Evaluation of Morphological and Mitochondrial Alterations of Mouse Fetus after Exposure to Methyl tert-butyl Ether

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Abstract

Although the biokinetics, metabolism, and chemical toxicity of methyl tert-butyl ether are well known, little attention was paid to the potential toxic effects of MTBE on reproduction and development in mammals. To evaluate the effects of MTBE on pregnant animals, two groups (control and test) of NMRI mice were chosen. In test group 500 and 1000 mg/Kg of it were administered intraperitonealy at 11 days of gestation and in control group no injection was made. Caesarean section was performed at 15 days of the gestation, and the fetus and placentas were examined externally. Based on our morphological results, MTBE caused significant increase (p < 0.05) in the weight of fetuses and the weight of placentas, the diameter of placentas and crown-rump length of fetuses. Also, our mitochondrial results showed significant (p < 0.05) increase in mitochondrial swelling, ROS formation and also significant (p < 0.05) decreased in MMP on mitochondria isolated from liver and brain in test group. These results suggest that MTBE through ROS formation may induce the mitochondrial dysfunction which in turn leads to inhibition of angiogenesis and morphological alterations in fetus of mouse.

Key words: MTBE, Morphology, Mitochondria, Mouse Fetus, placenta, Embryotoxicity

1. Introduction

Methyl tert-butyl ether (MTBE) is a volatile, colorless, and flammable liquid that is moderately soluble in water [1, 2]. MTBE had been used in USA gasoline since 1979 at low levels to eliminate lead as an octane booster and oxygenate [3]. Although banned in the United States since 2001, it remained a major environmental problem due to its remnants in groundwater [4]. Most of the people in the developing countries are exposed to MTBE from gasoline while fueling their cars or from the auto exhaust when driving. MTBE is also used in some medical procedures [5, 6].

No reproductive and developmental toxicity of MTBE in human isinvestigated in previous reports. There are limited numbers of animal developmental and reproductive toxicity studies and in all of them the inhalation route of exposure is used. Some information on

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reproductive organs also obtained from sub-chronic and chronic toxicity studies, and there are a few recent studies of possibleendocrine effects. While no effect on fertility endpoints is reported; these studies provide evidence for adverse effects of MTBE on development. Dose-dependent effects on fetal weight and fetal skeletal variations arereported in mice; no fetal effects are reported in the rats and rabbits [7]. Notably, developmental toxicity study in rat is conducted in a lower concentration range [8]. In rabbits, maternal toxicity is reported at the higher concentration (8,000 ppm) as reduced maternal food intake, maternal weight loss, hypoactivity, and ataxia during exposure and increased relative liver weights at term. However, no fetal effects related to exposure are reported in rabbits [9].

Due to the widespread use of gasoline as a fuel, contact with MTBE in various ways has been increased. Althoughthe biokinetics, metabolism, and chemical toxicity of MTBE on brain, liver and other organs, are well established, there is little data regarding the developmental toxicity associated with MTBE. In this study we proposedto study themorphological and mitochondrial alterations of mouse fetus after exposure with MTBE to explore the probable cellular and biochemical mechanisms.

2. Materials and Methods

2.1. Compounds

D-mannitol, sucrose, EDTA, Tris buffer, Coomassie blue, Mops, EGTA, $MgCl_2$, $CaCl_2$, KH_2PO_4, sodium succinate, KCl, HEPES, Rotenone, Rhodamine 123, cyclosporine A, butylated hydroxyl toluene, NaOH, methanol, malate, pyruvate, DMSO, chloroform, Dichlorofluorescein, di-Sodium hydrogen phosphate, Methyl tert-butyl ether, alcohol, toluene, paraffin with a melting point of 60-56 °C,antler glue, formalin, xylene, glacial acetic acid, picric acid, bouin's fixative solution, Haupt's gelatin, haematoxylin dye, eosin powder, gelatin, and ether, were purchased from Merck KGaA, (Darmstadt, Germany). All other chemicals were of the highest commercial grade available. Normal saline and distilled water were offered as a generous gift by DarooPakhsh Co. Ltd. Tehran, Iran.

