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Antioxidant potential of garlic oil supplementation prevents cyclophosphamide-induced oxidative testicular damage and endocrine depletion in rats

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HIGHLIGHTS

- Cyclophosphamide induced testicular oxidative stress and endocrine deficits in rats.
- Garlic oil attenuated cyclophosphamide-induced testicular damage and endocrine deficits.
- Garlic oil may possess beneficial health effect against cyclophosphamide toxicity in cancer patients.

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ABSTRACT

Cyclophosphamide is an alkylating anticancer agent with strong efficacy; however, its clinical use is constrained because of its off-target multiple organ toxicity, and one of them is testicular injury. We assayed to explore whether garlic oil (GO) could prevent cyclophosphamide (CYP)-induced testicular oxidative stress and hormonal deficit in male rats. Rats were pretreated with GO for 21 days before a single injection of CP (50 mg/kg, ip). The total phenol and flavonoids of GO were estimated as well as its antioxidant capacity using DPPH and FRAP assays. CYP induced prominent depression in testicular activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) level, whereas levels of malondialdehyde (MDA) markedly increased and confirmed by histopathological alterations. Serum levels of testosterone, FSH and LH were considerably reduced. Interestingly, the GO supplementation attenuated the biochemical changes in the testis, enhanced the hormone levels and alleviated the histological injury. The IC_{50} of GO in DPPH assay was comparable to that of standard. GO is capable of protecting the testis from CYP toxicity via its antioxidant property. The findings suggest GO beneficial effects in male cancer patients undergoing CYP chemotherapy.

1. Introduction

Chemotherapy strategies have led to improvement in the chances of surviving a diagnosis of cancer [1]. Chemotherapeutic agents kill rapidly proliferating cancer cells to destroy the neoplastic tissue in cancer patients. However, because of their low therapeutic index, they thereby exert damage to normal cells and tissues [2]. Therefore, the toxic damage of these agents to delicate organs in cancer patients is a matter of current concern to clinicians.

Cyclophosphamide is an efficacious anticancer agent widely used for the treatment of various cancers, as well as an immunosuppressive agent for organ transplantation, multiple sclerosis, systemic lupus erythematosus and other benign tumours [3]. However, the clinical utility of cyclophosphamide (CYP) is restrained because of several adverse effects associated with reproductive toxicity, hepatotoxicity, nephrotoxicity and cardiotoxicity in patients and animal models [4–6]. A

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number of earlier investigations indicate that CYP induces derangement in spermatogenesis and causes atrophy of seminiferous tubules and testosterone level depletion [7,8]. The biochemical and histological alterations in the testis and epididymis of humans and rats caused by CYP are also reported [3]. Studies have suggested that the reproductive deficits could be triggered by the generation of reactive oxygen species (ROS) and lipid peroxidation [4,9].

However, the underlying mechanism of CYP-induced testicular toxicity is unclear. CYP is metabolized by the hepatic cytochrome-P450 enzymes to produce the metabolites-acrolein and phosphoramide [2,10]. Phosphoramide is responsible for the CYP anticancer and immunosuppressive efficacy, whereas acrolein is associated with toxic effects on healthy cells. Robust evidence implicates acrolein in CYP-induced oxidative stress. Acrolein induces toxicity through excessive generation of ROS capable of damaging DNA and protein adduct formation (11). ROS also induces endoplasmic reticulum stress and immune dysfunction, cell membrane damage and mitochondrial disruption [11]. CYP thus provokes ROS and disrupts redox balance to engender oxidative stress. Literature has shown that CYP injection in animal models reduces activities of antioxidant enzymes, and increases malondialdehyde levels in the testis, liver and brain [2,3,10,12]. The biochemical basis of CYP toxicity has been related to free radicals generated in the testis. Several studies have shown that natural products possess potent antioxidant effect that could enhance fertility and mitigate oxidative stress-mediated toxicity. The search for natural products that may preserve the anticancer drug efficacy while mitigating its side effects is the goal of many studies. Recently, the beneficial health efficacy of natural oil is emerging in the literature, and they appear attractive to consumers because they are amenable to daily diet [4,13,14].

