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Induction of Oxidative Stress by Methotrexate in the Testicular Tissue of Swiss Albino Mice

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Abstract

Methotrexate toxicity has been reported to occur due to the prolonged usage of the drug for desired therapeutic outcome. Testicular dysfunction is one of the health problems associated with free radical induced oxidative stress leading to degeneration of spermatogenesis and other testicular damages. According to reports, increased production of free radicals as well as weakened antioxidant defense system result to oxidative stress. This study therefore was designed to investigate the potential induction of oxidative stress in the testicular tissue of male mice following methotrexate exposure in male Swiss albino mice. In this study, twenty mice of about equal age 4-5 weeks weighing 25 gm were divided into four groups (n=5). The group I served as the control group and received vehicle, group II, III and IV received 1 mg/kg, 3 mg/kg and 5 mg/kg MTX respectively for 21 days *via* intraperitoneal route. Testicular reproductive hormones, both enzymatic and non enzymatic anti-oxidant biomarkers were investigated. Reactive Oxygen Species (ROS) and sperm parameters were also evaluated. Marked increase in ROS and LPO levels with simultaneous depletion of CAT, SOD and GSH was seen confirming generation of oxidative stress leading to dysfunction and damage of testicular tissue of the mice treated with Methotrexate.

Keywords: Methotrexate; ROS; Oxidative stress; Testicular tissue; Hormones

Introduction

Methotrexate (MTX), a chemotherapy drug used in the cure of tumors and other diseases such as psoriasis has been reported by different scientists to have toxic effects [1-3]. Methotrexate has been reported to functions by interfering with cellular reproduction by inhibiting the enzyme dihydrofolic acid reductase, which catalyses the conversion of folic acid into an active form referred to as Folinic acid by binding to it hence inhibiting the synthesis of Thymine. MTX lead to a reduction in methionine synthesis and thus a decrease of S-Adenosyl Methionine (SAM) [1]. It has likewise been reported that the mechanism with which MTX induces hepatotoxicity includes oxidative stress which increase the generation of ROS and Nitrogen specie, inhibiting cytosolic NADP-dependent dehydrogenase and NADP malic enzymes which reduce the levels of glutathione, superoxide dismutase, catalase and ultimately reducing the effectiveness of the antioxidant defense system protecting the cells against ROS [2]. Imbalance between ROS production and antioxidant defense system is responsible for oxidative stress and oxidative stresses have been implicated in a number of pathological diseases [1,2]. It has been reported by Abd-Allah et al. [4] that MTX toxicity is characterized by marked inflammation and increased production of ROS and therefore increased oxidative stress [1,4]. The ability of MTX to induce oxidative stress in liver and kidney has been reported, this study therefore focused on evaluating its potential to induce oxidative stress in testicular tissue of Swiss albino mice.

Materials and Methods

Animals grouping and Treatments

The protocol was approved by the Institutional Animal Ethics Committee of College of Medicine, Ibadan. This study was carried out in strict accordance with the recommendations in the guide for the care and use of Laboratory Animals of the Institute. Male and female Swiss albino mice weighing about 25 g were obtained from the Laboratory Animal Division of the College. The animals were maintained under standard conditions of humidity (50 ± 5%), temperature (25 ± 2°C) and dark and light cycles (12 h each) with free access to food and water. Male mice were divided into four groups (n=5) and treated intraperitoneally as follows; Group I: Vehicle-treated control; Group II: MTX, 1 mg/kg/day for 21 days; Group III: MTX, 3 mg/kg/day for 21 days; Group IV: MTX, 5 mg/kg/day for 21 days.

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Testicular testosterone (T) and Luteinizing hormone (LH) concentrations

The testicular testosterone and luteinizing hormone levels in three mice from each group were measured [5]. Briefly, testicular proteins were extracted with phosphate buffer (50 mM, pH 7.4) and centrifuged at 10,000 rpm for 20 min. The supernatant was used to estimate T and LH levels using ELISA, and were expressed in ng/ml.

Biochemical estimations of testes tissue

Testicular tissue from each mouse was stored at -20°C for different biochemical assays of lipid peroxidation: Glutathione (GSH), Superoxide Dismutase (SOD) and Catalase (CAT). Protein quantity was estimated and according to Lowry's method [6]. 10% tissue homogenates (w/v) were prepared in chilled 100 mM Tris-HCl buffer (pH 7.4). The values were expressed per mg of protein.

