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Research Article

Epididymal Cytotoxicity and Protective Effect of Quercetin Following Epirubicin Exposure in Swiss Albino Mice

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Abstract

Epirubicin is an anthracycline drug used in the treatment of different cancers and in combination with other medications. Toxicity of this drug have been reported but little is known about its effect on epididymis thus, this study aimed at evaluating the possible adverse effect of epirubicin on the epididymis tissue using mouse model. Potential ameliorating effect of quercetin from onion extract was also investigated. In this study, the mice used were divided into six groups. Mice in group I received vehicle and served as control; group II-5 mg/kg/bwt/day, III-10 mg/kg/bwt & group IV- 20 mg/kg/bwt. Mice in group V received 10 mg/kg/bwt Quercetin (standard) + 20 mg/kg/bwt while group VI were co-treated with 10 mg/kg/bwt Onion extracts + 20 mg/kg/bwt for fourteen days. Activities of some vital biochemical biomarker enzymes, sperm parameters and epididymal histology were evaluated. In this study, significant ($p<0.05$) depletion of sperm count and motility was measured, raised ROS and MDA with simultaneous depletions of the antioxidant enzymes was evaluated. Disruption of epithelium and vacuolization were observed in the cauda epididymis. However, appreciable recovery was seen in both groups V and VI that were co-treated with either quercetin or onion extract. Conclusively, this study reports generation of oxidative stress in epididymis leading to spermotozoa cytotoxicity in epirubicin treated mice. The studies also revealed that quercetin from onion extract can help to reduce the deleterious effect of epirubicin treatment.

Keywords: *Epididymal Cytotoxicity; Quercetin; Epirubicin; Swiss Albino Mice*

Introduction

Chemotherapy and surgical techniques have been reported to enable cure for high percentage of cancer cases diagnosed. However, numerous studies have evaluated the adverse effect of these anticancer drugs on sperm parameters in which majority of them have been found to have deleterious effect of male reproductive system. Epirubicin an anthracycline agent, recently used as chemotherapy which act by intercalating DNA strands and inhibiting topoisomerase II, thereby inhibiting DNA replication and eventually, causing an interference with the synthesis of both RNA and protein [1] has been reported to generates free radicals that are detrimental to both the genetic material and the cells [2]. Though epirubicin usage is preferred to doxorubicin because it has been reported to show lesser toxicity compared to doxorubicin, however various side effects have been associated with epirubicin include cardiotoxicity [4,5] and genetic damage in animals and humans [6]. Therefore, it is necessary to investigate the safety of the drug.

Lately, regular intake of onions has been reported to lessen the danger of malignancy, DNA damage, vascular and heart diseases [7-9]. Onions are excellent source of antioxidants scavenging activity of phenolics [10], the report showed over 25 different varieties of flavonoid antioxidants [11] one of which is quercetin found in both fruits and vegetables. High attention is drawn to quercetin because of its health benefit especially, its antioxidant potential [16].

Male infertility remains a major challenge of anti-cancer drug usage, there is no literature available on the mitigating effect of quercetin and onion extract on epirubicin induced cytotoxicity in germ cells. Hence, the essence of this study is to ascertain the attenuation capacities of quercetin and onion extract on epirubicin-induced cytotoxicity in male germ cells. This study would be important in providing probable solutions in combating infertility as a long term side effect following treatment with epirubicin.

Method

Extraction and soxhlet polar fractionation of onion extract

Onions (*Allium cepa L.*,) purchased from a local market at Ado-Ekiti, Nigeria were washed with distilled water and allowed to air dry for 24 hrs. 200g of the powdered sample was extracted with 6L of 50% aqueous ethanol at 72°C, cooled to room temperature (25 ± 2°C) and filtered. Extract was then concentrated into paste using rotary evaporator, absorbed and air-dried. For soxhlet polar fractionation, the dried diatomaceous earth samples were sequentially extracted with ethyl acetate using a soxhlet extractor for 12 hrs [17].

Animals, grouping and treatments

Experimental animals for this study were approved by the University Animal Ethics Committee. The male Swiss albino mice (25g) purchased from the animal facility of the Ekiti- State University Ado-Ekiti were kept at room temperature (25 ± 2°C), with 50 ± 10% humidity and a cycle of 12 hrs light and 12 hrs dark.

Experimental procedures

Swiss albino mice were divided into six groups I-VI (n = 5) and treated with epirubicin and quercetin as shown in the table below. Experimental mice were autopsied after fourteen days of treatment; epididymis were removed and washed with phosphate-buffered saline (PBS) twice.

