Accepted: 15 November 2016
DOI: 10.1111/and.12782

ORIGINAL ARTICLE

WILEY andrologia
First International Journal of Andrology

6-Gingerol-rich fraction prevents disruption of histomorphometry and marker enzymes of testicular function in carbendazim-treated rats

M. Salihu | B. O. Ajayi | I. A. Adedara | E. O. Farombi

Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria

Correspondence
Ebenezer O. Farombi and Isaac A. Adedara, Drug Metabolism and Toxicology Research Laboratories, Department of Biochemistry, College of Medicine, University of Ibadan, Nigeria.
Emails: olatunde_farombi@yahoo.com and dedac2001@yahoo.co.uk

Funding information
University of Ibadan

Summary

Previous investigations demonstrated that 6-gingerol-rich fraction (6-GRF) prevented testicular toxicity via inhibition of oxidative stress and endocrine disruption in CBZ-treated rats. The influence of 6-GRF on alterations in histomorphometry and marker enzymes of testicular function in CBZ-treated rats which hitherto has not been reported was investigated in this study. The animals were orally administered either CBZ (50 mg/kg) alone or in combination with 6-GRF (50, 100 and 200 mg/kg) for 14 consecutive days. Histomorphometric analysis demonstrated that 6-GRF significantly prevented CBZ-mediated increase in the organo-somatic index of the testes and seminiferous tubular diameter as well as the reduction in epithelium height and tubular length of testes in the rats. Similarly, 6-GRF ameliorated CBZ-induced disruption in the epithelium height as well as in the proportion of tubule and interstitium of the epididymis the treated rats. Furthermore, 6-GRF prevented CBZ-mediated increase in testicular acid phosphatase activity and the decrease in testicular alkaline phosphatase, aminotransferases, glucose-6-phosphate dehydrogenase and lactate dehydrogenase activities. Moreover, 6-GRF ameliorated CBZ-induced reduction in the testicular and epididymal sperm count and sperm motility in the treated rats. Conclusively, 6-GRF enhances key functional enzymes involve in spermatogenesis and maintains histo-architecture of testes and epididymis in CBZ-treated rats.

KEYWORDS
6-gingerol-rich fraction, carbendazim, histomorphometry, marker enzymes, reproductive toxicity

UNIVERSITY OF IBADAN LIBRARY

1 | INTRODUCTION

Carbendazim (CBZ; methyl-2-benzimidazole carbamate) is a systemic broad-spectrum fungicide currently used in the control of various plant diseases (EFSA 2010). The general routes of human exposure to CBZ include inhalation of fumes during applications and ingestion of contaminated water, fruits and vegetables (Bakirci, Acay, Bakirci, & Otles, 2014). Regardless of the risks involved in the use of pesticides, they are still considered essential for farming because they enhance crop yield. The noxious effects of CBZ on mammals and birds include male reproductive toxicity, developmental toxicity and endocrine-disrupting activity (Lu, Liao, Kuo, Hwang, & Ueng, 2006; Lu et al., 2004). Previous studies demonstrated that CBZ induced testicular atrophy via sloughing of germ cells, inhibition of Sertoli cell microtubule function, induction of oxidative stress, apoptosis and DNA damage leading to infertility (Adedara, Vaithinathan, Jubendradass, Mathur, & Farombi, 2013; Aire, 2005; Bjørge et al., 1996; Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016; Yu, Guo, Xie, Liu, & Wang, 2009). Thus, recent investigations are aimed at protecting human health involve the use of antioxidants and naturally occurring substances to mitigate or inhibit oxidant-induced testicular damage due to excessive generation of free radicals from exposure to environmental contaminants.

 © 2017 Blackwell Verlag GmbH

Several herbal plants have been scientifically proven to possess pharmacological principles which make them useful as curatives for numerous ailments including infertility (D'Cruz, Vaithinathan, Jubendradass, & Mathur, 2010). *Zingiber officinale*, *which* is popularly known as ginger, is a globally marketed flavouring agent and cooking spice with a long history of human health benefits (Salihu et al., 2016). The focus on the human health benefits of ginger is on the increase due to its low toxicity and the presence of potent phytochemicals including gingerols, shogaols, zingerones and zingiberone (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016; Sekiwa, Kubota, & Kobayashi, 2000). Experimental evidence indicated that ginger has androgenic property (Kamtchouing, Mbongue-Fandio, Dimo, & Jatsa, 2002) and significantly improved reproductive function in male diabetic and hypertensive rats (Akinyemi et al., 2015; Shalaby & Hamowieh, 2010). 6-Gingerol (1-[4′-hydroxy-3′-methyoxyphenyl]-5-hydroxy-3-decanone) is the main pungent phenolic component in ginger well reported to exhibit the various pharmacological properties observed in ginger rhizome (Ajayi, Adedara, & Farombi, 2015). 6-Gingerol has been demonstrated to have numerous pharmacological activities including antioxidant, anti-inflammatory, antitumor and anticolitis activities (Ajayi et al., 2015; Chang & Kuo, 2015; Dugasani et al., 2010; Surh, 2002).

Morphometry assessments provide useful information on sensitive indicators of damage, target cells and the extent of toxicity (Almeida, Leal, & Franca, 2006; Blanco et al., 2007). Indeed, morphometric analysis of the testes and epididymis is valuable for correlations with physiological and biochemical results to understand the spermatogenesis process (Capucho et al., 2012; Costa, Faria, Fernandes, Silva, & Auharek, 2013). The seminiferous tubules of the testes are responsible for the production of sperm cells through a process known as spermatogenesis whereas the epididymis plays a pivotal role in sperm maturation, protection, transport, concentration and storage. Epididymal epithelium is involved in the secretion of different proteins and glycoproteins necessary for the maturation process (Hermo & Robaire, 2002). Thus, epithelium integrity is critical for normal spermatogenic process and its dysfunction has been linked directly to infertility (Jarvi, 2012). Moreover, numerous enzymes, including lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), glucose-6-phosphate dehydrogenase (G6PD), acid phosphatase (ACP) and alkaline phosphatase (ALP), are referred to as testicular "marker" enzymes because they play a significant role in the energy metabolism and stabilisation of testicular cells during spermatogenesis and maturation (Adedara et al., 2015; Gupta, Maikhuri, Dwivedi, & Setty, 1997; Srivastava, Sing, Srivastava, & Seth, 1990).

Although earlier investigations have demonstrated that 6-gingerol-rich fraction (6-GRF) prevented male reproductive toxicity via inhibition of oxidative stress and endocrine disruption in CBZ-treated rats (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016), it remains to be determined whether modulation of testicular and epididymal morphometry as well as the marker enzymes of testicular function contribute to the previously reported chemoprotective effects of 6-GRF on CBZ-induced reproductive dysfunction. Given these gaps in knowledge, this study analysed the epithelium height, total length and diameter of the seminiferous tubule as well as determined sperm output and marker enzymes of testicular function in adult rats co-treated with CBZ and 6-GRF to further study the health benefits of ginger in male reproduction.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Carbendazim (97% pure), thiobarbituric acid (TBA), 1-chloro-2,4-dinitrobenzene (CDNB), 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), hydrogen peroxide (H_2O_2), reduced glutathione and xylenol orange were sourced from Sigma Chemical Company (St. Louis, MO, USA). All other reagents were of analytical grade and were purchased from the British Drug Houses (Poole, Dorset, UK).

2.2 | Preparation and characterisation of 6-gingerol-rich fraction using Gas chromatography-mass spectrometry (GC-MS)

Fresh ginger rhizomes together with its foliage were purchased from a local vendor in Bodija Market, Ibadan, Nigeria. The plant samples were authenticated by Mr Dunatus Esimekhuai at the Department of Botany, University of Ibadan, where a sample (voucher specimen number UIH-22390) was deposited in the herbarium. 6-Gingerol-rich fraction was prepared from the ginger rhizomes and characterised using gas chromatography–mass spectrometry (GC-MS) as reported in our recent publication (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016).

2.3 | Animal model

Fifty adult male Wistar rats (10 weeks old; 195 ± 2 g) obtained from the Department of Biochemistry, University of Ibadan, were used for the present investigation. They were housed in plastic cages and allowed to acclimatise for a week before the commencement of the experiment. The animals were allowed access to rat chow and drinking water ad libitum and subjected to natural photoperiod of 12-hr light: 12-hr dark cycle. The experimental protocol was executed following approval by the University of Ibadan Ethical Committee and in conformity to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science USA.

