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Therapeutic Effect of Quercetin against Methotrexate - Induced Male Infertility

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Abstract

Methotrexate (MTX), a known chemotherapy drug has been reported to induce male reproductive toxicity leading to infertility in male mice. Therefore, current study was carried out to evaluate the ameliorative effect of Quercetin (a flavonoid) against methotrexate-induced male infertility. In this study, seventy five (75) Swiss albino miceweighing 25g (15 male and 60 female mice) were used. Fifteen male mice were divided into three groups I, II and III (n=5). Group I served as control and received vehicle, group II (5mg/kg Methotrexate) and group III (10mg/kgquercetin+5mg/kg Methotrexate) via intraperitoneal for 21 days. After completion of treatment, treated mice were allowed to mate with untreated female mice before autopsy. The study investigated effects on testicular tissue biochemical marker enzymes, hormones and sperm parameters including sperm count, motility and morphology. Effect on fertility was observed by evaluating incidence of pregnancy and implantation. The resultsshowed that treatment with methotrexate results in generation of oxidative stress in testicular tissue of mice resulting in infertility. This harmful effect can be reversed using a strong antioxidant such as quercetin as seen in this study.

Introduction

Methotrexate (MTX), an anticancer drug also used in the cure of other diseases such as psoriasis and certain inflammatory diseases functions by interfering with cellular reproduction. MTX is a chemical agent that acts as inhibiting the enzyme dihydrofolic acid reductase, which catalyzes the conversion of folic acid into an active form referred to as folinic acid by binding to it. Recently, MTX becomes drug of preference for most people who suffer from rheumatoid arthritis such as cases of juvenile rheumatoid arthritis which made it a disease modifying anti rheumatic drug (DMARD) for stated cases. Unfortunately, the drug is to be used for extended length of time for desirable therapeutic results [1] hence, increasing cases of toxicity levels in patients. Several reports have shown incidences of methotrexate toxicity in the testicular tissue of mice exposed to methotrexate [2-6]. These reports revealed that Methotrexate leads to impairment of male reproductive organs with lasting effects on reproductive health; hence the need for the reversal of its toxicity is necessary because of its therapeutic potentials. Quercetin (3,3',4',5,7-pentahydroxyflavone) in fruits and vegetables has attracted much attention for its beneficial health effect due to its potential antioxidant property. Different studies have suggested that the risk of various chronic health disorders such as tumour development, diabetes, cardiovascular disease, neurodegenerative disease and stroke may be reduced by daily intake of this substance [7-10]. Mechanisms such as antioxidant activity, anti-inflammation, interaction with receptors and other proteins, modifications of signal transduction pathway have been attributed to the beneficial effect of quercetin [11]. The phenolic hydroxyl group of quercetin is responsible for its antioxidant property [12]. Documentations of the beneficial health effect of quercetin against various diseases related to oxidative stress are available. Based on our knowledge there is not enough information about the ameliorative effect of quercetin on methotrexate induced infertility in sperm and testis of mammalian. Most of recent studies are about histological and cytotoxic effects of methotrexate on reproductive tract of male and the anti-oxidant properties of quercetin. Therefore the interest of this study was to determine the ameliorative potential of quercetin against methotrexate induced male infertility.

Keywords: Methotrexate Oxidative stress; Quercetin; Testicular toxicity; Infertility

Materials and Methods

Methotrexate**,** Quercetin**,** Dimethyl sulphoxide (DMSO), Nitro blue tetrazolium (NBT), Nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH), Nicotinamide adenine dinucleotide (reduced) disodium salt, (NADH), Oxidized Glutathione, Glutathione (reduced), 2-Thiobarbituric acid, Potassium dichromate, Acetone, Ethanol, Benzene, Formaldehyde, Hematoxylin and Eosin, Toluidine blue, 5,5, Dithiobis (2-nitrobenzoicacid),1-Chloro-2,4-dinitrobenzene, were purchased from Sigma Chemical CO (St Louis MO,USA). Lactate dehydrogenase kit was purchased from Merck (Spain), testosterone ELISA kit was purchased from DRG (Germany).

