International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 6, Issue 5 -2019

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.05.001

Quercetin controlled Cytarabine - induced testicular damage in Swiss albino mice

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Abstract

Treatments of antineoplastic drugs have been implicated in male infertility due to the cytotoxic effects of these drugs on spermatogonial stem cells which divides quickly. Among such drugs is cytarabine also known as Cytosine Arabinoside, an effective chemotherapeutic agent in the treatment of acute myeloid leukemia. This study clearly demonstrates the role of quercetin, a flavonol with strong potential to scavenge the free radicals and ameliorate the cytotoxic effect of cytarabine on male spermatogenic dysfunction. Swiss albino male mice (*Mus musculus*) are divided into three groups; the control group I received a normal saline, group II, 5mg/kg/bwt of cytarabine and group III, 5mg/kg/bwt of cytarabine and 10mg/kg/bwt of quercetin for 7 days. Results showed significant ($p<0.05$) decrease of SOD, GSH, CAT and GPx levels in the testes of mice in group II treated with cytarabine alone but elevated levels of these enzymes were seen in group III that received quercetin as co-treatment. Simultaneous increase level of lipid peroxidation in the group II confirmed generation of oxidative stress in the testicular tissue with cytarabine alone; however this adverse effect was reversed in the group III that received quercetin. Depletion of FSH, LH and testosterone level seen in group II was attenuated by quercetin in group III. Spermatogenic dysfunction was confirmed by the raised level of sperm morphology with decreased sperm count and motility. Evaluation of ultrastructural changes of leydig cell in the testes of mice treated with cytarabine showed condensed mitochondria, proliferated endoplasmic reticulum and increased number of lipid droplet but with addition of quercetin, the effect was reversed. This research work reveals the potential of quercetin to control cytarabine-induced testicular damage and may be a promising drug in the elimination and prevention of cytarabine toxicity if used along with the treatment

Keywords: Cytarabine; Quercetin; Testes; Oxidative stress; Leydig cells

1.0. Introduction

Cytarabine, also known as cytosine arabinoside is a chemotherapy medication used to treat acute myeloid leukemia, acute lymphocytic leukemia, chronic myelogenous leukemia and non-Hodgkins lymphoma. It is a synthetic pyrimidine nucleoside, which is converted intracellularly to the nucleotide, cytarabine triphosphate (8). Quercetin is a plant pigment abundantly occurs in many ethnic plants, especially onion and tea; therefore, it has importance in terms of ethno pharmacology such as its use as antioxidant, anticancer and neuroprotective (6). It has been reported that the combination of quercetin and cytarabine synergistically inhibits leukemic cell growth (28). It has also been demonstrated that quercetin (3,3',4',5,7-pentahydroxyflavone) inhibits the growth of several cancer cell lines and that the

anti-proliferative activity of this substance is probably mediated through a binding interaction with type II estrogen binding sites (type II EBS) (30). The effect of quercetin and cytosine arabinoside (Ara-C) in combination, was tested in these study, Quercetin significantly synergized the inhibitory activity of Ara-C on spermatogonial cell. The purpose of this study is to evaluate the ameliorative effects of quercetin against cytarabine- induced testicular damage in male mice.

2.0. Materials and Methods

2.1 Laboratory Animals

The study is carried out on Swiss Albino male mice (*Mus musculus*) obtained from laboratory animal division of college of medicine, Ibadan. Fifteen mice weighing 25g, were housed in conventional conditions at animal house laboratory in the college with free access to food and water.

2.2. Experimental Design

Fifteen male mice are divided into three groups (n=5 each), control group received normal saline, group II received subcutaneous Cytarabine at a dose of 5mg/kg and group III received a dose of 5mg/kg of cytarabine and 10mg/kg of quercetin. Group II and group III were sacrificed after 7 days of induction of the drug which was given intraperitoneally.

2.3. Testicular Testosterone, Follicle Stimulating and Luteinizing hormone concentrations

The testicular testosterone and luteinizing hormone levels in three mice from each group were measured (22). 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.

2.4. Biochemical estimations of testes tissue

Testis from each mouse was stored at -20^{o}C for different biochemical assays 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. Protein quantity was estimated according to Lowry's method (15).

2.4.1. 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^{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(19).

2.4.2. 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), phenazinemethosulfate (186 µM), nitrobluetetrazolium (300 µM) and NADH (780 µM) 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 10 min before the collection of butanol layer. The intensity of chromogen in butanol was measured at 520 nm (16).

2.4.3. Catalase (CAT) activity

Catalase activity was estimated by measuring the decomposition of hydrogen peroxide (H_2O_2). Assay mixture consisting of 0.01M phosphate buffer (pH 7), 0.2 M hydrogen peroxide and tissue homogenate was incubated at 37^{o}C 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 (1).

2.4.4. 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 (29).

2.5. Sperm Parameters

Caudal 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^{o}C. Sperm motility was estimated and expressed as percentage incidence (4). 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 (13). The sperm morphology was also evaluated (Wyrobek, Bruce 1975). 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. (35).

