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Long-term mequindox treatment induced endocrine and reproductive toxicity via oxidative stress in male Wistar rats

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ABSTRACT

Mequindox (MEQ) is a synthetic antimicrobial chemical of quinoxaline 1, 4-dioxide group. This study was designed to investigate the hypothesis that MEQ exerts testicular toxicity by causing oxidative stress and steroidal gene expression profiles and determine mechanism of MEQ testicular toxicity. In this study, adult male Wistar rats were fed with MEQ for 180 days at five different doses as 0, 25, 55, 110 and 275 mg/kg, respectively. In comparison to control, superoxide dismutase (SOD), reduced glutathione (GSH) and 8-hydroxydeoxyguanosine (8-OHdG) levels were elevated at 110 and 275 mg/kg MEQ, whereas the malondialdehyde (MDA) level was slightly increase at only 275 mg/kg. Furthermore, in LC/MS-IT-TOF analysis, one metabolite 2-isoethanol 4-desoxymequindox (M11) was found in the testis. There was significant decrease in body weight, testicular weight and testosterone at 275 mg/kg, serum follicular stimulating hormone (FSH) at 110 and 275 mg/kg, while lutinizing hormone (LH) levels were elevated at 110 mg/kg. Moreover, histopathology of testis exhibited germ cell depletion, contraction of seminiferous tubules and disorganization of the tubular contents of testis. Compared with control, mRNA expression of StAR, P450scc and 17β-HSD in testis was significantly decreased after exposure of 275 mg/kg MEQ while AR and 3β-HSD mRNA expression were significantly elevated at the 110 mg/kg MEQ group. Taken together, our findings provide the first and direct evidence *in vivo* for the formation of free radicals during the MEQ metabolism through N→O group reduction, which may have implications to understand the possible mechanism of male infertility related to quinoxaline derivatives.

Introduction

Oxidative stress can be defined as an imbalance between production of free radicals and reactive metabolites, so-called reactive oxygen species (ROS). In recent decades, there has been growing concern that repeated oxidative stress could produce serious reproductive hazards in the male reproductive milieu (Aitken and Baker, 2004; Ong et al., 2002). The germ line epithelium of the testis is highly vulnerable to oxidative stress due to abundance of unsaturated fatty acids and presence of ROS generating system. ROS and lipid peroxidation (LPO) are known to be the vital mediators in testis physiology (Kumar and Muralidhara, 2007; Turner and Lysiak, 2008). However, recent findings have led to the proposal that oxidative free radicals can involve in DNA damage in the germ cell line and serve as the important etiology of male infertility (Aitken and Baker, 2004, 2006; Aitken et al., 2004).

Quinoxaline 1, 4-dioxides (QdNOs) are potent agents with a wide range of biological properties like growth promoter, antibacterial, anticandida, antitubercular, anticancerous and antiprotozoal activities (Carta et al., 2005; Vicente et al., 2009). Carbadox (CBX), olaquindox (OLA) and cyadox are known members. The suspected toxicity of some QdNOs has raised a serious concern among governmental and international organizations. CBX and OLA are known genotoxic and possible germ cell mutagens. CBX and OLA have also reproductive toxic potential. Studies have shown that CBX reduces the fertility rate, disturbs spermatogenesis in mice and also causes toxic effects on embryos (Sykora et al., 1981; WHO, 1991a). It was also reported that the exposure to CBX on days 8 to 15 of pregnancy in rats resulted in

Abbreviations: AR, androgen receptor; CBX, carbadox; CNS,, central nervous system; FSH, follicle stimulating hormone; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; MEQ, mequindox; LH, lutinizing hormone; LPO, lipid peroxidation; OLA, olaquindox; QdNOs, quinoxaline 1, 4-dioxides; ROS, reactive oxygen species; SOD, superoxide dismutase; 8-OHdG, 8-hydroxydeoxyguanosine; StAR, steroidogenic acute regulatory protein.

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the decrease in fetal and maternal body weights, and teratogenic effects at doses as low as 25 mg/kg per day (Yoshimura, 2002). In a subchronic dietary study, OLA exposure resulted in an increased testicular weight in rats at 60 mg/kg body weight. In a dominant lethal assay, OLA has been found to cause the increase in frequency of dominant lethal mutations and abnormalities in spermatozoa (Chen et al., 2009; WHO, 1991b). Mequindox (MEQ), a new synthetic compound in QdNOs (Fig. 1), has been used as an antimicrobial feed additive in China. In our previous study of MEQ in Wistar rats, we have found that the high dose (275 mg/kg) can cause increase in testicular weight, MDA levels in serum and tubular cell degeneration after 90 days of feed (Ihsan et al., 2010). Furthermore, we also observed that administration of MEQ can reduce reproductive organ weights in both sexes, change in sperm morphology, decrease fertility, alter growth and development of next generation, and is a genotoxic agent (unpublished results). It is thought that reproductive toxicants can exert toxicity through oxidative damage to DNA in the germ cells and/or direct effects on the endocrine system, resulting in disruptive development and abnormalities. In recent *in-vitro* studies, our laboratory reported that the N→O group reduction was found to be the main metabolic pathway in QdNOs metabolism (Liu et al., 2008; 2010a; 2010b) and this reduction might lead to the formation of ROS and cell apoptosis (Huang et al., 2010). However, elevated levels of ROS have propensity to cause significant alteration in testis physiology *in vivo*, or induce oxidative damage to DNA, which is of potential risk to reproduction (Aitken and Baker, 2006; Doreswamy and Muralidhara, 2005; Kumar and Muralidhara, 2007; Toyokuni, 1999; Turner and Lysiak, 2008). An exclusive study examining adrenal toxicology by our group demonstrated that MEQ exposure at 110 and 275 mg/kg in diet resulted in adrenal cell damage and decline in serum corticosterone and aldosterone levels. Furthermore, we also found that same doses resulted in down-regulation of mRNA levels of P450scc, CYP11B1 and CYP11B2, but up-regulated the mRNA levels of CYP21 and 3βHSD genes (Huang et al., 2009). To date, no studies have examined whether MEQ induces testicular toxicity involving oxidative stress damage and dysregulation of hormone production. Thus, the aims of the present study were to test the hypothesis that MEQ exerts its toxicity in the testis by causing oxidative damage and steroidal gene expression profiles and to determine the mechanism of MEQ toxicity in the testis.

