**Fetal Malformations Due to Long Term Consumption of Sodium Benzoate in Pregnant Balb/c Mice**

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**ABSTRACT**

**Background:** Benzoic acid and its salts such as sodium benzoate (SB) are used as disinfectants and preservatives in numerous foods, pharmaceutical and cosmetic products. The aim of this study was to evaluate the teratogenic effects of SB on the skeletal system of Balb/c mice during pregnancy.

**Materials and Methods:** Thirty female Balb/c mice were divided into 3 groups, two experimental (I, II) that received daily intraperitoneal injection of 280 or 560 mg/kg of SB, respectively and one control group which received normal saline. All injections were carried out starting 10 days before mating and 6th to 15th of gestational days (GDs). Dams underwent Cesarean section on GD 18 and then morphological studies were done on the offspring’s. All malformed fetuses were stained with alizarin red S and alician blue and assessed with stereomicroscope. Data were analyzed by Anova, Tukey and Mann-Whitney U tests and using version 16 of SPSS software and differences less than 0.05 were considered significant.

**Results:** Various anomalies were detected in fetuses of experimental groups such as: severe skin hemorrhage, craniofacial deformities, vertebral column defects like scoliosis, limbs defects and neural tube defects.

**Discussion:** This study revealed that administration of SB before and during pregnancy probably can induce several malformations in the fetuses. Therefore, until further studies, the use of SB-containing preparations might need to be limited in pregnant women.

**Keywords:** Sodium benzoate (SB), Fetal malformation, Food additives, Mice.
INTRODUCTION
Sodium benzoate (SB) is an aromatic hydrocarbon and acid benzoic salt has antimicrobial properties and widely used as a food preservative. This salt is added to cans, ketchup and some drinks and tooth pastes for preventing fermentation (Maki and Takeda 1985, de Mendonça et al., 2001a, 2003b). Inhaling SB might give rise to allergy in eyes, hives on the skin, and also asthma and coughing (Shtenberg and Ignat’ev, 1970). Studies on laboratory animals have implied that intake of SB has adverse effects on organs such as heart, spleen, kidney, brain, and liver and causes alterations in plasma ions, congenital malformation, vertebral column deformity (Shtenberg and Ignat’ev, 1970, Toyoda et al., 1983, Fujitani, 1993) and eye malformation in chicken embryos (Daston, 1995), and even death (Shtenberg and Ignat’ev, 1970, Toyoda et al., 1983, Fujitani, 1993). Effects of SB in chronic exposure with mammals were limited to reduced food intake and growth (Tamaro et al., 1986, Tamaro et al., 2010). It has also been reported that SB at concentrations below 100 pg/ml and exceeding 500 pg/ml inhibited intracellular protein and DNA synthesis (Yılmaz et al., 2008). It was also reported in the study conducted by Stenberg et al. (1970) on rats that long-run oral intake of SB as much as 1800 mg/kg leads to decline in weight of rats and their internal body organs. Many biological studies concerning SB-induced toxicities have been reported. For example, the protozoan Tetrahymena pyriformis as a toxicological model revealed that the treatment with SB caused a statistically significant increase in DNA content, suggesting stimulation of the mitotic process (Stefanidou et al., 2003). Also, Yang et al. (1996) stated that SB can cause changes in genes expression through releasing free radicals. Villanueva et al. (1994) reported that many aromatic compounds (including benzoates family) are extremely carcinogen and are metabolized by living micro-organisms, producing active compounds which react with cellular DNA and alter cellular structure. Declining fetal body weight, increase in intraterine death, and generating fetal abnormalities such as dilated renal pelvis, skeletal variations and retarded ossification were observed in pregnant rats which were injected intraperitoneally with 100–1000 mg/kg of SB (Minor and Becker, 1971). Since the use of food additives has increased enormously in the last few decades, the purpose of this study was therefore to specifically evaluate the potential teratogenic effects of the food preservative SB on the skeletal system of Balb/c mice during embryonic development.

MATERIALS AND METHODS
Animals
Virgin female Balb/c mice, weighting 28-30 gram (8-9 weeks old) were used in this study. The animals were kept in a climate-controlled room under a 12 h alternating light/dark cycle (9.00- 21.00 h light), 20.1 to 21.2°C temperature and 50 to 55.5 % relative humidity. Dry food pellets and water were provided ad libitum. For mating, three females were caged with a male of the same strain overnight and the presence of vaginal plug in the following morning was considered as GD0. Maternal weights were measured throughout the experiment.