2.4 | Experimental protocol

Stock solution of 6-GRF (100 mg/ml) was prepared fresh every other day with corn oil during the investigation. The rats were assigned randomly to five groups of ten rats each and were treated for 14 consecutive days as follows: Group I (Control) rats received corn oil alone at 2 ml/kg. Group II (CBZ alone) rats were orally administered CBZ dissolved in corn oil at 50 mg/kg alone. Group III (CBZ + 6-GRF1) rats were orally co-administered with CBZ and 6-GRF at 50 mg/kg. Group

UNIVERSITY OF IBADAN LIBRARY

IV (CBZ + 6-GRF2) rats were orally co-administered with CBZ and 6-GRF at 100 mg/kg. Group V (CBZ + 6-GRF3) rats were orally co-administered with CBZ and 6-GRF at 200 mg/kg. The doses of CBZ (50 mg/kg) and 6-GRF were chosen from previously published studies (WHO, 1974; Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016). It is the lowest dose of CBZ that caused significant changes in reproductive parameters whereas 50, 100 and 200 mg/kg 6-GRF were the highest effective doses against CBZ toxicity within 14 days of exposure to rats during our preliminary studies (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016). Twenty-four hours following the last treatment, blood was collected from retro-orbital venous plexus using heparin containing tubes before the rats were sacrificed by cervical dislocation. The testes and epididymides were carefully removed, weighed and subsequently processed for biochemical determinations, histology and morphometric analysis. The organo-somatic index (OSI) of the testes or epididymis was calculated using the formula, OSI = 100 × gonad weight (g)/body weight (g).

2.5 | Testicular and epididymal morphometric analysis

The testes and epididymis were fixed with Bouin's solution and processed for histology according to a standardised protocol (Bancroft & Gamble, 2008). Briefly, the fixed testes and epididymis were dehydrated using increasing concentrations of alcohol, cleared by xylene and embedded in paraffin wax. Subsequently, the tissue samples were sectioned into 4-5 μm thickness by a rotary microtome (Leica, Germany), fixed on the slides and stained routinely with haematoxylin and eosin. All slides were coded before examination under a light microscope (Olympus CH; Olympus, Tokyo, Japan) and photomicrograph taken using a Sony DSC-W 30 Cyber-shot (Sony, Tokyo, Japan) by pathologists who were blinded to control and treatment groups. Histomorphometry was performed using an ocular micrometer and image analysis software (Image J, NHI, Bethesda, Maryland, USA). Each microscopic slide was assessed in 50 random histological fields at 250× magnification per animal during the measurement of seminiferous epithelium height, tubular diameter and length. The total length of the seminiferous tubules (LST) was calculated using the formula of Dorst and Sajonski (1974) which states that $LST = TSV/\pi R^2$. Where TSV is total volume of the seminiferous tubules, R is tubular diameter/2 and $\pi = 3.14$. The proportion of the epididymal components, namely epithelium tubule and interstitium, was determined in 20 tubules per animal at 250× magnification.

2.6 | Determination of activities of marker enzymes of testicular function

The right testes were carefully excised, rinsed in ice-cold saline and homogenised in 50 mM Tris–HCl buffer (pH 7.4). The resulting homogenate was centrifuged at 10,000 *g* for 15 min at 4°C. Marker enzymes of testicular function were determined in the testes supernatant according to established method (Malymy and Horecker 1966; Vanha-Perttula & Nikkanen, 1973) which is based on the hydrolysis of *p*-nitrophenyl-phosphate in acid and alkaline medium respectively. Briefly, acid phosphatase (ACP) activity was determined by adding 150 μl of the tissue homogenate to 750 μl buffer (1.0 ml *p*-nitrophenol phosphate (PNPP) in 0.1 M acetate buffer, pH 5.0) in an Eppendorf tube. Alkaline phosphatase (ALP) activity was assayed by adding 150 μl of the tissue homogenate to 750 μl buffer (1 mM PNPP in 1.0 M Tris buffer, pH 8.0) in an Eppendorf tube. The reaction mixture for either ACP or ALP was incubated at 37°C for 30 min in a water bath before termination using 100 μl of 0.1 M NaOH. The amount of *p*-nitrophenol liberated is measured spectrophotometrically at 420 nm. Glucose-6-phosphate dehydrogenase (G6PD) activity was assayed using NADP and glucose-6-phosphate as substrates according to the established method (Dawson, Thayer, & Desforges, 1958). Lactate dehydrogenase-X (LDH-X) activity was determined according to the method of described by Vassault (1983) which is based on the interconversion of pyruvate and lactate. An equimolar amount of NADH is oxidised to NAD during the reduction of pyruvate. The rate of decrease of absorbance at 340 nm is directly proportional to LDH activity in the sample.

2.7 | Marker enzymes of amino acid metabolism

Testicular and epididymal activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed according to Reitmann and Frankel (1957) using the Randox Kit (Random Laboratories Limited, Antrim, UK).

2.8 | Evaluation of sperm progressive motility, epididymal sperm count and testicular sperm number

The sperm progressive motility was assessed according to established protocol (Zemjanis, 1970). Briefly, epididymal spermatozoon obtained by cutting the cauda epididymis with surgical blades was released onto a sterile clean glass slide, diluted carefully with 2.9% sodium citrate dehydrate solution (37°C) and covered with a 24 × 24 mm coverslip. The sperm progressive motility was subsequently evaluated in 10 microscopic fields under a phase contrast microscope at 200× magnification.

The epididymal sperm count was determined according to established method (WHO 2010). Briefly, the caudal epididymis was minced in normal saline to release the spermatozoon followed by filtration using a nylon mesh. Subsequently, 5 μl of the spermatozoa was mixed with 95 μl of diluent (0.35% formalin containing 5% $NaHCO_3$ and 0.25% trypan blue) after which 10 μl of the diluted spermatozoa was placed in the hemocytometer, allowed to sediment by standing for 5 min in a humid chamber to avoid drying before counting using the improved Neubauer (Deep 1/ 10 m; LABART, Munich, Germany) chamber with a light microscope at 400×.

Testicular sperm number was determined using frozen left testes according to standardised protocol (Blazak, Trienen, & Juniewicz, 1993). Briefly, the testes were homogenised in 25 ml physiological saline containing 0.05% (v/v) Triton X-100 for 3 min. Subsequently, 5.5 μl of the

UNIVERSITY OF IBADAN LIBRARY

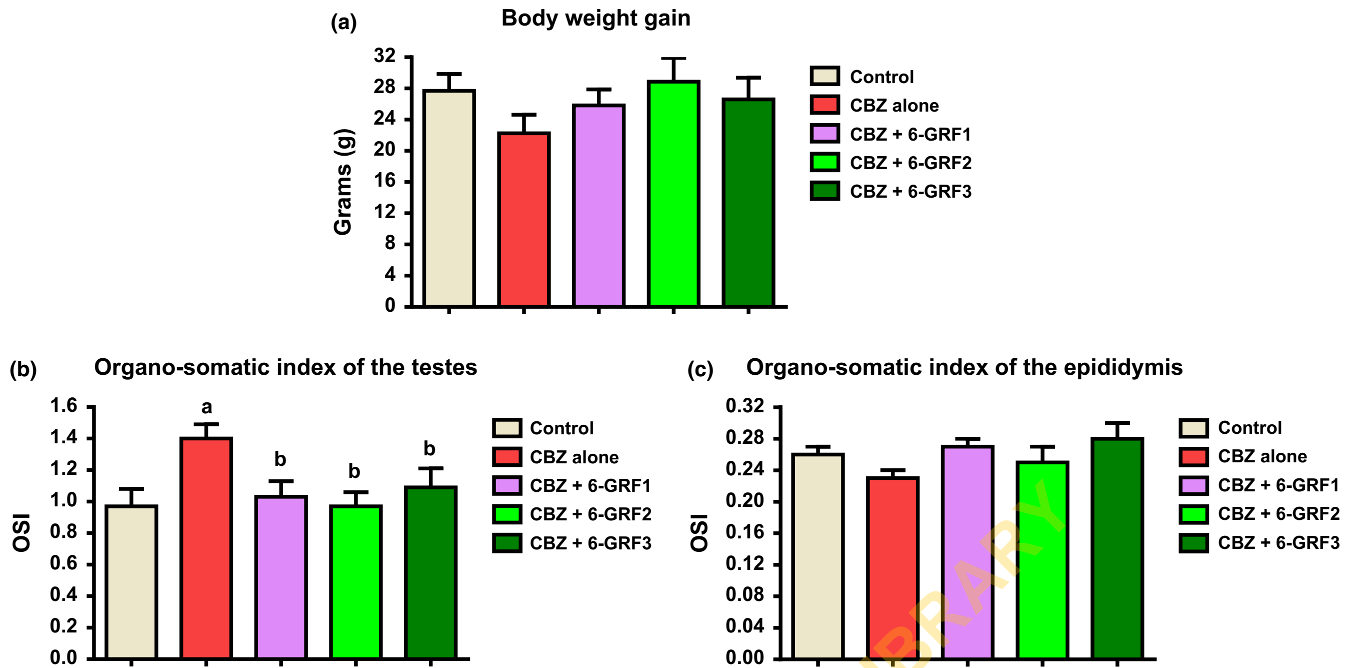

FIGURE 1 Influence of 6-GRF on body weight gain and organo-somatic index of the testes and epididymis in CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group following 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

