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Research Paper

Alteration of Biochemical Enzymes and Sperm Parameters by Quercetin In Chlorambucil-Treated Swiss Albino Mice

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ABSTRACT
The current study was carried out to evaluate the ameliorative potential of quercetin (QUC) against chlorambucil-induced male reproductive toxicity. In this study, male mice were divided into three groups I, II and III (n=5). Group I served as the control group and received vehicle, animals in group II were given 5mg/kg of CB while group III received 5mg/kg CB + 10mg/kg of quercetin via intraperitoneal treatment for 14 days. The study investigated effects on biochemical marker enzymes, hormones, and Sperm parameters including the count, motility and morphology; roles of apoptosis was also evaluated. The results showed that the treatment of chlorambucil lead to the generation of oxidative stress and induction of apoptosis in testicular tissue of mice. However, this harmful and deleterious effects of chlorambucil can be reversed or prevented by quercetin as seen in the study.

KEYWORDS*: Chlorambucil; Quercetin; Oxidative stress; Hormones; male reproductive toxicity.*

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I. INTRODUCTION

Male fertility can be directly related to the quality of sperm produced and its functionality. Male infertility issues arise when quality of sperm produced by the male testicular cells is altered. According to literature, drugs are part of the many causes of oxidative stress and damage (Khaki *et al*., 2009). Oxidative damage is an internal factor which can potently alter sperm parameters by manipulating spermatogenesis (1).

Chlorambucil (CB) is an anticancer and immunosuppressive drug used for a number of cancer and inflammatory-related conditions. Mechanistically, CB acts by causing interference in replication of DNA, inducing DNA damage which further leads to apoptosis ultimately (2). It has also been reported that CB is potent to induce oxidative stress in testicular cells of male rats, resulting in low quality of sperms produced (3). Interestingly, CB has been known to lack selective toxicity and as such tends to be toxic to many cells of the body. This has been linked with the inability of a CB to particularly differentiate between normal cells and tumor cells (2).

Quercetin (QUC) is one of the most abundant dietary flavonoids which have been studied over the years in a plethora of research studies. It is present in many fruits consumed in the world. It has a largely positive effect on human health, according to numerous researches where studies on QUC have been reported (3,4). It possesses antioxidative and anti-inflammatory properties (5,6). QUC is a potent scavenger of oxygen free radicals, while inhibiting lipid peroxidation. Asides the antioxidative and anti-inflammatory properties associated with quercetin, it is also beneficial against health conditions like hypertension, hyperlipidemia and thrombosis (7). This study sought to give insight to the effect of quercetin against Chlorambucil-induced testicular toxicity in male reproductive system.

II. METHODS

2.1 Animals grouping and Treatments

Specific pathogen-free, healthy young adult male mice of 10 to 12 weeks were used in this study. Male mice weighing about 25 g were obtained from a lab house of the institute. The animals were maintained under standard conditions of humidity (50 +/- 5%), temperature (25 +/- 2^{o}C) and dark and light cycles (12 hrs each) with free access to food and water. Male mice were divided into three groups of ten animals each and treated intraperitoneally for 14 days as follows;

Group 1: Control;
Group 2: CB, 5mg/kg
Group 3: CB, 5mg/kg + 10mg/kg Quercetin
Animal in each group was sacrificed after 14 days.

2.2 Biochemical estimations in tissue samples

Testis from each mouse were stored at -20^{0}C for different biochemical assays such as lipid peroxidation, glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT). Protein quantity was estimated and according to Lowry's method (Lowry, 1951). 10% tissue homogenates (w/v) were prepared in chilled 100mM Tris-HCl buffer (pH 7.4) using Cole Parmer tissue homogenizer. The values were expressed per mg of protein.

2.2.1 Determination of lipid peroxidation

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^{o}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 (8).

2.2.2 Measurement of superoxide dismutase 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 uM), nitroblue tetrazolium (300 uM) and NADH (780 uM) were diluted with appropriate enzyme in total volume of 3 ml. the mixture was incubated at 37^{o}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 10mins before the collection of butanol layer. The intensity of chromogen in butanol was measured at 520nm (9).

2.2.3 Catalase (CAT) activity

Catalase activity was quantified by measuring the decomposition of hydrogen peroxide (H_2O_2). Assay mixture consisting of 0.01M phosphate buffer (pH 7), 0.2M hydrogen peroxide and tissue homogenate was incubated at 37^{o}C for 1min. 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 15min. The absorbance was read at 570nm (10).

2.2.4 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 1000g for 5mins. 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 412nm. Glutathione content was expressed in µM/mg protein (11).

2.3 Testicular, LH and FSH Hormones Measurement

Testicular levels of testosterone, LH and FSH were measured according to the protocol. 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 measure testosterone, LH and level using ELISA method and were expressed in ng/ml. The absorbance of the product was read at 405 nm.