2.2. Animals

Mice with NMRI race used in this examination were purchased from Pasteur Institute of Iran All mice were settled in a room at a constant temperature of 25 ºC on a 12/12 hr light/dark cycle with food and water available. All experiments were performed according to ethical protocols and standards approved by the Committee of Animal

Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran. In this project following groups were considered and studied; control, 500 mg/kg and 1000 mg/kg groups.

2.3. Study Design

The mice were anesthetized at 15th day of gestation. After laparotomy, the uterus wasexternalized and the number and location of embryos and resorption were noted, then theweight of placenta (measured by digital balance), the weight of fetus and mother, size of thefetus by measuring C-R (Crown-Rump Length) and placenta diameter (measured microscopically by caliper). Then embryos were examined carefully for external abnormalities and afterwards, the sections (10 micrometer) of dissected embryos were stained by the haematoxylin-eosin (H & E) method and investigatedby stereomicroscope for skeletal defects. The incidence of skeletal defects and otherhistological lesions were determined and compared in the groups.

2.4. Isolation of Embryos Mitochondria

We detached embryos from mice uterus and then we isolated the liver and brain. The tissues were separately ground and homogenized with a glass hand-held homogenizer in ice-cold mitochondrial isolation medium (225 mM D-mannitol, 75 mM sucrose, and 0.2 mM EDTA, pH was set to 7.4). Mitochondria were isolated according to the procedure as reported by Pourahmad et al. The protein concentration was measured using Bradford method [10].

2.5. Quantification of Mitochondrial ROS Level

Isolated mitochondria were suspended in respiration buffer (10 mM Tris, 0.32 mM sucrose, 5 mM sodium succinate, 50 μM EGTA, 0.1 mM KH2PO4, 20 mM Mops and 0.5 mM MgCl2). The amount of mitochondrial H2O2 production was evaluated by Schimadzou RF-2500 fluorescence spectrophotometer (Japan) using DCFH-DA (final concentration, 10 μM). The excitation wavelength was 485 and the emission wavelength was 530 nm [11].

2.6. Determination of Mitochondrial Membrane

Rhodamine 123 was used as a cationic fluorescent dye to determine the mitochondrial membrane potential. Briefly, after isolation of mitochondria, 10 μM of rhodamine123 was added to the mitochondrial MMP buffer (68 mM mannitol, 5mM sodium succinate, 220 mM sucrose10mM KCl, 2 μM Rotenone, 2 mM MgCl2, 10 mM HEPES, 5 mM KH2PO4, 50 μM EGTA). The fluorescence was measured by Schimadzou RF-5000U fluorescence spectrophotometer (Japan) at the excitation and emission wavelength of 490 and 535nm, respectively [12].

2.7. Determination of Mitochondrial Swelling in Isolated Mitochondria

The mitochondrial swelling, as a result of colloidal osmotic effects of solute flux in and out of the mitochondrial matrix, was measured by monitoring the absorbance at 540 nm (A540) as described by Salimi et al. Briefly,

after incubating mitochondrial suspensions in swelling buffer (3 mM HEPES, 70 mM sucrose, 2 mM trisphosphate, 230 mM mannitol, 1 μM rotenone, 5mM succinate), the absorbance was measured at 540 nm for 60 min with an ELISA reader (Tecan). A reduction in the absorbance indicates an increase in mitochondrial swelling [13].

2.8. Statistical Analysis

Statistical significance between groups was verified using GraphPad prism program and compared by one-way analysis of variance (ANOVA). Chi-square test was used for binomial

data. The minimum level of significance was p < 0.05.

3. Results and Discussion

3.1. Histological Results

The photomicrographs of the H & E stained sections of a normal and MTBE treated mouse fetus was taken which some of them are displayed in figure 1. In the morphological and microscopic point of view, there were important findings in MTBE affected test groups were; necrosis, hyperemia and increased liver size (Figure 1 A). Brains of fetuses in MTBE affected test group were also larger than what was seen in the control group. Big size of telencephalon in the brain means failure to normal reduction of its compared to that of untreated safety control fetuses (Figure 1 B), occurrence of umbilical hernia (an obvious fetal damage) in dose of 500mg/kg (Figure 1 C), expansion of alveoli (Figure 1 D) and placental hyperemia (Figure 1 E-F) are

other signs of embryotoxicity in MTBE affected fetuses

3.1.1. Fetal Body Weight

The total weight of embryos in MTBE affected test groups were significantly (P < 0.05) higher than those of the control group (Figure 2 A).

