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Gallic acid protects against cyclophosphamide-induced toxicity in testis and epididymis of rats

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Summary

The protective role of gallic acid (GA) on reproductive toxicity induced by cyclophosphamide (CPA), an antineoplastic drug, was investigated in male Wistar rats. Sixty rats were grouped into 10 rats per group. Group 1 (control) received distilled water. Rats in groups 2 and 3 received GA alone at 60 and 120 mg kg^{-1} for 14 consecutive days, respectively. Group 4 received a single intraperitoneal dose of CPA at 200 mg kg^{-1} on day 1. Groups 5 and 6 received a single dose of CPA (200 mg kg^{-1}) intraperitoneally on day 1 followed by treatment with GA at 60 and 120 mg kg^{-1} for 14 consecutive days, respectively. In testes and epididymis of the treated rats, CPA administration resulted in significant elevation ($P < 0.05$) in malondialdehyde (MDA), nitrite and hydrogen peroxide levels. There was a significant decrease in the activities of superoxide dismutase and glutathione-S-transferase. Furthermore, there were significant reductions in plasma luteinising hormone (LH), follicle stimulation hormone (FSH) and testosterone levels, which were accompanied by significant decrease in sperm motility and viability in CPA-treated rats. Histological examination revealed marked testicular and epididymal atrophy in CPA alone treated rats and these aberrations were reversed by GA. In conclusion, GA has capacity to protect against reproductive toxicity induced by cyclophosphamide.

Introduction

The effectiveness of anticancer drugs is associated with cellular toxicity to one or more target tissues. Cyclophosphamide (CPA) is one of the commonly used antineoplastic and immunosuppressant drugs. It has been used for the treatment of variety of cancers and disorders such as acute and chronic leukaemia, multiple myeloma, systemic lupus erythematosus, rheumatoid arthritis and other benign diseases (Senthilkumar *et al.*, 2006; Perini *et al.*, 2007). Kenney *et al.* (2001) reported that male patients with cancer have increased evidence of oligospermia and azoospermia following administration of CPA. More so, CPA is commonly used in cancer chemotherapy; hence, occupational exposure by healthcare professional has become a normal occurrence (Rekhadevi *et al.*, 2007). Studies have also shown that treatment of adult male patients with CPA resulted in diminished sperm count and with concomitant lack of spermatogenic cycles in their testicular tissues (Kim *et al.*, 2012). Moreover, experimental studies have also revealed that decreased testicular weight, transitory oligospermia, impairment of sperm motility and its fertilising ability as well as abnormal changes of the testis and epididymis are evident following treatment of male rats and mice with CPA (Elangovan *et al.*, 2006; Selvakumar *et al.*, 2006a,b).

The therapeutic and toxic effects of CPA are dependent on its metabolic activation by the hepatic microsomal enzymes cytochrome P450 mixed function oxidase system that produces two active metabolites namely phosphoramide and acrolein. The former is associated with CPA immunosuppressive and antineoplastic effect whereas the latter is responsible for its toxic effect (Kern & Kehrer, 2002; Lindley *et al.*, 2002). Experimental evidence has shown that following CPA administration there is disruption of redox balance mediated by oxidative stress which generated biochemical and physiological disturbances (Das *et al.*, 2002; Ghosh *et al.*, 2002). Although the exact

mechanism by which CPA induces male reproductive toxicity is not fully known, however, it has been reported that acrolein induces the generation of reactive oxygen species that eventually leads to oxidative stress following CPA administration (Sudharsan *et al.*, 2006; Türk *et al.*, 2010; Liu *et al.*, 2012). However, it will be advisable that CPA administration be accompanied by an antioxidants or phytochemicals capable of minimising the biochemical and physiological disturbances associated with CPA administration.

Gallic acid, (GA, 3,4,5-trihydroxybenzoic acid), is an important polyphenolic substance found in green tea, grapes, red wine, mango, walnut, etc. (Korani *et al.*, 2014). The efficiency of antiradical and antioxidant activities of phenolic compounds is variable and related to many factors, such as number, site of bounding and mutual positions of hydroxyls on the aromatic ring. For example, an O-hydroxyl substitution on the aromatic ring has a beneficial effect on the antiradical and antioxidant activity of phenolic acids (Sroka & Cisowski, 2003). Studies have shown that GA has antioxidant, free radical scavenging ability, anti-inflammatory, antimicrobial, anti-allergic, anti-fungal and anticancer activities both *in vivo* and *in vitro* (Choi *et al.*, 2010; Bhouri *et al.*, 2012). GA has also been used as an antioxidant in food, cosmetics and in pharmaceuticals (Bhouri *et al.*, 2012). Studies have shown that GA has ability to scavenge superoxide anion radical and hydroxyl radical, thereby preventing the development of oxidative stress (Jadon *et al.*, 2007; Kazim *et al.*, 2013). The study was conducted to explore possible protection offered by GA against CPA-induced testicular and epididymal damage in male Wistar rats.