2.4. Sperm Parameters

Testes was removed from each mouse and cleaned, and minced in a pre-warmed Petri dish containing 500 µl phosphate buffer saline solutions (PBS, pH 7.4) at 37^{o}C. Sperm motility was estimated and expressed as percentage incidence. 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 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. (14).

2.5. CASP- 3 and CASP- 9 activities

Caspase 3 and Caspase 9 activities were estimated by using manufacturer protocol. Briefly, the assay was based on monitoring of DEV D-p- nitroaniline (pNA) through time. The temperature was maintained at

37ºC and the kinetics of the reaction were followed by 3 hrs by absorbance at 405 nm every minute on each well using the plate reader.

2.6 Statistical Analysis

All statistical comparisons between the groups were made using analysis of variance (ANOVA) by Prism statistics software. Results were presented as mean +/- SEM (Standard Error Mean). Values of $p<0.05$ were considered as statistically significant.

III. RESULTS

3.1 EFFECT on Total Protein Level

Chlorambucil treatment induced significant decrease ($p<0.01$) in the protein level. Quercetin caused recovery to a certain degree when compared to control.

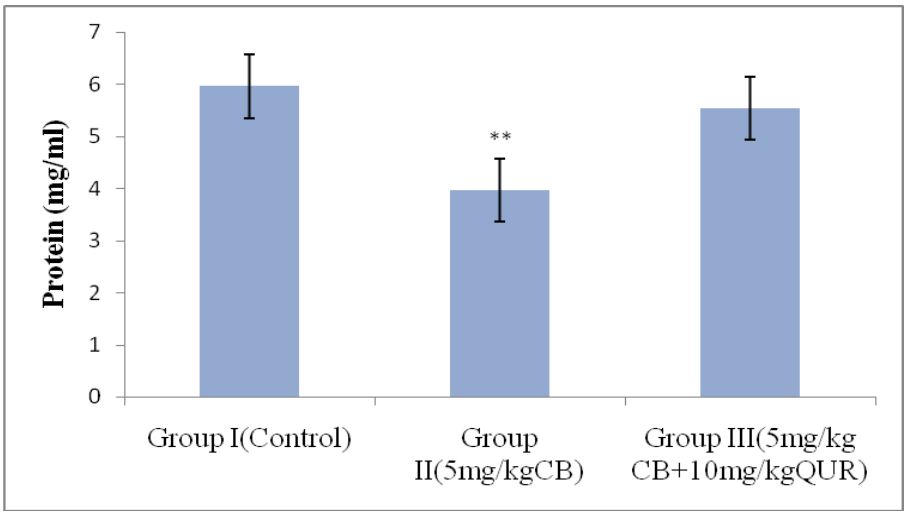

Fig 1: Bar chart showing the result of ameliorative potential of quercetin in chlorambucil-induced changes on the level of protein. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *($p<0.01$), ** ($p<0.05$).

3.1.2. Effect on Catalase Level

The level of catalase decreased significantly ($p<0.01$) following treatment with CB. However adverse effect of CB was reversed by co-administration of quercetin when compared with control.

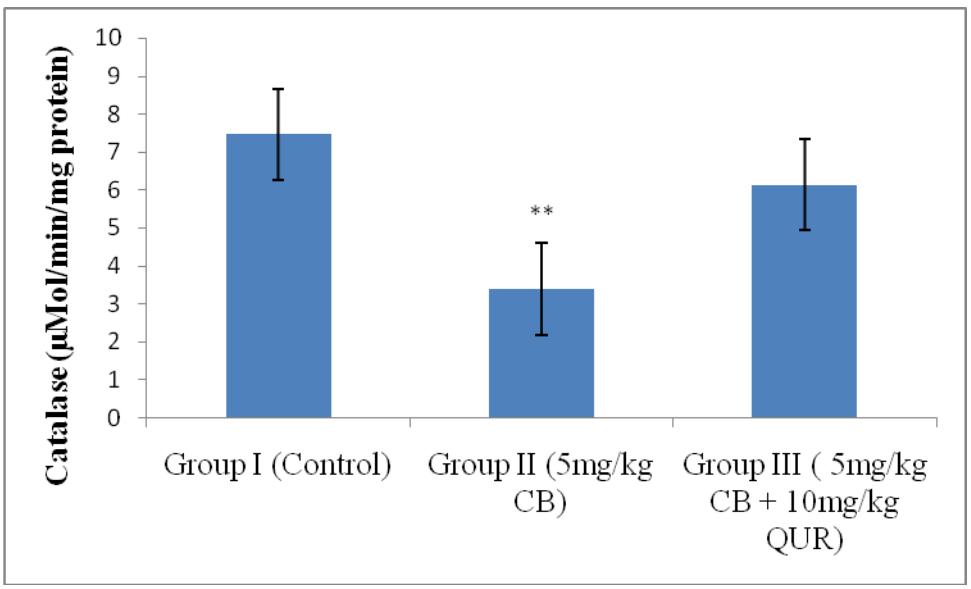

Fig 2: Bar chart result of ameliorative potential of quercetin in chlorambucil induced changes on the level of catalase. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *($p<0.01$), ** ($p<0.05$).

