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Comparative Analysis of the Protective Potentials of *Nigella sativa* and *Lepidium meyenii* on Alcohol-induced Testicular Toxicity in Adolescent Wistar Rats

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ABSTRACT

Alcohol, a psychoactive drug, is soluble in both water and lipids, due to which it can diffuse to all tissues and affect the normal functioning of the body. Gonadal toxicity is reported as one of the side effects of its long-term consumption. This study examined the possible comparable protective potentials of *Lepidium meyenii* (LM) and *Nigella sativa* oil (NSO) on the biological integrities of the testes after exposure to excessive alcohol. Thirty-six adolescent Wistar rats (60– 150 g) were randomly divided into six groups and treated orally for 56 consecutive days. Group treatments included normal saline for the control group, 40% alcohol, LM, NSO, LM+alcohol, and NSO+alcohol. On the 57th day, samples were collected to assess reproductive hormones, sperm analysis, testicular histology and proliferating cell nuclear antigen (PCNA) expression. The results revealed that excessive alcohol consumption affected the structural integrities of the testes by depleting the mature sperm cell population and actively dividing PCNA immune-reactive cells. The treatment with *Lepidium meyenii* does not show any significant protective effects on alcohol-induced structural distortion of rats' testes. However, NSO promises to be effective in protecting against alcohol-induced changes.

Keywords: testes, alcohol, germinal epithelium, *Lepidium meyenni, Nigella sativa*

INTRODUCTION

Infertility is a disease of the male or female reproductive system defined by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse [1]. Estimates suggest that between 48 million couples and 186 million individuals live with infertility globally [1]. In the male reproductive system, infertility is most commonly caused by problems in the ejection of semen, absence or low levels of sperm, or abnormal shape (morphology) and movement (motility) of the sperm[2]. However, this incidence varies from one region to the other, with the highest reports in the infertility belt of Africa, which includes Nigeria [3,4]. Unfortunately, about 50% of these infertility cases are male factor-related [5].

Alcohol has been reported as one of the causative factors of male infertility. Clinically, the most important endocrine consequences of long-term alcohol use are its effects on the gonads as it affects the synthesis of testosterone, consequently reducing sperm parameters [6] and nuclear maturity as well as the DNA integrity of spermatozoa[7] [8]. Many men with infertility consume alcohol. The big problem in the management of male factor infertility associated with alcohol consumption is the addictive nature and the rate of relapse among participants [9].

In this era of advanced biomedical technology, medical management of male infertility includes in-vitro fertilization (IVF), sperm freezing, donation techniques, and the use of pro-fertility drugs such as clomiphene citrate. In developing countries, medicinal

plants such as *Nigella sativa* and other traditional fertility supplements (such as maca) are used as alternatives to modern therapies [10].

Nigella sativa is a dicotyledonous of the Ranunculaceae family which is an amazing herb with a rich historical and religious background [11,12] found in southern Europe, northern Africa, and Asia Minor. Historically, it has been recorded that *Nigella sativa* seeds were prescribed by ancient Egyptian and Greek physicians to treat headaches, nasal congestion, toothache and intestinal worms, diuretics, and to increase milk production. The seeds of *Nigella sativa* have long been used in the Middle and Far East as a traditional medicine for a wide range of illnesses [13].

Lepidium meyenii (LM), popularly known as maca, is a Peruvian hypocotyl found in the central Andes. Traditionally, its roots have been used by people residing in the high altitudes of the Andean subregion as a nutrient, energizer, aphrodisiac and for its fertility-enhancing properties [14]. Several studies have reported that Maca root preparations improved sexual function in healthy adult males [15] and in males with drug-induced sexual dysfunction [16] or erectile dysfunction [17]. In rodents, there are reports that administration of LM enhances spermatogenesis, fertility and sexual behavior, likely due to its phytosterol or phytoestrogen content [18,19] and its androgen-like effects [17]. LM contains several bioactive compounds (maca nutrients, macaridine, glucosinolates, maca alkaloid, macamides and macaene), which have also been suggested to be responsible for their beneficial effects on sexual function [18,19]. The goal of this study is to compare the protective potentials of *Nigella sativa* and *Lepidium meyenii* on alcohol-induced testicular toxicity in adolescent Wistar rats

MATERIALS AND METHODS

Animal care

Thirty-six adolescent male Wistar rats weighing 60-150 g were collected from the University of Ilorin Biological Garden. They were kept under standard laboratory conditions (12 hour light/dark cycles at 25-28^{0}C) in the animal house facility of the Faculty of Basic Medical Sciences, University of Ilorin, Ilorin. They were fed daily with pelletized grower feed containing 15% crude protein, 7% fat, 10% crude fibre, 1% calcium, 0.35% phosphorus, and 2.55 kcal/kg of metabolized energy as indicated. Portable water was provided, and a hygienic environment was ensured. They were cared for and used following the standard laboratory animal care and use guide.