Animal groups	Treatment (mg/kg /bwt/day)
Group I	Control- Vehicle
Group II	5 mg/kg/day, EP
Group III	10 mg/kg/day, EP
Group IV	20 mg/kg/day, EP
Group V	QUC + EP (10 +20 mg/kg/bwt)
Group VI	Onion extract + EP 10 +20 (mg/kg/bwt)

Table 1: *Administration of quercetin, onion extract and epirubicin.*

Measurement of reactive oxygen species (ROS) level

Briefly, 50 µl of epididymis homogenate and 1400 µl sodium acetate buffer were transferred to a cuvette. After then, 1000 ul of reagent mixture (N, N-diethyl para phenylenediamine 6 mg/ml with 4.37 µM of ferrous sulfate dissolved in 0.1M sodium acetate buffer pH- 4.8)

was added at 37°C for 5 minutes. 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).

Measurement of malondialdehyde level

Epididymal tissue was homogenized in 0.05M phosphate buffer at pH = 7.4 and the concentration of 10% (w/v). The obtained solutions were centrifuged in 1000g and supernatants were used for the evaluation. Levels of lipid per oxidation were measured using cheeseman and Esterbauer method. For this purpose, 300 ml of trichloric acid 10% was added to 150 µl supernatant of centrifuged sample and then centrifuged for 10 minutes at 4°C and 1000g. 300 µl of supernatant were transferred to a test tube and was incubated with 300 µl of thiobarbituric acid 0.67% at 100°C for 25 minutes. 5 minutes after cooling the solution, the pink color due to the reaction of TBA-MDA appeared and was measured at a wavelength of 535 nm. Concentration of MDA was calculated using the coefficient of TBA-MDA complex absorption and was expressed as nMol/mg protein.

Biochemical enzymes assay

The epididymal superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes activities were measured according to standard procedure [18,19].

Sperm count and sperm head morphology

After the animal sacrifice, epididymis was removed and placed in a petri-plate containing 2 ml of phosphate buffer at room temperature. The epididymis was cut into small portions to allow the sperms to swim out. The solution containing the sperms was centrifuged at 1000 rpm for 3 minutes. After centrifugation, 1 ml of supernatant was taken and used for sperm count and sperm head morphology. The epididymal sperm count was determined by hemocytometer. The sperm count was expressed as number of sperms per milliliter. For sperm head morphology 0.5 ml of above solution containing the sperms and 0.5 ml of 2% eosin solution were mixed and kept for one hour to stain the sperm. Smears were prepared using 2 - 3 drops of the above solution, air dried and fixed with absolute methanol for 3 minutes. Two hundred sperms per animal were examined to determine the morphological abnormalities under oil immersion [18,19]. Sperm head morphology was scored under the category of normal, quasinormal and grossly abnormal as described by Burruel., *et al* [20]. Sperms missing rostral part of the acrosome and/or the posterolateral region of the acrosome are called quasi-normal heads. Grossly abnormal heads included collapsed and triangular heads with highly deformed acrosomal caps and nuclei. Data were shown in terms of normal to abnormal ratio of sperms.

Sperm motility

In order to observe mobility, 10 micro liters of semen was placed on a glass slide and covered with a lamella. Using a light microscope with a magnification of 400X, the number of sperm with rapid progressive forward movement (RPFM), slowly progressive forward movement (SPFM), Non-progressive motility (NPM) and motionless (ML) sperm cells were counted in several microscopic field of vision and percentage of motile and Immobile sperm cells was obtained.

Epididymis histology

Preparation and quantification of histological slides were done as standardized previously in our laboratory [21,22]. Both the testes were fixed in 10% formalin, dehydrated in ethanol and embedded in paraffin. Tissue sections (5m) were mounted on glass slide coated with albumin and dried at 30°C for 24h. The sections were then deparafinized with xylene, rehydrated with alcohol and water. The rehydrated sections were stained with haematoxylin and eosin (H&E), mounted with DPX and examined under microscope. Histological

quantification was performed by counting the normal number of seminiferous tubules in each slide. A graph was plotted between the normal numbers of seminiferous tubule at Y-axis vs. dose of epirubicin X-axis. Relative area was calculated by area of treatment group/ area of control group.