UNIVERSITY OF IBADAN LIBRARY

resulting homogenate was placed on the haemocytometer and counted twice at 100× magnification under a light microscope to determine the average number of elongated spermatid nuclei with spermatid characteristic of steps 17–19 of spermatogenesis. These values were subsequently used to obtain the total number of spermatids per gram of testes.

2.9 | Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Bonferoni's post hoc test to identify significantly different groups using GraphPad PRISM 5 software (Version 4; GraphPad Software, La Jolla, California, USA). Results are presented as mean ± standard deviation. Values of p <.05 were considered significant.

3 | RESULTS

3.1 | Influence of 6-GRF on body weight gain and organo-somatic index of the testes and epididymis in CBZ-treated rats

The body weight gain and organo-somatic index (OSI) of the testes and epididymis of control and treatment groups are presented in Figure 1. Following an exposure period, there were no treatment-related effects on the body weight gain and OSI of the epididymis of the rats tin all the groups. However, OSI of the testes was significantly ($p < .05$) increased in the rats exposed to CBZ alone when compared with the control. Co-treatment with 6-GRF at 50, 100 and 200 mg/kg ameliorated CBZ-mediated adverse effect on the testes by decreasing the OSI of the testes to near control value in the exposed rats.

3.2 | Influence of 6-GRF on morphometric characteristics of the testes and epididymis in CBZ-treated rats

Figures 2 and 3 show the influence of 6-GRF on the morphometric characteristics of the testes and epididymis of rats orally treated with CBZ for 14 consecutive days. Administration of CBZ caused a significant ($p < .05$) increase in the seminiferous tubular diameter, whereas the seminiferous epithelium height, total tubular length per testicle and tubular length per gram testicle were significantly decreased in treated rats compared with the control. Similarly, the epithelium height of the epididymis as well as the proportion of the tubule and interstitium of the epididymis was significantly reduced in CBZ-treated rats when compared with control. However, the reduction in testes and epididymis morphometric parameters was ameliorated in rats simultaneously treated with CBZ and 6-GRF at 50, 100 and 200 mg/kg when compared with those treated with CBZ alone.

3.3 | 6-GRF prevents CBZ-induced alteration of marker enzymes of testicular function

The effects of 6-GRF on the marker enzymes of testicular function were investigated to delineate its protective mechanisms against CBZ-induced testicular toxicity in the treated rats. Figure 4 shows the influence of 6-GRF on the marker enzymes of testicular function,

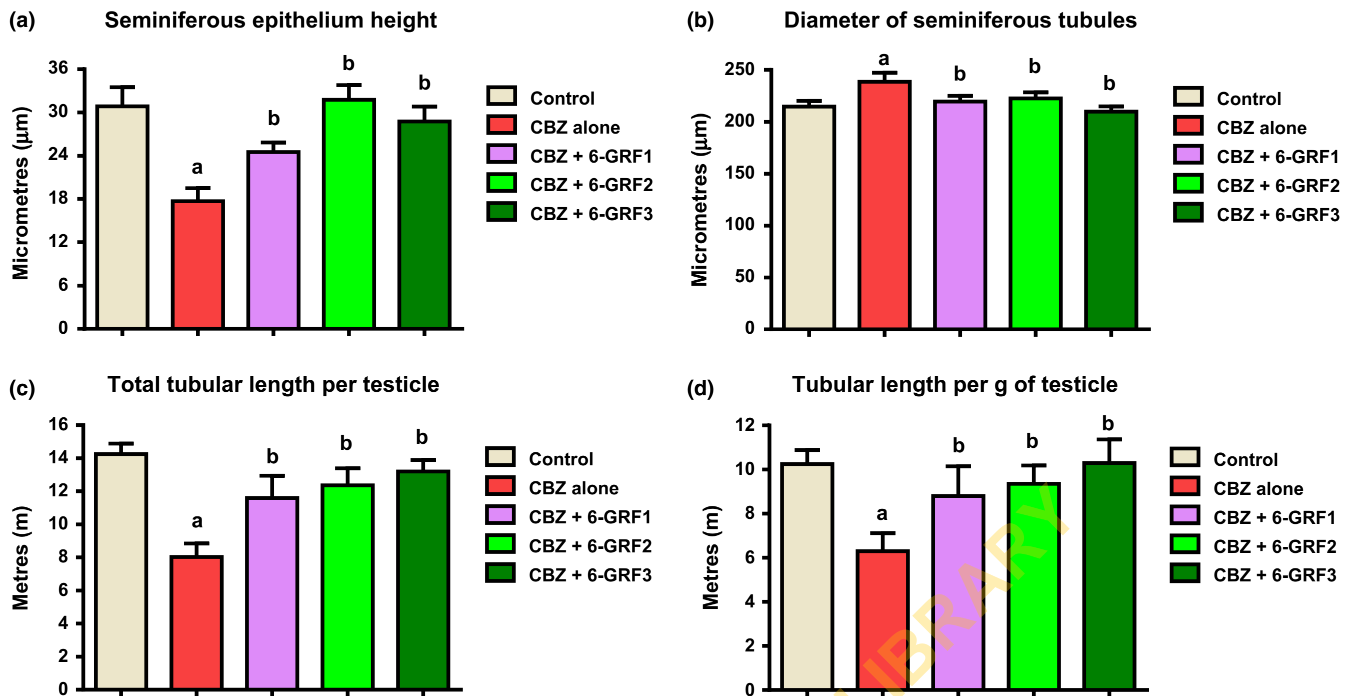

FIGURE 2 Effects of 6-GRF on seminiferous tubular diameter, epithelium height, total tubular length per testes and tubular length per gram testes of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