Measurement of reactive oxygen species (ROS) Level

The ROS assay was performed by the method of Hayashi et al. In brief, 50 µl of tissue homogenate and 1400 µl sodium acetate buffer were transferred to a cuvette. After then, 1000 ul of reagent mixture (N, N-diethyl paraphenylenediamine 6 mg/ml with 4.37 µM of ferrous sulfate dissolved in 0.1 M sodium acetate buffer pH- 4.8) was added at 37°C for 5 min. The absorbance was measured at 505 nm using spectrophotometer (Molecular Devices.) ROS levels from the tissue were calculated from a calibration of H_2O_2 and expressed as U/mg of protein (1 unit = 1.0 mg H_2O_2/L).

Lipid peroxidation (LPO)

The lipid peroxidation was estimated by a spectrophotometric method in terms of thiobarbituric acid reactive substances. Briefly, one volume of the homogenate was mixed with two volumes of stock solution (15% w/v trichloroacetic acid in 0.25N HCl and 0.375% w/v thiobarbituric acid in 0.25N HCl) in a centrifuge tube, vortexed and heated for 15 min at 95°C in water bath. The mixture was cooled and centrifuged at 5000 rpm for 5 min and the absorbance of the supernatant was read at 532 nm [7].

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity Superoxide dismutase (SOD) activity was estimated by a spectrophotometric method. Assay mixture containing sodium pyrophosphate buffer (pH 8.3, 0.052M), phenazine methosulfate (186 µM), nitroblue tetrazolium (300 µM) and NADH (780 µM) were diluted with appropriate enzyme in total volume of 3 ml. The mixture was incubated at 37°C for 90 sec and reaction was stopped by addition of glacial acetic acid. The reaction mixture was mixed vigorously by adding n-butanol and allowed to stand for 10 min before the collection of butanol layer. The intensity of chromogen in butanol was measured at 520 nm [8].

Catalase (CAT) activity

Catalase activity was quantified by measuring the decomposition of hydrogen peroxide (H_2O_2). Assay mixture consisting of 0.01 M phosphate buffer (pH 7), 0.2 M hydrogen peroxide and tissue homogenate was incubated at 37°C for 1 min. The reaction was stopped by the addition of potassium dichromate (5% w/v) and acetic acid. The remaining hydrogen peroxide was determined by measuring chromium acetate after heating the assay mixtures in a boiling water bath for 15 min. The absorbance was read at 570 nm [9].

Glutathione (GSH) content

Glutathione (GSH) content was estimated by centrifuging an aliquot of 10% homogenates of the tissues in 100 mM Tris-HCl buffer (pH 7.4) containing 0.16M of KCl at 1000 rpm for 5 min. The supernatant was used to measure the rate of reduction of 5'5'-dithiobis-(2nitrobenzoate) to 2-nitro-5-thiobenzoate. The absorbance was read at 412 nm. Glutathione content was expressed in uM/mg protein [10].

Table 1: Shows the T, FSH and LH levels in testes of mice.

Groups	Testosterone	LH	FSH
	µg/ml	µg/ml	µg/ml
Group I (Control)	7.314 ± 0.47	19.83 ± 1.69	4.83 ± 1.00
Group II (1 mg/Kg)	5.211 ± 0.21*	13.22 ± 0.12*	2.59 ± 1.01*
Group III (3 mg/kg)	5.634 ± 0.22*	9.93 ± 1.62*	3.76 ± 0.85*
Group IV (5 mg/kg)	2.785 ± 0.01**	5.23 ± 0.48**	1.67 ± 1.01**

Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

Sperm parameters

Cauda epididymidis was removed from each mouse and cleaned off from the epididymal fat pad, and minced in a pre-warmed Petri dish containing 500 µl phosphate buffer saline solutions (PBS, pH 7.4) at 37°C. Sperm motility was estimated and expressed as percentage incidence [11]. For sperm count, an aliquot of this suspension was charged into the Neubauer's counting chamber and the spermatozoa were counted under light microscope. Total sperm count was calculated as the average of the spermatozoa count (N) in each chamber X multiplication factor (10^6) X dilution factor and was expressed in millions/ml [12]. The sperm morphology was also evaluated [13]. Briefly, a smear of sperm was made on a clean slide and stained with hematoxylin and eosin and were examined under a light microscope with an oil immersion lens. The morphology of spermatozoa was scored according to Qureshi et al. [14].