Here, we evaluated the testicular toxicity of MEQ in male Wistar rats in feed for 180 days. The dose levels of this experiment were based on the results of our previous studies in rats (Huang et al., 2009; Ihsan et al., 2010). Specifically, we examined (1) the effect of MEQ on testicular weight as well as morphological changes in testis, (2) the effect of MEQ on the levels of MDA, SOD, and GSH *in vivo*, (3) whether MEQ causes oxidative DNA damage *in vivo* as evidenced by the presence of 8-hydroxydeoxyguanosine (8-OHdG), (4) LC/MS-IT-TOF analysis for metabolites of MEQ in testis, (5) the ability of MEQ to alter serum LH, FSH and testosterone levels, and (6) the effect of MEQ on the mRNA expression of different genes, responsible for cholesterol transport and testosterone biosynthesis. These genes included steroidogenic acute regulatory protein (StAR), cholesterol side-chain cleavage enzyme (P450scc), androgen receptor (AR), 3-beta-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450c17 subfamily a (CYP17a) and 17-beta-hydroxysteroid dehydrogenase (17β-HSD).

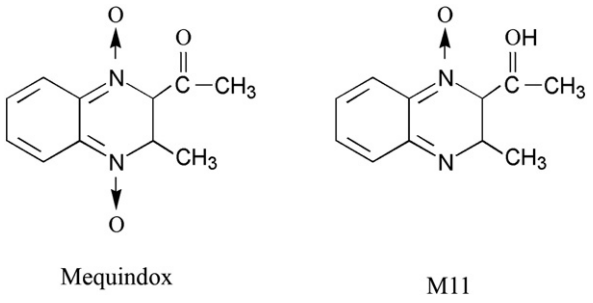

Fig. 1. Chemical structure of MEQ and 2-isoethanol 4-deosymequindox (M11).

Materials and methods

Materials. Mequindox (purity 98%) was purchased from Beijing Zhongnongfa Pharmaceutical Co. Ltd. (Huanggang, PR China). MDA, SOD, GSH and 8-OHdG kits were obtained from Shanghai Dobio Biotech Co., LTD (Shanghai, PR China). All other chemicals were purchased from Sigma (St. Louis, USA) unless otherwise stated.

Animals and diets. Male specific pathogen-free Wistar rats (4–5 weeks old), were procured from the Center of Laboratory Animals of Hubei Province (Wuhan, PR China). Rat room was maintained at a temperature of 22 ± 3 **°C**, a relative humidity of 50–70%, and a 12-h light/dark cycle. The animals were housed five rats per box. Hardwood shavings were used as bedding and were changed every week. This study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Huazhong Agricultural University).

Before initiation of dosing, all rats were quarantined for 2 weeks and evaluated for weight gain and any gross sign of disease or injury. After quarantine, rats were randomized on the basis of their body weights and divided into five treatment groups (n = 15 per group). One group was served as control and received standard commercial feed without any feed additive. Four groups were given 25, 55, 110 and 275 mg/kg MEQ in diet, respectively. The feed was offered *ad libitum* for 180 days. At the end of experiment, rats were fastened over night and anesthetized with diethyl ether. Blood samples were collected and set on ice. After clotting on ice, whole blood samples were centrifuged at 2000 × *g* for 10 min at 4 °C. Serum was stored at −20 °C until needed for hormone analysis. In addition, testes were dissected out and weighed immediately. Seven to nine left testes were frozen immediately in liquid nitrogen and stored at −70 °C for RNA isolation, oxidative stress indices and detection of metabolites and, right testis was fixed in Davidson's fixative for histopathology. After fixation, testes were sectioned to 5 μm thickness and stained with hematoxylin-eosin (HE). Slides were observed under an optical microscope (Olympus BX 41, Japan).

Preparation of tissue extract. The frozen testes were thawed and homogenized using a Potter–Elvehjem homogenizer. A 20% homogenate of the tissue was prepared in ice cold 0.2 M sodium phosphate buffer (pH 6.25). The homogenate was centrifuged at 10,000 × *g* for 1 h at 4 °C, and resultant supernatant was used for SOD, GSH, MDA and 8-OHdG assays.

Oxidative stress indices. The malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH) and 8-Hydroxy deoxyguanosine (8-OHdG) levels in testes were assayed by using commercial ELISA kits. Data were determined according to the manufacturer's instructions.

LC/MS-ITTOF analysis of MEQ and its metabolites in testes. To check whether MEQ was metabolized in the testes, the metabolites were detected using a hybrid ion trap/time-of-flight (IT/TOF) mass spectrometry combined with a high-performance liquid chromatography system (Shimadzu Corp., Kyoto, Japan). The liquid chromatography system (Shimadzu) was consisted of a solvent delivery pump (LC-20AD), an autosampler (SIL-20AC), a DGU-20A3 degasser, a photodiode array detector (SPD-M20A), a communication base module (CBM-20A) and a column oven (CTO-20AC).

