MALFORMATIONS INDUCED IN SKELETAL SYSTEM OF MOUSE FETUSES BY THE FOOD PRESERVATIVE SODIUM BENZOATE

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**ABSTRACT**

 The present study delt with the teratogenic effects of sodium benzoate (SB) which is used as a food preservative and antimicrobial agent on the skeletal system of the prenatal fetuses of mice on day 18 of gestation. The used low and high doses of SB were found to be 200 and 500 mg/kg b.wt. The pregnant mice were divided into three groups (G1, G2, G3) each subdivided into two sub-groups for treatment with low and high doses of SB.

 This study revealed that most elements of fetuses skull showed moderate to severe malformations. Ossification of the central of some cervical vertebrate was absent especially at the high dose in group2. The high dose of sodium benzoate showed some unossified caudal vertebrae especially in groups 2 and 3. The whole sternum and xiphoid cartilage became shorter than the control in all groups treated with both doses. The cartilaginous portion of the ribs exhibited less blue coloration than the control denoting reduction in its chondrification. Also, some ribs showed curled appearance.

 In addition, the scapula and clavicle of the pectoral girdle were shorter and less thickened in all treated groups. The long bones of the fore and hind limbs in the high dose treated groups were shorter in length and decreased in thickness compared to the control. In such case, the density of red coloration was less than the control.

**Key words**: Skeletal system, Sodium benzoate, Teratogenesis, Malformation.

**INTRODUCTION**

 Food additives have been increasingly used in food industry as a result of production technologies. Chemical preservatives are used in this condition and they seemed to be the most effective agent for a longer shelf-life. These chemicals are mainly used for adverse preferential taste of consumers. When the food additives are given to organisms in excessive amounts, they may cause toxic effects.

 Sodium benzoate (SB) is one of the food additives, and is widely used in food industry as a food preservative **(Beliveau, 1987)** and as a therapeutic agent in some medical purposes. SB becomes metabolized in the liver which involves the activation of benzoate into benzoyl CoA and the formation of hippurate from benzoyl CoA and glycine. It was reported that SB inhibits DNA synthesis and causes premature chromosome condensation leading to pyknotic nuclei and chromatin erosion and finally death of the cell **(Turkoglu, 2007).**

 In addition, SB has multiple applications on food chemicals. So, its biosafety and teratogenicity are important issues which should be studied. Due to these effects SB has been chosen for the present study as an example of the currently used preservatives in Egypt and in the world as a whole. So, the present study was undertaken to evaluate the possible teratogenic effects of SB in mouse fetuses. This study included analysis of the morphological anomaly and malformation of the fetal skeleton.

**MATERIALS AND METHODS**

 For the present study sodium benzoate was obtained from the Egyptian company for agriculture “ETACO”, Cairo, with a chemical formula C6H5COONA and molecular weight 144. Sodium benzoate was dissolved in distilled water.

 Virgin males and females of CD-1 pure inbred strain albino mice. *Mus musculus,* were obtained form Theodor Bilharz Research Institute, Cairo, with an a average body weight of 27-30 gm. Mating was performed by housing three females with one male overnight. At early morning, the presence of vaginal plug or smear was designated as day one of pregnancy. The approximate LD50 of the sodium benzoate was calculated by administrating of different doses sodium benzoate. Orally by gavage tube to female albino mice. The approximate oral LD50 of sodium benzoate was calculated by the method of Miller and tainter (1944) and was found to be 3000 mg/kg. wt.

 Non pregnant and pregnant female mice were performed to determine the highest and lowest oral dose of sodium benzoate for five successive days tolerated by the experimental animal without deaths. It was found that the highest and lowest oral doses of SB were 200 mg/kg. wt. and 500 mg/kg. b. wt. They were used to study the teratogenic effects on pregnant mice and their fetuses.

**Experimental animals**

 Seventy pregnant female mice divided into four groups, control group consisted of ten animals, Group1 consisted of twelve animals sub divided into two subgroups administrated by low and high doses from day 3 to day 7 of gestation, Group 2 administrated low and high doses of sodium benzoate from day 8 to day 12 of gestation and group 3 administrated low and high doses of sodium benzoate from day 12 to day 16 of gestation. The pregnant females of all groups were sacrificed on the morning of day 18 of gestation.

