Oxidative Effect of Metronidazole on The Testes of Wistar Rats

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Abstract: There are growing evidences that oxidative damage to the human spermatozoa membrane is an important pathophysiological mechanism in male fertility and following reports by previous researchers on the toxic effects of metronidazole on testicular functions, this study is designed to investigate further its direct oxidative effects and that of the introduction of vitamin E to metronidazole treated rats. It also looked at the natural reversal effects on abstinence from medication for 8weeks. A total of 105 adult male and 50 female wistar rats weighing 170±10g (70-90 day old) were used for the experiment. The rats were randomly divided into a control and experimental groups. There were four major groups with 5 subgroups consisting of 5 rats each. Varying doses of metronidazole were used depending on the experiment. Experiment 1; animals were fed with 7.5mg/kg of metronidazole, experiment 2; fed with 15mg/kg of metronidazole, experiment 3; administered with 200mg/kg of metronidazole and experiment 4; fed with 400mg/kg of metronidazole. Each experimental has 5 sub-groups; A control, B; group fed with the experimental dose, C; experimental dose with vitamin E, D; experimental dose with testosterone, E; fed with experimental dose, vitamin E and testosterone and sub-group F, a reversal group which was left for 8weeks after cessation of treatment. At the end of the experiment, animals were scarified and the antioxidant effects of metronidazole were investigated using malonildialdehyde concentration and catalase activity. Results showed that metronidazole at the therapeutic dose of 7.5mg/kg did not have significant negative effect on the testis in all the parameters assessed. At the dose of 15mg/kg, 200mg/kg and 400mg/kg, there was significant increase in lipid peroxidation and catalase activity. The results of this study indicate that metronidazole administration (15mg/kg, 200mg/kg or 400 mg/kg), for 8weeks, caused an oxidative stress on the testes of male rats.

Key words: anti-oxidant; lipid; metronidazole; peroxidation; oxidative-stress.

INTRODUCTION

Metronidazole is a nitromidazole anti-infective drug used mainly in the treatment of infections caused by susceptible organisms particularly anaerobic bacteria and protozoa such as amoeba. Metronidazole is also used in the treatment of dermatological conditions (Rosacea), where it is marketed by Galderma under the trade name Rozex and Metrogel (Rossi, 2006). Metronidazole can easily be purchased off the counter (OTC) in the pharmacy and sometimes in the open market in Nigeria. It is marketed by Pfizer under the trade name Flagyl in the US, by Sanofi-Aventis globally under the same tradename Flagyl, in Pakistan and Bangladesh it is also available with the brand name of Nidagyl manufactured and marketed by Star Laboratories. In Thailand it is marketed as Mepagyl by Thai Nakhorn Patana. They are also marketed in UK by Milpharm Limited and Almus Pharmaceuticals. Metronidazole was developed in 1960.

Metronidazole, taken up by diffusion, is selectively absorbed by anaerobic bacteria and sensitive protozoa. Once taken up by anaerobes, it is non-enzymatically reduced by reacting with reduced ferredoxin, which is generated by pyruvate oxido-reductase. This reduction causes the production of toxic products to anaerobic cells and allows for selective accumulation in anaerobes.

The metronidazole metabolites are taken up into bacterial DNA and form unstable molecules. This function only occurs when metronidazole is partially reduced and because this reduction usually happens only in anaerobic cells, it has relatively little effect upon human cells or aerobic bacteria (Eisenstein and Schaechter, 2007).

The greatest paradox of aerobic respiration is that oxygen, which is essential for energy production, may also be detrimental because it leads to the production of reactive oxygen species (Saleh and Agarwal, 2002). When levels of reactive oxygen species (ROS) overwhelm the body’s antioxidant defense system, oxidative stress (OS) occurs. The mitochondrion is the powerhouse of respiration. Hence, it is the major site of ROS generation, which is produced through the nicotinamide adenine dinucleotide–dependent oxido-reductase pathway (Gavella and Lipovac, 1992). In contrast, the sperm plasma membrane produces ROS through the nicotinamide adenine dinucleotide phosphate–dependent oxidase system (Aitken et al., 1992).

