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Lutein Attenuates Cyclosporin-induced Testicular Impairment in Male Rats through Modulation of Androgenic Hormones and Enzymes

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ABSTRACT

Background: Male reproductive toxicity has been linked to cyclosporine, a commonly used immunosuppressive drug for the prevention of organ rejection in patients undergoing renal transplant. The goal of this study was to elucidate how lutein protects male testicles from cyclosporine-induced damage.

Methods: Thirty adult male Wistar rats were randomly allotted to five groups, each with six animals. Rats in groups 1 and 2 were given saline (2 mL/kg/day p.o) and corn oil (2 mL/day p.o) respectively. Rats in groups 3 and 4 were given lutein (40 mg/kg/day p.o) and cyclosporine (40 mg/kg/p.o./day), while rats in group 5 were given a combination of cyclosporine (40 mg/kg/day p.o) and lutein (40 mg/kg/day p.o). At the end of the fourth week, sperm indices, serum hormones, testicular steroidogenic enzymes [3 and 17 beta-hydroxysteroid dehydrogenases (3β-HSD and 17β-HSD)] and enzyme markers of spermatogenesis [lactate dehydrogenase (LDH-X), sorbitol dehydrogenase (SDH), gamma glutamyl transferase (γ-GT), acid phosphatase (ACP) and alkaline phosphatase (ALP)] were assayed. The testis of each rat was also investigated for histopathological abnormalities and germ cell count.

Results: Lutein attenuated cyclosporine-induced sperm impairment. In rats treated with cyclosporine, lutein reduced LDH-X, SDH, ACP, γ-GT; raised LH, FSH, testosterone, 3ß-HSD, 17ß-HSD, ALP levels, and improved spermatogenesis.

Conclusion: These results suggest that lutein attenuates cyclosporine-induced testicular impairment through modulation of androgenic hormones and enzymes.

INTRODUCTION

Organ transplantation is often referred in mythological literatures as a preferred treatment strategy and cure for diseases (Wainwright et al., 1994; Thygesen et al., 2012; Tsuchimoto et al., 2019). Organ transplantation has increasingly become an accepted and viable strategy for the management of dysfunctional body organs. When the body's immune system attacks a foreign organ, rejection reactions occur, and several immunosuppressive medications are typically applied to avoid organ rejection (Pan *et al.,* 2018). Despite the fact that the modern era of pharmacotherapeutic immune-suppression began with the introduction of the anti-proliferative drug 6-mercaptopurine, the development of cyclosporine A, a novel anti-rejection agent has been the most significant advance in immune-suppression in the last three decades (Abdul-Hamid *et al.,* 2016).

Cyclosporine is a neutral and lipophilic cyclic polypeptide isolated from the fungus *Tolipocladium inflatum gums* (Turk et al., 2007). Aside from its anti-graft rejection application, it is clinically relevant as anti-rheumatoid arthritis, anti-uveitic, anti-psoriatic agent, and also for treating atopic dermatitis (Nussemblatt *et al.,* 1988; Yoshinoya *et al.,* 1988; Mihatsch *et al.,* 1992). However, its use has been linked with numerous deleterious effects consisting of renal and hepatic impairments as well as cardiotoxicity (Seethalakshmi *et al.,* 1990; Lee, 2013). Furthermore, there is evidence that long-term use of cyclosporine induces testicular damage leading to reduced sperm count and motility through a variety of mechanisms that involve oxidative stress (Lee, 2013; Pan *et al.,* 2018). Epidemiological and experimental investigations have shown that cyclosporine reduces testosterone synthesis, causes DNA damage, and raises the apoptotic index of germinal cells in seminiferous tubules (Turk et al., 2007, 2010; Pan et al., 2018; Ghazipoor et al., 2020; Khattab et al., 2020). It has recently been demonstrated that phytochemicals derived from herbs and spices like chamomile (Al-Dabbagh et al, 2019), curcumin (Huang *et al.,* 2018), lycopene (Ateşşahin *et al.,* 2007), N-acetylcysteine (Duru *et al.,* 2008), black grape extract (Ergüderİ *et al.,* 2005), morin (Ben-Azu *et al.,* 2018, 2020), lutein (Buscemi et al., 2018) and taurine (Hagar *et al.,* 2004; Oyovwi *et al.,* 2021) possess powerful antioxidant and neuroprotective activities. Therefore, supplementation with antioxidant molecules like lutein becomes essential in the light of this background (Johnson, 2002; He *et al.,* 2011). Also, potential of reproductive pharmacological activities of lutein dietary supplement for treatment of reproductive toxicant-related infertility has gained momentum (Fatani *et al.,* 2015; Li *et al.,* 2015).

Lutein is a plant-derived xanthophyll that naturally occurs in fruits and vegetables such as spinach, lima beans and orange (Sommerburg et al., 1998). Lutein is a potent antioxidant (O'Nell *et al.,* 2001; Johnson, 2002; He *et al.,* 2011; Kim *et al.,* 2012; Du *et al.,* 2015; Mammadov *et al.,* 2019) with various biological and protective effects including hepatoprotective (Li *et al.,* 2015), anticancer (Zhang *et al.,* 2018), neuroprotective (Binawade *et al.,* 2013), anti-diabetics (Neelam *et al.,* 2017), reproductive-protective (Li *et al.,* 2015), and anti-inflammatory (Chung *et al.,* 2017) properties. Moreover, the antioxidant activity of lutein has been linked to reduced rate of accelerated aging (He et al., 2011; Li et al., 2010). The mechanisms of lutein's antioxidant activity are well-known and documented in the literature (He et al., 2011; Stahl and Sies, 2012). Additionally, other studies also indicate that lutein has a potential role in the treatment of osteoarthritis (Qiao *et al.,* 2018), burn-induced multiple organ injury (AbuBakr *et al.,* 2018), and optic nerve injury (Karakurt *et al.,* 2019). Considering the antioxidant and anti-inflammatory properties of lutein, we hypothesized that it could be a beneficial therapeutic agent for preventing cyclosporine-induced toxicity in several organs. The current study was therefore designed to investigate the protective effects of lutein against cyclosporine-induced testicular damage in rats.

