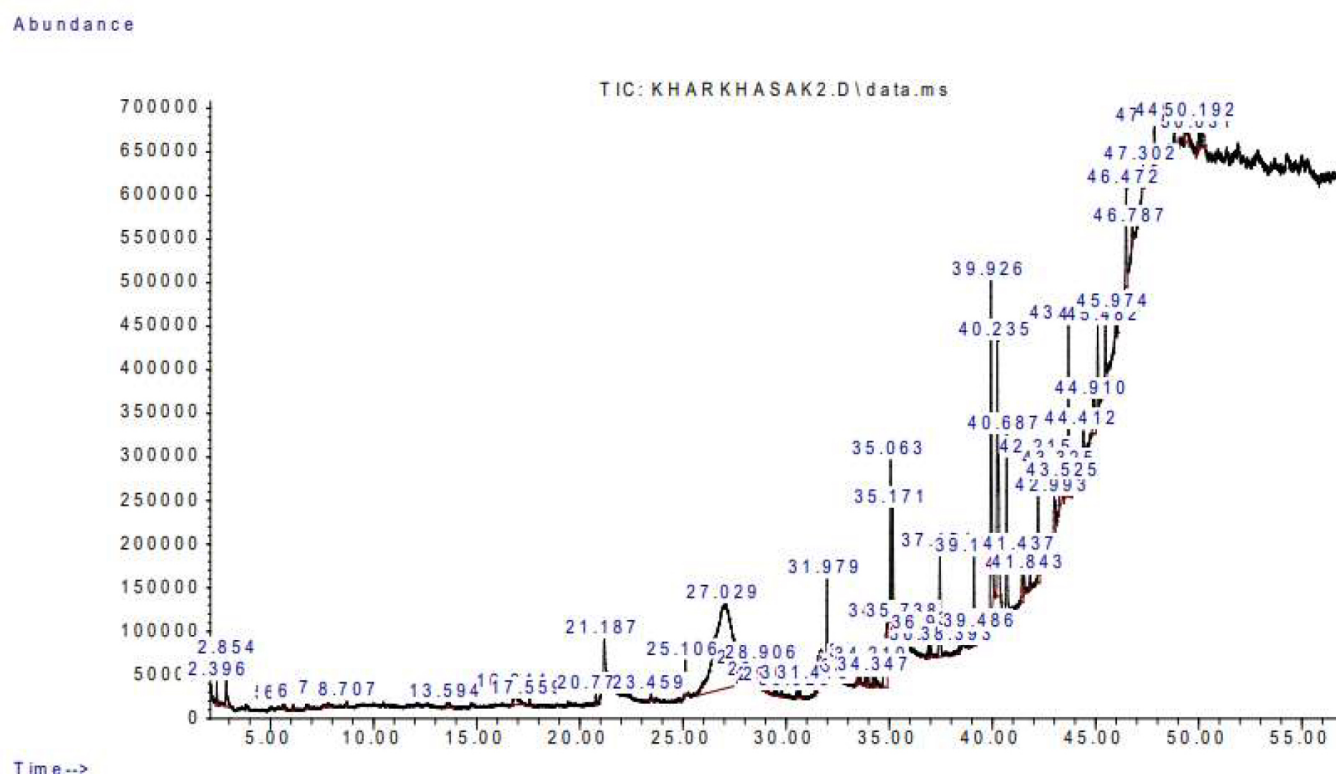


Figure 1

Qualitative analyses of the extract of *Tribulus Terrestris* extracts by using GC-MS. (Kharkhasak is the Persian name of *Tribulus Terrestris*)

