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Kolaviron protects against ethylene glycol monoethyl ether-induced toxicity in boar spermatozoa

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Summary

This study investigated the ameliorative effects of kolaviron (a biflavonoid from the seed of *Garcinia kola*) and vitamin C on ethylene glycol monoethyl ether (EGEE)-induced oxidative damage in boar spermatozoa *in vitro*. EGEE (1.0 mM) was incubated with boar spermatozoa for 3 h with or without either kolaviron (50 and 100 μM) or vitamin C (1.0 mM). Spermatozoa parameters were determined hourly during the incubation period, whereas aminotransferases and alkaline phosphatase activities and oxidative stress indices were assessed after the incubation period. Results showed a time-dependent decline in spermatozoa motility and viability with significant elevation in total abnormalities in EGEE-treated spermatozoa. Exposure to EGEE resulted in significant increase in aminotransferases, alkaline phosphatase and superoxide dismutase (SOD) activities, whereas it markedly decreased glutathione (GSH) level, catalase (CAT) and glutathione S-transferase (GST) activities with concomitant increase in hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) levels. Pre-treatment of spermatozoa with kolaviron or vitamin C significantly decreased H_2O_2 and MDA levels, improved spermatozoa characteristics and ameliorated oxidative damage in EGEE-treated spermatozoa. Taken together, EGEE exhibited its spermatotoxicity via induction of oxidative stress. The protective effects by kolaviron and vitamin C against EGEE-induced oxidative damage may be due to their intrinsic antioxidative potentials.

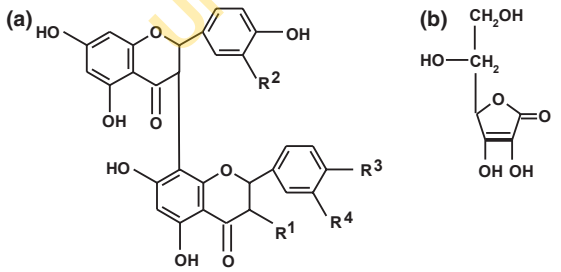
Introduction

Exposure of humans to environmental contaminants that negatively affect the male reproductive system is increasing (Sharpe, 1993; Chia, 2000). Ethylene glycol monoethyl ether (EGEE) is one of such chemicals that are used in a variety of industrial and household products. EGEE is a widely used solvent for nitrocellulose, dyes, inks, resins, lacquers, paints and varnishes (HSDB, 1996). It is also a component of many cleaning agents, epoxy coatings, paints and hydraulic fluid and is an anti-icing fuel additive in aviation. Animal and human studies have shown that EGEE can cause reproductive, developmental and haematological effects through inhalation, dermal absorption and ingestion (Cullen *et al.*, 1983; Vincent *et al.*, 1994; Yoon *et al.*, 2001; Starek *et al.*, 2008). EGEE significantly decreased total and progressive motility of spermatozoa in both the cauda epididymidis and sperm ducts of rats (Wang *et al.*, 2006). Previous studies from our laboratory demonstrated induction of oxidative damage in the testes and spermatozoa in rats exposed to multiple doses of ethylene glycol monoethyl ether (Adedara & Farombi, 2010).

It is undeniable that good quality semen is essential for reproductive success. This quality appears to have been directly affected in recent years, and evidently, there are now unfavourable trends in male reproductive health (Guillette & Crain, 2000). Some environmental chemicals exert their toxicity through generation of ROS (Sikka *et al.*, 1995; Saradha & Mathur, 2006). Reactive oxygen species (ROS) are highly reactive oxidising agents in biological systems. The functional implications of oxidative damage and its role in the development of male infertility have been reported (Aitken, 1995; Kumar *et al.*, 2002). The sperm plasma membrane contains a high amount of unsaturated fatty acids. Therefore, it is particularly susceptible to peroxidative damage. The lipid peroxidation destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with loss of motility and the defects of membrane integrity (de

Lamirande *et al.*, 1997; Sanocka & Kurpisz, 2004; Henkel, 2005). Antioxidants play a major role by continuously inactivating ROS to keep only a small amount necessary to maintain normal cell function (Oyagbemi *et al.*, 2010). Enzymatic antioxidant defences include superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, glutathione-S-transferase and catalase (CAT). Nonenzymatic antioxidants are represented by ascorbic acid (vitamin C), reduced glutathione (GSH), α-tocopherol, carotenoids and other antioxidants. Excessive ROS generation that overcomes the scavenging of ROS by human spermatozoa appears to be related to male infertility (Iwasaki & Gagnon, 1992).