3.1.2. Placental Weight

The weight of placenta in MTBE affected test groups were significantly (P < 0.05) higher than those of the control group (Figure 2 B).

3.1.3. Diameter of Placenta

The placenta diameter in MTBE affected test group seemed to be decreased at the dose of 500mg/kg but this decrease was not significant (p > 0.05) compared to thecontrol group. However, at the dose of 1000mg/kg,the placenta diameter in MTBE affected test group was significantly larger than the control group (Figure 2 C).
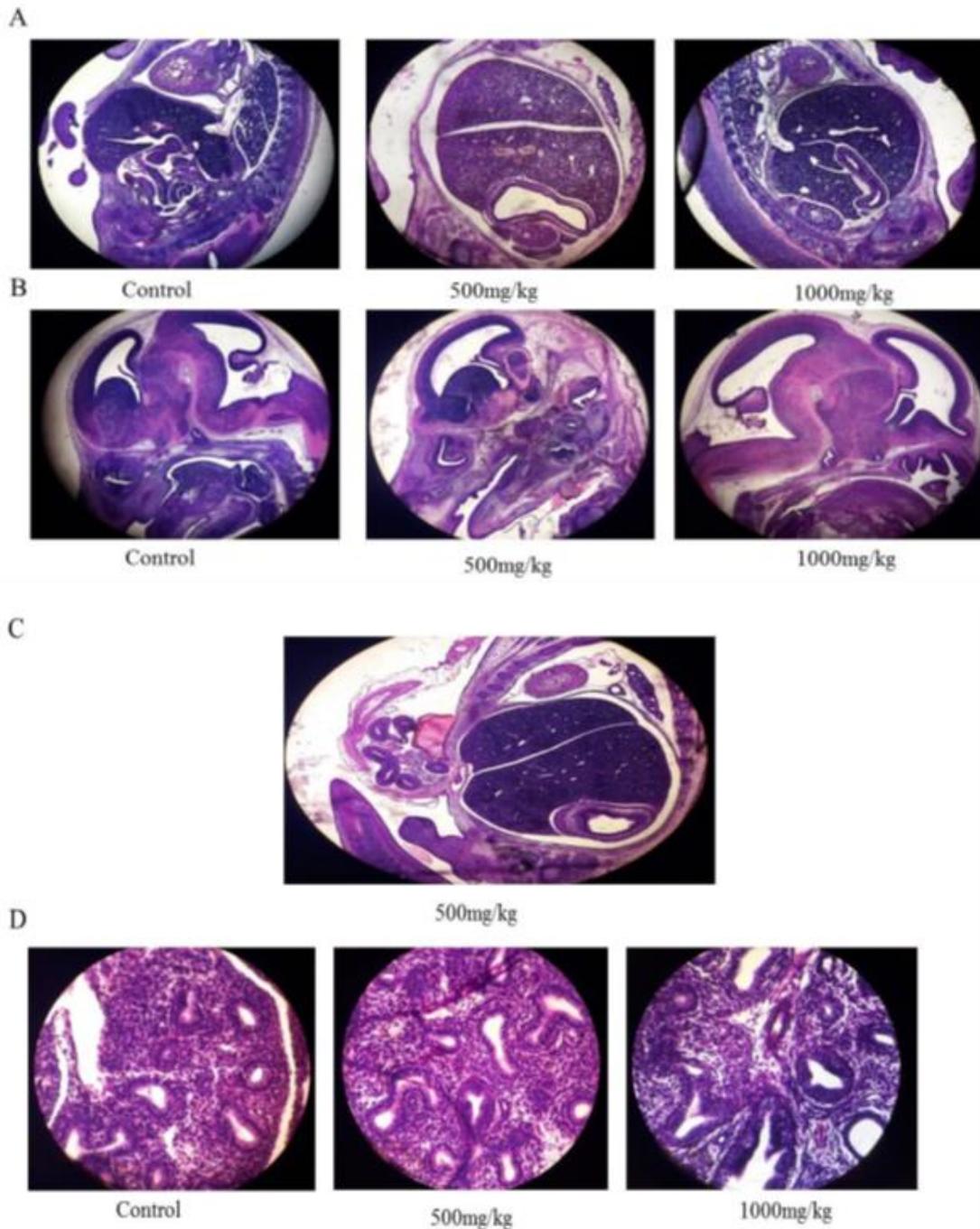
3.1.4. Length of Fetuses (Stature of Fetus)