Treatment
Thirty pregnant mice were randomly divided into 3 groups (10 mice in each group). Two experimental groups I, and II received daily intraperitoneal injections of 280 or 560 mg/kg/day of SB, respectively. The control group received normal saline. All injections were done starting 10 days before mating and on 6th to 15th days of gestation. There are few studies evaluating the teratogenic effects of SB and most experiments were carried out in rats (Fujitani 1993, Oyanagi 1987). In this study, we selected nearly half of the doses that were used in the previous studies. SB powder was obtained from Sobhan Darou Pharmaceutical Company in Tehran, Iran. Approval for this study was gained from the Animal Care and Ethics Committee of Birjand University of Medical Sciences.

Fetal assessment
On GD18, pregnant mice were sacrificed under ether anesthesia and each uterus was opened and the umbilical cord cut close to the fetus. The number of dead and living fetuses and absorbed embryos were counted; then, their weight (by Sartarious PT210, Switzerland) and crown-rump length (C-R-L) by a digital Electronic caliper (TCM, German) were measured. Thereafter, the fetuses were examined externally for detecting of abnormalities with a stereomicroscope (Olympus SZX, Japan). Finally, fetuses with external malformations were fixed in an alcoholic solution and were stained with alizarin red S and alician blue for detection of skeletal malformations (Kimmel and Trammell, 1981, Menegola et al., 2001).

Statistics
Data on fetal body weight and crown-rump length of the fetuses were reported as mean ± SD. Tukey test was done after ANOVA between each treated group and control group. With regards to the frequency of fetuses with skeletal anomalies the Mann Whitney U test when the frequency of each category was 5 or more was used, and with Fisher's direct probability test for other cases.
Statistical analyses were carried out by SPSS software (ver. 16) and differences less than 0.05 (P<0.05) were considered significant.

RESULTS

Maternal toxicity and pregnancy weight
In order to study the toxicity of SB in maternal mice organs, the experimental groups and the control group were sacrificed and then dissected, but no gross pathological alterations in the maternal organs and pregnancy weight reduction were diagnosed in any groups.

Externally-visible abnormalities
Various malformations were detected in fetuses of both experimental groups such as: hemorrhage, limbs defects, craniofacial defects, vertebral column deformities and neural tube defects (table 1).

Hemorrhage
One of the most common malformations observed in the fetuses of both experimental groups was hemorrhage that occurred in 13.1% and 31.2% in the fetuses of experimental group I and II, respectively. The difference in incidence of this malformation was statistically meaningful in comparing between the two experimental groups and control group and also in comparison between experimental group I and II (table 1). Hemorrhage was spread on the surface of the skin of fetuses (fig 1).

Craniofacial deformities
In this observation, craniofacial deformities such as calvaria deformity were detected in both experimental groups. The percentage of fetuses with craniofacial deformities was increased in experimental group II (fig2) and the difference of its incidence in fetuses of experimental group II was statistically higher as compared with the control group and experimental group I (table 1).
Vertebral column deformities
Another common malformation was observed in the SB-treated fetuses was vertebral column deformities (VCD) especially in the form of scoliosis (Fig3). The VCD rates were increased in both experimental groups I (4.7%) and II (6.7%) in comparison with the control group. Nevertheless, the difference between the two experimental groups was not statistically meaningful (table 1).

Limb defects
Limb defects such as meromelia, syndactyly and the others anomalies were detected only in experimental group I (table 1). The incidence of this malformation (fig 4) was not statistically meaningful as compared with control group and experimental group II (p=0.9).

Neural tube defects (NTDs)
NTDs occurred in fetuses of experimental groups I and II in the form of exencephaly (figure 5). The difference in the incidence of NTDs in fetuses of experimental group I and II was statistically increased significantly as compared with the control group and comparison between experimental groups was not statistically significant (table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>Treatment groups with SB (mg/kg)</th>
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<tbody>
<tr>
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<td>NO</td>
<td>NS</td>
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<tr>
<td>Litters</td>
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<tr>
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<tr>
<td>Fetuses examined</td>
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<td>Resorptions</td>
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<td></td>
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<tr>
<td>Hemorrhage</td>
<td>Fetus No.(%)</td>
<td>0(0)</td>
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<td></td>
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<tr>
<td>Limbs defects</td>
<td>Fetus No.(%)</td>
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</tr>
<tr>
<td>Vertebral column deformities</td>
<td>Fetus No.(%)</td>
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<tr>
<td>Exencephaly</td>
<td>Fetus No.(%)</td>
<td>0(0)</td>
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Table 1 - External Malformations in Balb/c Mice Treated With SB
Litters = number of litters having fetuses with finding (percentage of total litters);
Fetus = number of fetuses with finding (percentage of total fetuses)
*P<0.05 vs. control
**P<0.05 vs. Experimental groups.