Materials and methods

Potassium hydroxide, sodium hydroxide, reduced glutathione (GSH), thiobarbituric acid (TBA), xylenol orange, Trichloroacetic acid, ethanol, potassium iodide, sodium potassium tartrate, 1, 2-dichloro-4-nitrobenzene (CDNB), sorbitol, hydrogen peroxide (H_2O_2), sulphosalicyclic acid, N-(1-naphthyl) Ethylenediamine dihydrochloride, cyclophosphamide (CPA), 5′, 5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) and Gallic acid (GA) were purchased from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade.

Animal model

Sixty adult male Wistar rats (200–290 g) were obtained from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan. The animals were kept in wire mesh cages under controlled light cycle (12-h light/12-h dark) and fed with commercial rat chow and water *ad libitum*. All of the animals received humane care according to the criteria outline in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health, and the ethics regulations were followed in accordance with national and institutional guidelines for the protection of the animals' welfare during experiments (PHS, 1996).

Animal treatment

The animals were randomly divided into 6 groups of 10 animals per group. Group 1 received saline and served as control. Rats in groups 2 and 3 received GA alone at 60 mg kg^{-1} (GA1) and 120 mg kg^{-1} (GA2) for 14 consecutive days, respectively. Group 4 received a single intraperitoneal dose of CPA at 200 mg kg^{-1} on day 1. Rats in groups 5 (CPA + GA1) and 6 (CPA + GA2) received a single dose of CPA (200 mg kg^{-1}) intraperitoneally on day 1 followed by treatment with GA at 60 and 120 mg kg^{-1} for 14 consecutive days, respectively. Testicular and epididymal damage was achieved by single intraperitoneal administration of cyclophosphamide according to the report of Viswanatha *et al.* (2013).

Preparation of post-mitochondrial fractions of testes and epididymis

Rats were starved overnight and the blood from each group was drawn from retro-orbital venous plexus for hormonal assay before they were sacrificed by cervical dislocation. The testes and epididymis were removed, rinsed in 1.15% KCl and homogenised in potassium phosphate buffer (0.1 M, pH 7.4). The homogenates were centrifuged at 12 000 ***g*** for 15 min to obtain the post-mitochondrial fraction. The supernatants obtained were stored at −20 °C until the time of use.

Biochemical assays

The supernatants of the testes and epididymis were used for the following biochemical assays. Superoxide dismutase (SOD) was determined by measuring the inhibition of auto-oxidation of epinephrine at pH 7.2 at 30 °C as described (Misra & Fridovich, 1972) with slight modification from our laboratory (Oyagbemi *et al.*, 2014). The activity of Catalase (CAT) was determined according to the method of Shinha (1972). The reduced glutathione (GSH) content was measured at 412 nm as described by Jollow *et al.* (1994). The glutathione-S-transferase (GST) was estimated according to the method of Habig *et al.* (1974) using 1-chloro-2, 4-dinitrobenzene (CDNB) as

substrate. Lipid peroxidation level was measured according to the method described by Farombi *et al.* (2000). Lipid peroxidation in units mg^{-1} protein was computed with a molar extinction coefficient of 1.56×10^5 $M^{-1}Cm^{-1}$. The Glutathione peroxidase activity was measured as described by Buetler *et al.* (1963). Hydrogen peroxide (H_2O_2) generation was determined as previously described (Wolff, 1994). Protein concentration was determined by the method of Gornal *et al.* (1949).

Determination of plasma LH, FSH and testosterone concentrations

Assays for concentrations of testosterone, LH and FSH were performed using the commercial enzyme immunoassay kits according to the manufacturer's instructions (DRG Diagnostics GmbH, Marburg, Germany). The sensitivity of the testosterone assay was 0.08 ng ml^{-1} and with negligible cross-reactivity with other androgen derivatives, such as androstenedione, 5α-dihydrotestosterone and methyl testosterone. The intra-assay coefficients of variation for the testosterone assay were 6%. The sensitivity of LH was 0.05 ng at 83%, whereas FSH sensitivity was 0.04 ng at 94%. The intra-assay coefficients of variation were 3.5% for LH and 3.2% for FSH.

Sperm motility and viability assays

Sperm motility was assessed by the method described by Zemjanis (1970). The epididymal sperm that were in rapid forward movement (progressive motility) were evaluated microscopically within 2–4 min of their isolation from the cauda epididymis and data were expressed as percentages. A portion of the sperm suspension placed on a glass slide was smeared out with another slide and stained with 1% eosin and 5% nigrosine in 3% sodium citrate dehydrate solution for viability according to Wells & Awa (1970).

