Journal of Animal & Plant Sciences, 33(2): 2023, Page: 389-396
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2023.2.0629

EFFECTS OF DIFFERENT DOSES OF SHIITAKE MUSHROOM (*LENTINUS EDODES*) ON SPERM PARAMETERS AND TESTICULAR TISSUE IN RATS: AN EXPERIMENTAL STUDY

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ABSTRACT

Lentinus edodes has been cultivated and used as food and medicine in far eastern countries.The present study aimed to reveal whether *L.edodes* that is used for different purposes has a toxic effect on the male genital system and to investigate its relationship with male infertility by showing its effects on sperm parameters. Twenty-four male Wistar rats were randomly divided into three groups as Control (C), Low-dose (LD) and High-dose (HD). Rats in Control were administered saline physiological once a day for 7 days. The LD and HD groups received 100 and 400mg/kg of *L.edodes* extract, respectively, once daily for 7 days. At the end of 7 days of administration of treatments, semen analysis, micronucleus analysis, gene expression levels and testicular Johnsen scoring was performed. Comparisons between groups without normal distribution were analyzed using Kruskal–Wallis variance analysis and those between groups with normal distribution were analyzed using one-way analysis of variance, followed by Tukey's test. P values of <0.05 were considered statistically significant. It is demonstrated that both low and high doses of extract significantly reduced spermatogenesis in seminiferous tubules found in the testicles of rats. In addition, high-dose administration resulted in decreased total sperm counts, whereas the progressive and non-progressive sperm motility counts were significantly decreased in both experimental groups. The gene expression levels of Casp-3 and TNF-α were increased in both the LD and HD groups compared with that in the C. Oral administration of low and high doses of *L.edodes* led to reduced spermatogenesis in the testicles, tissue damage to the testicle and decreased motile and total sperm counts.

Keywords: Infertility, *Lentinus edodes*, Male Rat, Shiitake mushrooms, Testis

Published first online December 18, 2022 Published final March 24, 2023

INTRODUCTION

Shiitake mushroom (*Lentinus edodes*, SHI) is one of the white rot fungus species (Lee *et al.,* 2017). SHI has been cultivated and used as food and medicine in far eastern countries for thousands of years. Today, SHI is the world's second most consumed mushroom and one of the most cultivated edible mushroom species worldwide (Wasser, 2005).

SHI has an excellent nutritional value; its crude stems contain a total of water (88-92%), proteins, carbohydrates, lipids, vitamins, and minerals. Dried shiitake mushrooms are rich in carbohydrates (58-60%), protein (20-23% with digestibility of 80-87%), lipid (3-4%), and fiber (9-10%). They are also a good source of vitamins including 32.5% provitamin D2 (ergosterol)followed by B vitamins. The minerals found in SHI are Fe, Ca, Cu, P, K, Mg, Mn, Zn, and Cd. About 1–5% of the dry weight of SHI correspond to water-soluble polysaccharides (Wasser, 2005).

These mushrooms have been used as a food and medicinal product for thousands of years due to their biological activities, such as antitumor, antiviral, and anti-inflammatory. Lentinan, a β-1, 3-glucan polysaccharide isolated from SHI, has been medically used in Japan since 1980 due to its immunomodulatory and antitumor effects (Chihara *et al,* 1987). The antitumor effect of lentinan has been thought to be T-cell-mediated and has been shown to play an important role in the thymus-dependent immune mechanism (Wasser, 2002). However, recent studies have indicated that lentinan directly suppresses the proliferation of liver cancer cells, colon cancer cells, and murine skin carcinoma cells (Ng and Yap, 2002; Gu and Belury, 2005; Wang *et al*., 2017a; Wang *et al.,* 2017b). Lentinan administration reportedly enhances the antigen-presenting functions of dendritic cells, thereby stimulating tumor-specific cytotoxic T cells (Ina *et al.,* 2013). In a study of a rat model of colon cancer, different doses of SHI added to diets showed inhibition of tumor growth and metastasis through

antitumor effects (Frank *et al.,* 2006). Moreover, studies have shown that SHI extracts have antimicrobial properties (Hearst et al., 2009).

