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Lion's mane (*Hericium erinaceus*) as a potential protective against metronidazole-induced toxicity in brain and testes of male rats: Protein expression and biochemical evaluation

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Abstract

Lion's mane (*Helicium erinaceus*) is a highly medicinal fungus with diverse bioactive molecules that have been proven to be beneficial in neurodegenerative diseases. Thus, the current study's objective was to probe the potential function of this mushroom in the protection of the brain, and we screened its effect on testes in metronidazole-induced toxicity. Adult male Albino Wistar rats were divided into four groups, each containing six animals. In the control group, rats were administered distilled water; the second group was given metronidazole at a dose of 500mg/kg body weight; the third and fourth groups were administered Lion's mane at 1 and 1.5g/kg, respectively. Two hours later, metronidazole was given at a dose of 500mg/kg. Metronidazole and Lion's mane were given orally for 40 consecutive days. Results of open field test showed significant neurobehavioral alterations in Lion's mane-treated rats. Lion's mane at 1g/kg mitigated histological damage in the brain and preserved testosterone levels, whereas 1.5g/kg showed adverse effects on the testes. All treated groups showed relatively same intensity for expression of both p53 and Bcl-2 proteins. Notably, p53 expression was more intense at 1.5g/kg, while Bcl-2 expressed intensively at 1g/kg. We conclude from the current study that Lion's mane, at low doses, acts as a protective herb against metronidazole toxicity, exhibits an improvement of the histological changes, and exerts anti-apoptosis characteristics.

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Introduction

Lion's mane (*Hericium erinaceus*) is a well-known mushroom that can be found abundantly on various continents in the world, including Asia, North America, and Europe. It has been given different terms depending on the country; for instance, in China, it's called "Houtou", while in Japan, "Yamabushitake", and *Hericium erinaceus* in Latin countries (1). This mushroom gained popularity a long time ago as a source of therapeutic benefits and as a dietary supplement for human consumption because it is composed of a variety of biologically and physiologically active constituents that are responsible for the regulation of significant functions in the human body (2,3). These crucial and valuable constituents of the mushroom include diverse biological molecules such as carbohydrates, proteins, fats, phenols, flavonoids, terpenoids, sterols, minerals, and vitamins. All these molecules provide it with various properties and functions against certain pathological conditions (4-6). They have been shown to exhibit therapeutic characteristics against different health disturbances, improve conditions of chronic diseases such as diabetes, hypertension, hyperlipidemia, and cancer, and boost immunity in immunocompromised individuals (7), as

well as gastrointestinal abnormalities (8). The remedy properties of the mushroom are attributed to its contents of antioxidants, anti-inflammatory and anticancer molecules, in particular, phenolic and beta-glucan polysaccharides (9,10). Importantly, it has also been demonstrated that the fungus is neurons protective, enhancing their growth and preventing neurodegenerative changes related to Parkinson's disease, depression, and ischemic stroke (11,12). Therefore, scientists have recently paid deep attention to examining its function on the brain, which can be a promising active nutrient as a result of its therapeutic properties and health benefits in brain-related disorders, including Alzheimer's disease and mood suppression (13-17). Among essential *H. erinaceus* compounds that have been observed to be associated with a positive influence on the neurons in terms of protection and stimulation of nerve growth include erinacines A, hericenones, terpenoids, and sterol (18,19). These molecules possess the capacity to reduce oxidative stress, decrease inflammatory responses, and strengthen neuron growth, resulting in improvement and enhancement of neuronal integrity (20,21). Specifically, erinacines A was demonstrated to have a protective role in both in vivo using mice administrated 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's disease and in vitro using mouse N2a (Neuro-2a) cell culture against reactive oxygen species (ROS). Moreover, it has also been confirmed its ability to prevent neuronal cell death via decreasing both p21 and GADD45 levels, as well as stimulation of pathways involved in survival neurons through phosphorylation of certain enzymes such as extracellular signal-regulated kinases, PAK and LIM domain kinase 2 (22). To examine the potential benefits of lion's mane in the brain and testes organs, metronidazole was selected to trigger toxicity in the target tissues. Metronidazole is a frequently used antimicrobial agent against anaerobic bacteria and protozoal infections, which cause gastrointestinal disturbances in both human and veterinary medicine (23,24). It has been demonstrated to be a safe antibiotic when prescribed for a short course. However, it has been confirmed that long-term metronidazole administration may exhibit cytotoxic effects in both the brain and testes as a result of producing oxidative stress (25,26).

Depending on the displayed facts related to this antimicrobial agent, and due to the lack of information about the mushroom's effect on the testes, it was decided to use it as a model to trigger the possible damage in neurons and male reproductive organs in experimental adult male rats. Accordingly, the current study aimed to examine the potential beneficial influence of Lion's mane in the protection of the brain and testes against metronidazole-induced toxicity.

Materials and methods

Ethical approval

This research was undertaken in the Animal Unit, College of Pharmacy, Hawler Medical University, Erbil, Iraq. Members of the Scientific and Ethical Committee in the Health Sciences College approved the research proposal on 12/03/2024, which holds the number Sc.E.C.5A.

Animals

Twenty-four adult male Albino Wistar rats weighing between 170-210 grams were purchased from Pharmacy College, Hawler Medical University, Erbil, Iraq. Two weeks prior to the start of the experiment, animals were separated and acclimatized in standard plastic cages for rats. Animals were provided with regular food in the form of pellets, and the room temperature was constantly maintained at 23°C for the duration of the experiment. The light cycle consisted of twelve hours of light and twelve hours of darkness.