Much attention has been focused on the protective effects of antioxidants and naturally occurring substances against oxidative damage that results from excessive generation of free radicals in the body. The importance of antioxidants such as vitamins in protecting living organism against the toxic effects of environmental chemicals has been reported (Alpsoy *et al.*, 2009). Kolaviron (Fig. 1a), a bioflavonoid isolated from the seeds of *Garcinia kola* has been reported to possess anti-inflammatory, antioxidant, antigenotoxic and hepatoprotective activities in model systems via multiple biochemical mechanisms (Farombi *et al.*, 2000, 2004, 2005; Olaleye *et al.*, 2000; Terashima *et al.*, 2002). Moreover, we have demonstrated *in vitro* the ability of kolaviron to inhibit hydroxyl and superoxide anion radicals, which are known to play an important role in the process of lipid peroxidation (Farombi & Nwaokeafor, 2005). We have also demonstrated the ameliorative effect of kolaviron on di-n-butylphthalate-induced testicular damage in rats and attributed its intrinsic antioxidant properties to be responsible for this effect (Farombi *et al.*, 2007). Vitamin C (Fig. 1b) is a low-molecular-weight antioxidant that defends the cellular compartment against water-soluble oxygen and nitrogen radicals. It is an effective antioxidant of the hydrophilic phase (Jurczuk *et al.*, 2007).



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavanone	OH	H	OCH_3	OH

Fig. 1 Chemical structures of tested compounds: (a) kolaviron and (b) vitamin C.

To get a better insight into the mechanism(s) involved in EGEE-induced spermatotoxicity *in vitro* and the possible role of exogenous antioxidant in ameliorating the toxicity, we used boar spermatozoa and kolaviron, an isolated antioxidant from commonly consumed *Garcinia kola* seed, which till date has not been carried out.

Materials and methods

Chemicals

Ethylene glycol monoethyl ether (EGEE), epinephrine, glutathione, 5, 5′-dithio-bis-2-nitrobenzoic acid, hydrogen peroxide, thiobarbituric acid and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Isolation of kolaviron

Kolaviron was isolated according to published procedure (Iwu, 1985). Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60 °C) in a soxhlet for 24 h. The defatted dried marc was repacked and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6 × 300 ml). The concentrated ethylacetate yielded a golden yellow solid termed kolaviron. Kolaviron was identified by direct comparison of the 1H nuclear magnetic resonance (NMR), ^{13}C NMR and electron ionisation (EI)-mass spectral results with previously published data (Iwu, 1985). The purity of isolated kolaviron was 96%.

Incubation medium: Tris-citric-fructose extender

Semen incubation medium consisted of tris-(hydroxymethyl)-aminomethane (37.85 g l^{-1}), citric acid anhydrous (21.15 g l^{-1}) and D(−)fructose (10 g l^{-1}) (Roca *et al.*, 2000). Kolaviron was dissolved in dimethyl sulphoxide (DMSO) to produce stock solution of 100 mM. The DMSO was used after dilution to give a concentration of 0.2% that had no effect on the cell function. Stock solutions of EGEE (100 mM) and vitamin C (50 mM) were prepared with incubation medium. All stock solutions were freshly prepared at the beginning of the experiment. The preventive effect of kolaviron on EGEE-induced spermatotoxicity was compared with vitamin C as a standard antioxidant.