2.6. Ultrastructure of Leydig

The ultrastructural studies were done according to methods described in (24). Small pieces of testes were cut and fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium phosphate buffer (pH - 7.3) for 12 h at 4^{o} C. After wash in buffer, the samples were fixed in 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4^{o} C. The samples were dehydrated in an ascending grade of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thick Sections (1 µm) were cut with an ultramicrotome, mounted on to glass slides, stained with aqueous toluidine blue and observed under a light microscope for gross observation of the area and quality of the tissue fixation. For electron microscopic examination, thin sections of grey-silver colour interference (70-80 nm) were cut and mounted onto 300 mesh- copper grids. Sections were stained with alcoholic uranyl acetate and alkaline lead citrate, washed gently with distilled water and observed under a Morgagni 268D transmission electron microscope (FEI Company, Netherlands) at an operating voltage 80 kV. Images were digitally acquired by using a CCD camera (Mega view III, FEI Company) attached to the microscope.

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

3.0. Results

3.1. Effects on protein (mg/ml)

Protein level was significantly reduced (*p<0.05)* following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

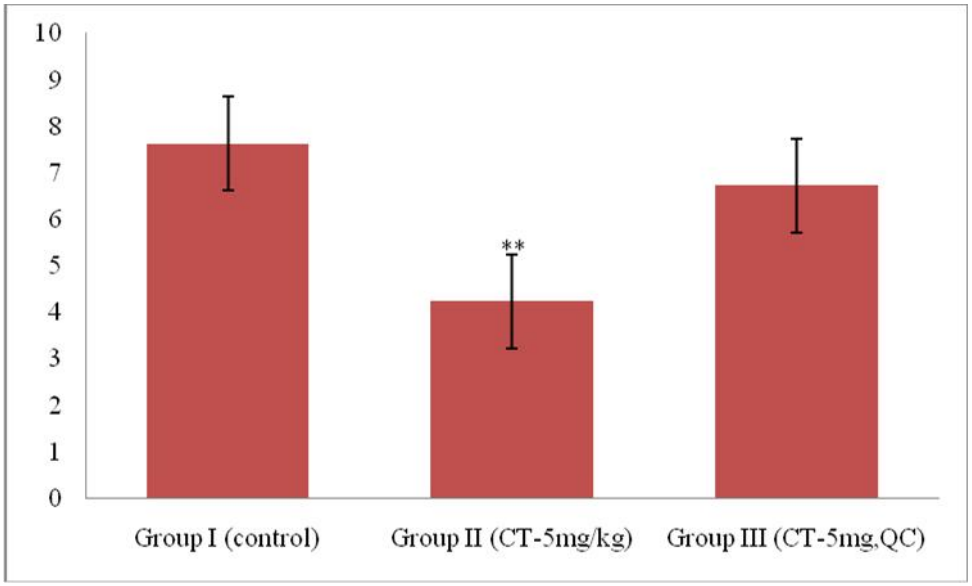

Fig. 1: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine- induced changes in protein level. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.2. Catalase activity (µMol/min/mg protein)

Catalase activity was significantly reduced (*p<0.05)* following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

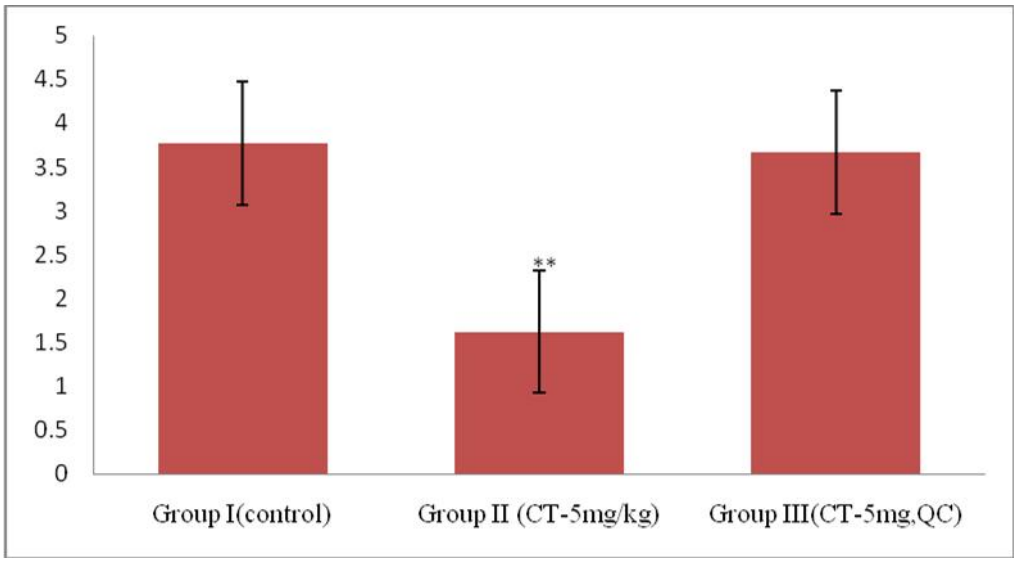

Fig.2: Showing bar chart representing result of ameliorative effect of quercetin in cytarabine induced changes in catalase activity. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.3. Superoxide dismutase (U/ml/min)

Superoxide dismutase activity was significantly reduced (*p<0.05)* following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

