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ORIGINAL RESEARCH

Aqueous Seed Extract of *Nigella Sativa* Ameliorated Metronidazole-Induced Testicular Damage Via Up-Regulations of the Antioxidant System

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Abstract

Background: Infertility is a condition characterized by the failure to achieve a pregnancy after 12 months or more of regular, unprotected sexual intercourse.
Objectives: To determine the effect of aqueous seed extract of *Nigella sativa* on testicular antioxidant levels in metronidazole-induced infertility in male Wistar rats.
Methods: A total of thirty-five (35) male Wistar rats (aged 7-10 weeks old) were randomly distributed into five different groups (n = 7 each); Group A (Control group), Group B (Metronidazole only), Group C (Metronidazole + aqueous seed extract of *Nigella sativa*), Group D (Metronidazole Recovery), and Group E (Metronidazole + aqueous seed extract of *Nigella sativa* + Recovery). Experimental animals received 500 mg/kg of metronidazole and 300 mg/kg of aqueous seed extract of *Nigella sativa* orally.
Results: Metronidazole treatment caused a significant ($p<0.05$) reduction in the level of testicular glutathione (Control group - 3.50±0.50; MTZ only - 2.00±0.30; MTZ+NS - 2.50±0.40; MTZ+ NS+ Recovery - 2.80±0.40). Similar reductions occurred in the activities of testicular superoxide dismutase (Control - 1.58±0.10; MTZ only - 0.23±0.06; MTZ+NS - 0.68±0.10; MTZ+ NS+ Recovery - 0.68±0.10) and catalase (Control - 2.70±0.35; MTZ only - 0.70±0.25; MTZ+NS - 1.23±0.20; MTZ+ NS+ Recovery: 1.25±0.30) which was followed by histo-architectural alterations in the testes of rats. Thereafter, aqueous seed extract of *Nigella sativa* treatment improved all the initial alterations caused by metronidazole treatment. Two - weeks recovery period was accompanied by a significant ($p = 0.017$) increase in antioxidant enzyme activities of group E when compared with group B and group D.
Conclusions: The aqueous seed extract of *Nigella sativa* exerts its ameliorative effect by improving antioxidant enzyme activities in the testes which then protect the testes against oxidative damage, enhancing sperm quality, preventing apoptosis, supporting testicular function, and aiding tissue repair.

Keywords: Anti-oxidants, Male infertility, Metronidazole, Nigella sativa, Superoxide dismutase.

Introduction

Infertility is a disease of the male or female reproductive system characterized by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. [1] It has significant psychological, economic, and medical implications, potentially resulting in trauma and stress. About 45% of cases of infertility involve exclusively males, while 20% involve both males and females. [2] Usually, male infertility can be diagnosed by simply carrying out an analysis of semen to determine parameters such as concentration of sperm, sperm number, sperm motility, and morphology. [3] Notably, infertility in males is usually characterized by abnormal sperm parameters, including low sperm count, poor sperm motility, and abnormal sperm morphology. Furthermore, illnesses, injuries, chronic health problems, lifestyle choices and other factors which includes genetic, environmental, and endocrine agents have all been implicated in the pathogenesis of male infertility. [4]

Nigella sativa is an herbaceous plant of the annual variety belonging to the Ranunculaceae family. It thrives in tropical and subtropical regions, particularly on loamy soils. [5] This plant, known for its delicate flowers and thread-like leaves, can reach a height of 20-60 cm. [6] The flowers themselves contain numerous seeds and come in various colors ranging from yellow to white. *Nigella sativa* is a branched herb erected with a tap root system, division of leaves, and attractive flowers. While the plant is generally pentamerous, its stamens can be found in abundance. The seeds are produced within large fruit. The cultivation of *Nigella sativa* is prevalent in the Middle East, Europe, Asia, and other Mediterranean countries, serving diverse purposes. [7] The cultivation typically takes place in spring and the harvest occurs in fall, although insignificant variations in the cultivation pattern may arise depending on the geographic location. The growth duration of *Nigella sativa*, from sowing to harvest, typically spans six months. The primary reason for cultivating this plant is its seeds, which are widely used as spices, flavoring agents, and medicinal remedies, particularly in South Asia.

