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Research Article

D-Ribose-L-Cysteine-rich Supplement Attenuates Doxorubicin-Induced Impaired Spermatogenesis, Testicular Steroidogenesis and Redox Status in Sprague-Dawley Rats

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ABSTRACT

Reproductive function is often impaired during chemotherapy and currently there is no universally approved drug/supplement that can effectively protect the testis from cellular assault due to chemotherapeutic agent such as doxorubicin (dox). This study was designed to investigate the efficacy of D-Ribose-L-Cysteine rich supplement, Cellgevity™ (cgt), in ameliorating some doxorubicin-induced testicular damage in Sprague-Dawley rats. At the end of the study, biochemical assays and sperm epididymal parameters analysis were done. Our results show that simultaneous administration of *cgt* and doxorubicin has ameliorative effect on the testis when compared to pre-treatment or post-treatment with cgt. Significant increase in testosterone, sperm concentration, progressive motility and significant decrease in immotility were observed in the cellgevity treated group. Also, simultaneous administration of *cgt* with *dox* reduced lipid peroxidation and boosted only glutathione level in testicular tissues. Nevertheless, pre-treatment or post-treatment with *cgt* did not play a significant role in modulating sperm parameters, hormones and tissue oxidative stress level. This result reveals that cellgevity has testiculo-protective effect against doxorubicin administration.

Keywords: *Cellgevity™; doxorubicin; antioxidant; testosterone, sperm parameters*

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INTRODUCTION

Chemotherapy is one of the available treatments for cancer. It involves the use of chemicals (anticancer drugs) to target cancerous or rapidly dividing cells (Bagnyukova *et al.*, 2010). One of the most popular and most active chemotherapeutic drugs is doxorubicin (*dox*) (Kumar *et al.*, 2014). It is an anthracycline antibiotic closely related to the natural product daunomycin and like all anthracycline it works by intercalating DNA and remains one of the most potent antineoplastic drugs (Awasthi *et al.*, 2003; Carvalho *et al.*, 2009). It is, therefore, common to use *dox* in the treatment of solid tumours and haematological malignancies, including breast, bile ducts, prostate, uterus, ovary, oesophagus, stomach and liver tumours, childhood solid tumours, osteosarcoma and soft tissue sarcoma, Kaposi's sarcoma as well as acute myeloblastic and lymphoblastic leukaemia and Wilm's tumour (Danesi *et al*, 2002; O'Shaugnessy *et al*, 2003; Gruber *et al.*, 2004; Petrioli *et al.*, 2008). The core characteristics of anthracyclines include the production of reactive oxygen species and it has been shown that these molecular characteristics are double-edged sword acting not only on neoplastic cells but also on multiple cellular targets throughout the body (Cummings *et al.*, 1991; Cappetta *et al*., 2017). Therefore, the mechanism of action of *dox* involves its oxidation to semiquinone, which is an unstable metabolite that is converted back to *dox* in a process that releases reactive oxygen species (Thorn *et al*., 2011).

Testicular dysfunction is amongst the most common long-term side effect of chemotherapy in men. This is because the testis, being a rapidly dividing organ in the body, is an easy target for cytotoxic chemotherapeutic drugs. Thus, male individuals undergoing chemotherapy stand the risk of temporary, long-term or permanent gonadal damage (Traila *et al.*, 2018). This, therefore, has raised concerns about the reproductive health of surviving patients since they eventually are at risk of persistent impaired spermatogenesis and infertility (Ishikawa *et al.*, 2004; Pectasides *et al.*, 2004;

Bieber *et al.,* 2006). Studies have reported that *dox* induces significant decline in testicular weight, sperm count, serum testosterone and increases in serum lactate dehydrogenase and increases lipid peroxidation in the testis (Quiles *et al.,* 2002; Patil *et al*, 2009, Saalu *et al.,* 2010).