The length of fetuses in MTBE affected test group at the dose of 500mg/kg was significantly (P<0.05) longer than the length of fetuses in the control group. However, at the higher dose of 1000mg/kg, the length of fetuses in MTBE affected test group seemed to be shorter than that of control group but this difference was not significant (p > 0.05). (Figure 2 D).
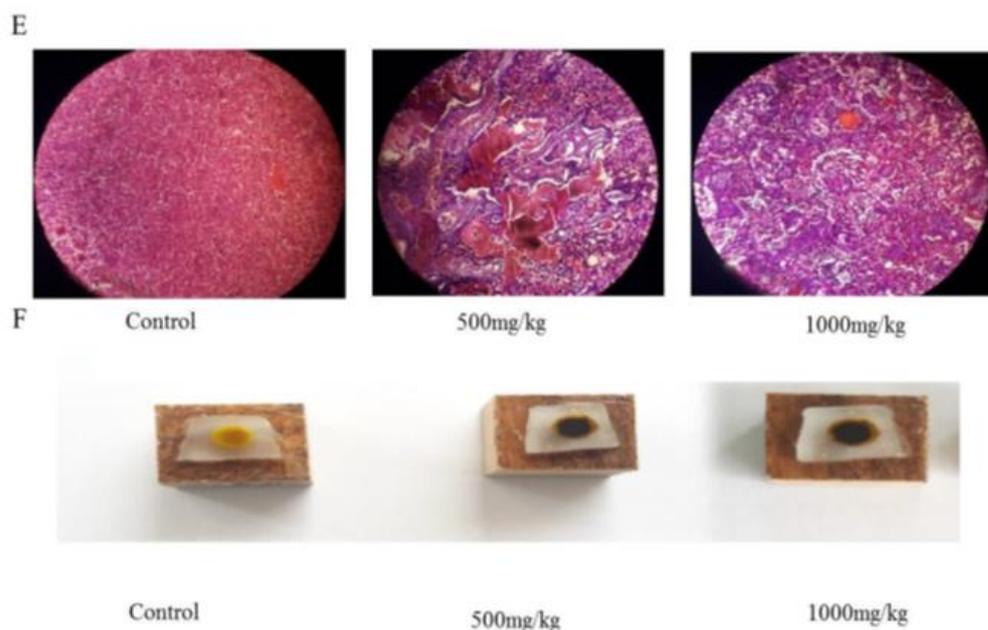
3.2. Mitochondrial Results

3.2.1. ROS Formation Assay

As shown in Figure, maternal MTBE (500, 1000mg/kg) exposure induced significant (p<0.05) increase in ROS (H2O2) formation on isolated mitochondria obtained from both liver (Figure 3 A) and brain (Figure 3 B) tissues in a concentration dependent mannercompared to the control group.

A

| Control | 500mg/kg | 1000mg/kg |

B

| Control | 500mg/kg | 1000mg/kg |

C

500mg/kg

D

| Control | 500mg/kg | 1000mg/kg |

Figure 1. morphological alterations in the liver of foetuses (A), the brain of foetuses (B) the umbilical hernia of fetus (C), the alveolar space of fetuses (D) and the placenta of mice. The A indicates necrosis, hyperemia and increased liver size in test groups (500 and 1000 mg/kg) compared control group. B shows that brains of fetuses in test groups were larger than control group. C indicates occurrence of umbilical hernia in dose of 500mg/kg. D showed the expansion of alveoli in test groups compared to control group. Finally, E and E show the alteration in placenta and placental hyperemia in test and control groups. The number in each group are 10 mice.