 For studying the skeletal malformations, fresh fetuses were skinned and fixed in 95% ethanol for 5 days, followed by acetone for 2 days. Then, the fetuses were stained for 3 days in 20 ml freshly prepared staining solution at 4° C. the staining solution is consisted of: (1 ml 0.3% Alcian blue 8 Gs in 70% ethanol, 1 ml 0.1% Alizarin Red-S in 95 ethanol, 1 ml glacial acetic acid, 17 ml 70% ethanol). After the staining process was completed, the fetuses were washed with tap water, and then the specimens were immersed in ascending series of glycerol and 1% aqueous KOH solution and then preserved in 100% glycerin for examination and photography.

**RESULTS**

**Gross morphology**

 With respect to the external features of the fetuses obtained from control and SB treated mothers by cesarean sections on day 18 of gestation, no detectable alternations were observed. Fetal growth retardation was the only change observed. This alternation was clearly visible at the level of all used doses in the three experimental groups. Hematoma was shown especially in the first group of fetuses maternally treated with low and high doses of SB during days 3-7 of pregnancy. This observation was not pronounced in the other two groups.

**Examination of fetuses skeleton**

 The mouse fetuses have two types of skeletal tissues, bones and cartilage. In relation to bones, there are two types, cartilaginous bones and membranous bones. The first type is formed at the beginning from cartilaginous tissues then transformed into bones by the process of ossification. The membranous bone is formed essentially as bone due to deposition of osteocytes directly at certain areas of the body to form most elements of the skeleton of the vertebrate body. However, at the beginning of development, the fetuses skeleton starts as cartilaginous tissue. Then, by progress of development, the cartilaginous tissues diminished gradually and disappeared completely in most areas, while some of them still persist as cartilaginous bones.

 Osteological observation of 18th day fetuses indicated that treatment of mothers with different doses of SB caused several undesirable effects ranging from moderate to severe malformations. These effects included both chondrogenesis and osteogensis processes that takes place in the formation of the skeletal system of the animal. In addition, treatments of pregnant mice with different doses of SB caused a considerable delay in development of a number of fetuses (Fig. 2).

 Generally, the skeletal system of the mouse consists of two main structures. Axial skeleton which comprises bones of the skull, vertebral column, ribs and sternum and appendicular skeleton which consists of bones of pectoral girdle and fore limb and pelvic girdle and hind limb (Fig. 3).

1. **Axial Skeleton**
2. **The skull**
	1. **Control animals**

 The skull elements of the control fetuses observed in the present study are in a good ossified condition (Fig. 4). The premaxilla is fused with the nasal and maxillary bones. The nasal bones are quadrangular in shape. The internasal and frontnasal sutures are clearly marked. The frontal bones are consisted of two halves surrounding the anterior fontanella. At the base of the cranium the ethmoid, presphenoid bones are seen in one plane. The mandible is well ossified with a slightly angulated ramus (Fig. 6).

* 1. **Sodium benzoate treated animals**

 Examination of the skull of fetuses obtained from mothers treated with different doses of SB showed that ossification of the skull components was moderate to heavy compared to the control untreated fetuses. In a significant number of cases no ossification was observed in some skull elements. Some components of the skull stain blue with alcian blue indicating that they are cartilaginous in nature and confirmed the delay in development of such bones. The bones of the skull that revealed retardation in ossification were parietal, interparietal, zygomatic process of squamosal one, tympanic bulla, squamosal, periotic, supraocciptal, palatine, pterygoid and ethmoid bones. This delay in ossification of the mentioned bones was different from the control. This effect progressed directly with the increment of the used dose (Figs. 2-9). The most evident decrement was deposition of bone material that starts with the first group, progresses with the second group and attained its final effect in the third group. The components of the skull still unchanged (well ossified) as illustrated in figures 6, 7 and 10, that belonging to the high dose treatment of the second and third groups. In addition to the decrease in ossification of some bones, the palatal bones shows different degrees of ossification according to the density of the red color of Alizarin dye. Furthermore, the fusion of the two palatal bones was incomplete in some places with small gaps between them. This result was evidenced with the high dose of SB (Figs. 5, 6 and 8) in the three studied groups.

**2- The vertebral column**

 The vertebral column of fetuses obtained from the control untreated pregnant mice consists of well ossified vertebrae which are represented by 7 cervical, 12 thoracic, 7 lumber, 4 sacral and at least 10 caudal vertebrae with clearly ossified body and arches (Fig. 11).

