Accepted: 17 May 2016

DOI: 10.1111/and.12658

ORIGINAL ARTICLE

WILEY ANDROLOGIA

6-Gingerol-rich fraction from *Zingiber officinale* ameliorates carbendazim-induced endocrine disruption and toxicity in testes and epididymis of rats

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Summary

This study evaluated the protective effects of 6-gingerol-rich fraction (6-GRF) from *Zingiber officinale* on carbendazim (CBZ)-induced reproductive toxicity in rats. Adult male rats were treated with either CBZ (50 mg/kg) alone or in combination with 6-GRF (50, 100 and 200 mg/kg) for 14 consecutive days. Gas chromatography–mass spectrometry (GCMS) analysis revealed that 6-GRF consists of ten bioactive chemical components with 6-gingerol being the most abundant (30.76%). Administration of 6-GRF significantly ($p < .05$) prevented CBZ-mediated increase in absolute and relative testes weights as well as restored the sperm quantity and quality in the treated rats to near control. In testes and epididymis, 6-GRF significantly abolished CBZ-mediated increase in oxidative damage as well as augmented antioxidant enzymes activities and glutathione level in the treated rats. Moreover, CBZ administration alone significantly decreased plasma levels of testosterone, thyrotropin, triiodothyronine and tetraiodothyronine, whereas follicle-stimulating hormone was significantly elevated without affecting luteinising hormone and prolactin levels when compared with the control. Conversely, 6-GRF ameliorated the disruption in the hormonal levels and restored their levels to near normalcy in CBZ-treated rats. Collectively, 6-GRF inhibited the adverse effects of CBZ on the antioxidant defence systems, hormonal balance and histology of the testes and epididymis in rats.

KEYWORDS

6-gingerol-rich fraction, carbendazim, oxidative stress, rats, reproductive toxicity

1 | INTRODUCTION

There is a growing public health concern about the frequent application of pesticides because epidemiological studies have related it with different diseases including cancers, endocrine dysfunction, neurological disorders, reproductive and developmental abnormalities (Garry, Holland, Erickson, & Burroughs, 2003; Garry et al., 2002; Hanke & Jurewicz, 2004; Lesmes-Fabian & Binder, 2013). Organic and inorganic fungicides are frequently applied in agriculture to control a large number of fungal pathogens during crop production (Ivic, 2010; Zubrod, Baudy, Schulz, & Bundschuh, 2014). Carbendazim (CBZ, a benzimidazole fungicide) is one of the most global environmental contaminants of great burden to human and animal reproductive health (Sakr & Shalaby, 2014). Human beings could be directly exposed to CBZ through oral, inhalation and dermal routes, whereas indirect routes include the consumption of contaminated fruits and vegetables, drinking water, as well as residential and occupational exposure (Bakirci, Acay, Bakirci, & Otles, 2014; Boobis et al., 2008). Subsequent to exposure, CBZ is readily absorbed and metabolised rapidly. The adverse testicular effects of CBZ exposure have been shown to include

decreased sperm numbers and motility, increased sperm abnormalities, oxidative damage, reduction in the rate and stability of microtubule assembly, and induction of apoptosis (Adedara, Vaithinathan, Jubendradass, Mathur, & Farombi, 2013; Pacheco et al., 2012; Rama, Bortolan, Vieira, Gerardin, & Moreira, 2014).

Zingiber officinale (Family Zingiberaceae), commonly called ginger, is cultivated throughout Africa and Asia and is used worldwide as a spicy seasoning in food. Traditionally, the root is used in Chinese, Ayurvedic, Tibb-Unani and African herbal medicines all over the world (Dugasani et al., 2010; Khan et al., 2012; Sekiwa, Kubota, & Kobayashi, 2000). Moreover, experimental evidence showed that ginger possesses androgenic activity (Akhlaghi et al., 2014; Kamtchouing, Mbongue-Fandio, Dimo, & Jatsa, 2002). Water and methanol extracts of ginger ameliorated reproductive dysfunction in male diabetic rats by increasing the weights of sexual organs, sperm quality and quantity, serum testosterone level and mitigated histopathological damage (Shalaby & Hamowieh, 2010). Dietary supplementation of ginger attenuated age-related subfertility by improving sperm characteristics in senescent male commercial broiler breeders (Akhlaghi et al., 2014) as well as restored systolic blood pressure and reproductive function in hypertensive male rats (Akinyemi et al., 2015).

6-Gingerol (1-[4′-hydroxy-3′-methyoxyphenyl]-5-hydroxy-3-decanone is the most pungent of all the active principles found in ginger and has been shown to possess anticancer, anti-inflammatory and antioxidant properties (Surh et al., 1999; Surh, 2002; El-Ghorab, Nauman, Anjum, Hussain, & Nadeem, 2010; Dugasani et al., 2010; Salihu, Ajayi, Adedara, & Farombi, 2016). However, as one of the principal bioactive components in ginger, there is paucity of information on the influence of 6-gingerol on CBZ-induced reproductive toxicity. Thus, considering the long history of human health benefits of ginger and particularly its antioxidant and androgenic activities, this study evaluated for the first time the possible protective effects of 6-gingerol-rich fraction on some biomarkers of reproductive function in CBZ-induced toxicity in male Wistar rats.

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 | Authentication and preparation of 6-gingerol-rich fraction

Ginger rhizomes were obtained from a local vendor in Bodija Market, Ibadan, Nigeria. The rhizomes were authenticated by Mr Dunatus Esimekhuai at the Department of Botany, University of Ibadan, Ibadan, where a sample (voucher specimen number UIH-22390) was deposited in the herbarium. Briefly, the fresh rhizomes were rinsed with distilled water, sliced, air-dried and powdered. The powder was percolated in 95% ethanol and allowed to stand for seventy-two hours. This process of extraction was repeated twice, and the extract was collected, filtered and concentrated under vacuum using rotary evaporator at 45°C. The crude ethanol extract was coded FA001.

2.3 | Column chromatography

The column chromatography was carried out according to previously published procedure (Almada da Silva et al., 2012) with some modifications. Briefly, 50 g of FA001 was dissolved in ethyl acetate, absorbed over silica gel (70-230), defatted with n-hexane and isocratically eluted with n-hexane and ethyl acetate (1:1), resulting in 10 fractions (250 ml each). Fractions 1-6 were rich in 6-gingerol when compared on thin-layer chromatography plate with the standard 6-gingerol (Natural Remedies Ltd., Bangalore, India). The fractions possessing the same Rf value with the standard were pooled, concentrated at reduced pressure to give a yellow liquid residue (6-gingerol-rich fraction, i.e. 6-GRF) which was subsequently subjected to gas chromatography–mass spectrometry (GC-MS) analysis.

2.4 | Gas chromatography–mass spectrometry (GC-MS) analysis of 6-GRF

The chemical constituents of 6-GRF was determined by GC-MS-QP2010 (Shimadzu Co. Ltd., Kyoto, Japan) using a capillary column (30.0 m × 0.25 mm RTX-5MS column; thickness: 0.25 μm). The 6-GRF samples were diluted using ethanol, collected into 1.5-ml vials specific for GC-MS which was subsequently used for the analysis. The operating conditions were split-injector at 250°C; helium at a constant flow rate of 5.4 ml/min and a linear velocity of 39.7 cm/s was employed as a carrier gas (Pressure: 68.2 KPa). The column oven temperature was programmed to have a starting temperature of 40°C, held for 1 min, and then raised to 300°C. The mass detector was operated in electron impact mode with an ionisation energy of 70 eV, a scanning range of 30–800 atomic mass units (amu). The result was expressed as molecular ion, and its fragments are described as the relationship between their atomic mass and corresponding charge (m/z), together with their per cent relative abundance (%).