Researchers during the last decade implicated oxidative stress as a mediator of sperm dysfunction and consequently, antifertility (Aitken et al., 1995; Sharma and Agarwal, 1996). It has been suggested that this
phenomenon was causally related to the ability of germ cells to generate reactive oxygen species (ROS), such as hydrogen peroxide, speroxide anion and hydroxyl radical. In normal circumstances, there is equilibrium between the generation of ROS and antioxidant strategies of the male reproductive tract, leaving only a critical amount of ROS required for normal sperm functions such as capacitation, acrosome reaction and fusion with the oocyte membrane (Griveau and Le Lannou 1997). Excessive production of ROS, however, results in destruction of the antioxidant capacity of spermatozoa and seminal plasma causing what is called oxidative stress (Lewis et al., 1995). Due to high polyunsaturated fatty acid content, spermatozoa plasma membranes are highly sensitive to ROS-induced damage (Sharma and Agarwal, 1996). There are growing evidences that oxidative damage to the human spermatozoa membrane is an important pathophysiological mechanism in male infertility. Therefore, this study is design to study the oxidative properties of metronidazole as it relates to infertility.

MATERILS AND METHODS

Study Site:
The experimental male wistar rats were bought and housed in the animal house located in the College of Health Sciences, Niger Delta University, Wilberforce Island. This study site was chosen because similar studies had been carried out successfully in this center such as the antifertility effects of *Abrus Precatorius* on the testes of male rats.

Metronidazole, vitamin E and Testosterone were purchased from Cynflac Pharmacy, hospital road, Yenagoa.

Animals and Treatment:
A total of 105 adult male and 50 female wistar rats weighing 170±10g (70-90 day old) were obtained from the Animal house of the College of Health Sciences, Niger Delta University, Wilberforce Island. There were maintained in 12 h light and 12h dark conditions at a temperature of 27°C-30°C in the animal house. The standard laboratory chew and tap water were available *ad libitum*. After the acclimatization period of two weeks, the rats were randomly divided into a control and experimental groups. There were four major groups with 5 subgroups consisting of 5 rats each.

Experiment 1: Using Adult Rats Fed With 7.5mg/kg of Metronidazole:
In experiment 1, adult male rats weighing 75g±5, about 70-90 days old at the commencement of the experiment were used. There were divided into a control group (Group1A), a group treated with 7.5mg/kg of metronidazole (1B), a group fed with 7.5mg/kg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 1C), a group treated with 7.5mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 1D), another group treated with 7.5mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 1E). A reversal group (Group 1F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group had 5 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 0.1ml of distilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Experiment 2: Using Adult Rats Fed With 15mg/kg of Metronidazole:
In experiment 2, adult male and female rats weighing 160g±0.5, about 70-90 days old at the commencement of the experiment were used. There were divided into a control group (Group2A), a group treated with 30mg/kg of metronidazole (2B), a group treated with 30mg/kg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 2C), a group treated with 30mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 2D), another group treated with 30mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 1E). A reversal group (Group 1F) was left for 8weeks after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group had 5 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 0.2ml of distilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility after the treatment.

Experiment 3: Using Adult Rats Fed With 200mg/kg of Metronidazole:
In experiment 3, adult male rats weighing 170g±0.9, about 70-90 day old at the commencement of the experiment were used. The rats five in each group were randomly divided into a control group (Group3A), a group treated with 200mg/kg of metronidazole (3B), a group fed with 200mg of metronidazole and 400mg/kg/day of vitamin E concurrently for 8weeks (Group 3C), a group treated with 200mg/kg of metronidazole and 0.36mg/kg/day of testosterone (Group 3D), another group treated with 200mg/kg of metronidazole, 400mk/kg/day of vitamin E and 0.36mg/kg/day of testosterone concurrently (Group 3E). A
reversal group (Group 3F) was left for 8 weeks after cessation of treatment with metronidazole to see whether
the observed effects were reversible. The metronidazole was delivered as a single dose in 0.625ml of distilled
water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for fertility
after the treatment.