MATERIALS AND METHODS

Procurement and Management of Experimental Animals

Adult male Wistar rats weighing 150 to 200 g (7-9 weeks old) were housed in Achievers University's animal holdings in Owo, Ondo State, under standard conditions of 25±2℃ and natural lighting conditions. The rats were allowed to acclimatize for minimum of 14 day prior to onset of experiments and experimental procedures were in line with international laboratory best practices for the use and care of rodents (NIH-Publication No. 85-23, revised). This study used six animals per group based on the previously reported principles of 3Rs (Fenwick et al., 2009).

Drugs and Chemicals

Cyclosporine (manufactured by Novartis but purchased from Kule Ara Pharmaceuticals, Ibadan, Oyo State, Nigeria) and lutein was bought from Carbone Scientific Co., Ltd, London, UK. The vehicle (corn oil) was manufactured by IndiaMART InterMESH Ltd but was obtained from Shoprite, Ibadan, Nigeria. Cyclosporine and lutein were freshly prepared independently in 20 mL of corn oil before they were administered orally. The doses and routes of the various treatments were obtained from previous reports: corn oil (Kunle-Alabi et al., 2017; Jang *et al.,* 2016; Oyovwi et al., 2021), cyclosporine at 40 mg/kg (Kabel *et al.,* 2020), and lutein at 40 mg/kg (Fatani *et al.,* 2015).

Experimental Groupings

Rats were allotted to 5 (n=6) groups. Group 1 was adopted as normal control and had 2 mL/kg/day of saline. Group 2 received 2 mL/kg/day of corn oil. Groups 3 and 4 had lutein (40 mg/kg/day) and cyclosporine (40 m/kg/day) respectively. Group 5 was administered a combination of cyclosporine (40 mg/kg/day) and lutein (40 mg/kg/day). Treatments were done orally between 8:00am and 9:00am once daily for the period of 28 days (Figure 1).

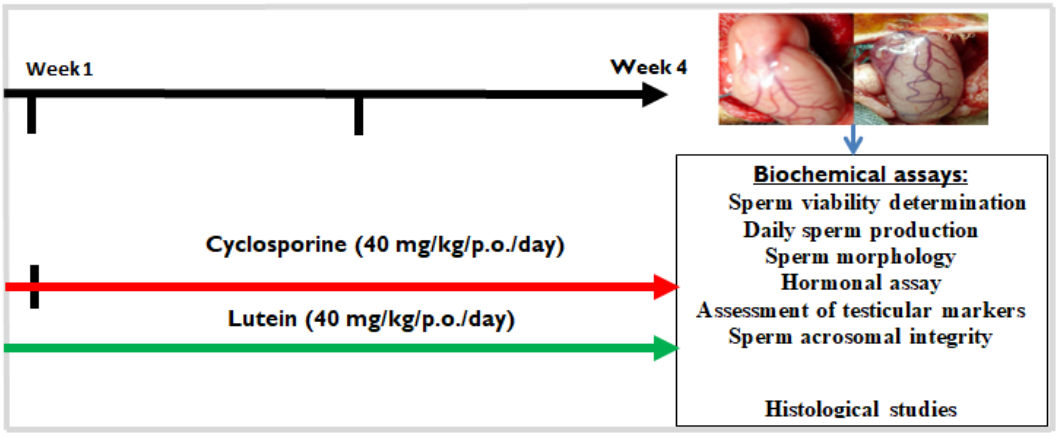


Figure 1: Experimental protocols

Collection and Preparation of Sample

Animals were anesthetically euthanized under ether anesthesia and samples of blood were obtained from each rat via the retro-orbital sinus into plain bottles. After centrifugation, the sera were separated from clots and were stored at −80°C. Cleaned testicular tissues were weighed. For sperm parameters (count, motility, viability and morphology), each animal's cauda epididymis was used. While one testis was used for daily sperm production (DSP) assay after homogenization and centrifugation at 14,000 *g* in a radioimmunoprecipitation buffer solution, the second testis was preserved in Bouin's fluid for histological examination

Determination of Sperm Indices

Sperm count and motility were assessed as previously described (Yokoi et al., 2003) using a Neubauer hemocytometer and x400 magnification phase contrast microscope (Yokoi et al., 2003). Hundred (100) sperms were observed in three different fields and classified as motile or non-motile. Motility was measured as a percentage of the total population. Sperm suspensions were assessed as described by Wyrobek et al. (1983) using 0.05% eosin-Y in a 2-min incubation period (25°C). Live sperm were not stained, whereas dead sperm were pink (Wyrobek et al., 1983). Each sample had 200 sperms counted and the percentage viability was calculated. The percentage of intact sperm cells was used to determine sperm viability (Elliasson, 1977). Each decapsulated and homogenized testis was gently mixed 1 min after settling based on earlier protocol (Blazak et al., 1993). Using the light microscope, DSP was calculated by counting the sperm heads (spermatid head at stage 19) number in four (4) compartment of a Neubauer hemocytometer preceding mixing of each sample. For sperm morphology, after processing on a clean slide, a thin layer of sperm was prepared and fixed with 95% ethanol (Wyrobek et al., 1983). On each slide, a total of 200 sperms were counted with sperm abnormalities expressed as percentages.

Measurements of Hormones, Enzymes and Testicular Markers

Follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, gamma glutamyl transferase (γ-GT), acid phosphatase (ACP), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), and lactate dehydrogenase-X (LDH-X) levels were assayed with ELISA kits based on the manufacturer's guidelines. The activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were determined as we previously reported (Oyovwi et al., 2021) and enzyme activity was expressed based on 0.001/min absorbance at 340 nm. Aminoff's (1962) method was used to calculate sialic acid. The molar extinction coefficient was used to calculate the level of sialic acid (70.79 x 103 $M^{-1}cm^{-1}$) expressed as g/mg of protein.

Evaluation of Sperm Acrosomal Integrity

Acrosomal integrity was assessed as described previously (Pope et al., 1991). After processing, for 1 min (37°C), 5 µL of each sample was incubated with the single-stain solution measured at 5 mL. The reaction mixture was withdrawn onto a clean slide, air-dried at 37°C, and examined under x1000 magnification by light microscopy. Acrosomal integrity was determined by counting 200

sperms per slide and categorizing them as intact or not intact.

