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ORIGINAL RESEARCH

The Effect of Thymoquinone on the Hypothalamic-Pituitary-Gonadal- Gonadal Axis in Metronidazole-Induced Infertility in Adult Male Wistar Rats

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Abstract

Background: Hypothalamus centrally modulates fertility by controlling the reproductive hormonal axis.
Objectives: To investigate the effect of thymoquinone on the hypothalamic-pituitary-gonadal (HPG) axis in metronidazole-induced infertility in adult male Wistar rats.
Methods: Forty-nine male Wistar rats were divided into seven groups: group A (Control), Group B (Metronidazole only), Group C (Metronidazole + Thymoquinone low dose), Group D (Metronidazole + Thymoquinone high dose), Group E (Metronidazole + Recovery), Group F (Metronidazole + Thymoquinone low dose + Recovery), and Group G (Metronidazole + Thymoquinone high dose + Recovery). Experimental animals received 500 mg/kg of metronidazole, 200 mg/kg of thymoquinone, and 400 mg/kg of thymoquinone. Hormonal levels (GnRH, FSH, LH, testosterone) and sperm parameters were assessed after specific treatment durations using enzyme-linked immunosorbent assay (ELISA) and standard sperm analysis techniques.
Results: Metronidazole significantly decreased reproductive hormones: GnRH (71.11 ± 0.78 pg/mL), FSH (24.12 ± 0.60 mIU/mL), LH (4.87 ± 0.75 mIU/mL), and testosterone (2.13 ± 0.54 ng/mL) compared to controls (GnRH: 94.44 ± 0.19, FSH: 36.41 ± 1.90, LH: 14.93 ± 0.05, testosterone: 6.84 ± 0.13). Thymoquinone administration notably restored hormone levels: Group C (GnRH: 99.50 ± 0.29, FSH: 38.32 ± 0.90, LH: 35.97 ± 0.54, testosterone: 6.99 ± 0.53) and Group D (GnRH: 95.50 ± 0.17, FSH: 40.18 ± 0.63, LH: 31.43 ± 1.10, testosterone: 6.89 ± 0.42). Sperm parameters improved significantly in treated groups, with viability at 75.00 ± 0.04%, count at 205.00 ± 0.87 million/mL, motility at 77.00 ± 0.40%, and morphology at 80.00 ± 0.19%. Histological analysis revealed regeneration of testicular tissues.
Conclusion: Thymoquinone effectively ameliorates metronidazole-induced infertility by restoring the HPG axis function, enhancing hormone levels, and improving sperm parameters.
Keywords: Hypothalamic-Pituitary-Gonadal Axis, Infertility, Luteinizing Hormone, Metronidazole, Testosterone, Thymoquinone.

Introduction

A clinical definition of infertility is a condition of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse. [1] The World Health Organisation (WHO) defines infertility as a disease of the male or female reproductive system characterised by the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. [2] American Society for Reproductive Medicine states that infertility is a disease, condition, or status characterised by the inability to achieve a successful pregnancy based on a patient's medical, sexual, and reproductive history, age, physical findings, diagnostic testing, or any combination of those factors. [3,4]

According to a study conducted in Nnewi, Nigeria, the infertility prevalence rate of 26.8% was observed, and the secondary infertility (55%) was the predominant type of infertility within the study population. In comparison, the remaining (45%) had primary infertility. [5] A cross-sectional survey conducted in North-Western Nigeria found that about 62% of women with infertility had received previous hospital treatments, highlighting the varied and often inadequate nature of fertility services available. [6] Many women also sought traditional therapies, which had a success rate of only 8.3% when used alone but were slightly more effective when combined with other methods. [7] Furthermore, a comprehensive analysis emphasises the psychological and social burden of infertility on Nigerian women, as many experience long durations of infertility, with significant impacts on their mental health and social status. [7]

The hypothalamus-pituitary-gonadal axis is pivotal in the modulation of reproductive activities and fertility. The hypothalamus synthesises gonadotropin-releasing hormone (GnRH), which is crucial for prompting the anterior pituitary gland to secrete two principal hormones: follicle-stimulating hormone (FSH) and luteinising hormone (LH). [8] GnRH initiates the release of FSH and LH, which subsequently stimulate the testes to generate testosterone. This mechanism is essential for sexual maturation, reproductive cycles, and overall fertility. FSH facilitates the development and maturation of germ cells, whereas LH triggers testosterone synthesis in males. [9] The HPG axis functions through intricate feedback mechanisms involving sex steroids, which modulate GnRH release. Testosterone is crucial for maintaining qualitative spermatogenesis and plays a vital role in the initiation of sperm production. The presence of testosterone in the testes is essential; without its receptor, spermatogenesis cannot progress beyond the meiosis stage, leading to infertility. [10] Perturbations in the HPG axis may result in conditions such as hypogonadism and infertility, underscoring its critical importance in reproductive health. [8]