Materials used

Alcohol (40%) was purchased from a local shop at Agbede in Ilorin. *Nigella sativa* oil and *Lepidium meyenii* (1000 mg) were purchased in the state. This research was approved by the University of Ilorin ethical review committee (UERC) (UERC/ASN/2019/856). The study was carried out upon the recommendation of the College of Health Sciences ethical review committee in compliance with the Institutional Animal Care and Use Committee (IACUC).

Experimental design

Thirty-six 8-week-old male Wistar rats were randomly placed into six groups of six rats each. They were subjected to an adaptation period of 14 days in the animal facility before the commencement of the experiment. The six groups include Group DW, which received distilled water; Alcohol group, received 3.2 ml/kg of alcohol; LM group received 100 mg/kg of *Lepidium meyenii*; NSO group received 0.5 ml/kg *Nigella sativa* oil; LM + alcohol group received 100 mg/kg *Lepidium meyenii* and 3.2 ml/kg of 40% alcohol; and NSO + alcohol received 0.5 ml/kg of NSO and 3.2 ml/kg of alcohol. All administrations were via the oral route. The rats were weighed weekly for dosage adjustments, and the experiment lasted 56 days.

Experimental termination and sample collection

The rats were euthanized a day after the treatments under anesthesia by administration of intraperitoneal ketamine, 100 mg/kg. Blood was collected for hormonal assay, while the testes were excised, weighed, fixed in Bouin's fluid and subsequently processed for histological and immunohistochemical analysis. Also, the cauda epididymis of each rat was excised and gently minced in 2 ml of normal saline. The resulting suspension was used for the determination of semen analysis. A drop of the suspension on the glass slide was viewed under the light microscope. The percentage of motile spermatozoa was derived by subtracting the number of still spermatozoa from the total sperm count according to the methods of Tjioe and Oentoeng [20]. Spermatozoa exhibiting any motion after the dilution were considered motile. Sperm concentration was determined by immobilizing cells in 3% saline. This was then thoroughly mixed and assessed using a Makler Counting Chamber (Sef- Medical Instruments Ltd Haifa) according to the method of Eliasson. [21] A sperm suspension of 10 µl was placed onto the Makler Chamber stage and counted for the number of sperms under a light microscope (Olympus).

Reproductive hormone assay

The serum reproductive hormones i.e. follicle-stimulating hormone (FSH), testosterone and Luteinizing hormone (LH) levels were assayed using a commercially available kit (IBL) and the manufacturer's instructions. The kit employs a competition-based solid-phase enzyme-linked immunosorbent assay (ELISA). The samples were plated in duplicate, and the concentrations of testosterone (catalogue no. CSB- E05100r), FSH (catalogue no. CSB- E12654r) and LH (catalogue no. CSB- E06869r) were determined using a standard curve with 5 standard values. Using a microplate reader [22], the optical density of the samples and testosterone, FSH and LH standards were quantified at 450 nm.

Histological and morphometric studies

For histopathological examination, fixed testicular tissues were dissected, processed through graded ethanol and embedded in paraffin. Microtome sections at 5 µm were obtained and stained with hematoxylin and eosin using a microtome (micron HM 315 microtome). The slices were examined and images were captured using an Olympus light microscope (Olympus BX51) and a camera (Olympus E330, Olympus Optical Co. Ltd.). Adopting a systematic random sampling, a total of eleven seminiferous tubules were carefully chosen for each rat, and Image J software was used to estimate and measure the lumen and total seminiferous tubule diameters. [23,24]