Experimental design

The experiment lasted 40 consecutive days and included four groups, each consisting of six adult male rats that received the following liquids or substances: the first group, control animals, were administered distilled water during the entire period of the experiment. The second group received only metronidazole at a dose of 500mg/kg of body weight orally. The third and fourth groups were administered Lion's mane at 1 and 1.5g/kg of body weight, respectively. After two hours, these two groups were administered metronidazole at a dose of 500mg/kg of body weight. The doses in this study were selected depending on a previous research study (14). Tablets of Lion's mane supplement and metronidazole were ground and dissolved in distilled water. The dose administered to the rats was measured as 10ml/kg of body weight. The third and fourth groups were treated with Lion's mane three days before starting the experiment to initiate its effect before inducing toxicity. The required volume was freshly prepared every day by mixing the Lion's mane powder with distilled water. This mixture was given to the rats at approximately 8:00 am. Two hours later, metronidazole was given.

Chemical compounds and pharmaceuticals

Lion's mane (*Hericium erinaceus*) mycelium was obtained from Erbil (DXN's Property, Malaysia) in the form of pills, each containing 300mg. Metronidazole tablets containing 500mg were purchased from a local pharmacy (Flamingo, England). Additionally, other chemicals were used, such as Diethyl ether (DEA) (Cario Erba Reactifs-SDS), 2% injection of xylazine (Xyla, Holand), and ketamine (SIR ALDAWA CO. Baghdad, Iraq).

The open field

A modified open box constructed from wood, measuring 100x100x50cm, comprised of twenty-five squares of identical area, each 20cm, was utilized to evaluate the behavior of the treated rats in different time points: days 15, 30, and 40 (27). The test was performed after two hours of metronidazole administration. This involved placing an animal in the center of the wooden box for five minutes and recording the behavior of the tested rat with a camera. The behavioral measurements that were taken included latency, square crossing, rearing, and grooming.

Biochemical assessment

On day forty-one, rats were anesthetized using diethyl ether, and blood samples were collected from the retro-orbital plexus into gel coagulant tubes. All tubes containing blood underwent centrifugation at 3500 revolutions per minute (RPM) for 14 minutes. The collected sera were utilized to assess testosterone levels employing the Testosterone II CalSet kit (Roche, Germany) and the Cobas® 6000 analyzer series (Roche, Germany).

Histopathological investigation

The brain and testes of the dissected animals were harvested as tissue samples and fixed in 10% neutral buffered formalin at room temperature for at least three days before proceeding to the next step, immersion of the tissue in increasing concentrations of ethyl alcohol (70%, 90%, and 100%) for dehydration. Following this, the samples were cleaned with xylene, embedded in paraffin wax, and then poured into blocks according to the desired shape. Afterward, a rotary microtome was utilized to create consecutive slices with a thickness of 4–5µm. These slices were then gathered on glass slides for the subsequent staining procedure. Several mounted slides were deparaffinized and stained with Harris Hematoxylin and Eosin (H and E) following standard protocols. While others were utilized for the immunohistological process. Histopathological examination was conducted by two experienced pathologists utilizing a 2.0 USB digital image camera (Omax ToupView 9.0-Megapexil, China), which was provided with image processing software. The software was calibrated to all lenses of Microscope Olympus-CX31 with the aid of a 0.01mm stage micrometer (ESM-11/ Japan) (28,29).

Immunohistochemistry analysis

Immunohistochemistry was employed to identify the B-cell leukemia/lymphoma 2 protein (Bcl-2) and tumor protein p53 (p53) exclusively in brain tissue (30,31). The immunostaining of Bcl-2 was conducted in this study using a FLEX monoclonal Bcl-2 oncoprotein (Dako, [Clone 124, Dako: IR614], Ready-to-Use (Link). For p53, a FLEX monoclonal p53 protein [Clone DO-7, Dako: IR616], Ready-to-Use (Link), was utilized in conjunction with the Dako EnVision FLEX detection system and autostainer Link. For pre-treatment, the Dako PT Link instrument was utilized to deparaffinize and rehydrate tissue sections, followed by heat-induced epitope retrieval (HIER) using the EnVisionTM FLEX target retrieval solution at high pH (Dako, DM828) for 20-40 minutes at 97°C. Subsequently, the slides were removed from the PT Link and subjected to washing with EnVisionTM FLEX wash buffer for 1-5 minutes. Subsequently, slides were positioned in the pre-programmed Autostainer Link instrument utilizing EnVisionTM FLEX DAB-Chromogen (Dako, DM827), and an aliquot of 100µ of primary antibodies (Ready-to-Use) was applied to the tissue sections. Following the wash with the buffer, an additional 100µ of EnVisionTM FLEX HRP conjugate (Dako, SM802) was introduced. Dehydration, clearing, and permanent mounting were conducted at the conclusion in accordance with the manufacturer's recommendations (30,31).

Statistical analysis

Data from testosterone levels, latency, square crossing, rearing, and grooming experiments were systematically collected and presented as means and standard error of the mean (M ± SEM). The normality of the dataset was assessed using the Shapiro–Wilk test, confirming a normal distribution. A one-way analysis of variance (ANOVA) was employed to evaluate significant differences among the experimental groups, followed by Tukey's post hoc test to pinpoint specific differences. All statistical analyses were conducted using GraphPad Prism 9 (USA) software, with a significance value of $P \leq 0.05$.