Statistical analysis

All statistical comparisons between the groups were made using Analysis of Variance (ANOVA) by Prism statistics software. Results were presented as mean ± S.E.M (Standard Error Mean). Values of $p<0.05$ were considered as statistically significant.

Results

Effect on T, FSH and LH Levels

The study shows the T, FSH and LH levels in testes of MTX -treated mice (Table 1). The activity of Testosterone significantly ($p<0.05$) decreased in the testes of mice treated with MT when compared with the control.

Effect of methotrexate on reactive oxygen species

Methotrexate shows significant raised level ($p<0.5$) of reactive oxygen species in dose dependent manner following exposure in male treated mice when compared to the control (Figure 1).

Effect of methotrexate on antioxidant activities and LPO level

MTX-treatment induced significant ($p<0.01$) decrease in the protein level of testis. Oxidative stress was induced by MTX treatment as confirmed by the simultaneous significant decrease ($p<0.01$) of GSH, CAT, GPx SOD activities and increased level of LPO in the testes of mice when compared with controls (Figures 2-6).

Effect on sperm parameters

MET treatment caused significant decrease (p<0.001) in epididymal sperm count and motility across the groups when

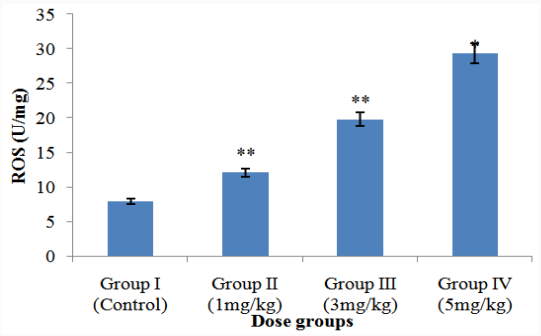

Figure 1: Barchat showing the effect of Methotrexate on ROS level. The data showed significant changes from untreated Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

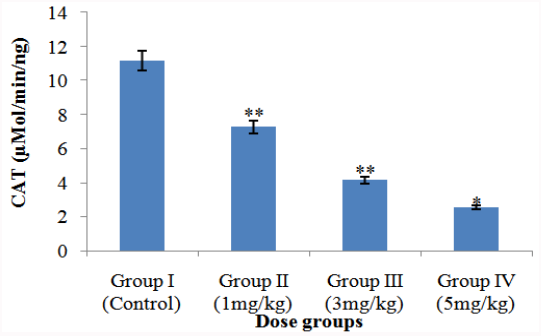

Figure 2: Barchat showing the effect of Methotrexate on Catalase activity. The data showed significant changes from untreated Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

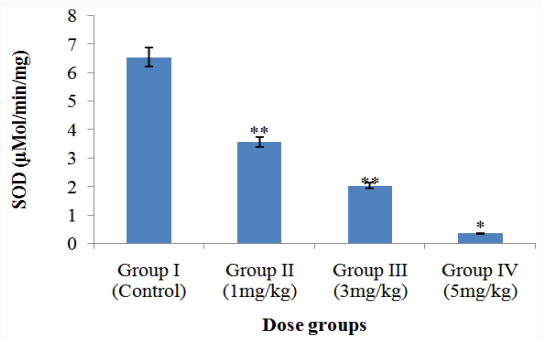

Figure 3: The effect of Methotrexate induced decrease changes in the superoxide dismutase level. The data showed significant changes from untreated Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

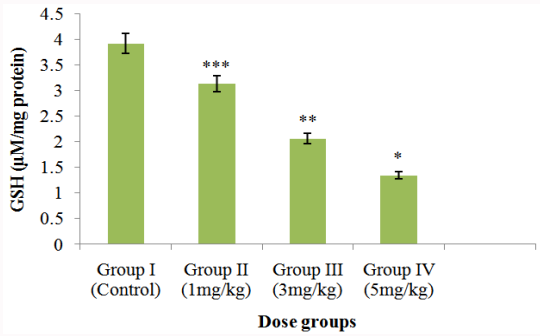

Figure 4: The effect of Methotrexate on Glutathione level. The data showed significant changes from untreated Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

