



## Effects of the Food Additive Sodium Benzoate on Developing Chicken Liver

Feryal KARAKAHYA, Yücel BAŞIMOĞLU KOCA\*

*Department of Biology, Faculty of Science and Art, Adnan Menderes University, Aydın, TURKEY*

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**Abstract.** In this study, the effects of sodium benzoate (E-211) (SB) on the liver tissues of chicken embryos which were exposed to different doses of SB and for different durations were histopathologically investigated. SB was injected into vitellus at three different doses (250, 500, 1000 mg/kg) on the 5<sup>th</sup> day of the incubation. The embryos of the control and experimental groups were collected on the 7<sup>th</sup> day (stage 31) and 10<sup>th</sup> day (stage 36) of incubation and weighed. It was statistically detected that SB caused rise to a significant decrease ( $P<0.001$ ) on the total weights of the embryos depending in the dose and duration. At microscopic level deterioration of vein structures, congestion, edema, sinusoidal dilation, deterioration of hepatocyte arrangements, swelling, vacuolization, chromatin condensation, karyolysis, shape deformation and eccentric located of the nuclei decrease in Nuclear Organization Regions (NOR) and mitotic division, stickiness of chromosomes, anaphase bridge, nuclei budding and the formation of micronuclei were observed.

**Keywords:** Food additive, sodium benzoate, chicken embryo, liver, histopathology

## Gelişmekte Olan Tavuk Karaciğeri Üzerine Gıda Katkı Maddesi

### Sodyum Benzoatın Etkileri

**Özet.** Bu çalışmada sodyum benzoata (E211) farklı doz ve sürelerle maruz kalan tavuk embriyosu karaciğer dokusuna etkileri histopatolojik yönden araştırılmıştır. Sodyum benzoat, inkübasyonun 5. gününde üç ayrı dozda (250, 500, 1000 mg/kg) vitellusa enjekte edildi. Kontrol ve deney gruplarına ait embriyolar inkübasyonun 7. (safha 31) ve 10. günlerinde (safha 36) çıkarıldı, ağırlıkları tartıldı. Yapılan istatistiki değerlendirmeler sonucunda sodyum benzoatın doza ve süreye bağlı olarak embriyoların toplam ağırlıklarında anlamlı bir azalmaya neden olduğu belirlendi ( $P<0.001$ ). Mikroskop düzeyinde venlerin yapısında bozulma, konjesyon, ödem, sinuzoidlerde genişleme, hepatosit düzenlenmelerinde bozulma, şişme, vakuolizasyon, kromatin kondensasyonu, karyolisis, nükleusun şekil bozukluğu ve eksentrik yerleşimi, Nükleolar Organizasyon Bölgeleri'nde (NOR) ve mitotik bölünmede azalma, kromozomlarda yapışıklık, kalgın kromozomlar, nükleusta tomurcuklanma ve mikronükleus oluşumu gözlemlendi.

**Anahtar Kelimeler:** Gıda katkısı, sodyum benzoat, tavuk embriyo, karaciğer, histopatoloji

## 1. INTRODUCTION

Biochemical, physiological, genotoxic and embryological studies related to food additives have increased in recent years <sup>[1-3]</sup>. It has been observed in previous studies that sodium benzoate SB adversely affects various physiological systems in different species <sup>[2,4,5]</sup>.

SB is a substance used as a food additive. SB has the chemical formula  $\text{NaC}_6\text{H}_5\text{CO}_2$ ; it is a widely used food preservative, with E number E211 <sup>[6,7]</sup>. SB is bacteriostatic and fungistatic under acidic conditions. It is most widely used in acidic foods such as salad dressings (vinegar), carbonated drinks (carbonic acid), jams and fruit juices (citric acid, pickles (vinegar) and condiments. It is also used as a

\* Corresponding author. Email address: ykoca66@yahoo.com

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preservative in medicines and cosmetics<sup>[8-10]</sup>, antimicrobial agents in edible coatings<sup>[11]</sup>. Also, benzoic acid and SB chemical intermediates are of great relevance in the chemical industry<sup>[12]</sup>.

There are also some embryotoxicity studies which examine the effects of SB on the development of different organisms<sup>[13,14]</sup>. In a study that was carried out on pregnant rats which were exposed to different doses of SB during pregnancy, an increase in maternal body alongside fetus weight and food intake and also a 100% mortality rate shortly after birth of offspring were reported skeletal system, brain, kidney and eye malformations and abnormalities, 1% double-sided eyeless fetus and 2% pyelectasis<sup>[15]</sup>.

Despite the fact that SB is allowed to be used as a preservative in foods, both WHO (World Health Organization) and FAO (Food and Agriculture Organization) are continuing to investigate the effects of SB on environment and human health<sup>[7,12,16]</sup>. This study aims to histopathologically determine the effects of SB on the liver tissue of chick embryos and give inside as to whether SB can be safely used and how food containing SB can be safely consumed especially in pregnancy.

