Heliyon 10 (2024) e24989

Contents lists available at ScienceDirect

Heliyon

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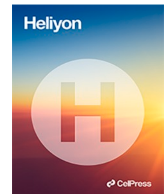

Research article

Possible mechanisms involved in the protective effect of lutein against cyclosporine-induced testicular damage in rats

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ARTICLE INFO

Keywords:
Lutein
Cyclosporine
Oxidative status
Inflammation
Apoptosis
Reproduction

ABSTRACT

Oxidative stress and aberrant inflammatory response have important implications in cyclosporin-induced reproductive functions. Previous studies have shown that agents with antioxidant and anti-inflammatory activities might be beneficial in reversing cyclosporin-induced reproductive impairment. Lutein is a naturally occurring compound with antioxidant and anti-inflammatory properties. However, the effect of lutein against cyclosporin-induced reproductive impairment remains in complete. Hence, we investigated the protective effect of lutein, specifically focusing on the role of nuclear factor erythroid 2 related factor-2 (Nrf2)/heme-oxygenase-1 (HO-1)/connexin-43 (Cx-43) upregulation system against cyclosporine-induced reproductive impairment. Six male Wistar rats were allotted into 5 groups and given daily gavage of cyclosporine (40 mg/kg) and/or lutein (30 mg/kg) for four (4) weeks or in combination, respectively. The testicular antioxidant scaffolds: superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), add to sulfhydryl (T-SH), non-protein sulfhydryl (NP-SH), glutathione reductase (GR), glutathione-S -transferase (GST), glutathione peroxidase (GSH-Px), thiobarbituric acid reactive substances (TBARS), myeloperoxidase (MPO), testicular proinflammatory cytokines, apoptotic related protein, nucleic acids, sialic acid, testicular proton pump ATPase, stress responsive protein, BTB-related protein and total protein levels in the testes were assayed thereafter. Cyclosporin significantly increased NOX-1, TNF-α, IL-1β, MPO, caspase-3 and -9 levels, which were reversed by lutein. Lutein reversed cyclosporin-induced decreases in Nrf2, HO-1, BCL-2, cytochrome C, with corresponding increase in CAT, SOD, GSH, T-SH, NP-SH, GST, GR, GSH-Px, and Cx-43 levels compared to cyclosporin groups. Lutein also abates cyclosporin-induced alterations Na + -K + -ATPase activities. Our findings showed that lutein's protective effect against cyclosporin-induced reproductive impairment might be associated with mechanisms linked to its antioxidant, anti-apoptotic, and anti-inflammatory properties, notably through up-regulation of Nrf2/HO-1/Cx-43 signaling and down-regulation of NOX-1 signaling.

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https://doi.org/10.1016/j.heliyon.2024.e24989
Received 23 June 2023; Received in revised form 7 January 2024; Accepted 18 January 2024
Available online 22 January 2024
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1. Introduction

Long-term chemotherapeutic agent-induced infertility has been linked to public health concern on male fertility [1]. A major global health problem is the harmful impact of pharmaceuticals and environmental toxins on reproductive functions and subfertility because damage to the testicular organs puts life itself in danger [1]. The hypothalamic, pituitary, and gonad axis are referred to as such because they function as a unified unit to induce hormonal changes that have both local and systemic consequences in the body [2]. This axis is essential for the regulation of growth, reproduction, and aging [2]. It is recognized that certain treatment regimens, such as immunosuppressive medications intended to avoid allergic reactions, might create changes in this axis that negatively affect and disrupt reproductive functioning [3,4].

Cyclosporine is most typically utilized as a potent immunosuppressant in transplantation surgery and the treatment of autoimmune illnesses [5]. This is based its high inhibitory activity on lymphokine synthesis, differentiation, and signal transduction pathways of cell T-receptors, the fungal peptide [5]. But studies have shown that cyclosporine can has toxic side effects, including vascular dysfunction [6], hepatotoxicity [5], ovarian damage [7], testicular injury [8], spermatozoal toxicity [9,10], cardiotoxicity [11]. It is widely accepted that oxidative stress contributes to the toxicity of cyclosporine [9]. Reproductive abnormalities are associated with elevated levels of oxidative stress that are caused by cyclosporine-mediated male infertility [12,13]. Chronic cyclosporine use causes oxidative stress, which impairs Sertoli cell phagocytic role and the hypothalamic-pituitary-gonadal axis directly [9,12,13]. Direct anomalies in the hypothalamic-pituitary-gonadal axis, Sertoli cell phagocytic function, testicular inflammation, and apoptosis, all of which are thought to be the underlying causes of testicular and sperm toxicity, may account for the diminished sexual performance [9]. Notably, pro-inflammatory cytokines like TNF-α and IL-1 inflammatory cytokines, as well as testicular caspase-3, have been identified as essential actors in cyclosporine-mediated oxidative stress-induced male reproductive problems [4,13]. The body's natural antioxidant defenses are overwhelmed by severe oxidative stress, as observed in cyclosporine toxicity, leading to oxidative damage [4]. Supplementation with antioxidant compounds have been proposed to mitigate cyclosporine-induced oxidative aberration [4,14].

Lutein is a tetraterpenoid that can be found in a range of fruits and vegetables, according to Sommerburg et al. [15]. Luetin has two hydroxyl groups in its structure, which provide it antioxidant capabilities [16]. Because it is not generated by the body, it must be received from the consumption of lutein-rich foods such as kale, spinach, kohlrabi, and collards [17]. Recent studies have highlighted lutein's antioxidant powers, as well as its benefits in treating a variety of eye illnesses, such as age-related macular degeneration [14]. Additionally, lutein as been shown to demonstrate anti-inflammatory impacts. The mechanisms of lutein's antioxidant activity are well recognized and extensively discussed in literature [18]. By blocking the formation of nuclear factor kappa B (NF-κB), lipid peroxidation, TNF-α, IL-1, as well as elevating GSH levels, lutein has found to demonstrate antioxidant and anti-inflammatory properties [19]. As a result, our previous studies showed that lutein reduced cyclosporine-induced reprotoxicity through modulation of androgenic hormones and enzymes in rats [4]. However, there is currently no published evidence of the involvement of Nrf2/HO-1/Cx-43 up-regulatory role in the reproductive enhancing effect of lutein in cyclosporine-induced increased NOX-1 in rat testes. Therefore, this experiment was designed to investigate the role of Nrf2/HO-1/Cx-43 in the reproductive enhancing effect of lutein in cyclosporine-induced elevated NOX-1-mediated testicular damage in rats.