Histological Evaluation of Testicular Tissues

Crossed sessions of testes were paraffin-embedded, fixed in a 10% formaldehyde solution and was stained using haematoxylin and eosin stain (H&E), mounted, and examined microscopically by a trained histopathologist in a blinded manner as previously described (Garu et al., 2011).

Evaluation of Germ Cell Count

The count of germ cells of the testes were performed as previously described by Akpantah et al. (2003) using Bouin's fluid, alcohol/chloroform clearance, molten paraffin wax fixation (20 min, 57°C) and staining with H&E. The tissues were oven-dried after being cleared in xylene. The evaluations were carried out using light microscopy at x400 magnification.

Statistical Analysis

The data was analyzed using GraphPad Prism (GraphPad Software, Inc., Lajolla, USA version 8.0). The mean ± standard error of the mean (SEM) is used to present all data. Inferential analysis was performed using one-way analysis of variance (ANOVA) followed by Benferroni post hoc test. P- values less than 0.05 indicated significant difference between compared data.

RESULTS

Protective Impacts of Lutein on Cyclosporine-mediated Perturbation on Serum Hormonal Functions

The protective impacts of cyclosporine-induced perturbation on serum hormonal indices as the consequence of reproductive toxicity were evaluated. When compared with control groups, cyclosporine significantly decreased LH [F (4, 25) = 39.93, *P*=0.0014] (Figure 2a), FSH [F (4, 25) = 6.99, *P*=0.0006] (Figure 2b), and testosterone [F (4, 25) = 19.10, *P*<0.0001] (Figure 2c). In comparison to cyclosporine-treated rats, co-treatment with lutein significantly reduced cyclosporine-induced perturbation in LH, FSH, and testosterone concentrations (Figure 2a-c). Also, lutein significantly increased LH and FSH compared to controls.

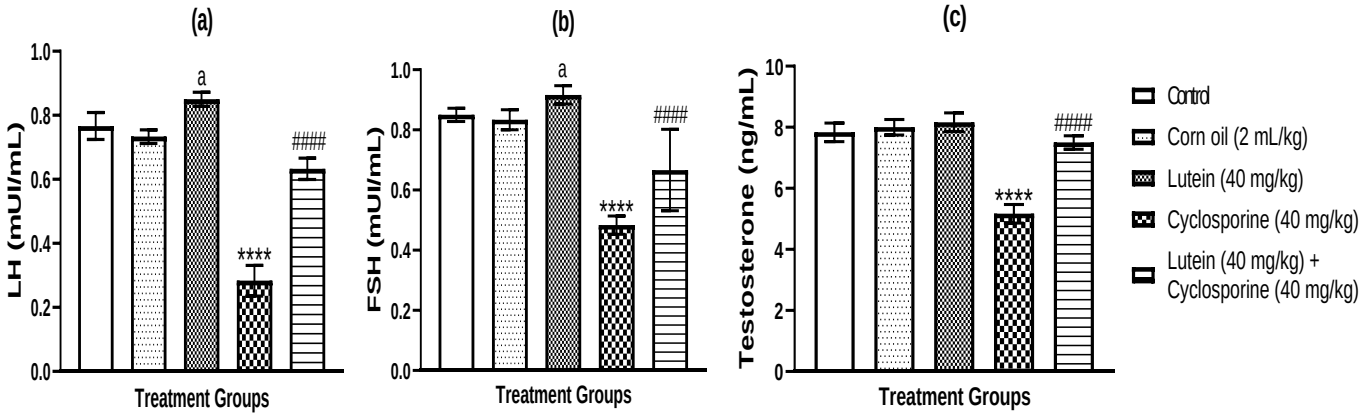


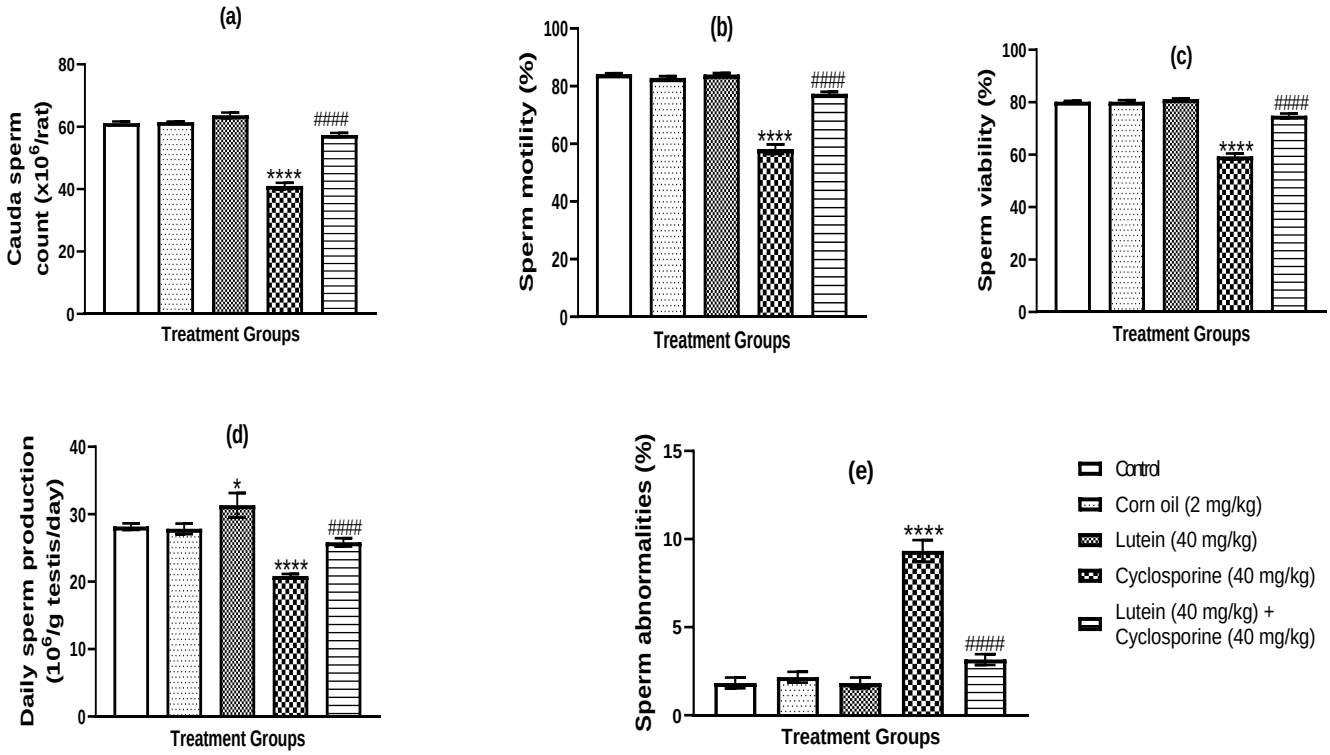
Figure 2: Protective effects of lutein on cyclosporine-induced perturbation on serum hormonal parameters in male Wistar rats: follicle stimulating hormone, FSH (a), luteinizing hormone, LH (b), and testosterone (c) in male Wister rats. Bars represent Mean ± S.E.M. (n = 6). $^{*}P < 0.05$, $^{****}P < 0.0001$ relative to control; $^{\#\#\#\#}P < 0.0001$ relative to cyclosporine group for ameliorating protocol of cyclosporine group.