Cellgevity™ (*cgt*), made up of mainly riboceine™ which contains cysteine molecularly coated with ribose. Following intestinal absorption, the ribose is transported into the cell where the cysteine is decoupled and used in the synthesis of gluthatione (Max International, 2017). Other constituents of *cgt* include alpha lipoic acid, broccoli seed extract, turmeric root extract, resveratrol, grape seed extract, quercetin, milk thistle, vitamin c, selenomethione, cordyceps, black pepper, and aloe extract (Max International, 2017). Recent studies have indicated that *cgt* may play some roles in lowering oxidative stress level in tissues (Falana *et al.*, 2017). Since *dox* has been shown to increases lipid peroxidation in the testis (Patil *et al*, 2009; Saalu *et al.*, 2010), the aim of this study was to determine whether glutathione-promoting D-Ribose-L-Cysteine-rich supplement as an adjunct to *dox* can prevent and/or ameliorate the effect of *dox* on testicular morphofunctional integrity in Sprague-Dawley rats.

MATERIALS AND METHODS

Cellgevity™ was purchased from *Max International*, Ikeja, Lagos, Nigeria while doxorubicin was purchased from Bernados Pharmacy, Lagos State, Nigeria. Experimental animals used for this research were obtained from the Animal House of College of Medicine of the University of Lagos, Lagos State, Nigeria. They were kept in the Animal House of the Department of Anatomy, College of Medicine, University of Lagos, Nigeria. Animals were allowed access to rat chow and water *ad libitum* under normal laboratory conditions of 12:12 light / dark cycle.

Experimental Design: At the beginning of the experiment, 30 male Sprague-Dawley rats (180-200 grams) were divided into six groups (n= 6 rats/group). Control group consist of animals that were given distilled water. The *dox* group was administered 1.8 mg/kg of *dox* only for 14 days. The *cgt* group was administered, based on human dose equivalent (Nair and Jacob, 2016), 150 mg/kg *cgt* only for 14 days. The *dox+cgt* group was administered 1.8 mg/kg body weight of *dox* and 150 mg/kg body weight of *cgt* simultaneously for 14 days. The 1 week *dox+cgt* group was administered 1.8 mg/kg body weight of *dox* the first week and 150 mg/kg of *cgt* the second week while the 1 week *cgt+dox* was administered 150 mg/kg of *cgt* the first week and 1.8mg/kg body weight of *dox* the second week. All administrations for the different durations were done orally.

Animal Euthanasia, Samples Collection and Hormone Assay: At the end of administrations, animals were sacrificed by cervical dislocation. On each animal, ventral abdominal incision was made following which blood was collected, through cardiac puncture, into anticoagulant-free sample bottles and centrifuged at 3000rpm for 15 minutes to obtain serum for hormonal assay of testosterone (TT) and estradiol (E_2) using ELISA kit obtained from Accubind Inc (USA). Manufacturer's instructions that accompanied assay kits were strictly adhered to. The left testis was homogenized in cold phosphate buffer and centrifuged at 3000rpm. The supernatant was collected into plain sample bottle, stored at -20^{o}C and was later used for hormonal assay and oxidative stress studies.

Semen Analysis Epididymal Sperm Count: Sperm count was done as previously described by Latchoumycandane *et al.* (2002). Briefly, 0.1 mL sample of semen was placed in 0.9 mL normal saline for sperm cells to swim out in the petri-dish. It was well shaken and the sample was taken to a counting chamber (improved Nuebauer hemocytometer; (Deep 1/10 m; LABART, Munich, Germany). After the sperm cells have settled on the grid, it was then viewed under the microscope and spermatozoa in five squares were counted. The number of sperms counted in five squares was multiplied by dilution factor and 0.05 and 10^6 to determine the number of sperm per mL.