Animals and collection of epididymal spermatozoa

Six healthy and sexually matured large white boars, 14–16 months old, obtained from Bodija Abattoir close to the University of Ibadan campus, were used for the study. The right and left epididymis were trimmed off the testes, and spermatozoa samples were collected as described by (Oyeyemi & Ubiogoro, 2005). Briefly, 1.0 cm incision was made with scalpel blade on the cauda epididymis and the spermatozoa flushed with 2–3 drops of the incubation medium kept at body temperature. Aliquots of spermatozoa from each epididymis were initially evaluated under a light microscope for concentration and motility. Sperm samples with highest motility (>70%) were applied in this study.

Ethylene glycol monoethyl ether and kolaviron treatments

The experiments were performed on eight groups consisting of the control, 50 μM kolaviron alone (KV1), 100 μM kolaviron alone (KV2), 1.0 mM Vitamin C alone, 1.0 mM EGEE alone, EGEE + KV1, EGEE + KV2, EGEE + Vitamin C. The concentrations of EGEE and kolaviron were selected based on the preliminary experiments carried out in our laboratory with various doses. The lowest concentration of EGEE that cause significant changes in stress parameter within 3 h of incubation were selected. The concentration of vitamins C was adopted from the study by Verma & Kanwar (1998). Kolaviron and vitamin C were added prior to the addition of EGEE. The pH was adjusted to 7.0 for all treatments at the beginning of incubation. All the experiments were repeated five times under the same conditions. The spermatozoa samples were incubated at 37 °C for 3 h. Motility, viability and morphological characteristics were estimated at 0, 1 and 3 h. Time zero for the study is approximately 1–2 min after the addition of EGEE.

Spermatozoa count and motility

Spermatozoa motility was assessed by the method described by Zemjanis (1970). The data were expressed as percentages. The spermatozoa were counted by haemocytometer using the improved Neubauer (Deep 1/10 m, LABART, Germany) chamber as described by Pant & Srivastava (2003). A total of 400 spermatozoa from each rat were examined for morphological examination.

Morphological abnormalities and percentage viability assay

A portion of the sperm suspension placed on a slide glass was smeared out with another slide and stained with Wells and Awa's stain (0.2 g of eosin and 0.6 g of fast green dissolved in distilled water and ethanol in the ratio 2 : 1) for morphological examination and 1% eosin and 5% nigrosine in 3% sodium citrate dehydrate solution for live/dead ratio for according to the method described by Wells & Awa (1970).

Biochemical assays

At the end of incubation, spermatozoa suspensions were homogenised at 4 °C with a glass Teflon homogeniser for 10 s and centrifuged at 2000 ***g*** for 10 min. The supernatant was collected for the estimation of catalase (CAT) activity using hydrogen peroxide as substrate according to the method of Clairborne (1995). Superoxide dismutase (SOD) was assayed by the method described by Misra & Fridovich (1972). The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al.* (1973). Glutathione-S-transferase (GST) was assayed by the method of Habig *et al.* (1974). Protein concentration was determined by the method of Lowry *et al.* (1951). Reduced glutathione (GSH) was determined at 412 nm using the method described by Jollow *et al.* (1974). Hydrogen peroxide generation was assessed by the method of Wolff (1994). Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Farombi *et al.* (2000) and expressed as micromoles of MDA/g tissue. Activities of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) according to Reitmann & Frankel (1957) and Alkaline phosphatase (ALP) according to Rec Gscc (1972) were all determined using the Randox Kit (Randox Laboratories Limited, UK).

Statistical analysis

Statistical analyses were carried out using one-way analysis of variance (ANOVA) to compare the experimental groups followed by the Student's *t*-test using SPSS (student version 17, SPSS Inc., UK) and values <0.05 were considered statistically significant.

