Testicular Oxidative Stress and Inducible Nitric Oxide Synthase Expression Following Metronidazole Administration in the Laboratory Mouse

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ABSTRACT

Background: Metronidazole (MTZ) is a nitroimidazole derivative, widely used as an antibacterial and antiprotozoal agent. The drug causes marked impairment in the fertility of mice by affecting the testis and epididymis. The possible mechanism involved in this impairment inflicted through oxidative stress remains to be elucidated. Objective: To evaluate oxidative stress-induced effects in the testis of mice administered with MTZ. Methods: Swiss strain adult male mice were treated with low (250mg/kgBW/day) and high (500mg/kgBW/day) doses of MTZ for 28 days. Recovery study was carried out 42 days after cessation of the drug treatment. Testis was dissected out for histopathological observation, i-NOS expression by immunohistochemistry and estimations of antioxidant enzyme activities, functional markers and LPO level by spectrophotometry. All the data were analyzed statistically by one way ANOVA followed by Newman-Keul’s test and were considered significant at \( p < 0.05 \).

Results: Histopathological observations of the testis revealed spermatogenic inhibition with intense i-NOS expression in the intertubular spaces following high dose of MTZ administration. Antioxidant enzyme activities (SOD, GPx and GR), functional markers (LDH and ALP) and LPO levels were significantly altered in the testis of mice administered with high dose of MTZ. The effects were reinstated similar to the controls, 42 days after the treatment withdrawal. Conclusion: The present study suggests MTZ (500mg/kgBW/day)-induced reversible direct effect in the seminiferous tubules of the testis, inflicted through oxidative stress.

INTRODUCTION

The rise in the percentage of male factor infertility due to the consumption of therapeutic drugs has made efforts to study their untoward side effects on the male reproduction. Prescribed drugs, for curing various diseases cause male infertility. Among them, adverse effects of certain derivatives of nitroimidazole such as ornidazole, metronidazole, tinidazole, nimorazole etc., on the fertility potential of the males have been widely reported.¹⁻²

Metronidazole, MTZ, (1-[2-hydroxyethyl]-2-methyl-5-nitroimidazole) is the first nitroimidazole to exert useful clinical activity. It is the drug of first choice, widely used as an antibacterial and antiprotozoal agent. Its clinical applications have been growing for the treatment of Helicobacter pylori infections, amoebiasis, giardiasis, trichomoniasis, Crohn’s disease, bacterial vaginosis and several other anaerobic bacterial infections and is being used as a prophylactic antibiotic in surgical interventions.³ The clinicians generally prescribe MTZ to be consumed maximum for seven to ten days, however, for the treatment of several peculiar complications like Chagas disease,⁴ Crohn’s disease,⁵ liver abscess,⁶ etc., this drug is advised to be consumed for 4-8 weeks.

Our earlier study has also reported marked impairments in the testis and epididymis and fertility of the mice administered orally with 250mg/kgBW/day and 500mg/kgBW/day of MTZ for 28 days, without affecting the level of testosterone, hence suggested its direct action.⁷ It is, thus, assumed that there might be some other factors responsible for causing impairment in fertility, induced by administration of MTZ. Oxidative stress is one of the factors that affects the fertility status and thus has been extensively studied in recent years.⁸ In line of that, the present study explores the possibility...
of oxidative stress responsible for spermatogenic inhibition following administration of MTZ.

**MATERIALS AND METHODS**

**Animal selection**
Twenty five Swiss strain adult (12 weeks) male mice weighing about 25-30g were used for the present investigation. The animals were housed under standard laboratory conditions and maintained on pelleted diet and water *ad libitum*. The animal study plan was approved by the Animal Ethical Committee, Banaras Hindu University, Varanasi, India (No. Dean/11-12/CAEC/263).