Determination of Sperm Motility: To assess motility, a drop of epididymal fluid was delivered onto a glass slide such that the spermatozoa were evenly distributed, covered by a 22x22 mm cover slip and examined under the light microscope at a magnification of x100 while several fields were evaluated. Sperm motility was classified as progressive motile, non-progressive motile or immotile. After assessing different microscopic fields, the relative percentage of motile sperm was estimated and reported to the nearest 5% using the subjective determination of motility (WHO, 2010).

Determination of Tissue Lipid Peroxidation and Antioxidant Enzymes activities: Malondialdehyde (MDA) was used as a proxy index for lipid peroxidation in testicular tissue homogenate and determined using the method of Mihara and Uchiyama (1978). It is based on the interaction of MDA with thiobarbituric acid (TBA) to form a pink complex and absorbance read at 535nm. Glutathione (GSH) level was determined in tissue PBS sample based on the reaction of Ellman's reagent 5,5' dithiobis (2-nitrobenzoic acid) DNTB) with the thiol group of GSH (Ellman, 1959). The level of activity of superoxide dismutase (SOD) was determined by its ability to inhibit the auto-oxidation of epinephrine determined by the increase in absorbance at 320nm. The reaction was carried out in 0.05N HCL (Sun and Zigman, 1978). Catalase (CAT) activity was measured in the tissue PBS samples by the method of Aebi (1984), which is based on the exponential disappearance of H_2O_2 by the action of catalase contained in the examined samples.

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. Data are expressed as mean ± standard error of mean (SEM). Graphpad Prism 5 was used to determine the statistical significance of the data. Values were considered significant at $p<0.05$.

RESULTS

Sperm Parameters

As shown in Figure 1, there was a significant decrease in sperm concentration in *dox*-treated group compared with control whereas *cgt*-treated group showed no significant difference compared with control. 1 WK *dox+cgt* and 1WK *cgt+dox* treated groups also showed significant decrease in sperm concentration compared with control. However, *dox+cgt* treated group showed significant increase in sperm concentration when compared to the *dox*, 1 WK *dox+cgt* and 1WK *cgt+dox* treated rats. In Figure 1b, the percentage of non-progressive motility was significantly increased in *dox*-, 1WK *dox+cgt*-, and 1WK *cgt+dox*- treated groups compared with control. Also, there was a significant decrease in non-progressive motility of *cgt* treated group when compared with control and *dox+cgt*. In Figure 1c, there was a significant decrease in progressive motility in *dox* treated group compared with control group whereas *cgt*-treated group showed no significant difference compared with control. When compared to the control group, 1WK *dox+cgt*, 1WK *cgt+dox* showed significant decrease in progressive motility. However, there was a significant increase in progressive motility in *dox+cgt* treated rats when compared with *dox*, 1WK *dox+cgt*, 1WK *cgt+dox* group. In Figure 1d, sperm immotility was significantly increased in *dox*-, *dox+cgt*-, and 1 WK *cgt+dox*- treated groups whereas there was a significant decrease in immotility of *cgt* treated group when compared with control and *cgt* group.