During analysis, 0.1 g of the tissue was homogenized with 5 ml of 40 **°C** distilled water at the 10,000 × *g* for 3 min in a model omni mixer

homogenizer 17106 (OMNI International, Waterbury, CT. USA). Then the homogenate was homogenized for 1 min after that 5 ml of 15% trichloroacetic acid was added. The homogenate was centrifuged at 10,000 × *g* for 15 min. After that the supernatant was collected, 10 ml acetonitrile was added and vortex-mixed for 5 min. After vigorous shaking, followed by centrifugation at 10,000 × *g* for 15 min, the supernatant was dried using N_2 at 40 °C with the water bath. The residue was reconstituted in 5 ml distilled water. The total supernatant was applied to the methanol and water pre-washed HLB 3 cm^3 cartridge (Waters Corporation, Milford, Mass USA). The samples were then sequentially washed with 3.0 ml water and 5% methanol in water. The extracts from tissue samples were eluted into plastic tubes with 5 ml methanol. The elution was evaporated to dryness under a gentle stream of nitrogen at 40 °C, reconstituted the residue with 500 µl solution [methanol: water (40:60 v/v)] and passed through 0.22 µm filter membrane for LC/MS-ITTOF. The extracted samples were analyzed using a VP-ODS column (150 mm × 2.0 mm I.D.; particle size 5 µm), with an injection volume of 20 µl. The mobile phase and analysis method were in accordance with the literature (Liu et al., 2010b).

Hormone assays. Serum LH, FSH and testosterone levels were quantified by sandwich enzyme linked immunosorbent assay (ELISA) using specific commercial kits (Shanghai Dobio Biotech Co., LTD, Shanghai, PR China) using Microplate Spectrophotometer µQuant system (BioTek Instruments, Winooski, VT, USA). Data were determined according to the manufacturer's protocols.

Real-time reverse transcription polymerase chain reaction. To investigate the relationship between oxidative stress, hormonal changes, histopathology and gene expression, we analyzed the expression of 6 genes using real time RT-PCR. Briefly, total RNA from the testes was extracted using the Trizol Reagent according to the manufacturer's instructions. One microgram of RNA was reverse transcribed to cDNA with the use of ReverTra AceTM First Strand cDNA Synthesis Kit (Promega, USA). cRNA was amplified by quantitative real-time PCR (Bio-Rad, USA) using SYBR® Premix Ex TaqTM RT-PCR kit. (Takara, Code DRR041 A, Japan). Rat specific primers were designed for the genes of interest: StAR, P450scc, AR, 3β-HSD, CYP17a and 17β-HSD (Table 1). Each 25 µl reaction mixture consisted of 12.5 µl SYBR® Premix Ex TaqTM, 0.5 µl of each primer (10 µm), 2 µl of cRNA, and 9.5 µl Rnase Free dH_2O. Cycling conditions were as follows: step 1, 30 s at 95 °C; step 2, 40 cycles at 95 °C for 5 s, 60 °C for 30 s; step 3, Dissociation stage. Data from the reaction were collected and analyzed by the complementary computer software. Relative quantitative analyses of RT-PCR gene expression were calculated using $2^{-\triangle\triangle Ct}$ data analysis method as previously described (Huang et al., 2009; Wang et al., 2011). The housekeeping gene β-Actin was employed for compression as a control.

Table 1
Primers used for Real time RT-PCR analysis.

Primer	Sequence (5′-3′)	Amplified region
StAR	Sense: GCCCCGAGACTTCGTAAG Anti-sense: CAGGTGGGACCGTGTTCA	537–668
P450scc	Sense: GTGTTGTATTTGGGGAGC Anti-sense: AGTCTGGAGGCATGTTGA	629–754
AR	Sense: CAAAGGGTTGGAAGGTGA Anti-sense: CGAGCGGAAAGTTGTAGT	939–1096
3β-HSD	Sense: CCTGCTGCGTCCATTTTA Anti-sense: TCTGCTTGGCTTCCTCCC	909–1054
Cyp17a	Sense: AGAAATCTGGTGGACATA Anti-sense: ACTTGAGCACAGTGGTAG	631–934
17β-HSD	Sense: ACCGCCGATGAGTTTGTT Anti-sense: GGGTGGTGCTGCTGTAGA	727–862
β-Actin	Sense: GAGATTACTGCCCTGGCTCCTA Anti-sense: ACTCATCGTACTCCTGCTTGCTG	1026–1175

Statistical analyses. Statistical analysis was performed using SPSS 15.0 for windows. All results were expressed as mean ± SE. Group difference was assessed by one-way analysis of variance (ANOVA) followed by least significance difference (LSD) test. A probability of $p<0.05$ was considered significant.

Results

Effects of MEQ on body and testicular weights

The final body and testis weight of male rats treated with MEQ for 180 days is shown in Table 2. The rats exposed to 275 mg/kg MEQ, the final body weight and testes weight were significantly reduced by 28% and 15.9% respectively ($p<0.01$). Though testes weight exhibited a depressive trend at 55 mg/kg MEQ, but no significant differences were observed with the control group. The relative testes weight was markedly increased at only highest dose ($p<0.01$).