The main chemical constituents found in *Nigella sativa* include thymoquinone, α-phellandrene, thymol, oleic acid, carbohydrates, and proteins. [8, 9] The taste of black cumin seeds is bitter, and their composition varies depending on the harvesting season, species, and the ecosystem in which they are cultivated. [10] Previous research has identified palmitic acid, oleic acid, trans-anethole, and linoleic acid as the main constituents of these seeds. [11] Additionally, phenolics and Quinones (such as thymol, thymoquinone, thymohydroquinone, and, dithymoquinone) have been found in the plant. [7] The oils of black seeds have been shown to contain various compounds, with thymoquinone being the most prevalent. [12] Monoterpene hydrocarbons were found to dominate the compounds yielded by *Nigella sativa seed*s. [13] Proteins, oils, phenols, and alkaloids have also been detected in these seeds. [14] Recent studies have highlighted the diverse biochemical makeup of the plant, including phenols, terpenes, and flavonoids. [15, 16]

Metronidazole is a foremost nitroimidazole [17] employed clinically for the management of anaerobic infections, usually involving the genital regions in both sexes. The anti-spermatogenic effect of metronidazole is well known. [17] The infertility mechanism of metronidazole arises from its action on the capacity of spermatozoa to finally generate ATP via the glycolytic pathway. [18] Spermatogenesis may be harmed by the escalation in inhibition of alpha-glycosidase malondialdehyde (MDA). At the same time, sperm motility might be diminished by the inhibition of energetic transferase or non-protein substance in the

epididymis. Metronidazole also has an excellent nervous tissue penetration. [7] Therefore, the aim of this study was to determine the effect and mechanism of exertion of the effects of aqueous seed extract of N*igella sativa* on metronidazole-induced infertility in experimental animals.

Methods

Study design
An experimental animal-based design.

Experimental Animals
Adult male Wistar rats (7-10 weeks old) weighing 150-200g were obtained from the Olabisi Onabanjo Animal House and were kept in wire plastic topped cages in a room under standard conditions of illumination with a 12-hour light-dark cycle at 25±1° C. They were also provided with water and fed *ad libitum*.

Sample size
Thirsty-five rats, divided into seven animals each in five groups.

Drug preparation
The drug (Metronidazole) was obtained from Forever Pharmacy Company, Nanjing Xin'gang Development Zone, Qixia District, Nanjing City. The anti-spermatogenic impact of metronidazole has been demonstrated to be acute. [17] The drugs were dissolved in isotonic saline solution and administered orally.
Preparation of Metronidazole (MTZ) [19]:
Dose: 500 mg/kg body weight. [19]
For a 200g of rat, 100mg of metronidazole was dissolved in 2ml of saline water and then administered. Stoke solution is 2000mg or 2g (400mg x 5tablets) dissolved in 40ml.
Preparation of aqueous seed extract of Nigella sativa [20]
Dose: 300 mg/kg body weight. [20]
For a 200g of rat, 60mg of powdered seed extract of *Nigella sativa* was dissolved in 2ml of saline water and was then administered. Stoke solution is 6000mg or 6g dissolved in 200ml.

Grouping and methodology
Rats were randomly divided into five groups:
Group A: Control group; received normal saline.
Group B: Received 500 mg/kg of metronidazole for 14 days.
Group C: Received 500 mg/kg of metronidazole for 14 days and 300 mg/kg of aqueous seed extract of *Nigella sativa* for 14 days.
Group D: Received 500 mg/kg of metronidazole for 14 days and was allowed to recover for 14 days.
Group E: Received 500 mg/kg of metronidazole for 14 days and 300 mg/kg of aqueous seed extract of *Nigella sativa* for 14 days and was allowed to recover for 14 days.
Experimental rats (Groups B, C, D, and E) received oral metronidazole for 14 days at the dose of 500 mg/kg/day body weight which will give acute effect, [19] and (Groups C and E) received aqueous seed extract of *Nigella sativa* for 14 days at the dosage of 300 mg/kg as described above. [20]

Animal sacrifice, collection, and preparation of samples
At the end of the study, each animal was sacrificed by cervical dislocation.