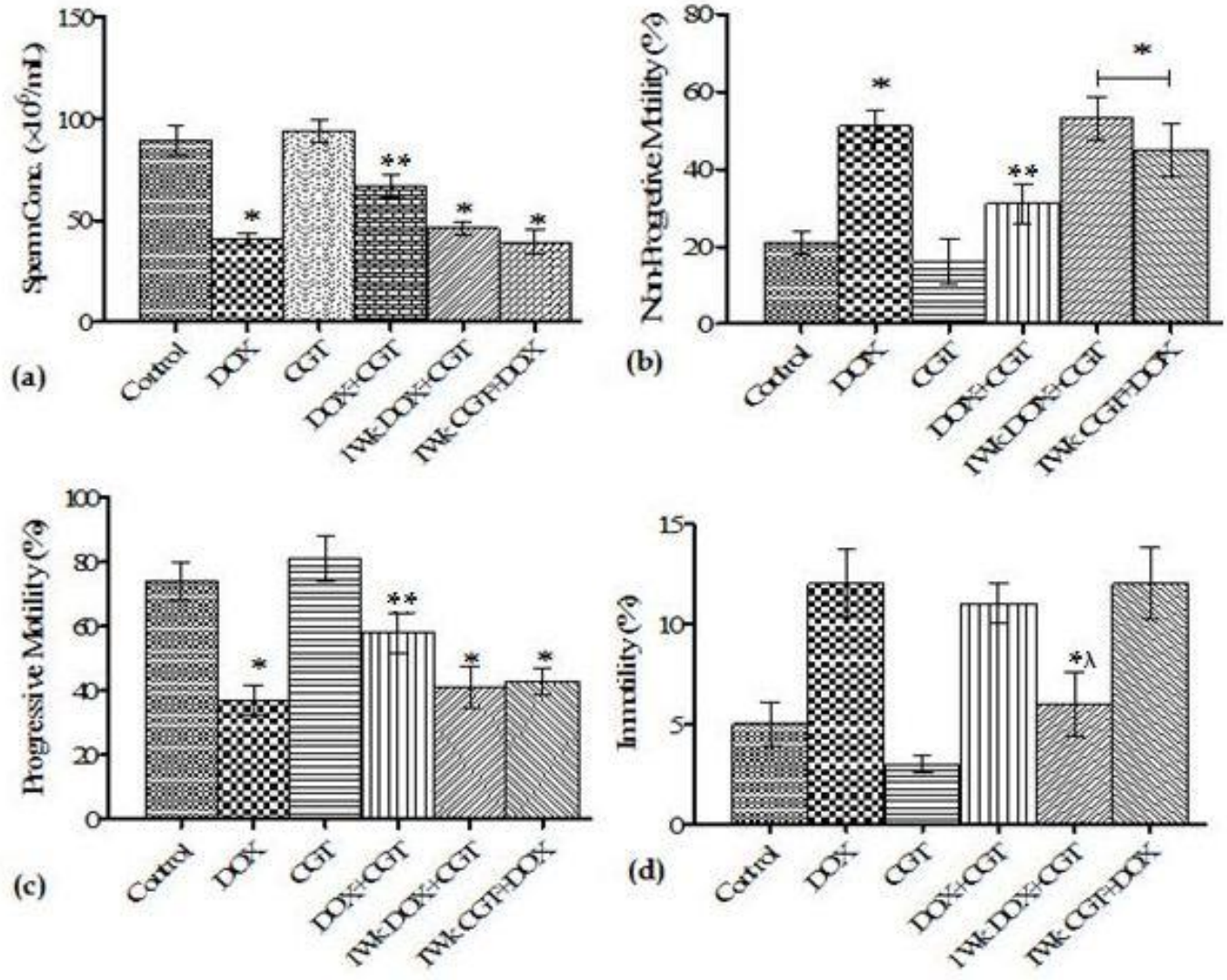

Figure 1

Comparison of sperm parameters between experimental groups.* shows significance difference compared with control and cgt; ** indicates significance difference compared with control and dox at $p<0.05$; λ indicates significance difference compared with dox, dox+cgt, 1 wk cgt + dox at $p<0.05$. Dox= Doxorubicin, cgt= CellgevityTM. 1Wk dox + cgt means animals receive dox for one week before treatment with cgt for another one week. 1Wk cgt + dox means animals were treated with cgt for one week before administration of dox for another one week.