3.1.3 Effect on Superoxide Dismutase (SOD) Activity

The concentration of superoxide dismutase increased significantly upon the treatment with CB at a dose 5mg/kg in comparison with the vehicle treated control, upon co-administration of quercetin an insignificant difference was recorded when compared with the control.

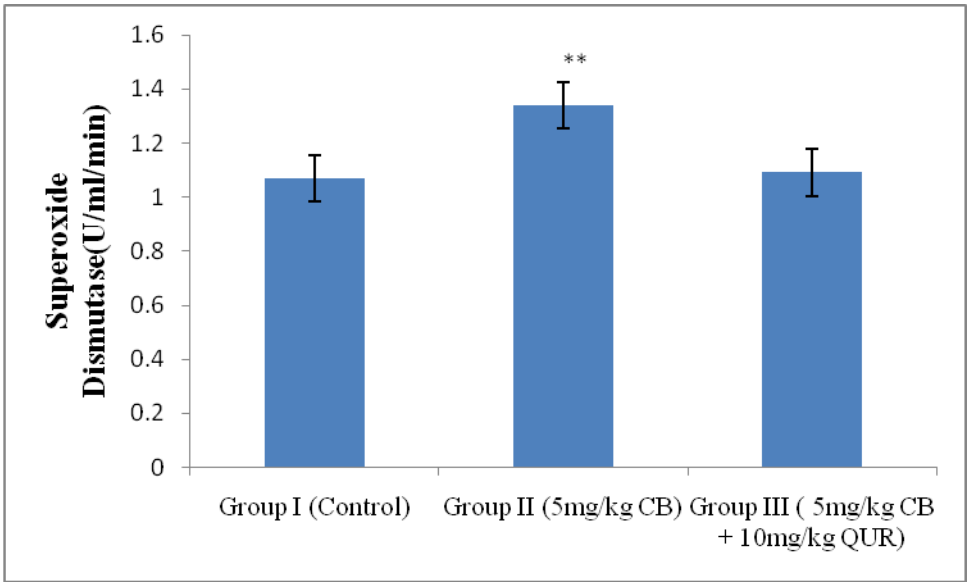

Fig 3: Bar chart result of ameliorative potential of quercetin in chlorambucil induced changes on the level of superoxide dismutase. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.4 Effect on Reduced Glutathione Levelo

The level of reduced glutathione decreased significantly ($p<0.01$) following treatment with CB. However adverse effect of CB was reversed by co-administration of quercetin.

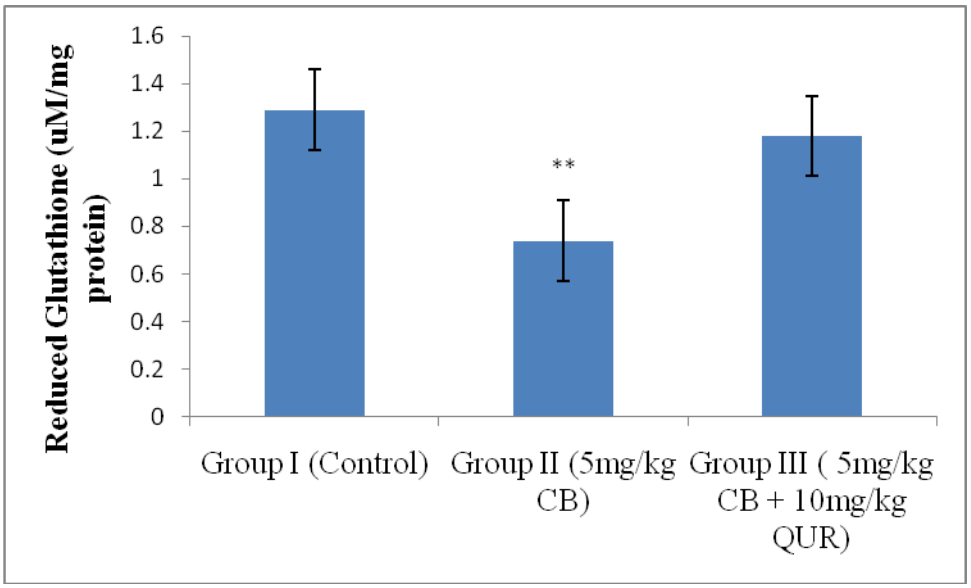

Fig 4: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of reduced glutathione. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.5 Effect on Glutathione Peroxidase activity

The activity of Glutathione peroxidase decreased significantly ($p<0.01$) following treatment with CB. However adverse effect of CB was reversed by co-administration of quercetin.

