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The protective effect of *Zataria Multiflora* on the embryotoxicity induced by bisphenol A in the brain of chicken embryos

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ABSTRACT

Bisphenol A (BPA), is one of the main industrial chemicals synthesized for various purposes. In the present study, the brain tissue of chicken embryos was used to evaluate the embryotoxic effects of BPA and also the preventive effects of Zataria multiflora. To that end, fertile eggs were categorized into four groups (n=10). The eggs air sacs of the experimental groups were injected BPA (200 ppm) after four days.. The Zataria multiflora (100 and 200 µg/egg) was administered into chick embryos 6 hours prior to administration of BPA. The control group simply was treated with olive oil. The eggs were incubated at 37°C and a humidity of 63%. After 20 days, the embryos were beheaded and the brains were gathered for biochemical measurements. The findings indicated that *Zataria multiflora* (200 µg/egg) significantly reversed the increased levels of MDA ($p<0.05$) and protein ($p<0.001$) in the brain of BPA-exposed group and also the decreased levels of total antioxidant and GSH as well as the CAT and SOD activities in the brain of BPA-exposed group. *Zataria multiflora* reversed the toxic effects of BPA on the embryonic development stages of the brain via modulating oxidative stress.

Keywords: *Zataria multiflora, bisphenol A, brain, chicken embryo, oxidative stress.*

1. INTRODUCTION

Chemical toxicants are recognized as major risk factors for the embryo's development and pregnancy [1]. Various toxic agents pose a major hazard to fetuses during development. Several studies have revealed the traces of chemicals in pregnant women, although some of them have been banned since the 1970s [1]. Chemical toxicants can cause structural and functional deficiencies, growth abnormalities, or fetus death [1]. Bisphenol A (BPA) is considered an environmental danger for human health that widely contaminates the environment [2]. BPA is applied in the resins and plastics production and has been suggested to be a leading warning to the primary periods of mammalian life [2]. As BPA penetrates into the placental barriers and blood-brain ; this agent is a serious threat to the developing nervous system in embryos and infants [3]. In the case of oral exposure, the proliferation of the cell cycle will be interrupted in the neural progenitor cells (IPCs) and neurogenesis in developing neocortex [4]. Furthermore, the BPA exposure to during prenatal and neonatal stages caused memory impairment, cognitive impairment, and sexual differentiation disorders in the infants of examined animals [5]. It has been proposed that exposure to BPA may interrupt brain development by increasing estrogen accumulation in the brain which inhibits the expressions of N-methyl-D-aspartate receptor (NMDAR)and estrogen receptor beta (ERbeta) [6] BPA may also affect ERs expression in the brain without a direct effect on serotonin neurons has also been indicated [6]. But the chronic exposure to BPA during the prenatal or neonatal period decreased tyrosine hydroxylase immunoreactive neurons only in the substantia nigra of female mice [7]. Several investigations have shown that BPA induced embryotoxicity via inducing oxidative stress [8,9]. Strong evidence indicated that antioxidants prevented embryo toxicity induced by oxidative stress [10]. Today, much attention is paid to the medical effects of an ancient medicinal plant, *Zataria multiflora*, and its main ingredients [11]. *Zataria multiflora* belongs to the family called *Lamiaceae* which wildly grows in Pakistan, Afghanistan, and Iran [12]. *Zataria multiflora* (in Persian called Avishan-e-Shirazi) is used as a flavoring agent in many foods [12]. It was indicated that *Zataria multiflora* possesses several beneficial effects including antioxidant, antimicrobial, antiseptic, anti-inflammation, anti-cancer and immunomodulation [13,14]. However, there is not strong enough evidence on the relationship between the impacts of *Zataria multiflora* on the embryotoxicity. Thus, the present study aims to investigate *Zataria multiflora* effects on embryotoxicity induced by BPA by measuring oxidative stress indices in the embryos brain of chicken.

2. MATERIALS AND METHODS

Chemicals. All chemicals and kits were supplied from Sigma Company, USA.

Preparation of extract. *Zataria multiflora* was gathered from pastures of Fars province, Iran, and its authenticity was confirmed

by botanists in the herbarium of the Ferdowsi University of Mashhad. One hundred grams of the plant was pulverized and the resulted powder was soaked in 300 mL of 80% ethanol (Merck) and incubated for 48 h. Then the extract was filtered and dried in a shaker incubator at 40 °C. The dried extracts were stored at 4 °C**.**

Experimental design.