Results

Sperm analysis

Figs 2–4 reveal the effects of EGEE alone and when coincubated with either kolaviron or vitamin C on the epididymal spermatozoa motility, viability and abnormalities for 0, 1, 2 and 3 h respectively. Treatment of spermatozoa with EGEE alone resulted in significant decrease in motility and viability with concomitant increase in abnormalities compared with the control. The most frequent

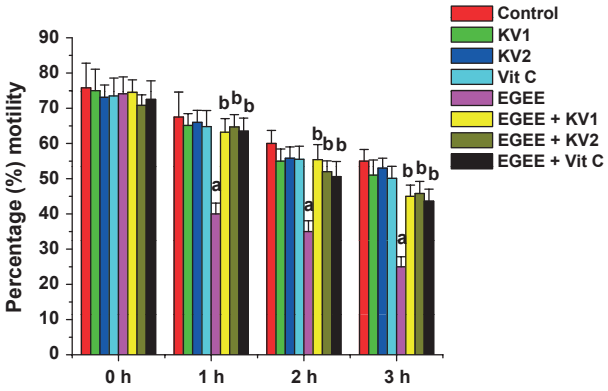


Fig. 2 Percentage motility of spermatozoa from boar after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

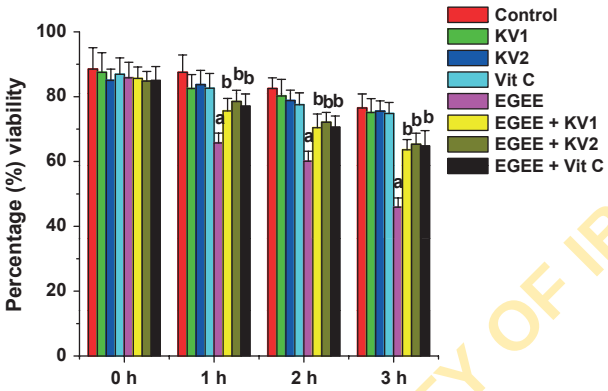


Fig. 3 Percentage viability of spermatozoa from boar after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

abnormalities observed in EGEE-treated spermatozoa are tailless heads, bent tails, curved mid-pieces and bent mid-pieces, whereas rudimentary tails occurred less frequently. While motility and viability significantly increase, there was a significant decrease in sperm abnormalities on simultaneous incubation with either kolaviron or vitamin C compared with the EGEE-treated spermatozoa.

Activities of antioxidant enzymes in boar spermatozoa after 3-hour incubation with EGEE, kolaviron and vitamin C

The activities of antioxidant enzymes, SOD, CAT, GST and GPx in the control and experimental groups are presented in Figs 5 and 6. EGEE-treatment significant decreased the activities of CAT, GST and GPx, whereas it

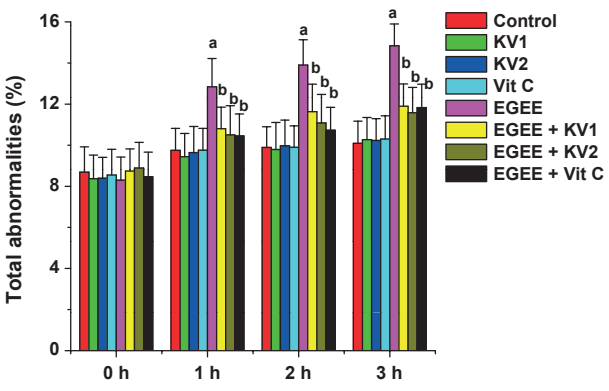


Fig. 4 Percentage morphological abnormalities of spermatozoa from boar after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

increased SOD activity when compared with the control. However, coincubation with either kolaviron or vitamin C shows restoration of normalcy by significant increase in the activities of CAT, GST and GPx as well as decrease in SOD activity compared with the group treated EGEE alone. Coincubation of either kolaviron alone or vitamin C alone exhibited no significant effect on the antioxidant enzymes compared with the control.

Levels of glutathione, hydrogen peroxide and lipid peroxidation

The levels of GSH, H_2O_2 and MDA in boar spermatozoa after 3 h incubation with EGEE, Kolaviron and vitamin C are presented in Figs 7 and 8. The levels of GSH drastically decreased, whereas H_2O_2 and MDA levels significantly increased in EGEE-treated spermatozoa. However, the levels of these parameters were restored in groups cotreated with either kolaviron or vitamin C compared with the group-treated EGEE alone. Alone, neither kolaviron nor vitamin C exhibited significant alterations in the levels of GSH, H_2O_2 and MDA levels compared with the control.