The magnification of pictures for A, B and E are 10 and for C and D are 40.

3.2.2. MMP Assay

The uptake of the cationic fluorescent dye, rhodamine 123, has been used for the measurement of mitochondrial membrane potential collapse. As shown in Figure 8, maternal MTBE (500, 1000mg/kg) exposure significantly decreased the MMP in a concentration and time-dependent manner in mitochondria obtained from liver and brain ($P < 0.005$) tissues compared to the control group (Figure 3 C and D).

3.2.3. Mitochondrial Swelling

The decreased absorbance at 540 nm (A540) was used as an indicator of mitochondrial swelling assay which is a criterion of mitochondrial membrane permeability. maternal MTBE (500, 1000mg/kg) treatment significantly ($P < 0.005$) increased mitochondrial swelling in isolated mitochondrial suspensions of liver and brain in a concentration-dependent manner compared to the control group (Figure 3 E and F).

3.3. Discussion

It is estimated that approximately 10–15% of congenital structural anomalies are the result of the adverse effect of environmental factors [14]. This means that approximately 1 in 250 newborn infants suffer structural
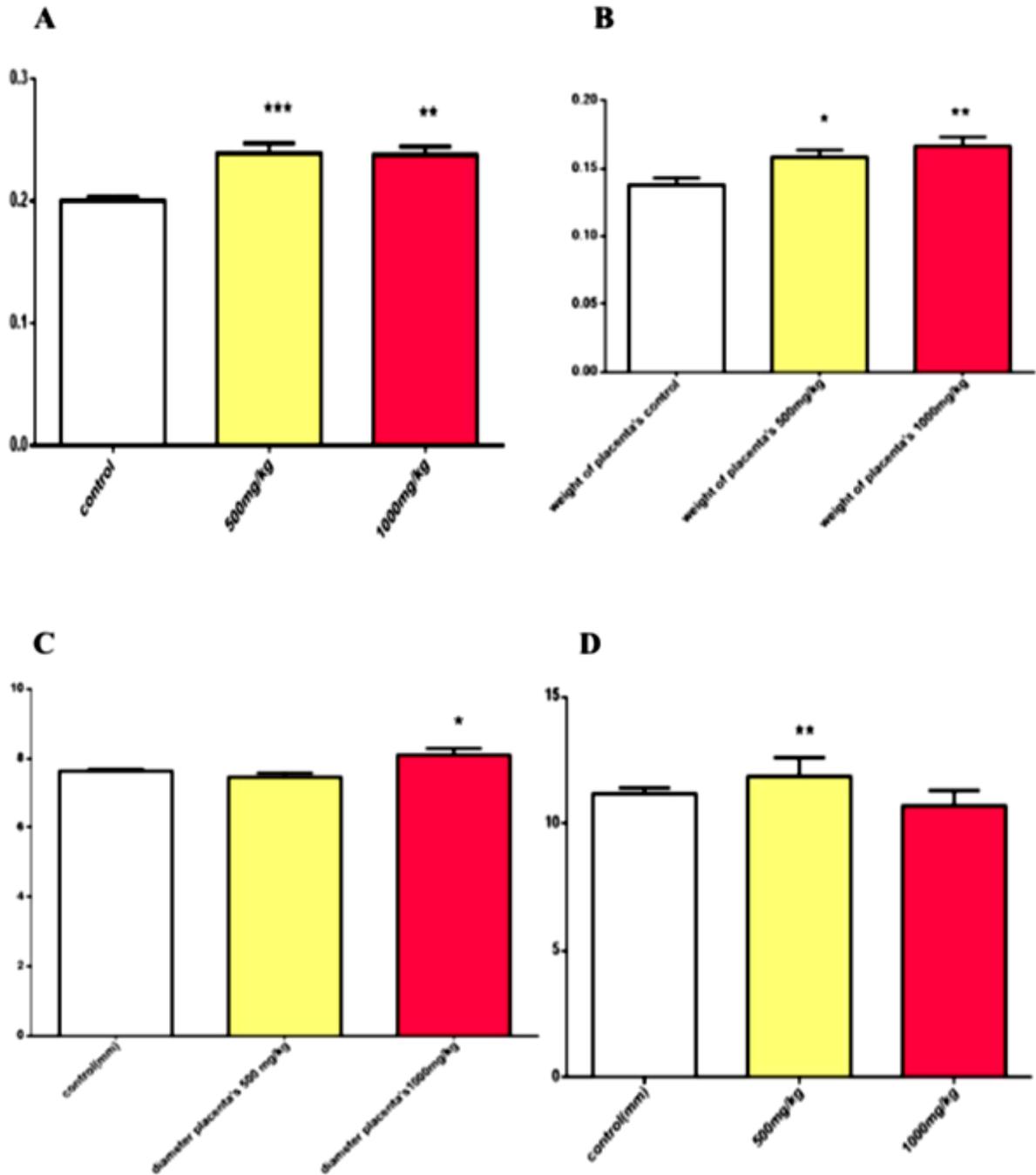
Figure 2. The effect of MTBE on fetal body weight (A), placental weight (B), diameter of placenta (C) and length of fetuses (D). A shows that MTBE treatment increased fetal body weight (g) in test group compared control group. B shows that significantly increased placental weight (g) in test group compared control group. C indicates the changes of diameter of placenta (mm) in test groups compared to control. D showed the change of length of fetuses (mm) in test groups compared to control.