**Experimental design, drug and dosage**
All the mice were divided into five groups of five each. Mice of Groups I and II served as untreated and vehicle-treated controls while that of Groups III and IV were administered with the doses of 250mg/kgBW/day and 500mg/kgBW/day of MTZ, respectively, for 28 days. Mice of Group V were administered with 500mg/kgBW/day of MTZ for the same duration followed by their sacrifice, 42 days after the drug withdrawal. MTZ (CDH, India) was dissolved in double distilled water and administered orally. The therapeutic dose of MTZ, used in human, was selected and translated to mice. The procedure for the oral administration of drug through gavage was based on the study of Noorafshan *et al*.

**Animal sacrifice and collection of samples**
All the animals were sacrificed by cervical dislocation and the testis was dissected out for the following studies:

**Histological Studies**
Bouin’s fixed testis was dehydrated and embedded in paraffin. Sections of 5μm thickness were taken from the mid portion of each testis, stained with Periodic Acid Schiff reagent followed by counterstaining with Ehrlich’s Haematoxylin.

**Inducible nitric oxide synthase (i-NOS) expression**
I-NOS expression was performed as described by Yadav *et al* and Singh *et al*. Bouin’s fixed testis was dehydrated and embedded in paraffin. Sections of 5μm thickness were taken from the mid portion of each testis. The sections were blocked using 2% normal goat serum blocking buffer followed by incubation with anti-i-NOS primary antibody (1:500, Santa Cruz) in PBS for 48 hours at 4°C. Sections were incubated with biotinylated secondary antibody (Santa Cruz) and streptavidin peroxidase complex. The colour was developed with 3, 3’ diaminobenzidine (DAB) and the sections were permanently mounted with DPX after dehydrating them in graded ethanol. The sections were examined under bright field microscopy (Nikon, eclipse P-200) and the images were captured at 40X magnification.

**Antioxidant enzyme estimations**
The testis from each mouse was dissected out carefully and washed with ice-cold physiological saline solution. The tissue was weighed and 10% homogenate was prepared in ice-cold phosphate buffer (0.05M, pH 7.0). The homogenate was centrifuged at 10,000 X g for 20 minutes at 4°C. The supernatant was used for the enzyme assays after estimating the protein content by the method of Lowry *et al*. Briefly, the assay mixture contained 2.4ml of Tris-HCl (50mM) containing 1mM EDTA (pH 7.6), 300μl pyrogallol (0.2mM) and 100μl enzyme source. The increase in absorbance was measured immediately at 420nm, against a blank containing all components except the enzyme source and pyrogallol, at 10 seconds interval for 3 minutes on a spectrophotometer. The enzyme activity was expressed as units per milligram protein.

**Catalase (CAT)**
Catalase (EC 1.11.1.6) was assayed by the method of Claiborne. Briefly, the assay mixture contained 2.4ml of phosphate buffer (50mM, pH 7.0), 10μl of H₂O₂ (19mM) and 50μl enzyme source. The decrease in absorbance was measured immediately at 240 nm, against a blank containing all the components except the enzyme source, at 10 seconds interval for 3 minutes on a spectrophotometer. The enzyme activity was expressed as micromoles H₂O₂ consumed per min per milligram protein.

**Glutathione peroxidase (GPx)**
Glutathione peroxidase was assayed by the method of Flohe and Gunzler. Glutathione peroxidase assay consisted of potassium phosphate buffer (0.1M, pH 7.0), 2.25mM NADPH in 0.1% NaHCO₃, glutathione reductase (7.1μl/ml), glutathione (11.52mg/ml) and 1.5mM of H₂O₂. Enzyme assay was carried out by pipetting 750μl of potassium phosphate buffer, 60μl of NADPH, 15μl of glutathione reductase and 25μl of reduced glutathione in 1.6 ml cuvette. Enzymatic reaction was started by adding 50μl of sample (supernatant) and 100μl of H₂O₂ and the extinction of the sample was recorded at 340 nm every minute for a period of 120 seconds in the spectrophotometer. The glutathione peroxidase activity was expressed in units per milligram of protein.

