Ethylene glycol monomethyl ether-induced testicular oxidative stress and time-dependent up-regulation of apoptotic, pro-inflammatory, and oncogenic markers in rats

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ABSTRACT

Ethylene glycol monomethyl ether (EGME) is a major component of paints, lacquers, inks, and automobile brake fluids. As a result, exposures to humans are inevitable. We therefore, investigated in this study, its effect on testicular cells in a time-course manner in male Wistar rats. Animals were orally administered 50 mg/kg body weight of EGME for duration of 7, 14, and 21 days. Following 7 days of the administration, levels of NO and GSH were significantly reduced, while levels of c-Myc, K-Ras, caspase-3, IL-6, TNF-α, and IL-1β were significantly increased compared with control. At the end of 14 days exposure, GPx, and SOD activities, as well as IL-10 level were significantly decreased, while levels of c-Myc, K-Ras, p53, Bax, caspase-3, IL-6, TNF-α, IL-1β, and GST activity were significantly elevated compared with control. After 21 days of EGME administration, Bcl-2, IL-10, and NO levels were significantly decreased, while levels of c-Myc, K-Ras, p53, Bax, caspase-3, IL-6, TNF-α, IL-1β, MDA and GST activity were significantly increased compared with control. After 7, 14, and 21 days of EGME administrations, testis histopathology showed severe loss of seminiferous tubules, the seminiferous epithelium revealed very few spermatocytes, spermatids, spermatagonia, spermatozoa, and Sertoli cells, while the interstitial tissue is eroded, with scanty abnormal Leydig cells, compared with the control that appeared normal. We therefore, concluded that EGME-induced testicular toxicity as a result of EGME administration could be via the disorganization of the endogenous antioxidant systems as well as up-regulation of pro-inflammatory, apoptotic and oncogenic mediators in rats.

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1. Introduction

EGME is a member of the chemicals known as the ethylene glycol ethers (EGEs). EGME It is a solvent used industrially and utilized extensively for the manufacture of cellulose acetate, stains, inks, paints, and resins. EGME is also used as an antifreeze in hydraulic fluids and jet fuels, and also as leather and upholstery cleaners [1,2]. EGME is readily absorbed through skin contact and inhalation, posing health risks to diverse animal species including humans [1]. Organs and tissues having cells that are rapidly dividing and high metabolism, such as thymus and testes, are reported to be particularly vulnerable [3–5]. Reproductive tissues, neurological and hematological abnormalities are reported consequences of occupational exposure to EGME in humans [6–11]. Damage to the reproductive system, including alteration in female fertility and testicular atrophy are the outcomes of EGME treatment already reported in laboratory animals [12–16]. Also, EGME can affect the immune system, leading to reduced spleen cell number, decreased thymus weight, and thymic atrophy [17].

Following testicular exposures, EGEs are activated in affected cells by the action of alcohol dehydrogenase (ADH) and aldehyde

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dehydrogenase (ALDH) respectively [18]. Methoxyacetic acid (MAA), a metabolite of EGME is responsible for the teratogenicity, hematotoxicity, gonadotoxicity, immunotoxicity, and embryotoxicity of EGME [5,19–21]. Severe effects of EGEs on sperm and testes as a whole have been documented in humans [5], but have not been fully investigated. In six EGME-exposed workers, smaller testicular size was reported compared with nine unexposed individuals [7]. In wider research involving 73 painters exposed to EGME and EGEE, high cases of azoospermia, oligozoospermia, and an increased odds ratio (OR) for a smaller sperm count per ejaculate were reported compared to workers not exposed [11]. In animal studies, EGME was reported to cause atrophy of testes and disruption of sperm synthesis [20,22]. The Sertoli cells were also affected [23], and the key target cell where disruption of nuclei or atrophic nuclear chromatin condensation was evident is the pachytene spermatocyte [22].

Previous studies have not checked the time-course effect of EGME on testicular cells. In the light of the above and a follow-up, this present study investigated the time-course effect of EGME on testicular markers of lipid peroxidation (MDA), oxidative stress (CAT, SOD, GPx, GST, GSH, and NO), inflammation (IL-10, IL-6, TNF-α, and IL-1β), apoptosis (caspase-3, p53, Bax, and Bcl-2) and proto-oncogenic markers (c-Myc and K-Ras) in male Wistar rats.