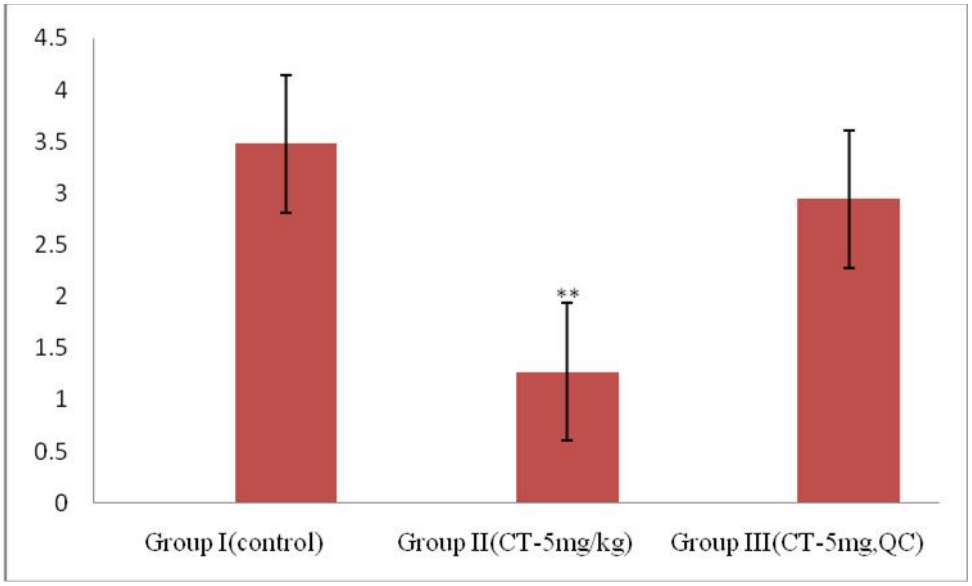

Fig. 3: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes in superoxide dismutase activity. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.4. Reduced glutathione level (GSH) (µM/mg protein)

GSH level was significantly reduced (*p<0.01*) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however the effect was reversed in group III (CT+QC) when compared to group I.

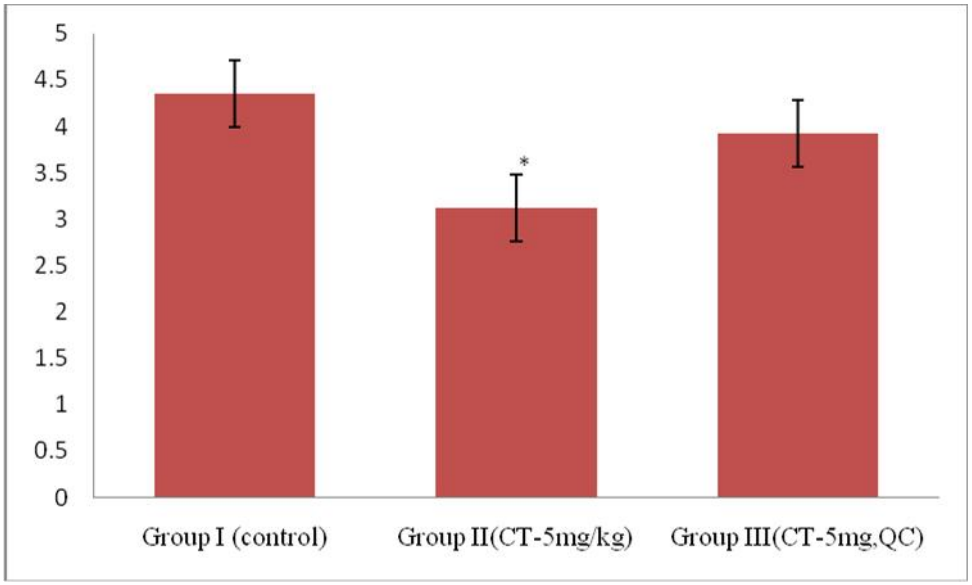

Fig. 4: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes in GSH level. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.5. Gluthathione peroxidase activity (GPX) (Unit/mg protein)

GPX activity was significantly reduced (*p<0.05*) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

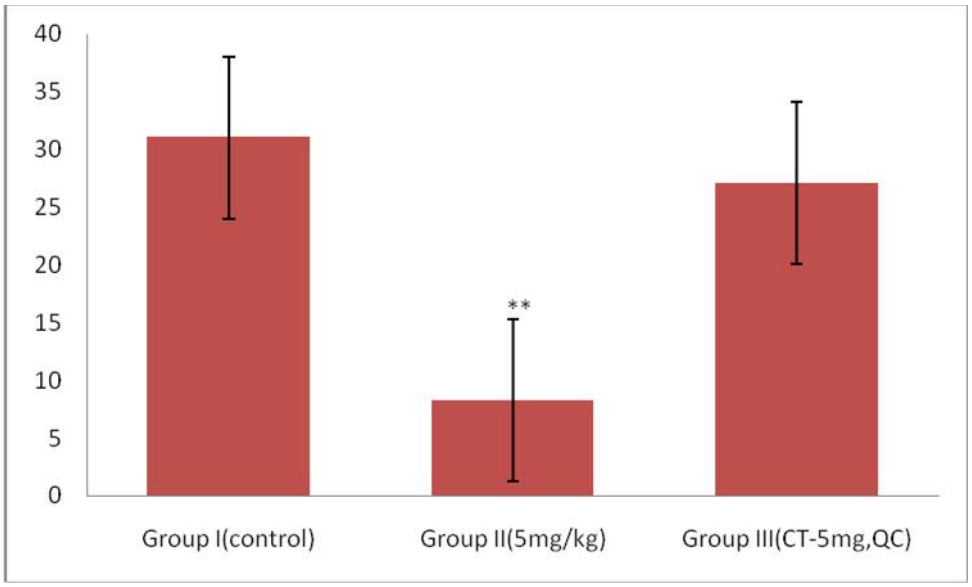

Fig. 5: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes in GPX activity. The data(n=5) are expressed as mean ± SD and significant changes from untreated control (Group 1) are reported. Note: * (P<0.01), ** (P<0.05).