** indicates significance with control group.
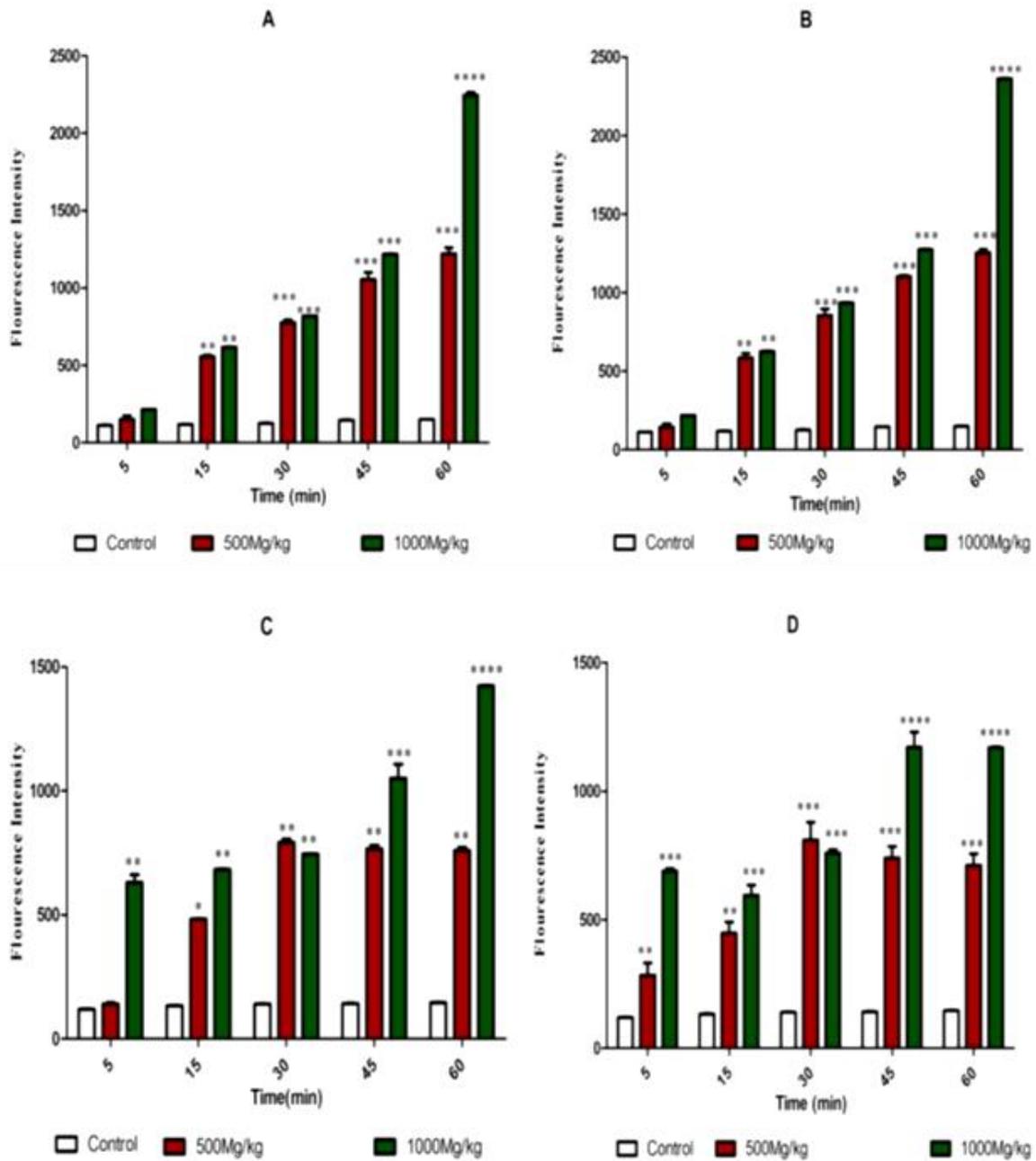
Figure 3. Mitochondrial alterations in isolated mitochondria obtained from liver and brain of fetus. A and B show that ROS formation significantly increase in isolated mitochondria obtained from test groups compared to control group in both organs. Also, C and D show that MMP collapse significantly increase in isolated mitochondria obtained from test groups compared to control group in both organs. E and F indicate that mitochondrial swelling occurs in both organs in test groups in comparison with control group.