Tissue preparation
The testicular tissues were transferred into 10% phosphate buffer (pH 7.4). The tissue was homogenized using a manual homogenizer. The broken cell debris were removed by centrifugation (3000rpm) for ten minutes to obtain the supernatant. The level of antioxidant enzyme activities as well as lipid peroxidation were measured by Thiobarbituric acid reactive assay. [21]

Histological examination
Tissue specimens from the testes of all experimental rats were collected at the end of the

study and fixed in neutral buffered formalin. They were processed by conventional method, embedded in paraffin, sectioned at 4-5µm and stained by Haematoxylin and Eosin. The histological analysis was done at the Histology Laboratory in Anatomy Department of Olabisi Onabanjo University, Sagamu Campus.

Statistical analysis

The results were presented as Mean ± S.E.M for comparative distinctions among all groups with the use of One-way ANOVA test, and examination for multiple comparisons using Turkey (HSD) Post HOC analysis and Duncan tests. Statistical values were considered to be of statistical significance when $p<0.05$.

Ethics considerations

The data collection and analysis were compiled following the principles of the Declaration of Helsinki. Ethical approval was obtained from the Health Research Ethics Committee of the Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria with the number OOUTH/ HREC/681/ 2023AP.

Results

Figures 1, 2, 3, and 4 show the antioxidant level of testis across all groups. In Figure 1 (SOD), there is a significant increase ($p = 0.01$) in Group C (MTZ + NS: 0.68±0.10) when compared to Group B (MTZ: 0.23±0.06) and a non-significant increase ($p = 0.07$) when compared to Group E (MTZ + NS + Recovery: 0.68±0.10). There was a significant decrease ($p = 0.03$) in Group B (MTZ: 0.23±0.06) when compared to Group A (Control: 1.58±0.10) and a non-significant decrease when compared to Group D (MTZ + Recovery: 0.30±0.09).

In Figure 2, [catalase (CAT)], there was a significant decrease ($p<0.05$) in Group B (MTZ: 0.70±0.25) when compared to Group A (Control: 2.70±0.35) and a significant increase ($p = 0.04$) in Group D (MTZ + Recovery: 0.75±0.28) when compared to Group B (MTZ: 0.70±0.25). There was a significant increase ($p = 0.002$) in Group C (MTZ + NS: 1.23±0.20) when compared to Group B (MTZ: 0.70±0.25) and a non-significant increase when compared to Group E (MTZ + NS + Recovery: 1.25±0.30).

In Figure 3, [Malondialdehyde (MDA)], there was a significant decrease in Group B (MTZ: 1.00±0.20) when compared to Group A (Control: 3.00±0.20) and a significant decrease in Group B (MTZ: 1.00±0.20) ($p = 0.012$) when compared to Group D (MTZ + Recovery: 1.50±0.40). There was a significant increase in Group C (MTZ + NS: 2.20±0.30) ($p = 0.001$) when compared to Group B and a significant decrease in Group C ($p = 0.014$) when was compared to Group E (MTZ + NS + Recovery: 2.50±0.40).

In Figure 4, [Glutathione (GSH)], there is a significant decrease in Group B (MTZ: 2.00±0.30) when compared to Group A (Control: 3.50±0.50) and a non-significant increase ($p = 0.091$) when compared to Group D (MTZ + Recovery: 2.20±0.30). There is a significant increase ($p = 0.031$) in Group C (MTZ + NS + Recovery: 2.80±0.40) when compared to Group B (MTZ) and a non-significant decrease ($p = 0.20$) when compared to Group E (MTZ + NS + Recovery: 2.00±0.30).