2. Materials and methods

2.1. Chemicals and kits

EGME (C₃H₈O₂; CAS# 109-84-4; 99.5% purity), is a product of BDH Laboratory Supplies, Poole, BH15 1TD, England. Rats IL-10, IL-6, TNF-α, IL-1β, caspase-3, p53, Bax, Bcl-2, c-Myc, and K-Ras enzyme-linked immunosorbent assay (ELISA) kits are manufactured by Cusabio Technology Llc, Houston, TX, USA. The rest of the reagents and chemicals used were obtained from a recognized chemical manufacturing company and were of standard and analytical grade.

2.2. Oral acute toxicity study of EGME

The mean lethal dose (LD₅₀) of EGME was performed according to the method of Lorke [24]. The first phase involved three groups of
three rats each and were orally administered 1000, 2000, and 3000 mg/kg body weight of EGME respectively. These doses were administered based on documented findings that the LD<sub>50</sub> of EGME in rat is around 1000 mg/kg body weight or more [25–27]. Rats were checked for signs of toxicity and/or mortality for a week. Following the first phase, another three groups of one rat each were orally administered 900, 950, and 980 mg/kg body weight of EGME respectively, based on the outcomes of phase one, and were monitored for signs of toxicity and mortality. LD<sub>50</sub> was calculated from the outcomes of the two phases.

2.3. Experimental animals and study design

Male Wistar rats (150 g; n = 20) were bought and kept in cages in the animal house facility of our department, where they were given unrestricted access to food and water. Experimental procedures were conducted by following the established protocol of the Institutional Animal Care and Use Committee that was approved by the Animal Ethical Committee of the Department of Biochemistry, Federal University of Agriculture, Abeokuta, Nigeria. At the expiration of one week of acclimatization, the animals were randomly separated into four groups containing five animals each. Animals in group one were the control and were served only rat chow and water throughout, while animals in groups two, three and four were administered 50 mg/kg EGME orally, once per day for 7, 14, and 21 days respectively. The administered dose is the 1/20th of the mean lethal dose (LD<sub>50</sub>) obtained in this research study.

2.4. Sample collections and preparations

Group 1 animals were sacrificed on day 0 before the commencement of EGME administration. EGME was administered for 7, 14, and 21 days, and 24 h after each of these days (days 7, 14, and 21); rats were sacrificed through cervical dislocation. They were handled by the international guidelines for the handling and utilization of laboratory animals [28]. The harvested testes were rinsed in cold saline (0.9% w/v) solution, dried and weighed. A portion of the testis was suspended and homogenized 0.1 M (pH 7.4) phosphate buffer. The homogenate was centrifuged at 5000 rpm for 10 min, and the resulting supernatant was kept in Eppendorf tubes and used for the estimations of the activities, levels or concentrations of biochemical parameters of interest.

2.5. Estimation of biochemical parameters

Testis concentrations of MDA, NO, GSH, and total protein were determined by following the method of Buege and Aust [29], Green et al. [30], Moron et al. [31], and Gornall et al. [32] respectively. Activities of testis GPx, GST, SOD, and CAT were estimated following the method of Rotruck et al. [33], Habig et al. [34], Misra and Fridovich [35], and Sinha [36] respectively.

2.6. Estimations of testicular levels of TNF-α, IL-1β, IL-6, IL-10, caspase-3, p53, Bax, Bcl-2, c-Myc, and K-Ras

All these were determined by following the protocols inserted in each of the ELISA kit manufactured by Cusabio Technology LLC.
Houston, TX, USA.

2.7. Histopathological examination

Another section of the testis was excised and suspended in phosphate-buffered formalin solution for 48 h. After the dehydration in elevated concentrations of alcohol and clearance in xylene twice, the testicular tissues were placed in paraffin, excised into sections, stained with hematoxylin-eosin dye, and finally viewed 100× magnification under a microscope.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used for data analyses, followed by the Tukey test for multiple comparisons among the groups of rats using Graph Pad Prism program version 6.0. Results were written as mean ± standard error of the mean. A p-value lower than 0.05 (p < 0.05) was taken to be significant statistically.