Histopathology

Samples from testes were fixed with Bouin's solution, sectioned and stained routinely with haematoxylin and eosin for microscopy. All slides were coded before examination with light microscope by investigators who were blinded to control and treatment groups.

Statistical analysis

All values are expressed as mean ± SD. One-way ANOVA with Dunnett's post-test was performed using GraphPad Prism version 4.00 (San Diego, California, USA). Statistical values with $P < 0.05$ were considered statistical significance.

Results

Testicular and epididymal antioxidant status

Figures 1 and 2 show the effects of CPA and GA on antioxidant enzyme activities and non-enzymic antioxidant (GSH) level in the testes and epididymis of the experimental rats. There was a significant ($P < 0.05$) decrease in the SOD and GST activities in testes and epididymis of CPA-treated rats when compared to the control. CPA treatment decreased catalase (CAT) activity in the testes, whereas epididymal CAT activity was not significantly altered. In addition, GSH level was not affected in both testes and epididymis of the treated rats. However, co-treatment with GA at 60 and 120 mg kg^{-1} reversed the CPA mediated decrease in antioxidant enzymes in the testes and epididymis of the treated rats.

Levels of nitrite and hydrogen peroxide generation in testes and epididymis

The levels of nitrite and hydrogen peroxide generation in the testes and epididymis of experimental rats are presented in Fig. 3. Administration of CPA caused a significant ($P < 0.05$) elevation in levels of nitrite and H_2O_2 generation in the testes and epididymis of the treated rats. However, co-treatment with GA at 120 mg kg^{-1} (GA2) significantly decreased levels of nitrite and H_2O_2 generation in testes and epididymis to near normal in the treated rats. Co-treatment with GA at 60 mg kg^{-1} (GA1) significantly decreased levels of nitrite, but not H_2O_2 generation, in testes and epididymis of the treated rats.

Lipid peroxidation level and sperm characteristics

The level of lipid peroxidation in the testes and epididymis as well as the sperm characteristics of experimental rats is presented in Fig. 4. Administration of CPA caused a significant ($P < 0.05$) elevation of malondialdehyde (MDA) content, a biomarker of lipid peroxidation, in the testes and epididymis of the treated rats. Furthermore, CPA treatment significantly decreased sperm motility and viability when compared with control. However, co-treatment with GA (60 and 120 mg kg^{-1}) reversed the CPA-induced levels of MDA in testis and epididymis, as well as the sperm characteristics to near-normal values.

Organ weights and plasma hormone concentrations

The effects of CPA on organ weights and plasma concentrations of FSH, LH and testosterone are presented in