Experiment 4: Using Adult Rats Fed With 400mg/kg of Metronidazole:
In experiment 4, adult male rats weighing 200g±5, about 70-90 week old at the commencement of the
experiment were used. There were divided into a control group (Group4A), a group treated with 400mg/kg of
metronidazole (4B), a group treated with 400mg/kg of metronidazole and 400mg/kg/day of vitamin E
concurrently for 8 weeks (Group 4C), a group treated with 400mg/kg of metronidazole and 0.36mg/kg/day of
testosterone (Group 4D), another group treated with 400mg/kg of metronidazole, 400mk/kg/day of vitamin E
and 0.36mg/kg/day of testosterone concurrently (Group 4E). A reversal group (Group 4F) was left for 8 weeks
after cessation of treatment with metronidazole to see whether the observed effects were reversible. Each group
had 7 rats randomly divided into the groups. The metronidazole was delivered as a single dose in 1.25ml of
distilled water by gastric gavage. Female rats were used to mate with the control and treated male rats to test for
fertility after the treatment.

Dose of Metronidazole:
The dose of 400mg/kg and 200mg/kg respectively was selected because the LD50 of metronidazole (p.o)
was determined and it was found to be 5000mg/kg (Ogunleye, 2007). The 400mgkg dose taken in this study is
less than 1/8 of the lethal dose and 200mg/kg is less than 1/16 of the lethal dose. Besides, from literature, several
other authors had used similar dose (Davood et al., 2007; Raji et al., 2007).

Route of Administration:
The tablet form of metronidazole and vitamin E were administered through the naso-gastric rout while the
testosterone injection was given intramuscularly.

Retrieval of Tissues:
At termination, the rats were anaesthetized with ketamine 1 mg/kg [intramuscularly (i.m.)]. Testicular
tissues were collected for malonildialdehyde (MDA) estimation and catalase activities.

Determination of Catalase Activities:
This was determined according to the method described by Akenes and Njaa (1981). The principle of the
test is that catalase in the sample reacts with hydrogen peroxide to produce a coloured solution which was
measured spectrophotometrically. 0.2ml of sample was added to 1.8ml of 30ml of Hydrogen peroxide (H₂O₂)
substrate in a 2ml curvette. The absorbance was read at 240nm at 1 minute interval for 3 minutes. Enzyme
activity calculated using the molar extinction coefficient of 40.0M⁻¹cm⁻¹

Determination of Malonildialdehide (MDA):
MDA level was determined in the supernatant of the gastric homogenates by the modified method of Buege
and Aust (1978). Concentration was calculated using the molar absorptivity of malondialdehyde which is 1.56 ×
100000 M. It is an index of the degree of oxidative damage in biological tissues. The inhibition of the rate of
peroxidation is calculated by using the formula: rate of peroxidation (%) = (1- mean × value of treatment
group/mean × value of control) × 100.

Statistical Analysis:
Data are expressed as mean ± SD and the test of significance analyzed by the student’s t-test. The
differences were considered significant at p < 0.05.

Ethical Considerations:
The research proposal was submitted to the Ethical Research Committee of the College of Health Sciences
of the University of port-Harcourt for consideration and approval before commencement of this research work.

RESULTS AND DISCUSSION

Catalase Activity:
The enzyme catalase activity was significantly increased in most of the experimental groups but the highest
was observed in experimental group 3 and 4 which were administered with 200mg/kg and 400mg/kg
respectively compared with the control. There was no significant reduction in catalase activity in the sub-group
treated with metronidazole and testosterone in comparison to those administered with either 200mg/kg or
400mg/kg of metronidazole alone. Catalase activities were not significantly higher in vitamin E treated groups. Values are as presented in table 1.

**Testicular Malondialdehyde (MDA) in Control and Rats Treated With Metronidazole:**

Malondialdehyde concentration, an index of lipid peroxidation was significantly increased in the following groups: group treated with 15mg/kg of metronidazole (2B), group treated with 200mg/kg of metronidazole (3B), group administered with 400mg/kg of metronidazole (4B) and groups treated with 200mg/kg of metronidazole and testosterone and group treated with 400mg/kg of metronidazole and testosterone in comparison to the control group. Groups in which metronidazole was concurrently treated with vitamin E did not show any significant difference. The reversal groups also did not reveal any statistically significant difference when compared to the control group (p>0.05). Values are as presented in table 2.