Results

Effect of Lion's mane mushroom on open field activity in metronidazole-induced Neurotoxicity

The effects of Lion's mane on the open field test were recorded at three different time points during the treatment; days 15, 30, and 40. Male rats on day fifteen of Lion’s mane administration showed no significant differences in latency, number of squares crossed, rearing, and grooming between treated and control groups (Figure 1). On day thirty, animals administered Lion's mane at 1g/kg of body weight showed a significant increase in number of grooming compared with metronidazole-treated rats (Figure 2). On the other hand, no significant alterations were observed in latency, number of squares crossed, and rearing between control and treated groups (Figure 2). On the last day of the experiment, rats treated with Lion's mane at 1.5g/kg of body weight revealed a significant increase in latency compared to the control, metronidazole-treated rats, and animals treated with Lion's mane at 1g/kg of body weight (Figure 3). Meanwhile, no significant changes were reported in the remaining measurements, including the number of squares crossed, the number of rearing, and the number of grooming (Figure 3).

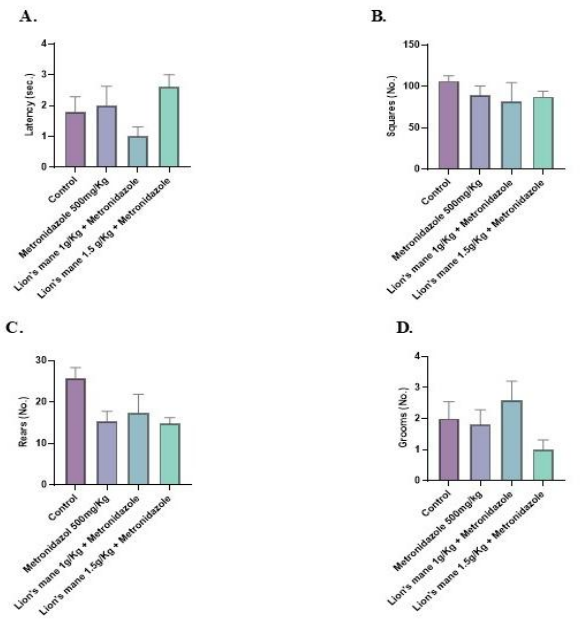

Figure 1: Effect of Lion's mane on open field test on day 15 of treatment. A. latency, B. number of squares crossed, C. number of rearing, D. number of grooming. Error bars represent mean± SEM. Sec=second. * Indicates significant differences at $P \leq 0.05$.

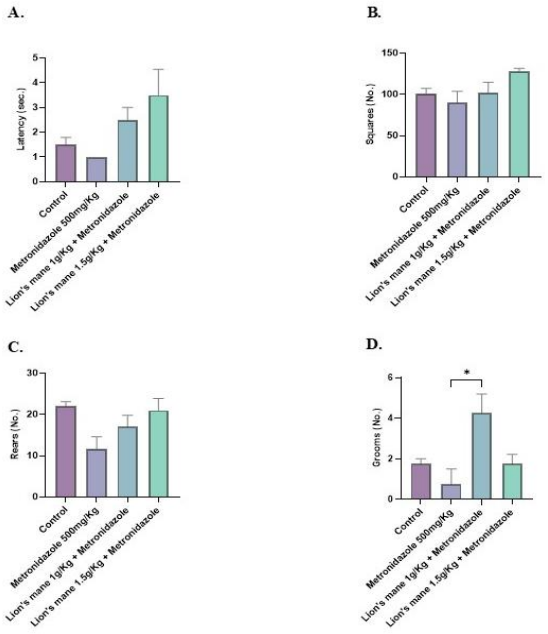

Figure 2: Effect of Lion's mane on open field test on day 30 of treatment. A. latency, B. number of squares crossed, C. number of rearing, D. number of grooming. Error bars represent mean± SEM. Sec=second. * Indicates significant differences at $P \leq 0.05$.

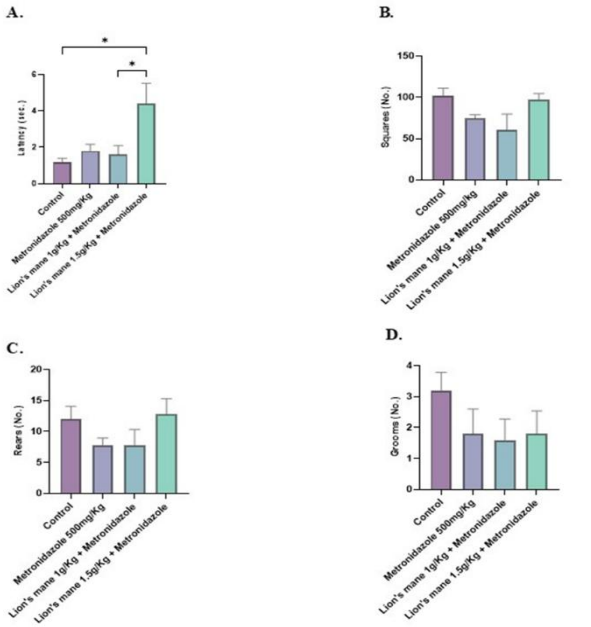

Figure 3: Effect of Lion's mane on open field test on day 40 of treatment. A. latency, B. number of squares crossed, C. number of rearing, D. number of grooming. Error bars represent mean± SEM. Sec=second. * Indicates a significant difference at $P \leq 0.05$.