Garlic (*Allium sativum*) is a common spicy flavoring agent and medicinal herb. It is used since ancient times for prevention and treatment of various ailments. Literature has revealed many pharmacological properties of garlic and its derivative compounds by epidemiological studies and animal experiments [15]. One of the garlic extracts is the garlic oil (GO) used for medicinal purposes due to its constituent compounds with several biological effects. Garlic oil (GO) has been shown to contain more than 30 organic sulfur-containing compounds including diallyl trisulfide, diallyl disulfide, and diallyl sulphide with well reported antioxidant properties [16,17]. According to published papers, GO shows beneficial effects against experimental hepatocarcinogenesis, acetaminophen hepatotoxicity and diabetes in both animals and humans [18–22]. In addition, GO was reported to inhibit proliferation and induces apoptosis in pancreatic cancer cell line [23]. Although considerable number of studies have evaluated the effects of garlic and GO on toxicity and diseases in different models, the effect of GO on anticancer drug-induced organ toxicity remains to be reported. Considering its wide spectrum of biological properties including antioxidant and anti-inflammatory effects, the present study evaluates the effect of GO on CYP-induced testicular toxicity and endocrine deficit in rats.

2. Materials and methods

2.1. Drug and chemicals

Cyclophosphamide was procured from Khandelwal Laboratories Pvt Ltd, Mumbai, India. Assay kits for antioxidant enzymes were purchased from Randox Laboratories Ltd., Crumlin, Antrim, UK. Thiobarbituric acid (TBA) was purchased from Hi Media Laboratories Pvt. Ltd, India. All other reagents used were obtained commercially and of analytical grade.

2.2. Animals

Twenty-four male Wistar rats, weighing 160–200 g, were supplied by Animal Breeder in Abakaliki, Nigeria. Animals were housed in an Animal Facility of the Department of Biochemistry, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo, in a room at 25 ± 2 °C with a lighting schedule of 12 h light and 12 h dark. Rats were fed with commercial pelleted rat growers mash diet (Vital Feed, Jos, Nigeria) and water *ad libitum*. The study was conducted following the ethical procedures and policies approved by the Ethics Committee of the Department of Biochemistry, Alex Ekwueme Federal University, Ndufu-Alike, Ikwo. All experimental procedures were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised in 1996).

2.3. Garlic oil preparation

Garlic oil was purchased from a local producer in Kaduna, Nigeria. The natural method adopted in the production was consistent with the distillation method previously described by Sheen et al. [24].

2.4. Total phenolic content determination

The total phenolic content in GO was determined by Folin–Ciocalteu colorimetric method and expressed as milligrams of gallic acid equivalent (GAE) per 100 g of GO (Wangensteen et al. [25].

2.5. Total flavonoid content determination

The estimation of total flavonoids in GO was done using the method of Ordonez et al. [26]. The value was expressed as milligrams of quercetin equivalent (QE) per 100 g of GO.

2.6. In vitro antioxidant assays

The radical scavenging activity of GO was evaluated using DPPH assay by the method of Blois [27] with vitamin C as the standard antioxidant agent. Ferric reducing antioxidant power (FRAP) of GO was determined by the method of Benzie and Strain [28] with gallic acid as the standard antioxidant agent.

2.7. Experimental design

Group 1 (Normal control): Male rats received normal saline (2 ml/kg BW) on day 21 only.

Group 2 (Garlic oil): Male rats received garlic oil (10% w/w) from day 1 to day 21 [29] with normal saline (2 ml/kg BW) on day 21 only.

Group 3 (Cyclophosphamide): Male rats received cyclophosphamide (50 mg/kg, ip) on day 21 only [2].

Group 4 (Garlic oil + Cyclophosphamide): Male rats received garlic oil (10% w/w) from day 1 to day 21 + cyclophosphamide (50 mg/kg, ip) on day 21 only.

The garlic oil-supplemented diet was prepared by thoroughly mixing 10 g of fresh garlic oil with 90 g of rat feed and served to the rats *ad libitum* [14]. On day 22, the weight of rats were measured and recorded before sacrifice. All rats were anaesthesized with diethyl ether to collect blood samples from the abdominal aorta. Serum from the blood was used for estimation of hormones. The animals were sacrificed by decapitation and the testis were carefully excised immediately and rinsed in ice-cold physiological saline, and weight determined. Testis tissues were homogenized in 0.1 M phosphate buffered saline (1:5 w/v, pH 6.4) and centrifuged (4000 g for 20 min). The supernatant separated was used to analyse for oxidative stress parameters. A portion of testis was preserved in 10% formalin for histological analysis.