Protective Impacts of Lutein on Cyclosporine-mediated Alteration in Spermatogenesis

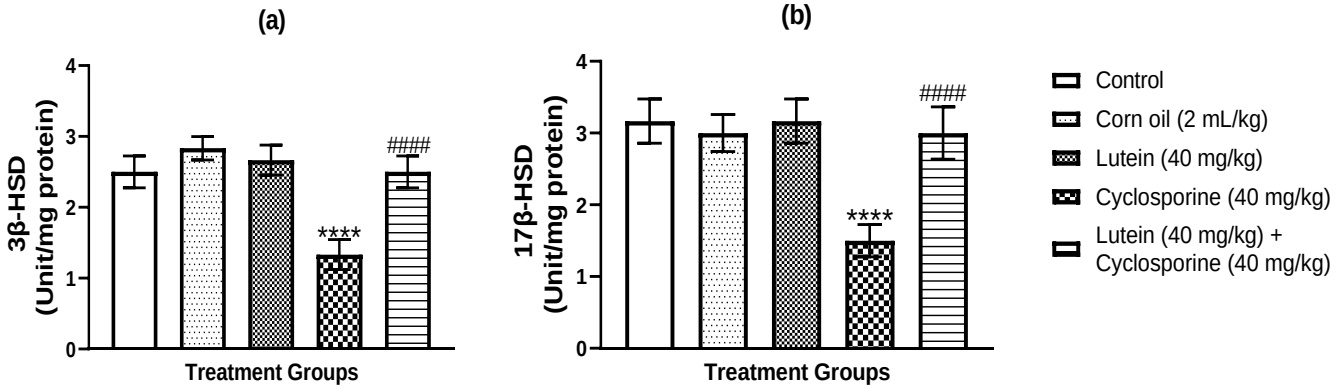
Cyclosporine significantly reduced cauda sperm count [F (4, 25) = 156.50, *P*<0.0001] (Figure 3a), sperm motility [F (4, 25) = 163.90, *P*<0.0001] (Figure 2b), sperm viability [F (4, 25) = 156.60, *P*<0.0001] (Figure 3c), and daily sperm production [F (4, 25) = 16.23, *P*<0.0001] (Figure 3d), but with corresponding alteration in sperm morphologies [F (4, 25) = 68.38, *P*<0.0001] (Figure 3e). Notably, lutein significantly improved cyclosporine-induced alterations in sperm parameters when compared to cyclosporine-treated rats; however, lutein administration alone had no effect on sperm spermatogenesis relative to vehicular control (Figure 3a-e).

Lutein Attenuates Cyclosporine-induced Alterations in Androgenic Enzymes Activities in Testes

As depicted in Figure 4, cyclosporine decreased 3ß-HSD [F (4, 25) = 8.14, *P*=0.0002] (Figure 4a) and 17ß-HSD [F (4, 25) = 5.79, *P*=0.0019] (Figure 4b) in comparison to vehicular controls. Luein alleviated cyclosporine-induced decreases in 3ß-HSD and 17ß-HSD (Figures 3a and 3b). However, when compared to the values from the control groups, lutein administration alone did not produce any significant changes in 3ß-HSD (Figure 4a) or 17ß-HSD (Figure 4b) levels.



Figures 3a-e: Protective effects of lutein on cyclosporine-induced alteration in sperm count (a), motility (b), viability (c), daily sperm production (d) and morphology (e). Data are expressed as mean ± S.E.M. (n = 6). $^{****}P< 0.0001$ when compared with control; $^{\#\#\#\#}P < 0.0001$ when compared with cyclosporine group



Figures 4a-b: Protective effects of lutein on cyclosporine-induced diminution of testicular 3ß-hydroxy steroid dehydrogenase (3ß-HSD) (a) and 17ß-steroid dehydrogenase (17ß-HSD) (b) activities in male Wistar rats. Data are expressed as mean ± S.E.M. (n = 6). $^{****}P< 0.0001$ when compared with control; $^{\#\#\#\#}P < 0.0001$ when compared with cyclosporine group