3. Results

3.1. LD₅₀ study

Animals showed toxicity signs which include decreased food and water consumption and death after about 12 h of EGME administration. Mortality was recorded in phase one after the administration of 1000 and 3000 mg/kg body weight of EGME, while in phase two, no mortality was recorded following administration of 900, 950, and 980 mg/kg body weight of EGME. Consequently, oral LD₅₀ of EGME was calculated using the formula: \( \text{LD}_50 = \sqrt{(D_0 \times D_{100})} \), and was found to be 990 mg/kg in rat, where \( D_0 = \) highest dose that gave no mortality (980 mg/kg) and \( D_{100} = \) lowest dose that produced mortality (1000 mg/kg).

3.2. Effect of EGME on testes relative weight

There was no significant (p > 0.05) difference in testes relative weight following 7 and 14 days of EGME exposures, but a significant (p < 0.05) decrease was recorded after 21 days compared with control, 7 and 14 days of administrations (Fig. 1).

3.3. Time course effect of EGME on testis MDA level

No significant (p > 0.05) effect was recorded for testis MDA after 7 and 14 days, but administrations for 21 days resulted in a significant (p < 0.05) increase compared with control, 7 and 14 days of EGME exposures (Fig. 2A).

3.4. Time course effect of EGME on testis NO level

Administrations of EGME for 7 and 21 days resulted in a significant (p < 0.05) decrease in testicular NO level compared with control and 14 days of administrations (Fig. 2B).

3.5. Time course effect of EGME on testis GSH level

Administrations of EGME for 7 days significantly (p < 0.05)
decreased the testis level of GSH compared with control, 14 and 21 days (Fig. 2C).

3.6. Time course effect of EGME on testis activity of GPx

Administrations of EGME for 14 days resulted in a significant (p < 0.05) decrease in testis activity of GPx compared with control and 7 days (Fig. 3A).

3.7. Time course effect of EGME on testis SOD activity

The testis SOD activity was significantly (p < 0.05) decreased following 14 days of EGME administrations compared with control, 7 and 21 days (Fig. 3B).

3.8. Time course effect of EGME on testis GST activity

For GST, both 14 and 21 days of exposure to EGME significantly...
epithelium and seminiferous tubules consisting of spermatocytes, spermatogonia, spermatooza, spermatids, and Sertoli cells, while the Leydig cells in the interstitia are appearing normal (Fig. 7). After 7 days of exposure, there was a mild loss of seminiferous tubules, and the seminiferous epithelium consisting of few spermatogonia, spermatocytes, spermatooza, Sertoli cells, and spermatids, while the interstitia have normal Leydig cells (Fig. 7). For 14 days of exposure, there was a severe loss of the seminiferous tubules, the seminiferous epithelium consisting of spermatids, spermatogonia, spermatocytes, Sertoli cells, and loss of spermatooza, as well as disrupted interstitial tissue with few Leydig cells (Fig. 7). Following 21 days of exposure, there was a severe loss of seminiferous tubules, the seminiferous epithelium consisting of very few Sertoli cells, spermatogonia, spermatids, spermatocytes, and spermatooza. The interstitial tissue is eroded, having scanty abnormal Leydig cells (Fig. 7).

4. Discussion

Greater doses of EGME cause 100% mortality and toxicity to the reproductive system, for all the major routes of exposure. The degree of EGME toxicity on the fetus is completely dependent on the period of exposure [37,38]. Following exposures in this present study, relative testes weight was significantly decreased (Fig. 1) after 21 days of EGME administrations, an indication of testicular toxicity in the rats over time [5,39].

Reactive oxygen species (ROS) generation and lipid peroxidation are the key players in testicular pathology and physiology [40]. Excessive ROS production that overcomes the mopping of these ROS by endogenous antioxidants is linked to male infertility [41]. It has been reported that the administration of EGES leads to a steady production of ROS [42] and alterations in the anti-oxidative systems by either raising or lowering the levels of antioxidant defense systems through ROS production [43]. In this study, the significant increase in testis MDA concentration after 21 days of EGME administrations (Fig. 2A) may be an indication of oxidative stress following the generation of reactive oxygen species. The generated free radicals may be responsible for the attack of electron-rich components of biological membranes, leading to their destruction and thereby jeopardizing the cellular integrity and functions [44,45]. In a study where furan was administered to rats, testicular MDA concentration was found to increase significantly [46], while cisplatin triggered testicular and epididymal oxidative stress in rats [47].