2.5 | Animal model

Fifty adult male Wistar rats (10 weeks old; 188 ± 6 g) obtained from the Department of Biochemistry, University of Ibadan were used for this study. They were housed in plastic cages placed in a well-ventilated rat house, provided rat chow and water ad libitum and subjected to natural photoperiod of 12-hr light:12-hr dark cycle. The experimental protocol was carried out after approval and in accordance with the guidelines set by the University of Ibadan Ethical

Committee, which conformed to the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Science and published by the National Institute of Health.

2.6 | Experimental protocol

Stock solution of 6-GRF (100 mg/ml) was prepared fresh every other day with corn oil during this study. The rats were assigned randomly to five groups of ten rats per group and were treated for 14 days as follows: Group I rats received corn oil alone at 2 ml/kg and served as control. Group II rats were orally treated with CBZ dissolved in corn oil at 50 mg/kg alone. Group III rats were orally co-treated with CBZ and 6-GRF at 50 mg/kg (6-GRF1). Group IV rats were orally co-treated with CBZ and 6-GRF at 100 mg/kg (6-GRF2). Group V rats orally were orally co-treated with CBZ and 6-GRF at 200 mg/kg (6-GRF3). The dose of CBZ (50 mg/kg) was chosen from previously published study (WHO, 1974), whereas 50, 100 and 200 mg/kg 6-GRF and the duration of treatment were selected from our preliminary studies (Salihu et al., 2016). Twenty-four hours after the last treatment, the rats were sacrificed by cervical dislocation, and blood was collected from retro-orbital venous plexus using heparin-containing tubes. Plasma samples obtained by centrifugation of the blood at 3000 g for 10 min were subsequently stored frozen at −20°C until the determination of hormones concentrations. The testes and epididymides were quickly excised, weighed and subsequently processed for biochemical assays and histology.

2.7 | Assay of plasma LH, FSH, prolactin and testosterone concentrations

The circulatory concentrations of LH (RPN 2562, Amersham, UK), FSH (RPN 2560, Amersham, UK), prolactin (ab113351, Abcam, UK) and testosterone (EIA-5179, DRG Diagnostics GmbH, Marburg, Germany) were assessed with the aid of an ELISA strip reader (Robonik India Private Limited, Mumbai, India) using the commercial enzyme immunoassay kits specific for rats according to the manufacturer's protocols. The sensitivity of LH was 0.07 ng at 78%, and FSH sensitivity was 0.06 ng at 95%, whereas prolactin sensitivity was 0.08 ng at 92%. The intra-assay coefficients of variations were 3.2%, 3.4% and 3.5% for LH, FSH and prolactin respectively. The sensitivity of the testosterone assay was 0.05 ng/ml with negligible cross-reactivity with other androgen derivatives such as androstenedione, methyl testosterone and 5α-dihydrotestosterone. The intra-assay coefficient of variation was 3.8%. All the samples were assayed on the same day to avoid the inter-assay variation.

2.8 | Assay of plasma concentrations of thyrotropin, triiodothyronine and tetraiodothyronine

Plasma concentrations of thyrotropin (TSH), triiodothyronine (T_3) and tetraiodothyronine (T_4) were assayed using with the aid of an ELISA strip reader (Robonik India Private Limited, Mumbai, India) using radioimmunoassay kit obtained from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD). The limit of detection for TSH was 0.25 ng at 85%, whereas the intra-assay coefficient of variation was 2.1% for TSH. The total plasma triiodothyronine (T3) and tetraiodothyronine (T4) concentrations were determined using the commercial enzyme immunoassay kits (DiaSorin, Sauggia, Italy) according to the manufacturer's instructions. Sensitivity of the assays was 225 pg/dl for total T3 and 53 pg/dl for total T4. Intra-assays coefficients of variation for total T3 was 3.2–4.4% while total T4 was 2.8–3.0%. All the samples were assayed on the same day to avoid the inter-assay variation. The total plasma T_3 and T_4 concentrations were expressed as ng/dl and μg/dl respectively.

2.9 | Antioxidant status of the testes and epididymis

The testes and epididymis of control and treated rats were homogenised in 50 mM Tris–HCl buffer (pH 7.4) containing 1.15% potassium chloride to obtain the homogenate which was centrifuged at 10,000 g for 15 min at 4°C. Subsequently, the supernatant was collected for the biochemical determinations. Protein concentration of the samples was determined according to the method of Lowry, Rosenbrough, Farr, and Randall (1951). Hydrogen peroxide (H_2O_2) generation was assayed according to the method of Wolff (1994). The level of malondialdehyde (MDA), an index of lipid peroxidation, was assayed according to the method described by Farombi, Tahnteng, Agboola, Nwankwo, and Emerole (2000). Superoxide dismutase (SOD) activity was determined according to the method of Misra and Fridovich (1972), whereas catalase (CAT) activity was determined using hydrogen peroxide as a substrate according to established protocol (Clairborne (1995). The level of reduced glutathione (GSH) was assessed according to the method of Jollow, Mitchell, Zampaglione, and Gillette (1974). Glutathione-Stransferase (GST) activity was determined according to the method of Habig, Pabst, and Jakoby (1974), whereas glutathione peroxidase (GPx) activity was determined according to the method of Rotruck et al. (1973).

2.10 | Evaluation of sperm progressive motility and epididymal sperm number

The sperm progressive motility was evaluated according to established protocol (Zemjanis, 1970). Briefly, epididymal spermatozoon was obtained by cutting the cauda epididymis with surgical blades. The spermatozoon was released onto a sterile clean glass slide, diluted properly with 2.9% sodium citrate dehydrate solution which had been pre-warmed to 37°C and covered with a 24 × 24 mm coverslip. Subsequently, the sperm motility was observed in 10 microscopic fields under a phase contrast microscope at 200× magnification. Sperm motility was calculated by scoring the number of progressive spermatozoa, followed by the nonprogressive and then the immotile spermatozoa in the same field. The result was expressed as percentage of sperm progressive motility. Epididymal sperm number (ESN) was determined according to established method (World Health Organization, 2010). Briefly, the sperm samples were obtained by

mincing the caudal epididymis in normal saline and then filtered using a nylon mesh. An aliquot of 5 µl of the spermatozoa was mixed with 95 µl of diluent (0.35% formalin containing 5% $NaHCO_3$ and 0.25% trypan blue). Subsequently, 10 µl of the diluted spermatozoa was transferred to the hemocytometer, allowed to sediment by standing for 5 min in a humid chamber to avoid drying prior to counting using the improved Neubauer (Deep 1/ 10 m; LABART, Munich, Germany) chamber with a light microscope at ×400.

2.11 | Sperm morphological abnormalities and percentage viability assay

Total sperm abnormalities and viability assay were determined according to the method of Wells and Awa (1970). Briefly, aliquot of the sperm suspension placed on a glass slide was smeared out with another slide and stained with Wells and Awa's stain (0.2 g eosin and 0.6 g fast green dissolved in distilled water and ethanol in a 2:1 ratio) for morphological examination. A total of 400 spermatozoa from each rat were used for morphologic examination. The sperm viability was determined using a different stain containing 1% eosin and 5% nigrosine in 3% sodium citrate dehydrate solution.