Figure 2

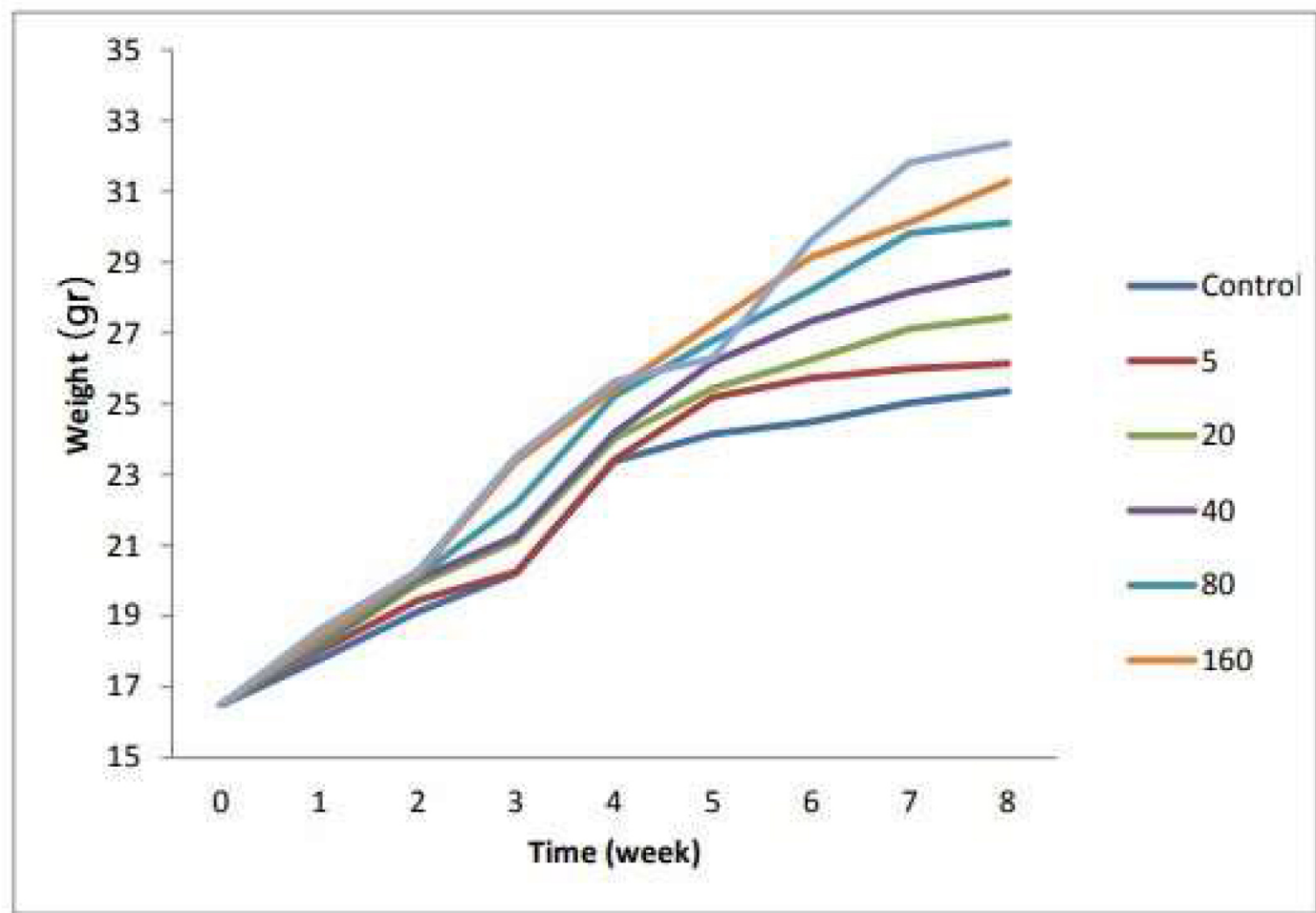


Figure 2

The comparison of the average weight of mice

Figure 3

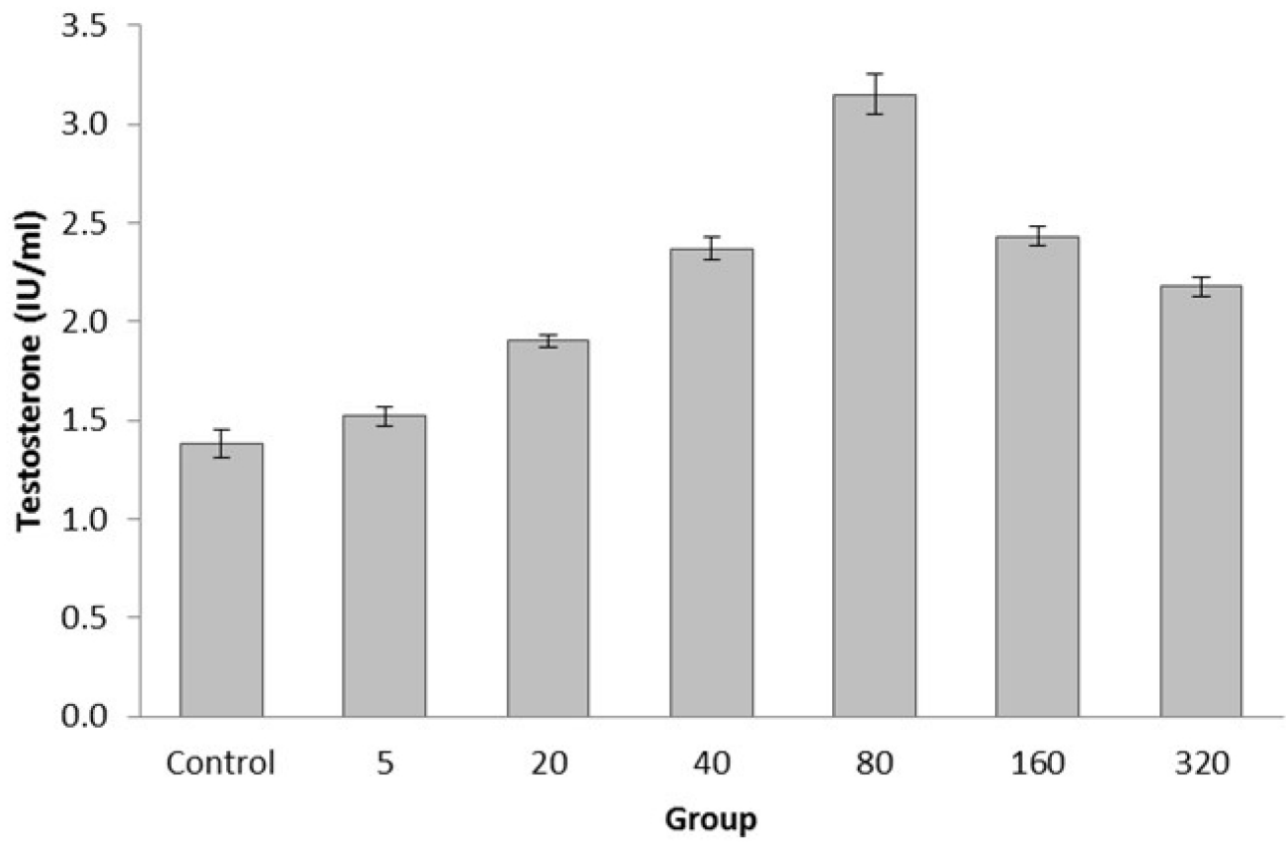


Figure 3

The comparison of mean testosterone level (Error bars: $\pm$ STDV)