Patients with cancer possess a great risk of infertility due to both the disease itself and the drugs used in the treatment (McBride and Lipshultz, 2018). It is important to understand the effects of SHI, which is frequently used by patients with cancer due to its antitumor activity, on testicular histology and spermatogenesis for prevention and treatment of infertility in these patients. Infertility is defined as the failure to conceive after 1 year of regular, unprotected sexual intercourse (Lundy and Vij, 2019). Male infertility is observed in one-third of all infertile couples. All living cells are normally exposed to some type reactive oxygen species (ROS), but if increase of ROS levels, oxidative stress (OS) occurs, which increases the rate of cellular injury. Many previous studies have shown that OS is one of the causes of male infertility. The majority of infertile men have a high level of seminal ROS. Sperm DNA damage, OS, and apoptosis are obviously involved in the pathogenesis of male infertility (Agarwal and Said, 2005). Malignant neoplasms begin to have multifactorial negative effects on normal spermatogenesis long before cancer is diagnosed.

Given the antioxidant and anticancer effects of SHI, to the best of our knowledge, no study has investigated these effects on infertility, especially male infertility, in the literature. Therefore, the present study aimed to reveal whether SHI that is used for different purposes has a toxic effect on the male genital system and to investigate its relationship with male infertility by showing its effects on sperm parameters using rats as experimental animals.

MATERIALS AND METHODS

Materials and experimental design: This study was approved by Institutional Animal Use and Care Committee of Canakkale Onsekiz Mart University (COMU) (Approval No: 2018/11-04) and performed in accordance with the Helsinki Declaration of World Medical Association recommendations on animal studies.

A total of twenty-four Wistar adult male rats were obtained from COMU Experimental Research Application and Research Center with a mean age of five months and mean weight of 280-300 g. The rats were housed in stainless steel cages in an animal room maintained at a standard humidity (45-55%) and temperature 22±2°C with 12 hour light/dark cycles. All animals were fed standard food and water. Twelve hours before the study procedure, feeding was stopped and the rats were only allowed to drink water. The entire experiment was conducted under half-sterile conditions.

***Lentinus edodes* extracts:** Extraction of *L. edodes* was performed according to previous literature (Khan *et al*., 2011). Dried mushroom powder was obtained from Agroma Mushroom Breeding Centre (Agroma Gıda Tarım Hayvancılık San. Tic. Ltd. Şti. Denizli/Turkey) where *L. edodes* is permitted to be dried according to Food and Feed Law No. 5996 by the Ministry of Food, Agriculture and Livestock of Turkey. Solvents, ethyl alcohol (Merck Milipore, Germany) and ultra purified water (Millipore Milli-Q water purification system),with different polarity were used to obtain extracts from the fungus of *L. edodes*. Before extracts were obtained, the dried samples were divided into 10-g packages with filter paper and extracted in the Soxhlet device (Wisd, Wise Therm). In this process, 300 ml of ethanol was used for 10 g of sample. After 12 h of extraction, the samples wrapped with filter paper were removed from the device and left to dry. The solvent-free sample was ready to be extracted with the ultra purified water, wherein extraction was conducted with ethyl alcohol (C_2H_6O, Boiling Point: 78.37°C) and ultra purified water (H_2O, Boiling Point: 100°C). Then, the solvents of the extracts were completely evaporated through the evaporator (Spectral, Heidolph, Laborota 4001). The resulting crude extracts were stored in the refrigerator at 0–4°C.

Experimental procedure: A total of 24 male Wistar rats were randomly divided into three groups with eight animals in each group (doses are determined based on previous studies (Grotto *et al.,* 2016):

Group 1: Saline physiological (Control, C) group (n = 8)
Group 2: SHI 100 mg/kg, low-dose (LD) group (n = 8)
Group 3: SHI 400 mg/kg, high-dose (HD) group (n = 8)

Rats in Group 1 (Control group, C) were administered 2 ml saline physiological (SF) through gavage once a day for 7 days. The LD and HD groups received 100 and 400 mg/kg of *L. edodes* extract, respectively, prepared every day in 2 ml of SF via gavage once daily for 7 days.