2. Materials and methods

2.1. Animals

The adult male Wistar rats used in this study weighed 150–200 g (7–9 weeks old) were acquired from the University Central Animal Facility. They were housed in plastic cages under typical conditions such as a 12:12 day/night cycle and an average temperature of 25 °C. The rats had unrestricted access to water and standard rat chow for at least 14 days prior to starting the experiment to allow them to get used to it. The animals used in the study were treated in compliance with the National Institutes of Health (NIH) guidelines for laboratory animal care and use (publication #85-23, amended) following experimental protocol approval by the university's research ethics committee (REC/FBMS/DELSU/18/62). In accordance with the three Rs, six (6) animals were employed in each group in this study (3Rs: Replacement, Reduction and Refinement).

2.2. Drugs and chemicals

Cyclosporine (Kule Ara Pharmaceuticals, Ibadan, Oyo State, Nigeria) and lutein used in the study were purchased from Carbone Scientific Co., Ltd. Bought in London, United Kingdom. Corn oil was purchased from Shoprite in Ibadan, Nigeria. Cyclosporine and lutein were individually dissolved in 20 mL of corn oil and orally administered immediately before use. The dosages and methods of corn oil [4], cyclosporine (40 mg/kg) [5,14] and lutein (40 mg/kg) were determined based on previous dose-response effects and early research [5,19]. However, saline (2 mL/kg, p.o.) and corn oil (2 mL/kg, p.o.) were administered to naïve rats in separate groups, which served as control and vehicle groups, with treatments spaced 30 min apart.

2.3. Experimental procedures

Five groups of six animals each based on calculated power analysis [20] and previous effect size [5], were formed by randomly dividing the animals. As a standard control, group 1 was given 2 mL/kg of saline. Throughout the trial, group 2 was given 2 mL/kg of

corn oil (Control Vehicle). Rats in group 3 received lutein (40 mg/kg/day) orally as the drug control group. Group 4 received oral gavage treatment with cyclosporine (40 m/kg/day). Lastl, cyclosporine and lutein were administered to group 5 rats for a period of four weeks.

2.4. Sample collection and preparation

To avoid significant chemical changes, all animals were euthanized twenty-four (24) hours after receiving their final dose of medication with ketamine anesthesia. Each animal's testicles were promptly removed, thoroughly cleaned of any connective tissues and adhering fat, and their grams of weight were recorded. Before being centrifuged at 14,000×*g* for 20 min, the testes were homogenized in a glass-Teflon homogenizer for 10 s at 4 °C in RIPA buffer, pH 7.4. As soon as possible, supernatants were taken and kept at 20 °C until needed for biochemical tests.

2.5. Determination of oxidative status

2.5.1. Estimation of thiobarbiturate acid reactive substance (TBARS) in testicular cells

Using a biochemical assay kit (ZeptoMetrix), the amounts of TBARS was evaluated based on the lipid peroxidation byproducts, malondialdehyde (MDA) equivalents (TBARS). Thus, 0.1 ml of testicular homogenate was mixed with reaction buffer and testicular homogenate from the kit, and the mixture was heated at 95 °C for 60 min. The supernatant's absorbance was assessed at 532 nm after cooling. nmol/mg protein was used to express the detected MDA levels [21].

2.5.2. Estimation of antioxidant enzyme activities in testicular cells

Superoxide dismutase activity was assayed as described by McCord and Fridovich [22]. The underlying idea is that superoxide anions cause the rapid auto-oxidation of adrenaline in aqueous solution to adrenochrome. CAT activity was assayed as described by Ben-Azu et al. [21] with a spectrophotometer at 420 nm.

2.5.3. Estimation of glutathione redox status (GST, GSH, GSH-Px and GR) in testicular cells

The amount of GSH-CDNB conjugate produced per minute per milligram of protein was estimated using the specific activity of the glutathione transferase (GST) enzymes. The method described above was used to measure GSH, or total reduced glutathione [23]. We also assayed for glutathione peroxidase (GSH-Px) and glutathione reductase (GR) levels based on previous procedure [23].

2.5.4. Estimation of total sulfhydryl (T-SH) and non-protein sulfhydryl NP-SH content in testicular cells

Using Sedlak and Lindsay's method [24], total amounts of sulfhydryl was assayed. 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.01 M Ellman's reagent, and 0.2 M Tris buffer (pH 8.2) were reacted with testicular homogenate (DTNB) and the extinction at 412 nm was determined after centrifugation of the clear supernatants at room temperature. To determine non-sulfhydryl levels, testicular homogenate was mixed with a 50 % trichloroacetic acid (TCA) solution. Samples were centrifuged for 15 min after intermittent mix for 0–15 min. After that, in less than 5 min, 0.1 mL of DTNB at 412 nm was add to 2 mL of the supernatant and 4 mL of 0.4 M Tris buffer (pH 8.9).

2.5.5. Estimation of nucleic acid (RNA and DNA) and total protein (TP) contents in testicular cells

The nucleic acids in testicular cells were determined using Bregmans and Bouman [25] method. An Iced-cold 10 % solution of trichloroacetic acid was used to suspend homogenates (TCA). Using 95 % ethanol, two extractions of the pellets were performed. In order to measure the amounts of DNA and RNA in nucleic acids, either diphenylamine or the orcinol reagent was used. To assess TP levels in testes using reference other than human plasma albumin, we used Finestone et al. [26] method.

2.6. Estimation of inflammation markers in testicular cells

To measure the levels of TNF-α and IL-1 in testis homogenate, we employed Thermo Fisher Scientific ELISA kits. The kits were applied in accordance with the manufacturer's recommendations. To gauge polymorphonuclear leukocyte assembly, testicular myeloperoxidase (MPO) activity was assayed. This test was based on the oxidation of guaiacol, which was dependent on hydrogen peroxide [27]. The MPO activity of the testes enzyme, which is mostly found in the azurophil granules of polymorphonuclear leukocytes, utilized as a marker for tissue accumulation of neutrophils, was measured based on the original method described by Bradley et al., [28] with minor modification [29] and expressed MPO activity U/mg tissue.