Statistical analysis

Statistical analysis was performed using the SPSS 20 version for Windows statistical package. All experiments were performed in triplicate, and the inter-group difference among three experiments was tested by analysis of variance (ANOVA). Data are expressed as means ± SEM of three different experiments, and $P < 0.05$ was considered statistically significant.

Result

Catalase (CAT) activity

The CAT activities in epirubicin-treated mice (group II, III and IV) was significantly decreased compared to control samples (group I). The groups co-treated with Quercetin and onion extract (group V and VI) showed significant recovery when compared with the groups that received epirubicin only (Figure 1).

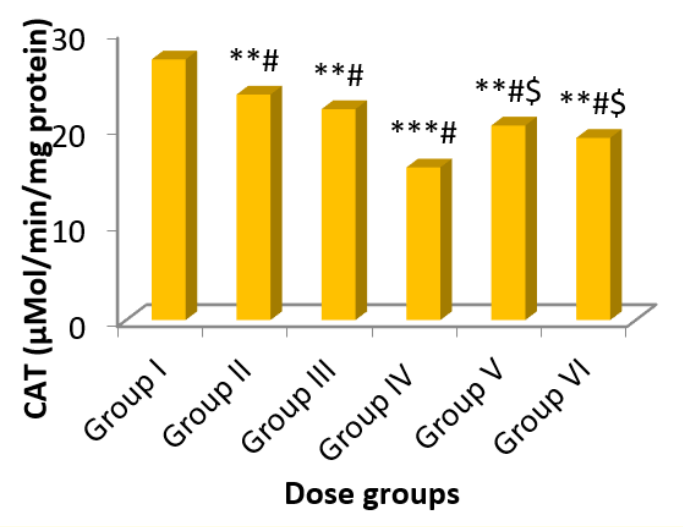

Figure 1: *Bar chart showing ameliorative effect of quercetin and onion extract. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

The effect on SOD activity

Significant depletion of superoxide dismutase enzyme activity was seen in the epididymis of epirubicin-treated mice across the groups (II, III and IV) in dose dependent manner. However, the adverse effect was significantly reversed in the groups co-treated with quercetin and onion extract (group V and VI) (Figure 2).

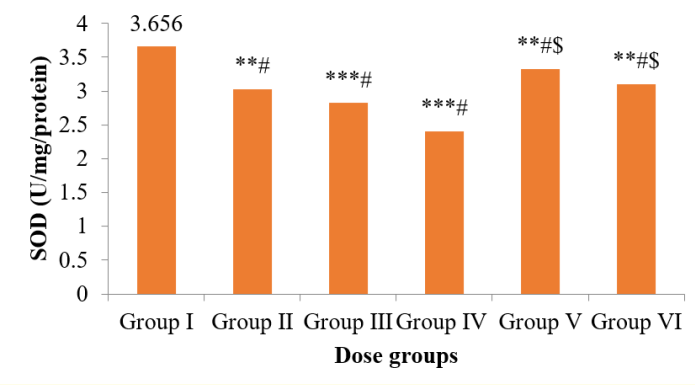

Figure 2: *Bar chart showing effect of Epirubicin in SOD activity. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

The effect on GSH level

Decreased level of non-enzymatic reduced glutathione was measured in the epididymis of mice treated with Epirubicin (Group II, III and IV) compared to control in dose dependent-pattern co-treated with Quercetin and onion (Figure 3).

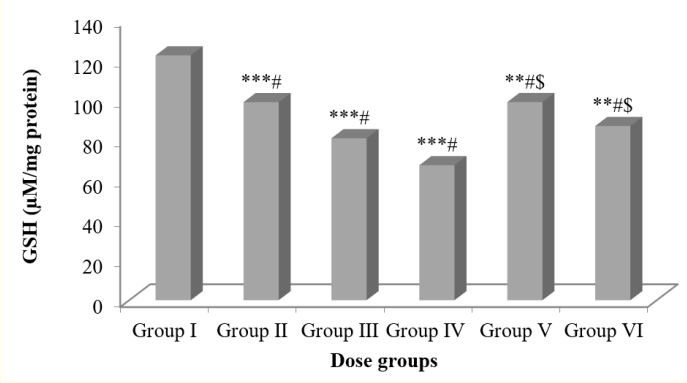

Figure 3: *Bar chart showing effect of Epirubicin on GSH levels. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

MDA concentration

Significant raised concentration of Malondialdehyde was measured in the epididymis of the mice (Group II, III and IV) treated with Epirubicin. Significant decrease in the groups co-treated with Quercetin and onion extracted (group V and VI) was also recorded when compared to groups treated with epirubicin alone confirming ameliorative effects of these flavonoids (Figure 4).