## 2. MATERIAL AND METHODS

### 2.1. Animals

Approval by the Experimental Animals Local Ethics Committee, Adnan Menderes University (2010/052) was taken. In this study, 200 Specific Pathogen Free (SPF) Legghorn type chicken eggs were obtained from the Republic of Turkey Ministry of Food, Agriculture and Livestock, Veterinary Control and Research Institute (Bornova-İzmir/TURKEY). The developmental stages of the embryos were determined according to Hamburger and Hamilton<sup>[17]</sup> scale and organ development was also determined according to this were assessed again using this scale.

### 2.2. Experimental design

The food additive sodium benzoate ( $C_7H_5O_2Na$ , Cas No: 532-32-1) was purchased from Carlo Erba (Italy). The study was divided into two groups; control and experiment. The control group was subdivided into two groups; intact (n: 20) and distilled water injected (sham group, n: 20) on the 5<sup>th</sup> day (stage 27) of the incubation.

The application dose of SB (0.1% by weight) was determined based on the suggested dose of WHO (World Health Organization) [CICADs (Concise International Chemical Assessment Documents)]<sup>[12,16]</sup>. SB in doses of 250, 500 and 1000 mg/kg was injected into the eggs containing embryo which were allocated for the experiment group on the 5<sup>th</sup> day of incubation. 0.1ml of distilled water and SB to the distilled water control and experiment groups were injected into the vitellus respectively using a G27 needle. Next, covered with parafilm, the eggs were put into an incubator machine (37.5<sup>o</sup>C, % 60-80 moisture) (Brinsea Octagon-40DX). The embryos belonging to groups which had 3 different dosages of

SB (total n: 60) and distilled control groups were collected (on 7<sup>th</sup> day of incubation) 2 days after the injection and the remained (on 10<sup>th</sup> day of incubation) 5 days after injection (total n: 60). All embryos were then separated from vitellus using saline (0.9% NaCl<sub>2</sub>).

### 2.3. Histopathological examination

For histologic preparation, 7 day embryos (stage 31) and 10 day embryos (stage 36) were fixed in Saint Marie's fixative (99 ml 95% alcohol + 1 ml glacial acetic acid) (at +4 °C for 24 hours), dehydrated through a graded series of ethanols, kept in xylol until transparent and embedded in paraffin. Sample sections were taken at a thickness of 5 µm from paraffin blocks on Rotary microtome (Leica RM 2145). The sections were stained with Hematoxylin-Eosin (Mayer's) for general structure, Periodic Acid Schiff (PAS) for glycogen and reticulin staining (Bio-Optica) for reticular fibres and Nucleolar Organization Regions and then closed with entellan <sup>[18]</sup>. The sections were examined and photographed using an Olympus BX51 microscope (Olympus E-330 digital camera).

### 2.4. Statistical analysis

Semi-quantative data were statistically analyzed using one way analysis of variance method (SPSS V.17) and P<0.05 was accepted as the level of significance.