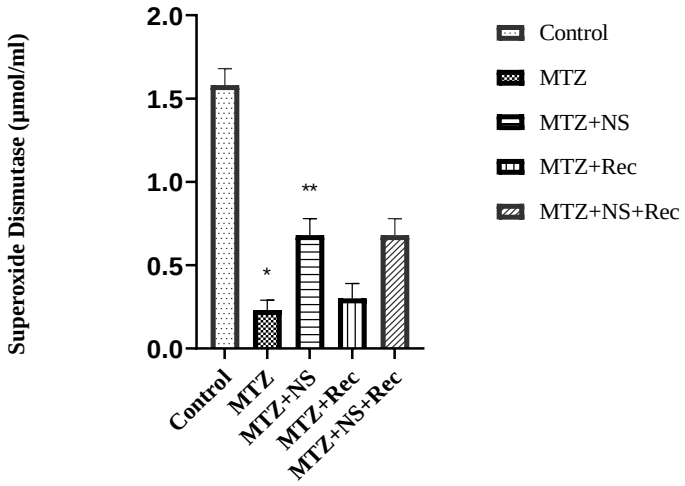

Figure 1: Effect of Nigella Sativa on testicular superoxide dismutase activity in rats.
MTZ = metronidazole, NS = *Nigella sativa* seeds, Rec = Recovery

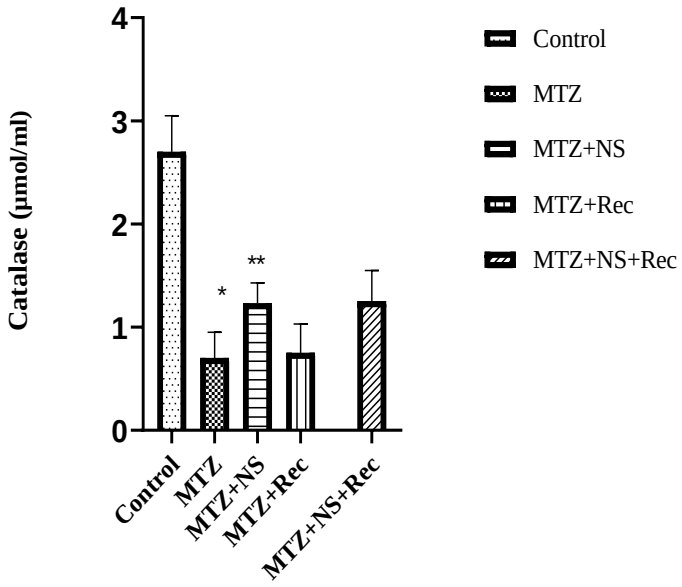

Figure 2: Effect of Nigella Sativa on testicular catalase activity in rats.
MTZ - Metronidazole, NS - *Nigella sativa* seeds, Rec – Recovery

Discussion

An antioxidant can be defined as a substance that can impede oxidative processes and hinder chemical reactions that transfer electrons or hydrogen to oxidizing agents. There are two categories of antioxidant systems, namely enzymatic systems like catalase (CAT) superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), as well as non-enzymatic

antioxidant systems like glutathione, uric acid, vitamin E, bilirubin, α-lipoic acid, vitamin C, and carotenoids. [22] Causes of oxidative stress include an imbalance of free radicals and the body's antioxidant defenses. [23] The inability to protect against the harmful effects of free radicals will result in the process of lipid peroxidation, which is characterized by the generation of MDA. It has been well-documented that elevated levels of MDA are linked to the development of numerous diseases that are connected to oxidative stress. [24]

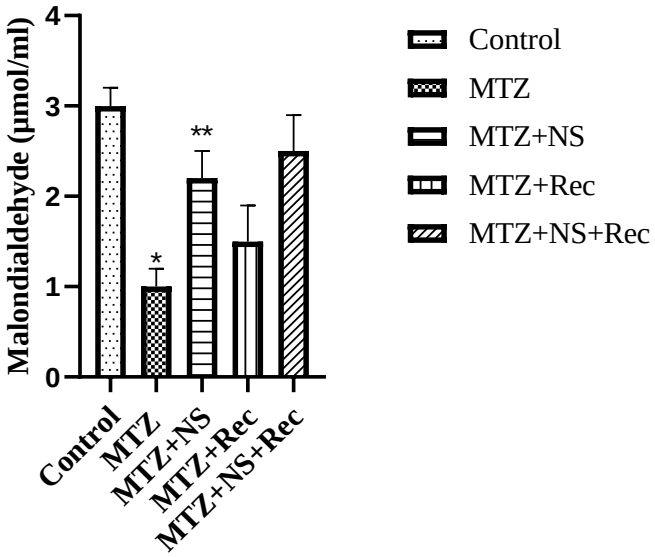

Figure 3: Effect of *Nigella Sativa* on testicular malondialdehyde (MDA) level in rats
MTZ - Metronidazole, NS - *Nigella sativa* seeds, Rec - Recovery