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 hr each) with free access to food and water. Male mice were divided into three groups of five animals each and treated intraperitoneally as follows; Group I: Vehicle-treated control; Group II: MTX, 5mg/kg/day for 21 days; Group III: 10mg/kg quercetin+5mg/kg Methotrexate. After completion of treatment, treated mice were allowed to mate with untreated female mice before autopsy.

General observations

Body and testes weights mice were observed daily for behavioural changes. Body weight was recorded daily prior to administration of MTX with the help of a mono pan balance. At autopsy, testicular tissues were removed, blotted free of blood and adhering tissues and weighed.

Testicular Testosterone (T), Follicle Stimulating (FSH) and Luteinizing hormone (LH) concentrations

The testicular testosterone and luteinizing hormone levels in three mice from each group were measured [13]. Briefly, testicular proteins were extracted with phosphate buffer (50 mM, pH 7.4) and centrifuged at 10,000 g 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 tissues from each mouse were stored at -20°C for different biochemical assays of lipid peroxidation: malonialdehyde (MDA), lactate dehydrogenase (LDH), glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). Protein quantity was estimated according to Lowry's method [14]. 10% tissue homogenates (w/v) were prepared in chilled 100 mMTris-HCL buffer (pH 7.4). The values were expressed per mg of protein.

Estimation of Lipid peroxidation (LPO) level

The lipid peroxidation was estimated by a spectrophotometric method in terms of thiobarbituric acid reactive substances. Briefly, one volume of homogenate was mixed with two volumes of stock solution (15% w/v trichloroacetic acid in 0.25 N HCL and 0.375% w/v thiobarbituric acid in 0.25 N 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 [15].

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was assayed 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 [16].

Catalase (CAT) activity

Catalase activity was estimated by measuring the decomposition of hydrogen peroxide (H2O2). Assay mixture consisting of 0.01M phosphate buffer (pH 7), 0.2 M hydrogen peroxide and tissue homogenate was incubated at 37ºC for 1 min. The reaction was stopped by 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 [17].

Glutathione (GSH) content

Glutathione (GSH) content was quantified by centrifuging an aliquot of 10% homogenates of the tissues in 100 mMTris-HCL buffer (pH 7.4) containing 0.16 M KCL at 1000 g for 5 min. The supernatant was used to measure the rate of reduction of 5' 5'dithiobis-(2 nitrobenzoate) to 2-nitro-5 thiobenzoate. The absorbance was read at 412 nm. Glutathione content was expressed in µM/mg protein [18].

Sperm Parameters

Caudaepididymidis was removed from each mouse and cleaned off from the epididymal fat pad, and minced in a prewarmed Petri dish containing 500 µl phosphate buffer saline solutions (PBS, pH 7.4) at 37°C. Sperm motility was estimated by putting a drop of sperm suspension on a clean slide and covered with a cover slip and analysed by the computer assisted sperm analyser (CASA) by Hamilton, Thorne. The motility was expressed as percentage incidence [19]. 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 [20]. The sperm morphology was also evaluated [21]. Briefly, a smear of sperm was made on a clean slide and stained with haematoxylin 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. [22].

Male fertility and dominant lethality

Male fertility was checked in all the groups after 21 days of MTX treatment according to standard method [23,24]. Each male was caged with five female per week for four weeks. The females were at proestrous when allowed with the males. Each female was checked for vaginal plug daily for confirmation of mating. Following confirmation of mating, females were separated from males. The females were sacrificed on 13 days of mating to check implantation. The number of pregnant mice was recorded to determine percent of fertility [25]. The incidence of pregnancy was established after counting the number of implants. The dead implants per female were determined to obtain the post-implantation loss [26]. Fertility index was calculated by the ratio of the number of pregnant females to number of females cohabited with males multiplied by 100.

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 $p < 0.05$, $p<0.01$, $p< 0.001$ were considered as statistically significant.

Results

General observations

The earliest toxic signs observed in few MTX treated mice were weakness and diarrhoea resulting in reduction of body weight of the treated mice when compared with the control (Table 1).

Effect on T, FSH and LH Levels

Table 2 Shows the T, FSH and LH levels in testes of MET-treated mice. The activity of Testosterone significantly ($p < 0.05$) decreased in the mitochondrial fraction of testes of mice treated with MTX, while the decrease observed in group III testis were not significant when compared with the control.