2.12 | Determination of daily sperm production (DSP) and testicular sperm number (TSN)

Daily sperm production was determined using frozen left testes according to established protocol (Joyce, Porcelli, & Cooke, 1993). Briefly, the testes were homogenised in 25 ml physiological saline containing 0.05% (v/v) Triton X-100 for three minutes. Subsequently, 5.5 µl of the 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 (Amann, Johnson, Thompson, & Pickett, 1976; Hess, 1990). These values were subsequently used to obtain the total number of spermatids per gram of testes. Moreover, as developing spermatids spend 6.1 days in rats, the values for the number of spermatids per testis were divided by 6.1 to obtain daily sperm production.

2.13 | Histological examination of the testes and epididymis

The testes and epididymis were fixed with Bouin's solution and processed for histology according to established procedure (Bancroft & Gamble, 2008). Briefly, the fixed testes and epididymis were dehydrated using increasing concentrations of alcohol, cleared by xylene and embedded in paraffin wax. The tissues were subsequently cut into 4- to 5-µm sections by a microtome, fixed on the slides and stained routinely with hematoxylin and eosin. All slides were coded before examination under a light microscope (Olympus CH; Olympus, Tokyo, Japan), and photomicrographs were taken with a Sony DSC-W 30 Cyber-shot (Sony, Tokyo, Japan) by pathologists who were blinded to the treatment groups.

2.14 | Statistical analysis

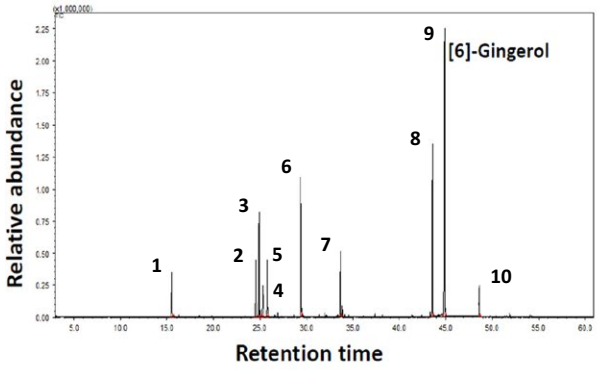
All the data in this study were tested and confirmed to be normally distributed and homoscedastic with the Levene test and residual distribution. Further statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Dunnett post hoc test to identify significantly different groups (SPSS for Windows, version 17). Results are presented as mean ± standard deviation. Values of $p < .05$ were considered significant.

3 | RESULTS

3.1 | Chemical characterisation of 6-GRF

The GC-MS fingerprinting of 6-GRF is presented in Fig. 1. The chromatogram of 6-GRF shows 10 different phytochemicals identified as

(a)



(b)

Peak	Ret. time	Area	Area (%)	Name*	Similarity* (%)	STRUCTURE
1	15.518	1095068	4.17	trans-2-Tridecen-1-ol	90	
2	24.539	1317701	5.02	alpha-Curcumene	88	
3	24.930	2565333	9.78	trans-alpha-Bergamotene	91	
4	25.318	959096	3.66	alpha-Farnesene	88	
5	25.786	1274253	4.86	beta-Sesquiphellandrene	92	
6	29.409	3809381	14.52	Zingerone	86	
7	33.665	1562260	5.96	Cyclopropanemethanol	84	
8	43.570	4440797	16.93	Zingibereno	82	
9	44.876	8070710	30.76	Gingerol	87	
10	48.612	1137820	4.34	Matairesinol	78	

* Name and similarity were based on NIST08 library

FIGURE 1 The gas chromatography–mass spectrometry (GC-MS) fingerprinting of 6-GRF (a). The table showing details of 10 different phytochemicals constituents of 6-GRF (b)

trans-2-tridecen-1-ol (peak 1, Rt 15.52 min), alpha-curcumene (peak 2, Rt 24.54 min), trans-alpha-bergamotene (peak 3, Rt 24.93 min), alpha-farnesene (peak 4, Rt 25.32 min), beta-sesquiphellandrene (peak 5, Rt 25.79 min), zingerone (peak 6, Rt 29.41 min), cyclopropanemethanol (peak 7, Rt 33.67 min), zingibereno (peak 8, Rt 43.57 min), gingerol (peak 9, Rt 44.88 min) and matairesinol (peak 10, Rt 48.61 min). The most abundant of the active principles of the fraction is 6-gingerol (30.76%), followed by zingiberone (16.93%) and zingerone (14.52%), whereas the least abundant phytochemical is alpha-Farnesene (3.66%).

3.2 | Effects of 6-GRF on body weight gain and relative organ weight in CBZ-treated rats

The data on the body weight gain and the relative organ weights are presented in Table 1. The results showed that, in comparison with the control rats, the absolute and relative weights of the testes were significantly ($p < .05$) increased in the rats exposed to CBZ alone, whereas there were no treatment-related effects on the body weight gain as well as the absolute and relative weights of the epididymis, seminal vesicle and prostate gland. Administration of 6-GRF at all the investigated doses restored the absolute and relative testes weights to near control. The body weight gain in the co-treatment groups had a trend to decrease although it was not statistically significant.

3.3 | Effects of 6-GRF on SOD and CAT activities and level of H_2O_2 in testes and epididymis of CBZ-treated rats

The modulatory effects of 6-GRF on SOD and CAT activities and the level of H_2O_2 in CBZ-treated rats are presented in Fig. 2. In comparison with control, administration of CBZ significantly ($p < .05$) reduced the CAT activity without affecting SOD activity in the testes, whereas it significantly decreased the activities of SOD and CAT in epididymis of the treated rats. Moreover, CBZ treatment caused a significant elevation in the level of H_2O_2 in the testes and epididymis of the treated rats. Carbendazim alone decreased testicular CAT activity from 37.72 ± 1.18 Unit/mg protein (control) to 22.34 ± 0.83 Unit/mg protein, and epididymal SOD activity was decreased from 0.83 ± 0.12 Unit/mg protein to 0.47 ± 0.11 Unit/mg protein, whereas epididymal CAT activity was decreased from 85.0 ± 1.06 Unit/mg protein (control) to 52.2 ± 1.81 Unit/mg protein. Testicular H_2O_2 level was increased from 41.33 ± 2.26 Unit/mg protein (control) to 63.67 ± 1.14 Unit/mg protein, whereas epididymal H_2O_2 level was elevated from 64.68 ± 2.88 Unit/mg protein (control) to 125.70 ± 3.81 Unit/mg protein following treatment with CBZ alone.

Conversely, co-treatment with 6-GRF1, 6-GRF2 and 6-GRF3 significantly reversed the CBZ-mediated decrease in the activities of SOD and CAT, and restored the H_2O_2 level to near control in testes and epididymis of the treated rats. 6-GRF1, 6-GRF2 and 6-GRF3 increased the testicular CAT activity (27.87 ± 0.98; 31.63 ± 1.07, 34.85 ± 0.75 Unit/mg protein), whereas it increased epididymal SOD (0.69 ± 0.11, 0.72 ± 0.13, 0.78 ± 0.11 Unit/mg protein) and epididymal CAT (71.02 ± 1.9, 80.02 ± 2.07, 81.04 ± 1.75 Unit/mg protein) respectively. 6-GRF1, 6-GRF2 and 6-GRF3 decreased testicular H_2O_2 level (50.12 ± 1.54, 46.36 ± 2.07, 48.2 ± 1.75 Unit/mg protein) and epididymal H_2O_2 level (84.32 ± 3.01, 70.31 ± 2.07, 74.52 ± 3.22 Unit/mg protein) respectively.