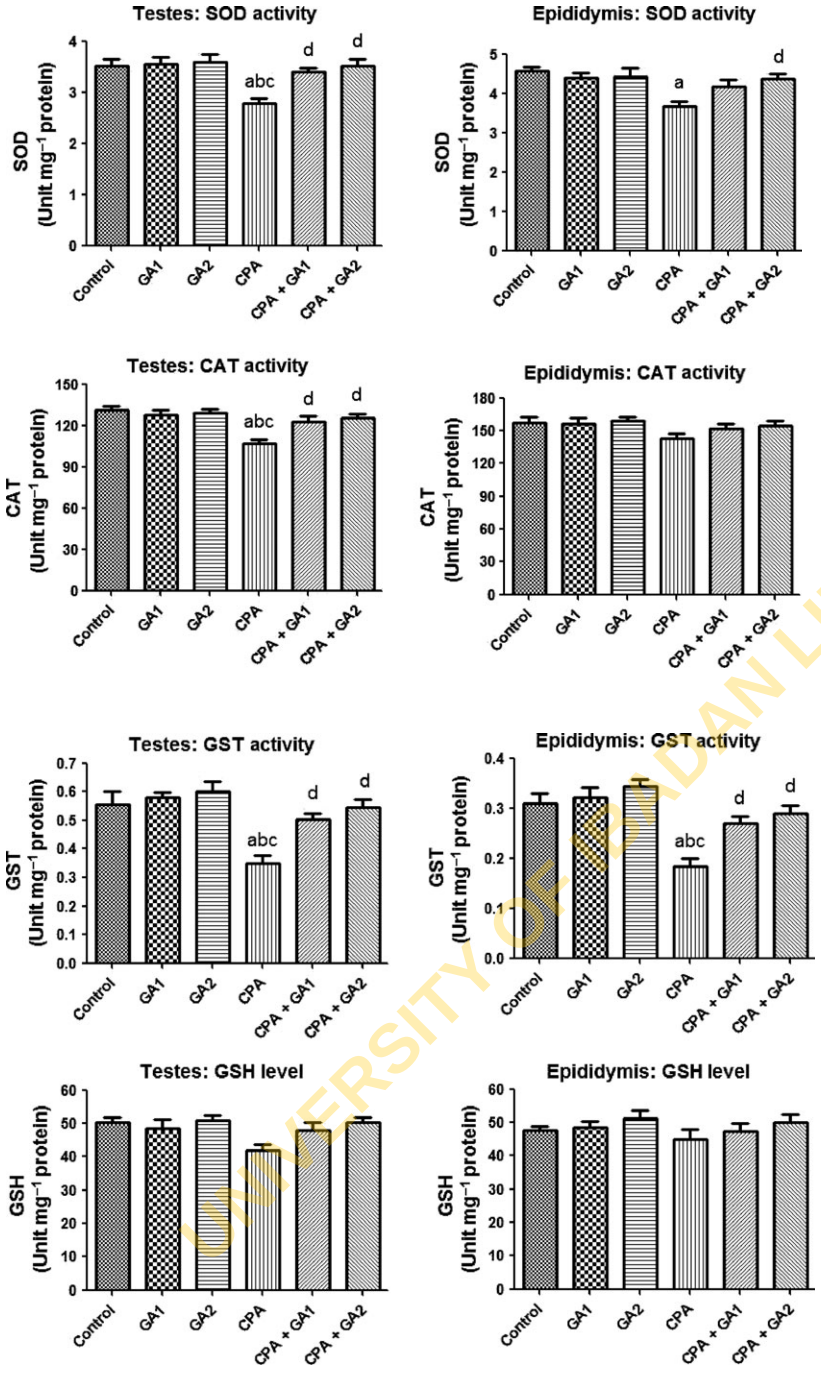

Fig. 1 Effects of gallic acid on superoxide dismutase (SOD) and catalase (CAT) activities in testes and epididymis of cyclophosphamide-treated rats. GA, gallic acid; GA1, (60 mg kg^{-1}); GA2, (120 mg kg^{-1}); CPA, cyclophosphamide. Values are expressed as mean ± SD of 10 rats. [a]: $P < 0.05$ versus Control; [b]: $P < 0.05$ versus GA1, [c]: $P < 0.05$ versus GA2; [d]: $P < 0.05$ versus CPA.

Fig. 2 Effects of gallic acid on glutathione-S-transferase (GST) activity and glutathione level (CAT) in testes and epididymis of cyclophosphamide-treated rats. GA, gallic acid; GA1, (60 mg kg^{-1}); GA2, (120 mg kg^{-1}); CPA, cyclophosphamide. Values are expressed as mean ± SD of 10 rats. [a]: $P < 0.05$ versus Control; [b]: $P < 0.05$ versus GA1, [c]: $P < 0.05$ versus GA2; [d]: $P < 0.05$ versus CPA.

Table 1. Administration of CPA caused a significant decrease in the weight of epididymis without affecting the testes when compared to the control. However, the plasma levels of FSH, LH and testosterone were markedly decreased in the CPA-treated rats. However, co-treatment with GA (60 and 120 mg kg^{-1}) reversed the CPA-induced epididymal weight reduction and FSH, LH and testosterone plasma concentrations to near-normal values.

Histology

Figures 5 and 6 represent the photomicrographs of the testes and epididymis of the experimental rats. The testes of control and rats treated with GA at 60 mg kg^{-1} (GA1) and 120 mg kg^{-1} (GA2) alone appear normal. However, the testes of CPA-treated rats revealed mild maturation of the sperm, whereas the epididymis shows severe atrophy with