Proliferative nuclear cell antigen (PCNA) studies

The PCNA study was done using immunohistochemical studies. Five blocks per testis for the PCNA study were immunostained in each group. The carefully chosen paraffin blocks for immunohistochemical studies were stained with monoclonal antibody PCNA (LabVision, avidin-biotin–peroxidase method). Deparaffinized sections were rehydrated with graded alcohols after being deparaffinized in xylol. They were then immersed in 0.01 M citrate buffer (pH 6) and microwaved (Gibson's oven) on high for 20 min before cooling for at least 20 min at room temperature. Endogenous peroxidase activity was inhibited by a 10-minute incubation in phosphate buffer saline with 3% hydrogen peroxide (PBS, 10 mM Na2PO4, 140 mM NaCl, pH 7.2). The sections were rinsed thrice for 2 minutes each before beginning the immunostaining process [25]. Histostainplus kit (Zymed) immunohistochemical staining was conducted using the streptavidin-biotin method, which contains 10% nonimmune serum and secondary antibody (biotinylated) and Streptavidin–peroxidase. Sections were rinsed multiple times with PBS before being treated with blocking serum for 10 min to inhibit unspecific binding. After removing the excess blocking serum, the histological sections were coated with primary antibody PCNA in dilution (1:50) for one hour at room temperature. For 10 min, the secondary antibody was utilized as a second layer. Then, the sections were washed for 10 min with PBS and streptavidin-peroxidase complex. Following that, the sections were rinsed with PBS, and the peroxidase signal was generated in phosphate buffer saline containing 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxide (H_2O_2). Before drying and mounting per mount, the sections were faintly counter-stained in hematoxylin. Negative control slides were created by replacing the primary antibody with PBS in identical areas. In the same run, slides with normal positive reactivity tissues of the antibody were also employed. Nuclear staining was observed in brown PCNA expression instances [25].

Statistical analysis

Statistical data were analyzed using GraphPad Prism 8.4.2 and SPSS version 20 (Statistical Package for the Social Sciences, version 20.0). Results were presented as mean ± SEM, and all statistical comparisons were performed using one-way analysis of variance (ANOVA). The test of significance was also performed to test any significant differences between different treatments (the level of significance was set at $P < 0.05$). The post-hoc test used to determine the test of significance was Turkey. Cell counting was done using Image J.

RESULTS

Alcohol-induced emaciation in rats

The weighting of rats revealed significant physical changes in the weight of the animals across the different groups. The rats in the Alcohol group experienced a substantial difference in weight gain when compared to groups NS, LM, and NSO. Groups LM + alcohol and NSO + alcohol displayed a significant increase in weight when compared to Alcohol. There was no significant difference in the relative testicular weight (see Table 1).

Table 1: Body weight changes in experimental animals

Groups	IBW	FBW	WC	TW	RTW
NS	81.33±2.12	137.20±2.24	55.87±3.22	2.40±0.13	1.74±0.07
Alcohol	124.50±5.63	173.33±13.03	48.83±7.70*	2.06±0.27	1.23±0.20
LM	124.00±6.57	202.83±13.50	78.83±16.11*ᵅ	2.57±0.12	1.28±0.06
NSO	127.50±6.72	208.00±13.41	80.50±9.93*ᵅ	2.54±0.16	1.22±0.04
LM+Alcohol	125.17±6.96	206.00±12.71	80.83±12.07*ᵅ	2.82±0.13	1.37±0.05
NSO+Alcohol	123.83±7.87	188.40±15.50	64.57±9.14	2.58±0.11	1.40±0.08

IBW=Initial body weight, FBW=Final body weight, weight changes, TW=Testicular weight, RTW=Relative testicular weight. * indicates a significant difference when compared to DW. ᵅ indicates a significant difference when compared to Alcohol.

Nigella sativa **oil prevented semen composite depletion in alcohol-induced testicular toxicity**

Repeated exposure to excessive consumption of 40% Alcohol caused significant ($p < 0.05$) reductions in the sperm population and a drop in their motility when compared to the control, LM + alcohol and NSO + alcohol-treated rats (Figure 1). The sperm concentration and motility were significantly increased ($p < 0.05$) in NSO and NSO + alcohol when compared to the rats only exposed to excessive alcohol (Figure 1).