2.7. Estimation of apoptotic, BTB-related protein, stress responsive protein markers and proton pumps (Na^+-K^+-ATPase and $Ca2^+$-ATPase) activities in testicular cells

Testicular apoptotic [cytochrome C, caspase 3 and 9, and Bcl-2], BTB-related [NOX-1 and Cx-43], and stress-responsive proteins [Nrf2 and HO-1] were obtained using commercial ELISA kits from BioVision, Inc. and Sigma-Aldrich, and Cusabio. The manufacturer's instructions were followed during the assays. Spectrophotometry was used to quantify the activity of the testicular Na+-K+-ATPase and Ca2+-ATPase, as previously described [30]. The homogenate was blended with two (2 mL) of the standard reagents and left to stand at room temperature for 30 min to allow for color change development. Spectrophotometer was then used to measure the

absorbance at 820 mm. Inorganic phosphate is thought to be produced as a result of the hydrolysis of adenosine triphosphate (ATP) in the presence of the appropriate cations. Calculating the amount of released inorganic phosphate involves using the ammonium molybdate-ascorbic acid system. The oxidation of ammonium molybdic acid by strong sulfuric acid gives inorganic phosphate, a yellow colour. Molybdic acid was then reduced by ascorbic acid to produce the recognizable blue tint.

Color intensity was directly correlated with the volume of inorganic phosphate released into the reaction media.

2.8. Statistical analysis

The data were examined using a biostatistics program (Graph Pad Prism 8). The mean and SEM values for all the data were provided. The statistics were then conducted using a one-way analysis of variance (ANOVA), followed by a post hoc test (Benferroni) for multiple comparisons. For each test, a significance level of p less than 0.05 was applied.

3. Results

3.1. Lutein abates cyclosporine-induced oxidative stress in rats testes

Fig. 1a–c shows the influence of lutein on oxidative stress induced by cyclosporine in rat testes. As seen in Fig. 1a–c, cyclosporine (40 mg/kg, i.p.) induced significantly ($p < 0.05$) oxidative stress, as evidenced by an increased level of TBARS ($F(4,25) = 226$, $p < 0.0001$), with simultaneous decrease in SOD (Fig. 1b) and CAT (Fig. 1c) levels compared to control groups. By reducing TBARS levels and enhancing SOD and CAT activities in rat testes under oxidative stress, lutein treatment (40 mg/kg, p.o.) considerably ($p < 0.05$) alleviated cyclosporin-induced oxidative damage.

3.2. Lutein protects against cyclosporine-induced changes in glutathione redox system in rats testicular

According to Fig. 2a–f, cyclosporine (40 mg/kg) significantly ($p < 0.05$) lowered glutathione redox status as seen by reducing GSH (Fig. 2a), GPx (Fig. 2b), T-SH (Fig. 2c), NP-SH (Fig. 2d), GR (Fig. 2e) and GST (Fig. 2f) in comparison to controls. The glutathione redox system was altered by cyclosporine, but treatment with lutein (40 mg/kg, p.o.) significantly ($p < 0.05$) alleviated this by increasing GSH, GPx, T-SH, NP-SH, GR and GST activity in the testes (Fig. 2a–f).

3.3. Protective impacts of lutein against cyclosporine-evoked changes in apoptotic related factors in rats

The changes in B-cell lymphoma-2 (Bcl-2), mitochondrial cytochrome C concentration and caspases −3 and −9 in response to cyclosporine treatment are shown in Fig. 3a–f. Cyclosporine significantly decreases Bcl-2 (Fig. 3a) mitochondrial cytochrome C content (Fig. 3b) with a corresponding increase in the activities of caspase-3 (Fig. 3c) and caspase-9 (Fig. 3d). respectively. However, in rats co-

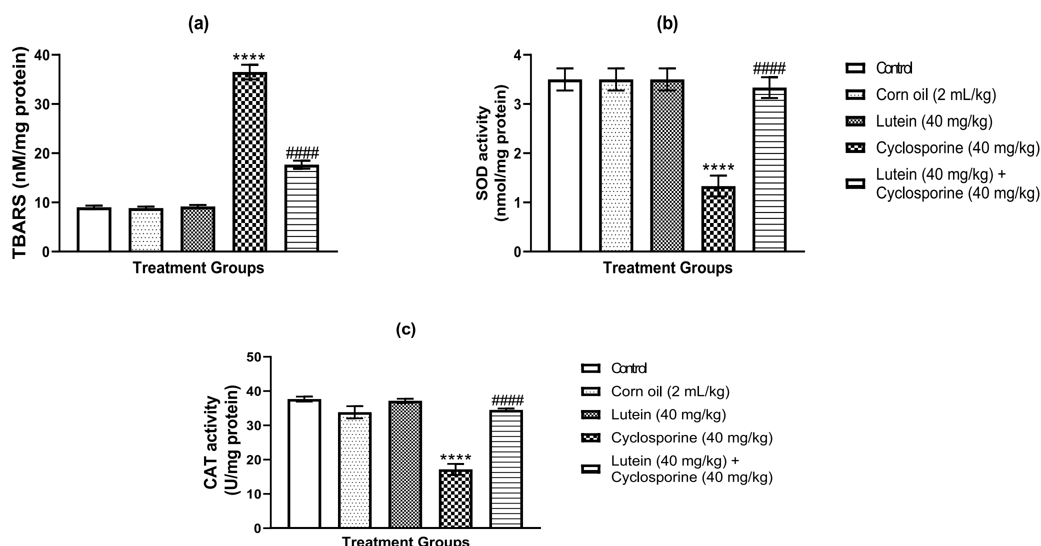

Fig. 1. a–c. *Lutein abates cyclosporine-induced oxidative stress in rats testes*. Thiobar-bituric reactant (TBARS) (a), superoxide dismutase (SOD) (b) and catalase (CAT) (c) are shown. The mean and S.E.M. are represented as bars ($n = 6$). There are significant differences between the different treatment groups according to a one-way ANOVA and a Bonferonnis post hoc test. ****$p < 0.0001$ vs. control group; ####$p < 0.0001$ versus the cyclosporine group.