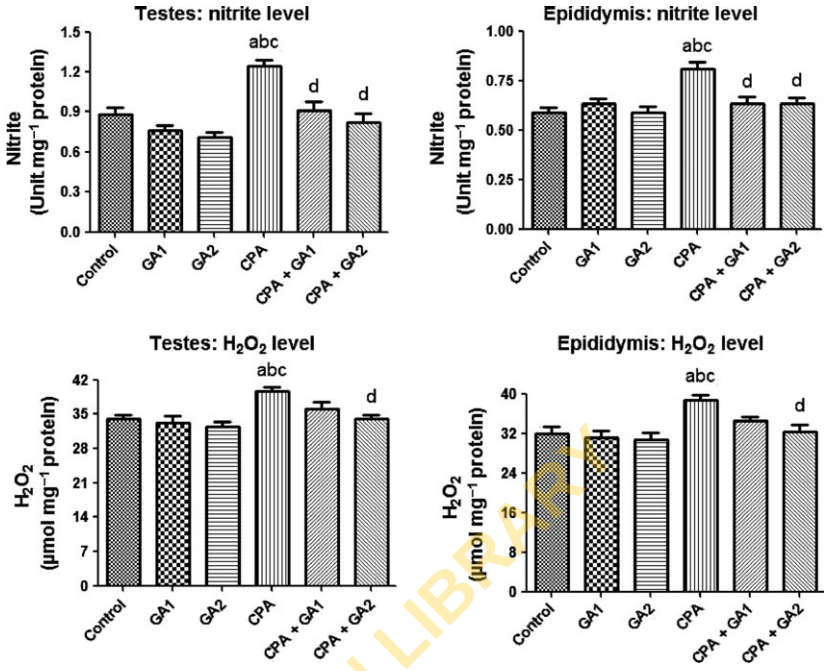

Fig. 3 Effects of gallic acid on nitrite and hydrogen peroxide (H_2O_2) generation level in testes and epididymis of cyclophosphamide-treated rats. GA, gallic acid; GA1, (60 mg kg^{-1}); GA2, (120 mg kg^{-1}); CPA, cyclophosphamide. Values are expressed as mean ± SD of 10 rats. [a]: $P < 0.05$ versus Control; [b]: $P < 0.05$ versus GA1, [c]: $P < 0.05$ versus GA2; [d]: $P < 0.05$ versus CPA.

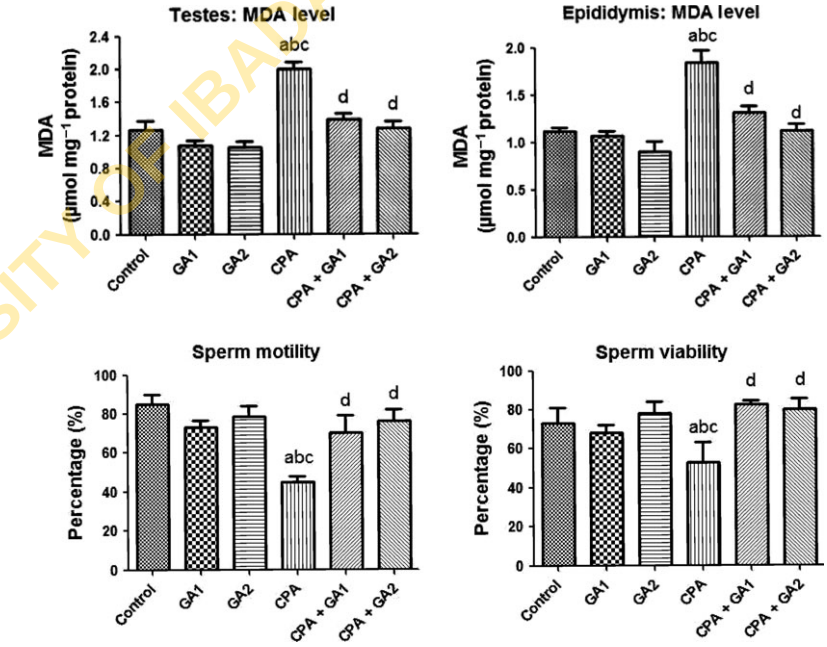

Fig. 4 Effects of Gallic acid on malondialdehyde (MDA) level and sperm characteristics in cyclophosphamide-treated rats. GA, Gallic acid; GA1, (60 mg kg^{-1}); GA2, (120 mg kg^{-1}); CPA, cyclophosphamide. Values are expressed as mean ± SD of 10 rats. [a]: $P < 0.05$ versus Control; [b]: $P < 0.05$ versus GA1, [c]: $P < 0.05$ versus GA2; [d]: $P < 0.05$ versus CPA.

thickening of the propia and columnar epithelium along with the absence of sperm in the lumen. Interestingly, the testes of rats co-treated with CPA and GA appear normal.

Discussion

Despite the advantage of CPA over other anti-cancer drugs, its use is limited due to its toxic effects, one of which is gonadal toxicity. However, the ability of CPA to induce oxidative stress through free radical generation following its treatment has been already established (Das *et al.*, 2002; Ghosh *et al.*, 2002). The results of the present study revealed that CPA administration was associated with marked oxidative stress in the reproductive tissues evident by significant increase in the levels of MDA, nitrite and H_2O_2. This result is in accordance with

previous findings that reported CPA treatment caused impairment of sperm and its fertilising ability in mice (Elangovan *et al.*, 2006; Selvakumar *et al.*, 2006a). Several research evidences suggest that nitric oxide (NO) plays a very prominent role in testicular injury and spermatogenesis. Hence, the result of the present study revealed a significant increase in nitric oxide level. Previous studies by Rosselli *et al.* (1995) showed a great correlation between nitrate/nitrite level concentration in seminal fluid and reduced infertility. Increased level of MDA, which is one of the end products of lipid peroxidation and a marker of oxidative stress, was observed to increase significantly in the testicular tissue.