 Examination of the vertebral column of fetuses obtained from pregnant dams treated with different doses of SB revealed that the atlas and axis vertebrae were not well ossified as seen in figure (11). The centra of some or all cervical vertebrae have lost their ossification. On the contrary, all the transverse processes of the cervical vertebrae were well ossified. Also, the centra of the thoracic, lumbar and sacral vertebrae were well ossified as illustrated in figure 11. However, sodium benzoate treatment led to poor deposition of the ossified material in most of the caudal vertebrae.

**3- Ribs**

 The control fetuses possess 13 pairs of ossified condition, while the distal end is remained cartilaginous in nature (Fig. 3).

 The ribs of fetuses obtained from mothers treated with different doses of SB showed no variations in number and in the ossified condition compared to the control (Figs. 3), but tend to be shorter than the control group. Also, both components of ribs (sternal and vertebral portions) were still chondrified and ossified, respectively in all treated groups. The most observed change was the appearance of curled - shapes ribs. Also, a decrease in size and length of ribs was observed in the first group treated with the high dose and in the second group treated with both doses (Fig. 2).

**4- Sternebrae**

 Most of the control fetuses possess 6 sternebrae and the last one of them is the xiphisterum. All these structures are founds in a good ossified form.

In the treated condition, the most ossified sternebrae were observed in the second group that treated with low and high doses of SB (Fig. 9). In such case the sternum and the xiphoid cartilage tend to be shorter than in the normal group.

1. **The appendicular skeleton**

**B.1. The pectoral girdle and fore limb**

 The pectoral girdle of the control untreated fetuses on day 18 of gestation comprises a well ossified scapula and clavicle, stained well with alizarin red “s”, while the suprascapula and epiphyscal cartilages still cartilaginous in nature and stained blue with alcian (Fig. 10). The fore limb components are the humerus, radius, ulna, phalanges with five digits and cartilaginous carpalia and metacarpalia.

 Exposure of pregnant mothers to different doses of SB showed that the degree of ossification of the scapula, clavicle, humerus, radius and ulna was slightly affected especially at the high dose level of SB in the three studied groups. The intensity of the stain in the second group was less dense than the other two groups. The length and thickness of these components were also affected. The clavicle bone showed a severe shortage in its length compared to the control. The scapula of the treated fetuses were subjected to a considerable decrease in its length at the level of low and high doses of SB as in some fetuses of the second and third groups compared to the control (Fig. 10).

**B.2. The pelvic girdle and hid limb**

 The pelvic girdle of the control fetuses consists of three well ossified bones; ilium, ischium and pubis. The pubic symphysis remains cartilaginous in nature. The hind limb consists of well ossified bones; these are the femur, tibia and fibula, a series of phalanges in the four digits and cartilaginous tarsals and metatarsals (Fig. 11).

 Examination of the pelvic girdle of fetuses obtained from SB treated mothers showed that the most evident change was the decrease in length of the components of the pelvic girdle that treated with the low dose. A severe shortage was observed with the high dose compared to the control. The cartilaginous portions exhibited less blue colouration than the control denoting less condrification (Fig. 11).

 Considering the hind limb, a severe shortage was observed in the length and thickness of the femur, tibia and fibula reaching about half the length of the control condition. Also, elements of the tarsal and metatarsal of the second treated group with the high dose of SB were non-chondrified. Most of phalanges of the second group treated with low and high doses of SB and those of the third group treated with the high dose showed some non-ossified and some non-chondrified elements when compared with the control (Fig. 11).

**DISCUSSION**

 Assessment of health hazards arising from occupational exposure of animal or human to chemicals or toxic substances in the environment has already been studied by many workers, but the effect of such substances on embryos or fetuses have got little attention.

 The present study deals with the effect of SB on the skeletal malformations produced in mice fetuses that examined on day 18 of gestation. It has been found that SB in the used doses caused variable deformities in some elements of the examined skeletal system. At the same time, non-ossified parts were seen in some components of the skeleton.

 The present results revealed that elements of the bony skull showed delay in development of a quite large number of bones of the skull according to the intensity of the red coloration denoting a decrease in the process of osteogenesis. These bones are parietal, interparietal, zygomatic process of squamosal, periotic, supraoccipital, palatine, pterygoid and ethmoid as well as the unchondrified premaxilla. These results coincided with that observed by other authors **(Brown *et al*., 1976; Mayura *et al*., 1980; Wangikar *et al*., 2004; Patil *et al*., 2006). Verret *et al*. (1980)** examined the effect of 80 food additives on the developing chick embryo and observed a significant numbers of abnormal birds and the incidence of abnormalities was a dose-related. The authors reported that the predominant abnormality was cleft palate and other abnormalities as well. Incomplete ossification of the skull bones was reported also by **Patial *et al.* (2006)** in wistar rat fetuses subjected to the toxicity of ochratoxin A which is a natural contaminant in various food or feed commodities resulting in toxicosis in animal and human population. In addition. **El-Makawy *et al.* (2008)** showed that stannous chloride (Sncl2), which is used as food additives and dyes, reduced ossification of all bones of the axial and appendicular skeletons of fetuses maternally treated with this substance. This effect was a sign of growth retardation or developmental delay. These abnormalities were not observed by **Abdel-Wahab *et al.,* (1999)** who reported that 7-methionine prevents developmental toxicity of ochratoxin in rat fetuses.