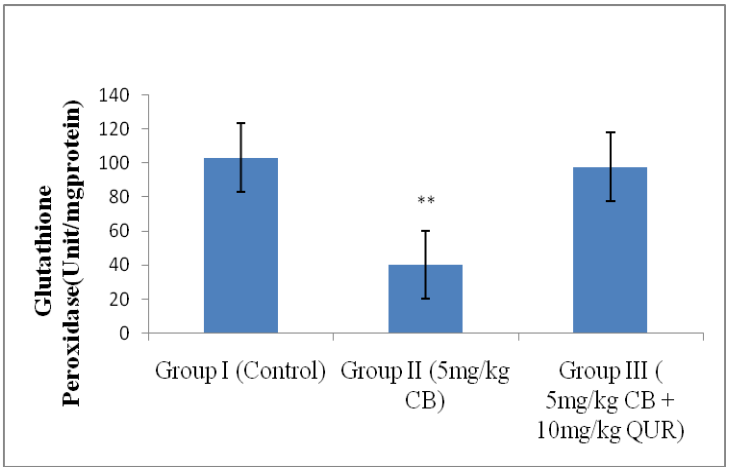

Fig 5: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of Glutathione peroxidase. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.6 Effect on Lipid Peroxidation Level

The result shows a significant increase in the lipid peroxidation levels when treated with chlorambucil at a dose of 5mg/kg when compared with the vehicle treated control. The co-administration with quercetin reversed the adverse effect of CB on lipid peroxidation level. However, the CB and quercetin treated group shows a significant difference when compared with the control group.

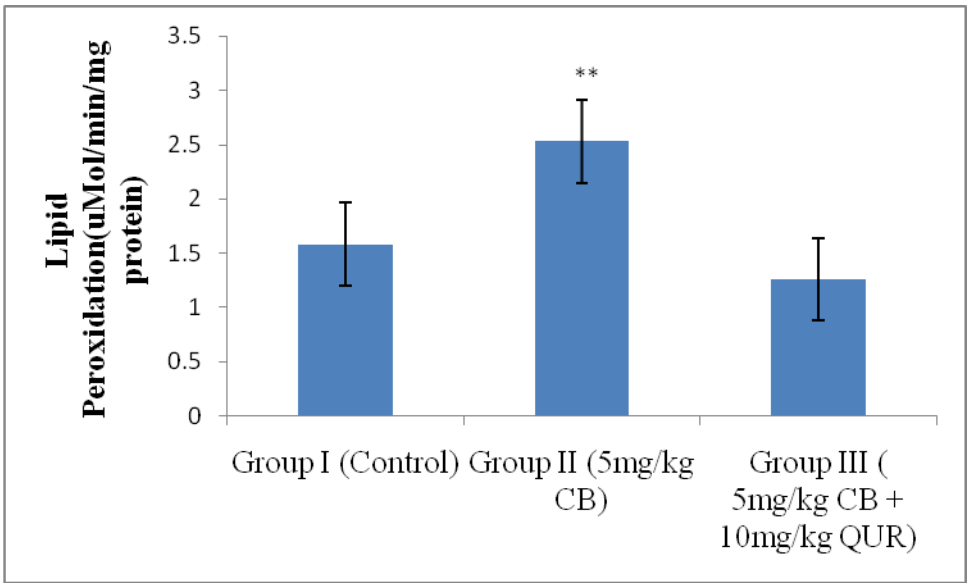

Fig 6: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of Lipid peroxidation. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.7 Effect on Percentage Sperm Count

Chlorambucil treatment caused a significant decrease in epididymis sperm count level across the chlorambucil treated group when compared with control. A significant difference was observed with the co-administration of quercetin at 10mg/kg in the testes spermatozoa.

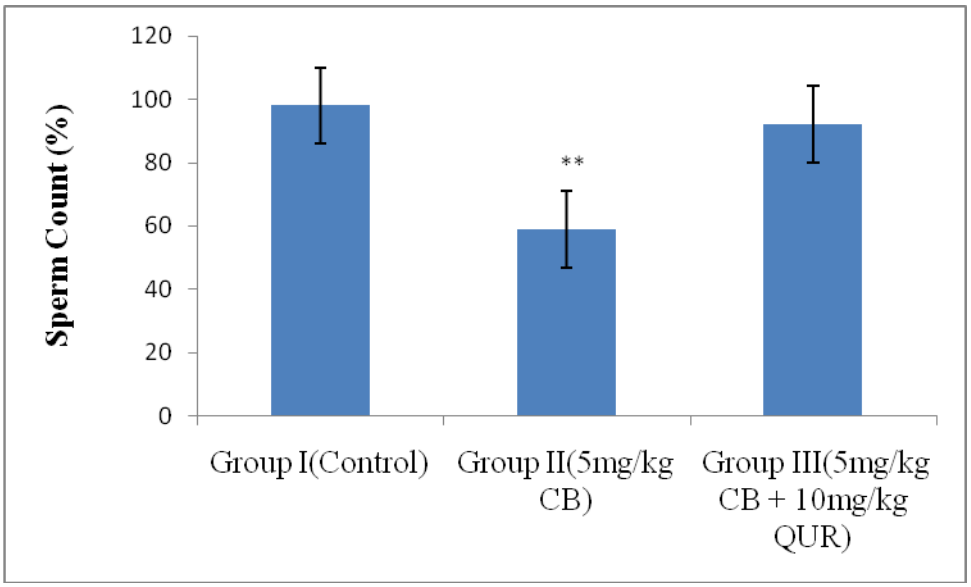

Fig 7: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on sperm count percentage. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.8 Effect on Sperm Motility