Organ harvesting and sperm collection: Organ harvesting was conducted as described previously in the literature (Naghdi *et al.,* 2016). Briefly, at the end of 7 days of administration, a single midline incision was made on the scrotal sac and the skin was separated from the muscle layer under combination of ketamine hydrochloride (35 mg/kg, Ketalar®, Pfizer Drugs Ltd., Sti, Istanbul, Turkey) and xylazine (10 mg/kg, Alfazyne 2%, Ege Vet San. Tic, Izmir, Turkey) anesthesia. Then, a similar transverse incision was made through the muscle, and the testicle was removed out. The cauda epididymis was separated from the testicle with the adipose layer and dissected in 1.5 mL of pre-warmed phosphate-buffered saline (pH = 7.4) at 37°C. The tissue sample was gently shaken in a petri dish to disperse the spermatozoa. Samples were incubated for 20 min at 37°C, and the

spermatozoa were examined in the Makler counting chamber under the microscope.

Johnsen scoring for testicular sections: After epididymal sperm collection, the total left testes of rats were removed and fixed in Bouin fixative. Following fixation and routine tissue tracking procedures, the testes were embedded in paraffin. Routine hematoxylin-eosin (H-E) staining protocol was performed using 5-µm sections from paraffin embedded tissues.

Two sections of each testicle obtained from the rats were selected, and a total of 100 seminiferous tubules were evaluated for each rat's section and scored from 1 to 10 according to the Johnsen criteria (Johnsen, 1970). Light microscopic assessments were conducted under the light microscope of Zeiss Axioscope A1, and ZenBlue program was used for imaging.

Semen analysis: Semen motility was analyzed according to World Health Organization (Fifth edition) criteria (Büyük *et al.,* 2021, Naghdi *et al.,* 2016). After the epididymal sperm sample was obtained and incubated as described above, a drop of 10 µL of semen samples was introduced in the Makler counting chamber. Under light microscope, 10 small squares were counted and total sperm, progressive sperm motility non-progressive sperm motility, and immotile sperm counts were determined and the data were numerically recorded.

Micronucleus (MN) analysis: Micronucleus, a special kind of chromosomal damage, is a chromatin occupying cell cytoplasm and containing a small globular body. Micronucleus amounts increase in physiological conditions such as aging, and in pathologic conditions which cause DNA damage or genomic instability. Evaluation of micronucleus after staining is one of the most used genotoxicity assessment method (Sommer *et al*., 2020). This method, named Micronucleus (MN) analysis, was used to evaluate the spermatids in this study.

The remainder of the epididymal sperm sample was spread onto a slide, and a smear was prepared. The slide was allowed to dry for 10 min, followed by washing with 70% ethanol. The slide was then soaked in 1% Periodic acid solution and stained with Schiff reagent. The stained slides were examined under light microscope at 1000× magnification, and spermatids with and without MN were counted (Lakshminarasaiah *et al.,* 2011).

Gene expression analysis: Changes in tumor necrosis factor-α (TNF-α) and Caspase-3 (Casp-3) expressions levels were detected in tissues subjected to low and high doses of SHI. At the end of the experimental process, approximately 10–30 mg of testicular tissue samples were homogenized in the homogenizer (RETSCHMM 400). Total RNA was isolated (Ambion PureLink RNA MiniKit) from homogenates, and RNA quantification and purity were measured with Nanodrop device; concentrations of the samples were equalized. Complementary DNA (cDNA) was synthesized (HighCapacity cDNA Reverse Transcription Kit) from the RNA samples. Polymerase chain reaction (PCR) conditions were as follows: Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min. The targeted regions were amplified from cDNAs using StepOne (Thermo Scientific, USA) real-time PCR in accordance with the Tagman qPCR Mastermix protocol. β-actin was used as a housekeeping gene. Gene expressions were determined using Ct values, and fold changes were evaluated by the $2^{-\Delta\Delta CT}$ method. The ID numbers of primers for Casp-3, TNF-α, and β-actin are Rn00563902_m1, Rn01525859_g, and Rn00667869_m1, respectively.

Statistical analysis: Statistical analysis of the data was performed using IBM SPSS Statistics Data Editor Version 21. Comparisons between groups without normal distribution were analyzed using Kruskal–Wallis variance analysis and those between groups with normal distribution were analyzed using one-way analysis of variance, followed by Tukey's test. P values of <0.05 were considered statistically significant.

RESULTS

Histological results according to Johnsen scores: According to the results of statistical analysis, the highest mean Johnsen score was identified for rats in Group C (the mean scores were 9.87± 0.12, 8.00± 0.32, and 7.37± 0.26 for the C, LD, and HD groups, respectively). When the C and LD groups were compared, a significant decrease in the Johnsen score was observed in the LD group compared with that observed in the C group ($p = 0.000$). Comparison of the C and HD groups showed a significant decrease in the Johnsen score in the HD group ($p = 0.000$). When the LD and HD groups were evaluated, a significant decrease in the Johnsen score was noted in the HD group ($p = 0.000$).