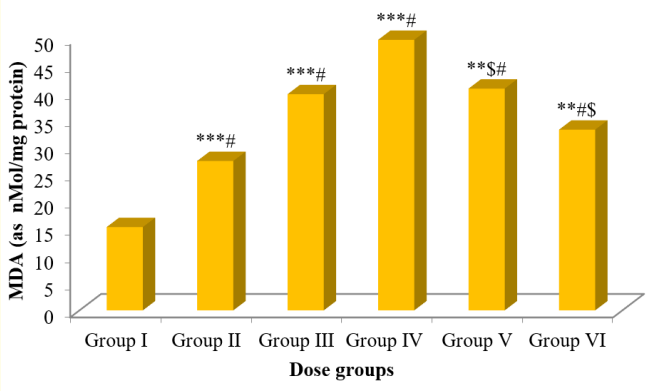

Figure 4: *Bar chart showing ameliorative effect of Quercetin and Onion Extract. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

The effect on reactive oxygen species level

Significant increased level of ROS in epididymis of mice treated with Epirubicin across the group. Co-treatment with Quercetin and Onion extract drastically attenuate the harm effect of epirubicin as seen in group V and VI (Figure 5).

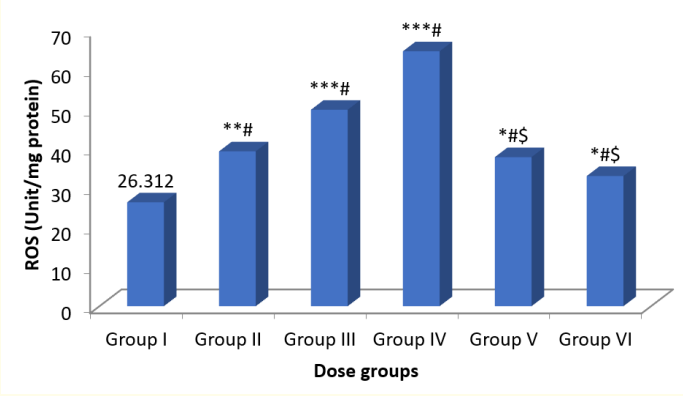

Figure 5: *Bar chart showing ameliorative effect of quercetin and onion extract on. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

The effect on sperm head count

Epirubicin treatment at dose of 10 and 20 mg/kg led to significant decrease in sperm head count as compared to control group. However, the adverse effect was reversed in the groups co-treated with quercetin and onion extract (Figure 6).

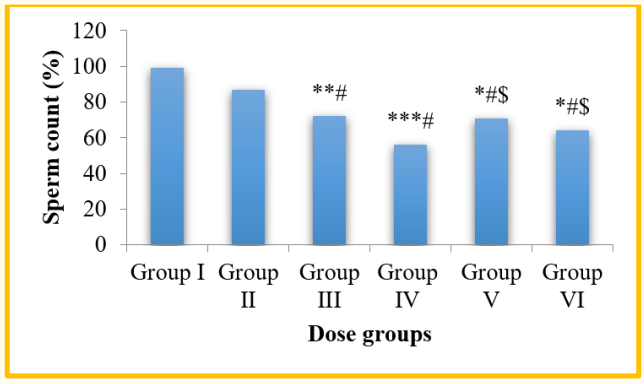

Figure 6: *Bar chart showing effects of Quercetin and Onion on sperm head count. All the values are expressed as mean ± SEM, (n=5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

Incidence of morphologically abnormal sperm

The incidences of morphologically abnormal sperm in all the epirubicin-treated groups were significantly increased compared with the control group. The epirubicin co-treated with Quercetin and Onion extract groups showed significantly deceased incidences compared with the epirubicin group (Figure 7).