UNIVERSITY OF IBADAN LIBRARY

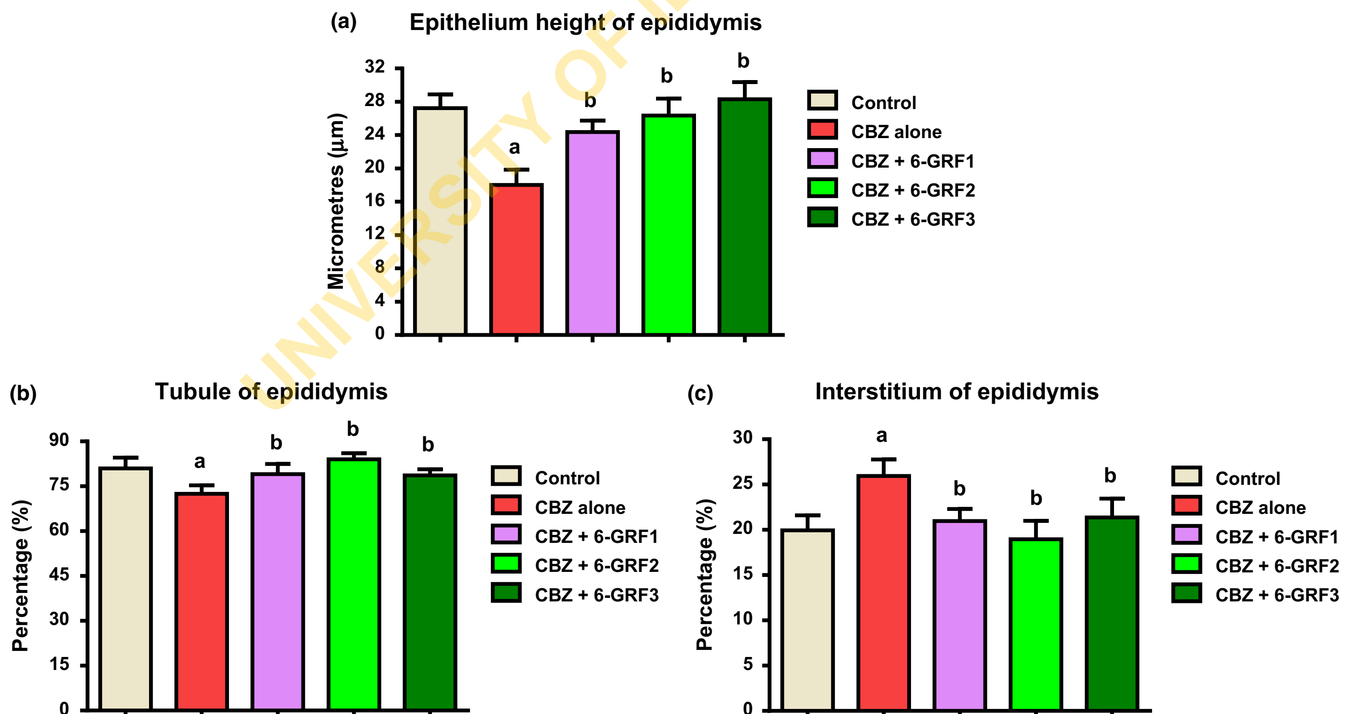

FIGURE 3 Effects of 6-GRF on epithelium height, the tubule and interstitium of the epididymis of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

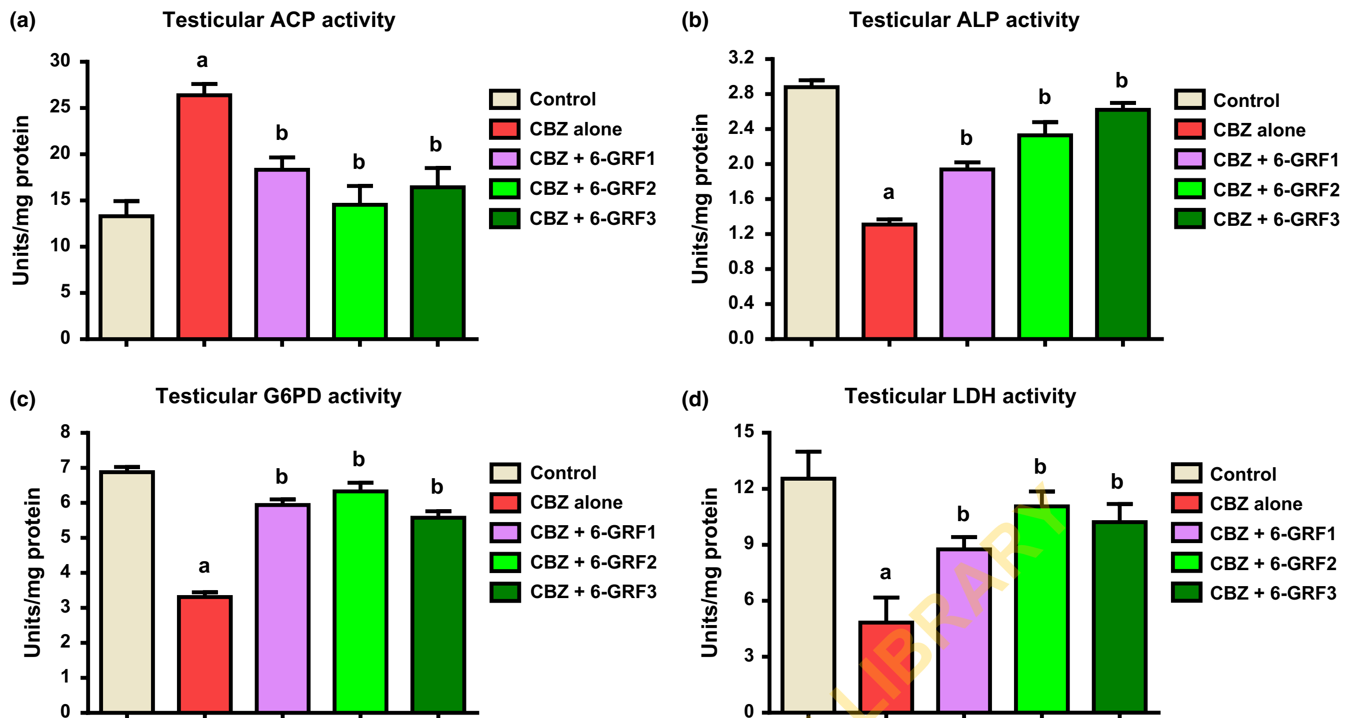

FIGURE 4 Effects of 6-GRF on ACP, ALP, G6PD and LDH in testes of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

namely ACP, ALP, G6PD and LDH in CBZ-treated rats. The testicular activity of ACP was increased significantly, whereas ALP, G6PD and LDH activities were significantly decreased in rats treated to CBZ alone when compared with control. However, co-administration of 6-GRF significantly prevented these alterations and restored the activities of these enzymes towards normalcy in CBZ-treated rats.

3.4 | Influence of 6-GRF on marker enzymes of amino acid metabolism in testes and epididymis of CBZ-treated rats

Figure 5 shows the effects of 6-GRF on the activities of ALT and AST in the testes and epididymis of the CBZ-treated rats. Administration of CBZ significantly decreased the activities of ALT and AST in the testes and epididymis of the treated rats when compared with control. However, co-administration of 6-GRF significantly increased the activities of AST and ALP in the testes and epididymis of the treated rats when compared with those exposed to CBZ alone.

3.5 | Influence of 6-GRF on sperm motility, epididymal sperm count and testicular sperm number in CBZ-treated rats

The effects of 6-GRF on sperm motility, epididymal sperm count and testicular sperm number of CBZ-treated rats are presented in Figure 6. Administration of CBZ significantly decreased sperm progressive motility, epididymal sperm count and the testicular sperm number when compared with the control group. However, co-administration of 6-GRF at 50, 100 and 200 mg/kg significantly restored these parameters to near control values in rats simultaneously treated with CBZ and 6-GRF when compared with the group treated with CBZ alone.

3.6 | Influence of 6-GRF on histological changes in testes and epididymis of CBZ-treated rats

The representative photomicrographs of the histological structures of the testis and epididymis are depicted in Figures 7 and 8. The histology of the seminiferous tubules of the control group shows the normal architecture of germinal cells, seminiferous epithelium (E), lumen (L) formation of the seminiferous tubules and the interstitial vessels (I) whereas those of the CBZ alone group showed degeneration of the seminiferous epithelium (E), the lumen (L) of some tubules and the interstitial vessels. Also, the epididymis of the control rats showed normal epithelial cells with adequate spermatozoa whereas epididymis from CBZ-treated rats presented degeneration of shape with very few sperm cells. However, histological structures of the testis and epididymis of rats co-treated with CBZ and 6-GRF at 50, 100 and 200 mg/kg showed relatively normal features.