H–E- stained images of the tissue sections of rats in the groups are presented in Figure 1. The mean and SD values of all experimental groups' Johnsen scoring can be seen in Table 1.

Table 1: Histopathological and sperm motility evaluations' Mean±SD values.

	Johnsen Score	Progressive Sperm Motility Counts (million/mL)	Non-Progressive Sperm Motility Counts (million/mL)	Immotile Sperm Counts (million/mL)	Total Sperm Count (million/mL)
C	9.87 ± 0.35	11.00 ± 3.38	15.00 ± 2.16	55.71 ± 3.94	80.37 ± 7.90
LD	8.00 ± 0.92$^{\alpha}$	1.50 ± 2.20$^{\alpha}$	5.28 ± 2.56$^{\alpha}$	69.28 ± 16.08	83.83 ± 12.27
HD	7.37 ±0.74 $^{\alpha,\beta}$	1.00 ± 1.41$^{\alpha}$	6.71 ± 3.09$^{\alpha}$	49.57 ± 12.29 $^{\beta}$	62.33 ± 8.59 $^{\alpha,\beta}$

α: Compared with C group, p< 0.05 , β: Compared with LD group, p< 0.05

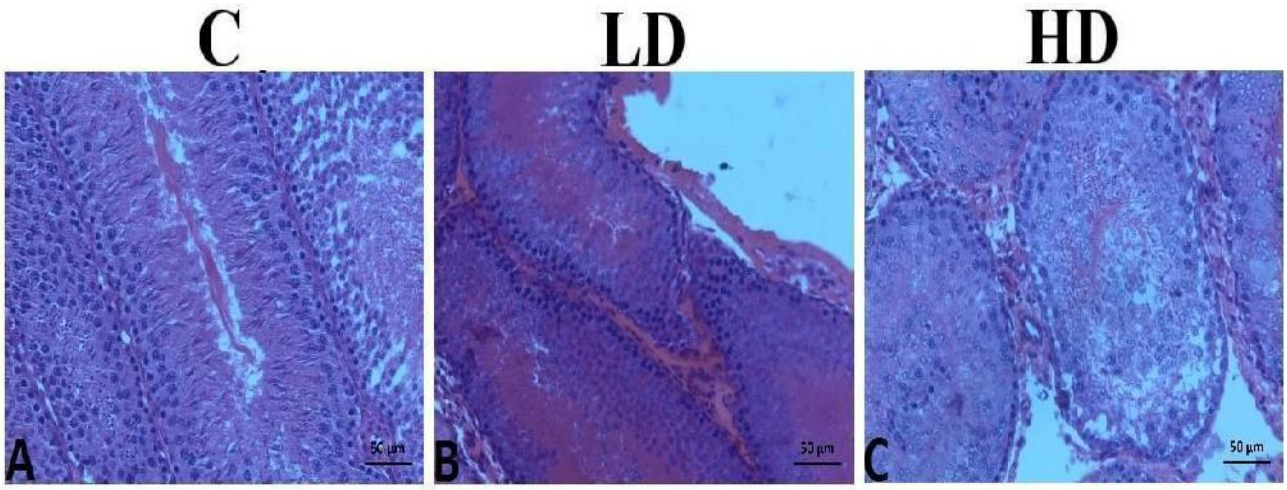

Figure 1: H-E staining microscopic photographs of Groups C, LD and HD can be seen in A, B and C, respectively (Magnification x100).

Semen analysis: In terms of total sperm count of the rats in the groups, the mean total sperm count was 80.37 ± 7.90 million/ml for the C group, 83.83 ± 12.27 million/ml for the LD group, and 62.33± 8.59 million/ml for the HD group. Intergroup significance was p = 0.002. While there was no significant difference between the C and LD groups (p = 0.785), a significant difference was noted between the C and HD groups, wherein a decreased count was noted in the HD group (p = 0.008). Total sperm count was significantly lower in the HD group than in the LD and HD groups (p = 0.003).