**Table 1:** Catalase Activity of Control and Treated Experimental Rats u/mg protein.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>DOSE 7.5mg/kg</th>
<th>DOSE 15mg/kg</th>
<th>DOSE 200mg/kg</th>
<th>DOSE 400mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>68.34±0.9</td>
<td>68.34±0.9</td>
<td>68.34±0.9</td>
<td>68.34±0.9</td>
</tr>
<tr>
<td>B</td>
<td>88.57±15.6</td>
<td>100.87±15.1*</td>
<td>156.54±10.8*</td>
<td>209.20±12.3*</td>
</tr>
<tr>
<td>C</td>
<td>84.66±13.4</td>
<td>70.87±14.4</td>
<td>86.76±07.9*</td>
<td>102.25±08.4*</td>
</tr>
<tr>
<td>D</td>
<td>90.98±11.6</td>
<td>123.76±07.9*</td>
<td>174.13±14.5*</td>
<td>189.60±21.1*</td>
</tr>
<tr>
<td>E</td>
<td>70.76±09.9</td>
<td>74.13±12.8</td>
<td>75.86±13.8</td>
<td>99.34±06.9</td>
</tr>
<tr>
<td>F</td>
<td>70.09±08.3</td>
<td>69.76±10.3</td>
<td>73.34±08.3</td>
<td>79.98±13.6</td>
</tr>
</tbody>
</table>

A; control, B; fed with metronidazole, C; fed with metronidazole and 400mg/kg of vitamin E, D; fed with metronidazole and 0.36mg/kg of testosterone, E; fed with metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, F; reversal. Values are expressed as mean ± standard deviation, n=5, *p<0.05 (significant)

**Table 2:** Malondialdehyde of control and treated experimental rats.

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>DOSE 7.5mg/kg</th>
<th>DOSE 15mg/kg</th>
<th>DOSE 200mg/kg</th>
<th>DOSE 400mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.67±0.03</td>
<td>0.65±0.03</td>
<td>0.65±0.03</td>
<td>0.65±0.03</td>
</tr>
<tr>
<td>B</td>
<td>0.78±0.07</td>
<td>0.86±0.05*</td>
<td>0.94±0.07*</td>
<td>1.04±0.50*</td>
</tr>
<tr>
<td>C</td>
<td>0.69±0.10</td>
<td>0.70±0.08</td>
<td>0.69±0.40</td>
<td>0.74±0.04</td>
</tr>
<tr>
<td>D</td>
<td>0.68±0.16</td>
<td>0.74±0.12</td>
<td>0.87±0.09*</td>
<td>0.83±0.90*</td>
</tr>
<tr>
<td>E</td>
<td>0.69±0.06</td>
<td>0.67±0.07</td>
<td>0.68±0.30</td>
<td>0.69±0.89</td>
</tr>
<tr>
<td>F</td>
<td>0.67±3.08</td>
<td>0.66±0.15</td>
<td>0.66±0.16</td>
<td>0.67±0.08</td>
</tr>
</tbody>
</table>

A; control, B; fed with metronidazole, C; fed with metronidazole and 400mg/kg of vitamin E, D; fed with metronidazole and 0.36mg/kg of testosterone, E; fed with metronidazole, 400mg/kg of vitamin E and 0.36mg/kg of testosterone, F; reversal. Values are expressed as mean ± standard deviation, n=5, *p<0.05 (significant)

**Discussion:**

Our findings show that both low and high dose administration of metronidazole had deleterious effects on testicular tissues. This is being demonstrated by the significant increase in malondialdehyde (MDA) and catalase activities in these groups. Only the group treated with 7.5mg/kg of metronidazole did not affect the MDA and catalase activities. However, simultaneous administration of metronidazole and vitamin E inhibited lipid peroxidation and reduced catalase activities in these groups. This implies that vitamin E prevents lipid peroxidation and conforms to earlier work done Farombi et al., (2002). Groups treated with metronidazole and testosterone concurrently did not interfere with either malondialdehyde concentration or catalase activities. This suggests that testosterone does not have anti-oxidative properties.