4 | DISCUSSION

Histomorphometric analysis and assessment of marker enzymes of testicular function are commonly used to quantify testicular injury

UNIVERSITY OF IBADAN LIBRARY

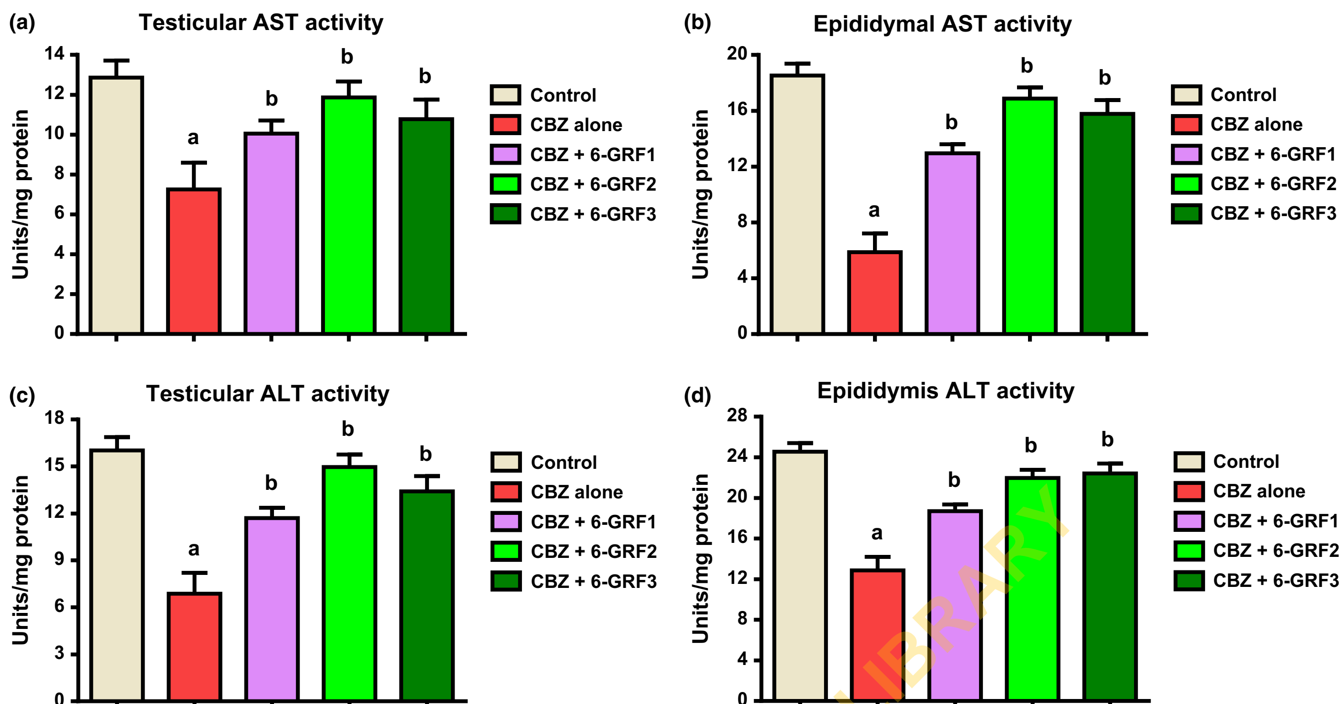

FIGURE 5 Effects of 6-GRF on AST and ALP in testes and epididymis of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

UNIVERSITY OF IBADAN LIBRARY

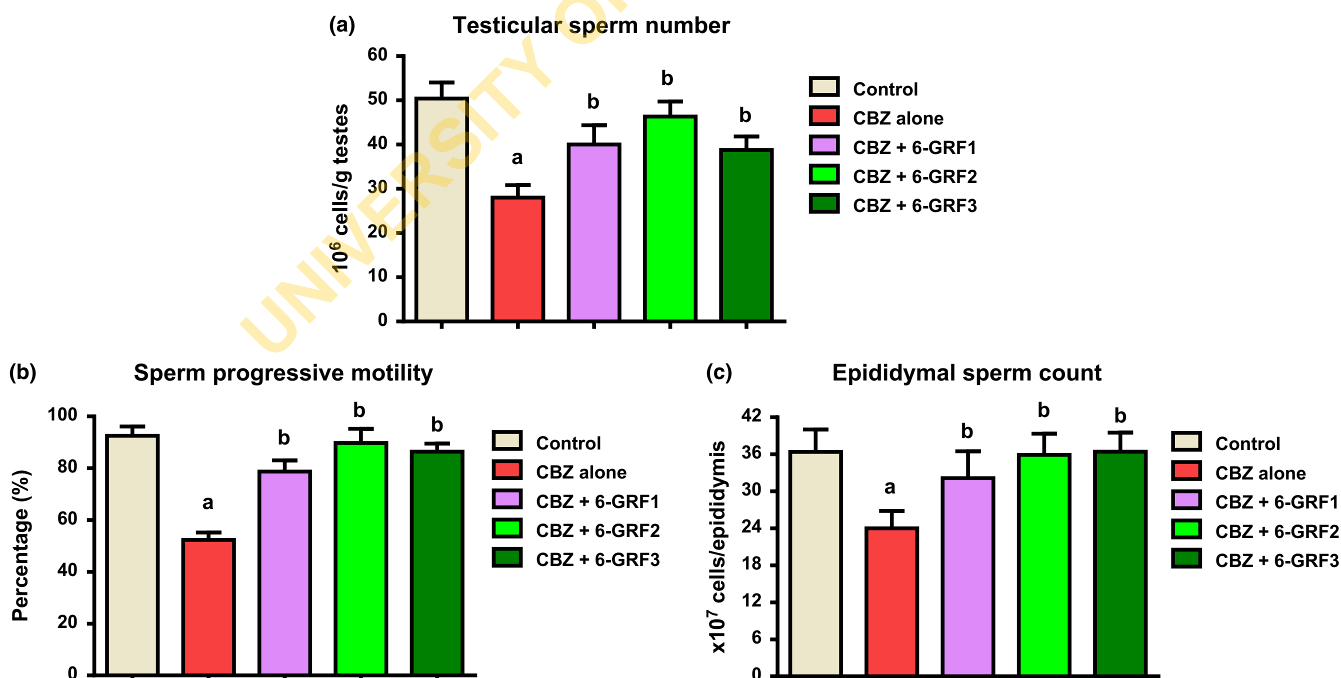

FIGURE 6 Effects of 6-GRF on testicular sperm number, sperm progressive motility and epididymal sperm count in CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]: Values differ significantly from control ($p < .05$). [b]: Values differ significantly from CBZ alone group ($p < .05$)