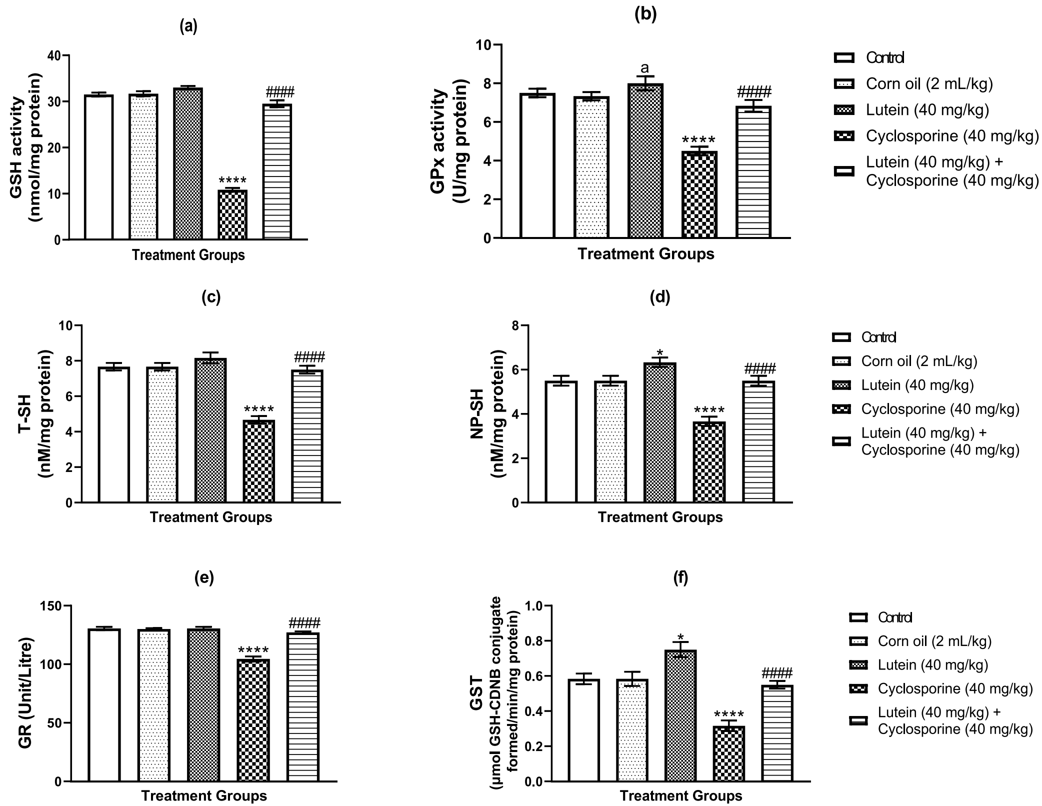

Fig. 2. a–f. *Lutein protects against cyclosporine-induced changes in glutathione redox system* in *rats testes*: reduction of glutathione, GSH (a), glutathione prooxidase, GSH (b), total sulfhydryl, T-SH (c), non -protein sulfhydryl, NP-SH (d), glutathione reductase, GR (e) and glutathione S-transferase, GST (f). Bars represent mean S.E.M (n = 6). There are significant differences between the different treatment groups according to a one-way ANOVA and a Bonferonnis post hoc test. ####p < 0.0001 compared to cyclosporine group; *p < 0.05, ****p 0.0001 compared to control group.

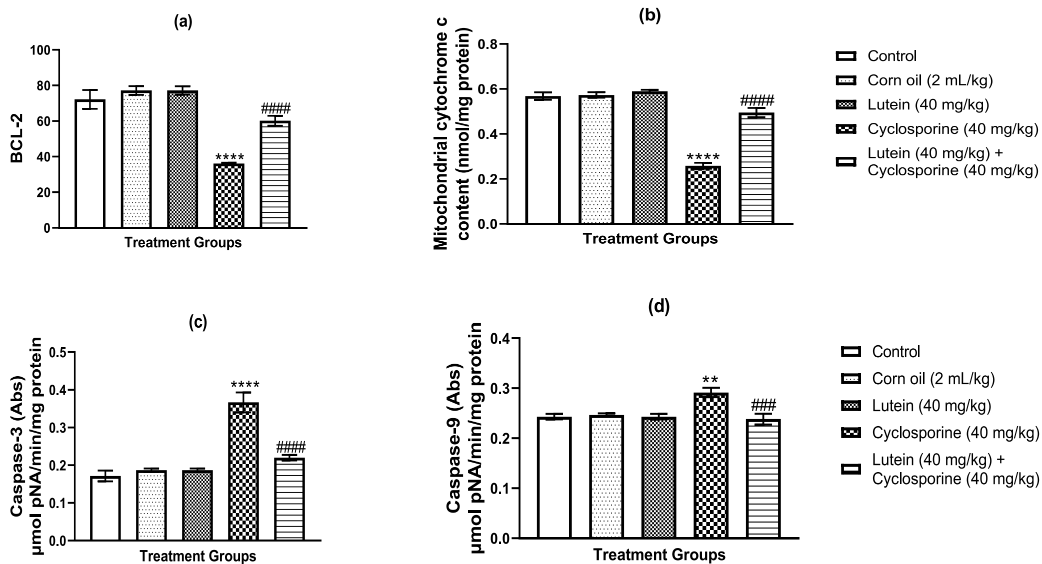

Fig. 3. a–d. Lutein reverses the effects of cyclosporin-induced changes in B cell lymphoma-2 (BcL-2) (a), mitochondrial cytochrome C content (b), caspase −3 (c), and caspase −9 (d) in rats testes. The bar represents the mean and S.E.M (n = 6). ****p < 0.0001 compared to controls; ####p < 0.0001 compared to cyclosporine.

treated with lutein, cyclosporine-induced decreases in Bcl-2 and mitochondrial cytochrome C levels and increases in caspase activities, were alleviated.

3.4. Protective effect of lutein on cyclosporine-induced alterations in testicular pro-inflammatory cytokines and enzyme in rats

Using one-way ANOVA and a post hoc test, Fig. 4 illustrates how cyclosporine significantly raised IL-1β (Fig. 4a) and TNF-α (Fig. 4b) levels in comparison to control animals. Additionally, as compared to the control group, cyclosporine-treated groups showed a marked increase in testicular MPO activity (Fig. 4c). Nevertheless, lutein supplementation significantly attenuates these changes (Fig. 4a–c).