Effect of MEQ on testicular histology

As shown in Fig. 2, marked changes in seminiferous tubules were observed at 275 mg/kg MEQ. Seminiferous tubules exhibited disintegration of germinal epithelium, degenerative changes of lining cells, germ cell depletion and reduction in the round sperms (~65%). Most tubules (~45%) examined showed degeneration and had no mature spermatozoa evident in contrast to controls. Reduction in the overall diameter of the seminiferous tubules was also observed in few areas.

Effects of MEQ on oxidative stress indices

Oxidative indices including MDA, SOD, and GSH levels as well as 8-OHdG were examined. As shown in Table 3, MDA levels were significantly higher in the 275 mg/kg MEQ ($p<0.01$). Significant increases in testicular SOD, GSH and 8-OHdG levels were observed in both 110 mg/kg ($p<0.05$) and 275 mg/kg MEQ groups ($p<0.01$), indicating activation of defense mechanisms against increased oxidative damage.

MEQ and its metabolites in testis

The representative HPLC chromatograms of MEQ metabolite are shown in Fig. 3. The metabolite of MEQ was identified on the base of the retention time and the associated fragment ions. Metabolite M11 with retention time of 5.0 min was detected in the all MEQ treated groups (Fig. 1). M11 was identified as 2-isoethanol 4-desoxymequindox.

Effects of MEQ on serum hormones

The effects of MEQ on serum hormones are shown in Fig. 4. For 110 mg/kg MEQ, LH levels were significantly higher when compared with the control ($p<0.01$). FSH was markedly decreased to 45% and

Table 2
Effects of MEQ on body and testicular weights in different treatment groups.

Groups	Final body weight (g)	Testicular weight (g)	Relative testicular weight (g)
Control	387.8 ± 9.17[a]	3.40 ± 0.10	0.87 ± 0.021
25 mg/kg	386.5 ± 7.91	3.34 ± 0.07	0.87 ± 0.017
55 mg/kg	379.3 ± 12.31	3.14 ± 0.12	0.88 ± 0.036
110 mg/kg	381.3 ± 8.04	3.29 ± 0.08	0.87 ± 0.025
275 mg/kg	279.1 ± 10.43**	2.86 ± 0.12**	1.02 ± 0.036**

[a] Data presented as mean ± SE of 10 animals in each group.
** Significantly different from control group at $p<0.01$.

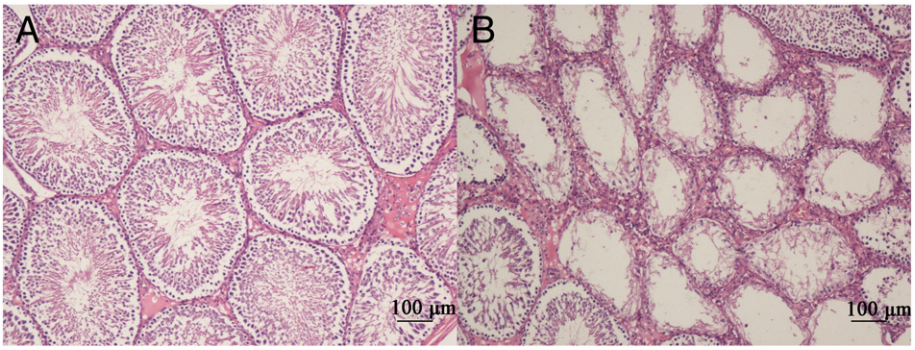

Fig. 2. Light micrographs of testicular sections stained with hematoxylin-eosin (HE) after 180 days. (A): Testis in a rat of control group. (B): Testis in a rat of 275 mg/kg MEQ group showing degeneration and disintegration of germinal epithelial cells.

40% for the control rats at doses of 110 and 275 mg/kg MEQ ($p<0.05$). Testosterone levels were significantly decreased to 49.8% only for the 275 mg/kg group ($p<0.05$).

Effects of MEQ on genes expression

The effects of MEQ exposure on mRNA expression of genes responsible for cholesterol transport and steroidogenesis in male rats were determined (Fig. 5). Compared with controls, expression of the gene for transporting plasma cholesterol to inner mitochondrial membrane, StAR, was significantly decreased after exposure of 275 mg/kg MEQ ($p<0.01$). The mRNA levels of P450scc and 17β-HSD were markedly reduced at dose of 275 mg/kg ($p<0.05$). Interestingly, significant differences between the control and 110 mg/kg MEQ were detected for AR and 3β-HSD mRNA expression ($p<0.01$). Testicular CYP17a mRNA levels were significantly increased by MEQ at 25 mg/kg ($p<0.01$).

Discussion

The ability of QdNOs to disrupt steroidogenesis and mechanisms by which these compounds interfere with the function of steroidogenic enzymes is a relatively unexplored area of toxicology. The present investigation is the first to determine the various targets of MEQ toxicity in an effort to understand the probable mode of action using male Wistar rats. The evaluation of testicular weight is an integral component in the assessment of reproductive toxicity. The weight of the testis is largely depending on the mass of the differentiated spermatogenic cells. Testosterone represents the key circulating androgen in the males. It also exerts a major role in the maintenance of structural and functional activates of the testis (Creasy, 2001a, b, c). In the present study, reduction in the body and testicular weights after MEQ treatment was indicative of toxicity at the high dose. Moreover, reduction in the diameter of seminiferous tubules with germ cell depletion was also observed. Reduction in the diameter of the seminiferous tubule might have occurred as a consequence of reduced secretion of seminiferous tubule fluid. Seminiferous tubule fluid is secreted by Sertoli cells under the influence of testosterone (Boujbiha et al., 2009; Creasy, 2001b). This result proposed an altered testosterone secretion and/increased oxidative damage in testis might be responsible for the reduction in testicular weight (Abd-Allah et al., 2000; Aitken and Baker, 2004, 2006).