Effect of Lion's Mane Mushroom on testosterone hormone in metronidazole-induced testicular damage

Rats treated with metronidazole at 500mg/kg of body weight for 40 consecutive days displayed a significant reduction in testosterone levels compared with control animals (Figure 4). Likewise, rats administered Lion's mane at both doses of 1 and 1.5g/kg of body weight showed a significant decrease in testosterone levels in comparison with animals administered distilled water. Although 1g of Lion's mane did not show a significant increase in testosterone level, this dose showed a higher level of the hormone compared with 1.5g Lion's mane and metronidazole-treated rats (Figure 4).

Histological changes in the brain

Histopathological examination showed the detrimental effect of metronidazole on the brain tissues of rats in comparison with the control group, which was represented by the presence of perivascular edema, vacuolization around the neurons, and satelliosis in the cortex. Congestion and hemorrhage of the blood vessels, particularly in the meninges and hippocampus, and the presence of diffuse gliosis and focal gliosis in some areas. In addition, necrosis of some cells in the Purkinje layer caused a reduction in the number of axons (cell bodies) in some areas of the cortex in comparison with the control group (Figure 5). Likewise, Lion's mane, given in a dose of 1.5g/kg, showed similar

changes to those described above in the metronidazole-treated group. These changes included perivascular edema, congestion of blood vessels, diffuse/focal gliosis, vacuolar degeneration, and obvious neuropil vacuolization.

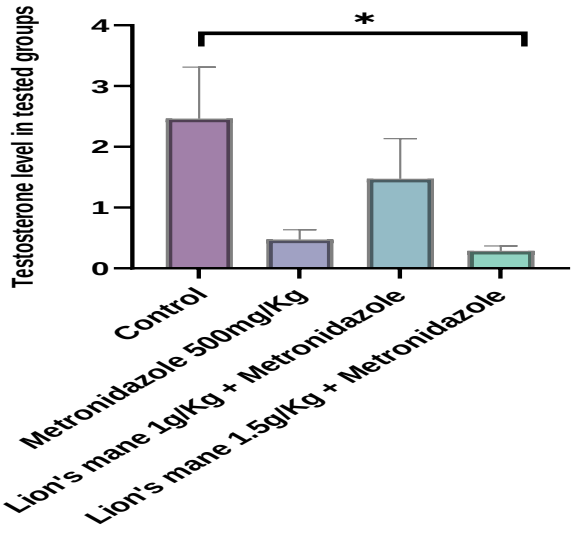

Figure 4: Impact of Lion's mane on testosterone level. Error bars represent mean± SEM. * indicates a significant difference at $P \leq 0.05$.

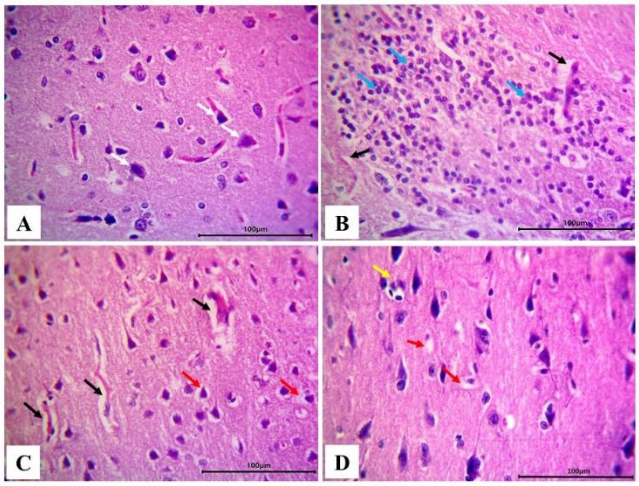

Figure 5: Histopathological findings in the brain tissue of treated rats. A. The control group shows the normal structure of pyramidal neurons in the cerebral cortex (white arrows), B. Metronidazole-treated group shows focal gliosis (blue arrows) and perivascular edema (black arrows). C. Lion's mane group at 1g/kg shows perivascular edema (black arrows) and vacuolization around the neurons (red arrows). D. Lion's mane group at 1.5g/kg shows mild satellitosis (yellow arrows) and mild vacuolization around the neurons (red arrows). Stain: HE, Scale Bar=100 µm.

Furthermore, reduction in the number of axons and cell bodies numbers, satelliosis in the cortical area, and thinning of the granular cell layer of the hippocampus. In addition, necrosis of some cells of the Purkinje layer (Figure 5). Rats administered Lion's mane in a dose of 1g/kg demonstrated similar changes. However, the histological changes were less severe than the changes observed in both groups of metronidazole-induced toxicity and Lion's mane given at 1.5g/kg. Notably, the neuropil and hippocampus were normal, and there was an appropriate number of axons and an intact Purkinje layer apart from the presence of some necrotic cells (Figure 5).