**Glutathione reductase (GR)**
Glutathione reductase was assayed by the method of Carlberg and Mannervick. One unit of enzyme activity is reported as 1.0 μl NADPH oxidized per minute assuming 6.22 X 10⁴ to be the molar absorbance of NADPH at 340nm. Glutathione reductase assay consisted of potassium phosphate buffer (0.2M, pH 7.0), 2.0 mM NADPH in 0.1% NaHCO₃ and oxidized glutathione (20mM). Enzyme assay was carried out by pipetting 600 μl of potassium phosphate buffer, 250 μl milli q water, 50 μl oxidized glutathione, 50 μl NADPH and 50 μl of sample (supernatant). The extinction of the sample was recorded at 340 nm every minute for a period of 120 seconds in the spectrophotometer. The glutathione reductase activity was expressed in units per milligram protein.

**Lipid peroxidation estimation (LPO)**
The level of LPO was measured in the tissue supernatant using malonaldehyde (MDA) concentration as a surrogate measure. The reaction mixture was formed by adding 100μl supernatant, 1400μl of 15% trichlo-roacetic acid containing thiobarbituric acid (0.375%) and 14μl of butylated hydroxytoluene (20mg/ml absolute alcohol). Samples were heated at 100°C for 15 minutes in water bath and the absorbance was measured at 532nm against blank containing all the reagents except the test sample. The values were expressed as nanomoles MDA produced per milligram protein.

**Testicular functional markers**

**Lactate dehydrogenase (LDH)**
The activity of lactate dehydrogenase was estimated in the tissue supernatant using LDH (P-L) kit (Mod. IFCC method).

**Alkaline phosphatase (ALP)**
The activity of alkaline phosphatase was estimated in the tissue supernatant using COGENT diagnostic kit.
Statistical analysis

All the data were analyzed statistically by one way ANOVA followed by Newman-Keul's test. Values were considered significant at \( p < 0.05 \).

RESULTS

Histological study of the testis

The testis of the control (Figure 1A) showed normal histological features. MTZ at the low dose induced mild regressive changes in the seminiferous tubules such as loosening and disorganization of the germ cells (Figure 1B). However, regressive changes in the seminiferous tubules appeared to be more pronounced in the testis of mice administered with high dose of MTZ (Figure 1C). The marked regressive changes were indicated by shrinkage of the seminiferous tubules, depletion, disorganization, intraepithelial vacuolization and exfoliation of germ cells with the appearance of few giant cells (Figure 1C). Forty two days after withdrawal of the drug treatment, regressive histological changes noticed in the seminiferous tubules were recovered and appeared comparable to that of the controls (Figure 1D).

Inducible nitric oxide synthase (i-NOS) expression in the testis

Immunohistochemical study showed normal expression of i-NOS in the testis of the control mouse (Figure 2A). By contrast an intense expression of i-NOS was observed in the intertubular space and germ cells of the seminiferous tubules in the testis of the mice administered orally with 500mg/kg BW/day of MTZ for 28 days as compared with the control.
controls (Figure 2B). However, the expression was decreased 42 days after cessation of the treatment as compared with MTZ-treated mice (Figure 2C), similar to that of the controls.

**Antioxidant enzyme activities in the testis**

**Superoxide dismutase (SOD)**

No significant alterations were noticed in the activity of SOD following low dose of MTZ administration as compared with the controls (Figure 3A). By contrast, high dose of MTZ resulted in a significant increase in the same as compared with the controls (Figure 3A). The increased activity of SOD declined significantly 42 days after cessation of the drug treatment as compared with the high dose of MTZ, thus attained the value of controls (Figure 3A).

**Catalase (CAT)**

No significant alterations were noticed in the activity of CAT following low dose of MTZ administration as compared with the controls (Figure 3B). However, the activity was decreased following high dose of MTZ administration (Figure 3B) although; the value was not significant as compared with the controls. The insignificant decrease following high dose of MTZ administration, was restored to the control extent, 42 days after cessation of the drug treatment (Figure 3B).
Glutathione peroxidase (GPx)
The activity of GPx was decreased significantly following both the doses of MTZ administration as compared with the controls (Figure 3C). The activity increased only to some extent, 42 days after cessation of the drug treatment as compared with high dose of MTZ (Figure 3C).