Table 3
Effects of MEQ on testicular MDA, SOD, GSH and 8-OHdG in different treatment groups.

Groups	MDA (nmol/mg protein)	SOD (Unit/g protein)	GSH (ng/mg protein)	8-OHdG (pg/mg protein)
Control	10.80 ± 1.25 [a]	583.4 ± 33.4	16.67 ± 2.51	123.6 ± 15.6
25 mg/kg	11.75 ± 1.06	689.8 ± 53.8	20.93 ± 2.56	155.2 ± 18.4
55 mg/kg	12.38 ± 1.01	690.9 ± 43.0	23.53 ± 1.63	250.3 ± 50.9
110 mg/kg	12.41 ± 0.84	772.4 ± 38.8*	25.15 ± 1.37*	256.5 ± 65.7*
275 mg/kg	15.78 ± 1.65**	1060.9 ± 119.8**	32.73 ± 4.78**	353.3 ± 48.9**

[a] Data presented as mean ± SE of 7 animals in each group.
* Significantly different from control group at p<0.05.
** Significantly different from control group at p<0.01.

In testis, steroidogenesis and spermatogenesis are two vital physiological processes which are under the control of endocrine system (Shi et al., 2007). Low levels of ROS play an essential role in cell signaling, proper cell function, sperm capacitation, acquisition of sperm-fertilizing ability as well as initiating a cascade of events that result in cytotoxicity (Aitken and Baker, 2006). It is believed that steroidogenesis itself produces the small amount of ROS, largely from the catalytic reactions of the steroidogenic cytochrome P450 enzymes and mitochondrial respiration (Aitken and Baker, 2004; Lee et al., 2007). Metabolism of MEQ involves the catalyzation by CYP reductase (Liu et al., 2010a, b). In a study, Liu et al. (2010b) detected ten possible metabolites of MEQ in the liver microsomes of different species. His results exhibited that MEQ was primarily metabolized to 1-desoxymequindox (M2) and bis-deoxymequindox (M6) by N→O group reduction in the rat liver microsomes. In our study, we proposed that MEQ was metabolized to 2-isoethanol 4-desoxymequindox (M11) by the hydrogenation of the 4-desoxymequindox (M1). During N→O group reduction reactions, ROS such as superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) are generated (Fig. 6) (Novave et al., 1986). Superoxide anion radicals attack DNA bases, producing damaged bases and single strand breaks or influence the enzyme activities and transcription and/transduction pathways. It oxidizes lipid and protein molecules, generating intermediates, which can react with DNA and form adducts (Kinoshita et al., 2002; Sikka, 1996, 2004). Whereas, excessive metabolism of H_2O_2 could be the cause of oxidized shift in the cellular redox state of glutathione and NADPH, impairing the ability to detoxify lipid peroxides (Doreswamy and Muralidhara, 2005; Huang et al., 2010; Murphy, 2009; Toyokuni, 1999). It is already reported that, under hypoxic conditions, mutagenicity of QdNOs is increased due to the presence of N→O group (Huang et al., 2010; Nunoshiba and Nishioka, 1989; Priyadarsini et al., 1996). Furthermore, recent studies have shown a clear correlation between genotoxicity and intracellular ROS generation (Zou et al., 2009). In present study, we determined the malondialdehyde (MDA) and 8-hydroxy-2-deoxyguanosine (8-OHdG) levels. Our results suggested that the increased MDA levels in testis were concurrent with significant oxidative damage to testicular DNA. This change might result from increased production of ROS during MEQ metabolism. This is the first report on the tendency of MEQ to induce LPO and DNA damage in rat testis *in vivo*, while there are many reports on the ability of QdNOs to induce DNA damage and mutagenicity in bacterial assays (Arimoto et al., 1980; Beutin et al., 1981; Negishi et al., 1980; Nunoshiba and Nishioka, 1989; Voogd et al., 1980; WHO, 1991a, b; Yoshimura et al., 1981), cell