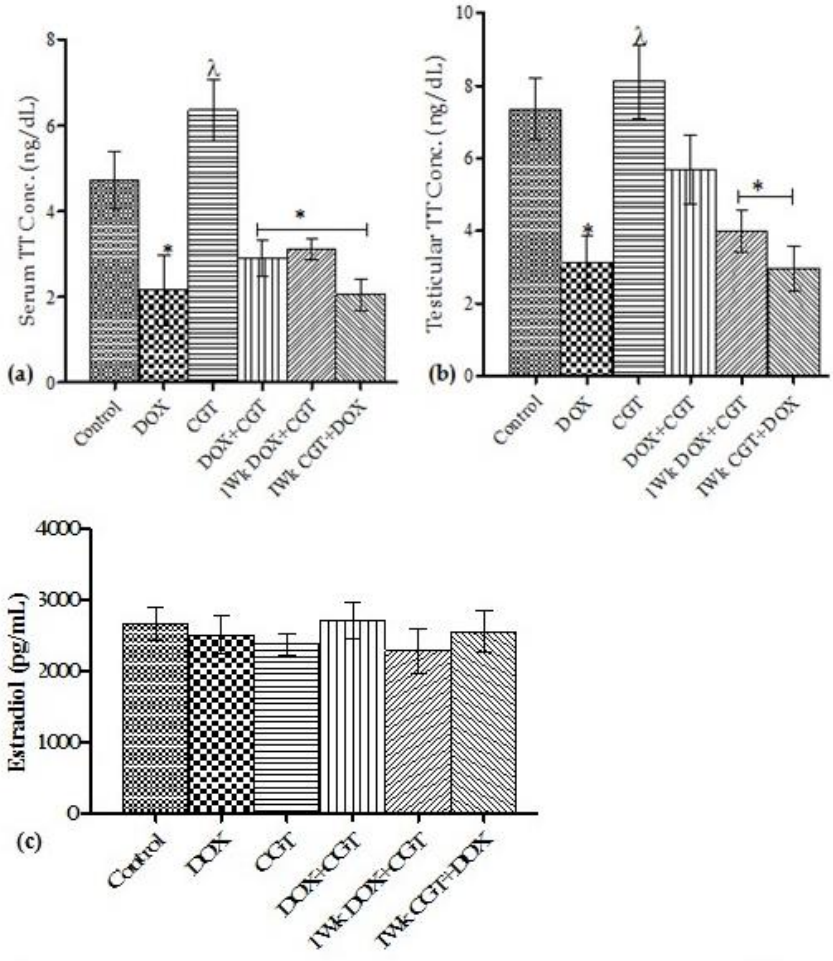

Figure 2
Comparison of Testicular Testosterone, Serum Testosterone and Serum Estradiol concentration between experimental groups. * indicates significant difference compared with control at $p<0.05$; λ shows statistical significance between cgvt group and other treated groups at $p<0.05$. Dox= Doxorubicin, cgt= CellgevityTM. 1Wk dox + cgt means animals receive dox for one week before treatment with cgvt for another one week. 1Wk cgt + dox means animals were treated with cgt for one week before administration of dox for another one week.

Concentrations of Testosterone and Estradiol: Serum testosterone following treatment with *dox* and *cgt* is shown in Figure 2a. Serum concentration of testosterone significantly reduced in *dox*- treated group compared with control group. Similarly, there was significant reduction of serum testosterone in *dox+cgt*, 1WK *dox+cgt*, and 1WK *cgt+dox* groups compared with control. However, significant increase in serum testosterone (TT) of the *cgt* treated group was observed when compared to the control group. Testicular testosterone following treatment with *dox* and *cgt* is shown in Figure 2b. There was a significant reduction in testicular TT of *dox*, 1WK *dox+cgt*, 1WK *cgt+dox* treated groups when compared with the control group. However, *dox+cgt* group showed no significant reduction in testicular TT compared with control. In *cgt* treated group, testicular TT increased significantly when compared with control. In Figure 1c, there was no significant difference in the level of serum estradiol of *dox*, *cgt*, 1WK *dox+cgt*, 1WK *cgt+dox* compared with the control group.