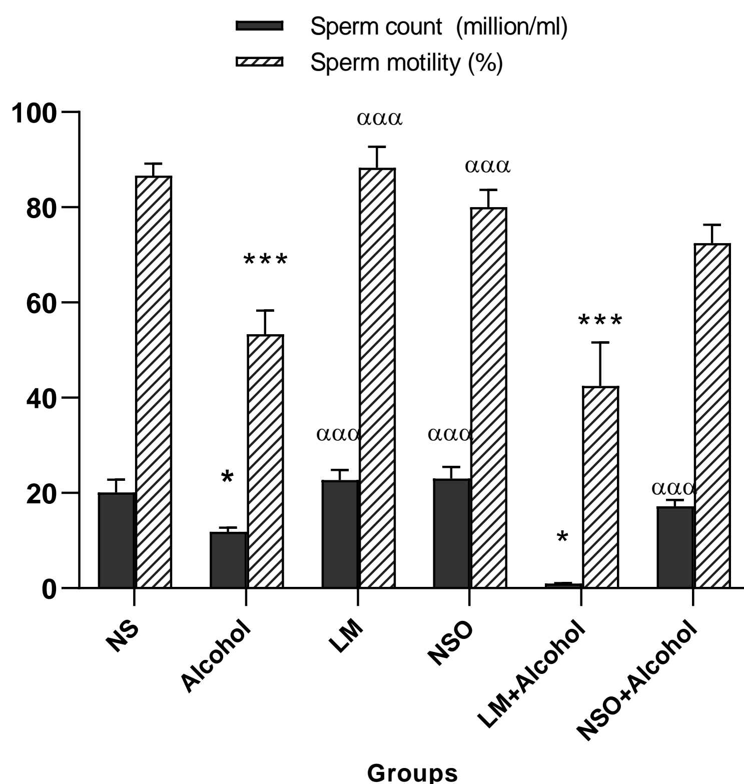

Figure 1: Semen analysis in rats

Rats exposed to normal saline (NS), 40% alcohol (Alcohol), *Lepidium meyenii* (LM), *Nigella sativa* oil (NSO), *Lepidium meyenii* + 40% alcohol (LM + alcohol), *Nigella sativa* oil + 40% alcohol (NSO + alcohol) respectively. Bars indicate mean ± SD, (*) and (***) represent statistically significant differences ($p < 0.05$ and $p < 0.001$ respectively) compared to DW; ααα indicate statistical difference ($p < 0.001$) between the groups and the Alcohol group.

Differential hormonal responses following administration of alcohol, *Nigella sativa* oil and *Lepedium meyenii*

The testosterone levels of the rats exposed to alcohol were significantly higher when compared to the control. Treatment with NSO + alcohol stimulated increased testosterone levels, while treatment with LM + alcohol was observed to reduce testosterone levels (Figure 2). The NSO group had testosterone levels comparable to that of the control. In contrast to the effects of the NSO on testosterone concentration, NSO and Alcohol significantly increased the luteinizing hormone concentration in treated rats (Figure 4), while the FSH concentrations were not significantly different in all groups (Figure 3).

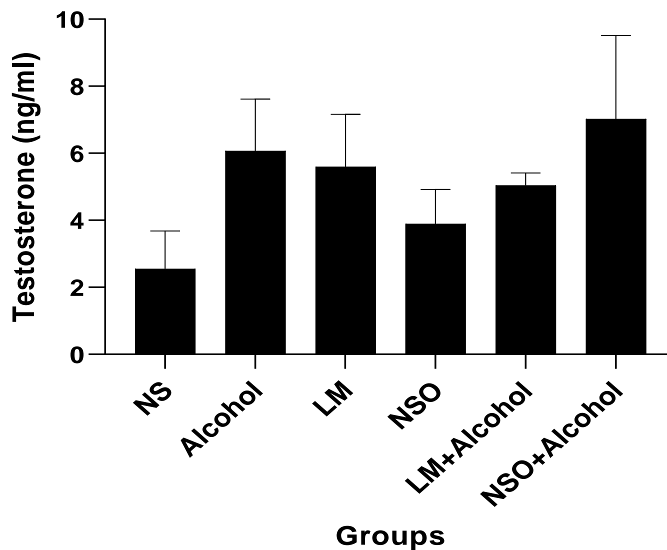

Figure 2: Serum testosterone in rats

Normal saline (NS), 40% alcohol (Alcohol), Lepidium *meyenii* (LM), *Nigella sativa* oil (NSO), *Lepidium meyenii* + 40% alcohol (LM + alcohol), *Nigella* s*ativa* oil + 40% alcohol (NSO + alcohol) respectively. Bars indicate mean ± SD