3.5. Protective impacts of lutein against cyclosporine-induced impairment in proton pump activities in rat testes

Testicular Na+-K+-ATPase (Fig. 5a) and Ca2+-ATPase (Fig. 5b) both showed a considerable decline based on control, suggesting proton pump impairment. However, lutein supplementation improved the activity of these proton pumps compared to cyclosporine groups.

3.6. Protective effect of lutein against cyclosporine-induced alterations in nucleic acid and protein activities in rats testicular cell

Testicular DNA (Fig. 6a), RNA (Fig. 6b), and TP (Fig. 6c) quantities in the cyclosporine groups significantly decreased, as shown by Fig. 6a–c. The lowered levels of testicular DNA, RNA, and TP were considerably increased in lutein-supplemented groups than compared to cyclosporine groups.

3.7. Protective effects of lutein onagainst cyclosporine-invoked changes in blood testes barrier (BTB)-related protein level in rats

Cyclosporine caused BTB destruction indicated by decreased connexin-43 (Cx-43) (Fig. 7a) and increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-1 (NOX-1) levels (Fig. 7b) concentrations in the testes compared to control groups. However, these alterations were abated with lutein compared to cyclosporine groups.

3.8. Protective effect of lutein against stress responsive protein in cyclosporine treated rats

Fig. 8a–b illustrates the effect of lutein on stress response protein in cyclosporine-treated rats. In contrast to the control group, cyclosporine considerably increased the protein levels of HO-1 (Fig. 8a) and Nrf2 (Fig. 8b) in the testes. Testicular Nrf2 and HO-1 levels were significantly elevated lutein compared to cyclosporine groups.

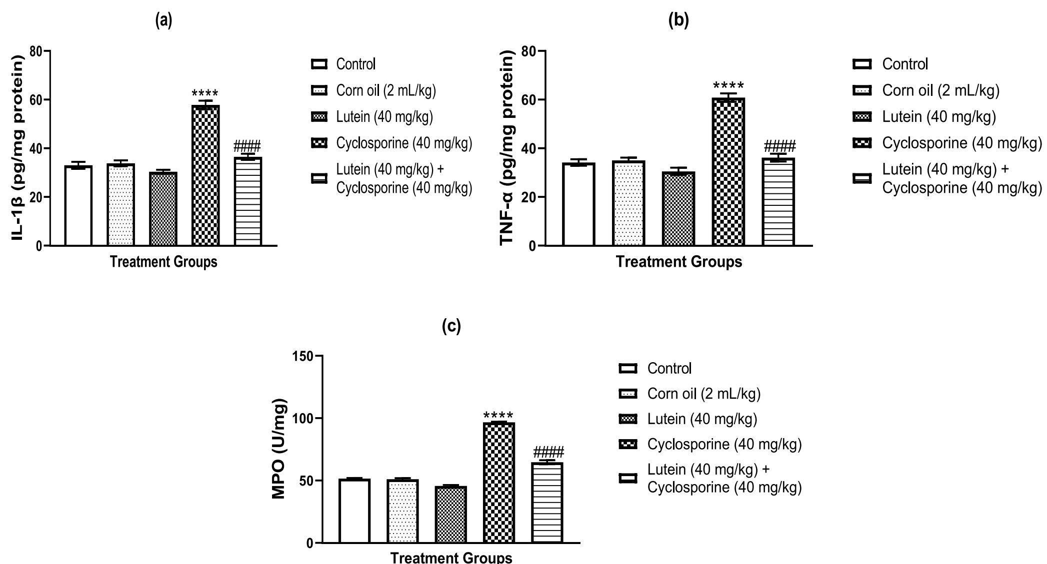

Fig. 4. a–c. Lutein reduces cyclosporine-induced changes in testicular proinflammatory cytokines and enzymes in rats: tumor necrotic factor-alpha (TNF-α) (a), interleukin-1 (IL-1) (b), and myeloperoxidase (MPO) (c) in rats testes. Bar was represented by mean S.E.M ($n = 6$) using one-way ANOVA followed by Benferroni post hoc test. ****$p < 0.0001$ compared to controls; ####$p < 0.0001$ compared to cyclosporine.

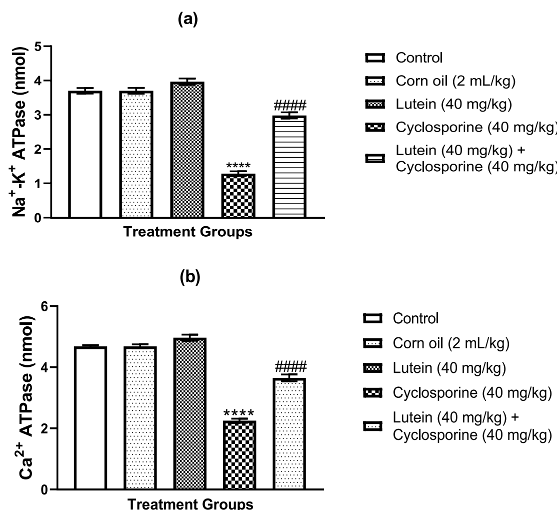

Fig. 5. a–b. Lutein reduced cyclosporine-induced impairments in testicular sodium-potassium adenosine trisphosphatase (Na + -K + -ATPase) (a), calcium adenosine trisphosphate (Ca2+-ATPase) (b) activities in rats. Data are presented as mean S.E.M. (n = 6). ****p < 0.0001 versus controls; ####p < 0.0001 versus cyclosporine.