Lipid Peroxidation and Antioxidant Enzymes Activities
Lipid peroxidation and antioxidant enzymes activities in testicular tissue are shown in Figure 3. There was a significant increase in MDA level of *dox* and 1WK *cgt+dox*-treated groups compared with control. There was no significant difference in the MDA level of *dox+cgt*, 1WK *dox+cgt*, and *cgt*-treated groups when compared with control (Figure 3a).
In Figure 3b, the level of GSH was significantly reduced in *dox*-treated group compared with control. However, GSH level significantly increased in *dox+cgt* treated group when compared to the control group. While there is no significant difference in GSH between the 1WK *dox+cgt* and 1WK *cgt+dox* treated groups, there is a significant decrease in GSH in *dox+cgt*, 1WK *dox+cgt*, 1WK *cgt+dox* when compared to *cgt*-treated group. In addition, there is a significant increase in GSH level of *dox+cgt* when compared with 1wk *cgt+dox*.
In Figure 3c, there was a significant decrease in catalase in *dox*, *dox+cgt*, 1WK *dox+cgt*, 1WK *cgt+dox* when compared to *cgt* and control group. However, there is a significant increase in the CAT levels of the control and *cgt* treated group when compared to *dox*, *dox+cgt*, 1 WK *dox+cgt*, 1WK *cgt+dox* treated group.

In Figure 3d, there was no significant difference in the level of SOD in the control when compared to *dox*, *cgt*, 1WK *dox+cgt*, 1WK *cgt+dox* treated group