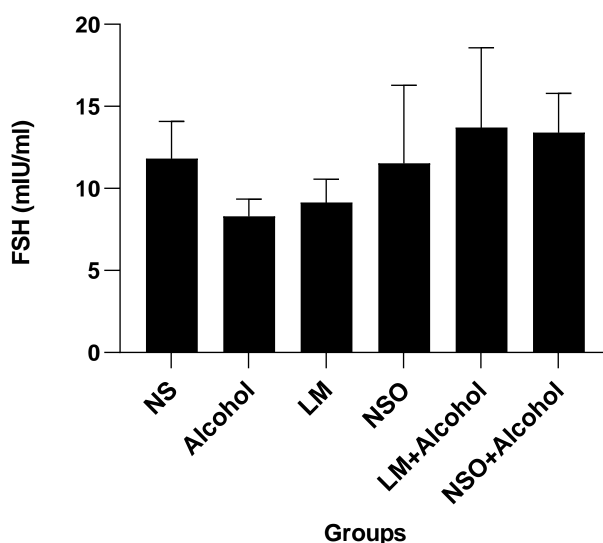

Figure 3: Serum Follicle Stimulating Hormone in rats

Normal saline (NS), 40% alcohol (Alcohol), Lepidium *meyenii* (LM), *Nigella sativa* oil (NSO), *Lepidium meyenii* + 40% alcohol (LM + alcohol), Nigella s*ativa* oil + 40% alcohol (NSO + alcohol) respectively. Bars indicate mean ± SD

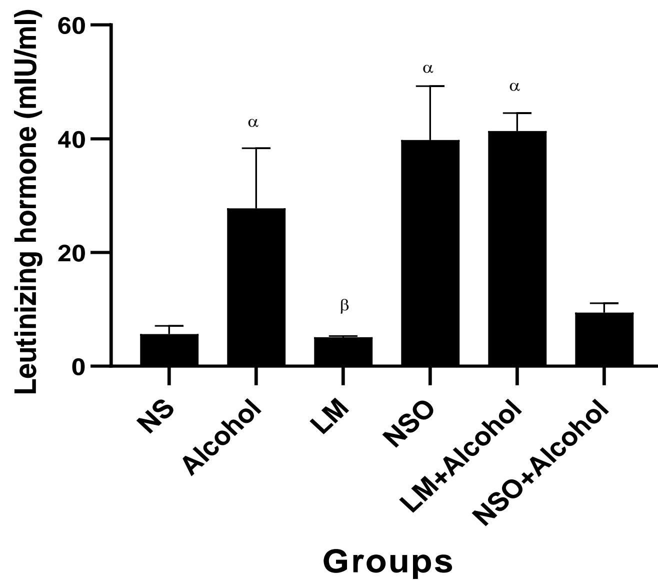

Figure 4: Serum Luteinizing Hormone in rats

Normal saline (NS), 40% alcohol (Alcohol), Lepidium *meyenii* (LM), Nigella *sativa* oil (NSO), *Lepidium meyenii* + 40% alcohol (LM + alcohol), *Nigella sativa* oil + 40% alcohol (NSO + alcohol) respectively. Groups Alcohol, LM + alcohol and NSO + alcohol displaced altered serum LH. Bars indicate mean ± SEM, α represents $p < 0.05$

Immunohistochemical studies of **proliferative nuclear cell antigen**

In Figure 5 below, early germ cells along the basement membrane are deeply stained (black arrow), whereas several spermatocytes show less intense (orange arrows) staining. Sertoli cells (green) and Leydig cells (red arrow) stain negative for PCNA (Original magnification X100).