Thymoquinone, the main active compound in *Nigella sativa* seeds, is best known for its therapeutic benefits, such as antioxidant, anti-inflammatory, anti-diabetic, and hepatoprotective effects, and for its low risk of side effects (some individuals may experience skin rashes, occasional nausea, vomiting, etc.). [11] *Nigella sativa*, commonly known as fennel flower or black seed, has a good history in traditional Middle Eastern medicine, where it is known as 'blessed seed' and is referred to as 'Asofeyeje' within the Yoruba community and 'Habatu sauda' within the Hausa community in Nigeria, West Africa. [12] It is known for therapeutic benefits such as anti-inflammatory, anti-diabetic, antioxidant, hepatoprotective properties, etc and was used across various places as a natural remedy to treat health issues, which include asthma, bronchitis, inflammatory conditions, hypertension, diabetes mellitus, headaches, fevers, seizures, gastrointestinal problems, and microbial infections. [11, 13]

A popular antimicrobial drug for treating a variety of anaerobic and protozoal diseases is metronidazole (MTZ). [14] The anti-spermatogenic effects of metronidazole have been demonstrated in various studies. [15] The

mechanism of metronidazole-induced infertility arises from its impact on the capacity of spermatozoa to generate ATP via the glycolytic pathway, leading to impaired sperm motility and hindering capacitation, which is crucial for fertilisation. [16, 17] There is a paucity of research on the effects of thymoquinone on the hypothalamic-pituitary-gonadal (HPG) axis, with little to no existing studies addressing this relationship. Therefore, this is a novel study that investigated the role of thymoquinone on the HPG axis in metronidazole-induced infertility in adult male Wistar rats and assessed its ameliorative effects. The investigation focused on both the brain and testes tissues of treated male rats. The testes were selected for analysis to evaluate the effects of thymoquinone treatment on sperm parameters, including morphology, motility, viability, and sperm cell count.

Methods

Experimental animals

In this study, forty-nine (49) male healthy Wistar rats, of average weight, 150-160 g, were obtained from Olabisi Onabanjo University Animal House, located in Sagamu Campus, Ogun State, Nigeria. The rats were kept for an acclimatised period of 14 days on pelletised rats' chow and water ad libitum in the Physiology Department section of Animal House at a room temperature of 27 - 30 °C, 12 hours' light and day cycle with well-defined maintenance and care for animals in compliance with ethical guidance of the Animal House. Ethical approval to conduct the study was obtained from the Olabisi Onabanjo University Teaching Hospital Health Research Ethics Committee (OOUTH HREC) [ID OOUTH/HREC/864/2024AP], and the experimental procedures followed the Institutional Animal Care and Use Committee (IACUC) Guidelines.

Experimental design

The animals were weighed and randomly assigned to seven groups of seven animals each. Control group A received 0.5 mL of normal saline, group B received 500 mg/kg of metronidazole (MTZ) for 14 days, group C (Metronidazole + thymoquinone low dose): Received 500 mg/kg of MTZ for 14 days, followed by 50 mg/kg of thymoquinone for another 14 days, group D (Metronidazole + thymoquinone high dose): Received 500 mg/kg of MTZ for 14 days, followed by 100 mg/kg of thymoquinone for another 14 days, group E (Metronidazole + Recovery): Received 500 mg/kg of Metronidazole for 14 days and was allowed to recover for 14 days. Group F (Metronidazole + thymoquinone low dose + Recovery): Received 500 mg/kg of MTZ for 14 days, followed by 50 mg/kg of thymoquinone for another 14 days. They were then allowed to recover without any administration for another 14 days. Group G (Metronidazole + thymoquinone high dose Recovery): Received 500 mg/kg of MTZ for 14 days, followed by 100 mg/kg of thymoquinone for another 14 days. They were then allowed to recover without any administration for another 14 days. The dosage of metronidazole was in accordance with the method of Abeer *et al*. (2004) [18], while that of Thymoquinone followed the technique of Bahaa *et al.* (2017) [11]

Sacrifice and sample collection

The rats were sacrificed on days 14, 28, and 32, corresponding to distinct phases of the experiment. On day 14, groups B and E, which had received 14 days of metronidazole (with group E allowed a subsequent 14-day recovery period), were sacrificed. Groups C and D were sacrificed on day 28, immediately after completing 14 days of metronidazole followed by 14 days of thymoquinone treatment at low and high doses, respectively. Finally, groups F and G, which underwent the same treatment as groups C and D but were given an additional 14-day recovery period without treatment, were sacrificed on day 32. Blood samples were obtained from the orbital venous sinus of the rats through ocular puncture using capillary tubes. [19] The collected blood was then

transferred into plain sample bottles and centrifuged at 4000 revolutions per minute for 15 minutes. The bottles were labelled correctly, and the serum was carefully collected using a micropipette and corresponding tips, then transferred into plain sample bottles.

Hormone assay

Enzyme-linked Immunosorbent Assay (ELISA) Kits were used to determine the concentrations of GnRH, testosterone, LH, and FSH in the collected serum samples.