Previous studies have been agreed to the present protocol applied in embryotoxicity of chicken [15]. The procedure consist of an affordable and rapid toxicity test with high sensitivity to reveal the effects of chemical substances on teratogenicity, growth restriction, embryonic lethality, metabolism and also immunopathological impact and systemic toxicity [16]. The eggs were selected which fertilized. They were supplied from Iran Farm, Khorasan Razavi, Iran on the second day of hatch. After dissolving BPA powder in olive oil it was injected into the eggs on the fourth day. BPA (200 ppm) concentration was administered within yolks of egg whenever the control eggs injected olive oil. To study embryo protective effect of *Zataria* against BPA induced toxicity in chick embryos experimental set up was divided into four groups of ten embryos: group 1: control (olive oil), group 2: BPA (200 ppm), group 3: (100 µg of extract/egg+ 200 ppm of BPA), group 4: (200 µg of extract/egg+ 200 ppm of BPA). The extract was administered into chick embryos 6 hours prior to administration of BPA. The eggs were then incubated at 37°C. After 20 days of incubation, the embryos were beheaded and the embryo brains were gathered for biochemical measurements.

Measurement of total antioxidant activity. To evaluate total antioxidant activity, Ferric reducing ability of serum (FRAP) assay was used. In order to reduce Fe3+ to Fe2+, the antioxidant capacity of blood serum was evaluated by assessing the amount of blood serum. A combination of Fe2+ and 2,4,6-tripyridyl-s-triazine (TPTZ) causes in blue color with a highest absorbance at 593 nm. **Measurement of lipid peroxidation.** In order to measure lipid peroxidation, MDA levels were measured. In the presence of thiobarbituric acid (TBA), MDA reacts as thiobarbituric acid reactive substance (TBARS) which leads to the production of a red colored complex that its peak absorbance will be at 532nm. After adding 1ml TBA (0.6%) and 3 ml phosphoric acid (1%) to 0.5 ml of brain homogenate in a centrifuge tube. In a boiling water bath, the combination for 45 min was heated . N-butanol (4 ml) was applied to the combination after cooling and using a vortex mixer it was mixed for 1 min. The mixture then was centrifuged at 20000 rpm for 20 min. After transferring the organic layer to a fresh, the absorbance was assessed at 532 nm and the results were compared with values obtained from MDA standards. The data was demonstrated as nmol/mg protein [15].

Measurement of GSH. After mixing the samples with 20% trichloroacetic acid they were centrifuged. The ratios of supernatant and Tris were 4:3. Next, after adding 1mM DTNB to the mixture, it was incubated for 30 minutes. The absorbance was measured at 412 nm [16].

Measurement of enzymes. Marklund and Marklund 1979 method was used to measure SOD activity; applying pyrogallol autoxidation inhibition at pH8. The units per mg protein per minute is considered for the specific activity of SOD as.17 CAT activity was measured by H2O2 consumption, using the method of Aebi's and modified by Pieper et al. (1995) [18].

Measurement of protein content. Using the Bradford method, and taking Bovine serum albumin (BSA) as control [19], the protein content was measured.

Statistical analysis. All evaluations were done in duplicate. One-way analysis of variance (ANOVA) and Dunnett's *post-h*oc test was used to compare the experimental groups against the control group. InStat 3.0 program was also used for statistical analysis. The results are considered as mean ± SEM. The results were based on individual brain analysis. Then, Linear correlation tests were conducted, which showed a significant difference at$p<0.05$.

3. RESULTS

The antioxidant levels in the brain of BPA-exposed group versus the control group showed a significant difference ($p<0.001$) (Figure 1). The levels of MDA were higher in the brain of BPA-exposed group than the control group ($p<0.01$) (Figure 2). The brain levels of GSH in of BPA-exposed group were significantly lower than the control group ($p<0.01$) (Figure 2). The CAT and SOD activities significantly increased in the BPA-exposed group compared with the control group ($p<0.01$) (Figure 3). Protein concentration in the BPA-exposed group was significantly higher than control ($p<0.001$) (Figure 4). However, extract treatment (200 µg/egg) significantly decreased the levels of MDA and protein in the brain of BPA-exposed group ($p<0.05$, $p<0.001$, respectively). In addition, extract administration significantly increased the GSH and total antioxidant levels (($p<0.01$, $p<0.05$, respectively) as well as the CAT and SOD activities ($p<0.05$) in the brain of BPA-exposed group (Figure 1-4).

Discussion. Oxidative stress has vital role in the pathophysiology of many complications of human pregnancy and this subject has now become a major focus of investigations [20].

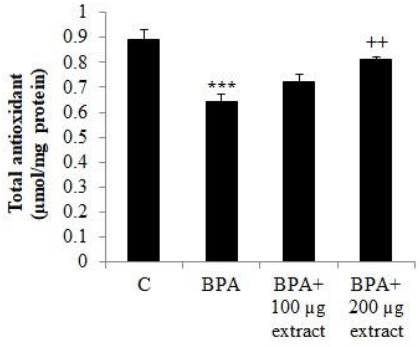

Figure 1. Total antioxidant the brain of the control group, BPA(200 ppm)-exposed group, two treatment groups exposed to BPA (200 ppm) which received *Zataria multiflora* (100 and 200 µg) (n=10, for each group).

Values are expressed as the mean ± SEM.

Significant difference between the control group vs BPA group: ***; $p<0.001$.

Significant difference between the two treatment groups vs BPA-exposed group: ++; $p<0.01$.