Anti-oxidant status in testes

MTX-treatment induced significant ($p <0.01$) decrease in the protein level of testis. QUE caused recovery to a certain degree when compared with control. Oxidative stress was induced by MTX treatment

Table 1: Reproductive organs weight of mice following 21 days.

Groups &Treatment (mg/kg/day)	Body weight (g)		Reproductive Organ weight (Mean ± S.E.)		
	Pre-dose	Post-dose	Testes	Seminal vesicle(s)	Epididymides
Group I (Control)	20.02 ± 0.23	28.04 ± 1.95	1.06 ± 0.10	0.94 ± 0.09	0.13 ± 0.02
Group II (MTX-5mg/kg)	22.92 ± 0.73*	21.76 ± 0.73*	0.60 ± 0.02***	0.59 ± 0.03***	0.09 ± 0.01*
Group III (MTX+QUE-10mg/kg)	22.08 ± 1.26	27.58 ± 0.56	0.99 ± 0.09**	0.77 ± 0.04	0.09 ± 0.01

Note: **, and *** Indicate significantly different as compared to controls at ($p < 0.01$), and ($p < 0.001$) respectively

Table 2: Effect on T, FSH and LH Levels.

Groups	T (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Group I (Control)	7.314 ± 0.47	9.83 ± 1.69	4.83 ± 1.00
Group II (MTX-5mg/kg)	2.785 ± 0.01*	2.523 ± 0.48*	1.67 ± 1.01*
Group III (MTX + QUE-10mg/kg)	5.954 ± 0.52	7.33 ± 1.62	3.76 ± 0.85

Note:* Indicate a significant difference as compared to control at ($p < 0.05$)

as confirmed by the simultaneous significant decrease ($p < 0.01$) in GSH, CAT, and SOD levels and increase MDA level in the testes when compared with controls. Co-administration with QUE significantly lowered MTX-induced increase in MDA level (Table 3).

Effect on Sperm Parameters

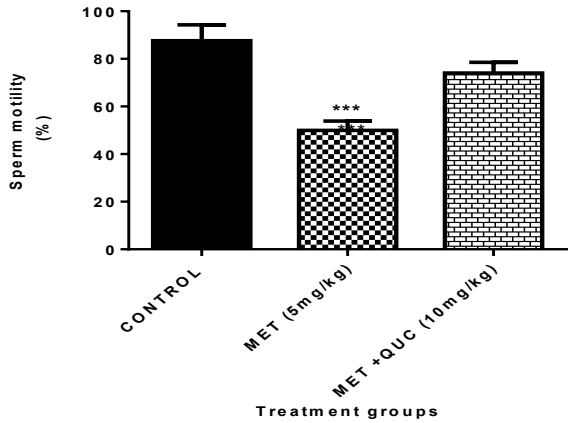
MTX treatment caused a significant decrease ($p < 0.001$) in epididymal sperm count and motility (Figures 1& 2) in group II, a significant increase in epididymal sperm count and motility was observed in mice treated with QC compared to group II. A marked increase ($p < 0.01$) in abnormal morphology was observed in epididymal spermatozoa (Figure 3) in MTX only treated mice, a marked increase in recovery of normal morphology in epididymal spermatozoa of groups treated with QUE was likewise observed as compared to MTX only treated mice.

Male fertility and dominant lethality

The MTX treatment decreased fertility in male mice, as there was no incidence of pregnancy and implantation in females mated to these males. Infertility was observed in males treated with 5mg of MTX. It was observed that QUE caused a reversal of the infertility caused by MTX treatment and there was a significant incidence of pregnancy and implantation in females mated to these males (Table 4).