Sperm analysis

Sperm count

To count the sperm cells, a small amount of prepared epididymal sperm suspension was diluted with formaldehyde fixative (10% formalin in phosphate-buffered saline). We diluted 400 µL of the sperm suspension with formaldehyde (Sigma, USA), and approximately 10 µL from the diluted solution was transferred into a Neubauer chamber using a Pasteur pipette (Thoma, Assistant, Sondheim/Rhön, Germany). The solution was allowed to remain for 7 minutes. Then, the sperm cells at the four corners of the central square were counted. [20]

Sperm viability

Sperm viability was evaluated using eosin and nigrosin staining (5% in saline). Fresh sperm suspension (40 µL) was placed on a glass slide, mixed with 1% eosin, and observed by a light microscope (×400) after the smear was allowed to air-dry on a glass slide. Live sperms remained unstained following staining. At least 250 sperms were counted from each sample in ten fields, and the ratio of live sperms was verified [20].

Sperm motility

The percentage of motile sperm was evaluated using a light microscope (Olympus Co., Tokyo, Japan) at 400× magnification. For this process, one drop of sperm suspension was placed on the chamber. Sperm motility was divided into four levels according to specific criteria: slowly progressive forward movement, rapid progressive forward movement, residual motion, and motionless. Sperm were counted in several microscopic fields, and the percentages of motile and immotile sperm cells were obtained. Motility estimates were obtained from five different fields in each sample. The mean of the five successive estimations was used as the final motility score. [20]

Sperm morphology

The sperm morphology was evaluated by analysis of sperm smears made from the left cauda epididymis. An aliquot of the sample was used to prepare smears for assessing spermatozoa deformities. The Papanicolaou method was used to estimate spermatozoa morphology. A total of 300 spermatozoa were analysed per slide (3000 cells per group) for abnormalities of the head and tail. [21]

Histological studies

Germinal layer thickness

After the hypothalamus, pituitary, and testes were preserved in formalin, histological processing, including dehydration, clearing, and embedding, was performed. The microscopic sections (5 µm) were prepared, and the hematoxylin and eosin staining method was used [22]. The histological analysis was performed at the histology laboratory in the Anatomy Department of Olabisi Onabanjo University, Sagamu Campus.

Statistical analysis

All measurements were analysed using SPSS version 25. The results were presented as Mean ± S.E.M. for comparative distinctions among all groups through the implementation of One-way ANOVA, and examination for multiple comparisons using Turkey (HSD) Post HOC analysis and Duncan tests. Values were considered to be of statistical significance when $p<0.05$.

Results

Table I shows the hormone levels across all groups. For GnRH, there was a significant increase in Group C (MTZ + TQ Low Dose: 99.50±0.29) and Group D (MTZ + TQ High Dose: 95.50± 0.17) when compared to Group B (MTZ: 71.11± 0.78) and a significant increase when compared to Group F (MTZ + TQ Low Dose + Recovery: 89.50± 1.29) and Group G (MTZ + TQ High Dose + Withdrawal: 85.11± 0.91). There was a significant decrease in Group B (MTZ: 71.11 ± 0.78) when compared to Group A Control (94.44 ± 0.19) and a significant decrease when compared to Group E (MTZ + Recovery: 79.78± 1.08).

Concerning FSH, there was a significant increase in Group C (MTZ + TQ Low Dose: 38.32± 0.90) and Group D (MTZ + TQ High Dose: 40.18± 0.63) when compared to Group B (MTZ: 24.12± 0.60) and a significant increase when compared to Group F (MTZ + TQ Low Dose + Recovery: 34.12± 0.11) and Group G (MTZ + TQ High Dose + Recovery: 31.73± 0.36). There was a significant decrease in Group B (MTZ: 24.12± 0.60) when compared to Group A Control (36.41± 1.90) and a non-significant decrease when compared to Group E (MTZ + Recovery: 28.25± 0.20).

For LH, there was a significant increase in Group C (MTZ + TQ Low Dose: 35.97± 0.54) and Group D (MTZ + TQ High Dose:31.43± 1.10) when compared to Group B (MTZ: 4.87± 0.75) and a significant increase when compared to Group F (MTZ + TQ Low Dose +Withdrawal: 28.64± 0.16) and Group G (MTZ + TQ High Dose + Recovery: 11.97± 0.47). There was a significant decrease in Group B (MTZ Only: 4.87± 0.75) when compared to Group A Control (14.93± 0.05), and a significant decrease when compared to Group E (MTZ +Withdrawal: 8.98± 0.42).

There was a significant increase in Testosterone levels in Group C (MTZ + TQ Low Dose: 6.99± 0.53) and Group D (MTZ + TQ High Dose: 6.89± 0.42) when compared to Group B (MTZ: 2.13± 0.54) and a significant increase when compared to Group F (MTZ + TQ Low Dose +Withdrawal: 5.89± 0.04) and Group G (MTZ + TQ High Dose + Withdrawal: 5.15± 0.48). There was a significant decrease in Group B (MTZ Only: 2.13± 0.54) when compared to Group A Control (6.84± 0.13), and a significant decrease when compared to Group E (MTZ + Recovery: 3.76± 0.09).