3.6. lipid peroxidation (LPO) (µMol/min/mg protein)

LPO level was significantly increased (*p<0.05*) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

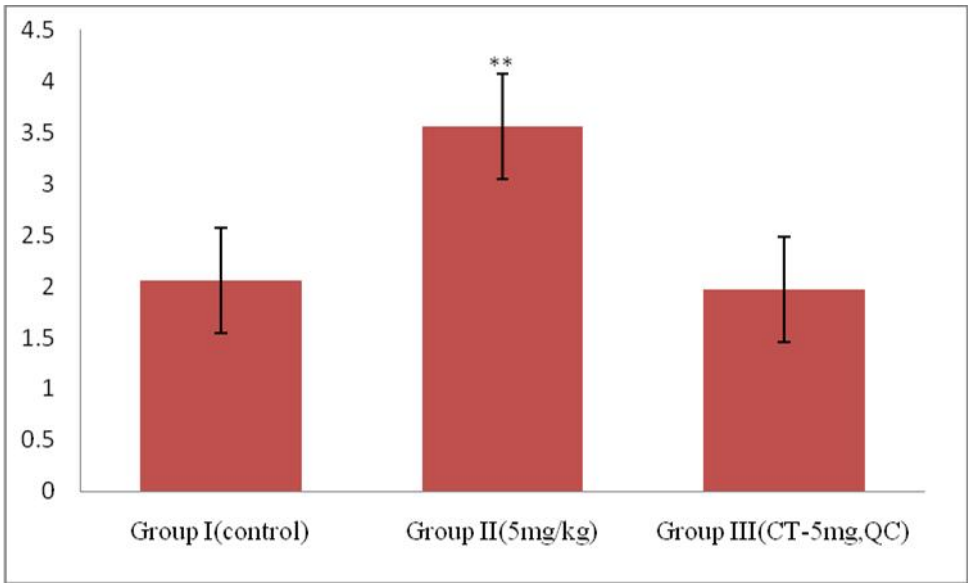

Fig. 6: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes in LPO level. The data (n=5) are expressed as mean ±SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.7. Sperm Motility (%)

The epididymal sperm motility was significantly reduced (*p<0.05*) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

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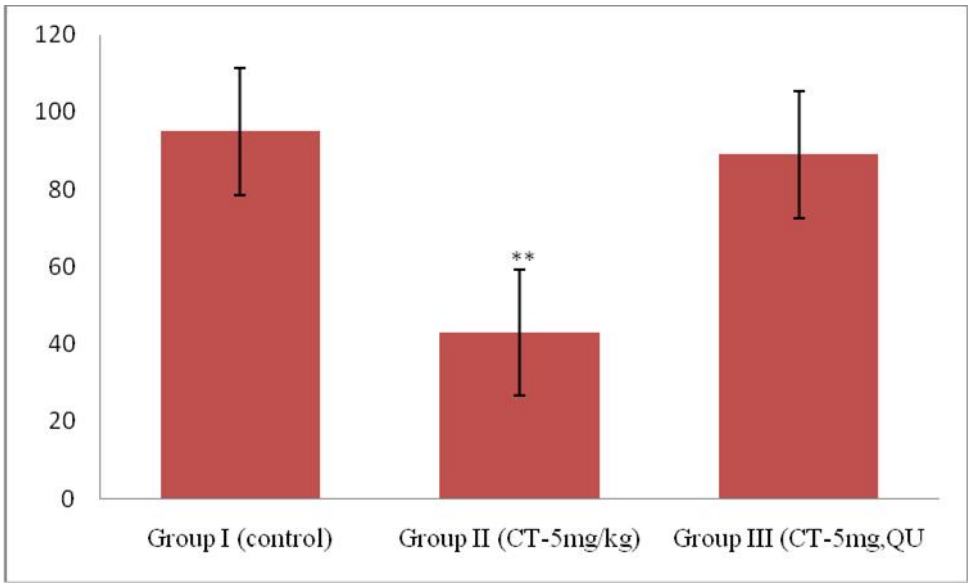

Fig. 7: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on sperm motility. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.8. Sperm Count (%)

The epididymal sperm count was significantly reduced ($p<0.05$) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