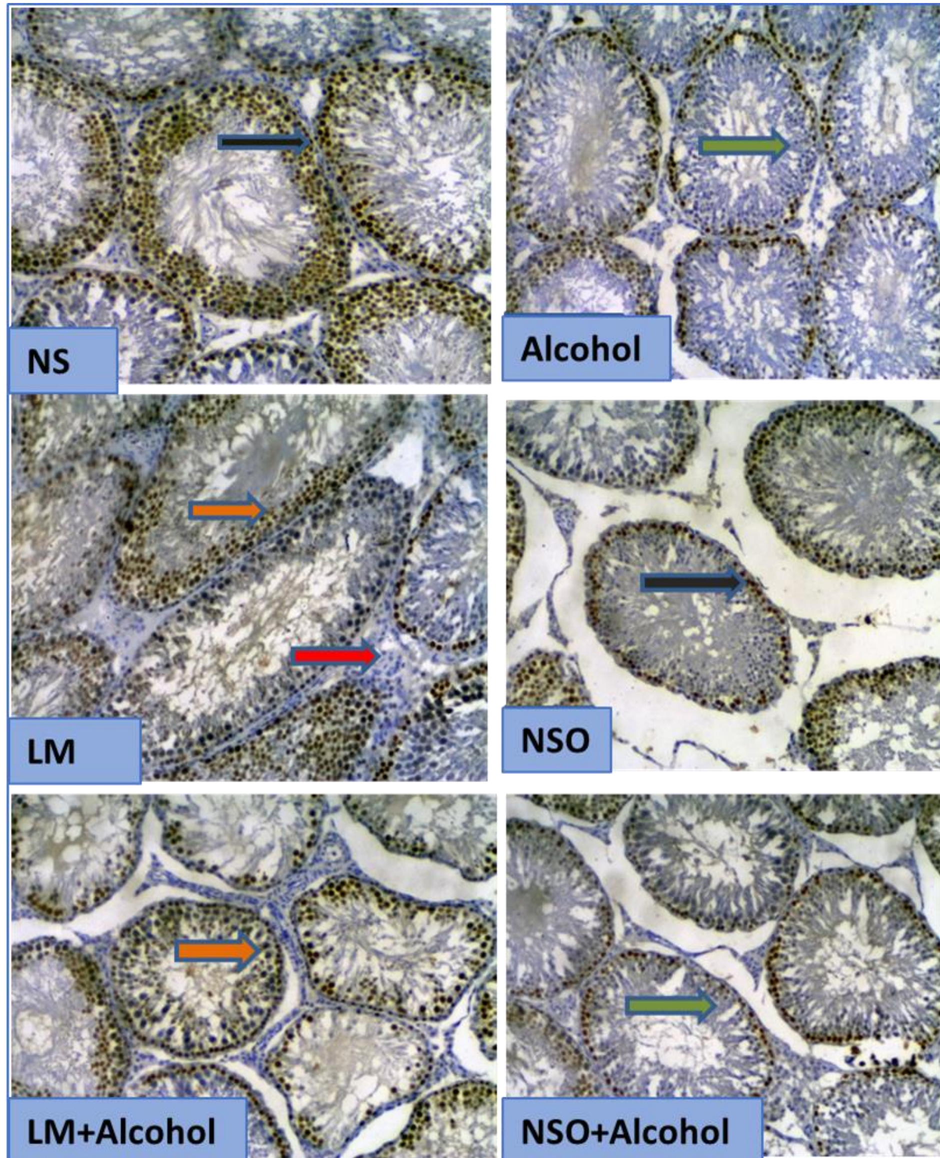

Figure 5: Photomicrograph of the testes showing the Proliferative cell nuclear antibodies

The control group and experimental groups. (Control group (NS), 40% Alcohol (Alcohol), *Lepidium meyenii* (LM), *Nigella sativa* oil (NSO), *Lepidium meyenii* and 40% alcohol (LM + alcohol), *Nigella sativa* oil and 40% alcohol (NSO + alcohol). Green arrow (secondary spermatocyte), (Orange arrow (primary spermatocyte), Blue arrow (seminiferous tubule), Red arrow (Leydig cells). Depletion of PCNA-stained cells in Alcohol, NSO, LM + alcohol and NSO + alcohol groups**.**

Table 2: Population of PCNA-stained cells

Animal Groups	Cell Counts
NS	1702 ±132.3
Alcohol	995 ± 377.0*
LM	1887 ±249.7[α]
NSO	1074 ± 173.5
LM + Alcohol	1555 ±270.7 [α]
NSO + Alcohol	1256 ±161.3 [α]

*indicates a significant difference compared to NS

[α] indicates a significant difference compared to Alcohol

Histological observations

Observations from the basic histology using light microscopy revealed normal seminiferous tubules in all groups, with a normal progression of the cells of the germinal epithelium. However, there was a widened interstitium in the NSO group, but the sizes of the seminiferous tubules were maintained compared to the control (NS) group. See Figure 6.