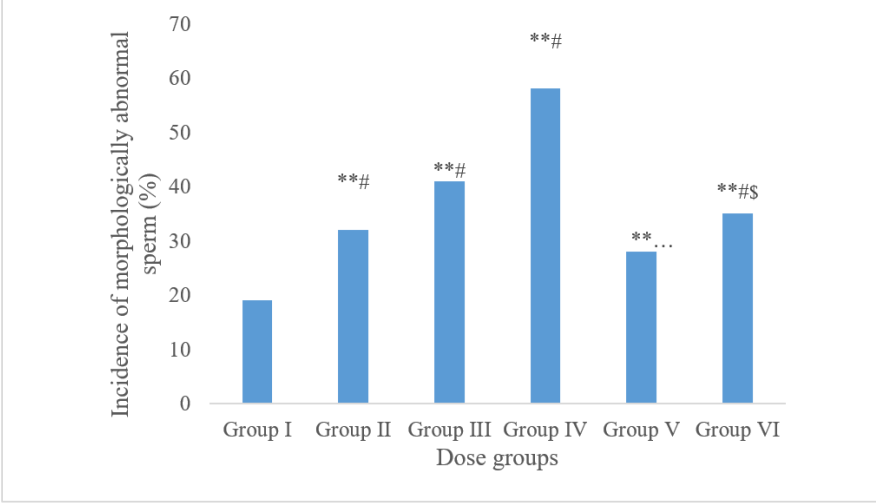

Figure 7: *Effects of quercetin and onion on sperm morphological ebnormalities in Epirubicin-treated mice. All the values are expressed as mean ± SEM, (n = 5), ***P < 0.001, **P < 0.01 and *P < 0.05, # vs. control and $ vs. EP-20.*

Sperm motility

Compared to the control group, mice in epirubicin treated group showed a remarkable increase in the percentage of slowly progressive forward movement (SPFM), Non-progressive motility (NPM) and motionless (ML) sperm cells. Mice treated with epirubicin alone showed a decrease in the percentage of motile sperm in comparison to the control group. This increase was statistically significant (p = 0.05). There was also a remarkable difference between epirubicin co-treated with Quercetin and onion extract and control group in terms of the percentage of immotile sperm (p = 0.05).

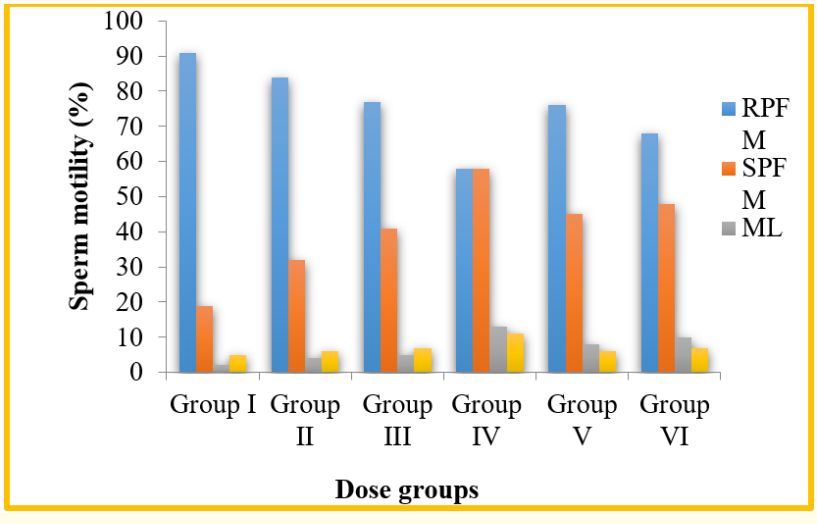

Figure 8

Histology observation

Severe disruption of epithelium with occurrence of vacuolization were observed in histology of epididymis with the disappearance of spermatozoa in the lumen of caput corpus and cauda epididymis.

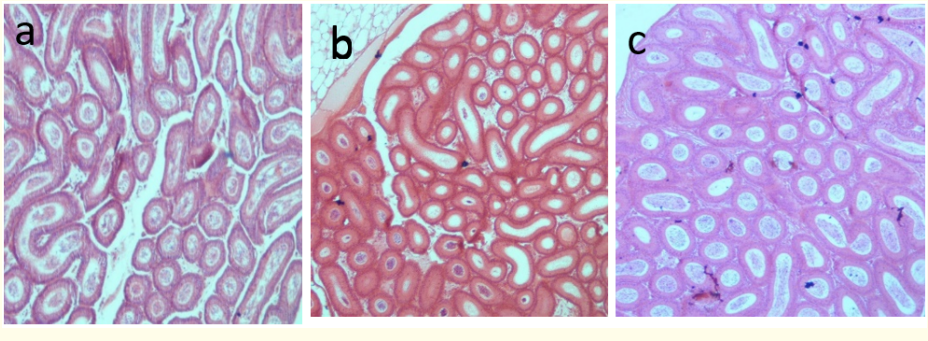

Figure 9: *Photomicrographs of Transverse section of epididymis of mice through cauda following epirubicin treatment. (a) Control (normal saline), (b) CP treatment (20 mg/kg) and (c) EP as well as Onion extract (20 and 10 mg/kg respectively.*