Table I: Effect of Thymoquinone on the Hypothalamic - Pituitary - Gonadal Axis Hormones

Groups	*GnRH (pg/mL)*	*FSH (mIU/mL)*	*LH (mIU/mL)*	*T (ng/mL)*
A: Negative control	94.44 ± 0.19	36.41 ± 1.90	14.93 ± 0.05	6.84 ± 0.13
B: Positive Control (MTZ Only)	71.11 ± 0.78 [a]	24.12 ± 0.60 [a]	4.87 ± 0.75 [a]	2.13 ± 0.54 [a]
C: MTZ + TQ Low Dose	99.50 ± 0.29 [ab]	38.32 ± 0.90 [ab]	35.97 ± 0.54 [ab]	6.99 ± 0.53[b]
D: MTZ + TQ High Dose	95.50 ± 0.17 [b]	40.18 ± 0.63 [ab]	31.43 ± 1.10 [ab]	6.89 ± 0.42 [b]
E: MTZ +Withdrawal	79.78± 1.08 [b]	28.25 ± 0.20[b]	8.98± 0.42 [b]	3.76± 0.09 [b]
F: MTZ + TQ Low Dose +Withdrawal	89.50 ± 1.29 [be]	34.12 ± 0.11[be]	28.64 ± 0.16 [be]	5.89 ± 0.04[be]
G: MTZ + TQ High Dose +Withdrawal	85.11 ±0.91 [be]	31.73 ±0.36 [be]	11.97 ± 0.47 [be]	5.15 ± 0.48 [be]

[a]Statistically significant compared to Control; [b]Statistically significant compared to the Metronidazole group. [c]Statistically significant compared to Metronidazole + Recovery group.

In Table II, there was significant increase in sperm viability in Group C (MTZ + TQ Low Dose 75.00 ± 0.04) and Group D (MTZ + TQ High Dose: 75.00 ± 0.00) when compared to Group B (MTZ only: 43.00 ± 0.75) and a significant decrease when compared to Group F (MTZ + TQ Low Dose + Recovery: 72.00 ± 0.34) and Group G (MTZ + TQ High Dose + Recovery: 73.00 ± 0.11). There was a significant decrease in Group B (MTZ only: 43.00 ± 0.75) when compared to Group A Control (75.00 ± 0.30) and a significant decrease when compared to Group E (MTZ + Recovery: 54.00± 0.45).

Sperm count was significantly increased in Group C (MTZ + TQ Low Dose: 205.00 ± 0.87) and Group D (MTZ + TQ High Dose: 199.00 ± 0.63) when compared to Group B (MTZ only: 72.00 ± 0.83) and a significant decrease when compared to Group F (MTZ + TQ Low Dose +

Recovery: 189.00 ± 0.36) and Group G (MTZ + TQ High Dose + Recovery: 188.00 ± 0.20). There was a significant decrease in Group B (MTZ only: 72.00 ± 28.83) when compared to Group A Control (207.00 ± 0.93) and a significant decrease when compared to Group E (MTZ + Recovery: 102.00± 0.65).

Sperm motility was significantly increased in Group C (MTZ + TQ Low Dose: 77.00 ± 0.40) and Group D (MTZ + TQ High Dose: 74.00 ± 0.97) when compared to Group B (MTZ only: 14.00 ± 0.36) and a significant decrease when compared to Group F (MTZ + TQ Low Dose + Recovery: 70.00 ± 0.35) and Group G (MTZ + TQ High Dose + Recovery: 68.00 ± 0.51). There was also a significant decrease in Group B (MTZ only: 14.00 ± 0.36) when compared to Group A (Control): (75.00 ± 0.00) and a significant decrease when compared to Group E (MTZ + Recovery: 40.00± 0.78).

Sperm morphology was significantly increased in Group C (MTZ + TQ Low Dose: 80.00 ± 0.19) and Group D (MTZ + TQ High Dose: 75.00 ± 0.00) when compared to Group B (MTZ only: 40.00 ± 0.23) and a significant decrease when compared to Group F (MTZ + TQ Low Dose + Recovery: 73.00 ± 0.80) and Group G (MTZ + TQ High Dose + Recovery: 76.00 ± 0.63). There was also a significant decrease in Group B (MTZ only: 40.00 ± 2.23) when compared to Group A (Control): (78.00 ± 0.32) and a significant decrease when compared to Group E (MTZ + Recovery: 40.00± 0.23).

Table II: Effects of Thymoquinone on sperm parameters

Groups	*Sperm viability (%)*	*Sperm Count (10^6/ml)*	*Sperm motility (%)*	*Sperm morphology (%)*
A: Control	75.00 ± 0 .30	207.00 ± 0.93	75.00 ± 0.00	78.00 ± 0.32
B: MTZ only	43.00 ± 0.75 [a]	72.00 ± 0.83 [a]	14.00 ± 0.36 [a]	40.00 ± 0.23 [a]
C: MTZ +TQ Low dose	75.00 ± 0.04 [b]	205.00 ± 0.87 [b]	77.00 ± 0.40 [ab]	80.00 ± 0.19 [ab]
D: MTZ + TQ High dose	75.00 ± 0.00[b]	199.00 ± 0.63[b]	74.00 ± 0.97[b]	75.00 ± 0.00[b]
E: MTZ + Recovery	54.00± 0.45 [b]	102.00± 0.65 [b]	40.00± 0.78 [b]	49.00± 0.34 [b]
F: MTZ + TQ + Recovery Low dose	72.00 ± 0.34 [be]	189.00 ± 0.36 [be]	70.00 ± 0.35 [be]	73.00 ± 0.80 [be]
G: MTZ + TQ + Recovery High dose	73.00 ± 0.11[be]	188.00 ± 0.20 [be]	68.00 ± 0.51[be]	76.00 ± 0.63[be]

[a] Statistically significant compared to Control; [b] Statistically significant compared to Metronidazole group; [e] Statistically significant compared to Metronidazole + Recovery group.