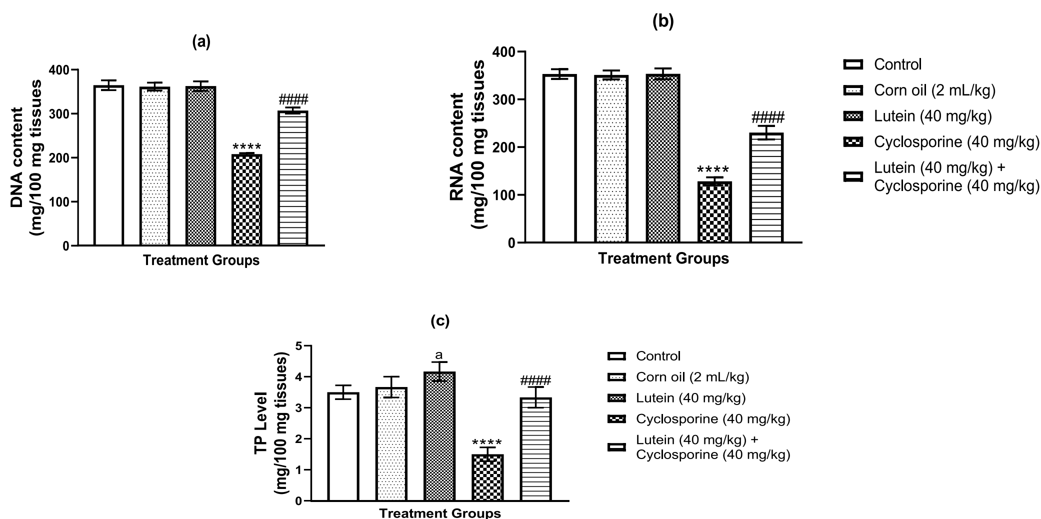

Fig. 6. a–c. Lutein assuages cyclosporine-induced impaired testicular deoxyribonucleic acid (DNA) (a), ribonucleic acid (RNA) (b), total protein (TP) (c) concentrations in rats testes. Data are presented as mean S.E.M. (n = 6). ****p < 0.0001 versus controls; ####p < 0.0001 versus cyclosporine.

4. Discussion

The use of alternative medicine to treat cyclosporine-induced toxicities in several organs has grown in popularity [5,14]. The increase of proinflammatory cytokines, apoptosis, lipid peroxidation, and oxidative stress following disruption of oxidant-antioxidant equilibrium has been established in multiple studies to be the most plausible mechanism of cyclosporine-induced deleterious effects on the reproductive system [14]. Thus, this experiment was aimed to examine the involvement of Nrf2/HO-1/Cx-43 up-regulatory role in the reproductive protective effect of lutein in cyclosporine-induced testicular damage in rats. In this investigation, lutein ameliorated cyclosporine-induced impaired testicular functions in rats as evidenced by increased testicular Nrf2/HO-1/Cx-43, proton pumps ATPase concentrations, activation of testicular immune system, reduction of testicular oxidative injury, normalization of antioxidant-redox status, with inhibition of testicular apoptosis influx. More specifically, lutein inhibits testicular oxidative injury and apoptotic-inflammatory influx by upregulation of Nrf2/HO-1/Cx-43 signaling pathway, as well as reducing the activation of testicular

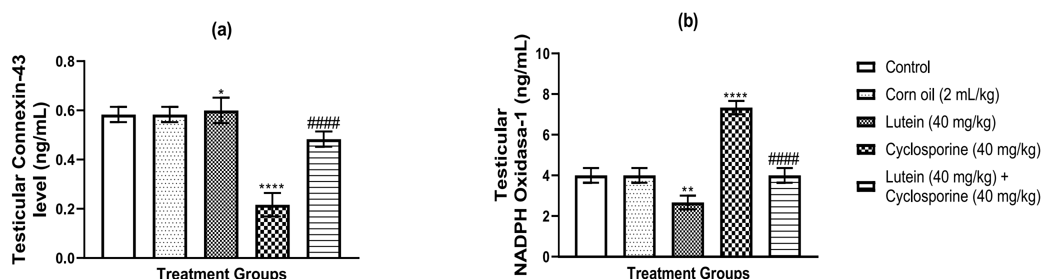

Fig. 7. a–b. Lutein reverses cyclosporine-induced alterations in testicular levels of connexin-43 (Cx-43) (a) and NADPH oxidase (NOX-1) (b) in rats testes. Data are presented as mean S.E.M. (n = 6) *p < 0.05, **p < 0.01, ****p < 0.0001 versus control; ####p < 0.0001 cyclosporine.

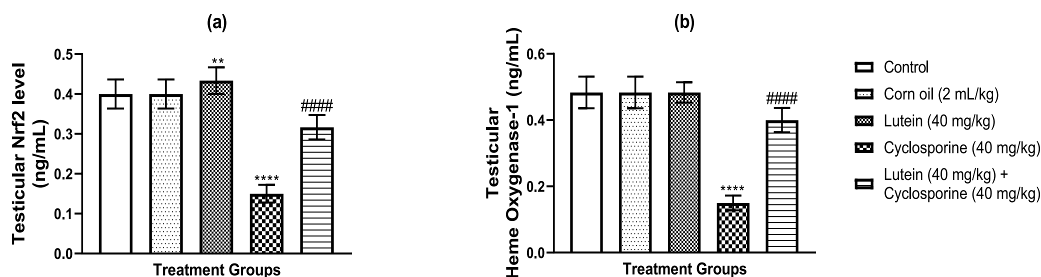

Fig. 8. a–b. Lutein increases the levels of testicular nuclear factor erythroid 2 (Nrf2) (a) and Heme oxygenase-1 (HO-1) (b) in cyclosporine-treated rats testes. Data are presented as mean S.E.M. (n = 6) (One way ANOVA followed by Benferroni post hoc test). **p < 0.01, **p < 0.01, ****p < 0.0001 versus control; ####p < 0.0001 versus cyclosporine group.

immune reactivity and antiapoptotic related protein markers. Notably, we measured that lutein raised the levels of protein and nucleic acid activity in rat testicular cells.

Numerous studies have demonstrated that cyclosporine exposure may impair tissues' redox balance, indicating that oxidative stress may be the cause of biochemical and physiological abnormalities associated with cyclosporine-induced reproductive impairment [14]. Since ROS-derived pro-oxidants cause cellular damage, oxidative stress is a condition marked by an increased rate of cellular damage [13]. One of the primary mechanisms of cyclosporine side effects is the induction of excessive ROS generation, which results in changes in the cellular antioxidant defense system and ultimately affects susceptibility to oxidative stress [15]. These increased levels of free radicals and suppressed antioxidant defense may cause cell instability and oxidative damage to the cell membrane, increasing sensitivity to LPO [31]. The endpoint biomarkers of oxidative stress-inducing harmful effects of medicines and toxicants are damage to membrane lipids, protein, and DNA [4]. More specifically, depleted GSH, which is a tripeptide for example has been implicated in reproductive impairment [9,10].