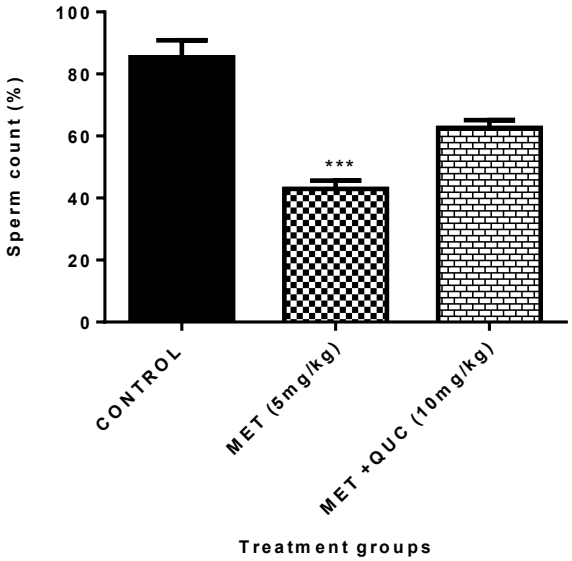
Discussion

The present study demonstrated that treatment with Methotrexate resulted in the generation of oxidative stress in the testis and caused a



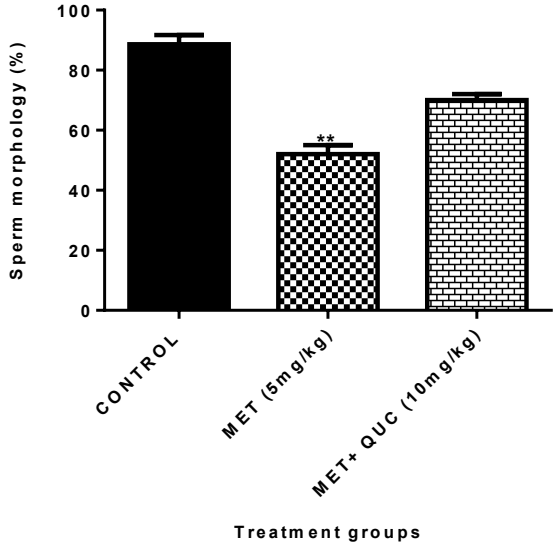
***p>0.01versus the control group.

Figure 2: Ameliorative effect of quercetin on the decreased sperm motility of methotrexate treated mice. Note: all data are presented as mean ± SD (n=5).



***p>0.01versus the control group.

Figure 1: Ameliorative effect of quercetin on the decreased sperm count of methotrexate treated mice. Note: all data are presented as mean ± SD (n=5).



**p>0.01versus the control group.

Figure 3: Ameliorative effect of quercetin on the abnormal sperm morphology of methotrexate treated mice. Note: all data are presented as mean ± SD (n=5).

Table 3: Effect on PRO, CAT, SOD, GSH and LPO.

Groups	PRO Mg/ml	CAT µMol/min/mg protein	SOD U/ml/min	GSH µM/mg protein	LPO µMol/min/mg protein
Group I (Control)	0.611	0.408	0.518	0.478	0.318
Group II (MTX-5mg/kg)	0.322**	0.242**	0.142**	0.296**	0.737***
Group III (MTX+QUE-10mg/kg)	0.442	0.352	0.445	0.475	0.555

Note: **, and *** Indicate significantly different as compared to controls at ($p < 0.01$), and ($p < 0.001$) respectively

Table 4: Effect on MTX on Male fertility and dominant lethality

Groups & Treatment s(mg/kg)	Pregnant female	Male Fertility Index (%)	Implantations/pregnant female ± S.E.M		
			Total	Live	Dead
Groups (Control)	16/20	80	8.69 ± 0.29	8.38 ± 0.16	0.30 ± 0.13
Group I (MTX-5mg/kg)	0/20	0	0	0	0
Group II (10mg/kg QUE +5mg/kg MTX)	12/20	30	6.88 ± 3.08**	3.38 ± 1.74***	3.50 ± 1.34**

Note: **, and *** Indicate significantly different as compared to controls at ($p < 0.01$), and ($p < 0.001$) respectively

series of testicular dysfunctions resulting in infertility and this can be ameliorated by treatment with quercetin. Malignancy has been a major source of concern as it pose a great threat to health, this has led to the development of chemotherapeutic drugs to cause drastic improvement in the illness of patient as well as increase the life expectancy of cancer patients. Methotrexate one of such chemotherapy drugs is a folate antagonist inhibiting the proliferation of malignant cells by inhibition of dihydrofolatereductase enzyme. It is an antitumor agent used for the treatment of a wide range of disease ranging from childhood acute lymphoblastic leukemia (ALL) to rheumatoid arthritis [27,28].