With the findings of this present study, we strongly believe that oxidative stress would have accounted for the reduction in fertility potentials in the earlier study which tested parameters such as fertility test, sperm analysis and hormonal assay (Ligha, 2011). This is evidenced by the high level of MDA and catalase activity in the metronidazole treated groups. Reactive Oxygen Species (ROS) are free radicals that play a significant role in many of the sperm physiological processes such as capacitation, hyperactivation and sperm-oocyte fusion (Aitken et al., 2004; Allamaneni et al., 2004; de Lamirande et al., 1998). However, they also trigger many pathological processes in the male reproductive system and these processes have been implicated in cancers of the bladder and prostate, as well as in male infertility (Bankson et al., 1993; Hietanen et al., 1994). Spermatozoa are sensitive to OS because they lack cytoplasmic defenses (Donnelly et al., 1999; Saleh and Agarwal, 2002). Moreover, the sperm plasma membrane contains lipids in the form of polysaturated fatty acids, which are vulnerable to attack by ROS. ROS, in the presence of polysaturated fatty acids, triggers a chain of chemical reactions called lipid peroxidation (Kobayashi et al., 2001; Zalata et al., 2004).

There are two main sources of ROS in semen: leukocytes and immature spermatozoa (Garriodo et al., 2004). Of these, leukocytes are considered to be the primary source (Aitken et al., 1992). Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production that ultimately leads to sperm dysfunction (Hendin et al., 1999; Ochsendorf, 1999; Pasqualotto et al., 2000; Saleh et al., 2002).

Spermatozoa produce ROS mainly when a defect occurs during spermiogenesis that results in retention of cytoplasmic droplets (Gomez et al., 1996; Zini et al., 1993).
Oxidative stress may also be limited by using chain-breaking antioxidants such as vitamin E and vitamin C as drug supplements. Vitamin C is a major chain-breaking antioxidant and is present in the extracellular fluid. It neutralizes hydroxyl, superoxide and hydrogen peroxide radicals and prevents sperm agglutination (Agarwal et al., 2004). In addition, it also helps recycle vitamin E (Sies et al., 1992). Vitamin E is found in reduced quantity in the seminal plasma of infertile men (Lewis et al., 1997). Vitamin C increases sperm counts in vivo in infertile male patients with oral doses ranging from 200–1000 mg/day. They conserve the alpha-tocopherol content of low-density lipoprotein and delay the onset of lipid peroxidation. The principal chain-breaking antioxidant vitamin E is present within the cell membrane. It neutralizes H₂O₂ and protects the plasma membrane from lipid peroxidation (Dawson et al., 1987). In a randomized cross-over study, oral administration of 600mg/day of vitamin E improved sperm function as assessed by the zona binding test (Kessopoulou et al., 1995).

A strong positive correlation exists between immature spermatozoa and ROS production, which in turn negatively affects the sperm quality (Saleh et al., 2002). The two main sites of ROS production are the mitochondrion and the sperm plasma membrane.

In vitro supplements used during sperm preparation and assisted reproductive technique also help to protect spermatozoa against ROS. Moreover, adding antioxidants to the culture media neutralizes ROS produced by the leukocytes and immature spermatozoa and improves sperm-oocyte fusion (Irvine, 1996). In an invitro study performed on samples from 25 male partners of infertile couples, in vitro supplementation of vitamin E prevented lipid peroxidation of the sperm plasma membrane by ROS and contributed to the recovery of high-quality spermatozoa after freezing–thawing procedures (Rossi et al., 2001). All these studies further show that metronidazole has oxidative properties and addition of vitamin E alleviates the deleterious effects of metronidazole on the testis.

Conclusion:

It is worthy to note that spermatozoa are under a continuous influence of oxidative stress (OS) because of the excessive generation of Reactive Oxygen Species. Although spermatozoa are affected in different ways by OS, there are sufficient antioxidant protections that can decrease the progression of the damage. However, when an imbalance exists between levels of ROS and the natural antioxidant defenses, various measures can be used to protect spermatozoa against the OS-induced injury. Diet forms an important component of the antioxidant protection system; it supplies the major antioxidants such vitamin C, vitamin E and carotenoids. Therefore, food rich in these elements should form a part of the daily diet. For those patients who are suspected to have high levels of ROS, antioxidant supplements can be considered. Nevertheless, further studies are required to validate their use in this group of patients. In certain cases, it is also essential to modify certain lifestyle behaviors because many habits and environmental factors increase the production of ROS and affect fertility. The results of this study indicate that metronidazole administration (200 or 400 mg/kg), for 8weeks, caused a harmful effect on the testes of male rats.

REFERENCES