Due to a high concentration of polyunsaturated fatty acids and a lack of antioxidant activity, male germ cells can be sensitive to oxidative damage [20]. According to previous research, oxidative stress is a major cause of male reproductive disorders. Recent research suggests that the formation of ROS contributes to the organ damage caused by cyclosporine [15,17]. It was shown that the total endogenous antioxidants and sulfhydryl counterparts were present at lower quantities in the cyclosporine-treated rats in the current study. There are many biological functions for endogenous sulfhydryl molecules, including cellular defense against oxidation through strong nucleophilic activity that shields proteins, nucleic acids, and other macromolecules from pro-oxidants [32]. This is because lower levels of T-GSH and NP-SH in this study's cyclosporine-treated rats' testicular tissues signal lower levels of nucleic acids and testicular proteins (TP). Therefore, this modification raises the possibility of cytotoxicity and oxidative damage to the male reproductive system. Another component that ensures the development of spermatozoa is TP, which is androgen dependent. However, the large drop in TP may be attributable to a reduction in protein synthesis because of the harmful effects of cyclosporine. The findings of the current investigation demonstrated that testes had much higher levels of peroxidation.

In our analysis, we found that cyclosporine reduced GSH level with decreased CAT, SOD, GSH-Px, GST, and GR activities relative to normal controls, indicating a long-lasting effect. Previous studies have implicated the role of decreased antioxidant system, linking LPO to testicular damage in rats challenged with cyclosporine [9,10]. The decrease in GSH levels and other markers of oxidative stress is consistent with previous animal studies showing that treatment with cyclosporine reduces antioxidant system [9,10]. While decreased in the intracellular antioxidant, GSH is known to be responsible primarily for cyclosporine's toxic effect, GSH is however believed to serve a variety of biological purposes, including protecting cells from oxidation [32]. The ability of GSH's sulfhydryl group to act as a potent nucleophile, offering antioxidant defense, and protection of DNA, proteins, and other macromolecules from ROS

makes this one of the compound's most crucial roles [32]. Accordingly, an increase in GSH suggests an increase in antioxidant capacity and a decrease in membrane lipid peroxidation, as shown by a drop in the concentration of TBARS/MDA [20]. Importantly, previous studies have substantially proven that synthesis of antioxidant machineries is dependent on the levels of Nrf2 [33]. Remarkably, reduced level of Nrf2 has been repeatedly used an important denominator to denotes severity of symptoms of many diseases [33–35]. Lutein has been shown to effectively protect proteins, lipids and DNA oxidative damage and intercepts ROS directly via other cellular antioxidant pathways [33]. However, the current study found that treatment with rat lutein abates cyclosporine-induced inhibition of testicular sulfhydryl group contents. Similar to this, after lutein treatment, the activity of the antioxidant enzymes SOD and CAT that have been suppressed were increased, suggesting its antioxidant capacity. A stress-responsive protein called HO-1 is quickly activated by both oxidative stress and several unpleasant stimuli. The cytoprotective action of HO-1 has been recognized as being resistant to oxidative damage [33]. It has also been established that the Nrf2 antioxidant system, which causes the expression of proteins and cytoprotective enzymes, is a key therapeutic target against oxidative stress [34,35]. One of the reasons that has been linked to decreased epididymal sperm motility in Nrf2 knockout mice is oxidative stress [36]. When the stress signal is produced, Nrf2 transports it to the nucleus, where it activates several genes that produce phase II antioxidant enzymes and stress-responsive proteins like HO-1 [35]. In this study, we found that lutein elevated both Nrf2 and HO-1 in the homogenate of testicular tissue. Therefore, our findings imply that lutein enhances antioxidant defense by activating Nrf2/HO-1 and upregulating antioxidant enzyme activity. Our findings may point to lutein's potential to reduce chemotherapy-induced cytotoxicity by triggering antioxidant machineries.

Mounting evidence have shown that the resting potential of the cell membrane, the transport of different solutes and water across the cell surface, the excitability of muscle and neuronal cells, and the Na+-coupled secondary transport of H+, Ca2+, glucose, amino acids, and neurotransmitters across the plasma membrane are believed to be generated by membrane-bound enzymes like ATPases [37]. Indeed, ion-activated adenosine triphosphatases, or ATPases, are also required for the development of sperm, sperm motility, and other testicular functions [37]. In fact, divalent cations found in ejaculated seminal fluids necessitate the activation of ATPase pumps [30,37]. However, it has been demonstrated that lipid peroxidation modifies the Na+/K+ ATPase in a selective manner at certain places [30]. According to our investigation, the sulfhydryl content of ATPase pumps was inhibited in cyclosporine-treated rats, which may have contributed to the severe decrease in proton pump activity and disruption of the lipophilic domains of the human sperm membrane [37]. Therefore, our findings are consistent with previous experimental studies [38] on animals showing that cyclosporine induced renal change with reduced Na+-K+-ATPase and Ca2+-ATPase functions compared with normal controls. Na^{+}-K+-ATPase and Ca2+-ATPase which is however, reduced in many diseases associated with reproductive impairment have also been used as a biomarker for the onset of testicular oxidative stress and decrease motility of sperm cells [39]. Together, our data offer more proof that cyclosporine increases the production of free radicals and suppresses antioxidant activity by impairing testicular Na+-K+-ATPase and Ca2+-ATPase functions. The seminiferous tubule and epididymis' luminal fluid may become alkaline if proton pump action is inhibited because acidification is impeded [39]. However, lutein cotreatment eliminated these modifications, which further suggest its protective role in sperm integrity and fertilization capacity.