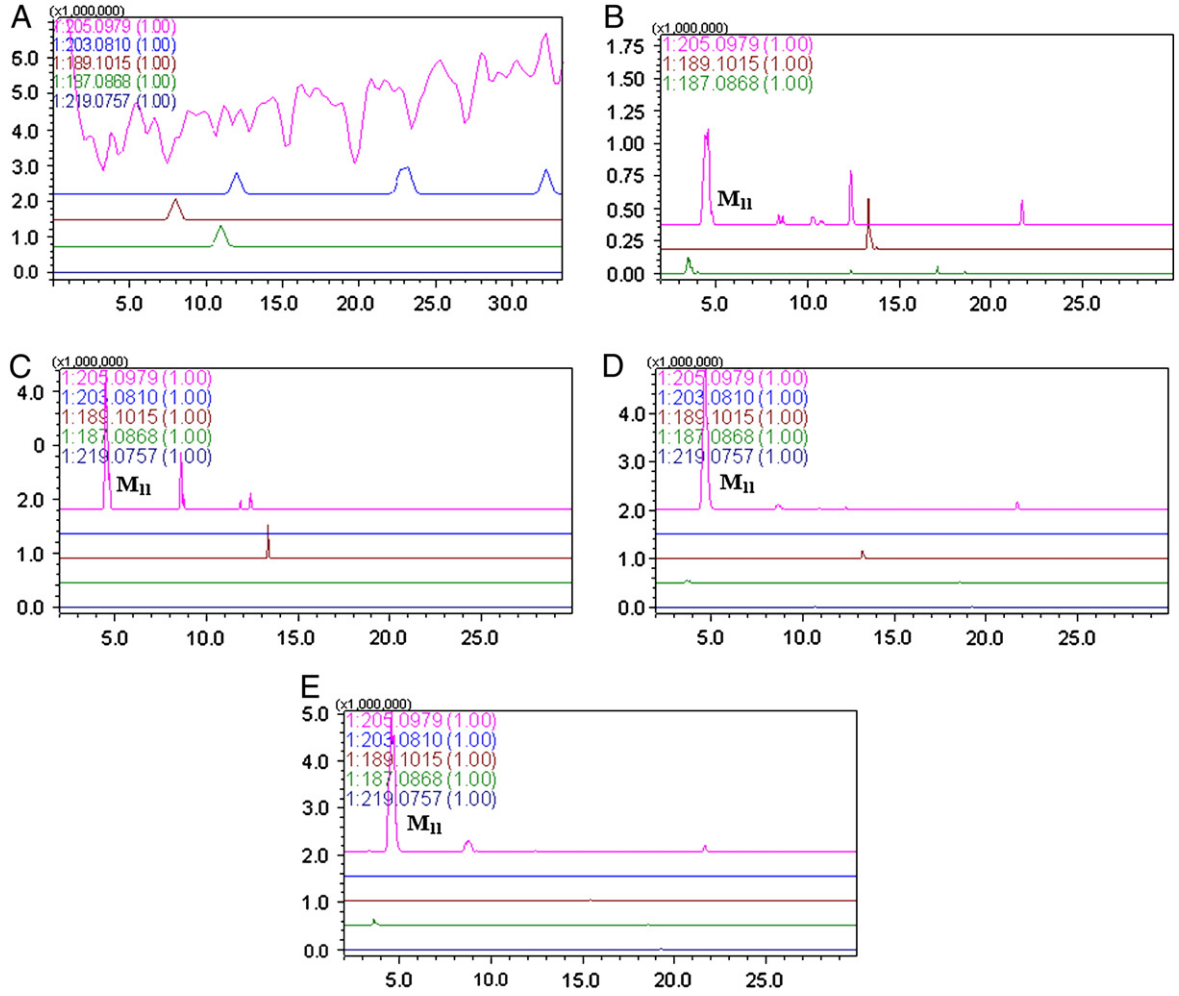

Fig. 3. The accurate extracted mass chromatograph of MEQ metabolite in control (A), 25 mg/kg (B), 55 mg/kg (C), 110 mg/kg (D) and 275 mg/kg (E).

culture models (Chen et al., 2008; Markovic et al., 2000; Scheutwinkel-Reich and vd Hude, 1984) and human hepatoma G2 cells (Zou et al., 2009).

To prevent peroxidation of membrane lipids during conditions of elevated H_2O_2, the rat testis has several antioxidant enzymes that scavenge and metabolize these free radicals (Peltola et al., 1992;

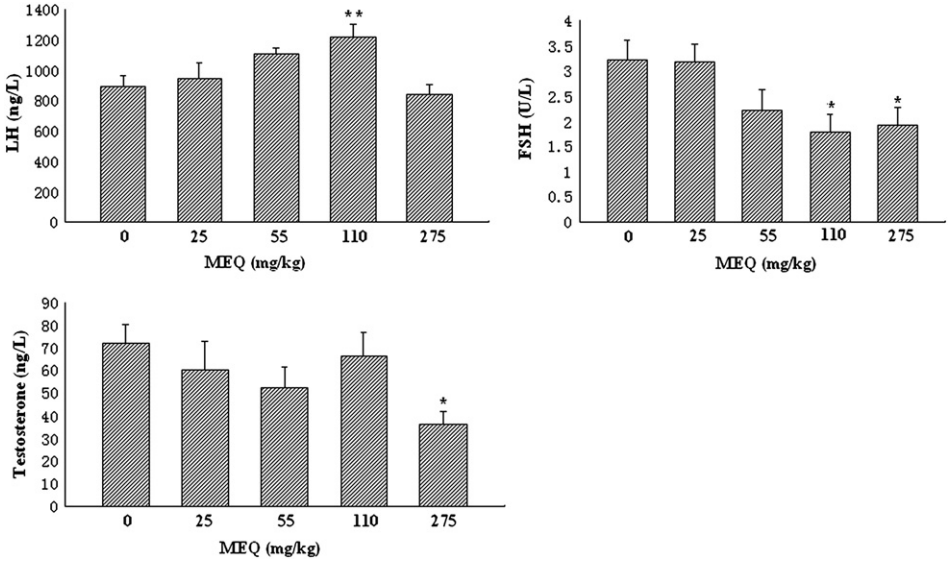

Fig. 4. Hormonal changes in different groups after 180 days MEQ diet.