2.8. Biochemical analyses

2.8.1. Assessment of oxidative stress indices

The homogenate supernatant was used for oxidative stress indices.

Testicular superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund [30], catalase (CAT) activity analysed by method of Aebi [31], glutathione peroxidase (GPx) activity determined by Paglia and Valentine method [32], while reduced glutathione (GSH) level was analysed using Beutler method [33]. Lipid peroxidation in the testis was analysed and expressed as malondialdehyde (MDA) by Ohkawa et al. [34].

2.8.2. Assessment of reproductive hormones

The serum was used to analyse luteinising hormone (LH), follicle stimulating hormone (FSH) and testosterone concentrations. The serum concentrations of LH, FSH and testosterone were estimated by ELISA assay kits (DRG Diagnostics Marburg, Germany), according to the kit manufacturer's instructions.

2.9. Histopathological examination

Testis sample fixed in 10% buffered formalin for 48 h was dehydrated in ethanol and embedded in paraffin. Sections were cut by rotary microtome and stained with hematoxylin and eosin (H and E) for microscopic histopathological changes. The slides were viewed and examined under with Motic™ compound light microscope.

2.10. Statistical analysis

The values were presented as mean ± standard error of mean (SEM). Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by Tukey's Post hoc test. The value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. Total phenolic and flavonoids contents of garlic oil (GO)

Table 1 shows the contents of total phenol and total flavonoids present in a sample of GO. Total phenol content in GO was 90.1 ± 8.6 mg GAE/100 g of oil, while total flavonoids was 201.4 ± 0.29 mg QE/100 g of oil of triplicate determinations.

3.2. Antioxidant activity of GO and standards

Table 2 depicts the scavenging potential of GO to reduce the radical DPPH as well as ability to reduce ferric ion to ferrous ions. At various concentrations (μg/ml), GO scavenged DPPH as expressed in percentage DPPH inhibition. In the DPPH assay, the IC_{50} of ascorbic acid was 29.32 μg/ml, while that of GO was 39.31 μg/ml. In the FRAP assay, the ferric reducing capacities of GO and gallic acid expressed as IC_{50} were higher than 100 μg/ml.

3.3. Effect of GO on rat body weight and testis weight

Table 3 presents the effect of GO-supplemented diet on rat body weight and testis weight. At sacrifice, the weight of rats injected with CYP was insignificantly ($p > 0.05$) higher than normal control. Also, weight of rats in GO + CYP was insignificantly ($p > 0.05$) lower compared to weight of rats in CYP group. On the other hand, CYP reduced the weight of rat testis insignificantly ($p > 0.05$) compared to normal group in this study. However, the supplementation of GO in GO + CYP increased the testis weight comparable to normal control, although it was not significant ($p > 0.05$) in comparison with CYP group.

Table 1
Total phenolic and flavonoids contents of garlic oil (GO).

Sample	Total phenol (mg GAE/100 g)	Total flavonoids (mg QE/100 g)
Garlic oil	90.1 ± 8.6	201.4 ± 0.29

Values represent mean ± SEM of triplicate analysis. GAE: gallic acid equivalent per 100 g oil; QE: quercetin equivalent per 100 g oil.

Table 2
DPPH scavenging potential and FRAP of garlic oil (GO).

Conc (μg/ml)	% DPPH Inhibition		FRAP (mM Fe^{2+}/g)	
	GO	Vitamin C	GO	Gallic acid
2.5	54.6 ± 1.56	67.4 ± 0.18	0.66 ± 0.03	0.047 ± 0.006
5	58.2 ± 5.86	73.3 ± 2.44	0.80 ± 0.12	0.055 ± 0.002
10	57.9 ± 0.18	92.6 ± 1.78	0.80 ± 0.10	0.056 ± 0.001
20	58.8 ± 0.93	93.2 ± 2.49	0.79 ± 0.06	0.059 ± 0.001
40	62.9 ± 4.08	94.2 ± 0.61	0.86 ± 0.08	0.060 ± 0.001
80	76.9 ± 2.62	93.2 ± 1.63	0.88 ± 0.06	0.070 ± 0.001
IC_{50} (μg/ml)	**39.31**	**29.32**	**> 100**	**> 100**

Values are represented as mean ± SD (n = 3). DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; GO: Garlic oil.