NO is a major and vital biological molecule formed in cells, involved in the regulation of many important physiological processes, including immune response, blood pressure and neural communication [48]. Overproduction of cellular NO can cause the generation of peroxynitrite that can eventually destroy tissues [49,50]. From this study, the significant decrease in testicular NO level (Fig. 2B) after 7 and 21 days may be attributed to EGME-induced oxidative stress that may have led to excessive production of ROS. Increased ROS concentrations lower functional NO concentrations via chemical deactivation. This is referred to as NO mopping, a major outcome of oxidative stress [48].

Enough evidence has been gathered on the deleterious effects of environmental contaminants and toxicants on the male reproductive system [51,52]. Free radicals are research focus due to their contributions in cellular pathogenesis and physiology of various disorders including male and female infertility [53,54]. GST, a key multifunctional non-enzymatic antioxidant, is also a protein in which diverse that are thiol-dependent depend on. GPx is a key peroxidase enzyme that is involved in the detoxification of hydroperoxides by catalyzing the GSH-dependent reduction of hydrogen peroxide and lipid hydroperoxides [55]. GST belongs to
one of the phase two enzymes of drug metabolism that catalyzes the conjugation of GSH with xenobiotics to form water-soluble products, readily excreted from the body [56]. The mutual and synergistic actions between SOD and CAT, another two endogenous antioxidant enzymes, against the overwhelming generation of ROS helps to detoxify superoxide and peroxyl radicals by converting them into non-toxic forms [56]. The significant decrease in testicular GSH level (Fig. 2C), GPx (Fig. 3A) and SOD (Fig. 3B) activities, as well as significant increase in GST (Fig. 3C) activity following EGME administrations, can be attributed to testicular response to EGME-induced free radical generation and oxidative stress. It has been reported that an increase in the activity of GST is known to serve as protective responses to eliminate xenobiotics [43,57]. Also reported was that exposure to ethylene glycol monoethylether (EGEE), a member of the ethylene glycol ether family, resulted into a decrease in the testicular level of GSH [19,56,58], activities of GPx [58], and SOD [19,58], as well as a significant increase in testicular GST [19,58] activity in rats. Following the dissipation of the superoxide radicals, the resulting H2O2 is broken down to H2O and O2 by CAT [59]. GSH is the substrate for GPx, and as the former is been oxidized, there is a concomitant conversion of H2O2 to non-toxic products [60]. Also, the non-significant effect of EGME on testis CAT activity recorded in this study (Fig. 3D), suggested that scavenging of H2O2 may be through the glutathione family of antioxidants as stated above, and not by CAT.

Cytokines are involved in immune cell activities [61]. Within the testes, numerous factors including tumor necrosis factor and interleukins coordinate immune cell function. These cytokines are also formed by non-immune cells to promote and maintain sperm production [61]. In this study, the significant increase in testicular levels of TNF-α (Fig. 4A), IL-1β (Fig. 4B), and IL-6 (Fig. 4C), as well as decreased level of IL-10 (Fig. 4D) as a result of 7, 14 and 21 days of EGME administrations, is an indication of EGME-induced testicular damage, causing the spermatogenic and testicular somatic cells to produce the inflammatory cytokines in response to the damage. Also, it could be as a result of the immune response, leading to the stimulation and secretion of inflammatory cytokines by immune cells to the affected site where they initiate inflammation [62]. In our previous study, we reported that administration of methyl cellosolve significantly increase the levels of inflammatory cytokines after 7, 14 and 21 days of administration in rats [63]. Also, Khosravi et al. [64] reported an increase in the levels of inflammatory cytokines in streptozotocin-induced diabetic rats.