Aminotransferases and alkaline phosphatase activities

In Figs 8 and 9, spermatozoa AST, ALT and ALP activities significantly increased following 3 h incubation with EGEE compared with the control group. Alone, neither kolaviron nor vitamin C had significant effect on the activities of these enzymes. Interestingly, coincubation with either kolaviron or vitamin C significantly decreased the activities of these enzymes when compared with the EGEE-treated group alone.

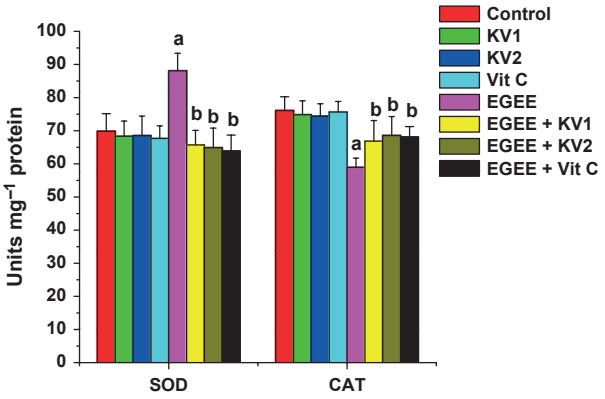


Fig. 5 Activities of SOD and CAT in boar spermatozoa after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

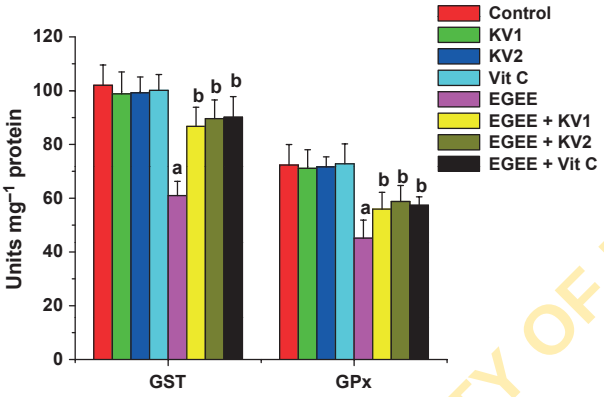


Fig. 6 Activities of GST and GPx in boar spermatozoa after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

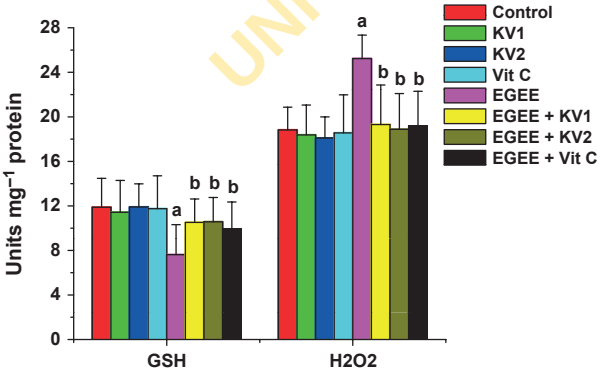


Fig. 7 Levels of GSH and H_2O_2 in boar spermatozoa after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

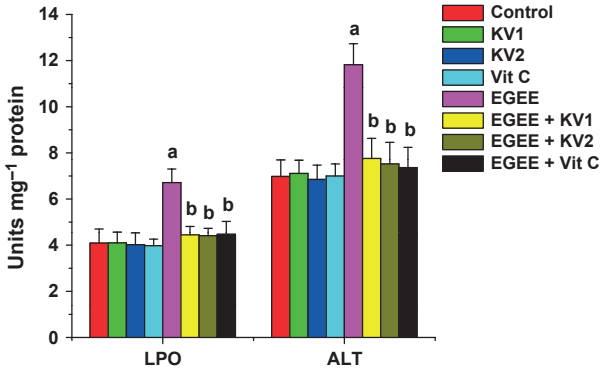


Fig. 8 Levels of LPO and ALT in boar spermatozoa after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