Significant difference at level $p \leq 0.05$

Figure 4

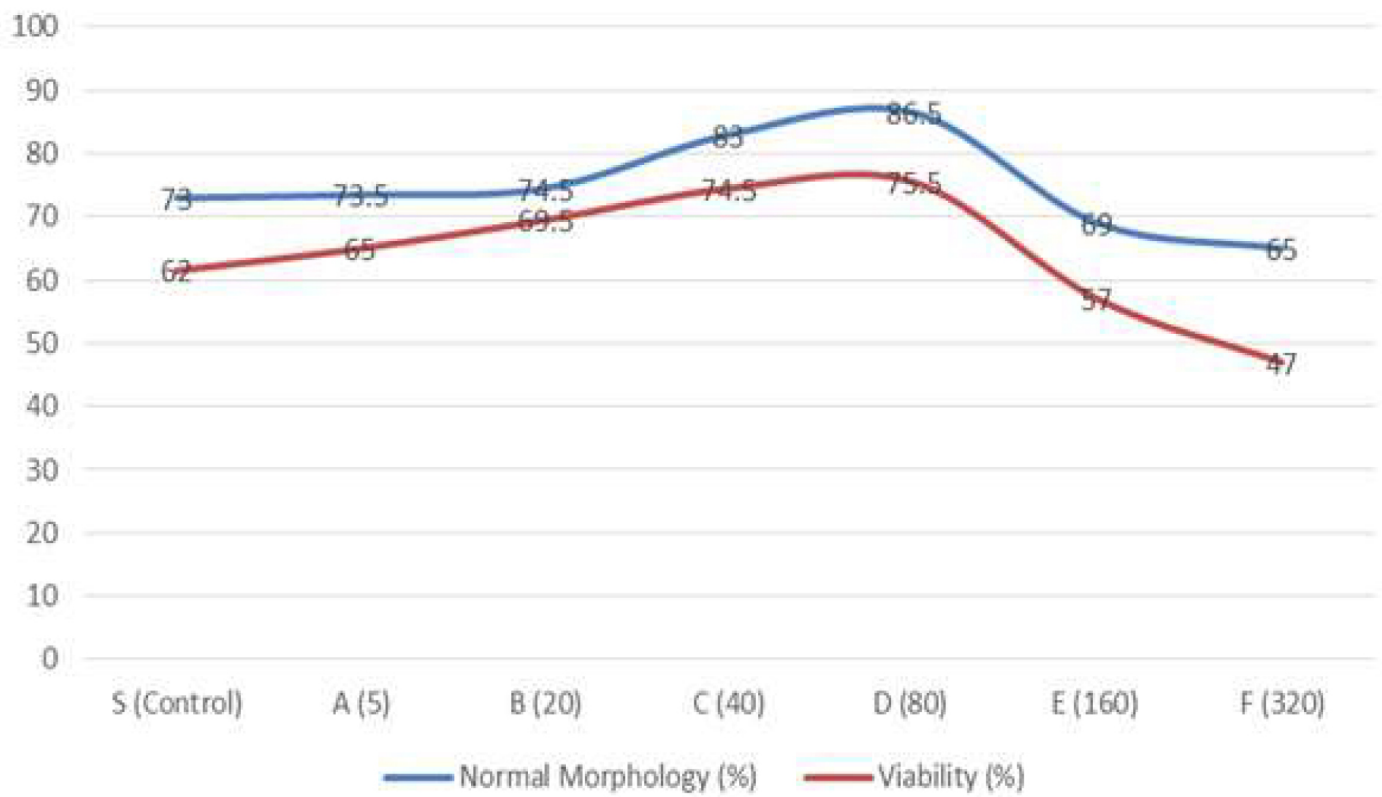


Figure 4
Comparison of average sperm morphology and viability

Figure 5

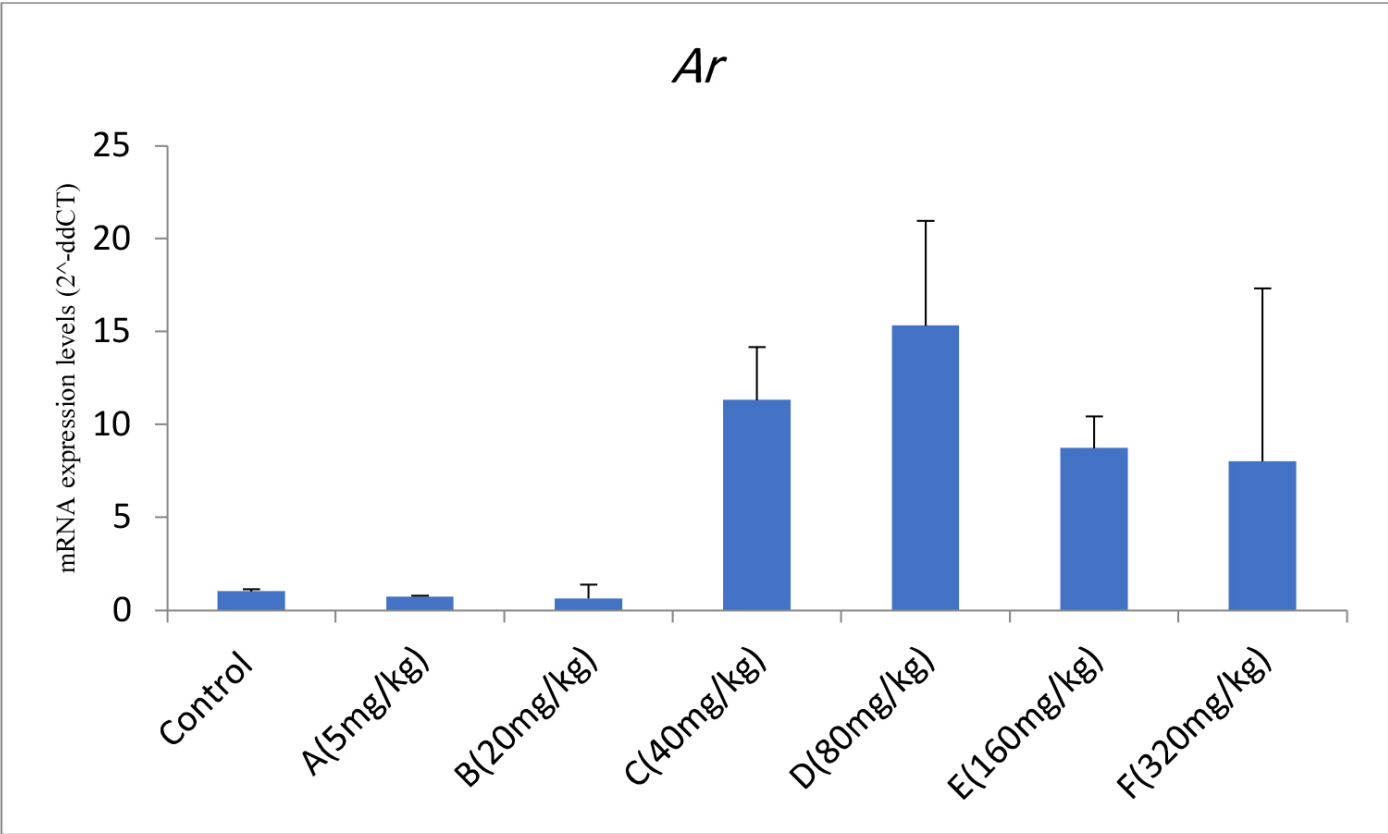


Figure 5

The comparison of *Ar* expression in in different groups with the control group using real-time PCR. (Data analyzed by One Way ANOVA Error bars: $\pm$ STDV). Significant difference at $p \leq 0.05$

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.docx](#)
- [Table2.docx](#)