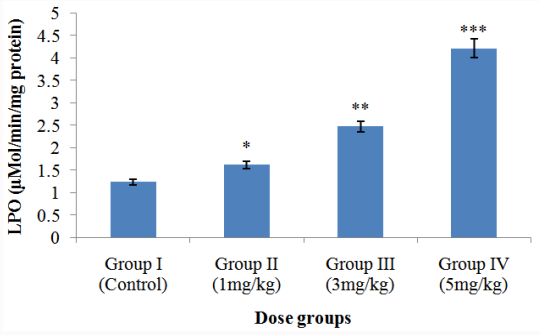

Figure 5: The effect of Methotrexate on lipid peroxidation level. The data showed significant changes from untreated Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at (p<0.01), and (p<0.05) respectively

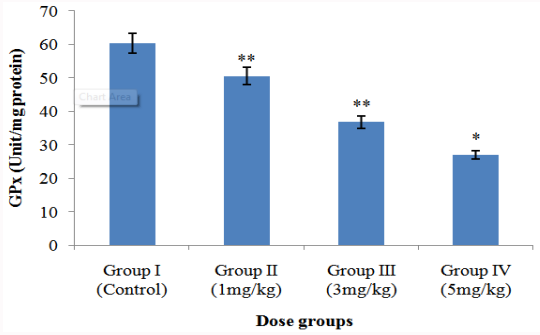

Figure 6: The effect of Methotrexate on glutathione peroxidase level. The data showed significance changes.
Note: *, and ** Indicate significantly difference as compared to controls at (p<0.01), and (p<0.05) respectively

compared with the vehicle control and a significant (p<0.05) abnormal sperm morphology (Figure 7).

Discussion

This present study investigated the induction of oxidative stress by methotrexate in the testicular tissue of mice. Oxidative stress reflects an imbalance between ROS and cellular anti-oxidant defense system. The metabolism drugs have likewise been reported to have the capacity to induce oxidative stress, methotrexate being one of such drugs. This current study showed signs of oxidative stress as shown by increased MDA activity in the testes of the MET-treated mice, the drug-treated mice also showed a defective antioxidant response as evident from diminished activity of antioxidant enzymes such as CAT, SOD, and GSH. Similar reports have been likewise been made available [15]. Oxidative stress is a common pathology that has been implicated in male infertility. Induction of oxidative stress can be as result of increasing the free radical generation in testis and epididymis resulting in degeneration of spermatogenesis and ultimately infertility [16].

GSH a non-enzymatic antioxidant functions includes removal

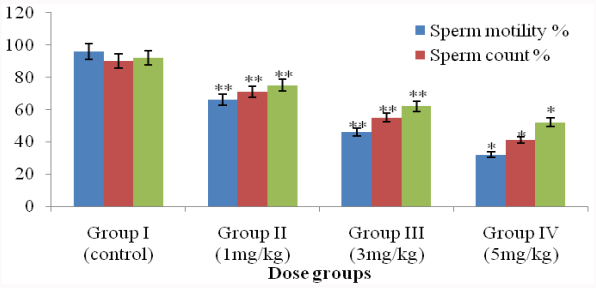

Figure 7: Barchat representing the effect of Methotrexate on sperm motility count and morphology levels in percentage. The data showed significant changes from Group I (control) to Group IV (5 mg/kg).
Note: *, and ** Indicate significantly different as compared to controls at ($p<0.01$), and ($p<0.05$) respectively

of free radicals such as H_2O_2 and superoxide anions, maintenance of membrane protein thiols and acting as a substrate for GPx and GR [1,17]. Thus, significant decrease in GSH level observed after treatment with MTX, leads to a defective antioxidant response thereby causing the cells to be susceptible to Reactive Oxygen Species [18]. GSH serves as an important marker of oxidative stress and it plays an important role in maintaining the integrity of the cell because of its essential role in several detoxification reactions in the organism and it is one of the most prominent non-enzymatic antioxidants [1,19-22]. Similar observations have also been reported to support this observation of decreased levels of GSH in the small intestine and testes of mice treated with Bleomycin and Salinomycin and in liver following MTX administration [8,9,18,20].

There significant reduction seen in the testosterone, LH and FSH level of testicular tissue of the mice exposed to MTX further confirmed that the tissue was undergoing oxidative stress which is responsible for the dysfunction of testes [9,10,23,24].

Conclusion

The present evaluated the potential of Methotrexate to induce oxidative stress in testicular tissue of Swiss albino mice. This study also that the treatment of MTX resulted in reduced level of FSH, LH, testosterone hormones. Oxidative stress was induced by MTX treatment as confirmed by the simultaneous significant decrease in SOD, GSH and CAT activities with simultaneous increase level of ROS and LPO. The study further confirmed the previous reports on MTX ability to generate reactive oxygen species.

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