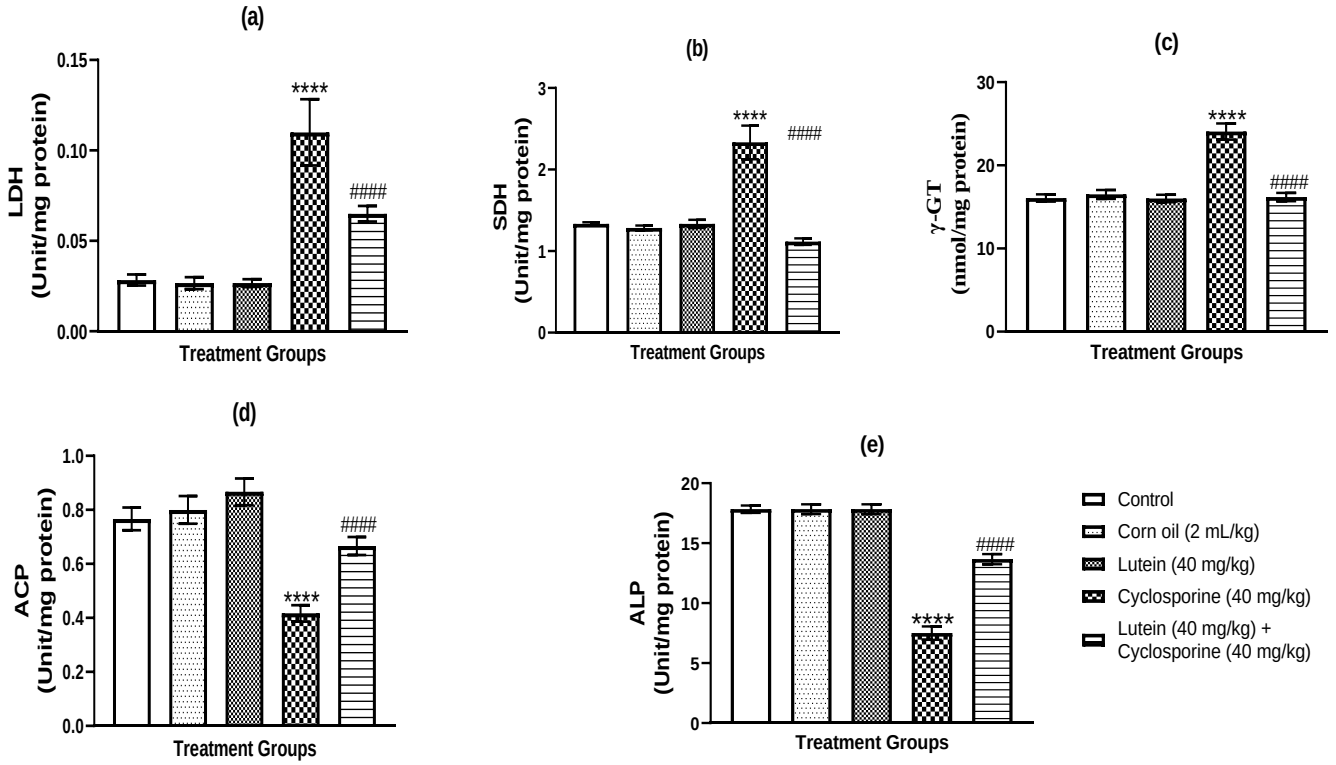
Protective Impacts of Lutein on Cyclosporine-mediated Diminution in Testicular Marker Enzyme Activities

As shown in Figure 5, cyclosporine significantly increased the activities of lactate dehydrogenase F (4, 25) = 17.84, P<0.0001] (Figure 4a), sorbitol dehydrogenase [F (4, 25) = 24.59, P<0.0001] (Fig. 5b), gamma glutamyl transferase [F (4, 25) = 32.89, P<0.0001] (Figure 5c) with corresponding decrease in acid phosphatase [F (4, 25) = 17.27, P<0.0001] (Figure 4d) and alkaline phosphatase [F (4, 25) = 112.60, P<0.0001] (Figure 4e) as compared to control group. However, lutein reversed these alterations in a significant manner in comparison with cyclosporine-treated rats (Figure 5a-e).

Protective Impacts of Lutein on Cyclosporine-mediated Defects in Sperm Acrosomal Integrity

As shown in the Figure 6, acrosomal integrity was significantly impaired by 28.67% in the cyclosporine treatment groups [F (4, 25) = 87.75, P<0.0001]. However, in lutein groups, the number of sperms with intact

acrosomes was increased in the cyclosporine group. Furthermore, rats exposed to vehicle or lutein alone had acrosomal integrity that was statistically comparable to the control group.



Figures 5a-e: Protective effects of lutein on cyclosporine-mediated diminution of testicular lactate dehydrogenase-X (LDH-X) (a), sorbitol dehydrogenase (SDH) (b), gamma glutamyl transferase (γ-GT) (c), acid phosphatase (ACP) (d) and alkaline phosphatase (ALP) (e) activities in rats. Data are expressed as mean ± S.E.M. (n = 6). $^{****}P< 0.0001$ when compared with control; $^{####}P< 0.0001$ when compared with cyclosporine group.

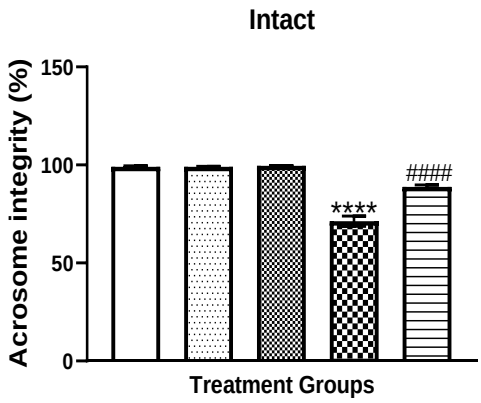


Figure 6: Protective effect of lutein on cyclosporine-induced alteration in sperm acrosomal integrity in rats. Data are expressed as mean ± S.E.M. (n = 6). $^{****}P< 0.0001$ when compared with controls; $^{####}P< 0.0001$ when compared with cyclosporine group.

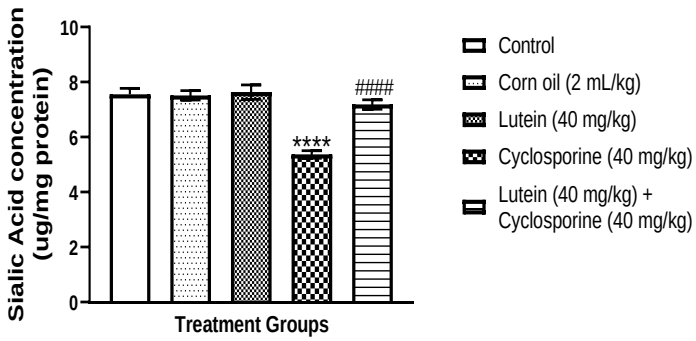


Figure 7: Protective effect of lutein on cyclosporine-induced decrease in sailic acid concentration in rats. Data are expressed as mean ± S.E.M. (n = 6). $^{****}P< 0.0001$ when compared with controls; $^{####}P < 0.0001$ when compared with cyclosporine group.