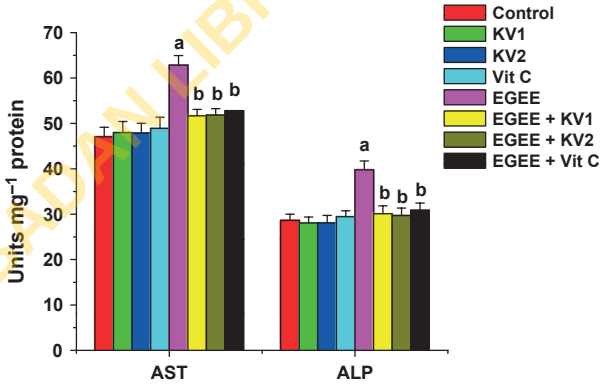


Fig. 9 Levels of AST and ALP in boar spermatozoa after 3 h incubation with kolaviron, vitamin C and EGEE. The data are expressed as mean ± SD. a: Values differ significantly from control ($P < 0.05$). b: Values differ significantly from EGEE ($P < 0.05$).

Discussion

Substantial evidence has piled up portending the adverse effects of environmental toxicants on male reproduction (Aitken *et al.*, 2004; Mathur *et al.*, 2008). Free radicals and reactive oxygen species are subject of research interest because of their active roles in cellular physiology and pathogenesis of number of diseases including infertility (Mehta *et al.*, 2009; Farombi *et al.*, 2012). The existence of a mutually supportive relationship between enzymatic antioxidants, SOD and CAT, against accumulation of ROS inactivates the superoxide anion and peroxide radicals by converting them into water and oxygen. In the present investigation, SOD activity significantly increased, whereas activities of CAT declined drastically in EGEE-treated spermatozoa. The increase in spermatozoa SOD suggests a means of protecting spermatozoa from

oxidative stress at a point when it can be particularly disruptive to their integrity. However, the decrease in the activity of CAT may reflect enzyme inactivation resulting from excessive generation of H_2O_2 from SOD activity. The restoration of SOD and CAT activities upon coincubation of EGEE-treated spermatozoa with either kolaviron or vitamin C demonstrates their potent antioxidative effect. This corroborates with our earlier reports where kolaviron and vitamin C pre-treatment improved the di-n-butylphthalate- and tetracycline-induced testicular toxicities *in vivo* respectively (Farombi *et al.*, 2007, 2008).

Glutathione (GSH) is a multifunctional intracellular nonenzymatic antioxidant on which many of the thiol-dependent enzymes rely. Glutathione peroxidase (GPx) is the most important peroxidase for the detoxification of hydroperoxides. It catalyses the glutathione-dependent reduction of lipid hydroperoxides and of hydrogen peroxide (Jee & Kang, 2005). GST is a family of isoenzymes that catalyse the conjugation of GSH with a wide variety of organic peroxides (including lipid peroxides) to form more water-soluble compounds products that are readily excreted from the system. The decrease in GPx and GST activities observed in EGEE-treated spermatozoa may be due to the decrease in the availability of substrate (GSH) and also alterations in their protein structure under oxidative condition. The insufficiency of defence capacities against ROS is characteristic of oxidative stress state. Administration of kolaviron increases the activities of GST and GPx as well as increased GSH level in EGEE-treated spermatozoa and thus indicating the beneficial action of kolaviron against oxidative stress caused by the EGEE.

The decreased activities of antioxidant enzymes in EGEE-treated spermatozoa was accompanied by increased levels of H_2O_2 and MDA, an end product of lipid peroxidation. In addition, EGEE treatment resulted in decreased spermatozoa motility and viability, whereas it significantly increased spermatozoa abnormalities. Lipid peroxidation is oxidative deterioration of polyunsaturated lipids, and it involves ROS and transition metal ions (Bhatia & Jain, 2004). Peroxidation of spermatozoa lipids destroys the structure of the lipid matrix in the membranes of spermatozoa, and it is associated with the rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece morphological defects and may completely inhibits spermatogenesis in extreme cases (Sikka, 1996; Sanocka & Kurpisz, 2004; Vernet *et al.*, 2004).