3.4 | Effects of 6-GRF on GSH level and activities of GSH-dependent enzymes in testes and epididymis of CBZ-treated rats

Figure 3 depicts the effects of CBZ exposure and 6-GRF administration on GSH level and activities of GSH-dependent enzymes in the testes and epididymis of experimental rats. In the testes, administration of CBZ significantly decreased GPx and GST activities and the level of GSH in the treated rats. Furthermore, CBZ exposure caused a significant decrease in GST activity and GSH level without affecting GPx activity in epididymis of the treated rats. Co-administration of 6-GRF1, 6-GRF2 and 6-GRF3 ameliorated the decrease in the activities of these antioxidant enzymes and GSH level, and restored their normalcy in CBZ-treated rats.

TABLE 1 Effects of 6-GRF on body weight gain and organ weights of CBZ-treated rats

	Control	CBZ alone	CBZ + 6-GRF1	CBZ + 6-GRF2	CBZ + 6-GRF3
Body weight gain	39.23 ± 4.00	40.22 ± 2.00	38.68 ± 5.46	36.50 ± 1.73	34.60 ± 3.62
Testis (g)	2.39 ± 0.11	3.97 ± 0.13[a]	2.41 ± 0.10[b]	2.46 ± 0.14[b]	2.38 ± 0.17[b]
Testis (g/100 g bw)	1.52 ± 0.03	1.82 ± 0.02[a]	1.57 ± 0.03[b]	1.58 ± 0.01[b]	1.56 ± 0.03[b]
Epidydimis (g)	1.23 ± 0.02	1.23 ± 0.05	1.21 ± 0.04	1.25 ± 0.08	1.22 ± 0.05
Epidydimis (g/100 g bw)	0.21 ± 0.01	0.21 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.21 ± 0.02
Seminal vessicle (g)	0.61 ± 0.15	0.53 ± 0.21	0.58 ± 0.15	0.57 ± 0.09	0.59 ± 0.11
Seminal vessicle (g/100 g bw)	0.27 ± 0.02	0.25 ± 0.02	0.26 ± 0.01	0.28 ± 0.02	0.27 ± 0.01
Prostate gland (g)	0.34 ± 0.09	0.31 ± 0.08	0.33 ± 0.10	0.32 ± 0.05	0.33 ± 0.01
Prostate gland (g/100 g bw)	0.12 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.11 ± 0.02	0.13 ± 0.01

Carbendazim (CBZ). Values are expressed as 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 ($p < .05$).

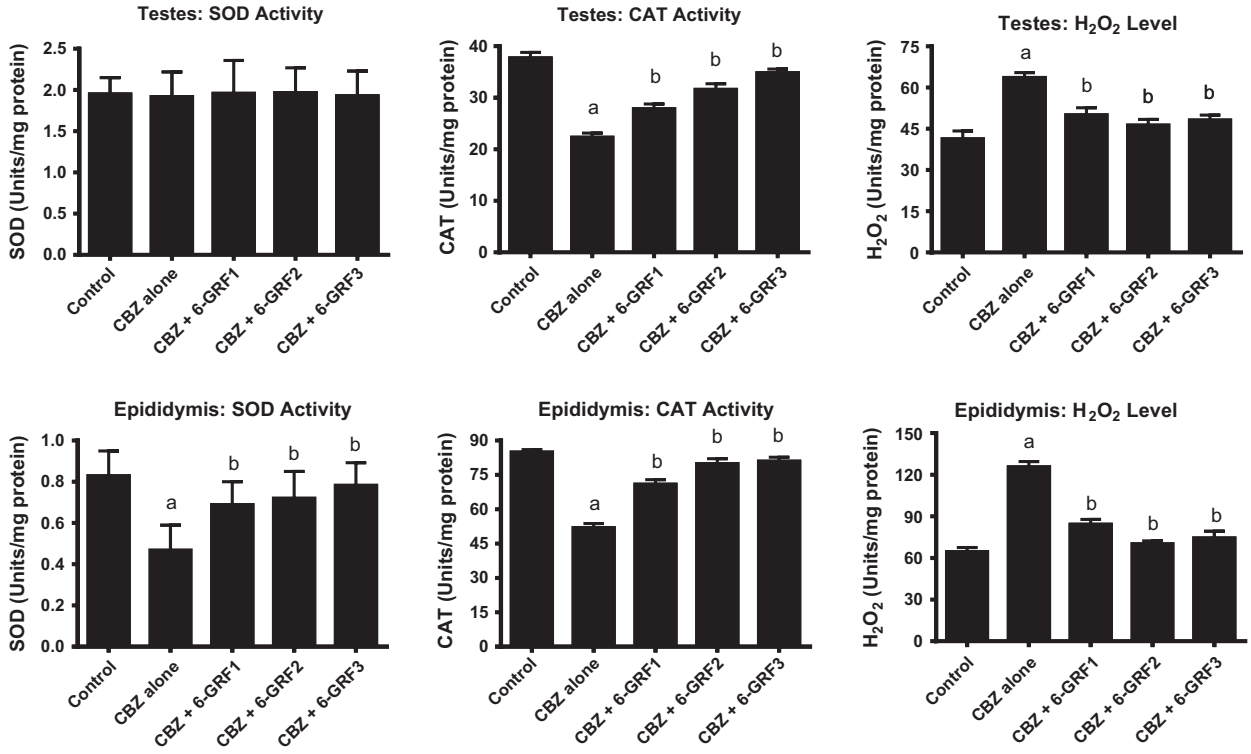


FIGURE 2 Effects of 6-GRF on SOD and CAT activities and the level of H_2O_2 in testes and epididymis of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment period. [a]Values differ significantly from control ($p < .05$). [b]Values differ significantly from CBZ alone group ($p < .05$)