Lutein Reverses Cyclosporine-mediated Decrease in Testicular Sialic acid Concentration

Cyclosporine decreased sialic acid [F (4, 25) = 8.14, P=0.0002] in the treatment protocols relative to control groups. Luein alleviated the cyclosporine-induced decreased sailic acid concentration. Treatment with lutein alone did not produce any significant changes in sailic acid levels relative to normal control groups (Figure 7).

Lutein Abates Cyclosporine-mediated Depletion in Germ Cell Count

Figure 8 presents the impacts of lutein on the numbers of testicular germ cell count in cyclosporine-treated rats. Cyclosporine decreased germ cell count [F (4, 25) = 96.53, P<0.0001] in the co-treatment protocols relative to control groups. Luein alleviated the cyclosporine-induced decreased germ cell count but treatment with lutein alone failed to alter the levels of germ cell count, although there was an increase in the germ cell count relative to normal control groups.

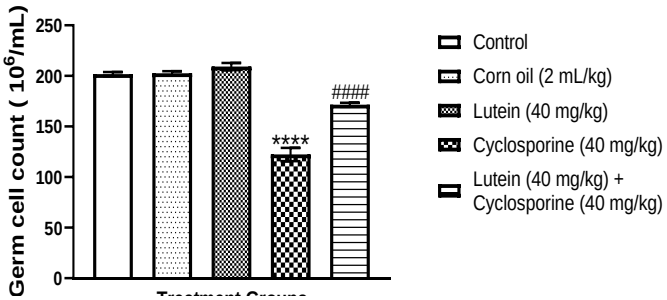
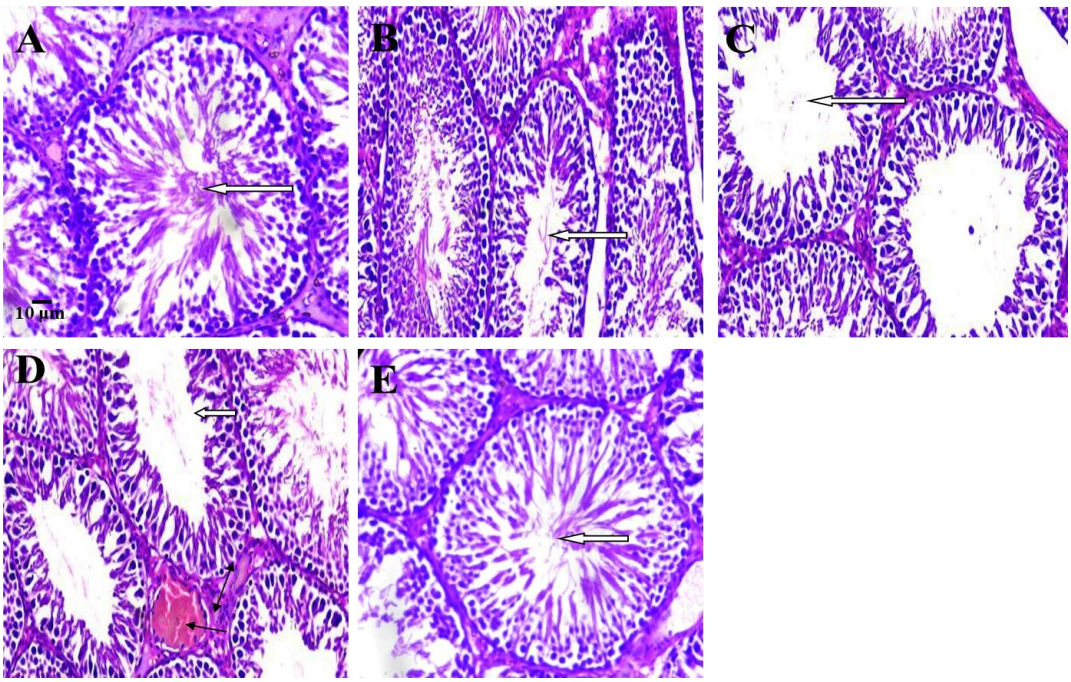


Figure 8: Protective effect of lutein on cyclosporine-induced depletion in germ cell count rats testes. Bar represents mean ± S.E.M (n = 6). $^{****}P$ < 0.0001 when compared with controls; $^{####}P$ < 0.0001 when compared with cyclosporine (ameliorating protocol of cyclosporine administration for 4 weeks followed by co-treatments with lutein).

Protective Effect of Lutein on Cyclosporine-mediated Histopathological Changes of Testes

Lutein alone produced no changes on the testicular architecture, seminiferous tubules and spermatogenesis relative to the vehicle-treated control group. On the other hand, cyclosporine reduced spermatocyte levels in the seminiferous tubules, as shown by degenerated seminiferous tubules and necrosis with presence of atrophy, few dead pyknotic cells, homogenous and vascular congestion when compared with normal controls. However, as shown in the co-treatment protocol, lutein, ameliorated cyclosporine-induced changes on testicular architecture, seminiferous tubules and spermatogenesis relative to control groups (Figure 9).



Figures 9b-e: Photomicrographs showing the protective effects of lutein on cyclosporine-induced histopathological changes in the testes of rats**.** A: Control (Normal saline); B: Corn oil (2 mL/kg); C: Lutein (200 mg/kg); D: cyclosporine (50 mg/kg); E: Cyclosporine (40 mg/kg) + Lutein (40 mg/kg). For figure 1, slides A, B and C revealed normal normal testicular architecture with seminiferous tubules, normal sperm maturation stage. Slide D revealed some degenerated germ cell layer and less presence of spermatozoa within their lumen and also shows presence of pinkish homogenous mass and vascular congestion. several atrophic seminiferous tubules, thickened pyknotic propria enveloping the tubular cells, degenerated and necrotic germ cells of the seminiferous tubules. Slide E are associated with showing normal testicular architecture with a mild degenerated seminiferous tubules containing slough cell. Slender arrow– presence of pinkish homogenous mass and vascular congestion; Spanned–degenerated germ cell layer; White arrow– seminiferous tubule with normal sperm maturation. Haematoxylin-eosin stain: Original magnification x400, Calibration bar = 0.01 mm (10 µm) for all mcirograghs.