Glutathione reductase (GR)
No significant alterations were noticed in the activity of GR in the group treated with low dose of MTZ as compared with the controls, however, administration of high dose of MTZ caused significant reduction in the activity of GR comparable to that of the controls (Figure 3D). Forty two days after cessation of the drug treatment, the activity of GR was restored significantly as compared with high dose of MTZ, similar to the control value (Figure 3D).

Lipid peroxidation level (LPO)
The level of lipid peroxidation remained unaltered following low dose of MTZ administration as compared with the controls (Figure 3E). MTZ administered with the dose of 500 mg/kgBW/day elevated the lipid peroxidation level, as indicated by the significant increase in the activity of MDA, comparable to that of the controls (Figure 3E). The elevated level of MDA was restored to the control values, 42 days after cessation of the drug treatment as compared with the high dose of MTZ (Figure 3E).

Testicular functional markers
Lactate dehydrogenase (LDH)
No significant alterations were noticed in the activity of LDH following low dose of MTZ administration, however, an increased activity was noted in the group administered with the dose of 500mg/kgBW/day as compared with the controls (Figure 4A). The increased activity of LDH declined significantly 42 days after cessation of the drug treatment as compared with the high dose of MTZ, thus attained the control value (Figure 4A).

Alkaline phosphatase (ALP)
The activity of testicular ALP remained unaltered following administration of low dose of MTZ (Figure 4B). By contrast, the activity increased significantly in the group treated with high dose of MTZ as compared with the controls (Figure 4B). The activity declined significantly 42 days after cessation of the drug treatment as compared with the high dose of MTZ (Figure 4B).
affecting the level of serum testosterone have already been reported in MTZ-induced regressive changes in the seminiferous tubules without MTZ, hence attained the control value (Figure 4B). After cessation of the drug treatment as compared with the high dose of MTZ, hence attained the control value (Figure 4B).

**DISCUSSION**

MTZ-induced regressive changes in the seminiferous tubules without affecting the level of serum testosterone have already been reported in our previous finding. The present study reveals the possible involvement of oxidative stress and alterations in the testicular functional markers inhibiting spermatogenic activity in MTZ-treated mice.

Free radicals are highly reactive intermediates produced in normal cellular metabolism. The oxygen radical generating systems are present in all types of cells including endothelial cells, adipocytes and germ cells. In a healthy condition there is a balance between the reactive oxygen species (ROS) formation and elimination. However, when ROS cellular overproduction overwhelms intrinsic antioxidant capacity, oxidative stress occurs followed by the damage to the biomolecules of normal cells and tissues.

To protect against the potentially damaging effects of ROS, cells are equipped with several antioxidant enzymes such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione reductase (GR) which neutralize the damaging effects of ROS by inactivating them. The synergistic actions of the antioxidant enzyme system are essential in protection against oxidative damage. These antioxidant enzymes, therefore, serve as the defense system of the cell. However, the antioxidant defense system may be overwhelmed by various pathological or environmental factors so that a fraction of ROS may escape destruction and form more reactive hydroxyl radicals.

Earlier, some authors have reported MTZ-induced oxidative stress in the testis. The results of the present study also reveal significant alterations in the activities of testicular antioxidant enzymes, functional markers and LPO level indicating the MTZ-induced oxidative stress in the testis. The level of LPO products have been widely used as an index of oxidative stress. The significant increase in the level of testicular LPO is in accordance with the findings reported in rats following administration of 15mg/kgBW/day, 200mg/kgBW/day and 400mg/kgBW/day of MTZ for 8 weeks. The elevated LPO level, as indicated by increased activity of MDA in the testis of MTZ-treated mice, is suggestive of the membrane damage of the germ cells. Beside this, the OH- radical has been proposed to be an initiator of LPO. Therefore, the increased level of LPO noticed in the present study, is also indicating elevation of OH-radical which itself is an ROS and damages the cell membrane. It is also reported that hydrogen peroxide (H2O2) increases lipid peroxidation. Therefore, the increased LPO level as noticed in the present study might be due to the excessive generation of H2O2 which is a superoxide anion and causes the tissue injury.