Histological changes in the testes

Both groups of rats administered metronidazole and Lion's mane in a dose of 1.5g/kg demonstrated similar histological changes. However, the changes were less severe in the metronidazole-treated group (Figure 6). The changes included sub-chronic orchitis, which is characterized by thickening in the tunica albuginea layer infiltration of mononuclear inflammatory cells, particularly the multinucleated giant cells, and the presence of sero-fibrinous edema between the tubules. Furthermore, some circulatory changes were observed, such as congestion and hyperemia of blood vessels, hemorrhage under the capsule, and edema between the seminiferous tubules. Additionally, degeneration and necrosis of spermatocytes and sloughing of some of these spermatocytes into the lumen, atrophy, and disarrangement of some seminiferous tubules. In addition, decrease in the number of Sertoli cells (Figure 6). Similarly, tissue sections of testes taken from rats administered 1g of Lion's mane showed mild alterations in comparison to the rats administered 1.5g of Lion's mane with the presence of some characteristic multinucleated giant cells (Figure 6).

Effect of Lion's mane mushroom on protein expression of p53 and Bcl-2 in metronidazole-induced neurotoxicity

Metronidazole-treated rats for 40 consecutive days showed positive moderate expression of nuclear p53, particularly in the cortex area of the brain tissue with a diffuse staining pattern compared with the control group, which revealed mild expression (Figure 7). Also, the expression was moderate in Lion's mane-treated rats in both doses of 1 and 1.5g/kg, with a similar pattern to that of the metronidazole-treated group. However, the positive staining was more intense in the brains of rats administered Lion's mane at the dose of 1.5g/kg of body weight (Figure 7). Compared with the control group, which revealed mild expression (Figure 8), all treated groups showed moderate cytoplasmic expression of Bcl-2. However, the positive expression was more intense in Lion's mane-treated rats at 1g/kg in comparison with the metronidazole-treated group and Lion's mane at a dose of 1.5g/kg (Figure 8).

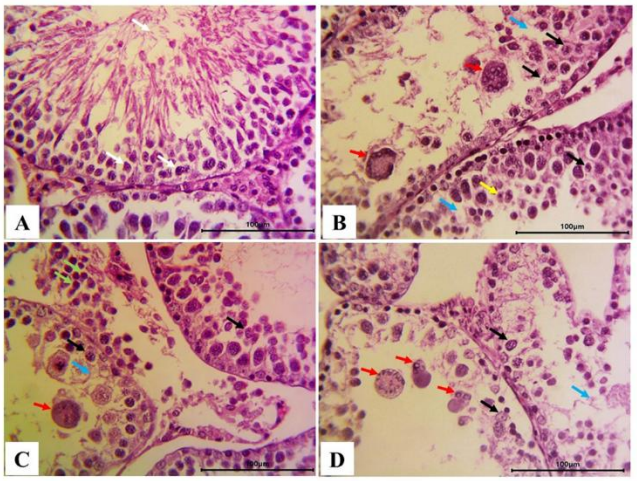

Figure 6: Histopathological findings in the testes of treated rats. A. The control group shows the normal structure of a seminiferous tubule represented by different stages of spermatogenesis (white arrows). B. Metronidazole-treated group shows necrosis of spermatocytes (black arrows), sloughing of spermatocytes into the lumen (blue arrows), presence of characteristic multinucleated giant cells (red arrows), and decrease in the number of Sertoli cells (yellow arrows). C. Lion's mane group at 1g/kg shows necrosis of spermatocytes (black arrows), sloughing of spermatocytes into the lumen (blue arrows), presence of characteristic multinucleated giant cells (red arrows), infiltration of mononuclear inflammatory cells in the interstitial tissue (green arrows). D. Lion's mane group at 1.5g/kg shows necrosis of spermatocytes (black arrows), sloughing of spermatocytes into the lumen (blue arrows), and the presence of characteristic multinucleated giant cells (red arrows). Stain: HE, Scale Bar=100 µm.

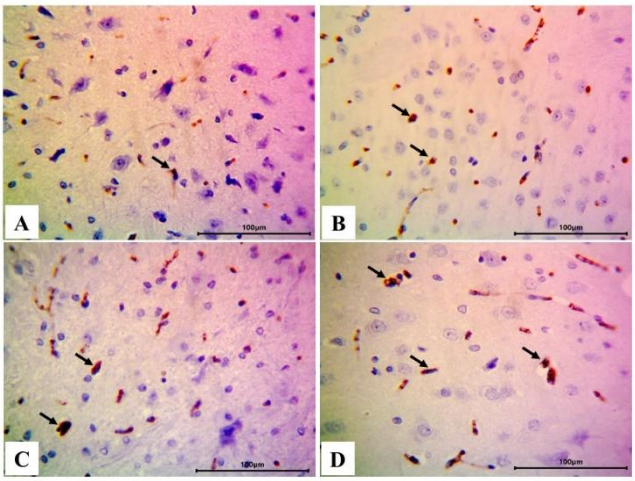

Figure 7: Immunohistochemical examination of p53 expression in the brain. A. Control group (mild expression), B. Metronidazole-treated group (moderate expression), C. Lion's mane group at 1g/kg (moderate expression), D. Lion's mane group at 1.5g/kg (intense expression). Black arrows indicate the positive expression, scale bar=100 µm.