DISCUSSION
This study showed that intake of SB by pregnant mice would lead to emergence of a series of abnormalities including: skin hemorrhage, disorder in vertebral column, calvaria deformities, limbs defects and NTDs. Food additives such as SB are used widely for various purposes, including preservation, coloring and sweetening. Some food additives, however, have been prohibited from use because of their toxicity (Sasaki et al., 2002). Benzyl alcohol, benzoic acid and its sodium and potassium salts can be considered as a single category regarding human health, as they are all rapidly metabolized and excreted via a common pathway within 24 hrs. Systemic toxic effects of similar nature (liver, lung and kidney) were observed. Also, SB at concentrations below 100 pg/ml and exceeding 500 pg/ml inhibited intracellular protein and DNA synthesis (Shitenberg and Ignat’ev, 1970, Oyanagi, 1987, Villanaueva et al., 1994, Yang et al., 1996). It has been reported that benzoic acid enhanced chromosomal aberrations, sister chromatid exchanges and micronucleus prevalence (200 and 500 mg/ml) in human lymphocytes without manipulating the pH of the medium (Minor and Becker, 1971). The most common aberrations are chromatid breaks which indicates benzoic acid caused DNA double strand breaks and sister chromatid union which is the breakage followed by reunion of both sister chromatids at an identical site (Murli, 2003). Findings of the current research are in agreement with those of the most former studies on teratogenic effects of benzoates and some other teratogens. As an example, in a study by Fujitani (1993) on embryo-toxic and phyto-toxic effects of SB in different species, the doses which poisoned the mother rats could also cause disorders in the embryo as well. Studies by Prater et al. (2006) on methylxanthine (MNU) - a nitrogenous product of digesting meat, treated meats, and marine foods - indicated that this nitrogenous compound could bring about occurrence of webbed fingers, syndactyly, and oligodactyly. The reason is believed to be due to impact
of reactive oxygen species (ROS) and sudden increase in oxygen amount or hyperoxia. Some reports also suggest that formation of ROS results in serious oxidative damages to cellular macro-molecules and redirection of signaling path along fetal growth trend, offering use of anti-oxidants as an effective method for elimination of these free oxygen radicals and alleviation of oxidative stress (Huang et al., 2004). Also, this mechanism has been suggested as one of the major teratogenic mechanisms of some antiepileptic drugs (Azarbayjani and Danielsson, 1998). According to investigations by Burke et al. (2006), nitric acid (as one of nitrogenous compounds) was released from metabolites produced by one of the benzoate derivatives. As a result, a possible mechanism involved in the observed defects caused by intake of SB, including abnormalities of limbs and vertebral column defects and cranio-facial anomalies might be attributed to release of nitrogenous products such as nitric oxide and also effect of ROS.

One of the observed abnormalities in this study was skin hemorrhage. Kreindler et al. (1980) showed that high dose of SB can cause release of histamine from mast cell granules and histamine affecting H1 receptors available in endothelial cells causing increase of artery permeability into different elements and leakage of blood plasma and inflammation and even hemorrhage in tissues. Therefore, perhaps we can attribute hemorrhage and injury of physical tissues observed in the skin of embryos with SB through this mechanism, permeation of SB inside the vessels, effect on genes interfering in blood coagulation factors and finally permeation in spaces between tissues.

Other findings of the current study regarding teratogenic effects of SB on fetus showed variety of abnormalities such as cranio-facial abnormalities mostly in the form of mandibular hypoplasia and calvarial deformity, deformation of vertebral column especially in the form of scoliosis, and NTDs. It was contended in a study that daily administration of high doses of SB could lead to genotoxic and teratogenic changes such as disorder in nervous system (Yang et al., 1996).