The present result demonstrates that kolaviron or vitamin C treatment ameliorated EGEE-induced adverse effects on sperm motility, viability and morphology as well as protected against induction of oxidative stress evidenced by decreased H_2O_2 generated and MDA level in the spermatozoa compared with the EGEE nontreated group. Kolaviron has been reported to elicit its protective action by acting as membrane stabiliser and thus can break the chain reaction of the lipid peroxidation initiated by hydroxyl radicals (Iwu *et al.*, 1990).

Transminases and phosphatases in semen play an important role in transamination and phosphorylation processes in spermatozoa metabolism (Dhami *et al.*, 1994). Alkaline phosphatase (ALP) hydrolyses 6-phosphoglucose, a glycolytic intermediate, to give free glucose. Spermatozoa AST, ALT and ALP activities significant increased following incubation with EGEE. An increase in transaminases activities suggests a depletion of amino acid reservoir in the spermatozoa (Ikegwuonu *et al.*, 1980). Also, the increase in AST and ALT activities may be attributed to some structural alterations in the mitochondria in the mid-piece region, thereby causing leakage of these enzymes in the medium (Strzegek, 1988). The increase in ALP activity suggests a modification of the role of the enzyme in the phosphorylative pathway of spermatozoa re-utilisation of glucose following exposure to EGEE. The reduction in the activities of AST and ALT upon treatment with kolaviron or vitamin C reflects their abilities to maintain the cellular integrity, thereby preventing leakage of these marker enzymes in the spermatozoa.

The observed protective effects of kolaviron and vitamin C against EGEE-induced spermatotoxicity appear to correlate with the structural variations in the two natural antioxidants. In mechanistic term, the reduction potential of vitamin C may be related to its ability to readily donate electrons or hydrogen ions to regenerate other antioxidants including glutathione, a major thiol-disulphide redox buffer of the cell, as well as to reduce numerous reactive oxygen and nitrogen species. Ascorbic acid also acts to protect membranes against peroxidation by enhancing the activity of a vitamin E, the chief lipid soluble and chain-breaking antioxidant (Bhatia & Jain, 2004). Early studies on the structure–activity relationships of phenolic compounds related to kolaviron (Fig. 1a) revealed that the presence of a hydroxyl group in the position three (3-OH) of the C-ring can make it a potent inhibitor of lipid peroxidation (Hudson & Lewis, 1983; Cook & Samman, 1996; Mora *et al.*, 1997). Furthermore, antiperoxidative effect of kolaviron may be linked to the presence of a C-4 carbonyl and C-5 and C-7 hydroxyl groups of the A-ring (Farombi *et al.*, 2007). These structural features confer on kolaviron the antioxidant activity of radical scavenging utilising its many hydroxyl groups on the dual B-rings and metal ion chelation utilising an abundant availability of 3- or 5-hydroxyl and 4-ketosubstituents or hydroxyl groups in the ortho-position in the B-ring (Farombi & Nwaokeafor, 2005). We therefore

hypothesise that kolaviron and vitamin C, by way of maintaining spermatozoa antioxidant capacity, are to able combat EGEE-induced oxidative damage. Vitamin C does not appear to afford a better protective effect than kolaviron in its action against EGEE-induced toxicity in the present investigation.

Taken together, the present study indicates that kolaviron and vitamin C were effective in protecting against EGEE-induced oxidative damage in the spermatozoa by replenishment of glutathione stores, as well as scavenging of ROS and as such maintains spermatozoa quality. Kolaviron supplementation may therefore be beneficial in medicine for protection against EGEE-induced reproductive dysfunction. In addition, as part of livestock management, kolaviron supplementation may be beneficial if pigs are to be raised for commercial purposes (breeding, meat production etc.).

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