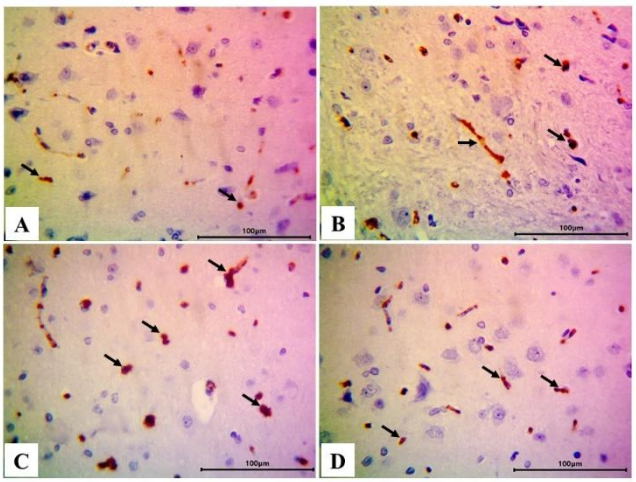

Figure 8: Immunohistochemical examination of Bcl-2 expression in the brain. A. Control group (mild expression), B. Metronidazole-treated group (moderate expression), C. Lion's mane group at 1g/kg (moderate expression), D. Lion's mane group at 1.5g/kg (moderate expression). Black arrows indicate the positive expression, scale bar=100 µm.

Discussion

Recently, Lion's mane has gained obvious attention from scientists as a potential therapeutic substance for neurological disorders, including Alzheimer's disease, depression, and Parkinson's disease. In the current study, we probed its efficacy in the protection of the brain of rats administered metronidazole. Our trial aimed to detect its effects on rat's reproductive organs as well. Neurotoxicity or neurological disorder is basically measured by a variety of neurobehavioral tests. An open field test is one of the neurobehavioral examinations that assess locomotor activity, anxiety, environmental exploring, and toxicity of chemicals in rodents and chicks (32-35). During the experimental period, it was obvious that metronidazole exhibited no neurological changes at the level of neurobehavioral examination compared to the control rats. Our findings showed that lg of Lion's mane caused a significant increase in grooming compared with rats treated with metronidazole.

Also, animals that were administered 1.5g of the mushroom showed a significant rise in the time spent in the center of the open field, which reflects changes in the response of rats to move quickly. A recent study has revealed that metronidazole caused pre-transcriptional alteration in the expression of apoptotic genes neurotransmitters, as well as an increase of inflammatory mediators in rats' brains,

administered 250mg/kg for 30 days (26). In order for metronidazole to trigger behavioral changes, it seems that animals are required to administer the drug for a longer period. In a previous study, genetically modified mice were utilized to examine the anti-depressive effect of Lion's mane (14). Rodriguez and his colleagues have examined the effect of Lion's mane on mice induced with Alzheimer's disease, which is characterized by the expression of Tau protein in their brains. They demonstrated that mice administered the mushroom at 1g and 550mg of mycelium polysaccharide displayed a significant increase in the time spent in the center of the open field, which enhanced the exploratory behavior and reflected its antidepression effects (14). An additional study also demonstrated that a combination of chlorella extract and Lion's mane improved depression in 6-month-old male mice (SAMP8). Open field results exhibited that aging animals showed a significant increase in the distance crossed in positive control, as well as mice who received 6 and 12mg of Lion's mane for three weeks. This study again supports the Lion's mane ability in improving neurobehavioral activity in aging animals (13).

Results of histopathology examination demonstrated the deleterious effect of long-term oral administration of metronidazole on the brain. Likewise, other studies proved the neurotoxicity effect of oral administration of metronidazole in rats, which could be attributed to its capacity to pass the blood-brain barrier (BBB) and, therefore, having a remarkable impact on numerous biomarkers in the brain by altering their enzymatic activity. Another reason for this neurotoxicity is its ability to interfere with antioxidants and activate both apoptosis and nitric oxide synthesis (36). The neurotoxicity of metronidazole on the brain has been noticed in several species, including cats, dogs, rats, and humans. Besides, several studies revealed the destructive effects and neurotoxicity of the long-term oral administration or high dose of metronidazole not only in the central nervous system but also in the endocrine system in rats (37-39). Besides, El-Moslemany *et al*. (26) showed histological findings similar to those of the current study. However, the results of the present work were more intensive, and this is due to the differences in the dose and duration of the experiments (26).

From the results of the current study, it's been noticed that administration of Lion's mane at 1g/kg in combination with metronidazole exhibited neuroprotective activity and a notable improvement in brain damage. One of the explanations for this protective role of the Lion's mane is its anti-oxidant and anti-inflammatory activities. These latter properties have been demonstrated in a stroke rat model that administered Lion's mane and its competent erinacine A (40). Erinacine A, in particular, has shown a remarkable reduction in oxidative biomarker inducible nitric oxide synthase (iNOS), as well as p38 mitogen-activated protein kinases (p38 MAPK). In addition to its antioxidant effects, it has been revealed that erinacine A decreased level of inflammatory mediators, including tumor necrosis factor á, interleukin-6 as well as interleukin-1β (40). The improvement of the brain damage in which the neuroprotective function of Lion's mane and its constituent, erinacine A, was confirmed against MPTP, a neurotoxic chemical both in vivo and in vitro through exhibiting anti-oxidant and anti-inflammatory effects (41). The evidence of Lion's mane in the prevention of neuron death in vivo was represented by a significant increase in nerve growth factor, neuronal glutathione, and dopamine in MPP^+-treated mice. Also, the mushroom and erinacine A has been demonstrated to inhibit signaling pathways involved in cell apoptosis; inositol-requiring enzyme type 1(IRE1), c-Jun N-terminal kinases (JNKs), nuclear factor kappa B (NF-κB) (41). There is more evidence that Lion's mane has been shown to reduce ROS and inflammatory mediators in cell lines, including HT22 cells (mouse hippocampal neurons) and BV-2 cells (a murine microglial cell line). Lion's mane extract has been shown to reduce oxidative stress and inflammation induced in HT22 cells and BV-2 cells by H_2O_2 and lipopolysaccharide, respectively. The neuroprotection activity of Lion's mane was indicated by maintaining neuron survival against H_2O_2-treated cells, protecting mitochondria against toxicity, decreasing ROS, and increasing the activity of antioxidant enzymes, in particular, catalase and glutathione (20). These findings are aligned with our results regarding neuronal protection against oxidative stress and inflammation caused by chemicals.