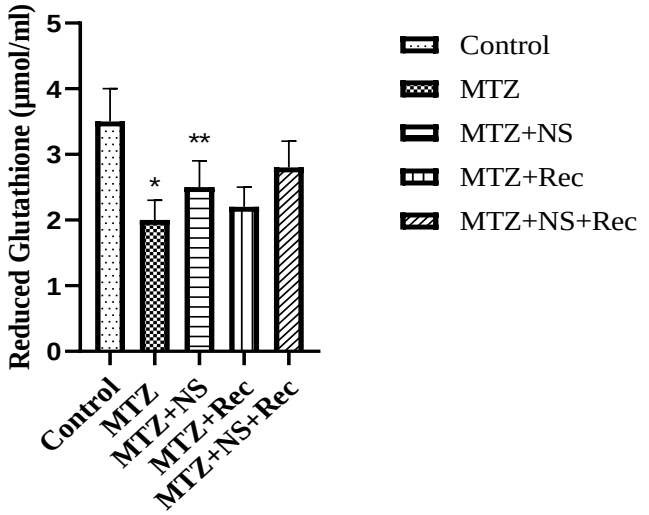

Figure 4: Effect of NS on testicular glutathione (GSH) levels in rats
MTZ - Metronidazole, NS - *Nigella sativa* seeds, Rec - Recovery

Testicular Histology

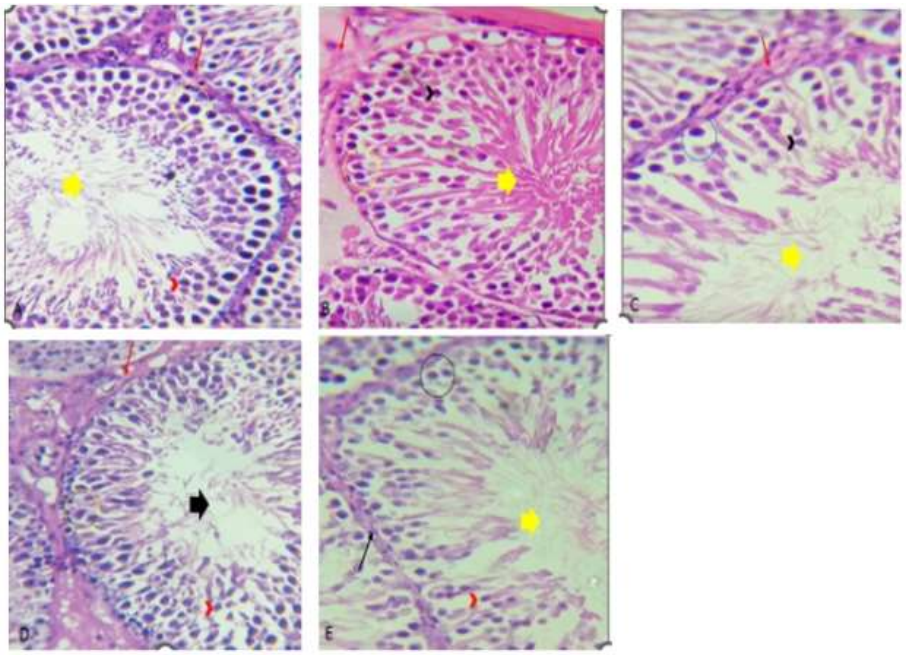

Plate 1: Photomicrograph of testicular tissue

Control group (A), MTZ group (B), MTZ + NS group (C), MTZ + NS + Rec group (D), MTZ recovery group (E), (H/E X400). Spermatogonia cells: yellow circle, lumen: yellow thick arrow, Sertoli cells: red arrow head, Leydig cells: red thin arrow, reduced Sertoli cells: black arrow head, spermatogonia cells: blue circle. Control group showing well differentiated and normal spermatogonia cells (yellow circles), lumen (yellow thick arrows), Sertoli cells (red arrow head) and Leydig cells (red thin arrow) on the interstitial layer.