Table 3
Effect of GO diet on rat body weight and testis weight.

Group	Rat body weight (g)		Testis weight (g)
	Initial	Final	
Normal	170.7 ± 8.15	199.6 ± 10.03	1.22 ± 0.15
GO	166.9 ± 9.10	196.9 ± 8.11	1.13 ± 0.05
CYP	195.1 ± 8.24	215.2 ± 10.58	0.93 ± 0.09
GO + CYP	178.5 ± 10.40	202.3 ± 9.98	1.20 ± 0.07

GO: Garlic oil; CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).

3.4. Effect of garlic oil on serum reproductive hormones in CYP-treated rats

Table 4 shows the effect of garlic oil-supplemented diet on LH, FSH and testosterone in rats injected with CYP. The results show that CYP significantly ($p < 0.05$) reduced the serum levels of LH, FSH and testosterone compared to normal control. However, the garlic oil supplemented diet in GO + CYP group improved the levels of LH, FSH and testosterone in the serum significantly ($p < 0.05$) in comparison to CYP group.

3.5. Effect of garlic oil on testicular oxidative stress indices in CYP-treated rats

Figs. 1–5 present the effect of garlic oil (GO)-supplemented diet on testicular activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and malondialdehyde (MDA). We observed that CYP significantly ($p < 0.05$) reduced the activities of SOD, CAT, GPx in the testis of rats compared to

Table 4
Effect of garlic oil-supplemented diet on LH, FSH and testosterone in CYP-injected rats.

Group	LH (mU/L)	FSH (mU/L)	Testosterone (ng/mL)
Normal	19.76 ± 3.21	29.33 ± 3.93	151.6 ± 7.60
GO	18.63 ± 1.77	28.80 ± 3.92	151.8 ± 9.90
CYP	8.90 ± 2.60*	11.79 ± 4.57*	94.65 ± 8.43*
GO + CYP	14.82 ± 1.33#	26.33 ± 3.72#	134.1 ± 9.17#

GO: Garlic oil; CYP: Cyclophosphamide; LH: Leutenizing hormone; FSH: Follicle stimulating hormone; Values are expressed in mean ± SEM (n = 6).
*$p < 0.05$: significant when compared to normal control group.
#$p < 0.05$: significant when compared to CYP group.

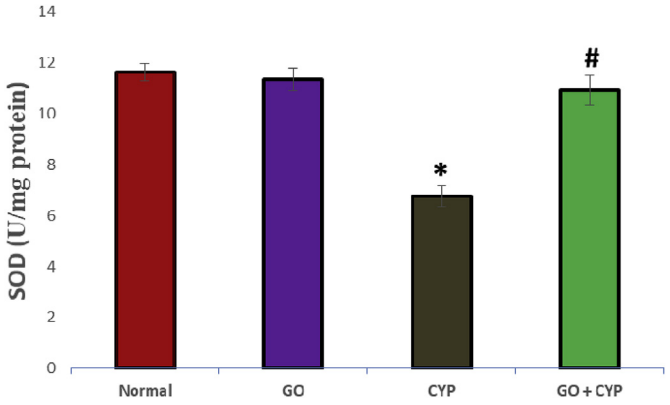

Fig. 1. Effect of garlic oil (GO) on testicular superoxide dismutase (SOD) activity in CYP-treated rats. CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).
*p < 0.05: significant when compared to normal control group.
#p < 0.05: significant when compared to CYP group.

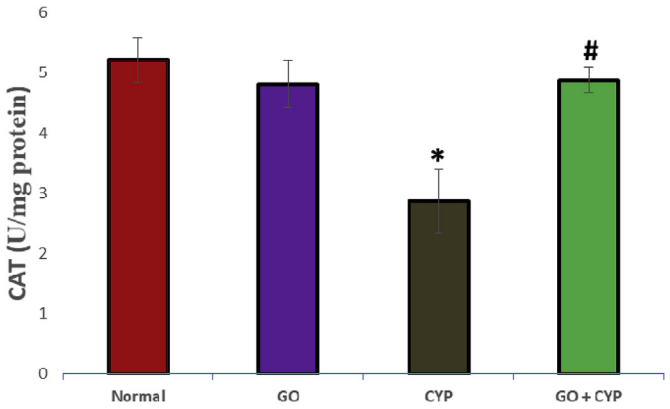

Fig. 2. Effect of garlic oil (GO) on testicular catalase (CAT) activity in CYP-treated rats. CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).
*p < 0.05: significant when compared to normal control group.
#p < 0.05: significant when compared to CYP group.