Histological observations

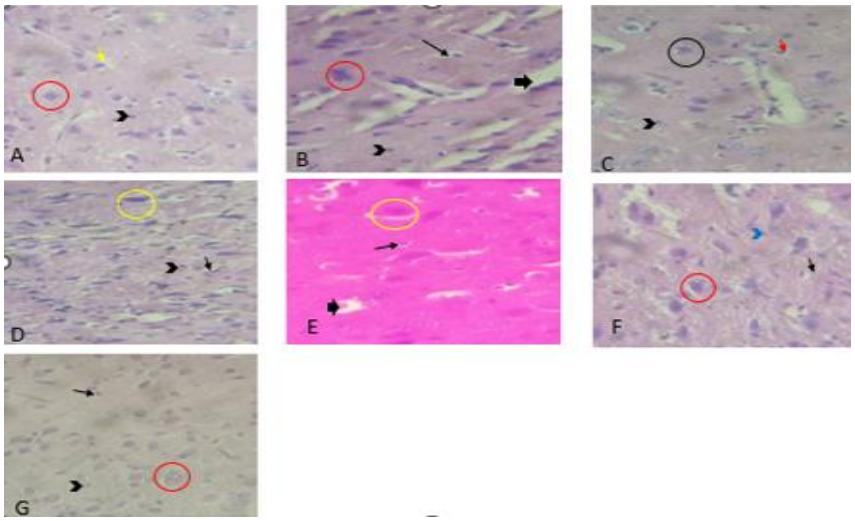


Figure 1: Photomicrograph of hypothalamic tissue.
Haematoxylin and Eosin stain X 400
A: Control group, B: MTZ Only, C: MTZ +50 mg/kg Thymoquinone, D: MTZ+ 100 mg/kg Thymoquinone, E: MTZ + Recovery, F: MTZ+ 50 mg/kg Thymoquinone+ Recovery, G: MTZ+ 100 mg/kg Thymoquinone + Recovery.

A - Control group showing well differentiated and organised neurons (red circle), basophil cells (black thin arrow), acidophil cells (blue thin arrow) and chromophobes (yellow arrowhead).
B - Induced with MTZ. The plate shows severe distortion of the neurons (red circle), atrophy of basophil cells (black thin arrow), acidophil cells (red thin arrow) and chromophobe cells (black arrowhead)
C - Induced MTZ and treated with TQ at a low dose. The plate shows regenerated neurons (yellow circle), chromophobe cells (black arrowhead) and basophil cells (black thin arrow).
D - Induced MTZ and treated with TQ at a high dose. The plate shows regenerated neurons (red circle), chromophobe cells (black arrowhead) and basophil cells (black thin arrow)
E - Induced MTZ and Recovery. This shows slight regeneration of neurons (yellow circle), oligodendrocytes (black arrowhead), and microglia cells (black thin arrow).
F - Induced MTZ and treated with TQ low dose recovery. The plate shows regenerated neurons (yellow circle), vacuolated chromophobe cells (black arrowhead) and basophil cells (red thin arrow).
G - Induced and treated with TQ at a high dose and Recovery. The plate shows regenerated neurons (red circle), chromophobe cells (black arrowhead) and basophil cells (blue thin arrow). H/E X 400