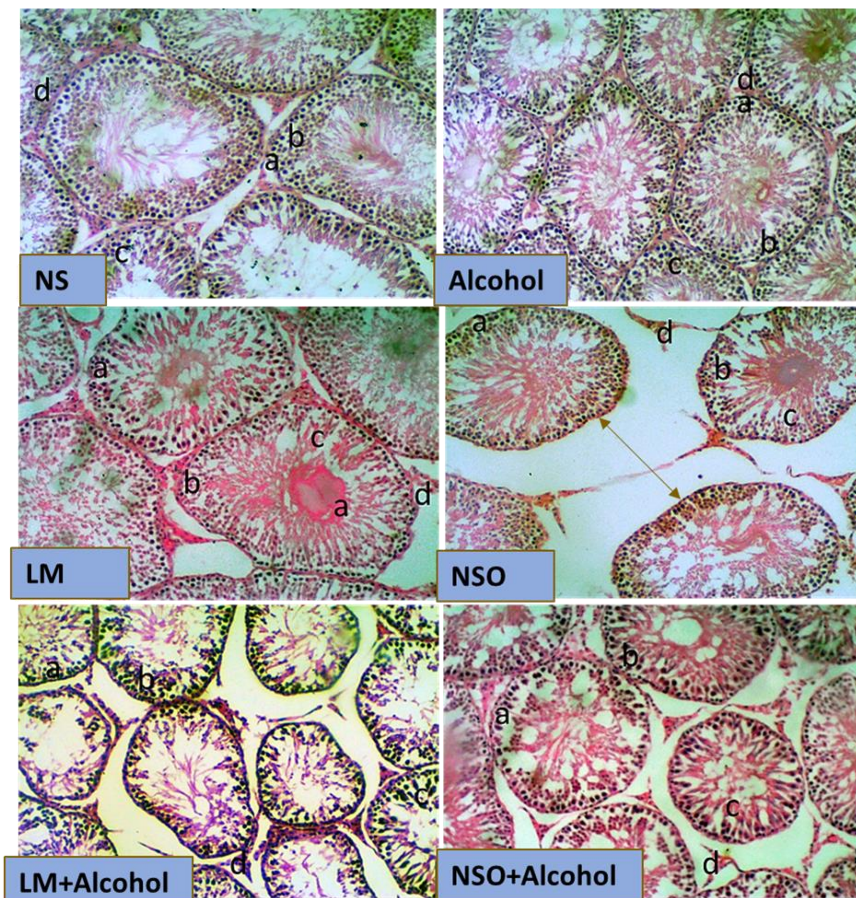

Figure 6: Representative photomicrographs of H&E stained rats' testis

Normal saline (NS), 40% alcohol (Alcohol), *Lepidium meyenii* (LM), *Nigella sativa* oil (NSO), *Lepidium meyenii* + 40% alcohol (LM + alcohol), *Nigella sativa* oil + 40% alcohol (NSO + alcohol) respectively. a- Lumen, b- Seminiferous tubule, c- Primary spermatocyte, d- Interstitium/interstitial space, e- Spermatogonia. H&E stain, ×100

DISCUSSION

Sperm cell development begins with the differentiation of spermatogonia in the seminiferous tubules, which then change into mature spermatozoa after a sequence of events. Spermatogenesis must proceed unobstructed and undisturbed to create viable spermatozoa with reproductive potential. Due to the sensitivity and delicate features of sperm cells, an insult to the organism, particularly when directed to the reproductive organs, such as the testes, could distort spermatogenesis and impair reproductive abilities [26]. The structural state and functionality of the testis were evaluated in this research work to determine the toxic effect of alcohol and comparable protective roles of *Lepidium meyenii and* NSO in the reproductive parameters of male Wistar rats.

Previously, testicular atrophy has been noted as the consequence of chronic alcohol usage in humans as well as in experimental animal models, and alcoholism has been linked with reduced testicular volume and size [27]. While many researchers have observed atrophy in testicular weight, some have reported an increase [28]. However, the report from this study reports that neither alcohol, *Lepidium meyenii*, nor NSO-treated rats displayed an appreciable change in the relative testicular weight. This is similar to a recent finding where no change in weight was observed in both ethanol and *Nigella sativa*-treated animals [29]. It was experiential that treatment with alcohol did not affect the relative weights of the reproductive organs. Results from previous studies revealed decreased testicular weight and a reduced organ coefficient as an indication of alcohol-induced testicular atrophy [30]. A reduced organ coefficient might be due to increased apoptosis in the respective organs as increased apoptosis is associated with weight loss in mice testis [30]. Moreover, NSO restored the alcohol-induced reduction in body weight and organ coefficient.