 In relation to the vertebral column, the present study showed absence of the centra of the last five cervical vertebrae (3-7), while the acute malformation was observed in some unossified caudal vertebrae of mice fetuses. Also, the present study reported that the sternum and ribs were shorter than the control and the ribs became curled in appearance. These findings were in agreement with that observed by other workers in rats and mice **(Brown *et la.,*  19976; Ogata *et al.,* 1999; George *et al.,* 2001; Adeeko *et al*., 2003; Wangikarr., *et al*., 2004; Patil *et al*., 2006; El-Makawy *et al*., 2008)**. These authors pointed out that the skeletal defects involved a severe reduction in abnormalities and ossification of ribs and sternum. However, no abnormalities in the skeletal structures were observed by **Abdel-Wahab *et al*., (1999)** in rat fetuses maternally treated with methionine.

 In addition, **Meyer and Hansen (1975)** studied the effect of the food color ponceau 4R in rats and investigated skeletal and internal malformations; these alternations included rudiments of extra ribs and other skeletal malformations such as scoliosis and kyphosis. Also, **George *et al*. (2001)** studied the developmental toxicity of the perfumery and flavoring agent isoeugenol in rats and showed some skeletal variations including delayed development of the thoracic or lumbar centra. These authors also manifested that the unossdified sternebrae were significantly increased at high doses of isoeugenol. Furthermore, **Patil *et al*., (2006)** investigated rudimentary ribs, ribs fusion and waviness, malformed sternebrae, delayed development of the thoracic and lumbar centra and unossified caudal vertebrae in rat fetuses maternally subjected to the effect of ochratoxin A.

 In addition, the current study revealed that treatment of pregnant mice with high doses of SB caused a pronounced shortage in the length and size of the pectoral and pelvic girdles elements such as scapula, clavicle, ischium, ilium and pubis. However, the pubis symphysis remains cartilaginous as it is. These observations were in agreement with the findings of **Brown *et al*. (1976), Mayura *et al*. (1982), Wangikar *et al*. (2004) and Patil *et al*. (2006).** These authors demonstrated a severe reduction in the length and size elements of the pectoral and pelvic girdles of fetuses maternally exposed to high doses of ochratoxin.

 Furthermore, the bones of the fore and hind limbs of fetuses obtained from SB treated mothers designated shortage in the length and sometimes in size of long bones and components of the digits. In some cases, the carpalia, metacarpalia and phalanges of the fore limb were severely affected and remain cartilaginous or not stained with both dyes of the skeletal tissue. These findings were coincided with the observations reported by several authors **(Brown *et al*., 1976; Mayura *et al*., 1982; Wangikar *et al*., 2004; Patil *et al*., 2006).**  These authors revealed a severe shortage in the length of the long bones of fetuses limbs which was not presented in the study of **Abdel-Wahab *et al*., (1999)** who showed that SB exits no effect on the long bones of fetuses obtained from treated mothers. Moreover, **Adeeko *et al*. (2003)** studied the effect of the food preservative tributyltin chloride on pregnancy in rats and showed delayed ossification of the fetal skeleton after treatment with 10 or 20 mg/kg tributylin chloride.

 Thus, from the findings of the present study and other studies, one can expected that the food preservative SB produced its malformations effect either through the direct action on organs related to bone formation or indirectly by altering calcium metabolism in various embryonic tissues especially preventing calcium precipitation in the cartilaginous structures in fetuses body.

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**EXPLANATION OF FIGURES**

Figure (1): Gross morphology of alive fetuses on day 18 of gestation Control and SB treated dams. Low and high (LD and HD) doses treated groups (G1, G2 and G3).

Figures (2 & 3): Skeletal system preparation of fetuses of control and SB treated mothers. Low and high doses treated groups (G1, G2 and G3).

Figures (4 & 5): Skeletal system preparation of the skull of fetuses of control and SB treated pregnant mice. Ventral view.