Correspondingly, Lion's mane was used to screen its potential protective effect on testes during long-term oral administration of metronidazole. Hence, this antimicrobial drug can trigger damage in male reproductive tissue, which was obvious through the harmful effects on the testicular tissue during the present experiment. Several studies verified that 28- and 60-day oral administration of the drug in a dose of 500 and 100 mg/kg, respectively, in mice, caused necrosis and sloughing of the epithelial cells lining the seminiferous tubules, resulting in inhibition of spermatogenesis (42-44). Other researchers recorded similar findings in rats, which aligned with our results (37,45-47). Recently, a study carried out by Raji *et al*. (48) using rabbits who were administered metronidazole in a dose of 400 mg/kg for 30 consecutive days showed disarrangement and degeneration of seminiferous tubules with necrosis of spermatocytes (48). Several mechanisms have explained the histological alterations caused by metronidazole. One of these suggests that metronidazole can trigger damage in male reproductive tissue since it has been proven its ability to generate ROS in rats and mice, which in turn cause detrimental effects in testicular tissue, including epididymis, seminiferous tubules, sperms, and testosterone hormone (25,43,44,49). One example of these free radicals is nitric oxide (NO), which is synthesized by nitric oxide synthase (NOS). Enormous production of NO can result in adverse consequences on living tissues (43). Interestingly, the main source of NO in

testes is activated macrophages (50). This fact interprets the intensive histological changes in the testicular tissue in the current work by the side of the proliferation of multinucleated giant cells.

Additionally, metronidazole is capable of damaging DNA, leading to disruption of the helical structure of the genetic material and, thus, the suppression of protein synthesis and, subsequently, cell death (37). Daily administration of lion's mane at 1.5g/kg was declared to have no improvement in both organs, the brain and testes. One essential composition of Lion's mane is phenolic compounds that can transform from antioxidant effect to prooxidant activity that may exhibit deleterious effects that involve damaging macromolecules, proteins, lipids, and DNA (51). In spite of the antioxidant activity of natural compounds in scavenging free radicals produced in the body, high doses of antioxidant substances can convert to prooxidant activity, which contributes to oxidative stress. Therefore, antioxidant concentration has to be limited depending on oxidative status needs (52).

As a result of testicular damage, testosterone levels remarkably declined during the experiment. This observation is consistent with a previous study performed by Sohrabi *et al*. (53), which showed low levels of testosterone in rats treated with metronidazole at 200 and 400mg/kg for two months (53). Additionally, several investigations have demonstrated resemblant results, which indicated decreased testosterone hormone concentration in rats who were administered the drug at different doses and durations (37,46,54). Again, in accordance with our results, a couple of studies have revealed that the administration of metronidazole caused impairment of fertility in male rats, which was reflected by damaging of the cells responsible for producing germ cells and the reduction of testosterone levels (25,45,46). They have reported a significant decrease in testosterone quantities in male rabbits administered metronidazole at 400mg/kg for thirty consecutive days.

In contrast to our results, the administration of metronidazole at 500mg/kg for 4 weeks did not cause any significant alteration in testosterone levels in male mice compared with the control group (48). This could be attributed to the dissimilarity in the duration of metronidazole administration between the two studies since rats in the current study were treated with the drug for 40 consecutive days rather than 28 days. Thus, prolonged administration of metronidazole can cause the accumulation of a significant amount of free radicals of the drug, which leads to massive damage to the testicular tissue and subsequently ends up with a decline in testosterone hormone level. Another hypothesis is the ability of metronidazole to penetrate the blood-testis barrier with an unambiguous toxic effect of the drug on the Leydig cells, which results in reduced testosterone production (55).

On the other hand, there is a lack of information about the effect of a lion's mane on testosterone. Therefore, we aim to perform this study to prove whether or not this mushroom has any effect on this hormone. Lion's mane at 1 and 1.5g/kg failed to maintain testosterone levels compared to the control rats. Although rats treated daily with Lion's mane at 1g/kg exhibited higher levels of the hormone than rats treated with metronidazole only and rats treated with 1.5g/kg, this was not significant. Previous studies revealed its beneficial functions in a variety of biological systems, including the cardiovascular, gastrointestinal tract, immune organs, and neurons, since it shows antioxidant and anti-inflammatory characteristics (6,42). Although it has been demonstrated to have antioxidant efficacy, a high concentration of Lion's mane has reversed its effect from antioxidant to prooxidant and resulted in testicular tissue damage (52).