The mean progressive sperm motility counts were 11.0± 3.38, 1.5 ± 2.20, and 1.0 ± 1.41 million/ml for the C, LD, and HD groups, respectively. A significant decrease was observed in the mean progressive sperm motility counts in the LD group compared with that observed in the C group (p = 0.001). Significantly lower counts were observed in the HD group than that in the C group (p = 0.001). Although there was a decrease in the counts in the HD group compared with that in the LD group, no statistically significance difference was observed.

The mean non-progressive sperm motility counts were 15.0 ± 2.16, 5.28 ± 2.56, and 6.71± 3.09 million/ml for the C, LD, and HD groups, respectively. When the groups were compared, there was a significant decrease in the motility counts in the LD group compared with that observed in the C group (p = 0.00). Significantly lower counts were found in the HD group than that in the C group (p = 0.00). No statistically significance difference was observed between the LD and HD groups (p = 0.577).

The mean immotile sperm counts were 57.71 ± 3.94, 69.28 ± 16.08, and 49.57± 12.29 million/ml for the C, LD, and HD groups, respectively. There was no significant difference between the Group C with LD and Group C with HD. (p = 0.192 and 0.424, respectively). There was a significant decrease in the sperm counts in the HD group compared with that observed in the LD group (p = 0.016). The semen analysis results of all the groups are presented in Figure 2. The results of all experimental groups' sperm motility rates can be seen in Table 1.

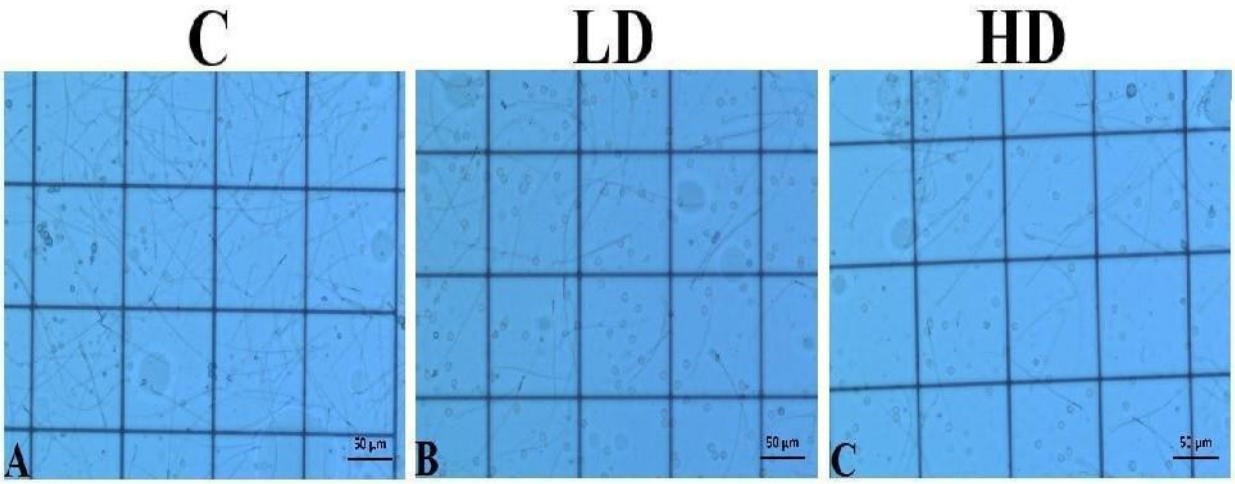

Figure 2: Light microscobic photographs of sperm motility assessment on Macler counting chamber of Groups C, LD and HD can be seen in A, B and C, respectively (Magnification x100).

MN analysis: DNA damage of spermatid was evaluated with MN analysis. The examinations showed no statistically significant number of spermatids containing MN (p=0.32). All groups had similar frequently MN in cytoplasmic areas. The MN amount were not increased in LD and HD groups.

Gene expression levels: The mean gene expression levels of Casp-3 were 0.96 ± 0.06, 2.02 ±0.32, and 1.68±0.11 in the C, LD, and HD groups, respectively. These levels increased significantly in the LD group compared with that in the C group (p = 0.004). There was a statistically significant increase in the HD group compared with that in the C group (p = 0.000), whereas no statistically significant difference was noted in these levels between the LD and HD groups (p = 0.113).