Furthermore, the significant increase in MDA, NO, H_2O_2 levels was accompanied by concomitant decrease in the activities of SOD, GST and catalase enzymes, and

Table 1 Organ weights and plasma hormone concentrations

Parameters	Control	GA1	GA2	CPA	CPA + GA1	CPA + GA2
Testes (g)	1.92 + 0.26	2.00 + 0.37	2.09 + 0.44	1.80 + 0.50	2.01 + 0.12	2.00 + 0.26
Epididymis (g)	0.39 + 0.05	0.31 + 0.08	0.39 + 0.07	0.24 + 0.05^{abc}	0.31 + 0.04^{d}	0.30 + 0.07^{d}
LH (pg ml^{-1})	10.50 ± 2.12	10.50 ± 0.71	9.33 ± 0.58	7.33 ± 0.57^{a}	9.00 ± 1.41^{d}	8.50 ± 0.71^{d}
FSH (pg ml^{-1})	6.50 ± 0.71	7.50 ± 2.12^{a}	8.00 ± 1.41^{a}	4.50 ± 0.71^{abc}	4.67 ± 0.57^{d}	7.0 ± 1.41^{d}
Testosterone (pg ml^{-1})	3.85 ± 0.35	4.05 ± 0.07	5.00 ± 0.14^{a}	2.73 ± 0.21^{abc}	3.23 ± 0.15^{d}	3.50 ± 0.71^{d}

GA, Gallic acid; GA1, (60 mg kg^{-1}); GA2, (120 mg kg^{-1}); CPA, cyclophosphamide; LH, Luteinizing hormone; FSH, Follicle stimulating hormone. Values are expressed as mean ± SD of 10 rats.
[a]$P < 0.05$ versus Control.
[b]$P < 0.05$ versus GA1.
[c]$P < 0.05$ versus GA2.
[d]$P < 0.05$ versus CPA.