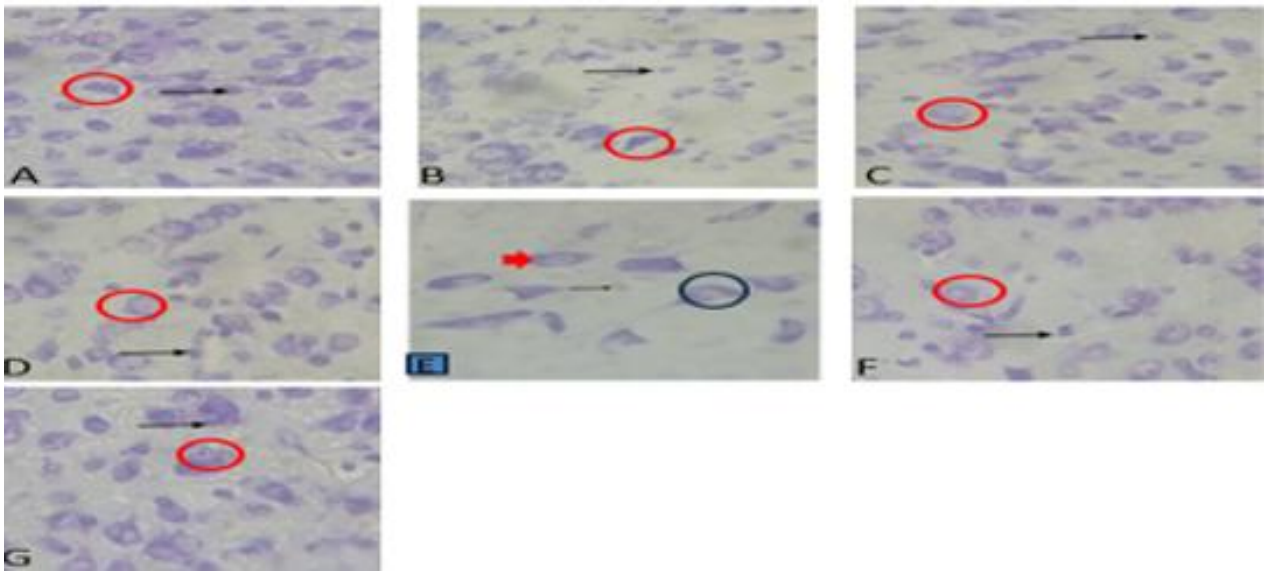


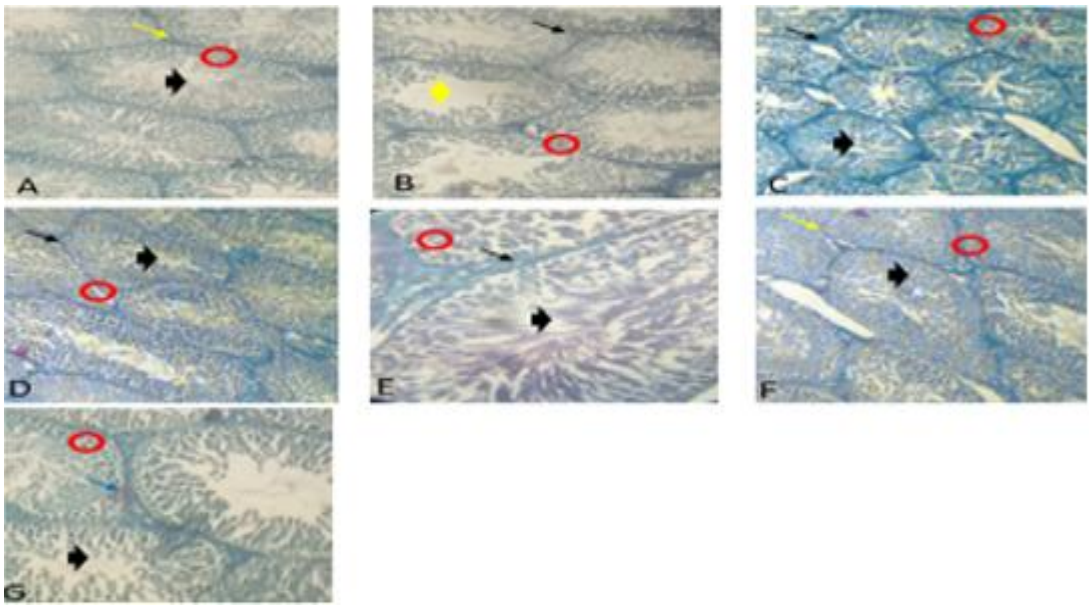
Figure 2: Photomicrograph of the pituitary gland
Cresyl violet stain X 400
A - Control group, B - MTZ only, C - MTZ + 50 mg/kg Thymoquinone, D - MTZ+ 100 mg/kg Thymoquinone, E - MTZ + Recovery, F - MTZ+ 50 mg/kg Thymoquinone+ Recovery, G - MTZ+ 100 mg/kg Thymoquinone + Recovery.

A - Control group. The plate shows differentiated and organised neurons(red circle), oligodendrocytes and microglia cells(black thin arrow)
B - Induced with the MTZ group. This shows severe necrotic distortion of neurons (red circle), oligodendrocytes, and microglial cells (black thin arrow).
C - Induced MTZ and treated with TQ at a low dose. This shows a typical neuron (red circle) and a microglial cell (black thin arrow).
D - Induced MTZ and treated with TQ at a high dose**.** This shows regenerated neurons (red circle), oligodendrocytes and microglia cells (black thin arrow).
E - Induced with MTZ and the recovery group. This plate shows slightly regenerated neurons

(black circle), oligodendrocytes, and microglial cells (black thin arrow).

F - Induced MTZ and treated with TQ low dose recovery**.** This plate shows regenerated neurons (red circle), oligodendrocytes and microglia cells (black thin arrow).

G - Induced MTZ and treated with TQ high dose recovery. This shows regenerated neurons (red circle), oligodendrocytes, and slightly vacuolated microglial cells (black thin arrow). Cresyl violet X400.