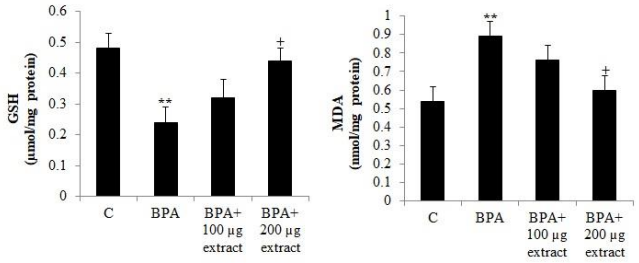

Figure 2. GSH and MDA levels in the brain of the control group, BPA(200 ppm)-exposed group, two treatment groups exposed to BPA (200 ppm) which received Zataria multiflora (100 and 200 µg) (n=10, for each group).

Values are expressed as the mean ± SEM.

Statistical significance for the difference between the control group vs BPA group: **; p <0.01.

Statistical significance for the difference between the two treatment groups vs BPA-exposed group: +; p <0.05.

We indicated the BPA exposure induces a remakable increment in the levels of MDA and total protein as well as a decrease in the levels of total antioxidant, GSH, SOD and CAT in the experimental group versus the control group. However, *Zataria multiflora* ameliorated these changes in the brain of BPA-exposed group.

Several scientific works of the literature indicated that teratogen agents disturb the developing embryo by inducing oxidative stress and result in severe embryonic injuries. According to the literature, the reason the embryos have a weak antioxidant defense system especially at the early stages of organogenesis [21]. Similarly, Lin and colleagues showed BPA raised dose-dependently the intracellular ROS generation and significantly decreased the levels of SOD and GSH and also increased MDA in the dopaminergic neuronal cells of 14 to 15-days-old SD embryos. They suggested that oxidative stress might play a critical role in neurotoxicity induced by BPA [22]. It has also been indicated that increased MDA levels in the brain of fetus or infant exposed to BPA during embryonic stages caused underdevelopment of the brain [23]. One of the basic mechanisms of BPA toxicity, in animal models, is oxidative stress. Kabuto and colleagues indicated that BPA administration was not changed the MDA levels in the mouse tissues. However, the CAT and SOD activities decreased after BPA administration. Another study has indicated that BPA increased MDA levels and also decreased the levels of GSH in the male rats brain [24]. Study on the BPA impact on oxidative stress and cognitive functions in rat brain has indicated that BPA leads to an increase in MDA level and a decrease in GSH level in the rat brain. In addition, the study indicated that the co-administration of N-acetylcysteine antioxidant ameliorated these changes [25]. The current study confirmed that *Zataria multiflora, a natural* antioxidant, could ameliorate oxidative stress induced by BPA in the brain of embryos. Previous studies indicated that *Zataria multiflora* prevented diabetes, asthma, neurodegenerative disease, etc., due to its antioxidant activities [13, 26]. To sum up, the findings of the study show that *Zataria multiflora* acts as a protective agent against BPA-induced embryotoxicity by modulating oxidative stress.

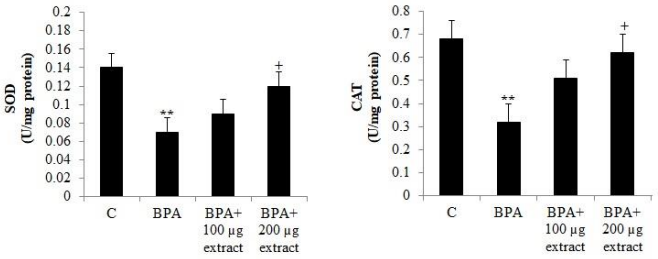

Figure 3. SOD and CAT in the brain of the control group, BPA(200 ppm)-exposed group, two treatment groups exposed to BPA (200 ppm) which received Zataria multiflora (100 and 200 µg) (n=10, for each group).

Values are expressed as the mean ± SEM.

Significant difference between the control group vs BPA group: **; p <0.01.

Significant difference between the two treatment groups vs BPA-exposed group: +; p <0.05.

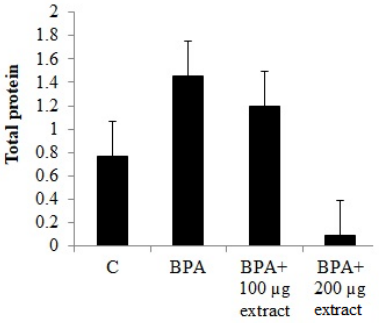

Figure 4. Total protein in the brain of the control group, BPA(200 ppm)-exposed group, two treatment groups exposed to BPA (200 ppm) which received Zataria multiflora (100 and 200 µg) (n=10, for each group).

Values are expressed as the mean ± SEM.

4. CONCLUSIONS

The present study indicated that *Zataria multiflora* could prevent BPA-induced brain damage by modulating oxidative stress in the brain of chick embryo. *Zataria multiflora* may be act as antidote against embryotoxic agents.

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6. ACKNOWLEDGEMENTS

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