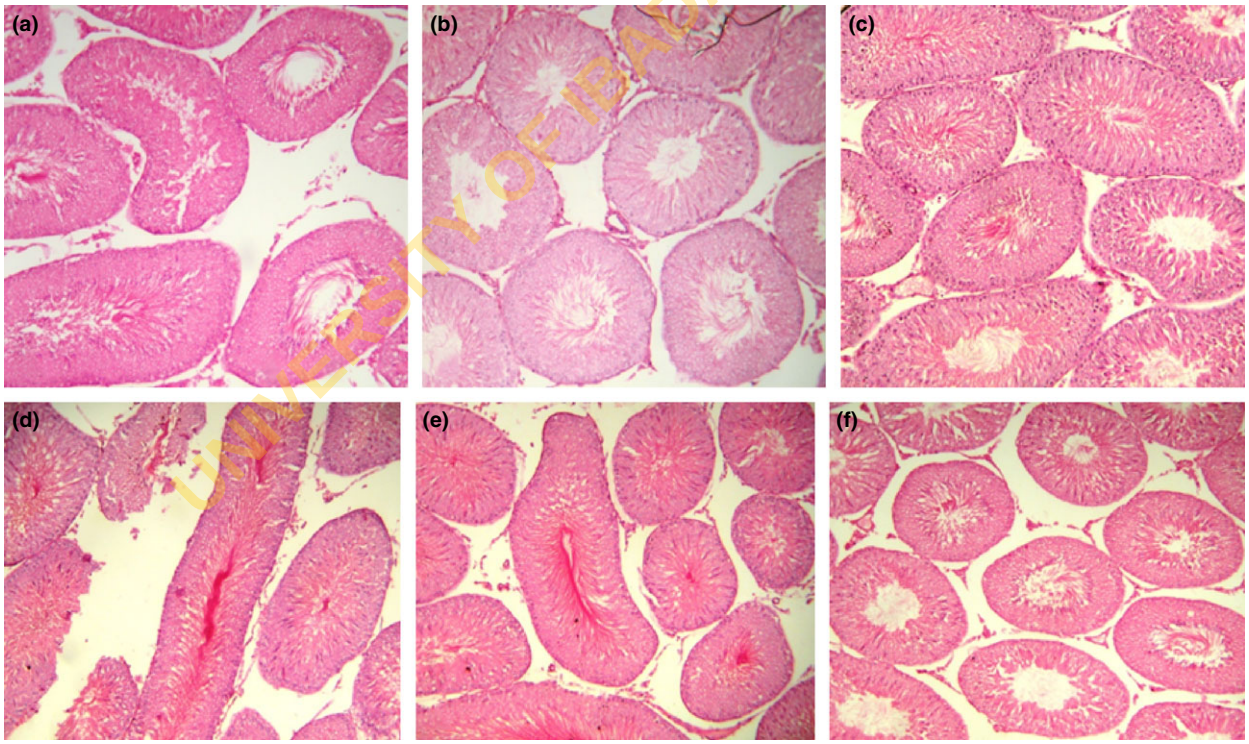

Fig. 5 Representative photomicrographs of testes from experimental rats: Photomicrograph of the testicular tissue shows circular and oblong seminiferous tubules that are filled with spermatogenic cells in different stages of maturation. Sertoli cells and Leydig cells look normal (a). Photomicrographs of the testicular tissue of rats treated with cyclophosphamide show mild maturation of the spermatozoon with the presence of spermatogenic cells in different stages of maturation (b). The photomicrographs of the testicular tissue of rats pre-treated with gallic acid (GA) and gallic acid alone show moderate maturation of the spermatozoa with the presence of spermatogenic cells, and the lumen is moderately filled with spermatozoa (c, d, e and f). The tissues are stained with H & E (×40 objectives).

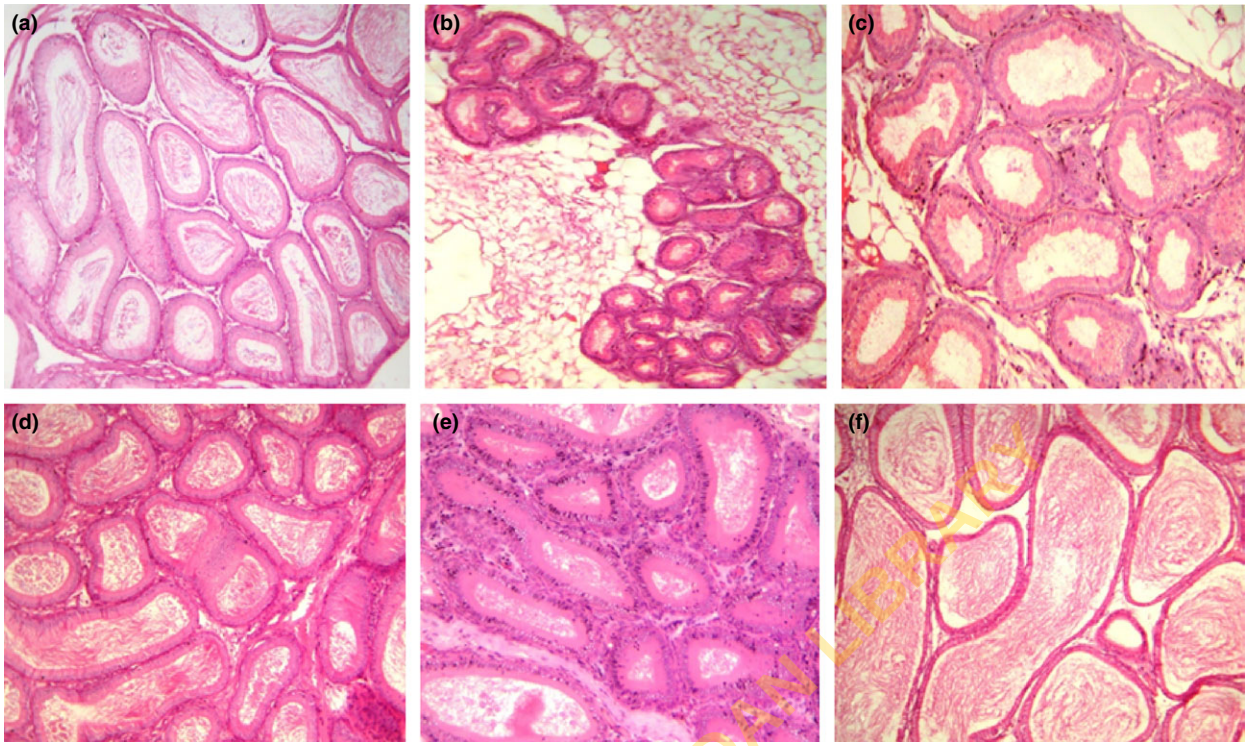

Fig. 6 Representative photomicrographs of epididymis from experimental rats: The photomicrograph of the epididymal tissue shows circular and oblong epididymal tubules that are lined with stereociliated columnar epithelium. There are adequate numbers of spermatozoa in the lumen of the tubules (a). Photomicrographs of the epididymal tissue of rats treated with cyclophosphamide show severely atrophied epididymis with thickening of the propia and columnar epithelium with the marked absence of spermatozoa in the lumen (b). The photomicrographs of the epididymal tissue of rats pre-treated wit gallic acid (60 & 120 mg kg^{-1}) show oval and irregularly shaped epididymal tubules with the marked absence of spermatozoan in the lumen of the epididymal tubules (c) with thickened of the duct propia (d). The rats that received gallic acid alone, the epididymal tissues show that lumens are completely filled with spermatozoa (e, f). The tissues are stained with H & E (×40 objectives).