MTZ-only shows distortion of the interstitial layer with reduced Leydig cells (red thin arrow), constricted lumen (yellow thick arrows), reduced Sertoli cells (black arrow head) and spermatogonia cells (yellow circle).

MTZ + NS show differentiated Leydig cells (red thin arrows), Sertoli cells (black arrow head), lumen with spermatocytes (yellow thick arrow) and spermatogonia cells.

MTZ + NS + recovery shows well differentiated spermatogonia cells (yellow circle), lumen with spermatocytes (black thin arrow) and Leydig cells (red thin arrow).

MTZ + recovery group shows well organized spermatogonia cells (black circle), lumen (yellow thick arrow), Leydig cell (black thin arrow) on the interstitial layer.

Mohajeri's study also found that *Nigella sativa* improved testicular damage and antioxidant status in mice. [25] This study collectively suggests that the extract of *Nigella sativa* seed may have a positive effect on the testes' antioxidant levels in male Wistar rats with metronidazole-induced infertility which is in line with the findings in this research. *Nigella sativa*'s anti-oxidative and anti-inflammatory properties likely contribute to the improvements in sperm quality, creating a conducive environment for efficient spermatogenesis. This study is similar to the findings on a study of the methanolic leaf extract of *Vernonia amygdalina* which eradicated the adverse effects of Nitrobenzene on the antioxidant enzymes, markers of testicular oxidative damage, endocrine and testicular structure in rats. [26] The anti-spermatogenic impact of metronidazole has been demonstrated. [17] Metronidazole induction cause oxidative stress which decrease SOD, CAT, GSH activity, leading to an accumulation of superoxide radicals, increased hydrogen peroxide levels and deplete glutathione levels; these effects are harmful to testicular cells and results in testicular damage. [27, 28]

The extracts and essential oils of *Nigella sativa* produce fertility and antioxidant effect. Mohajeri reported that *Nigella sativa* treatment improved spermatogenesis similar to the findings in this study where differentiated leydig cells, sertoli cells, lumen with spermatocytes and spermatogonia cells were demonstrated; these changes tend to promote spermatogenesis in the male Wistar rats. [25] It was reported by Mosbah *et al*. and Prairna *et al*. that *Nigella sativa* directly affect the reproductive cells and tissues' antioxidant defense mechanisms by increasing the activity of antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase. In consequence, these putative antioxidant actions reduce the production of lipid peroxidation and reactive oxygen species (ROS) in reproductive cells and tissues, thereby encouraging pregnancy and childbirth. [29, 30] Ardiana *et al*. reported that *Nigella sativa* has been proven to have antioxidant capabilities by reducing the production of reactive oxygen species (ROS) and malondialdehyde (MDA). [31]

This study was limited to the evaluation of testicular antioxidant (CAT, SOD, MDA, and GSH) activities and histological examination. Further study may consider investigating the effect of aqueous seed extract of *Nigella sativa* on the hypothalamic-pituitary gonadal axis in metronidazole-induced infertility in adult male Wistar rats, delving into its effect on hormonal levels (gonadotropin releasing hormone, follicle stimulating hormone, luteinizing hormone, and testosterone), and sperm parameters.

Conclusion

The aqueous extract derived from the *Nigella sativa* seeds is discovered to possess a wide range of advantageous effects on the testes and its associated systems. Notably, it has demonstrated the capacity to control the presence of oxidative stress markers within the testes, thus indicating its potential protective properties. Furthermore, it was suggested that the extract of *Nigella sativa* seeds might promote spermatogenesis through its potential to alleviate metronidazole infertility effects.

Authors' Contributions: OIO participated in the design of the study and drafted the manuscript. OIO, ADB, OBO, OAA, and OSO analyzed and interpreted the data. OBA, EVB, OOA, and TDO contributed to the conception and design of the study, and revised the manuscript for sound intellectual contents. All the authors approved the final version of the manuscript.
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