Photomicrograph of testicular (Leydig cell) using Masson trichrome stain. X 400

A - Control group, B: 500 mg MTZ only, C: 500 mg MTZ +50 mg/kg Thymoquinone, D: 500 mg MTZ +100 mg/kg Thymoquinone, E: 500 mg MTZ + Recovery, F: 500 mg MTZ +50 mg/kg Thymoquinone Recovery, G: 500 mg MTZ+ 100 mg/kg Thymoquinone + Recovery

A - Control group showing well differentiated and organised spermatogonia cells (red circle), seminiferous tubules (black thick arrow), with adequate sperm cells, and Leydig cells on the interstitial layer (yellow thin arrow) with adequate and well differentiated collagen fibre.

B - Induced with the MTZ group**.** This shows severe distortion of the spermatogonia cells (red circle), clear and distorted seminiferous tubules with loss of spermatocytes (yellow thick arrow), and a thickened interstitial layer with loss of Leydig cells (black thin arrow) with thickened collagen fibre.

C - Induced with MTZ and treated with TQ at a low dose. This shows the regenerated and normal distribution of collagen fibres (black thin arrow), spermatogonia (red circle), and seminiferous tubules with sperm cells (black thick arrow).

D - Induced with MTZ and treated with TQ at a high dose**.** This plate shows regenerated, well-aligned collagen fibres (black thin arrow), spermatogonia cells (red circle), and seminiferous tubules containing sperm cells (black thick arrow).

E - Induced with MTZ and Recovery. This shows slightly regenerated, well-distributed collagen fibres (black thin arrow), spermatogonial cells (red circle), seminiferous tubules (black thick arrow) with few sperm.

F - Induced with MTZ and treated with TQ at a low dose and Recovery. This shows regenerated, well-distributed collagen fibres (yellow thin arrow) housing Leydig cells and spermatogonia (red circle), and seminiferous tubules (black thick arrow) containing sperm.

G - Induced with MTZ and treated with TQ at a high dose and Recovery. This shows a regenerated collagen fibre (blue thin arrow) with Leydig cells, seminiferous tubules (black thick arrow) with a slight loss of sperm cells,

and spermatogonia cells (red circle)—Masson Trichrome stain X40.

Discussion

In this study, metronidazole induction resulted in a significant decrease in reproductive hormones (gonadotropin-releasing hormone, follicle-stimulating hormone, luteinising hormone, and testosterone) in adult male Wistar rats. Low or disrupted levels of GnRH, FSH, LH, and testosterone can cause male infertility by impairing the hormonal regulation essential for spermatogenesis, testicular function and reduced sperm count, contributing to infertility. [23 - 25]
The mechanism involves the metabolism of metronidazole (MTZ) into two primary metabolites, 2-methyl-5-nitroimidazole-1-acetic acid (AAM) and 1-(2-hydroxyethyl)-2-hydroxy-5-nitroimidazole (HM). These metabolites generate reactive oxygen species (ROS) via nucleophilic substitution reactions, disrupting DNA and causing oxidative damage. [26, 27]

This oxidative damage leads to damage to cells (of the hypothalamic nucleus that produces GnRH, the pituitary gland that produces FSH and LH, and the epithelial lining of the seminiferous tubule of the testes). [28] The decrease reported in this study agrees with previous investigations on the suppressive and anti-spermatogenic effects of metronidazole. [14, 15] Reduction of these hormones leads to infertility. Hassan *et al*. stated that regressive histological changes in testicular and epididymal tissues demonstrated the significant side effects of MTZ, including decreased sperm count and per cent total sperm motility, as well as significant reductions in serum levels of free testosterone and FSH levels. [14] Metronidazole has an excellent central nervous system penetration. [28] Furthermore, metronidazole can increase the production of free radicals by activating lipid peroxidation, thereby inhibiting antioxidant enzymes and promoting the formation of reactive oxygen species (ROS). [29] These free radicals cause impairment of the cell membrane and DNA fragmentation. [30] It is imperative to have deduced that thymoquinone (TQ) administration ameliorated metronidazole effects and thereby enhanced reproductive hormones. In this study, there was a significant increase in the levels of reproductive hormones, including gonadotropin-releasing hormone, follicle-stimulating hormone, luteinising hormone, and testosterone, across all TQ-treated groups. Previous studies have shown that thymoquinone improves reproductive hormones and reproductive success. Gholamnezhad *et al*. (2016) reported that TQ positively alters key fertility indices and, as a result, improves reproductive success. [31]