The study investigated the beneficial effects of lutein on cyclosporine-induced testicular injury. Based on the inhibitory effects of cyclosporine, a fungal peptide, on the down-regulation of immunomodulatory cytokines, it is commonly used as an immune suppressant drug during organ transplantation and treatment of autoimmune diseases (Rezzani, 2006). Cyclosporine has the ability to alter the blood-testis barrier (BTB), which protects the reproductive tissues (Hughes et al., 1998), and thus exerts its toxic effects on reproductive functions by inhibiting p-glycoprotein expression, which is required for toxicant excretion. The ability of cyclosporine to penetrate both the BTB and the blood-brain barrier has long been established based on its bio-accumulation and translocation mechanisms (Hughes et al., 1998).

Male reproductive hormones are essential for initiating and maintaining male reproductive function (Meeker et al., 2007), and gonadal damage however, is a common adverse effect of many toxicants and chemotherapeutic drugs, affecting both the endocrine and exocrine functions of the testis (Spermon et al., 2006; Oyovwi et al., 2021). The pulsatile secretion pattern of GnRH causes the cyclic release of LH and FSH. It is worth noting that blocking GnRH has been linked to lower LH and FSH levels (Oyovwi et al., 2021). The interstitial cells of Leydig in the testes produce testosterone, the primary male gonadal hormone. It is also a major indicator of androgenicity.

The presence of testosterone and FSH in the testes is required for the regulatory balance of spermatogenesis and seminiferous tubules physiology. In this study, we found that cyclosporine-treated rats had lower levels of testosterone, LH, and FSH, which is consistent with previous findings (Chen et al., 2013). The depletions of testosterone and FSH in males have been associated with profound reproductive impairment, although the link between cyclosporine-induced endocrine system impairment and hormonal imbalance has not been well characterized. Of note, cyclosporine has been shown to directly impair the activities of Leydig and Sertoli cells by inducing oxidative stress, apoptosis, and inflammation (Chen et al., 2008; Fatani et al., 2015) or by inhibiting the activity of enzymes involved in testosterone synthesis (Oyovwi et al., 2021). However, our findings show that lutein attenuates the negative effects of cyclosporine on hormonal concentrations and this might be associated with its ability to reduce oxidative stress, apoptosis, and inflammation-related activities (Fatani et al., 2015).

Spermatogenesis is a hormonally controlled process that involves the production of gonadotropins such as LH and FSH, which promote the physiological activities of Leydig and Sertoli cells (Oyovwi et al., 2021). For normal intratesticular testosterone production, communications between Leydig cells and Sertoli cells are also required (Oyovwi et al., 2021). The testosterone produced by Leydigs cell is required in the seminiferous tubules to promote spermatogonia differentiation into spermatozoa (Oyovwi et al., 2021). Changes in the synthesis of testosterone could be caused by a variety of pathological or experimental conditions (Dufau et al., 1979; Oyovwi et al., 2020, 2021). As a result of normal Leydig and Sertoli cell activity, normal spermatogenesis is assisted by intratesticular testosterone levels that are 100 times greater than those seen in blood plasma (Dufau et al., 1979;; Russell et al., 1998).

As a highly differentiated haploid cell, a matured mammalian spermatozoon plays an important role in fertilization; thus, its impairment is regarded as a major contributory factor in male infertility. The flexibility and functional abilities of spermatozoa are due to their highly specific lipid composition of plasma membrane (Aitken et al., 1989). However, due to the unique structure of the sperm membrane, mammalian spermatozoa appear to be particularly vulnerable to the damage caused by increased pathological ROS generation (Agarwal et al., 2008). In sperm cell membranes, high levels of ROS cause lipid peroxidation, a cascade of autocatalytic chemical reactions that can lead to dysfunction and death of cell. Notably, the lower sperm count observed in this study suggests that cyclosporine affects spermatogenesis via peroxidation of polyunsaturated fatty acids in spermatozoal plasma membranes or changes in intracellular redox state, as indicated by previous reports (Rezzani 2006; Turk et al., 2007; 2010). The reduced sperm levels in cyclosporine-treated rats could be due to the down-regulation of steroidogenesis gene and decreased 19 spermatids/stage VII step, resulting in a lower testosterone concentration, as testosterone regulates sperm production, as well as sperm maturation in the testes and epididymis respectively (Abarikwu et al., 2012; Shukry et al., 2020). Also, the reduced sperm motility by cyclosporine could be attributed to its ability to impair sperm flagellum, an important machinery for sperm cell motility. Furthermore, the reduction in daily sperm production in cyclosporine-treated rats corresponded to a decrease in serum testosterone concentrations, which is also alleged to occur via mechanism related to interference with testosterone biosynthesis (Abarikwu et al., 2012). However, lutein co-treatment effectively prevented these changes, implying that lutein has testicular protective properties. These results suggest that lutein may protect against cyclosporine-induced reproductive toxicity in clinical setting. These findings support a previous study showing that lutein reduces arsenic-induced reproductive toxicity in male mice by improving the anti-oxidant-Nrf2 signaling pathway (Li et al., 2015). More studies are needed to

characterize the precise molecular mechanism relating to this beneficial effect.