Figures (6 & 7): Skeletal system preparation of the skull of fetuses of control and SB treated mothers. Lateral view.

Figure (8): Skeletal system preparation of the vertebral of fetuses of control and SB treated dams. Ventral view.

Figure (9): Skeletal system preparation of the sternum and sternebrae of fetuses of control and treated pregnant mice. Ventral view.

Figure (10): Skeletal system preparation of the pectoral girdle and for limb of control and SB treated fetuses.

Figure (11): Skeletal system preparation of the pelvic girdle and hind limb of control and SB treated fetuses.

**ABBREVIATIONS**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| AT. | : | Atlas | P. | : | Parietal  |
| AX. | : | Axis | P.M.X. | : | Pre-maxilla  |
| B.S. | : | Basi-sphenoid  | P.S. | : | Pre-sphenoid  |
| BO.  | : | Basi-occipital  | PB. | : | Pubis  |
| C.D. | : | Caudal vertebrate | PH. | : | Phalanges  |
| C.P. | : | Condyle process | PL. | : | Palatine |
| C.V. | : | Cervical vertebrae | PS | : | Pre-sphnoid  |
| CA | : | Carpales | R.  | : | Radius  |
| CL. | : | Clavicle | S.SC. | : | Supra-scapula. |
| D. | : | Dentery  | S.V. | : | Sacral vertebrae |
| EO. | : | Exo-occipital. | SC. | : | scapula |
| F.L. | : | Fore limb | SO.  | : | Supra-occipital |
| FB | : | Fibula | SQ. | : | Squamosal  |
| FM | : | Femur | SR. | : | Sternum.  |
| FOM.M. | : | Formaen magnum.  | SR.P.R. | : | Sternal portion of ribs |
| FR. | : | Frontal | SV | : | Sacral vertebrae |
| H. | : | Humerus  | T.B. | : | Tympanic bula |
| H.L. | : | Hind limb | T.V. | : | Thoracic vertebrae |
| IL  | : | Ilium  | TB | : | Tibia  |
| IP | : | Interparietal | TS | : | Tarsalia  |
| IS  | : | Ischium  | U. | : | Ulna  |
| L.V. | : | Lumbar vertebrae | V.P.R. | : | Vertebral portion of ribs  |
| MC. | : | Metacarplia  | XI.C. | : | Xiphoid cartilage |
| ML.C. | : | Meckel’s cartilage | Zy. A. | : | Zygomatic rach |
| MT | : | Metatarsalia | Zy. P. J. | : | Zygomatic process of jugal |
| MX | : | Maxilla  | Zy.P.Sq | : | Zygomatic process of squamosal  |
| N. | : | Nasal |  |  |  |

**التشوهات الناتجه من تأثير بنزوات الصوديوم**

**على الجهاز الهيكلي لأجنة الفئران البيضاء**

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 يقوم هذا البحث بدراسة تأثير بنزوات الصوديوم على الجهاز الهيكلي لأجنة الفئران البيضاء ***mus maculus*** . وتعتبر هذه المادة هي أحد المضادات الميكروبات في صناعة الطعام في العصر الحاضر. وقد تم تحديد أقل وأعلى جرعة من بنزوات الصوديوم والتي تبقي الحيوان على قيد الحياة طوال فترة الحمل وقد وجد انها تبلغ 200 و 500 ملجم / كجم من وزن الحيوان. وقد أظهرت الدراسة كثير من التشوهات في أجزاء مختلفة من الجهاز العظمي لاجنة الفئران موضوع الدراسة. كما أوضحت الدراسة عن تشوهات متوسطة إلى شديدة بعناصر الجمجمة كما أنه لوحظ عدم تعظم أجسام بعض الفقرات العنقية وبعض الفقرات الذيليه.

 كما أوضحت الدراسة أيضاً قصر الغضروف السيفي بالمقارنة بالحالة العادية للأجنة في اليوم الثامن عشر من الحمل. وبالنسبة لعظام الحزام الصدري والحوض فقد لوحظ قصر هذه العظام مع نقص أحجامها بالمقارنة بالأجنة العادية وهذه العظام هي اللوحي، الترقوى، الفخدي والعظم القصبي الشطبوي. كما أن بعض هذه العظام تظهر نقصاً في شدة الصبغة الحمراء مما يدل على أنها أقل كثافة مع إنعدام ترسيب الخلايا العظمية بشكل طبيعي بالمقارنة بالحالة العادية. 