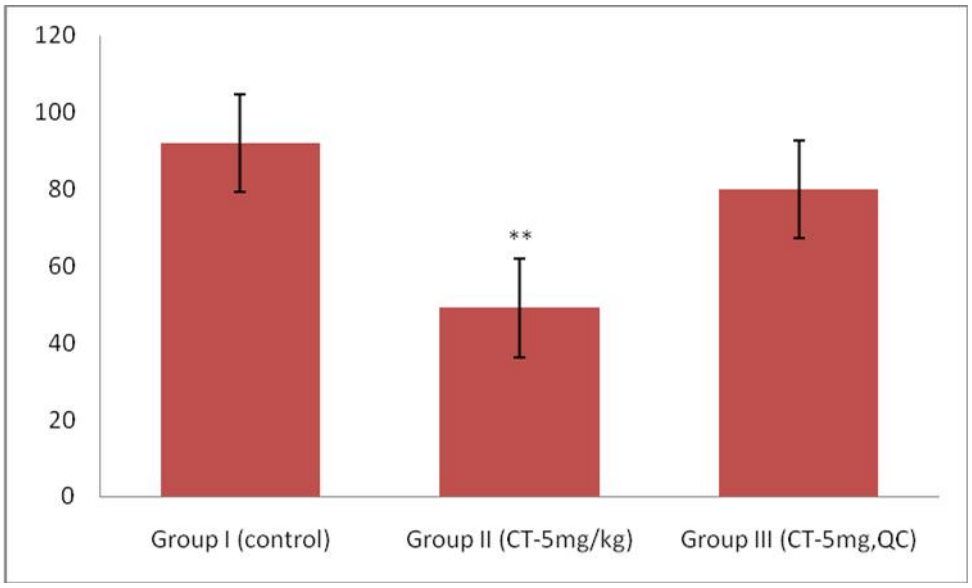

Fig. 8: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on the sperm count. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.9. Sperm Morphology (%)

The sperm morphology level was significantly reduced ($p<0.05$) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

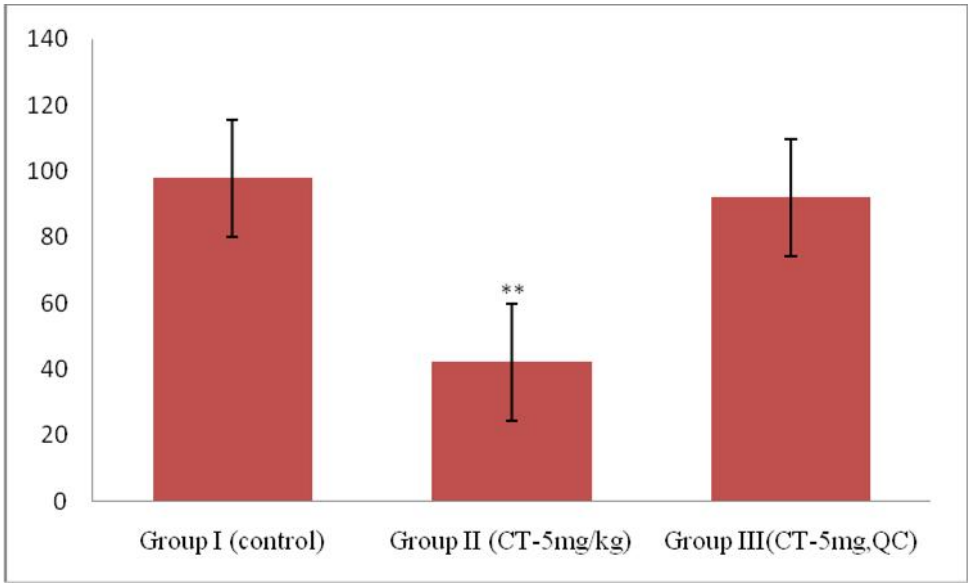

Fig. 9: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on the sperm morphology. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.10. Testosterone Concentration (ng/ml)

Testosterone concentration was significantly reduced ($p<0.05$) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

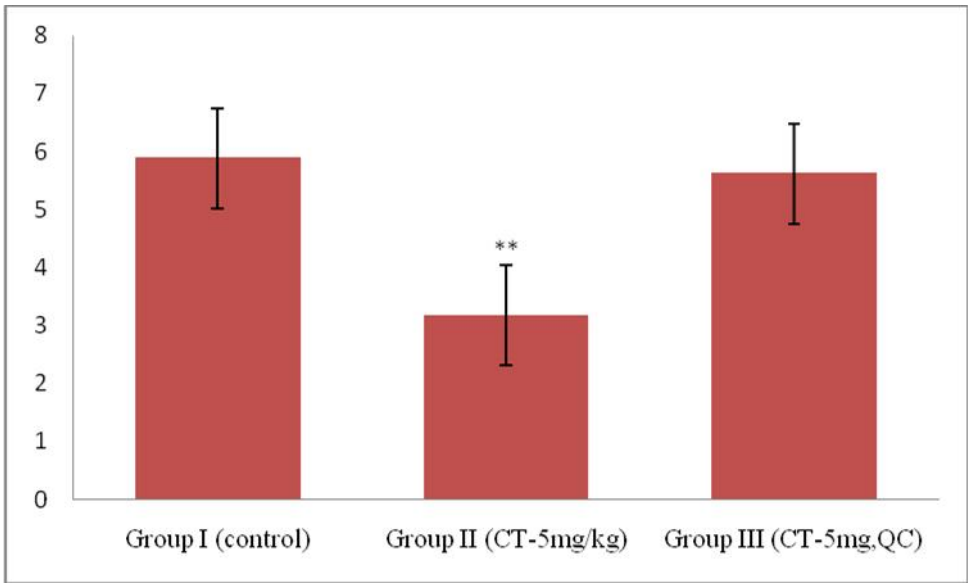

Fig. 10: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on testosterone concentration. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.11. Luteinizing Hormone (LH) (ng/ml)