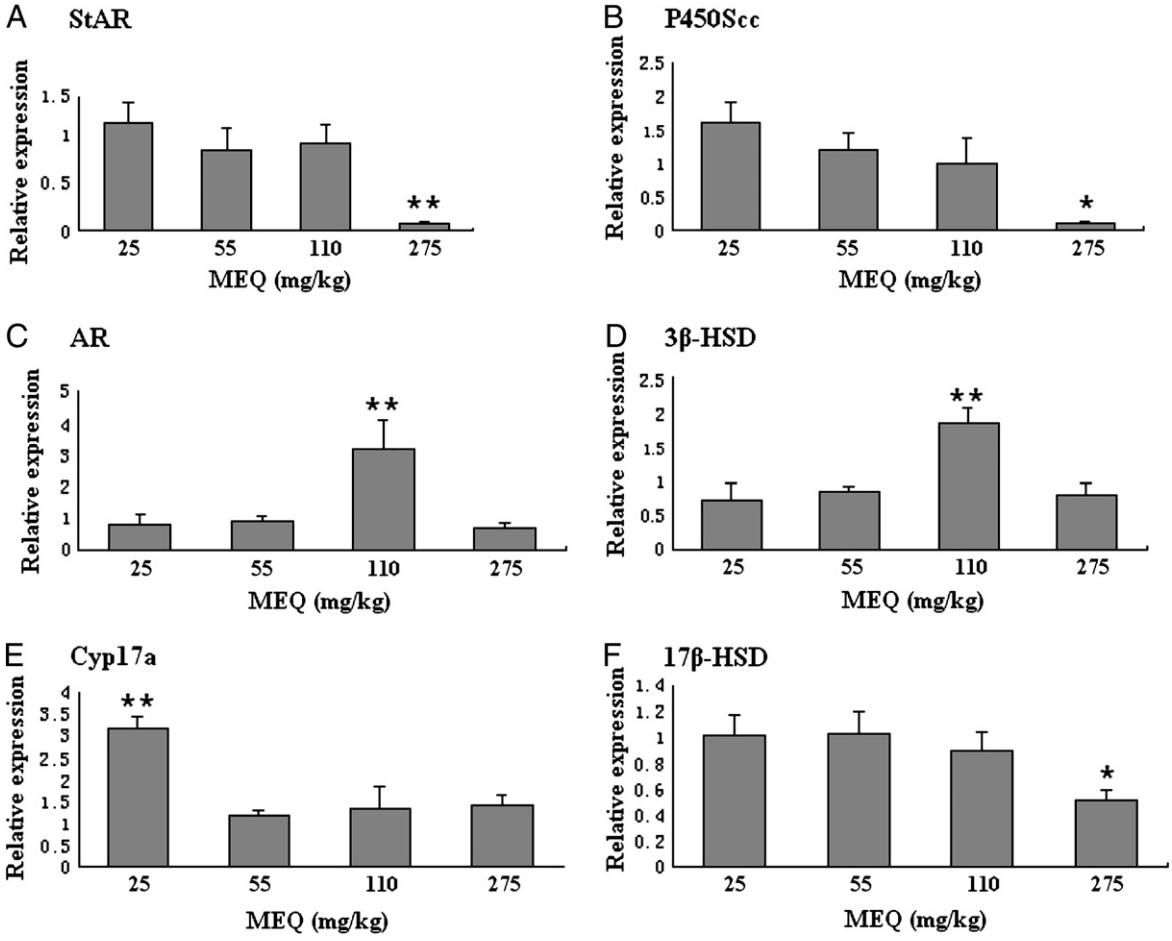

Fig. 5. Changes in gene expression for testosterone biosynthesis by MEQ diet after 180 days.

Sikka, 2004). Among the antioxidant defenses of testis, SOD is the first and most important line of defense. In mammalian testis, SOD plays a major role in male germ cell protection as well as differentiation (Bauche et al., 1994; Peltola et al., 1992). GSH is one of the most prominent antioxidant defense components in the testis (Ayer et al., in press). Besides serving as a substrate for glutathione-related enzymes such as GPx, GSH acts as a free radical scavenging ROS (Bauche et al., 1994; Doreswamy and Muralidhara, 2005; Gu and Hecht, 1996). Our findings showed an obvious increase of testicular antioxidant system. Induction of SOD and GSH levels in rat testis were correlated with increased MDA and 8-OHdG levels. This suggests a compensatory rise in antioxidant enzymes in response to H_2O_2 accumulation resulted from oxygen free radicals during progress of N→O group reduction (Huang et al., 2010; Kheradmand et al., 2009; Sikka, 2004).

Genes responsible for cholesterol transport and testosterone synthesis in rat testis include StAR, P450scc, 3β-HSD, CYP17a, and 17β-HSD (Shi et al., 2007). The level of testosterone in serum was significantly decreased to 50% of the control for the 275 mg/kg MEQ. In addition, adverse histopathological changes in the testis were also observed in male rats exposed to MEQ. These results differ from those obtained in another study, in which increased serum testosterone levels were observed in male swine treated with cyadox in the diet (Zhu et al., 2006). Moreover, an accompanying decline was also observed for mRNA levels of genes responsible for cholesterol transport and steroidogenesis, including StAR, P450scc, and 17β-HSD. The significantly decreased level of mRNA expression for StAR appeared to excess H_2O_2 generation and decreases testosterone synthesis (Diemer et al., 2003). Decreased expression of P450scc and 17β-HSD might be due to the reduced expression of StAR as inhibition of the expression of StAR gene results in the dramatic decrease in steroid synthesis and next intermediate may have been down-regulated (Fig. 6) (Clark et al., 1995; Stocco, 1998, 2001).