Similar to previous findings (Shukry et al., 2020), rats treated with cyclosporine had significantly lower 3ß-HSD and 17ß-HSD enzyme activities in this study. These steroidogenic enzymes are commonly found in the gonads and adrenal glands, are key regulatory enzymes involved in androgenesis, as well as steroid and xenobiotic metabolism (Oyovwi et al., 2021). In the testicular seminiferous tubes, 17ß-HSD converts androstenedione to testosterone to influence fertilization of gametes (Kabbaj et al., 2003). Remarkably, the low testosterone levels observed in this study following cyclosporine exposure may in part, be due to the low 3ß-HSD and 17ß HSD activities, thereby resulting in altered synthesis of testosterone rather than a deficiency of cholesterol, a synthetic precursor. However, further studies are needed to elucidate this possibility. Interestingly, previous reports have shown that reduced steroidogenesis in experimental rats may result from lower activities of testicular steroidogenic enzymes including 3ß-HSD and 3ß-HSD, which may result in reduced reproductive capacity. Exposure of rats to a series of drug toxicity and xenobiotics has been observed to reduce the activity of testicular 17ß-HSD with accompanying reproductive impairment (Oyovwi et al., 2021). In this study, we found that cyclosporine significantly reduced 3ß-HSD and 17ß-HSD activities, suggesting decreased androgen formation, reduced the conversion of 17-pregnenolone to 17-hydroxyprogesterone. Co-treatment with lutein, on the other hand, restored testicular androgenic enzyme activities, implying that lutein has an androgenic enhancing effect against cyclosporine-induced reproductive inhibition.

Furthermore, the levels of γ-GT, LDH-X, and SDH were found to be significantly higher in the cyclosporine-treated rats. However, these findings contradict those of Shukry et al. (2020), who found that cyclosporine administration caused a decrease in LDH-X and SDH activities in male rats. LDH and γ-GT are testicular markers enzymes linked to germ cell maturation and associated with specific cell types. LDH-X is a Leydig cell function marker while γ-GT is a membrane-bound enzyme that catalyzes the γ-glutamyl group transfer between peptides and amino acids (Shukry et al., 2020). Moreover, γ-GT also plays a role in the secretion of fluid into the seminiferous tubules that transport spermatozoa into the rat testis (Shukry et al., 2020). Of note, Sertoli cells are known to act as mechanical support for germinal cell elements and also for promoting their differentiation. The induction of LDH-X and γ-GT activities by cyclosporine may indicate germ cells depletion in the seminiferous tubule, as well as the possibility of interference with Sertoli cell physiology. When compared to the cyclosporine-treated rats, the rats co-treated with lutein showed a significant reduction in γ-GT, LDH-X, and SDH levels.

In addition, our histopathological findings also revealed vascular congestion, seminiferous tubule degeneration, spermatogenesis disruption, and Sertoli cell degeneration. Sertoli cell damage has been suggested as a possible cause of degenerated germ cell (Murphy and Richburg, 2015). The production of fewer spermatozoa could be caused by changes in the marker testicular enzymes activities linked with changes in histopathology. Previous reports (Khattab and Mansoury, 2020; Shukry et al., 2020) have shown that cyclosporine causes histological changes in the testes, including germinal cell atrophy, necrosis, and degeneration, interstitial edema, vascular congestion, and spillage of immature spermatogonia and spermatocytes into the lumen of seminiferous tubules. The group co-treated with lutein showed improved testicular histopathological features which agree with the findings of Fatani et al. (2015) and Li et al. (2015), demonstrating that lutein treatment restores testicular function and normal spermatogenesis.

A significant reduction in testicular alkaline phosphatase (ALP) and acid phosphatase (ACP) activities was observed after various doses of cyclosporine were administered. The amount of chemo-toxicological assault on organs and tissues can be determined by measuring the tissues and body fluid activities of marker enzymes such as ALP and ACP (Yakubu et al., 2003). Alkaline phosphatase is a protein that helps the accessory sex structure cells and spermatozoa in the seminal fluid mobilize carbohydrates and lipid metabolites for usage by the spermatozoa (Suzuki et al., 1998). Less carbohydrates and lipid metabolites may be mobilized for utilization by the accessory sex structure if testicular ALP activity is reduced. Acid phosphatase is found throughout the testes and is involved in sperm physiology, cell metabolism, autolysis and differentiation (El-Demerdash et al., 2013). The toxic effects of cyclosporine can also be linked to decreased ACP activity. Indeed, increased ROS production has been attributed to decrease in testicular ACP as shown by decreased sialic acid levels in the cyclosporine-treated rats (El-Demerdash et al., 2013). In line with this, cells of the seminiferous tubules which contain sialic acid, have been shown to act as a hydrogen peroxide scavenger by converting it to H_2O and a non-toxic carboxylic acid, affecting oxidative damage defense. It is worth noting that the androgen-dependent testicular sialic acid content also acts as a "lubricant" to help sperm move around by reducing friction between spermatozoa. As a result, a decrease in testicular ALP and ACP activities as well as sialic acid contents could indicate changes in sperm physiology, due to low accessory sex structure and cellular

stress thereby resulting to poor sperm penetration into the zona pellucida (Makhluf et al., 2006). In this study, alteration in sperm acrosomal integrity in cyclosporine-treated group suggests a reduction in the spermatozoa's fertilization capacity of the rats. The ability of lutein to normalize the altered ALP, ACP, sialic acid content as well as sperm acrosomal integrity however, suggests improvement in acrosomal integrity, decreased cellular stress and enhanced spermatogenesis (Maselli et al., 2014).

CONCLUSION

Our findings suggest that lutein attenuates cyclosporine-induced testicular dysfunction through increase testicular hormones (teststoterone, FSH, LH), androgenic enzymes activities (3ß-HSD and 17ß-HSD), testicular markers (ALP, ACP, sialic acid, LDH-X, SDH and γ-GT), germ cell count and reduced testicular alteration thereby resulting in enhanced spermatogenesis in rats testes.

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CONFLICTS OF INTEREST

Authors declare that they have no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study and the development of the manuscript. MOO, TPE, BBA and RRA designed the study and wrote the protocols; MOO, TPE, VE and RRA performed the experiments; MOO, TPE, VE and RRA did the biochemical assays; MOO, BBA, KEN, KEE and OGA analyzed the data and did the literature searches. All authors contributed reagents/materials/analysis tools; MOO, BBA, VE and OGA wrote the first draft of the manuscript; MOO, BBA, VE and OGA completed the final draft of the manuscript.

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