## 3. RESULTS

### 3.1. Histological results

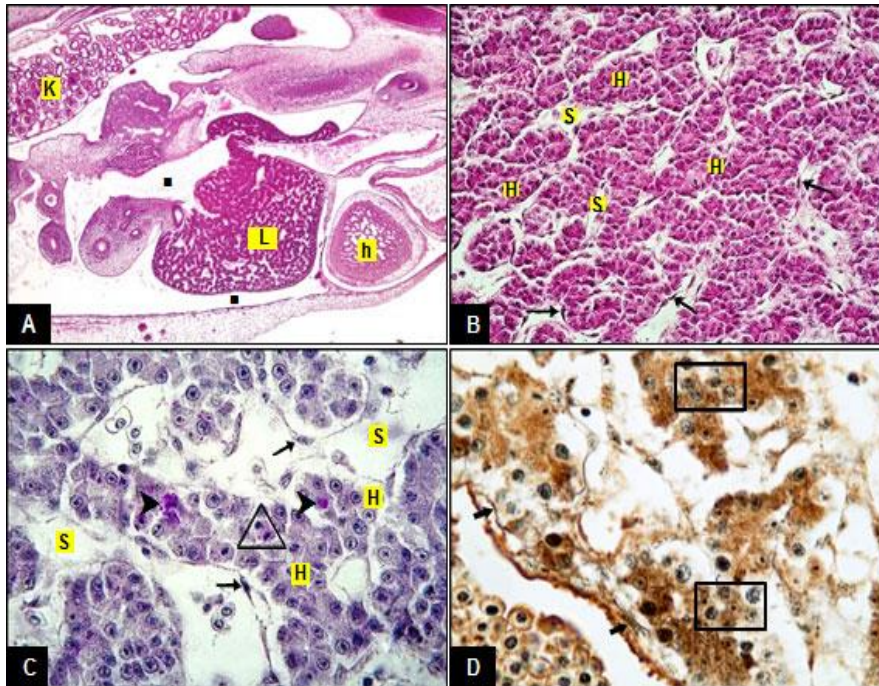
#### 3.1.1. Control groups

There was no histological difference found between the normal control and distilled water-control groups in the histological analyses. For this reason the control group as the basis for a normal control group and in histopathological evaluation was carried out in terms of this. It was determined that the liver was formed in the ventral of mid-intestine which is close relation with the hearth in cardio-hepatic area in the 7<sup>th</sup> (stage 31) and 10<sup>th</sup> days (stage 36). Additionally, in the stage 36 the liver was observed to consist of two lobes (*Fig. 1A, 2A*). It was observed that hepatocytes constituted the liver parancyma in the form of irregular cell groups at the 31<sup>st</sup> stage and endothelial cells which were among the hepatocytes formed in the lumen of sinusoids on the walls of blood cells (*Fig. 1B,C*).

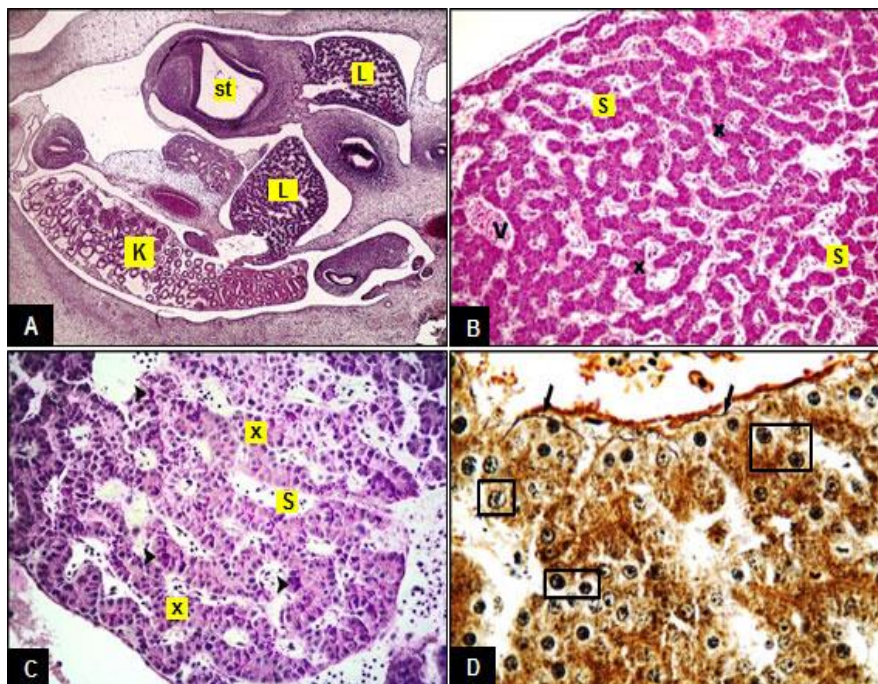
The liver parancyma was a more regular shape at stage 36. The parancyma contained the hepatocyte cords which were arranged in dendriform and of two cell thickness along with sinusoids anastomising with each other among the cords. At this stage the hepatic lobul structure was not distinct. The parancyma vena centralists were of different sizes and at different distances (*Fig. 2B,C*). At both stages, the hepatocytes were in low prismatic or cuboidal form. Moreover, they contained glycogen,

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especially at the 36<sup>th</sup> stage, generally around vena centralis (*Fig. 1C, 2C*). Reticular fibers were observed at both stage, but to a greater extent at the 36<sup>th</sup> stage, in particular more intensely around the veins and on the sinusoid walls that had started to form (*Fig. 1D, 2D*). Moreover, a significant number of NOR regions were detected in the hepatocytes nuclei of both stages.



**Figure 1.** Control group (7 day embryos-stage 31) **A.** Liver (L), heart (h), kidney (K), hepatic cavity (■). **B,C.** Hepatocyte (H), sinusoid (S), endothelium (→), hepatocytes containing glycogen (►). **D.** reticular fibers beginning to take shape around the veins (◄), hepatocyte nuclei containing NOR regions (□), mitotic figure in hepatocytes (△). **Staining:** **A,B.** H&E, **C.** PAS, **D.** Reticulum. **Magnification:** **A.** 4X, **B.** 40X, **C,D.** 100X.