Chlorambucil treatment caused a decrease in epididymal sperm motility level across the chlorambucil treated group. However, an insignificant difference was observed with co-administration of quercetin in the epididymal spermatozoa when compared with the control.

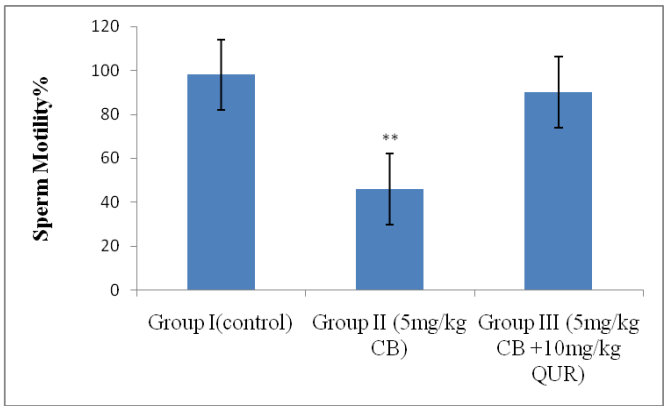

Fig 8: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on sperm motility percentage. The data are expressed as mean ± SD, (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.9 Effect on Sperm Morphology

The level of sperm morphology decreased significantly when treated with CB at a dose of 5mg/kg, co-administration with quercetin to the CB treated mice results in a significant increase.

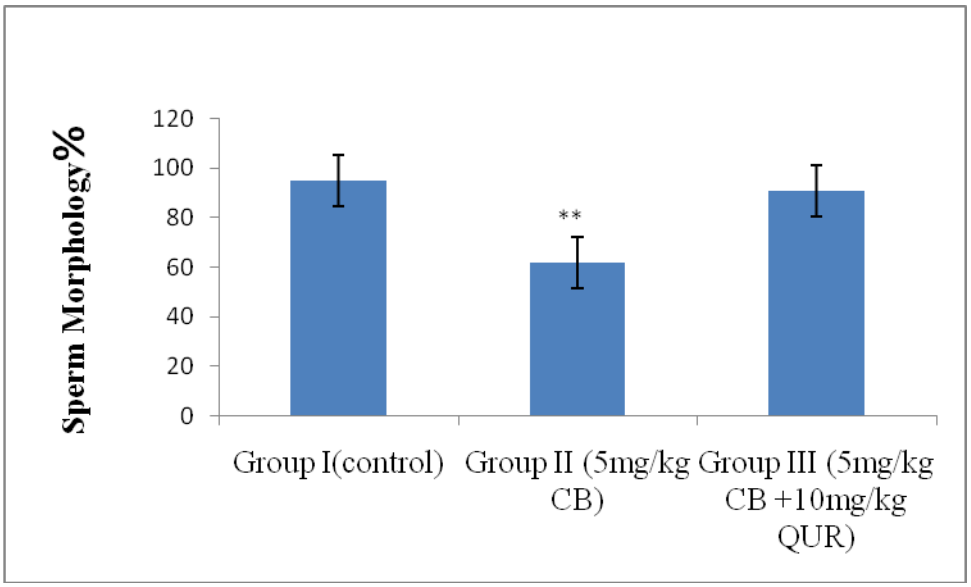

Fig 9: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on sperm morphology percentage. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.10 Effect on Testicular Testosterone

Testicular testosterone level decreased after treatment with CB (p<0.01), however co-administration of quercetin with the CB treated group results in an increased level of testosterone.