level of the non-enzymatic antioxidant, GSH, in the present study. The antioxidant defence system comprising of the enzymatic and non-enzymatic antioxidants confer protection on biological tissues, including testes by their direct involvement in the removal of free radicals (Adedara & Farombi, 2010). Superoxide dismutase, the first line of defence against oxygen-derived radicals is responsible for the dismutation of superoxide radicals to H_2O_2, whereas catalase metabolically removes H_2O_2 from the intracellular environment, thereby further reducing the H_2O_2 and hydroxyl radical generation (Adedara & Farombi, 2012). The present study shows a significant reduction of testicular and epididymal SOD, catalase and GST activities in CPA-treated rats, thus suggesting the inactivation and impairment of these enzymes activities in combating the noxious effects of ROS in the reproductive tissues. The decrease in catalase activity might contribute to the increased concentration of H_2O_2 seen in the testes. However, the lack of significant decrease in the level of GSH in both testes and sperm may indicate an efficient GSH-mediated detoxification pathway to mitigate the increased production of ROS during CPA exposure. The decrease in the antioxidant enzymes activities following CPA administration in rats has been previously documented (Motawi *et al.*, 2010).

Studies have shown that in the male mammalian reproductive system, luteinising hormone stimulates the Leydig cells to produce testosterone, which is very important for the initiation and maintenance of spermatogenesis via Sertoli cell androgen receptor. Furthermore, FSH plays a very essential role in normal spermatogenesis in pubertal rats (Sriraman *et al.*, 2005). The present study reveals that the plasma concentrations of LH, FSH and testosterone were significantly reduced in the CPA-treated group. Luteinising hormone, follicle stimulating hormone and testosterone are known hormonal biomarkers of androgenicity (Walton *et al.*, 1995; Adedara & Farombi, 2013). The increase in the concentration of FSH and LH was accompanied by a significant increase in testosterone level in the groups treated with gallic acid alone (GA1 and GA2) in the present investigation, thus indicating the androgenic potential of the gallic acid. This observation

may indicate that gallic acid has stimulatory influence on the hypothalamic–pituitary–gonadal axis of the male rats. Hence, GA stimulated the HHT axis in this experimental study over a limited period of time.

This therefore suggests that CPA has a deleterious effect on the anterior pituitary gland that plays an important role in the secretion of these hormones. Das *et al.* (2002) reported that decline in serum testosterone could be due to CPA-induced membrane lipid peroxidation in the testes and this is similar to the results obtained in the present study. Studies have also shown that increased oxidative stress resulted in ROS-induced damage to the macromolecule such as DNA, proteins and key enzymes important for testicular steroidogenesis and spermatogenesis (Sen *et al.*, 2004).

From this study, there was significant reduction of sperm progressive motility in CPA-treated rats which indicates sperm damage following CPA administration. Our finding of spermatotoxicity in CPA-treated rats is in accordance with previous studies that reported CPA and cisplatin-induced testicular damage in rats (Ilbey *et al.*, 2009). Histological examination revealed that mild maturation of the sperm with the presence of spermatogenic cells in different stages of maturation are seen in the testicular tissue of CPA-treated rats. Severe atrophy of the epididymis with thickening of the propia and columnar epithelium and marked absence of sperm in the lumen are seen in the epididymal tissue of rats treated with CPA alone. However, rats pretreated with GA showed moderate-to-normal maturation of the sperm in the testes and without epididymal damage.

It is an already established fact that CPA metabolism results in the generation of free radical; therefore, the use of compounds that have antioxidant potential will be beneficial to prevent CPA-induced male reproductive toxicity. This study revealed that GA supplementation has ability to ameliorate the male reproductive toxicity induced by CPA via inhibition of lipid peroxidation, enhancement of antioxidant status and, reproductive hormone levels, as well as preservation of testicular and epididymal histology to near-normal levels.

In conclusion, GA has the potential to prevent CPA-induced male reproductive toxicity through its inherent antioxidant and free radical scavenging activity. Gallic acid may be a source of antioxidant supplement in patients undergoing chemotherapy such as CPA treatment. Further studies are needed to using GA as antioxidant supplement during chemotherapy with CPA.

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