Testosterone is produced in the interstitial section of testis, under the influence of LH secreted from the pituitary gland (Nilsson, 2000; Tahka, 1989). In the current experiment, serum LH levels were significantly increased in the 110 mg/kg MEQ group. Interestingly, expression of AR and 3β-HSD in 110 mg/kg MEQ was also markedly increased. The reasons for these alterations in mRNA gene expression remain unclear. It might be the mutations in AR gene which resulted in hormonal imbalance and high levels of LH hormone at 110 mg/kg. Serum FSH levels were markedly decreased to 45% and 40% for male rats that had been exposed to 110 and 275 mg/kg MEQ, respectively. It is believed that FSH increases Sertoli cell synthesis of an androgen binding protein required for maintenance of high concentration of testosterone (Kackar et al., 1997). We assumed that MEQ might have an effect on the function of Sertoli cells and resulted in the decreased levels of FSH secretion. But additional studies are still required to clarify the influence of MEQ.

Expression of CYP17a in the 25 mg/kg MEQ group was markedly increased. We supposed that it might be an adoptive response of the body towards the MEQ (Huang et al., 2009). However, the reasons for these alterations in gene expression remain unknown. Additional studies are still needed to clarify its influence on low levels of MEQ on this gene.

In conclusion, our studies provide the first and direct evidence *in vivo* for the formation of free radicals during the progress through N→O group reduction. The present findings also reveal that relative high

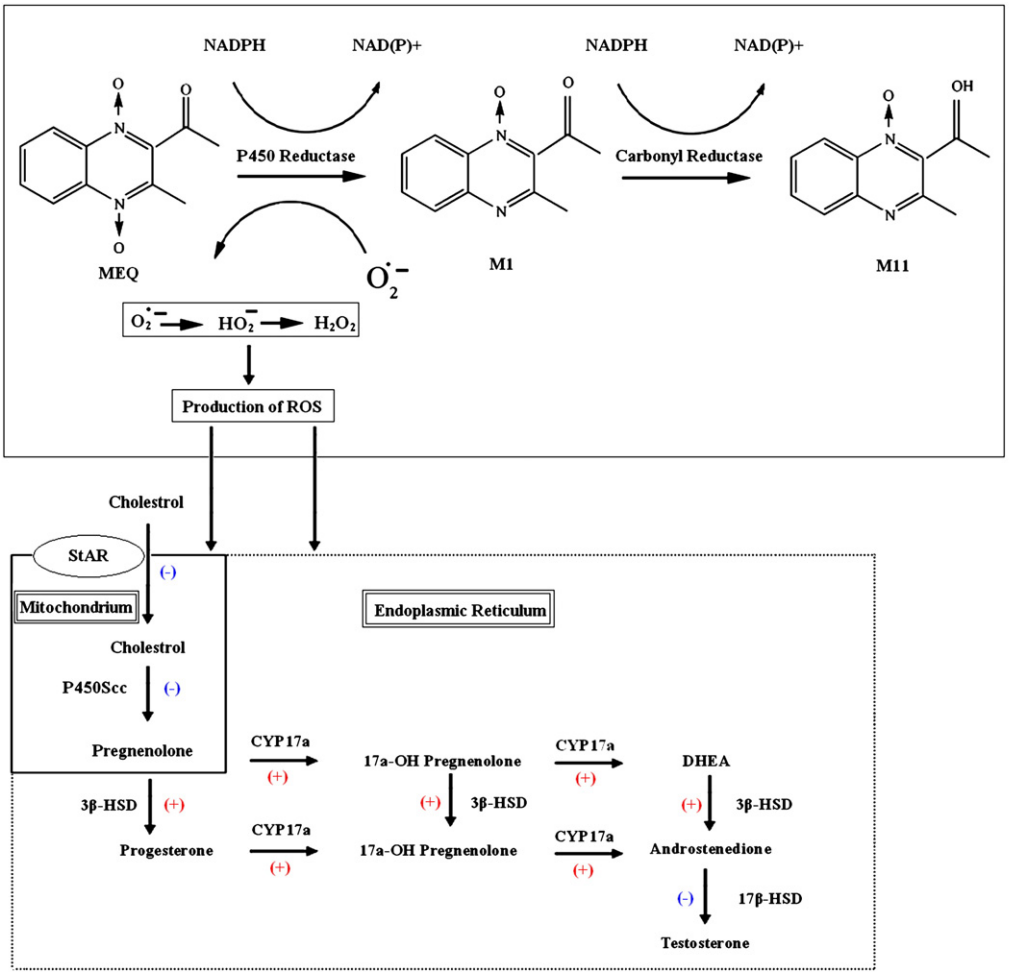

Fig. 6. Testosterone biosynthetic pathways and potential metabolism of MEQ in testicular cells. The schematic diagram shows the reactions leading from cholesterol to testosterone including the names of the corresponding enzymes and the organelles in which the reactions are carried out. (StAR): steroidogenic acute regulatory protein; (P450scc): cholesterol side-chain cleavage enzyme; (AR): androgen receptor; (3β-HSD): 3-beta-hydroxysteroid dehydrogenase; (CYP17a): cytochrome P450c17 subfamily a; (17β-HSD): 17-beta-xhydroxysteroid dehydrogenase; (+): up-regulated; (−): down-regulated; M1: 4-desoxymequindox; and M11: 2-isoethanol 4-deosymequindox.

levels of MEQ in diet can result in oxidative changes associate with DNA damage, disturb secretions of testosterone, LH and FSH with a concomitant reduction in expression of key genes responsible for cholesterol transport and steroidogenesis in male rats. Because these markers are important for male reproductive toxicity, disturb secretion of testosterone, oxidative stress and dramatic decrease expression of StAR, P450scc, and 17β-HSD genes could be the possible mechanism of male infertility related to MEQ treatment.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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