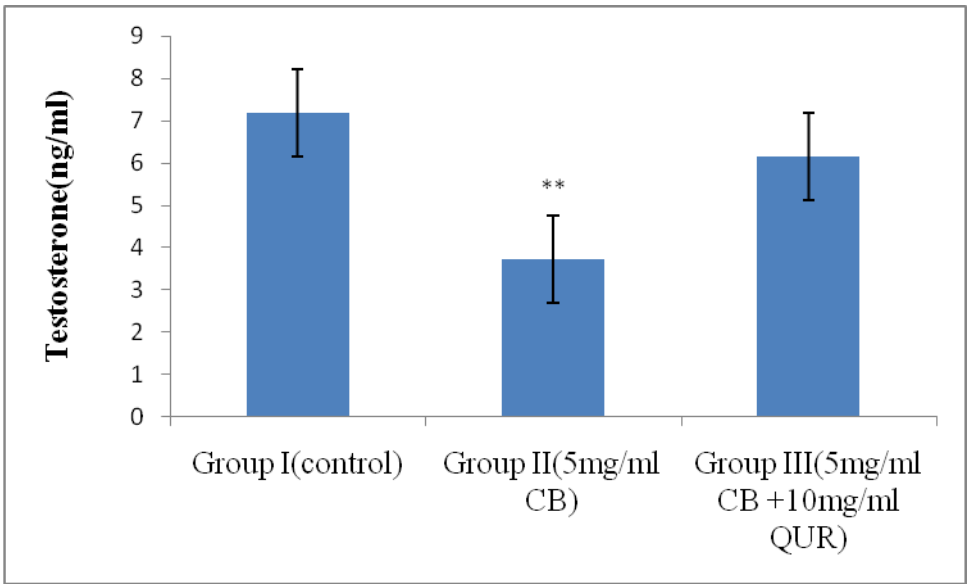

Fig 10: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of testicular testosterone. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.11 Effect on The Level of Luteinizing Hormone

The administration of CB results in a significant difference (p<0.01). However, co-administration of quercetin at 10mg/kg to the CB treated group results in an insignificant difference when compared to the vehicle treated group.

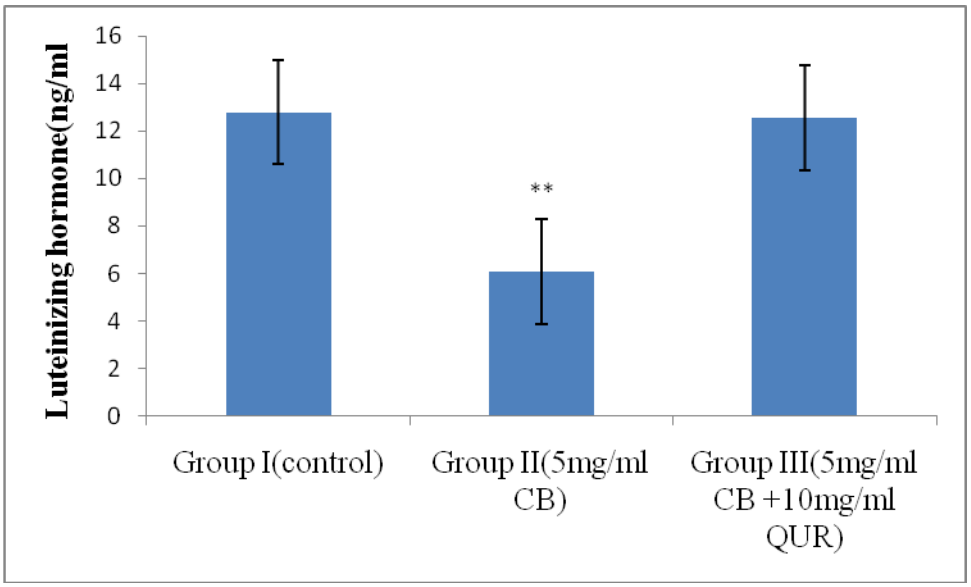

Fig 11: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of luteinizing hormone. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.12 Effect on the Level of Follicle Stimulating Hormone

The administration of CB results in a significant decrease (p<0.05) as compared with the control. However, administration of quercetin to the CB treated group results in a significant difference when compared to control.

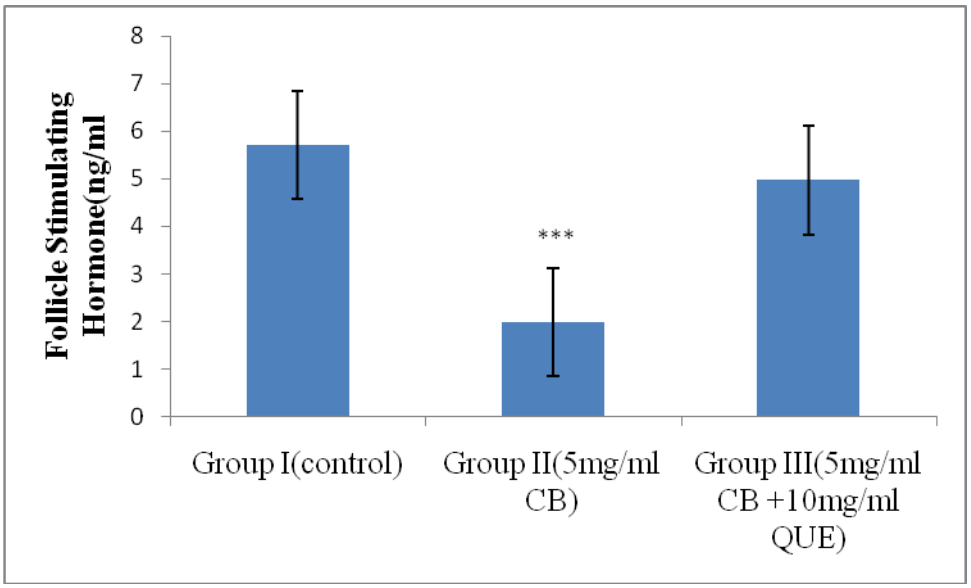

Fig 12: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of follicle stimulating hormone. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.13 Effect On Caspase-3

The administration of CB results in a decrease in caspase-3 levels, however, the co-administration quercetin at 10mg/kg results in an increase.