defects caused by an environmental exposure [15]. MTBE has been shown to disrupt normal angiogenesis in two embryonic piscine models [16]. Zebra fish embryos exposed to MTBE were shown to exhibit a dose dependent increase in vascular lesions, including pooled blood in the common cardinal vein, cranial hemorrhages, and abnormal intersegmental

vessel formation [17]. These data suggest that MTBE specifically targets the developing vasculature in zebrafish embryos. In order to study the effect of MTBE on prenatal development in mice as sensitive mammals species we decided to design the current research. According to another study, fetal body weight was reduced following the exposure to MTBE in mice. In this study, authors also reported that there was a significant increase in incidence of cleft palate and pooled external and visceral malformations and reduced relative maternal liver weights [8, 9]. However, there is another study by Conaway et al that mentioned no significant effects on external or soft-tissue or skeletal abnormalities in MTBE affected pregnant mice [18]. In our study we observed morphological changes in fetuses of mice compared to the control group (Figure 1).

One study performed by Kozlosky et al in 2013, showed that MTBE acts as an anti-angiogenesis in both in vitro and in vivo mammalian model systems 16. Angiogenesis is an energy consuming process, requiring endothelial cells to switch from a quiescent state to a migratory state for the formation of new blood vessels [19]. Angiogenesis participates in a wide range of ovulatory-related and non-ovulatory-related reproductive processes. Endothelial mitochondria have emerged as signaling hubs that modulate a wide range of endothelial functions, including angiogenesis, by coordinating reactive oxygen species formation, calcium signaling, metabolism and apoptosis. In the context of pregnancy, mitochondrial dysfunction has been associated with increased rates of preterm delivery, stillbirth, intrauterine growth restriction (IUGR), and sudden infant death [20]. In this work, we showed that prenatal exposure to MTBE induces mitochondrial alterations in fetus of mouse (Figure 3). This is another evidence to previous studies suggesting that MTBE induces mitochondria damages [21, 22].

Proper placental development and function are central to the health of both the fetus and the mother during pregnancy. Poor vascularization of the placenta can lead to preeclampsia, fetal growth restriction, and in some cases fetal death [23]. One recent study discussed how oxidative stress has been associated with aberrant angiogenesis and placental dysfunction resulting in adverse pregnancy outcomes [24]. In this study, we showed that mitochondrial ROS formation increases significantly compared to the control group that is associated with placenta alteration.

Finally, our results in this study suggested that MTBE through ROS formation may induce mitochondrial dysfunction which in turn leads to inhibition of angiogenesis and morphological alterations.

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