The mean gene expression levels of TNF-α were 1.01 ± 0.08, 1.42± 0.17, and 1.40±0.06 in the C, LD, and HD groups, respectively. These levels increased significantly in the LD group compared with that in the C group (p = 0.002). There was a statistically significant increase in the HD group compared with that in the C group (p = 0.025), whereas no statistically significant difference was noted in these levels between the LD and HD groups (p = 0.614). The gene expression levels of rats in all the groups are listed in Figure 3.

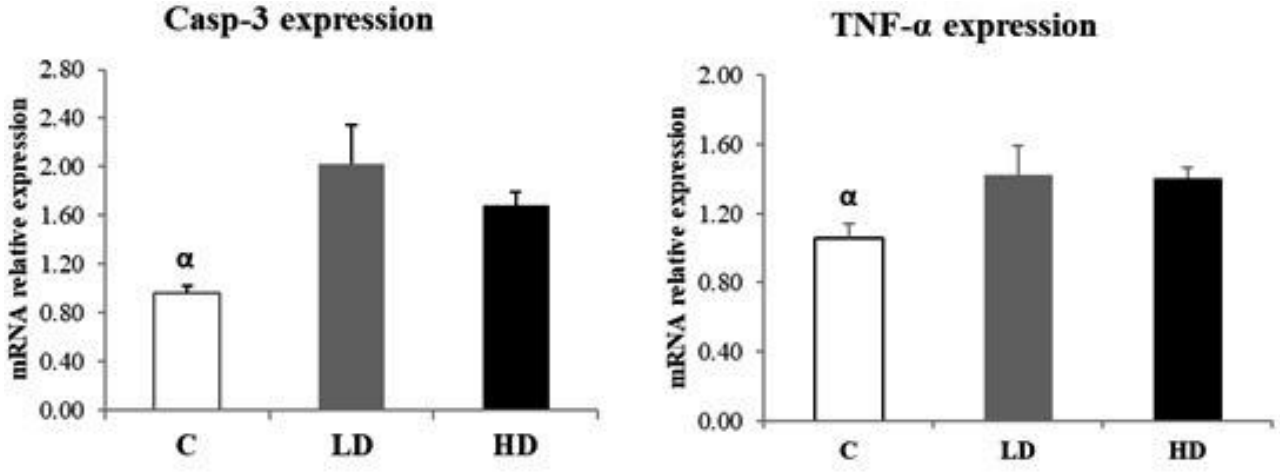

Figure 3. Gene expression levels of Casp-3 (A) and TNF-α (B) in all groups of rat testis tissues. After normalized the mRNA level with β-Actin, it is showed as a $2^{-(\Delta\Delta CT)}$ relative expression. One-way ANOVA was used to compare gene expression levels between groups and results were evaluated according to the post hoc Tukey's test.

α**:** different from Group C and Group LD, and HD $p<0.05$ statistically different.

DISCUSSION

The present study demonstrated that both low and high doses of SHI extract significantly reduced spermatogenesis in seminiferous tubules found in the testicles of rats. In addition, high-dose administration resulted in decreased total sperm counts, whereas the progressive and non-progressive sperm motility counts were significantly decreased in both the LD and HD groups. Immotile sperm counts showed a greater decrease in the HD group compared with that observed in the LD group. The gene expression levels of Casp-3 and TNF-α were increased in both the LD and HD groups compared with that in the C group.

As described above, infertility is the inability to conceive after regular sexual intercourse for 12 months. In approximately 50% of infertile cases, the cause is the male factor[12]. Male infertility can be caused by exposure to environmental factors, especially air pollution, various chemicals, endocrine disruptors, bisphenol A, phthalates, pesticides, herbicides, organophosphates, and heavy metals (Jurewicz *et al.,* 2018, Mima *et al,* 2018). Diagnosis of male factor infertility is based on the result of semen analysis. The World Health Organization defined cut-off values to distinguish between normal and abnormal semen counts. The most important fertility parameters determined after semen analysis are progressive and non-progressive sperm motility counts. It is desirable that progressive sperm motility counts should not to be below the cut-off values for healthy fertilization of the egg (Hamilton *et al.,* 2015). A decrease in the progressive sperm motility count is an indicator of male infertility. In this study, both total sperm and total motile sperm counts were significantly decreased with the administration of low and high doses of SHI, which have been previously determined in the literature. These results revealed that SHI, which is used both as a nutritional supplement and for therapeutic purposes, may cause infertility and should therefore be used with caution. Patients with decreased spermatogenesis and decreased sperm motility counts after SHI administration may require treatment to conceive, thereby reducing their quality of life and requiring additional treatment costs.