The clinical use of Methotrexate, as an anticancer drug for a countless number of human malignancies, has been impeded due to its detrimental effects, the major toxicity being infertility. Present study is to investigate if there is any ameliorative effect of quercetin on the major toxicity of MTX administration which is infertility. The earliest toxic signs observed in few MTX treated mice were weakness, salivation, diarrhoea and paralysis of hind limb.

Treatment with MTX for 21 days significantly reduced the testosterone in the testis of the mice. This could have been the cause of infertility in males as reported by previous studies [29] since testosterone is essential in the regulation of sexual behaviour, accessory sex organ functions, epididymal sperm maturation, and spermatogenesis [30] and drugs implicated in cytotoxicity of the testis either inhibit biosynthesis of testosterone or its secretion thus having intense effects on the processes required for the timely deposition of viable spermatozoa into the reproductive tract of the female. Leydig cells found adjacent to the seminiferous tubules are responsible for the production of testosterone in the presence of luteinizing hormone (LH). As LH level is significantly low in the testis of the MTX-treated mice compared to the vehicle control, this might be the reason for the decreased level of testosterone in the testis of the mice administered with MTX in a dose dependent manner. However, it was observed that the co-administration of quercetin elevated the testosterone, LH in MTX-treated mice, hence there was no significant difference between the quercetin-treated group and the vehicle control.

In this present study, we noticed signs of oxidative stress as shown by increased MDA activity in the testes of the MTX-treated mice, the drug-treated mice also showed a defective antioxidant response as evident from diminished activity of antioxidant enzymes such as CAT, SOD, GSH. Similar observations have been likewise reported [31]. However, it was observed that there was no significant oxidative damage in the quercetin-treated mice when compared to the vehicle-control. Quercetin, a flavonoid has been known for its antioxidative properties; hence we can presume that quercetin could emerge as anantioxidative agent against MTX induced oxidative stress. 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 dysregulation of spermatogenesis and fertility [32].

In addition to this, decrease in sperm count was observed in the caudal epididymidis of MET treated mice indicating cytotoxicity of MTX in the testes of the mice .The effect of MTX on the early germ cells in the spermatogenic cycle led to its abnormality, thereby increasing the normal of abnormal spermatozoa. MTX treatment resulted in an adverse effect on spermatozoa function, significant decrease of sperm motility was observed. MTX treatment resulted in the distortion of the normal sperm morphology, significant amount of abnormal spermatozoa was observed in MTX-treated mice. Oxidative stress in the epididymis might be the reason for this effect on the sperm parameters of the MTX-treated mice. Increased lipid peroxidation in the testis might have also contributed to abnormality of spermatozoa resulting in infertility. Similar observations have also been reported to support this observation [33-35]. As stated above, quercetin is a potent anti-oxidant; it was observed that there was no significant effect on sperm parameters in the quercetin treated mice when compared to the vehicle-control. Oxidative stress has been suspected to be the major reason responsible for the decrease in sperm count, impaired sperm motility and abnormal spermatozoa. The induction of oxidative stress and its effect was found to be prevented by quercetin treatment.

There was no incidence of pregnancy and implantation in the female mice that mated with the MTX treated male mice. The alterations in spermatogenesis resulting in abnormal spermatozoa having reduced motility could be correlated with the inability of the female mice to get pregnant and no incidence of implantation in the female mice mated with MTX treated males. However, it was observed that the co-administration of quercetin caused a reversal of the infertility caused by MTX treatment and there was a significant incidence of pregnancy and implantation in females mated to these males. This could be as a result of the ability of quercetin to reverse the effect of MTX on sperm parameters. However, the particular mechanism remains unknown.

In view of the present observations as well as the above cited reports, it is relevant to presume that quercetin, being an antioxidant, effectively ameliorate the induction of oxidative stress which is known to be a major cause of infertility in men, and hence, could be used as an effective inhibitor of the methotrexate-induced male infertility.

Conclusion

This present study show that therapeutic dose of Quercetin has the capacity to ameliorate the toxicity caused by Methotrexate in the testes through enhancement of antioxidant status, decrease in the level of inflammatory cytokines and sperm parameters. We therefore suggest that use of methotrexate should be accompanied by therapeutic dosage of Quercetin in order to eliminate infertility in patients.

Conflict of Interest Statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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