Albina et al. (2002) studied effects of 60 and 120 mg/kg of caffeine on embryo during pregnancy at GD5 to 18. Caffeine is used as a stress inhibitor in nonalcoholic and cacao drinks. Due to hydrophobic characteristic, it passes through the placenta and blood–brain barriers leading to delay in formation of ossification in occipital bone. At the same time, some researchers tried to study abnormal and normal development of skeletal system and neural tube using some small mutated mammals such as Loop-tail mice (Shum and Copp, 1996). By generalizing these findings about effect of some teratogeneses on development of skeletal and neural system, perhaps one can interpret effect mechanism of some compounds such as SB which threatens the growing genome by producing some metabolites and endangers morphogenesis of skeletal system probably due to passage through placental barrier. Studies of Rengasamy and Padmanabhan (2004) and Vajda et al. (2005) showed that incidence of some abnormalities such as vertebral column deficiencies are dose-dependent defects. In the present study, dose-dependent effects were found in the incidence of skeletal abnormalities such as vertebral column deficiencies. One can refer to mesenchyme constituting sclerotomes (which play role in axial ossification in the body) and notochord growth (vertebra progenitor) each having different interactions with growing neural tube during the primary embryonic periods (Hayes et al., 2001). Therefore, it seems that SB makes the subsequent differentiations problematic for migration of mesenchymal cells and their accumulation around the notochord in order to form vertebra progenitors leading to abnormalities. Reports of Kessel and Gruss (1991), regard the role of Hox gene important for development of vertebra and relate identification of vertebra to this gene expression in embryonic paraxial mesenchyme.

Shim and colleagues (2010) have reported that cyclooxygenase -2 (COX-2) transgenic fetuses exhibit severe skeletal malformations and die shortly after birth. Skeletal malformations are localized along the entire vertebral column and rib cage and are linked to defective formation of cartilage anlagen. COX-2 is a rate-limiting enzyme in the arachidonic acid cascade and catalyzes the conversion of arachidonic acid to prostaglandin H2, which is then converted to various prostaglandins by cell-specific prostaglandin synthases. COX-2 expression is observed in rat fetal organs, including skin, heart, cartilage, and kidney, during GD 15–20 (Stanfield et al., 2003, Shim et al., 2010). There are two isoforms of cyclooxygenase. COX-1 (cyclooxygenase-1) is constitutively expressed in many tissues and is thought to play roles in tissue homeostasis. However, its expression is highly inducible by many stimuli, such as cytokines, growth factors, and xenobiotics. In addition, the sclerotomal accumulation of p53 protein is observed in transgenic embryos, suggesting that COX-2 may induce apoptosis via the up-regulation of p53 (Stanfield et al., 2003). Also transgenic expression of COX-2 interferes with normal embryonic development, resulting in a severely malformed axial skeleton (Stanfield et al.,
Therefore, our results about occurrence of fetal abnormalities by SB match the information reported by others, hence, we suggest that SB can cause changes in some or all regions genes expression and induce congenital malformations.

As an affirmation, Villanaueva et al. (1994) reported that many aromatic compounds (including benzoates family) are extremely carcinogen and are metabolized by living micro-organisms, producing active compounds which react with cellular DNA and alter cellular structure. This assertion was in alignment with other researches in this field (Toth, 1984, Hu et al., 2008).

Also, Yang et al. (1996) stated that SB can cause changes in genes expression through releasing free radicals. In a study by Tsay et al. (2007), oral medication by SB in zebra-fish larvae was reported to cause disarrangement of muscular fibers, disorder in neurotransmission of motional neurons, intestinal abnormalities, and kidney deformation as the administered dose increased; in fact, SB results in these complications by influencing expression of genes and causing dysfunction in performance of enzyme (Tsai et al., 2007). In addition, Yilmaz et al. (2009) recognized injection of benzoic acid at high doses into blood lymphocytes as the cause of double strand DNA break, genetic poisoning, and influence on expression of genes. Thus, one of probabe mechanisms is that SB as a derivative of benzoic acid and an aromatic hydrocarbon can lead to skeletal abnormalities by affecting cellular DNA and changing genetic expression which play role in the process of differentiation of skeleton and organs through generating growth factors and regulating tissue homeostasis. In addition, it was asserted in a report that benzoate estradiol has a significant role in dynamics, physiology, and pathology of bones by affecting estrogen hormones via reducing performance of adenohypophysis; this compound was reported to cause excessive ossification in long bones (Lippman et al., 1942).

In conclusion, SB is a teratogenic additive in pregnant mice. This might be due to its effect on growth factors, cell cycle and gene expression and can induce congenital malformations. However, the mechanisms of the teratogenic effects of this substance need to be clarified by more detailed studies. All food additives must be kept under continuous observation and must be re-evaluated whenever necessary, in the light of changing conditions of use and new scientific information (Council Directive of 21 December 1988). Further studies on the teratogenic properties of SB, with the help of other tests for genotoxicity, should be conducted.

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