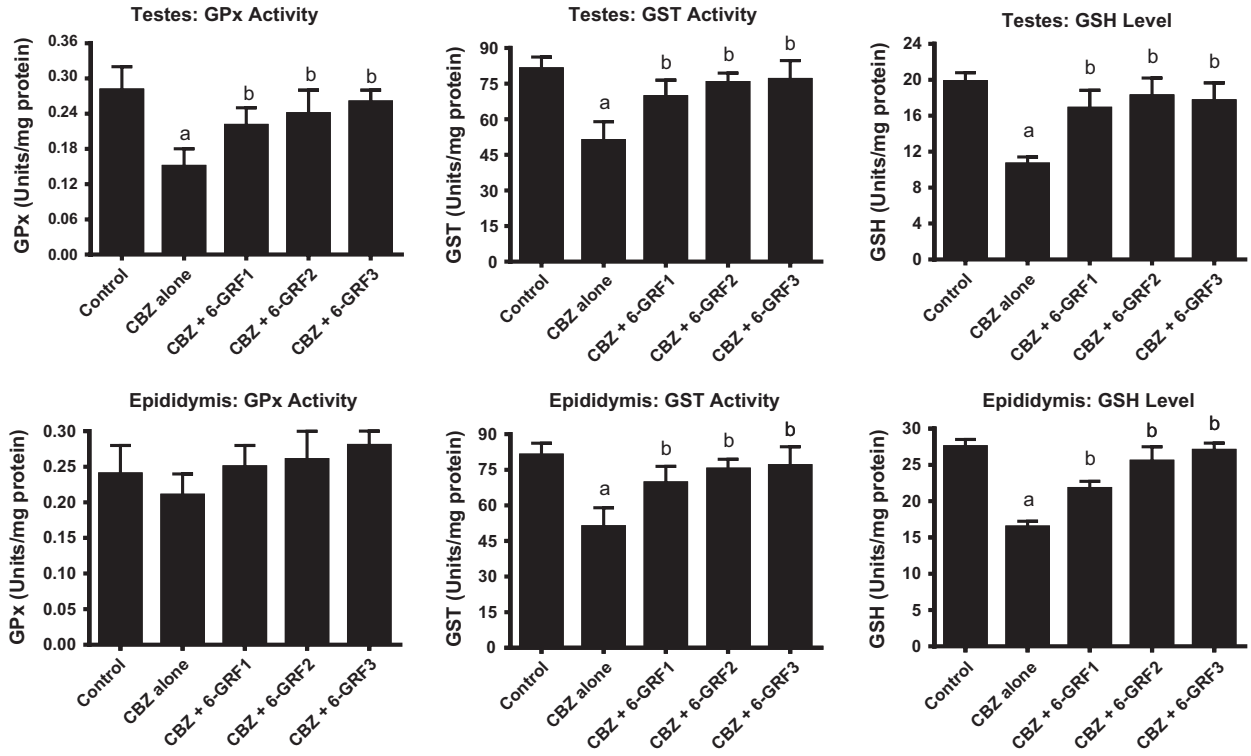


FIGURE 3 Effects of 6-GRF on GSH level and activities of GSH-dependent enzymes in testes and epididymis of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group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

3.5 | Effects of 6-GRF on lipid peroxidation level in testes and epididymis of CBZ-treated rats

Figure 4 shows the effects of 6-GRF on lipid peroxidation in testes and epididymis of the CBZ-treated rats. Administration of CBZ caused a significant increase in the level of malondialdehyde (MDA), an index of lipid peroxidation, in the testes and epididymis of the treated rats. However, co-administration of 6-GRF significantly decreased the MDA level in the testes and epididymis of the treated rats when compared with those exposed to CBZ alone. Exposure to CBZ elevated MDA level in the testes and epididymis by 89.7% and 103.4%, respectively, when compared with the control rats. However, testicular MDA level was reduced to near control level by 64.1%, 82% and 84.6%, whereas epididymal MDA level was reduced to near control by 48.3%, 79.3% and 93.1% in CBZ-treated rats following co-administration of 6-GRF1, 6-GRF2 and 6-GRF3 respectively.

3.6 | Effects of 6-GRF on sperm characteristics, testicular sperm number and sperm production in CBZ-treated rats

The effects of 6-GRF on spermiogram of CBZ-treated rats are presented in Fig. 5. Compared with the control group, CBZ treatment significantly decreased sperm progressive motility, epididymal sperm

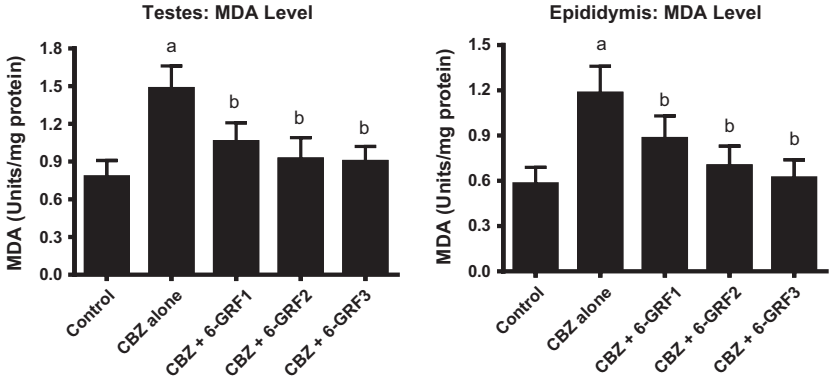


FIGURE 4 Effects of 6-GRF on LPO level in testes and epididymis of CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after 14 consecutive days of oral treatment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

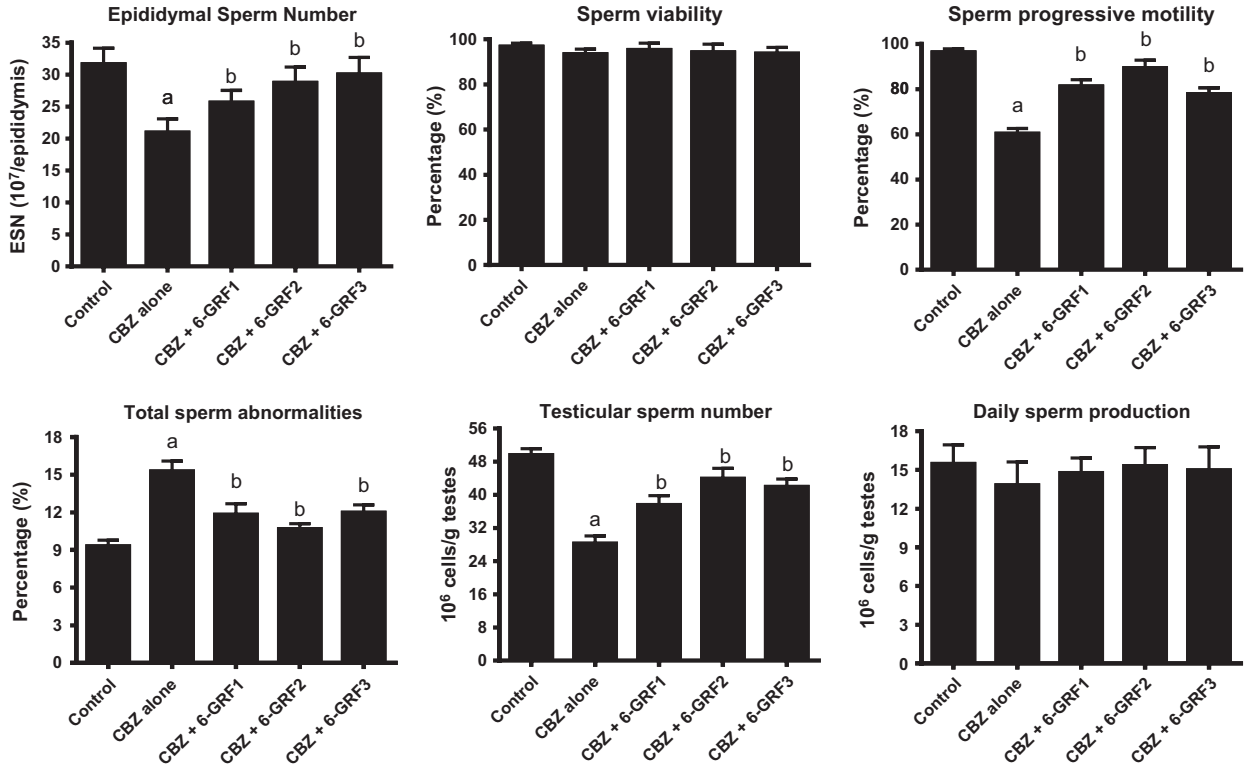


FIGURE 5 Effects of 6-GRF on sperm characteristics, testicular sperm number and daily sperm production in CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents mean ± SD of ten rats per group after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

number (ESN) and the testicular sperm number (TSN), whereas sperm abnormalities increased significantly in the treated rats. The major sperm morphological defects observed in the CBZ-treated rats include tailless heads, curved mid-pieces and bent mid-pieces. Carbendazim alone decreased ESN from 31.72 ± 2.07 × 10^7 per epididymis (control) to 21.01 ± 2.13 × 10^7 per epididymis, motility was decreased from 96.54 ± 1.36% (control) to 60.5 ± 2.21% and TSN was decreased from 49.67 ± 1.45 × 10^6 cells per g testes (control) to 28.31 ± 0.83 × 10^6 cells per g testes, whereas sperm abnormalities was increased from 9.33 ± 0.45% (control) to 15.34 ± 0.78%.