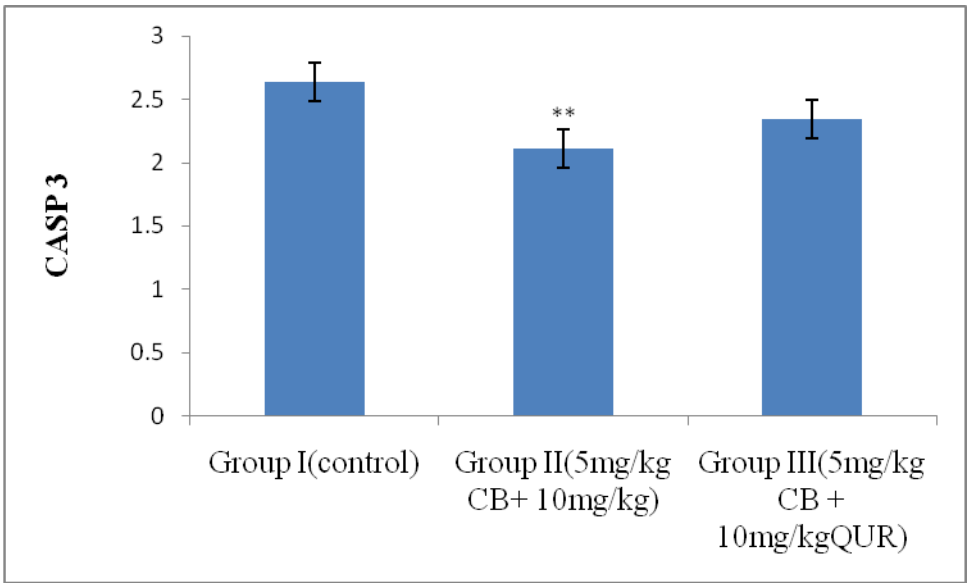

Fig 13: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of caspase 3. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

3.1.14 Effect on Caspase- 9 Level

The administration of CB results in a significant increase in caspase-9 levels. However, co-administration with quercetin results in a significant difference as compared with the control and a decrease when compared with the CB treated group.

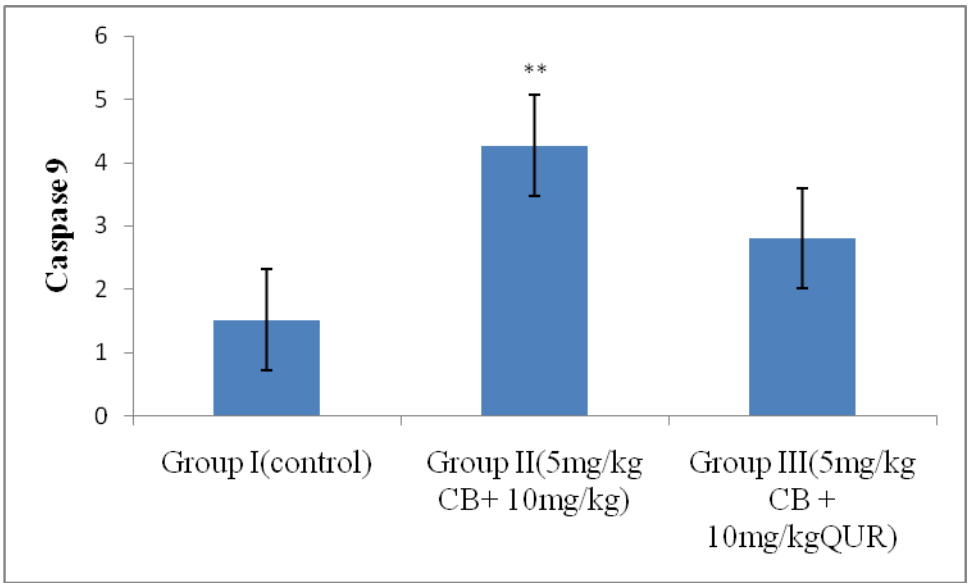

Fig 14: Bar chart representing result of ameliorative potential of quercetin in chlorambucil induced changes on the level of caspase 9. The data are expressed as mean ± SD (n=5) and significant changes from untreated control group (Group I) are reported *(p<0.01), ** (p<0.05).