Luteinizing hormone concentration was significantly reduced ($p<0.05$) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

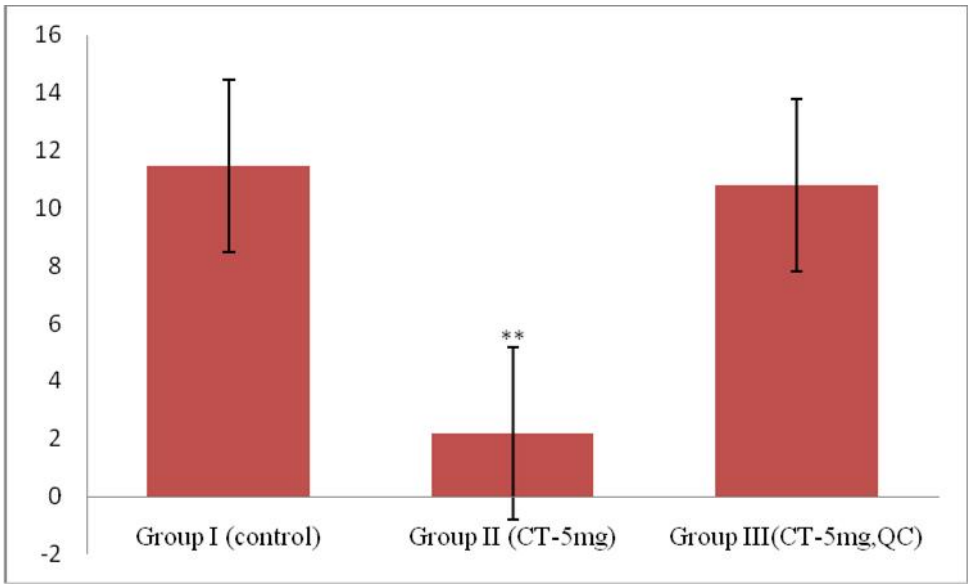

Fig. 11: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on luteinizing hormone concentration. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported. Note: * (P<0.01), ** (P<0.05).

3.12. Folicle Stimulating Hormone Concentration (FSH) (ng/ml)

Serum FSH concentration was significantly reduced ($p<0.05$) following treatment with cytarabine at a dose of 5mg per kg when compared with the vehicle treated control, however, the effect was reversed in group III (CT+QC) when compared to group I.

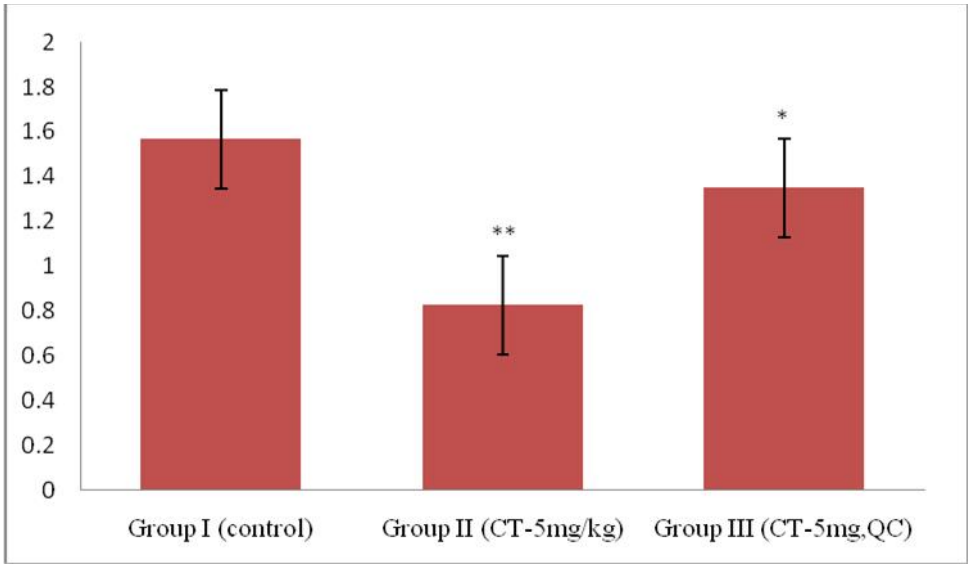

Fig. 12: Showing bar chart representing result of ameliorative effect of Quercetin in cytarabine induced changes on follicle stimulating hormone concentration. The data (n=5) are expressed as mean ± SD and significant changes from untreated control (Group I) are reported * (P<0.01), ** (P<0.05).

3.13. Effects of Cytarabine on ultrastructure Leydig cells

Leydig cells showed few lipid droplets in the small volume of cytoplasm in the control. However, the number of lipid droplets in Leydig cells in Cytarabine treated groups increased with 5mg/kg/bwt doses of Cytarabine (Fig 13 A&B). Also cytoplasm of those cells became irregular, hypertrophied and partly disorganized. Their nuclei appeared shrunken. The mitochondria were dark and condensed (A&B). Some degree of proliferation of smooth endoplasmic reticulum was also observed however the deleterious effect was reversed with quercetin as seen in fig 13 D.