Analysis of inflammatory markers further showed that cyclosporine-induced reproductive impairment was associated with inflammation. In fact, numerous experimental studies have suggested and demonstrated the significance of toxicant and drug-induced oxidative impairments and inflammatory cascade in the pathogenesis of male reproductive harm [40,41]. Increased oxidative stress characterized by free radical production along with an upregulation of pro-inflammatory cytokines (TNF-, IL-1) and apoptotic related proteins (cytochrome C, Bcl-2 and caspase 3 and 9) have been linked to cyclosporine-induced reproductive toxicity [42,43]. For instance, TNF-α and IL-1 are crucial for preserving healthy testicular function. However, elevated levels of TNF-α and IL-1, with upregulation of inflammatory genes have been shown to play prominent role in testicular dysfunction [44,14]. The current study confirmed prior findings by showing a significant increase in TNF-α and IL-1 levels in the cyclosporine-treated rats 14, 15]. Additionally, as seen by increased testicular MPO activity, our current investigation showed that cyclosporine-induced heightened testicular-inflammation was also linked to neutrophil recruitment, evidenced by increased release of testicular MPO. Notably, the recruitment of immune cells to inflammatory areas also stimulates the release of viscous cytokines and reactive oxygen intermediates [44,14]. However, the current investigation validated lutein's anti-inflammatory properties, which Oyovwi et al. [30] had previously described. We show that the ability of lutein to reduce reproductive impairment caused by testicular inflammation is explained by its ability to reduce ciclosporin-induced inflammatory flow, as evidenced by reduced levels of TNF-α, IL-1 and MPO. Selective germ cell death occurs regularly in the testes and is critical to maintaining spermatogenesis [30]. A slight increase in the percentage of testicular cell/germ cell death could lead to defective spermatogenesis and infertility [45]. Numerous types of physical or chemical injuries to the testes mostly result in increase in the incidence of germ cell death [46]. Here, we demonstrated that cyclosporine treatment-induced reduction in Bcl-2 was significantly followed by release of cytochrome C from the mitochondria into the cytosol compared with normal control groups. Consequently, elevated levels of caspase-9 with increased caspase-3 were measured in the testes, thus suggesting the long-lasting negative impacts of cyclosporine on testicular cells. Previous studies have repeatedly implicated the role of testicular apoptosis as one of the main causes of cell death in the testes, notably leading to decrease sperm count [44,14]. In this study, we measured that cyclosporine drastically altered the expression of proteins associated with apoptosis while also significantly reducing the expression of antiapoptotic factors such as BCL-2 in spermatogenic cells when compared to the control group [14,47]. Interestingly, we also showed that treatment with lutein significantly attenuated cyclosporine-induced apoptosis evidenced by reduced caspase-3 and -9, as well as cytochrome C with complementary increase in BCL-2 when compared with cyclosporine groups.

Furthermore, previous investigations have shown that BTB contributes to the maintenance of normal male fertility by limiting the entry of dangerous chemicals and stifling the development of sperm-specific antibodies [48]. As a result, the BTB's existence makes

treating many diseases more challenging. The BTB serves as a refuge, protecting germ cells from xenobiotic toxicants [49]. In relation to the integrity of the BTB, Cx43 is essential for the development of gap junctions for BTB integrity. Additionally, according to Gerber et al. (2016), NOX-1 has been identified as a key source of ROS linked with disruption of BTB integrity, whereas CX43 in Sertoli cells is required for BTB structure and function [50]. The decrease in Cx-43 protein level and the increase in NOX-1 concentration in the testes were both consistent with cyclosporine's deleterious effects on BTB-related protein levels. Of note, this finding is in line with the earlier reports on cyclosporin's induction of renal tubular dysfunction and afferent arteriolar morphological alteration [51,52]. However, lutein supplementation was shown to counteract these effects by lowering the NOX-1 concentration with corresponding decrease increase in Cx-43 protein in the homogenate, notably indicating inhibition of free radical generation. The modulation of Cx-43/NOX-1 pathway further reinforces the protective role of lutein against cyclosporine-induced alterations in BTB-related protein level in rat testes. Accordingly, among other mechanisms, increased Cx43 with reduced NOX-1 levels might be one of the protective primary means influencing BTB integrity in lutein treated rats.

Overall, previous studies have demonstrated that lutein interacts directly with Nrf2 [53], resulting in the increased expressions of several antioxidant and cytoprotective proteins such as HO-1 and Cx-43 [54–56]. Specifically, it is known that the activation of Nrf2 is triggered by the interaction of lutein with nuclear receptors, which then binds to ARE/EpRE sequence in HO-1 and Cx-43 genes, notably increasing their expressions [57]. The increased expression of antioxidant and cytoprotective proteins complementarily contributes to the protective effects observed with lutein supplementation, as these proteins play important roles in the protection against oxidative stress and cell injury. It is also believed that lutein-mediated upregulation of HO-1/Cx-43 pathway increases cell survival, particularly stimulating the production of new proteins, thereby reducing cell death. Remarkably, the increase expression of cytoprotective enzymes and increased cellular antioxidant activity, thus provide protection against oxidative stress [54–57].

Generally, the findings of this study strongly suggest that lutein demonstrates protective effects against cyclosporine-induced testicular damage in rats. In particular, the findings indicate that lutein can inhibit the up-regulation of oxidative and inflammatory pathways associated with cyclosporine-induced testicular damage. These findings could potentially have important clinical implications for health management in patients utilizing cyclosporine or other drugs with similar mechanisms of action. However, it is important to note that the findings of this study are based on preclinical findings in rats. Therefore, there is still need for further investigations, particularly clinical correlates to determine the feasibility of translating these findings to human subjects. Further studies are also expected to focus on establishing the safety profile and efficacy of lutein supplementation in humans, specifically determining the optimal dosage and timing of lutein supplementation for maximum efficacy. Overall, the findings from the present preclinical investigation showed that that lutein attenuates cyclosporine-induced testicular impairment via mechanisms associated with inhibition of Cx-43/NOX-1-mediated oxidative stress, inflammation, apoptosis, and normalization of BTB-related proteins in rats. In-line with previous studies, we showed that lutein's protective effects against cyclosporine-induced testicular dysfunction might also include the involvement of Nrf2/HO-1/Cx-43 pathway. However, more studies are required to substantiate this claim.

CRediT authorship contribution statement

Obukohwo Mega Oyovwi: Conceptualization, Data curation, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Benneth Ben-Azu:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. **Edesiri Prince Tesi:** Conceptualization, Data curation, Supervision, Writing – review & editing. **Victor Emojevwe:** Data curation, Funding acquisition. **Rume Arientare Rotu:** Funding acquisition, Resources, Software. **Goodies Emuesiri Moke:** Formal analysis, Methodology, Resources, Writing – review & editing. **Emuesiri Umukoro:** Funding acquisition, Resources, Software, Validation, Writing – review & editing. **Jerome Ndudi Asiwe:** Funding acquisition, Software, Supervision, Writing – review & editing. **Kingsley Eze Nwangwa:** Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The technical assistance provided by the Department of Physiology at Achievers University over the course of the study is greatly appreciated by the authors.

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