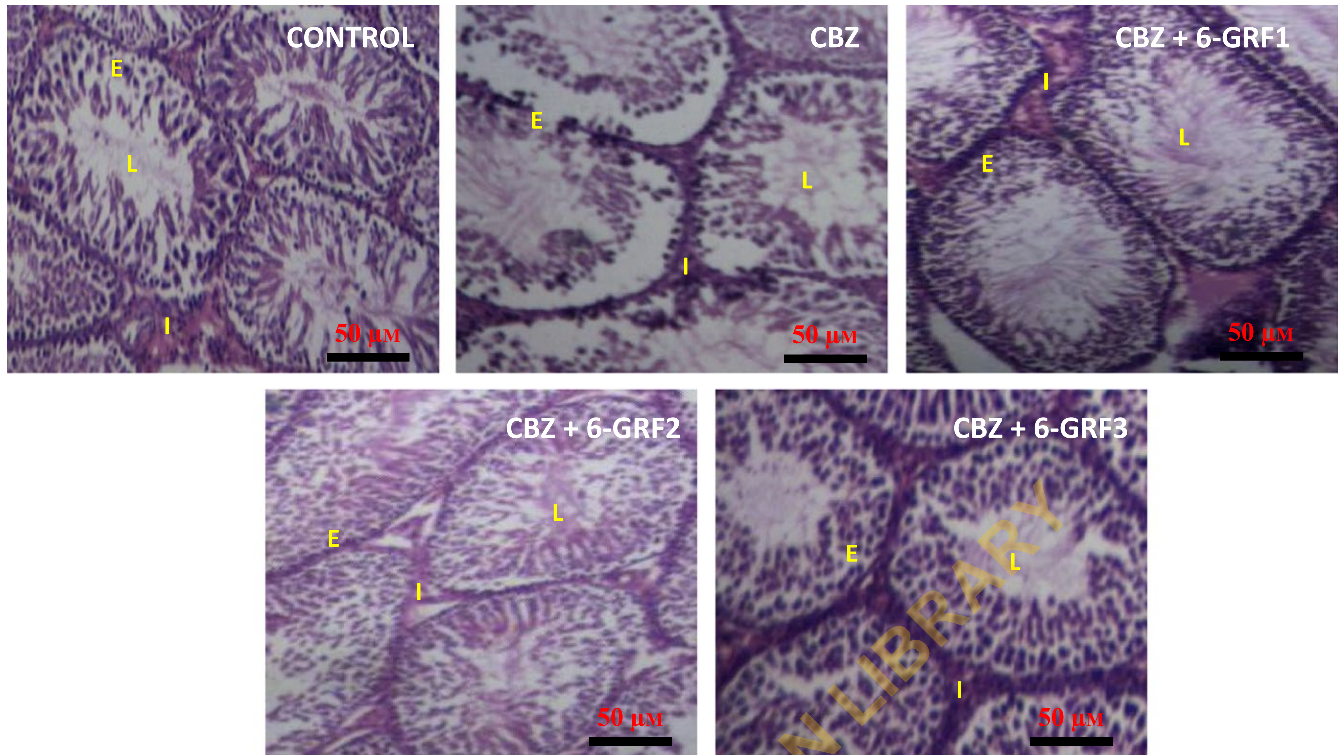

FIGURE 7 Photomicrographs showing the effects of 6-GRF on histological changes in testes of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction; Seminiferous epithelium (E), lumen (L) and the interstitial tissue (I) respectively. Control testes showing normal cytoarchitecture of the seminiferous epithelium, lumen and the interstitial tissue. CBZ alone-treated rats showing degenerated seminiferous epithelium, lumen and the interstitial tissue. Testes of rats from CBZ + 6-GRF1 showing normal cytoarchitecture with mild congestion of the interstitial vessels. Testes of rats from CBZ + 6-GRF2 and CBZ + 6-GRF3 showing normal seminiferous epithelium, lumen and the interstitial tissue. Original magnification: 250×

UNIVERSITY OF IBADAN LIBRARY

(Blanco et al., 2007; Srivastava et al., 1990). As evidenced in the biochemical, morphometry and histological analyses, the present study contributes to the dearth of knowledge about the efficacy of 6-GRF in ameliorating the modifications in histomorphometry and marker enzymes of testicular function in CBZ-treated rats.

Carbendazim is known to induce sloughing of the apical seminiferous epithelium via cleavage of the apical Sertoli cell cytoplasm leading to a cluster of sloughed spermatids with an attached portion of Sertoli cell cytoplasm (Nakai & Hess, 1994). The sloughed seminiferous epithelium fragments consequently block the efferent ducts and increased testes weight which eventually lead to marked testicular atrophy (Markelewicz, Hall, & Boekelheide, 2004). The increase in the organo-somatic index of the testes in CBZ-treated rats is attributable to fluid retention due to the impaired seminiferous tubule fluid passage from the testes to the epididymis by the germ cells that sloughed into the lumen (Nakai et al., 1992). Histomorphometrical evaluations demonstrated that 6-GRF significantly prevented alterations in the testes and epididymis architecture in CBZ-treated rats. Administration of CBZ significantly increased the seminiferous tubular diameter, whereas it decreased the seminiferous epithelium height, total tubular length per testicle and tubular length per gram testicle in the treated rats. The increase in the seminiferous tubular diameter is correlated with the accumulation of fluid previously reported (Nakai et al., 1992). The reduction in the length of seminiferous tubules and epithelium height of the testes is related primarily to the sloughing of the germ cells located in the seminiferous epithelium. Thus, the stabilisation of the organo-somatic index of testes and the restoration of seminiferous tubular diameter, epithelium height and tubular length in rats co-treated with 6-GRF signify an improvement and prevention of CBZ-induced germ cells sloughing and seminiferous tubule fluid retention in the treated rats.

Moreover, CBZ exposure significantly decreased the epithelium height and the tubule of the epididymis, whereas the interstitium of the epididymis was significantly increased in the treated rats. Sperm maturation involves the reaction of the spermatozoon with proteins that are produced and secreted in the different region of epididymis epithelium. Thus, the preservation of the epididymis integrity is vital because spermatozoon undergoes maturation and acquires progressive motility and the ability to fertilise ova in the epididymis (Cornwall, 2009). The alteration in the proportion of the tubule and interstitium of the epididymis reflects modification of its structure which could adversely impact the functional and viable state of the spermatozoon during migration and storage in the epididymis. The decrease in epithelium height of the epididymis is indicative of damage to the morphological integrity and inhibition of its functional role of proteins and glycoproteins synthesis. Interestingly, the epithelium height, the tubule and interstitium of the epididymis were improved in rats co-administered with CBZ and 6-GRF. These findings suggest the

ANDROLOGIA First International Journal of Andrology WILEY

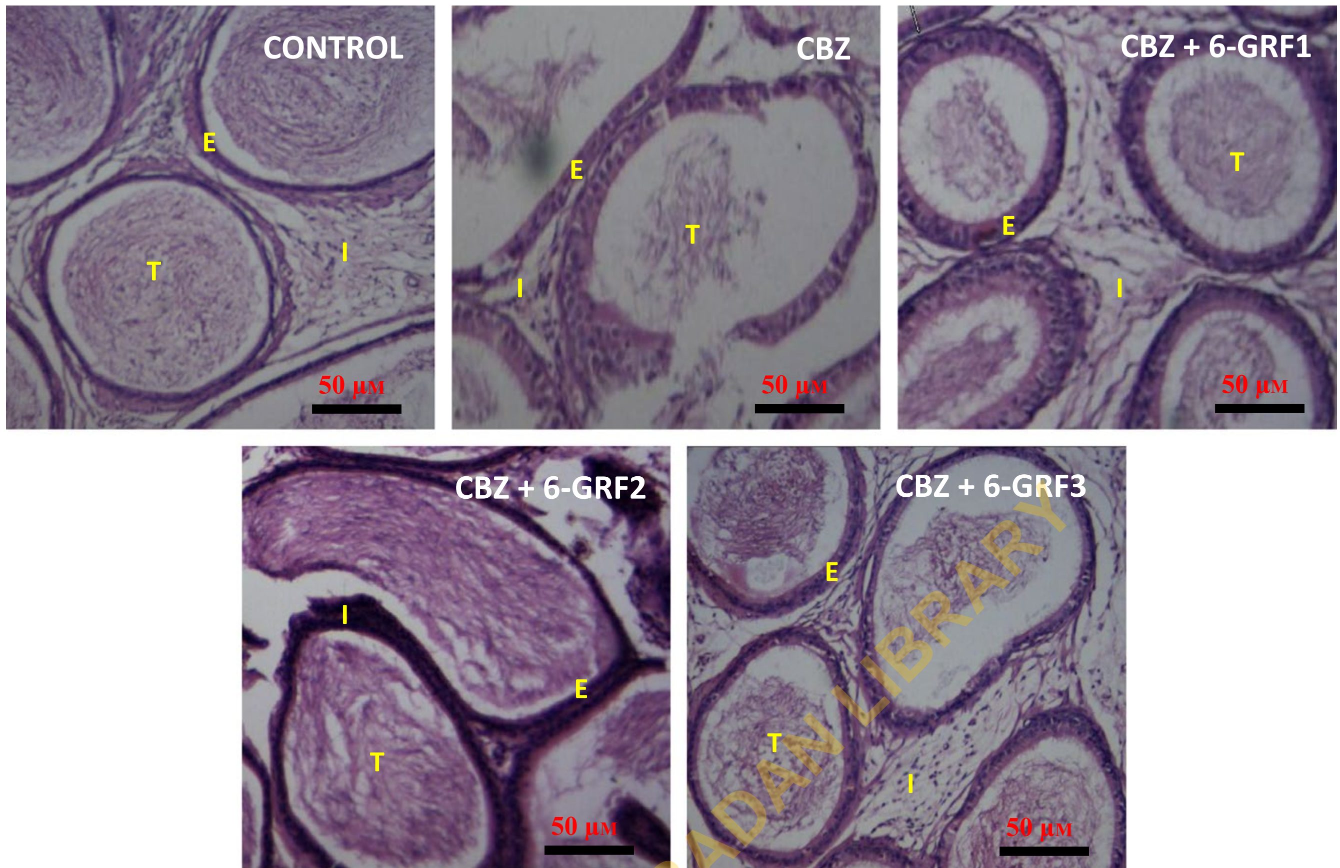

FIGURE 8 Photomicrographs showing the effects of 6-GRF on histological changes in epididymis of CBZ-treated rats. of experimental rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-gingerol-rich fraction; seminiferous epithelium (E), tubule (T) and the interstitial tissue (I) respectively. Control epididymis showing normal architecture. CBZ alone-treated rats showing degenerated epididymal lumen with inadequate sperm cells. Epididymis of rats treated with CBZ + 6-GRF1, 6-GRF2 and 6-GRF3 showing normal architecture. Original magnification: 250×

UNIVERSITY OF IBADAN LIBRARY

preservation of epididymal histomorphometry and the restoration of cellular integrity and function by 6-GRF in CBZ-treated rats.