However, co-administration of 6-GRF1, 6-GRF2 and 6-GRF3 significantly prevented CBZ-mediated decrease in TSN and sperm functional parameters in the treated rats when compared with the group exposed to CBZ alone. 6-GRF1, 6-GRF2 and 6-GRF3 increased ESN (25.7 ± 1.83, 28.81 ± 2.41, 30.1 ± 2.62 × 10^7 per epididymis), motility (81.42 ± 2.84, 89.5 ± 3.2, 78.2 ± 2.66%) and TSN (37.65 ± 1.13, 44.05 ± 2.43 and 42.03 ± 1.81 × 10^6 cells per g testes), whereas sperm abnormalities was decreased (11.85 ± 0.83, 10.75 ± 0.43 and 11.88 ± 0.65%) respectively. There were no treatment-related effects on the sperm viability and daily sperm production (DSP) in the rats following administration of CBZ alone or in combination with 6-GRF-1, 6-GRF2 and 6-GRF3.

3.7 | Effects of 6-GRF on circulatory hormone concentrations in CBZ-treated rats

The levels of LH, FSH and prolactin were assessed to determine the functionality of the pituitary, whereas TSH, T_3 and T_4 levels were assayed to determine thyroid functionality. Moreover, steroidogenic function of the testes was determined by measuring the testosterone level. The modulatory role of 6-GRF in CBZ-induced imbalance in hormone concentrations in the treated rats are presented in Figs 6 and 7. Exposure to CBZ significantly decreased testosterone level and increased FSH level but did not affect LH and prolactin levels in the treated rats when compared with control. However, co-treatment with all doses of 6-GRF significantly reversed the CBZ-induced alterations in the plasma FSH and testosterone concentrations to near control. Moreover, there was a significant decrease in levels of TSH, T_3 and T_4 but increased the T_3/T_4 ratio in CBZ-treated rats. The decrease in the TSH, T_3 and T_4 levels in CBZ-treated rats was 27.3%, 21.8% and 29.8%, respectively, when compared to the control group. However, 6-GRF ameliorated the diminution in the thyroid hormones and restored their levels to near normalcy in CBZ-treated rats. However, 6-GRF ameliorated the diminution in the thyroid hormones and restored their levels to near normalcy in CBZ-treated rats. The percentage restoration to control level following co-administration of 6-GRF1, 6-GRF2 and 6-GRF3 was 89.4%, 97.4% and 96% for TSH level, 91.8%, 90% and 94.5% for T_3 level and 87.6%, 95.6% and 94% for T_4 level, respectively, in the treated rats.

3.8 | Histopathological observations

Figures 8 and 9 represent the photomicrographs of the testes and epididymis of experimental rats. The testes of control rats appeared structurally and functionally normal. Testes of rats treated with CBZ alone showed degenerated seminiferous epithelium with few sperm cells, sloughing of spermatogenic cells into the lumen of some tubules as well as severe congestion of the interstitial vessels. However, the

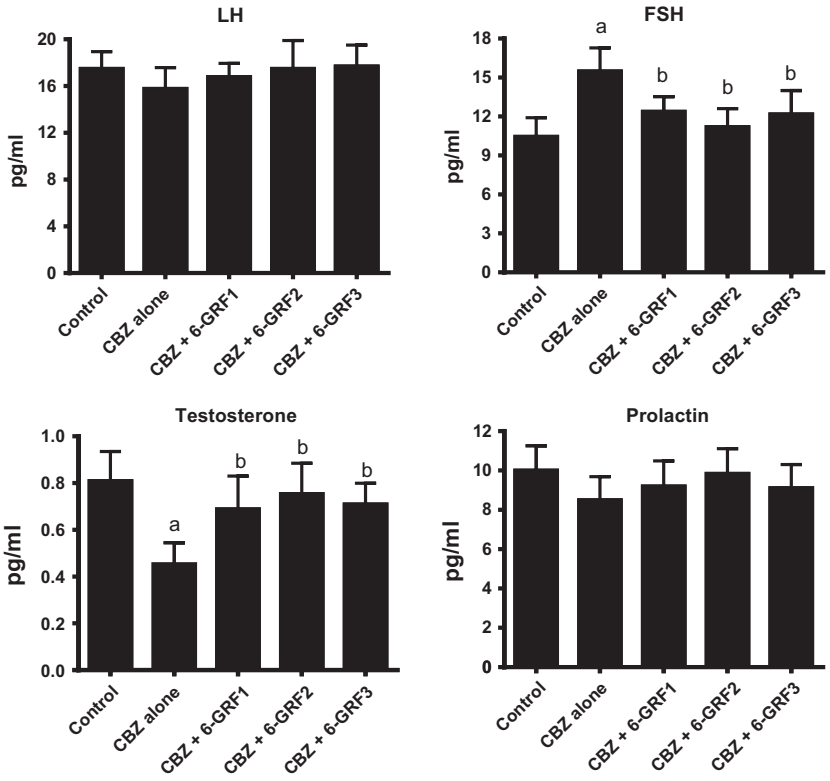


FIGURE 6 Effects of 6-GRF on plasma concentrations of luteinising hormone (LH), follicle-stimulating hormone (FSH), testosterone and prolactin in CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents 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 ($p < .05$)

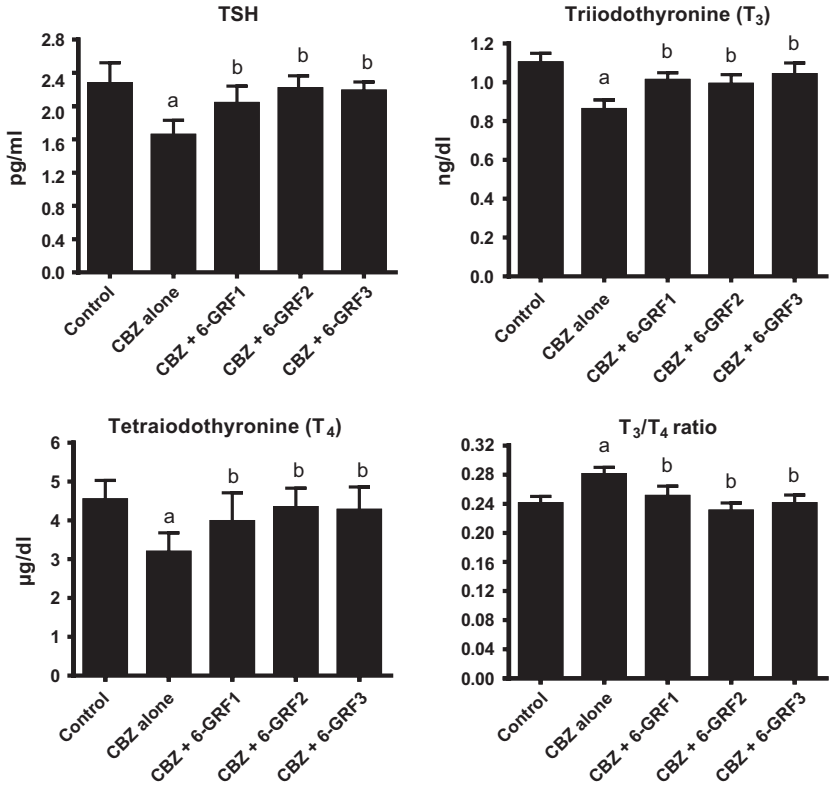


FIGURE 7 Effects of 6-GRF on plasma concentrations of thyrotropin (TSH), triiodothyronine (T_3) and thyroxine (T_4) levels in CBZ-treated rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Each bar represents mean ± SD of 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 ($p < .05$)