IV. DISCUSSION

This study demonstrates the role of Quercetin, an important flavonoid against Chlorambucil-induced male reproductive toxicity. Quercetin possesses the ability of scavenging the free radicals and chelating the transition metal ions in order to prevent the oxidation of lipoproteins. Donation of proton of quercetin to radical converts it into a radical which is very low in energy because of resonance stabilization. The result showed that quercetin reversed the adverse effect of chlorambucil in the reproductive system of the male mice. The effect of quercetin on male reproductive toxicity in chlorambucil treated mice was assessed by investigating oxidative

stress marker enzymes, catalase (CAT), Superoxide dismutase (SOD), reduced glutathione (GSH), It was observed that GSH, CAT and SOD levels were decreased in chlorambucil-treated mice. However, the co-administration of quercetin with chlorambucil significantly raised the activates of these enzymes. Therefore, quercetin administration reversed the effect of CB on oxidative stress.

Lipid peroxidation (LPO) of group treated with 5mg/kg CB co-administered with 10mg/kg of quercetin is reduced when compared to the control; this is because the hypolepidemic effect of quercetin is due to the fact that quercetin reduces de novo synthesis of fatty acids and consequently cholesterol biosynthesis and lipoprotein formation (15). It was reported that quercetin rich supplementation reduces concentrations of total cholesterols and increases concentrations of HDL-cholesterol (16). Also, quercetin was found to restore the lipid profiles to normal.

The present result clearly indicates that co-administration of quercetin with CB induced spermatogenic function at a dose of 10mg/kg as indicated by a marked increase in the testosterone, luteinizing and follicle stimulating hormone when compared to the chlorambucil treated mice. Similarly, it was found that quercetin increased the testosterone levels and had beneficial effects on sperm parameters in streptozotocin-induced diabetic male rats (1). The positive effect of quercetin at 10mg/kg on sperm characteristics was observed on rat (17), it was reported that quercetin improved sperm motility, viability and sperm count.

Follicle Stimulating Hormone (FSH) was found to be reduced upon addition of Chlorambucil(CB) at a dose of 5mg/kg and also the testosterone level also dropped upon CB treatment, this might have resulted in infertility in the mice since testosterone essentially regulates sexual behavior, accessory sex organ functions, epididymal sperm maturation, spermatogenesis and toxic agents which inhibit testosterone biosynthesis or secretion have profound effects on any of these processes required for the timely deposition of viable spermatozoa into the female reproductive system. At a dose of 5mg/kg/bwt, there was an increase in lipid peroxidation (LPO) and depletion in reduced glutathione when compared to control which clearly demonstrates failure of the anti-oxidant defense system of the mice. Treatment with CB at 5mg/kg/bwt results in decrease in catalase level which indicates accumulation of hydrogen peroxide and an increase in caspase 9 which is an initiator thereby initiates apoptosis, which is a programmed cell death.

Decrease in sperm count observed in CB treated mice indicates cyto-toxicity of CB in the testis. The early germ cells in the spermatogenic cycle were affected by CB and became abnormal thereby increasing the number of abnormal spermatozoa. Significant decrease in sperm motility indicated adverse effects of CB on spermatozoa function probably through the structure and function of testis. Although the damaged germinal epithelium in the testis appears to be the main reason for impaired sperm quality. Increased lipid peroxidation in the testis might have contributed to abnormality of spermatozoa which may result in infertility. Upon co-administration of quercetin, a significant increase was found in the sperm count, motility and morphology and also in the antioxidant properties of their defense system. This shows that quercetin reversed the toxicity of chlorambucil in the reproductive system of the treated male mice. The present result shows that the co-administration of quercetin results in an increased level of follicle stimulating hormone which induces Sertoli cells which plays a critical role in spermatogenesis by providing physical support, nutrients, and hormonal signals necessary for successful spermatogenesis, thereby improving fertility.

In addition, Robaszkiewicz (18) showed that quercetin decreased production of reactive oxygen species in testicular germ cells, and it was also investigated that quercetin has no deleterious effects on oxidative damage (19). The consequences of the antioxidant efficacy of quercetin that were confirmed by histological examinations showed that administration of quercetin caused highly regular seminiferous tubules with normal interstitial tissue with high number of spermatozoa in the lumen of the seminiferous tubule at low dose of quercetin (10mg/kg).

The bioactivity of quercetin is attributed to its specific molecular structure, in which the oxygen active group, phenolic hydroxyls and 2,3-unsaturated double bond give quercetin strong anti-oxidant ability not only from accepting oxygen free radicals but also by forming metal chelation compounds (20). Furthermore, quercetin was an effective antioxidant because of its ability to interact with and penetrate lipid bilayers. Its higher diffusion rate into the membranes allows it to scavenge oxyradicals at several sites throughout the lipid bilayer (21).

4.1 Conclusion

Quercetin is seen to confer its anti-oxidative properties on chlorambucil -induced male toxicity and may be a promising antioxidant (flavonoid) in the prevention of toxicity and adverse effect caused by Chlorambucil in the male reproductive system of mice. These results indicate that antioxidants from foods such as onion, apple could improve quality of sperm health.

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

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