In addition, activities of G6PD, LDH, ACP and ALP were assessed to further delineate the protective role of 6-GRF in CBZ-induced toxicity in the present study. The involvement of these marker enzymes in the energy metabolism and stabilisation of the testes is central to spermatogenesis and sperm maturation (Yan, Yue, Luo, Jin, & Xu, 2010). Testicular G6PD activity in the pentose phosphate pathway furnishes reducing equivalent nicotinamide adenine dinucleotide phosphate (NADPH) for the hydroxylation of steroids necessary for spermatogenesis (Abd El Tawab, Shahin, & AbdelMohsen, 2014). The decrease in intracellular activity of G6PD in the CBZ-treated rats indicates its adverse effect on metabolic pathways in the testicular system which may decrease the generation of NADPH and consequently impair the hydroxylation of steroids. Our previous studies have indicated that CBZ decreased steroidogenesis in the exposed rat (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016). Besides, the decreased G6PDH activity could also reduce GSH production due to reduced NADPH generation as well as decrease the production of 5-carbon sugar (i.e. ribulose-5-phosphate) required for DNA biosynthesis leading to impaired DNA replication and perturbed spermatogenesis. The lactate that is produced by the Sertoli cells is primarily utilised by the germ cells for ATP production during spermatogenesis (Bajpai, Gupta, & Setty, 1998). Thus, the observed decrease in the testicular LDH activity, a germ cell marker enzyme, is indicative of impairment in the lactate metabolism and consequently insufficient ATP generation in the spermatogenic cells due to CBZ exposure. However, administration of 6-GRF significantly restored these marker enzymes of testicular function in CBZ-treated rats to near control. These findings may indicate the spermatogenic property of 6-GRF. We hypothesise that 6-GRF, by way of maintaining the activities of G6PD and LDH, was able to mitigate CBZ-induced testicular damage.

Alkaline or acid phosphatase is a biomarker for primordial germ cells (Yan et al., 2010). The disruption in their testicular activities suggests modification of their roles in the phosphorylative pathway of testicular re-utilisation of glucose in germ cells following CBZ exposure. However, the restoration of ACP and ALP activities in rats co-treated with 6-GRF suggests the protection of the testicular germ cells from CBZ-induced degeneration. Moreover, amino transaminases namely AST and ALT are biochemical markers for amino acid metabolism due to their key role in transamination processes (Farombi, Adedara, Akinrinde, Ojo, & Eboh, 2012). Administration of CBZ markedly

decreased AST and ALT activities in the testes and epididymis of rats. The decrease in the activities of these enzymes following CBZ exposure suggests an inhibition of protein synthesis in the testes and epididymis. The reversal in the CBZ-mediated decrease in the activities of these aminotransferases by 6-GRF is indicative of its chemoprotective role in CBZ-induced testicular and epididymal dysfunction.

The noxious effects of CBZ on the biochemical and histomorphometry are well supported by the spermiogram from the treated rats. The current data indicated significant reduction in testicular sperm number, epididymal sperm count and sperm progressive motility in CBZ-treated rats compared with control. These findings demonstrated that CBZ exposure adversely impacted sperm motility and storage in epididymal milieu. The reduction in sperm quality and quantity observed in CBZ-treated rats in this study may be attributed to an outcome of increased oxidative stress in testis and epididymis (Salihu, Ajayi, Adedara, de Souza et al. 2016; Salihu, Ajayi, Adedara, & Farombi, 2016; Saradha & Mathur, 2006). Interestingly, improvement by 6-GRF was evident by the reversal of CBZ-induced diminution of spermiogram and degeneration of the testicular and epididymal architecture the treated rats.

Taken together, the present study highlights the chemoprotective role of 6-GRF in ameliorating the biochemical and histomorphometry aberrations induced in the testes and epididymis of rats by CBZ administration. The data presented herein are novel and revealed that the 6-GRF chemoprotective role in CBZ-induced male reproductive toxicity is associated with enhancement of key functional enzymes involve in spermatogenesis and maintenance of testicular and epididymal histo-architecture in rats.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ACKNOWLEDGEMENTS

This work was supported partly by the Multidisciplinary Research Grants under the Staff Training and Research Capacity Building Programme of the John D. and Catherine T. MacArthur Foundation Grant (USA) endowment from the University of Ibadan, Nigeria, awarded to EOF. The technical assistance of Dr. Ganiyat K. Oloyede and Mr. C.C. Nwabueze of the Department of Chemistry, University of Ibadan, is appreciated.

REFERENCES

Abd El Tawab, A. M., Shahin, N. N., & AbdelMohsen, M. M. (2014). Protective effect of *Satureja montana* extract on cyclophosphamide-induced testicular injury in rats. *Chemico-Biological Interactions*, *224*, 196–205.

Adedara, I. A., Awogbindin, I. O., Adesina, A. A., Oyebiyi, O. O., Lawal, T. A., & Farombi, E. O. (2015). Municipal landfill leachate-induced testicular oxidative damage is associated with biometal accumulation and endocrine disruption in rats. *Archives of Environmental Contamination and Toxicology*, *68*, 74–82.

Adedara, I. A., Vaithinathan, S., Jubendradass, R., Mathur, P. P., & Farombi, E. O. (2013). Kolaviron prevents carbendazim-induced steroidogenic dysfunction and apoptosis in testes of rats. *Environmental Toxicology and Pharmacology*, *35*, 444–453.

Aire, T. A. (2005). Short-term effects of carbendazim on the gross and microscopic features of the testes of Japanese quails (*Coturnix coturnix* japonica). *Anatomy and Embryology*, *210*, 43–49.

Ajayi, B. O., Adedara, I. A., & Farombi, E. O. (2015). Pharmacological activity of 6-Gingerol in Dextran sulphate sodium-induced ulcerative colitis in BALB/c mice. *Phytotherapy Research*, *29*, 566–572.

Akinyemi, A. J., Adedara, I. A., Thome, G. R., Morsch, V. M., Rovani, M. T., Mujica, L. K. S., … Schetinger, M. R. C. (2015). Dietary supplementation of ginger and turmeric improves reproductive function in hypertensive male rats. *Toxicology Reports*, *2*, 1357–1366.

Almeida, F. F. L., Leal, M. C., & Franca, L. R. (2006). Testes morphometry, duration of spermatogenesis and spermatogenic efficiency in wild boar (*Sus scrofa scrofa*). *Biology of Reproduction*, *75*, 792–799.

Bajpai, M., Gupta, G., & Setty, B. S. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. *European Journal of Endocrinology*, *138*, 322–327.

Bakirci, G. T., Acay, D. B. Y., Bakirci, F., & Otles, S. (2014). Pesticide residues in fruits and vegetables from the Aegean region, Turkey. *Food Chemistry*, *160*, 379–392.

Bancroft, J. D., & Gamble, M. (2008). *Theory and practice of histology techniques*, 6th ed.. Philadelphia, PA: Churchill Livingstone Elsevier.

Bjørge, C., Brunborg, G., Wiger, R., Holme, J. A., Scholz, T., Dybing, E., & Søderlund, E. J. (1996). A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. *Reproductive Toxicology*, *10*, 509–519.

Blanco, A., Moyano, R., Vivo, J., Flores-Acuña, R., Molina, A., Blanco, C., … Monterde, J. G. (2007). Quantitative changes in testicular structure in mice exposed to low doses of cadmium. *Environmental Toxicology and Pharmacology*, *23*, 96–101.

Blazak, W. F., Trienen, K. A., & Juniewicz, P. E. (1993). Application of testicular sperm head counts in the assessment of male reproductive toxicity. In R. E. Chapin, & J. Heindel (Eds.), *Methods in toxiciology*, Vol. *3A* (pp. 86–94). San Diego: Male reproductive toxicology Academic Press.

Capucho, C., Sette, R., Predes, F. S., Juliana de Castro Monteiro, J. C., Pigoso, A. A., Barbieri, R., … Severi-Aguiar, G. C. D. (2012). Green Brazilian propolis effects on sperm count and epididymis morphology and oxidative stress. *Food and Chemical Toxicology*, *50*, 3956–3962.