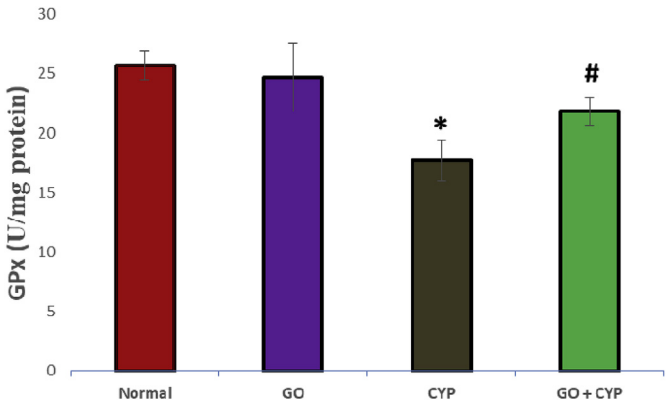

Fig. 3. Effect of garlic oil (GO) on testicular glutathione peroxidase (GPx) level in CYP-treated rats. CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).
*p < 0.05: significant when compared to normal control group.
#p < 0.05: significant when compared to CYP group.

normal control rats. Also, the level of GSH reduced considerably ($p < 0.05$), whereas MDA level increased markedly ($p < 0.05$) compared to normal control. The supplementation of GO in the diet (GO + CYP) significantly ($p < 0.05$) increased the activities of SOD,

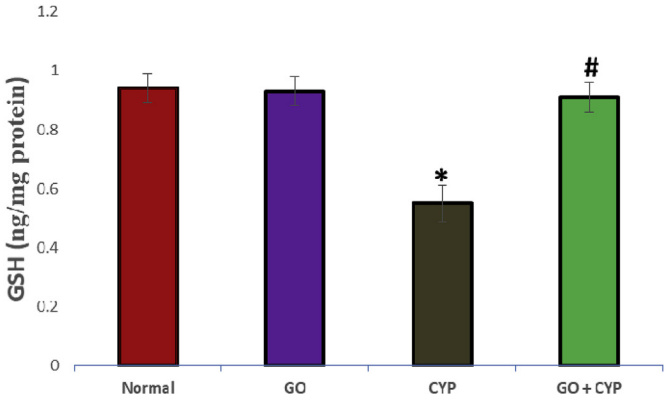

Fig. 4. Effect of garlic oil (GO) on testicular reduced glutathione (GSH) level in CYP-treated rats. CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).
*p < 0.05: significant when compared to normal control group.
#p < 0.05: significant when compared to CYP group.

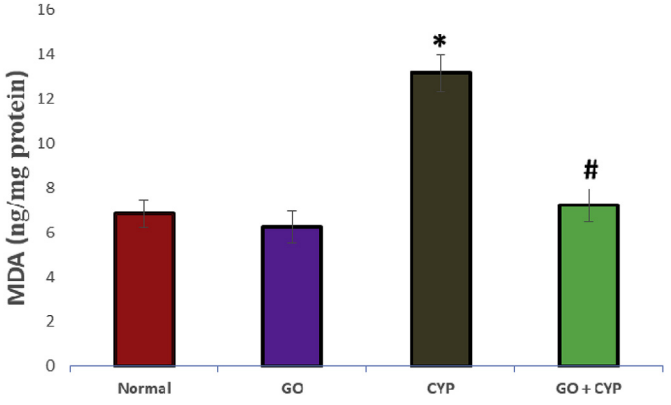

Fig. 5. Effect of garlic oil (GO) on testicular malondialdehyde (MDA) level in CYP-treated rats. CYP: Cyclophosphamide. Values are expressed in mean ± SEM (n = 6).
*p < 0.05: significant when compared to normal control group.
#p < 0.05: significant when compared to CYP group.

CAT, GPx, as well as GSH level when compared with CYP group. Interestingly, the level of MDA significantly ($p < 0.05$) reduced when compared with CYP group.