Testosterone level is a good indication of the reproductive functioning in males. Exposure to alcohol in this study is associated with an increased level of testosterone. Similarly, prior studies have shown that modest consumption of alcohol increases serum testosterone levels, particularly in short-term exposures [31,32], as observed in the present study. This has been attributed to the increased hepatic enzyme activity during such exposures [33]. However, in humans and animal models, during chronic exposures to excessive alcohol, the production of testosterone drops [34,35].

The serum luteinizing hormone levels displayed reverse effects, as they dropped with alcohol exposure. On the other hand, alcohol, *Lepidium meyenii*, and NSO did not affect the follicle-stimulating hormone levels. This is similar to the findings of other researchers [35,36].

From the result of this study, sperm count and sperm motility in rats treated with 40% alcohol were reduced. The co-administration of NSO with alcohol markedly reversed the adverse effect induced by alcoholism to within control levels and this probably results from the antioxidant activities of its bioactive components. However, treatment with *Nigella sativa* reversed the toxicity caused by alcohol. This is similar to the results from previous studies [29].

The semen parameters are a measure of both endocrine and testicular health. It also has a direct relationship with the reproductive capabilities of males. Insults to the testes are easily picked by an assessment of the sperm concentration, motility and percentage of sperms with normal morphology. Previous studies have also shown that changes in sperm parameters might be due to the direct effect of alcohol on testicular tissue which led to impaired sperm parameters [37]. Moreover, the decrease in sperm count in alcohol-treated rats might be due to decreased testosterone levels observed in chronic use [34] and increased oxidative stress. Additionally, reduced sperm motility might be a result of increased head-tail breakage in alcohol-treated rats as explained by Anderson *et al* [38]. The improvement in sperm parameters observed with concomitant use of NSO might be attributed to its anti-oxidative properties [39]. However, results from this study show that the co-administration of alcohol with *Lepidium meyenii* has not proven to be beneficial.

Alcohol has been found to increase the epithelial barrier permeability and affect tight junction-associated protein expression [40]. This might be a reason behind the profound destruction of seminiferous tubules, disorganized lining of seminiferous tubules and sloughing of germ cells. The histopathological examinations of testes showed that Alcohol had a deleterious effect on the germinal epithelium and co-administration with NSO was unable to alleviate the adverse effects induced by Alcohol. Data from this study supports those obtained by Hala who reported that pre-treatment of male rats with *Nigella sativa*, and celery oils for 4 weeks produced a protective effect against testicular injury induced by sodium valproate [41]. This effect was manifested by increased weight of the testis, improved semen quality and quantity, elevated serum testosterone level, decreased lipid peroxidation in the testis, and alleviation of degenerative changes in the testes of rats given sodium valproate. The mechanism for this protective effect of NSO against the toxic effects of alcohol can be attributed to its free radical scavenging activity and increased antioxidant enzymes in rats [39].

The role of proliferating cell nuclear antigen (PCNA) in the testes is well established. It plays an essential role in nucleic acid metabolism, cell replication and repair. The expression of PCNA in the Alcohol-treated groups was diminished. This is suggestive of reduced cellular replication and damage to the actively dividing portion of the germinal epithelium lined along the basement membrane of the seminiferous tubules – spermatogenic cells and the primary spermatocytes. Damage to the mitotic germinal epithelium is also indicative of DNA insults. While some researchers believe that the expression of PCNA indicates a good response to impaired spermatogenesis[42], others suggest that it is an indication of well-regulated spermatogenesis [43].

Nigella sativa oil has been proven to display a wide range of favourable biological activities, the most prominent being antioxidant and anti-inflammatory agents. Co-administration with Alcohol was shown to limit these structural and biochemical changes in the gonads. Many researchers have reported gonadal protection against insults following testicular exposure to different varieties of insults [44,45].

Conclusion

Besides being an important public and social issue, alcohol consumption can also significantly impact male reproduction by inducing testicular damage evidenced by abnormal sperm parameters. However, treatment with Nigella *sativa* oil protected against alcohol-induced testicular toxicity. This result shows that *Nigella sativa* oil could be a promising supplement for preventing male infertility induced by excessive alcohol consumption.

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