Discussion

Phytonutrients play a major role in cancer therapy and cancer chemoprevention. Flavonoids such as Quercetin and Onion extract have been proven to ameliorate the effect of chemotherapeutic drugs like Bleomycin, Methotrexate, Capecitabine [23-25]. Nevertheless, the effect of Quercetin and Onion extract on epirubicin-induced genetic cytotoxicity in germ cells has not yet been reported. Therefore, the aim of this research was to determine whether co-administration of Quercetin and Onion extract would ameliorate the epirubicin-induced genetic cytotoxicity in male germ cells. This study showed that Quercetin and Onion extract were able to ameliorate the observed epirubicin-induced genetic cytotoxicity as detected by reduction in the incidence of morphologically abnormal sperm as well as increased sperm count and motile sperms.

One of the major concerns of chemotherapy treatment over the years is the increase in the risk of the incidence of infertility attached to this particular treatment which has been proven by several reports. Consequently, it is imperative the male population subjected to chemotherapy treatment be educated on the possibility of them being infertile as a result of this treatment as one of the proven implications over time is diminished sperm quality or quantity [26]. In this study, it was observed that epirubicin caused a dose-dependent aberration in the sperm quality and quantity of the sperm cells, and the effect was significant at the two highest tested doses. Co-treatment of the animals with Quercetin and Onion extract significantly reduced epirubicin-induced cytotoxicity in the sperm germ cells. The results of our *in vivo* study on the effects of epirubicin treatment were in agreement with results of an earlier report on the utility of dexrazoxane for the attenuation of epirubicin-induced genetic alterations in mouse germ cells [27]. Mice administered with epirubicin showed poor sperm quality with increased abnormal sperm morphology and decreased total sperm number and motility as it was observed that percentages of sperm motility and count significantly decreased in epirubicin-treated mice. Incidences of high percentage of immotile or poorly motile sperm might likely result in infertility [28]. Additionally, epirubicin significantly elevated the frequency of abnormal sperm while co-treatment with Quercetin and Onion extract ameliorated the declined percentage of sperm count and motility. Furthermore, the occurrence of abnormal sperm was reinstated ensuing co-treatment with Quercetin and Onion extract. The observed declined sperm quality after treatment with epirubicin was steady with the report on the utility of dexrazoxane for the attenuation of epirubicin-induced genetic alterations in mouse germ cells [27]. The specific mechanisms by which Quercetin and Onion extract ameliorates epirubicin-induced cytotoxicity in germ cells are not fully understood. It has been proven that the metabolism of epirubicin results in the production of free radicals [29], so a likely explanation for this attenuation is that co-treatment with Quercetin and Onion extract (these flavonoids are known for their anti-oxidative properties) resulted in the scavenging of free radicals produced by epirubicin before they could cause DNA breakdown and result in genetic damage. Excessive production of free radicals is a pointer to a faulty anti-oxidant defense system and likewise a pointer to oxidative stress.

Oxidative stress was confirmed by increased MDA and ROS levels with simultaneous decline in the activity of Catalase and SOD as well as GSH levels and this was observed in epirubicin treated mice. SOD catalyzes the dismutation of superoxide radical into water and hydrogen peroxide while catalase decomposes hydrogen peroxide into water and oxygen, these two enzymes including GSH are important in protecting cellular organisms from damage resulting from ROS. In fact, it has been proven that oxidative stress results in sperm dysfunction and sperm DNA fragmentation [30]. Sekar., *et al*. reported that oxidative stress affects motility by modifying axoneme structure which results in decline in sperm motility as well as abnormality in sperm tail [31-35]. However, co-treatment with Quercetin and Onion extract caused a recovery of the anti-oxidant system by causing a significant decline in MDA and ROS levels as well as significantly increasing the activity of Catalase and SOD as well as increasing GSH level. Consequently, the antioxidant property of quercetin and Onion extract might be responsible for ameliorating the genetic cytotoxicity induced by epirubicin in the germ cells.

Conclusion

This study successfully shows that administration of high concentration of epirubicin (20mg/kg/bwt) leads to generation of oxidative stress resulting in the cauda epididymal sperm morphology changes and depletion of sperm number observed. Therefore, this study reports the cytotoxicity effect of epirubicin and ameliorative potential of *Allium* cepa extract.

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