3.6. Histopathological examination

Fig. 6 shows the histological observations of the effect of GO on CYP-injected rats. The testis histology of rats in normal and GO groups show normal architecture of the testis with Sertoli cells (SC), Leydig cells (LC) and normal tissue for spermatogenesis (SG). In rats injected with cyclophosphamide (CYP) only, testicular tissues were sloughed, necrotic Leydig cells (NLC) and severe arrest of spermatic tissue (SAS). In GO + CYP group, the adverse effects inflicted on the tissue by CYP were ameliorated by GO supplementation, although mild necrosis (MN) was observed.

4. Discussion

Cyclophosphamide is a cytotoxic alkylating agent clinically applied in cancer chemotherapy or as an immunosuppressant [35]. However, multiple side effects underlined chiefly by oxidative stress, including testicular toxicity are a major challenge that limits its use for patients. Various studies show that antioxidant agents can modulate chemotherapy toxicity and also minimize the adverse side effects [36].

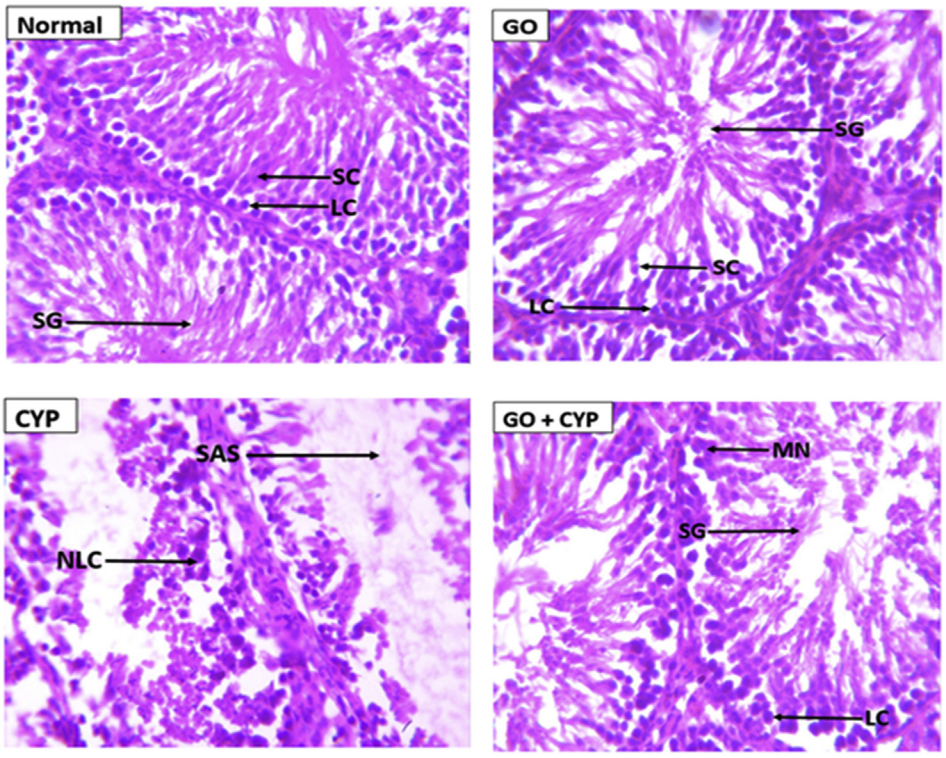

Fig. 6. Histological study of testes in control and experimental groups of rats. Normal and GO groups showing normal testicular architecture. CYP group showing severe arrest of spermatic tissue (SAS) and necrotic Leydig cells (NLC). GO plus CYP showing histological structure similar to normal and GO groups.

Thus, we examined the effect of natural medicinal oil, GO with antioxidant properties on CYP testicular toxicity and endocrine changes in rats.