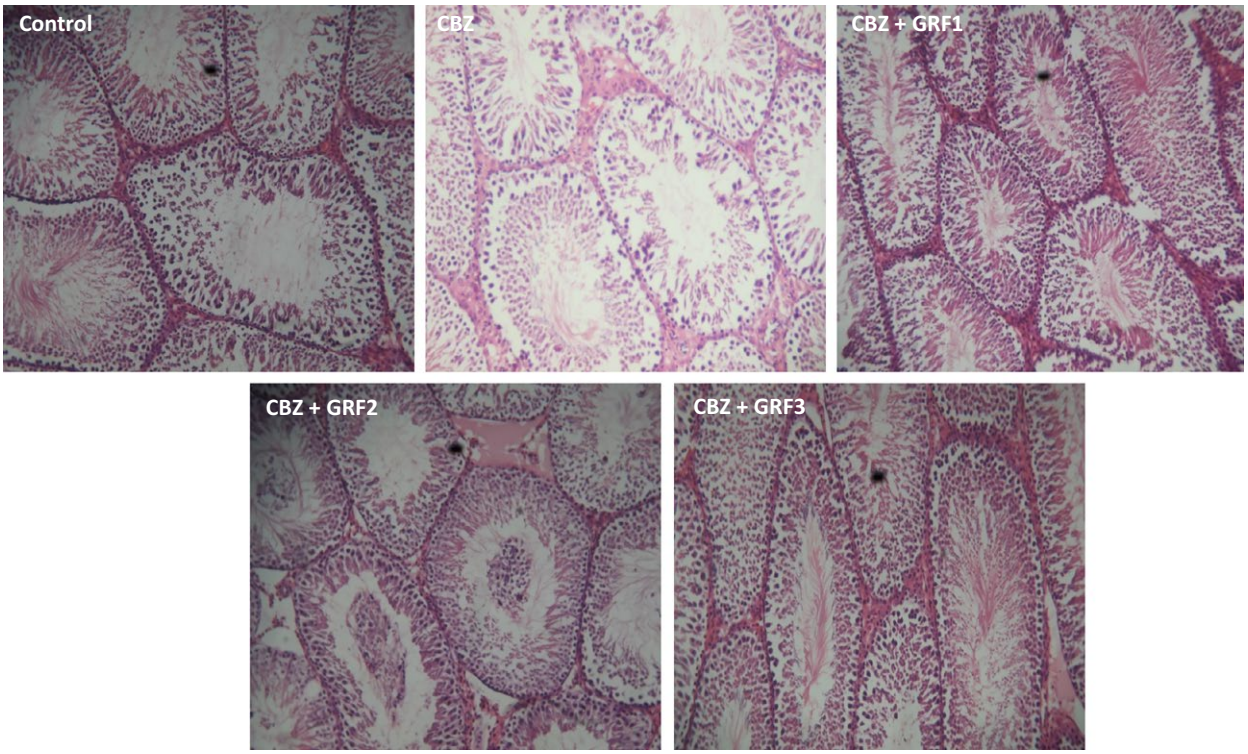


FIGURE 8 Photomicrographs of the testes of experimental rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Control testes showing normal architecture. CBZ alone-treated rats showing degenerated seminiferous epithelium with few sperm cells, sloughing of spermatogenic cells into the lumen of some tubules as well as severe congestion of the interstitial vessels. Testes of rats from CBZ plus 6-GRF1 appeared normal but with mild congestion of the interstitial vessels. The seminiferous epithelium of rats treated with CBZ + 6-GRF2 and CBZ + 6-GRF3 appeared normal and comparable with the control. Original magnification: 240×

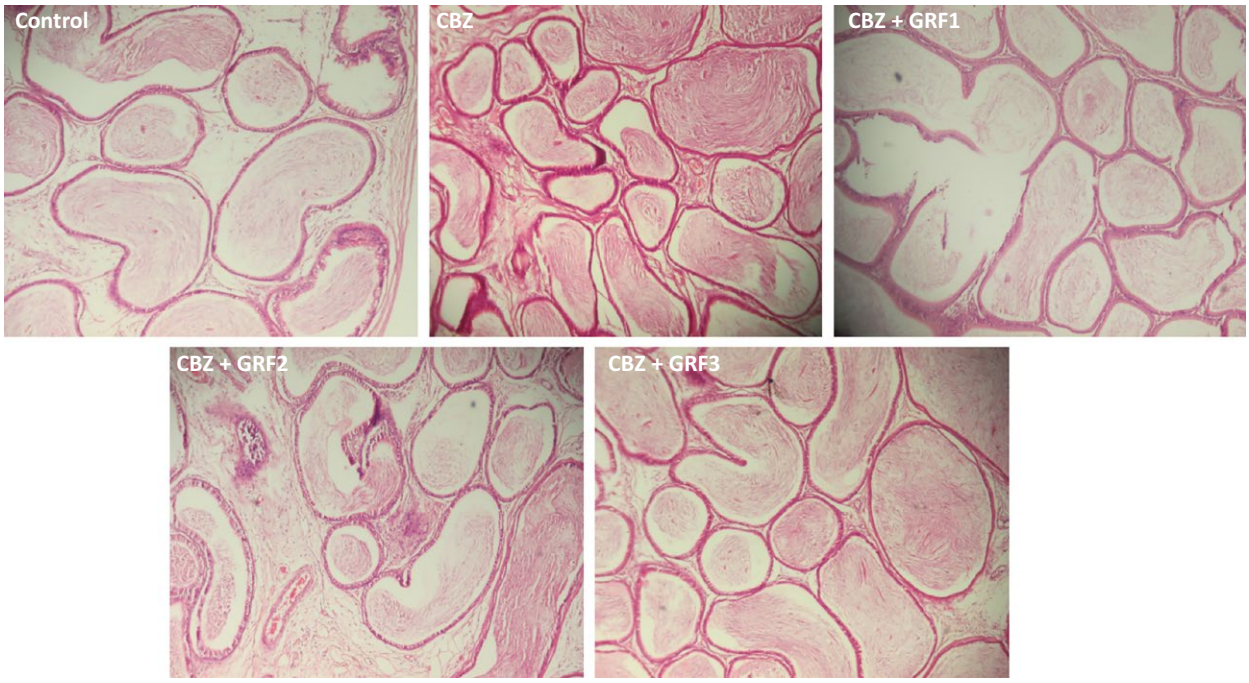


FIGURE 9 Photomicrographs of the epididymis of experimental rats. CBZ, 50 mg/kg carbendazim; 6-GRF1, 6-GRF2 and 6-GRF3 denote 50, 100 and 200 mg/kg of 6-Gingerol-rich fraction respectively. Control epididymis showing normal epithelial cells and abundance of spermatozoa. Epididymis of CBZ alone-treated rats showing irregularly shaped epithelial cells with few sperm cells. Epididymis of rats treated with CBZ + 6-GRF1, 6-GRF2 and 6-GRF3 appeared normal somewhat comparable with the control. Original magnification: 240×

testes of rats from CBZ plus 6-GRF1 appeared normal but with mild congestion of the interstitial vessels. The seminiferous epithelium of rats co-treated with 6-GRF2 and 6-GRF3 appeared normal and comparable with the control. Similarly, the ducts of epididymis from the control rats contain abundant spermatozoa with normal epithelial cells. Conversely, the ducts of epididymis from CBZ-treated rats were irregularly shaped and contained few sperm cells, whereas the ducts of epididymis in rats co-treated with 6-GRF1, 6-GRF2 and 6-GRF3 appeared normal somewhat comparable with the control.