The mechanism of neuroprotection provided by Lion's mane was further inspected by using the IHC technique to quantify the expression of two apoptosis-related genes; p53 and Bcl-2. Despite its role as a cancer suppressor, p53 is considered an essential transcription factor responsible for many functions, such as coordinating different genes in order to control a wide range of cellular mechanisms and cell stability. Other functions include engagement in most mechanisms in the neurons due to their diffuse expression in the brain tissue. Examples of these mechanisms are physiological cell death, gene repair, dendrite production, free radical damage, arrest of cell division, and autophagocytosis (56). In the current study, the metronidazole-treated group and both groups of rats treated with Lion's mane at 1 and 1.5g/kg exhibited relatively similar intensity of p53 expression. Nonetheless, a more intense reaction was seen in rats treated with 1.5g of Lion's mane than the other groups included in the experiment. Under normal circumstances, p53 expresses in low levels. During any stress factor or cell injury, such as damage to DNA, p53 is activated and tends to assemble in the nucleus to initiate its role as a pro-apoptotic (57). This finding leads us to the fact that Lion's mane serves as neuroprotective by stimulating p53 expression to induce apoptosis in the neurons. An in vitro study conducted by Zan *et al*. (58), who used human cancer cells from the stomach, showed the ability of Lion's mane to promote cell death by upregulation of p53 expression. Studying the effect of Lion's mane and its substantial function with p53 protein expression during drug toxicity needs to be further investigated to outline the possible mechanisms of this correlation in the course of brain damage (58).

It's well known that Bcl-2 plays an essential role in tumorigenesis and resistance against quite a few drugs through inhibiting the programmed cell death, as well as overexpression and phosphorylation of this protein, which helps in the modulation of cell division (59). Using the IHC technique revealed moderate expression of anti-apoptotic Bcl-2 in all treated groups. Our results agreed with that achieved by Chaturvedi *et al.* (36), who found that rats treated orally with metronidazole at 135mg/kg demonstrated

lower Bcl-2 expression in the brain tissue in comparison with the other animals' groups included in the experiment (36). This finding is attributed to the fact that metronidazole can increase the inflammatory markers within the brain. Similarly, El-Moslemany *et al*. (26) showed that oral administration of metronidazole in a dose of 200mg/kg body weight for 30 days caused a significant downregulation of Bcl-2 mRNA in rats (26). This result confirms the capability of metronidazole to trigger cell death in the brain tissue.

On the other hand, rats treated with 1g/kg of Lion's mane exhibited more intense expression than the remaining groups. Accordingly, another study conducted by Kushairi *et al*. (20), who used mouse hippocampal neurons treated with a combination of Lion's mane and H_2O_2, demonstrated an elevation of Bcl-2 activity in the cultured cells via qPCR technique. This neuroprotection was through its anti-apoptotic function (20). The variation in the gene expression of both p53 and Bcl-2 in relation to the doses of Lion's mane used in this work can be linked to dose-dependent apoptosis modulation (60,61). Nonetheless, more studies are necessary to illuminate this neuroprotection by using molecular biology techniques in order to outline the mechanistic pathways involved in this role.

Conclusion

This study concludes that Lion's mane alleviated neuronal and testicular damage caused by metronidazole, in particular at low doses. Whereas high doses resulted in deterioration of the reproductive organ and testosterone level. The results of this research provided novel evidence of mushroom's ability in testicular protection. Further work is needed to elucidate the definite effect of apoptotic genes in correlation with Lion's mane.

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Conflict of interest

The authors declare there are no conflicts in this study.

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إمكانية عرف الأسد كوقائي ضد سمية الميترونيدازول المحدثة في دماغ وخصى ذكور الجرذان: تقييم التعبير البروتيني والكيموحيوي

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الخلاصة

عرف الأسد هو فطر طبي مكون من جزئيات بايوحيوية متنوعة والتي أثبتت فائدتها في أمراض التنكس العصبي. لذلك كان هدف الدراسة التحقق من الوظيفة المحتملة لهذا الفطر في حماية الدماغ، وتم دراسة تأثيره على الخصى من خلال سمية الميترونيدازول المستحثة. قسمت الحيوانات الى أربع مجاميع; كل مجموعة مكونة من ست حيوانات. مجموعة السيطرة تم تجريعها بالماء المقطر، المجموعة الثانية تم إعطائها الميترونيدازول بجرعة ٥٠٠ ملغم/كغم، المجموعة الثالثة والرابعة تم إعطائها ١ و١,٥ غم/كغم من عرف الأسد على التوالي. بعد ساعتين، تم تجريعها الميترونيدازول بجرعة ٥٠٠ ملغم/كغم. تم تجريع الميترونيدازول وعرف الأسد عن طريق الفم لمدة أربعين يوماً متتالية. أظهرت نتائج الدراسة وجود تغييرات سلوكية عصبية معنوية في الحيوانات المعاملة بعرف الأسد. عرف الأسد بجرعة ١ غم/كغم قلل من الضرر النسجي في الدماغ وحافظ على مستويات هرمون التستوستيرون بينما أظهرت جرعة ١,٥ غم/كغم تأثيرات عكسية على الخصية. جميع المجاميع المعاملة أظهرت شدة متشابهة في تعبير كل من البروتين الثالث والخمسون والبروتين الورم الليمفاوي للخلايا البائية من النوع ٢. اللافت للنظر، كان تعبير البروتين الثالث والخمسون أكثر شدة بجرعة ١,٥ غم/كغم، بينما تعبير وبروتين الورم الليمفاوي للخلايا البائية من النوع ٢ كان أكثر شدة بجرعة ١ غم/كغم. نستنتج من الدراسة الحالية، إن عرف الأسد بالجرعة القليلة يعمل كنبات حامي ضد سمية الميترونيدازول، يظهر تحسين في التغييرات النسيجية، وله خصائص ضد موت الخلايا المبرمج.

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