Gonadotropin-releasing hormone (GnRH) is a crucial substance in the hypothalamic-pituitary-gonadal (HPG) axis in humans. Production of GnRH occurs in the neurons of the hypothalamus and causes the downstream production of sex hormones by the gonads. The binding of GnRH to the gonadotropin-releasing hormone receptor initiates the downstream signalling of the primary gonadotropins: follicle-stimulating hormone (FSH) and luteinising hormone (LH). [8] FSH acts on the Sertoli cells, promoting their proliferation and differentiation. Sertoli cells, in turn, provide the necessary physical and nutritional support to germ cells and help in their transition to mature spermatozoa [32]. FSH is involved in the regulation of spermatogenesis by controlling the rate of germ cell division and helps coordinate the stages of spermatogenesis, ensuring the production of mature and functional spermatozoa. [33] LH induces the proliferation and maturation of interstitial Leydig cells, and these cells end up secreting testosterone [9]. Testosterone is essential for the maintenance of qualitative spermatogenesis. It is the androgen in the testis that is required for the initiation of spermatogenesis, and the production of mature

sperm is highly dependent on the action of the androgen in the testes. So, in a case where there is an absence of testosterone or its receptor, spermatogenesis does not go beyond the meiosis stage, and this ultimately results in infertility. [10] Hence, in this study, thymoquinone enhanced the hormones of the hypothalamic-pituitary-gonadal axis through its regulatory function, helping to cure the infertility problem. Thymoquinone exerts antioxidant activity by scavenging metronidazole-induced reactive oxygen species (ROS), thereby mitigating oxidative stress. [34] This protective mechanism preserves the structural and functional integrity of hypothalamic-pituitary-testicular tissues, which is critical for hormone regulation, as demonstrated by histological improvements in the HPG axis revealed in this study.

The findings from this study showed that metronidazole significantly reduced sperm count, sperm motility, sperm viability, and sperm morphology, which leads to infertility effects. The mechanism of metronidazole-induced infertility arises from its impact on the capacity of spermatozoa to generate ATP via the glycolytic pathway ultimately. [16] Spermatogenesis cells may be harmed by an increase in alpha-glycosidase inhibition, as indicated of increased malondialdehyde (MDA). At the same time, sperm motility might be diminished by the inhibition of energetic transferase or non-protein substances in the epididymis. In this study, after administration of thymoquinone at the doses of 50 and 100 mg/kg for 14 days, there was a significant increase in the level of sperm parameters following the initial significant decrease induced of metronidazole. This study shows that thymoquinone is a promising agent to counteract metronidazole-related infertility, with important implications for reproductive health and therapeutic strategies.

This result correlates with the report from previous research. Several studies have explored the impact of thymoquinone on male infertility. It was found that TQ administration in rodent models improved sperm count, motility, and morphology, potentially by reducing oxidative stress and enhancing the testicular antioxidant defence system. [35] This was corroborated by a study by Khan *et al*., which demonstrated that TQ supplementation reduced testicular damage in rats exposed to toxins. [36] Thymoquinone has been shown to counter oxidative damage in the brain by scavenging free radicals and enhancing the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). It was found that TQ enhanced the activity of these enzymes, thereby reducing oxidative stress across different tissues, including the brain and testes. [34]

In this study, thymoquinone increased antioxidant levels by scavenging ROS induced by a high metronidazole dose (500 mg/kg/day for 14 days). This effect obviously generated reactive oxygen species (ROS) via nucleophilic substitution reactions, disrupting DNA and causing oxidative damage, thereby reducing sperm parameters. This effect was neutralised by thymoquinone, confirming its antioxidant properties.

Testicular histology showed severe distortion of the spermatogonia cells, clear and distorted seminiferous tubules with loss of spermatocytes, and a thickened interstitial layer with loss of Leydig cells, with thickened collagen fibres because of metronidazole induction for 14 days, which leads to the elevation of ROS. Metronidazole adversely affected testicular cytoarchitecture, particularly at high doses. In studies involving male rats, administration of metronidazole (500 mg/kg/day) resulted in significant degeneration of the seminiferous epithelium, leading to decreased testicular and epididymal weights, reduced spermatid counts, and abnormal sperm morphology. Histological evaluations revealed severe damage to germ

cells, with many tubules devoid of primary and secondary spermatocytes.

After the administration of thymoquinone at doses of 50 mg/kg and 100 mg/kg, testicular cytoarchitecture revealed regenerated and normal distribution of collagen fibres, spermatogonia, seminiferous tubules with sperm cells, and regenerated, well-aligned collagen fibres, spermatogonia cells, and seminiferous tubules with sperm cells, respectively. This is because thymoquinone (TQ) has been reported to have antioxidant properties that combat oxidative stress in several studies. Both the 50 mg/kg and 100 mg/kg administered doses of thymoquinone significantly mitigated histopathological damage caused by metronidazole in the hypothalamic-pituitary axis and testes. The 100 mg/kg dosage showed enhanced regenerative outcomes, characterised by improved neuronal organisation, well-aligned collagen fibres, and reduced cellular vacuolation compared with the 50 mg/kg dosage. Chromophobe vacuolation was noted in the 50 mg/kg recovery cohort but was markedly less pronounced at 100 mg/kg, suggesting improved cellular protection at this higher dosage. In summary, thymoquinone displayed a dose-dependent protective and restorative influence on metronidazole-induced reproductive toxicity.

Conclusion

The findings of this study revealed the effect of thymoquinone on the hypothalamic-pituitary gonadal axis in metronidazole-induced infertility in adult male Wistar rats. Thymoquinone enhanced hypothalamic-pituitary gonadal axis hormones and sperm parameters, confirming its antifertility properties. Findings also revealed the ameliorative and restorative effects of TQ, as evidenced by its cytoprotective action on the brain and testes.

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