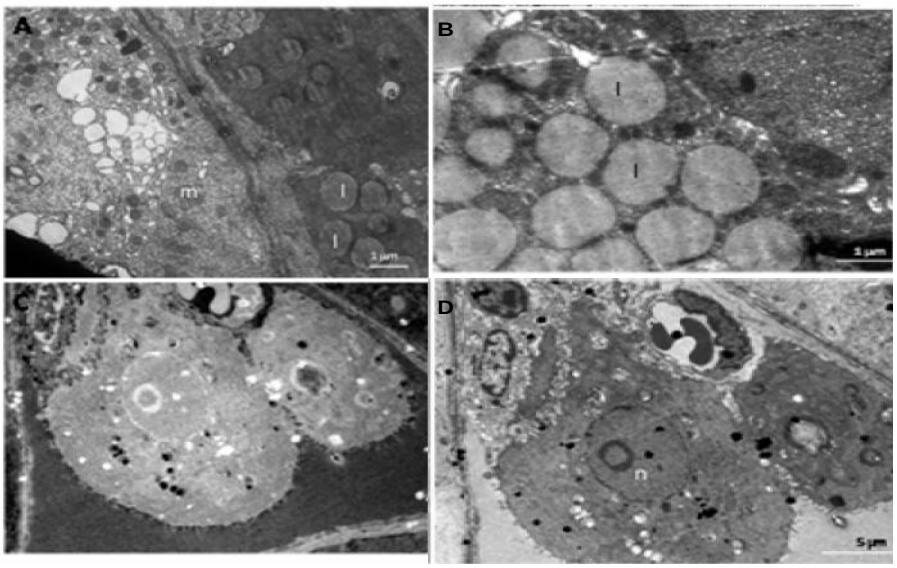

Fig. 13. Electron micrographs of testes sections focusing on Leydig cells in Cytrabine treated mice (A&B), Control (C) and Cytarabine +Quercetin mice (D). Note: Mitochondria (m) Lysosomes (l) of different shape and size are more indicating damage/toxicity at higher dosage. However, reversed is seen in group III that received quercetin as shown in figure (D).

4.0. Discussion

In the present study, protective effects of quercetin were investigated against the toxic effects induced by Cytarabine (CT) in reproductive tissues of male mice. Treatment with 5mg/kg/bwt CT indicates decrease in the antioxidant enzymes activities of SOD, GSH, GPx and CAT on the other hand there was an increase in level of lipid peroxidation lead to increased oxidative stress in the testicular tissues observed in groups treated with CT. Besides Cytarabine therapeutic effect, it has been reported toxic to the reproductive tissues in male (18). CT exerts its hazardous effect by increasing the level of Reactive Oxygen Species (ROS) thus reducing antioxidant enzymes status leading to alteration in testicular machinery. Co-treatment with quercetin protected the testicular tissues against detrimental effects of Cytarabine and reduced the oxidative stress in the tissue. Quercetin has been reported as a flavonol with efficient free radical scavenging potential. It is also known as an important anticancer and neuroprotective compound (6; 7; 26).

Decrease of SOD activity and simultaneous increase in LPO in mice treated with cytarabine was in agreement with a previous study in which it was reported that anticancer agent reduces antioxidant enzymes (36). Reduced levels of SOD might lead to increase in the generation of ROS and reactive nitrogen species (RNS), which cause oxidative damage by increasing lipid peroxidation in testes leading to a disruption in spermatogenesis and steroidogensis. Co-treatment with quercetin resulted to a significant increased in the activity of SOD which indicates that quercetin reversed cytarabine induced SOD reduction. As presented above, CAT activity was also reduced in CT-treated group as compared to the control group, This is in accordance with previous literature (27). However, CAT levels were significantly higher in the cytarabine plus quercetin treated group as compared to the cytarabine alone treated group. This suggests a protective role of quercetin against the cytarabine induced reduction in CAT within the testis. The cellular role of catalase is to protect the cell from oxidative damage by reactive oxygen species such as hydrogen peroxide of various metabolites and toxins including formaldehyde, formic acid, phenols, acetaldehyde and alcohols. Hydrogen peroxide is a harmful byproduct of many normal metabolic processes; to prevent damage to cells and tissues, it must be quickly converted into other less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (10).