Apoptosis is a process of programmed cell death characterized by biochemical and morphological alterations, as well as changes in genomic expression [65,66]. p53 is actively involved in the response to cellular discomfort by serving as a major hindrance to carcinogenesis [67]. A significant increase in testicular p53 level (Fig. 5A) after 14 and 21 days of exposure is an indication of EGME-induced testicular cell damage. The damage could be attributed to EGME-induced testicular oxidative stress and inflammation recorded in this study, which may have stimulated p53 activation. Upon activation, p53 may have initiated cell cycle arrest and activation of apoptotic genes to facilitate testicular apoptosis. Apoptosis is a strictly regulated process controlled by several signaling pathways, such as the mitochondrial pathway and caspase cascade [68–70]. p53 is a positive regulator of the Bad, Bak, and Bax pro-apoptotic proteins to stop Bcl-2 capture. Free Bad, Bak and Bax eventually attach to the mitochondrial membrane to cause mitochondrial membrane damage and cellular apoptosis [71–73]. This study reveals a significant increase in testicular Bax (Fig. 5B) after 14 and 21 days and a decrease in Bcl-2 (Fig. 5C) levels (after 21 days) following EGME administrations, suggesting a p53-induced testicular programmed cell death. In response to testicular damage, Activated p53 recorded in the study, may have up-regulated Bax and down-regulated Bcl-2 expressions, which are two players of apoptosis, and p53 targets. An increase in the cytosolic level of free Bax may have subsequently attacked the mitochondrial membrane; creating pores in it, causing mitochondrial membrane damage and the release of cytochrome c that propagate the testicular apoptosis with other downstream mediators of apoptosis (Apafl, caspases-3 and 9). Past studies have reported that p53 stimulates the transcription of Bak and Bax, which controls the outflow of cytochrome c from the mitochondrion, resulting in cellular apoptosis by stimulating the excision of caspase-3 and caspase-9 [72,73]. Cellular apoptosis is a complex biological process related to complex signaling pathway responses. The activation of cysteine proteases, particularly the caspases, is a major intracellular coordinator of cellular apoptosis [74,75]. In this study, an increased level of testis caspase-3 (Fig. 5D) was recorded after 7, 14 and 21 days of EGME administrations. The released cytochrome c into the cytoplasm following the attack of Bax on the mitochondrial membrane may have interacted with downstream apoptotic mediators (Apafl-1, caspase-9) to form apoptosome that cleaved the executioner caspases including caspase-3, thereby facilitating the testicular apoptotic process. The result of caspase-3 obtained in this study is corroborated by the findings of Adedara et al. [76] who reported that exposure to ethylene glycol monoethylether (EGEE) resulted into a significant increase in the expressions of testicular stress-inducible proteins (active caspases, Fas and Fas-L) in rats, while 14 and 21 days of methyl cellosolve in rats led to the significant increase in renal caspase-3 in rats [63].

K-Ras is a proto-oncogene and the widely accepted mutated oncogene, that is constantly associated with some of the worst forms of cancer [77]. It has been reported that Myc is essential for K-Ras-driven cancer [78] and activation of Ras stabilizes Myc [79], enabling Myc to render cells vulnerable to DNA damage and apoptosis [80]. c-Myc is another proto-oncogene, a strong pleiotropic transcription factor known to coordinate cell cycle growth, progression, adhesion, differentiation, metabolism, proliferation, and apoptosis [81–83]. Testicular c-Myc (Fig. 6A) and K-Ras (Fig. 6B) levels after 7, 14 and 21 days of EGME administrations were significantly increased, which may be an indication of EGME-induced mutations in these oncogenes by amplification or translocation to areas of high transcriptional activities, resulting into their activations and subsequent generation of reactive oxygen species that may have resulted into DNA damage. Activation of these oncogenes may further explain the significant increase in the levels of apoptotic mediators, p53, Bax, caspase-3, recorded in this study that facilitated apoptosis and prevented tumor initiation and progression that may ensue as a result of uncontrolled proliferation of damaged cells.

Testis histopathology (Fig. 7) revealed the toxic effect of EGME on the testicular cells. The severe loss of the seminiferous tubules, a very few spermatogonia, spermatocytes, spermatids, spermatooza, and Sertoli cells, as well as the erosion of the interstitial tissue with scanty abnormal Leydig cells, confirmed the outcomes of other findings in this study, and a clear indication that exposure to EGME over time may result into male infertility, hence, difficulty in childbearing.

We therefore, concluded that EGME-induced testicular toxicity may be via the disorganization of the endogenous antioxidant systems, causing the up-regulation of pro-inflammatory, apoptotic and oncogenic mediators in rats.

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CRediT authorship contribution statement

Oluwatosi T. Somade: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration.

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Declaration of competing interest

None to declare.

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