Chang, K. W., & Kuo, C. Y. (2015). 6-gingerol modulates proinflammatory responses in dextran sodium sulfate (DSS)-treated Caco-2 cells and experimental colitis in mice through adenosine monophosphate-activated protein kinase (AMPK) activation. *Food & Function*, *6*, 3334–3341.

Cornwall, G. A. (2009). New insights into epididymal biology and function. *Human Reproduction*, *15*, 213–227.

Costa, D. S., Faria, F. J. C., Fernandes, C. A. C., Silva, J. C. B., & Auharek, S. A. (2013). Testis morphometry and kinetics of spermatogenesis in the feral pig (*Sus scrofa*). *Animal Reproduction Science*, *142*, 63–70.

Dawson, J. P., Thayer, W. W., & Desforges, J. F. (1958). Acute hemolytic anaemia in the newborn infant due to naphthalene poisoning: Report of two cases with investigations into the mechanism of the disease. *Hematology*, *13*, 1113–1125.

D'Cruz, S. C., Vaithinathan, S., Jubendradass, R., & Mathur, P. P. (2010). Effects of plants and plant products on the testis. *Asian Society of Andrology*, *12*, 468–479.

Dorst, V. J., & Sajonski, H. (1974). Morphometrische untersuchunhen am tubulus system des schweinehodens wahrend der postnatalen entwicklug. *Monatshefte fur Veterinarmedizin*, *29*, 650–652.

Dugasani, S., Pichikac, M. R., Nadarajahc, V. D., Balijepallic, M. K., Tandraa, S., & Korlakunta, J. N. (2010). Comparative antioxidant and anti-inflammatory effects of [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol. *Journal of Ethnopharmacology*, *127*, 515–520.

UNIVERSITY OF IBADAN LIBRARY

First International Journal of Andrology ANDROLOGIA — WILEY

European Food Safety Authority. (2010). Conclusion on the peer review of the pesticide risk assessment of the active substance carbendazim. *EFSA Journal*, *8*, 1508.

Farombi, E. O., Adedara, I. A., Akinrinde, S. A., Ojo, O. O., & Eboh, A. S. (2012). Protective effects of kolaviron and quercetin on cadmium-induced testicular damage and endocrine pathology in rats. *Andrologia*, *44*, 273–284.

Gupta, G., Maikhuri, J. P., Dwivedi, A. K., & Setty, B. S. (1997). Effect of antispermatogenic compound CDRI-84/35 on marker enzymes of rat testis cells. *Contraception*, *56*, 395–400.

Hermo, L., & Robaire, B. (2002). Epididymal cell types and their functions. In B. Robaire, & B. T. Hinton (Eds.), *The epididymis—From molecules to clinical practices* (pp. 81–102). New York: Kluwer Academic/Plenum Publishersds.

Jarvi, K. (2012). Hairless or fertile? Finasteride leads to epididymal changes and infertility in rats. *Fertility and Sterility*, *97*, 1298.

Kamtchouing, P., Mbongue-Fandio, G. Y., Dimo, T., & Jatsa, H. B. (2002). Evaluation of androgenic activity of *Zingiber officinale* extract in male rats. *Asian Journal of Andrology*, *4*, 299–301.

Lu, S. Y., Liao, J. W., Kuo, M. L., Hwang, J. S., & Ueng, T. H. (2006). Antagonistic and synergistic effects of carbendazim and flutamide exposures in utero on reproductive and developmental toxicity in rats. *Journal of Food and Drug Analysis*, *14*, 120–132.

Lu, S. Y., Liao, J. W., Kuo, M. L., Wang, S. C., Hwang, J. S., & Ueng, T. H. (2004). Endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats. *Journal of Toxicology and Environmental Health, Part A*, *67*, 1501–1515.

Markelewicz, R. J. Jr, Hall, S. J., & Boekelheide, K. (2004). 2,5-hexanedione and carbendazim coexposure synergistically disrupts rat spermatogenesis despite opposing molecular effects on microtubules. *Toxicological Sciences*, *80*, 92–100.

Malymy, M., & Horecker, B. L. (1966). Alkaline phosphatase. *In Methods of Enzymology*, Vol. IX, pp. 639–642. Academic Press, New York.

Nakai, M., & Hess, R. A. (1994). Morphological changes in the rat Sertoli cell induced by the microtubule poison carbendazim. *Tissue and Cell*, *26*, 917–927.

Nakai, M., Hess, R. A., Moore, B. J., Guttroff, R. F., Strader, L. F., & Linder, R. E. (1992). Acute and long-term effects of a single dose of the fungicide carbendazim (methyl 2-benzimidazole carbamate) on the male reproductive system in the rat. *Journal of Andrology*, *13*, 507–518.

Reitmann, S., & Frankel, S. (1957). Colorimetric method for the determination of serum transaminase activity. *American Journal of Clinical Pathology*, *28*, 56–68.

Salihu, M., Ajayi, B. O., Adedara, I. A., de Souza, D., Rocha, J. B. T., & Farombi, E. O. (2016). 6-gingerol-rich fraction from *Zingiber officinale* ameliorates carbendazim-induced endocrine disruption and toxicity in testes and epididymis of rats. *Andrologia*, doi:10.1111/and.12658

Salihu, M., Ajayi, B. O., Adedara, I. A., & Farombi, E. O. (2016). 6-gingerol-rich fraction from *Zingiber officinale* prevents hematotoxicity and oxidative damage in kidney and liver of rats exposed to carbendazim. *Journal of Dietary Supplements*, *13*, 433–448.

Saradha, B., & Mathur, P. P. (2006). Effect of environmental contaminants on male reproduction. *Environmental Toxicology and Pharmacology*, *21*, 34–41.

Sekiwa, Y., Kubota, K., & Kobayashi, A. (2000). Isolation of novel glycosides from ginger and their antioxidative activity. *Journal of Agriculture and Food Chemistry*, *8*, 373–379.

Shalaby, M. A., & Hamowieh, A. R. (2010). Safety and efficacy of *Zingiber officinale* roots on fertility of male diabetic rats. *Food and Chemical Toxicology*, *48*, 2920–2924.

Srivastava, S., Sing, G. B., Srivastava, S. P., & Seth, P. K. (1990). Testicular toxicity of di- n- butyl phthalate in adult rats: Effect on marker enzymes of spermatogenesis. *Indian Journal of Experimental Biology*, *28*, 67–70.

Surh, Y. J. (2002). Anti-tumor promoting potential of selected spice ingredients with antioxidative and anti-inflammatory activities: A short review. *Food and Chemical Toxicology*, *40*, 1091–1097.

Vanha-Perttula, T., & Nikkanen, V. (1973). Acid phosphatases of the rat testis in experimental conditions. *Acta Endocrinologica*, *72*, 376–390.

Vassault, A. (1983). Lactate dehydrogenase. UV-method with pyruvate and NADH. In H. U. Bergmeyer (Ed.), *Methods of enzymatic analysis*(3rd ed). Volume *III*. (pp. 118–125). New York: Plenum.

WHO/JMPR. (1974). Evaluation of some pesticide residues in food. FAO/AGP/1973/M/9/1. WHO Pesticide Residues Series, No. 3.

World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th ed.). ISBN 978 92 4 154778 9, Geneva: World Health Organization.

Yan, L., Yue, D., Luo, H., Jin, X., & Xu, X. (2010). Effect of vitamin E supplementation on the enzymatic activity of selected markers in Aohan fine-wool sheep testis. *Animal Reproduction Science*, *122*, 264–269.

Yu, G., Guo, Q., Xie, L., Liu, Y., & Wang, X. (2009). Effects of subchronic exposure to carbendazim on spermatogenesis and fertility in male rats. *Toxicology and Industrial Health*, *25*, 41–47.

Zemjanis, R. (1970). Collection and evaluation of semen. In R. Zemjanis (Ed.), *Diagnostic and therapeutic technique in animal reproduction*, 2nd ed. (pp. 139–153). Baltimore: The William and Wilkins Company, Waverly Press Inc.

How to cite this article: Salihu M, Ajayi BO, Adedara IA, and Farombi EO. 6-Gingerol-rich fraction prevents disruption of histomorphometry and marker enzymes of testicular function in carbendazim-treated rats. *Andrologia*. 2017;49:e12782. https://doi.org/10.1111/and.12782

UNIVERSITY OF IBADAN LIBRARY