The antioxidant enzyme activity in the high dose of MTZ-treated group indicates an increased activity of SOD while decreased activity of catalase, GPx and GR. The results suggest that increased activity of SOD is probably facilitating higher conversion of the generated ROS, i.e., superoxide anion (O2-) to hydrogen peroxide (H2O2). Further, it appears in the present study that due to reduced CAT and GPx activities, H2O2 is probably not getting detoxified, subsequently resulting in high level of LPO in the testis. Griveau and Lannou reported that ROS such as H2O2 appear to be a key agent in causing cytotoxic effects in spermatozoa, and in addition to its direct effect on the cellular constituent, produces oxidative stress by decreasing the enzymatic defenses. Therefore, it may be possible that excessive production of H2O2 along with the reductions in the activities of CAT, GPx and GR is a critical factor in inducing oxidative stress in the testis. Likewise, the inhibition in the activities of GPx and GR might have led to decreased GSH/GSSG ratios that probably have rendered cells more susceptible to oxidative damage. The alterations noticed in the antioxidant enzyme activities and lipid peroxidation level may serve as a major cause of spermatogenic inhibition through the oxidative stress. The results are in accordance with Raji et al. and Ligha and Paul, suggesting increased activity of SOD and decreased activity of CAT in the testis after MTZ treatment.
Superoxide dismutase.

Nitric oxide (NO) is a free radical synthesized by nitric oxide synthase (NOS). Its excessive production is detrimental on the physiological system via the production of peroxynitrite, after interacting with oxygen and superoxide. It is derived in the testis from activated testicular macrophages, which are having high level of induced nitric oxide synthase (i-NOS). In the present study, the increased expression of i-NOS in the intertubular space of testis following administration of high dose of MTZ is, therefore, suggesting increased level of NO in the tissue. Since, NO is having small molecular size and is diffusible in nature, its site of action is distant away from the site of its production. Hence, it appears likely that the NO produced in the intertubular space might have diffused in the seminiferous tubules, causing detrimental effects directly on the germ cells.

Forty two days after cessation of the drug treatment, the rebalancing of the antioxidant enzyme activities and testicular functional markers as well as restoration in the level of LPO, as evidenced by the decreased MDA activity, might have reinstated the spermatogenic activity, as noticed in the histoarchitecture of the testis. These restorations could be the possible cause for the decreased expression of i-NOS in the testis, after cessation of the drug treatment.

CONCLUSION

It can, therefore, be concluded that MTZ-induced spermatogenic arrest is attributed to oxidative stress, thus suggesting the direct action of the drug on the spermatogenic activity. The alterations in the oxidative stress are reversible after withdrawal of the drug.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

ABBREVIATION

ALP: Alkaline phosphatase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase; i-NOS: Inducible nitric oxide synthase; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; MDA: Malonaldehyde; MTZ: Metronidazole; O.D.: Optical density; ROS: Reactive oxygen species; SOD: Superoxide dismutase.

REFERENCES

Kumari et al.: Metronidazole Inhibiting Spermatogenesis through Oxidative Stress

GRAPHICAL ABSTRACT

SUMMARY

- MTZ is a widely used antibacterial and antiprotozoal agent which causes marked impairment in the fertility of male mice following its administration at the dose of 500mg/kgBW/day for 28 consecutive days.
- The drug inhibits spermatogenic activity, inflicted through oxidative stress, revealed by intense i-NOS expression and significant alterations in the activities of antioxidant enzymes (SOD, GPx and GSH), functional markers (LDH and ALP) as well as LPO level in the testis.
- Forty days after cessation of the drug treatment, the rebalancing of the antioxidant enzyme activities and testicular functional markers as well as restoration in the level of LPO is suggested to reinstate the spermatogenic activity of the testis.
- These restorations might be the possible cause for the decreased expression of i-NOS in the testis, after cessation of the drug treatment.
- MTZ-induced spermatogenic arrest is attributed to oxidative stress, thus suggesting the reversible direct action of the drug on the spermatogenic activity.

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