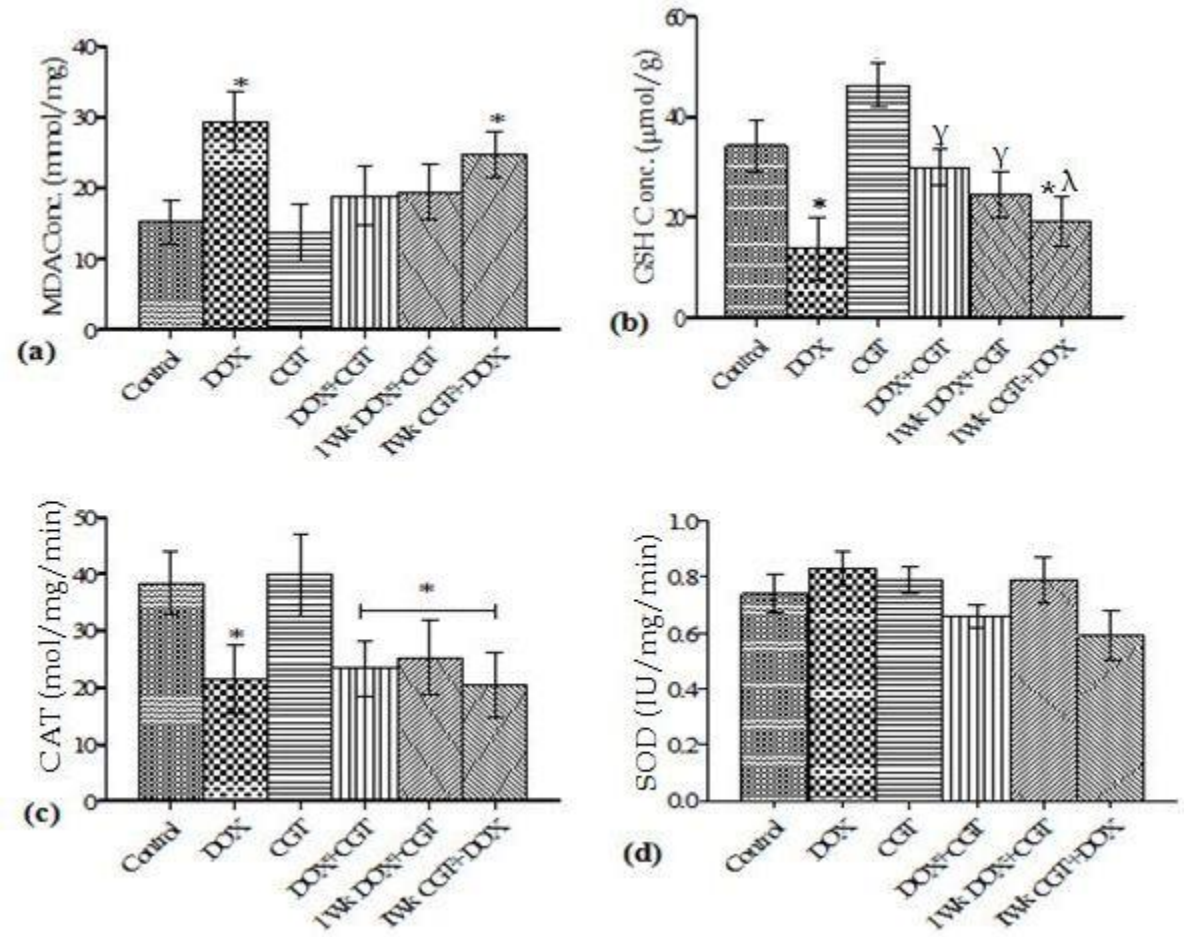

Figure 3
Malondialdehyde concentrations and antioxidant enzymes activities between the experimental groups. * indicates statistically significant diffences compared with control and cgt treated groups while γ shows statistically significant diffences compared with dox and λ indicates statistically significant diffences compared with dox+cgt and 1 Wk dox+cgt ($p < 0.05$). Dox= Doxorubicin, cgt= CellgevityTM. 1Wk dox + cgt means animals receive dox for one week before treatment with cgt for another one week. 1Wk cgt + dox means animals were treated with cgt for one week before administration of dox for another one week

DISCUSSION

The results of this investigation reveal that supplementation with *cgt* might be a beneficial adjunct therapy for men receiving *dox* medication against cancer. In the present study, semen parameters improved significantly in the group that received *cgt* simultaneously with *dox.* Specifically, *dox+cgt*-treated group showed significant increase in sperm concentration when compared to the group treated with *dox* only, the group treated with *dox* for one week followed by another week of *cgt* administration, and the group treated with *cgt* for one week before administration of *dox* another week. This implies that *cgt* can boost sperm concentration; when administered simultaneously with *dox*, it minimizes the deleterious effect of *dox* on the sperm concentration when compared to the groups that were administered with *cgt* a week before or a week after. In addition, there was a significant increase in progressive motility in *cgt* group when compared to the *dox*, 1WK *dox+cgt*, 1WK *cgt+dox*. This shows that *cgt* increases progressive motility in the animal and also modulates progressive motility in *dox* challenged animals. The negative effect of *dox* on semen parameters observed in this study is consistent with the findings of previous investigations (Saalu *et al.,* 2010; Badkoobeh *et al.,* 2013, Uyeturk et al., 2014). Overall, simultaneous administration of *dox* and *cgt* proves to yield better results of improving sperm motility when compared to the other treatment groups that were administered *cgt* a week before and after *dox*.

From this study, the mechanism through which *cgt* improves semen parameters appears to be mediated through increase steroidogenesis and boosting testicular anti-oxidant defense system. There is a significant increase in testicular TT in *dox+cgt* compared with *dox*, 1WK *dox+cgt*, 1WK *cgt+dox*-treated groups. But there was no significant increase in serum levels of TT in all the treated groups except *cgt* only group (Figure 2). This suggests that *cgt* can attenuate *dox*'s interference in Leydig cells' testosterone production capacity. On estradiol levels, however, there was no significant difference in all the groups indicating that neither *dox* nor *cgt* has major interference with estradiol steroidogenesis. Elevated level of estradiol has been shown to impede testicular

function (Schulster *et al.,* 2016); however, our present findings indicate that the mechanism of *dox* testicular insult is independent of estradiol level.