MN is formed as a result of chromosome damage and is characterized as a chromatin particle derived from acentric chromosomal fragments, which are not incorporated into the daughter nucleus after mitosis, or from entire chromosomes. MN assay is a worldwide recognized technique for assessing the potential chromosome damage caused by exposure to various chemical and physical agents (Fenech, 1993). It is also widely applied to different types of cells to assess the clastogenic and aneugenic effects of various agents (Fenech *et al.,* 1999). Compared with other DNA damage detection techniques, the MN assay provides some advantages. These include rapid application and sample preparation, ease of analysis, routine application, capability to evaluate different cell types, and ability to distinguish between clastogens and aneugens (Fenech, 1997). In the present study, no statistically significant micronucleated spermatid count was observed. This may indicate that in the doses and times applied herein, SHI does not have a toxic effect that could cause DNA damage to sperm. The results of the present study do not mean that MN formation will never occur but suggests the potential to increase MN formation depending on the dose and time.

Johnsen scoring is a method of evaluating spermatogenesis in seminiferous tubules and gives an idea of the adequacy of spermatogenesis and the number of mature spermatozoa (Johnsen, 1970). In this scoring method, the histological structures of the sertoli cells and the seminiferous tubules as well as the spermatozoa, spermatids, and other cells of the spermatogenic series in the testicle are examined in detail. Thus, sperm counts as well as changes in the tissue architecture of the testicles, which are germinal organs, are revealed (Johnsen, 1970). In the present study, a significant decrease was noted in the Johnsen scores in the LD and HD groups compared with that observed in the C group, indicating that the applied SHI has a toxic effect on the testicular tissue of rats and that it negatively affects spermatogenesis. Healthy male individuals who use SHI as a food supplement due to its beneficial health effects may experience toxic effects on germinal cells in the testicle. Therefore, while these individuals do not have any health problems, they are more likely to encounter infertility problems suddenly. This will reduce the quality of life of patients and increase their health expenditure. While studies have revealed the antitumor, antimicrobial, and anti-inflammatory effects of SHI, patients who use SHI to benefit from these effects are likely to develop conditions such as infertility, even if they achieve improvement in their underlying disease. This can lead to additional biological, psychological, and social problems for such patients. Therefore, it may be appropriate for patients and healthy individuals who use SHI as food to be careful while using this medicinal mushroom, in particular, those who want to conceive should avoid using SHI during this period.

Caspases are a large family of cysteine proteases and are involved in a number of cascades that are important in apoptosis. One of these cascades activates Casp-3. Casp-3 activation mediates apoptosis and is responsible for killing cells, harvesting macrophages, and delivering an “eat-me” signal (Nagata, 2018). In the present study, the gene expression levels of Casp-3 were studied to evaluate apoptosis in testicular tissue, and these expression levels were significantly increased in the testicular tissue of rats in the LD and HD groups. This suggests that SHI increases apoptosis in the testicles not

only at high doses of SHI but also at low doses. Increased apoptosis indicates the toxic effects of SHI on the testicle.

TNF-α is an inflammatory cytokine released mainly from macrophages. In acute inflammation, its release increases and plays a role in the progression of inflammation (Sethi *et al.,* 2008). In the present study, the gene expression levels of TNF-α increased in the SHI-treated groups compared with that observed in the control group. This result shows that SHI triggers inflammation in the testicles independent of the dose. Inflammation can also result in tissue damage, thereby promoting histopathologically shown damage in the testicles.

In the present study, the oxidation potential of SHI was not determined by biochemical examination of ROS in testicular tissue. This is the limitation of the study.

Conclusion: Oral administration of low and high doses of SHI for 7 days led to reduced spermatogenesis in the testicles, tissue damage to the testicle, and decreased motile and total sperm counts. In addition, elevated gene expression levels of Casp-3 and TNF-α both triggers inflammation and induces apoptosis. The findings of our study indicate that this mushroom leads to infertility. Therefore, individuals who want to conceive must refrain from using SHI, especially during these periods.

Acknowledgements: This work was supported by Canakkale Onsekiz Mart University Scientific Research Coordination Unit with Project Number: THD-2019-2852.

Conflict of interest: Authors declare no conflict of interest.

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