4 | DISCUSSION

Chemoprotective strategies against reproduction toxicity are essential not only to inhibit the progression of testicular degeneration but also restore the reproductive function of the toxicant exposed individual. The present study demonstrated for the first time that 6-gingerol-rich fraction possesses protective activity against CBZ-induced testicular and epididymal toxicity in rats. Exposure to CBZ did not affect the body weight gain thus signifying the normal general metabolic functions of the rats in the present investigation. Conversely, CBZ treatment significantly increased the absolute and relative testes weight, whereas the weights of the epididymis, seminal vesicle and prostate gland remained unaffected. The increase in testes weight in CBZ-treated rats in the present study is in agreement with previous investigations (Adedara, Vaithinathan, et al. 2013; Moffit, Bryant, Hall, & Boekelheide, 2007; Nakai et al., 1992) and has been related to impaired seminiferous tubule fluid passage from the testis to the epididymis by the germ cells that sloughed into the lumen following CBZ treatment (Nakai et al., 1992). Testis weight is a valuable index of testicular health and indirectly reflects alterations in seminiferous tubule fluid retention or germ cell loss (Adedara et al., 2016; Creasy, 2002). Thus, the restoration of the absolute and relative testes weight to near normal following treatment with the 6-GRF may indicate an improvement and prevention of CBZ-induced germ cells sloughing into the lumen and, consequently, enhance the seminiferous tubule fluid passage from the testis to the epididymis in the treated rats.

The twin functions of the testes, namely spermatogenesis and steroidogenesis, are normally protected from oxidative stress by the antioxidant defence system consisting SOD, CAT, GPx, GST and GSH (Adedara et al., 2016). The biochemical mechanism of antioxidant enzymes involves the rapid conversion of superoxide anion to H_2O_2 by SOD, thus preventing the production of highly pernicious hydroxyl radicals via Haber–Weiss reaction (Adedara, Ebokaiwe, et al. 2013; Aitken & Roman, 2008). The significant decrease in the activities of SOD and CAT with significant elevation in H_2O_2 level in CBZ-treated rats indicates enzymes inhibition and a state of oxidative stress in the treated rats. Thus, the decrease in H_2O_2 level following 6-GRF treatment is attributed to the enhancement of the SOD and CAT activities to scavenge free radicals and reactive oxygen species in the CBZ-treated rats.

Reduced glutathione is a vital tri-peptide necessary for the intracellular defence against reactive oxygen species. The level of GSH and activities of GSH-dependent enzymes including GPx and GST activities in the testes and epididymis of CBZ-treated rats were significantly

decreased and thus, indicates a state of oxidative stress. The depletion in the antioxidant status of the testes and epididymis of rats treated with CBZ resulted in oxidative damage as evidenced by the significant elevation in the levels of MDA, an index of lipid peroxidation and histological alterations. However, 6-GRF administration ameliorated the noxious effects of CBZ on the redox system by augmenting GSH level and GSH-dependent enzymes as well as improved the histology of the testes and epididymis in the treated rats. One of the reported mechanisms for the antioxidant effect of 6-gingerol is the enhancement of antioxidant status (Ajayi, Adedara, & Farombi, 2015; Dugasani et al., 2010).

Moreover, CBZ exposure decreased ESN, sperm motility and TSN but increased sperm abnormalities without affecting sperm viability and DSP in the treated rats. These findings suggest that while the observed testicular atrophy was not sufficient to prevent sperm production within the time of this investigation, CBZ treatment adversely impacted sperm motility and storage in epididymal compartment. Thus, the significant decrease in sperm quality and quantity observed in CBZ-treated rats in this study may be attributed to an outcome of increased lipid peroxidation of polyunsaturated fatty acids in the sperm plasma membrane, which may cause a loss in the fertilising potential of spermatozoa. However, co-treatment of 6-GRF at all doses efficiently ameliorated CBZ-induced decline in sperm functional characteristics in the treated rats. This improvement in the sperm parameters in rats co-treated with 6-GRF is attributable to its ability to prevent excessive production of free radicals and/or augment the epididymal antioxidant defence system to competently decrease CBZ-induced oxidative damage in the epididymis of the treated rats.

The hypothalamic–pituitary–gonadal axis is critical in the regulation of reproduction in both animals and humans. Normally, gonadal steroids act on the hypothalamus to control gonadotropin-releasing hormone pulses, whereas the secretion of the gonadotropin (LH and FSH) is regulated at pituitary level (Adedara et al., 2015). During spermatogenesis, LH stimulates Leydig cell to synthesise and secrete testosterone, whereas both FSH and LH cooperate to promote spermatogenic cell survival by suppressing proapoptotic signals (O'Shaughnessy, Monteiro, Verhoeven, De Gendt, & Abel, 2010). Thus, the significant alterations in the FSH and testosterone levels in the present investigation may be associated with decreased TSN and ESN in the CBZ-treated rats. Moreover, CBZ has been reported to induce oxidative damage in Leydig cells of rats (Rajeswary et al., 2007). Thyroid hormones play a regulatory role in the testicular basal metabolic activity, steroidogenesis and spermatogenesis (Maran, 2003; Wagner, Wajner, & Maia, 2008). The present investigation showed that exposure to CBZ significantly decreased the plasma levels of TSH, T_3 and T_4 in the treated rats. The decreased thyroid hormones indicate inhibition of their synthesis and/or increase in their metabolism as well as defectiveness in the thyroid pathway to activate the negative feedback to the hypothalamus and pituitary in the CBZ-treated rats. Hypothyroidism reportedly impaired fertility by compromising sperm count and progressive motility of the spermatozoa (Krajewska-Kulak & Sengupta, 2013). Moreover, the restoration of pituitary and thyroid hormones level to near normalcy following co-treatment with 6-GRF indicates an improvement in the hypothalamic–pituitary–testicular axis and thyroid gland function in the CBZ-treated rats.

In conclusion, the present investigation demonstrated the ameliorative potential of 6-GRF against CBZ-induced reproductive toxicity in male rats. Mechanistically, 6-GRF chemoprotective role in CBZ-induced male reproductive dysfunction involves significant reduction in oxidative stress, augmentation of antioxidant enzymes and hormonal level along with enhancement of sperm characteristics and histological damage in the testes and epididymis in the treated rats. The findings from this study suggest that 6-GRF could be an effective chemoprotective agent against reproductive toxicity resulting from exposure to fungicide CBZ.

ACKNOWLEDGEMENTS

This work was supported in part 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.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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