The impact of *cgt* on testicular steroidogenesis may derive from reduced level of free radicals that may otherwise impede testosterone elaboration by Leydig cells resulting in many more spermatogenic cells surviving to spermatozoa stage. Studies have shown that free radical generation, which is a natural by-product of testicular cellular activity, reduces spermatogenic efficiency (Yokoi and Mayi, 2004; Pranhalatan et al., 2005; Attesahin et al., 2006, Priestman, 2008). With the current work, it might be possible to increase sperm production in apparently normal individual by lowing testicular free radical contents.

Furthermore, the activities of oxygen free radical, superoxide radical and oxidative stress have been strongly accepted as essential factors in the pathogenesis of *dox*-induced cytotoxicity (Chularojmontri *et al.,* 2005, Prahalatan *et al.,* 2005; Saalu *et al*., 2010). This is confirmed in this study as significant elevation of MDA was observed in *dox* only group which provides evidence of increased lipid peroxidation in the testis of *dox* challenged animals. GSH exhibits the first line of antioxidant defence and primarily regulates redox status in cells (Schafer and Buettner, 2001; Suntres, 2011). GSH is involved in scavenging hydroxyl radical and detoxification of hydrogen peroxides and lipid peroxides through the action of glutathione peroxidase (Kern and Kehrer, 2005). In this present study, concomitant administration of *cgt* with *dox* significantly improved GSH level in comparison with sole administration of *dox* (Figure 3) suggesting that *cgt* has the capacity to boost cellular GSH production in the presence of pro-oxidant-generating *dox*. However, while animals that were treated with *cgt* either one week before or after *dox* administration exhibited higher GSH levels than the *dox* group; their GSH levels did not attain that of the control's. This is in accordance with a previous study which shows that *dox* reduces GSH levels in the testis (Saalu *et al.,* 2010). The significant increase in GSH level of *cgt+dox* group compared with 1 WK *dox+cgt* and 1WK *cgt+dox* implies that simultaneous administration of *cgt* with *dox* is the most effective way of preventing significant depletion of GSH not pre- or post-treatment. But *cgt* was unable to prevent *dox*-induced GSH depletion in the animals that where administered one week before *dox* and one week after *dox*. This is responsible for the elevation in MDA levels observed in these groups.

SOD and CAT are important scavengers of superoxide ion and hydrogen peroxide. These two enzymes prevent the generation of hydroxyl radical and protect cellular constituents from oxidative damage (Sivaraj *et al.,* 2011). SOD is involved in the rapid dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). The H_2O_2 generated from this dismutation process is also a powerful membrane penetrating oxidant which has to be rapidly removed from the cell. The elimination of H_2O_2 in the cell can be handled by catalase in reactions that produce water and molecular oxygen (Trachootham *et al.,* 2008). In this study, *cgt* had no significant impact on the activities of SOD and CAT. In all the groups treated with *dox,* the activity of catalase was significantly reduced while the activity of SOD remained unaffected. This suggests that neither *dox* nor *cgt* affected SOD levels remarkably. The incidence of chemotherapy-induced elevation in tissue oxidant level has been well reported as a major mechanism by which chemotherapy agents destroy cancer cells; and in the process normal cells become collaterally damaged (Priestman, 2008). Spermatozoa constitute one of the body cells that are most likely to undergo *dox*-induced oxidative damage. This was prevented by *cgt* as many more spermatogenic cells survive to spermatozoa stage.

In conclusion, this study demonstrates *dox* impairs spermatogenesis and testicular steroidogenesis by increasing cellular level of pro-oxidants evident by elevated MDA level. However, concomitant administration of *cgt* with *dox* suppresses *dox*-induced elevated oxidative stress and improved sperm parameters. This indicates that *cgt* possesses significant testiculoprotective effects, especially during simultaneous administration of *dox*. Since the testis is susceptible to chemotherapy-induced oxidative damage, the current investigation provides evidence that may open a new pathway for the management of cancer in such a way that allows for the preservation of male reproductive functions.

Conflict of Interest Disclosure:
The authors declare that they have no competing interest

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