In the present study, single intraperitoneal injection of CYP induced marked redox disturbances in the testis of rats. The disturbance was demonstrated by considerable depression in the SOD, CAT and GPx activities in the testis as well as significant reduction in GSH and increase in MDA level. The histopathological examination revealed severe arrest of spermatic tissue and necrosis of cells involved in spermatogenesis and testosterone production. Cyclophosphamide induces a broad spectrum of toxicities and tissue dysfunctions constituting a serious health hazards to cancer patients [2,3,12,37]. Literature strongly suggests that it interferes with antioxidant defense mechanisms by copious ROS generation associated with its toxic hepatic metabolite, acrolein, known to promote oxidative stress [37,38]. Oxidative stress is known to be a consumer of antioxidant enzymes (SOD, GPx, CAT) activities as confirmed in our findings here. Presence of omega 3 polyunsaturated fatty acids within the membrane of testicle makes this organ more susceptible to oxidative stress [39]. Therefore, the ROS generated by CYP might overwhelm GSH to expose cell membranes, SOD, CAT and GPx to ROS attack leading to lipid peroxidation indicated by increased levels of MDA in this study. Our findings are consistent with previous studies showing CYP-induced depression of antioxidant enzyme activities and lipid peroxidation in testes [3,5,10]. Interestingly, the dietary supplementation of GO to rats injected with CYP protects the testicular antioxidant defences as indicated by significant restoration of SOD, CAT, and GPx activities and level of GSH comparable to normal control. In corollary, the lipid peroxidation was inhibited which resulted in prominent decrease in MDA level. Previous studies have shown the antioxidant property of GO in acetaminophen toxicity and diabetic rats [20,21]. Garlic oil contains sulfhydryl group (–SH)-based compounds which directly scavenge ROS and modulate cell redox state [40]. Allin, allicin and other diallyl trisulfides are reported present in GO (20, 40]. Garlic oil is a natural extract from garlic bulbs and our estimation in this study further shows that it contains certain amount of phenols and flavonoids (Table 1). Diverse phenolic compounds and flavonoids which are increasingly being associated with numerous pharmacological effects, including antioxidant, anti-inflammatory, antimutagenic, anticarcinogenic, antidiabetic, anti-hypercholesterolemic and cardioprotective effects [41]. However, the antioxidant effects of these natural compounds were demonstrated in DPPH and FRAP assays estimated in this study. The antioxidant potential of GO is attributed to the GO organosulfur compounds and inherent phytochemicals that enhance glutathione level and glutathione-S-transferase activity [42]. The constituents of GO may conceivably be responsible for the restorative antioxidant effect observed in this study.

Toxicity induces disturbances in testosterone levels and gonadotropin secretion leading to testicular dysfunction [5,43,44]. Our study also reveal that CYP significantly decreased LH, FSH and testosterone levels in rats. The LH and FSH are anterior pituitary hormones which are key players in spermatogenesis. It is known that alterations in LH and FSH secretion could affect quality and quantity of sperm and testosterone regulation [45]. The oxidative testicular damage by CYP may be related to the depletion in testosterone level and feedback on the hypothalamus and the pituitary that control the secretion of LH and FSH. A study has shown that CYP inhibits Leydig cell steroidogenesis [46]; thus, the decreased testosterone level could be attributed to impaired Leydig cells in the current study. The decreased testosterone induced arrest of spermatogenic tissues as shown in the histopathological results. Although the secretion of testosterone is dependent upon the secretion of LH by the pituitary gland, the lowered level of testosterone may inflict a negative feedback mechanism on pituitary gland which may result in reduced level of LH and FSH observed in this study. Our data show that injection of CYP adversely affects testicular function by decreasing pituitary LH and FSH secretion and reducing testosterone level. Our findings are consistent with the previous studies showing the toxicity of CYP and cadmium by reducing the levels of testosterone, LH and FSH in preclinical studies [5,43]. The diet containing GO markedly improved the levels of LH, FSH and testosterone. The potential of GO to improve the hormones suggests a mechanism that may be related to

oxidative stress underlying the CYP-induced endocrine deficits. The histopathological alterations were ameliorated by GO dietary supplementation comparable to rats in control group. Our results are in consonance with the earlier studies of El-Akabawy and El-Sherif [41] and Elghaffar et al. [47] showing that GO is capable of reducing testicular oxidative stress and endocrine depletion in rat testis.

5. Conclusion

To our knowledge, this study is the first to report that GO exerts a protective effect against anticancer drug- or CYP-induced testicular damage. The protective effect of GO was evidenced by the clear restoration of antioxidant enzymes mechanism, modulation of serum testosterone LH and FSH levels and amelioration of induced histopathological changes in the CYP-treated testes of Wistar rats. These results suggest GO as a promising dietary supplement to protect the testes against CYP toxicity in males undergoing chemotherapy.

Authors statement

The authors declare no conflict of interest. All the authors agreed that the manuscript be submitted to Journal of Nutrition and Intermediary Metabolism.

Signed: The Corresponding Author, Dr Ademola C Famurewa.

Declaration of competing interest

The authors declare no conflict of interest.

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