Cytarabine has been reported to damage the male reproductive organs, leading to reduced spermatogenesis, testicular testosterone production. This is mediated by disturbing the testicular antioxidant defense system (25) by increasing ROS that leads to impaired steriodogenesis. Reduced androgen levels due to depleted testis antioxidant enzymes results in reduced spermatogenesis. In this study, reduction in the tissue antioxidant enzymes as well as testosterone concentration in the cytarabine treated mice was observed. It was speculated that depleted antioxidant enzyme levels may have reduced the testicular testosterone concentration by some unknown mechanism that needs to be determined. Defense are provided by antioxidant enzymes (SOD, GPx and CAT) which prevent biological molecules (lipids, proteins, DNA) from damage and decrease lipid peroxidation by inhibiting ROS formation. Hydrogen peroxide (HO), nitric oxide (NO), superoxide anion and hydroxyl radical (OH) are central reactive oxygen and nitrogen species (11; 33). SOD counteracts the toxic effects of the superoxide anion. Levels of antioxidant enzymes are important because dismutation of the superoxide anion to form HO is catalyzed by SOD while HO is converted to water molecules by CAT and glutathione peroxidase (GPx) while reduced GSH is used as an electron donor in such reactions (12; 31). Similarly, the level of GSH is retained by the thiol containing non-protein compound GSR. GSR regenerates GSH (reduced form) from GSSG (oxidized form) for the constant activity of GPx (32). NADPH oxidases are specialized enzymes that can generate superoxide anion which can be eliminated by CAT, GPx, and SOD, decreasing LPO to protect spermatozoa from oxidative stress (2; 27). Quercetin co-administrated with cytarabine resulted in a significant increase in CAT, GPx and SOD. These findings are in accord with previous literature reporting that quercetin increases antioxidant enzyme levels (GPx, SOD, CAT, GSH) and reduces lipid peroxidation (5). In this present study, quercetin significantly reduces oxidative stress in the testis mice. Male mice exposed to cytarabine (5 mg/kg/bwt) had a dramatic reduction in intratesticular testosterone, an effect that was largely reversed by treatment with Quercetin. Cytarabine treatments also decrease testicular luteinizing hormone (LH) and follicle stimulating hormone levels. The cytarabine-induced changes in testosterone were associated with a decreased number of LH receptors on Leydig cells and with inhibited P450 side-chain cleavage activity *(17)*.

A gonadotropin-releasing hormone (GnRH) challenge test led to prompt multifold increases in testes LH as well as to increases in intratesticular testosterone levels, indicating that low levels of LH could not still stimulate steroidogenesis. These cytarabine -induced changes in the hormonal regulation of spermatogenesis were interpreted as a primary effect on Leydig cells, with relative sparing of the hypothalamus and pituitary. Increase in number of lipid droplets in the Leydig cell cytoplasm in Cytarabine administered mice is indicative of cholesterol accumulation for testosterone synthesis. Hypertrophied Leydig cells with shrunken nuclei, disorganized cytoplasm, condensed mitochondria and proliferated smooth endoplasmic reticulum indicated impairment of functions like steroidogenesis resulting in reduced utilization of cholesterol. The use of free cholesterol in the process of steroidogenesis draws off substrate, thereby reducing the number of lipid droplets, whereas the blocking of this process would build up lipid droplets as cholesteryl ester storage. A declined secretory activity correlated with an increased number of lipid droplets, reduced ER and giant whorl-like smooth endoplasmic reticulum has been demonstrated in mouse Leydig cells following streptozotocin treatment (37). Melatonin has also been shown to alter the ultrastructure of mouse Leydig cells and decrease nuclear volume (21).

The results of this study revealed cytarabine administration significantly decreased sperm count, sperm motility and sperm morphology of the mice due to damage caused by free radicals from CT–treated .The changes observed in the above agree with the previous reports, which demonstrated that anticancer drugs (for example etoposide) impairs testicular function (9) . lipid peroxide is an essential process in many pathological events and is caused by oxidative stress , lipid peroxide is considered as one of the basic mechanisms of cell damage caused by free radicals after being reacted with lipid causing oxidation , Which results in the release of products such as malondialdehyde (MDA), Hydrogen Peroxide (H_2O_2) and Hydroxyl Radicals (20). These intermediates may be responsible for destruction of the structure of sperm plasma membranes and makes it more fragile as a result of the breakdown of unsaturated fatty acids and are very sensitive to the ROS , and it is associated with sperm cluster and its non-motility, decrease in the percentage of live sperms and increased percentage of dead sperms , morphologically abnormal sperms and impairment of spermatogenesis , which leads to infertility (14) . ROS causes oxidation of group (-SH) in proteins and DNA, this alter the sperm production and function and increase its susceptibility to attack by means of attachments (38), study by Said *et.al.*, 2005 suggested that abnormal sperm morphology combined with elevated ROS production may serve as a useful indicator of potential damage to sperm DNA . Many of cytarabine dose-limiting toxicities occur due to its generation of toxic oxygen species, resulting in oxidative stress, which negatively affects the cell division leading to cell death (18; 3). Co-treatment of cytarabine with quercetin leads to increase in sperm count, motility and morphology. In summary, cytarabine exposure in male mice is capable of producing long-lasting azoospermia and testicular atrophy. Animal studies have provided evidence for cytarabine-induced dysfunction of leydig cell and germ cells as a result of generation of oxidative stress. Therefore, it is likely that cytarabine targets multiple cell types and molecular pathways while producing testicular injury. However, this study has shown that combination of quercetin, a flavoid with cytarabine treatment can reverse the deleterious effect of the drug.

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

Acknowledgments

Authors acknowledge all research P4 members and staff of Chemical pathology Department College of Medicine Ibadan for their contributions to the success of this study.

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DOI:10.22192/ijarbs.2019.06.05.001

How to cite this article:
Olajumoke Omolara Ojo, Omojola Omolola O and Ogunbiyi Babafemi Tosin. (2019). Quercetin controlled Cytarabine- induced testicular damage in Swiss albino mice. Int. J. Adv. Res. Biol. Sci. 6(5): 1-13.
DOI: http://dx.doi.org/10.22192/ijarbs.2019.06.05.001