**Figure 2.** Control group (10 day embryos-stage 36) **A.**Liver with two lobes (L), kidney (K), stomach (st). **B,C.**Hepatocyte cords arranged in dendriform (x), sinusoid (S), vena centralis (V), glycogen stored in the form of particles in hepatocytes (►). **D.** Reticular fibers around the veins (►), (hepatocyte nuclei containing NOR regions (○)). **Staining:** **A,B.** H&E, **C.** PAS, **D.** Reticulum. **Magnification:** **A.** 4X, **B.** 20X, **C.** 40X, **D.** 100X.

### 3.1.2. Experimental groups

In the groups exposed to SB for two days (Stage 31) and five days (Stage 36) were macroscopically observed that vitelline vascularisation decreased depending on dose (*Fig. 3*).

#### 3.1.2.1. The groups exposed to sodium benzoate for two days (Stage 31)

Sinusoidal dilation, severance at some sinusoid endothelials, deterioration in the wall structure of vein (*Fig. 4A,B,E,F,I,K*), indistinctness of the cell boundaries of most of the hepatocytes, foamy look at their cytoplasm and dispersion, swelling, pyknotic nuclei, chromatin condensation and karyolysis were detected (*Fig. 4F,G,J,K*) in the experiment groups belonging to stage 31 depending on the dose increase. In addition, a decrease in most hepatocyte NOR regions was observed along with chromosome stickiness, lagging chromosome, damages such as anaphase bridge in dividing cells (*Fig. 4C,D,H,L*). Glycogen was not observed at the every dose of this stage (*Fig. 4B*). Reticular fibrils were not detected in the reticular stain (*Fig. 4C,D,H,L*).

#### 3.1.2.2. The groups exposed to sodium benzoate for five days (Stage 36)

In all the experimental groups of the 36<sup>th</sup> stage, in parallel to increasing dose, an increase especially in degeneration of the hepatocyte sequences on the liver periphery and necrotic appearance in this region,



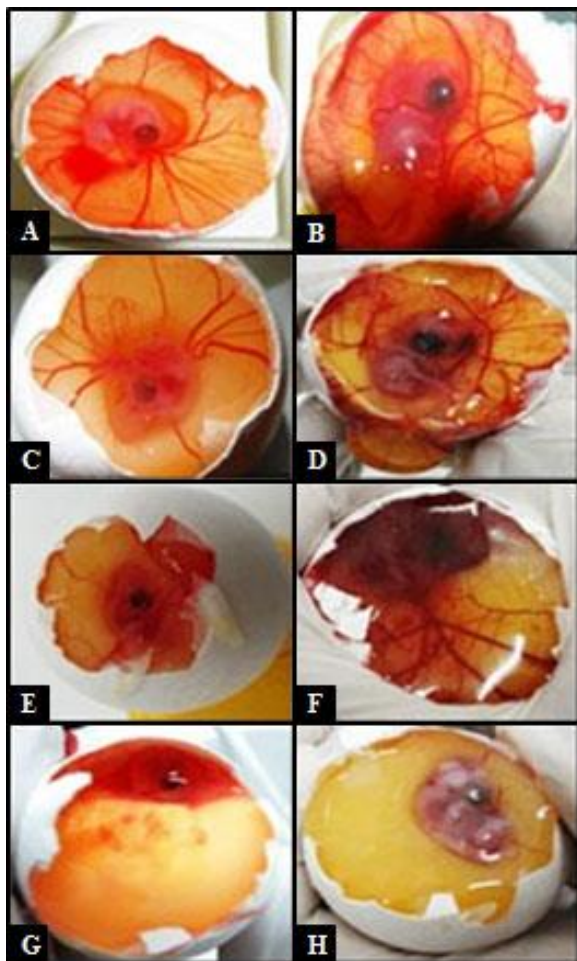
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severe sinusoidal dilation, edema in some veins, congestion and wall-thickening were identified. Hepatocytes forming paranchyma create cords of two cell thickness, however the this arrangement was observed to be imparied (*Fig. 5B,F,G,K*).

Severe vacuolisation and decomposition/dissolution, shape defects of nuclei, indistinction of the boundries and bud formation, pyknotic nucleus, eccentric located nucleus, swelling on the some hepatocythes and chromatin condensation, karyloysis and nucleus formation in erythrocytes are the mostly encountered findings (*Fig. 5B,C,F,G,J,K*). At this stage, as in the other ones, while the hepatocythes containing glicogen were not observed, reticular fibrils were not detected as well (*Fig. 5D,H,L*). In the groups which were given 250mg/kg and 500mg/kg of this stage, the hepatocytes undergoing mitosis were encountered even a few however they were not detected at the dose of 1000 mg/kg. Besides, decreasing NOR regions starting from low dose even no NOR regions some of them were found (*Fig. 5D,H,L*).

### **3.2. Statistical results**

When the average weight of the embryos which were exposed to SB for 2 and 5 days was compared with the control groups a decrease related to increasing substance amount occured and this decrease was found to be significant statistically ( $P<0.001$ ) (*Fig. 6*).



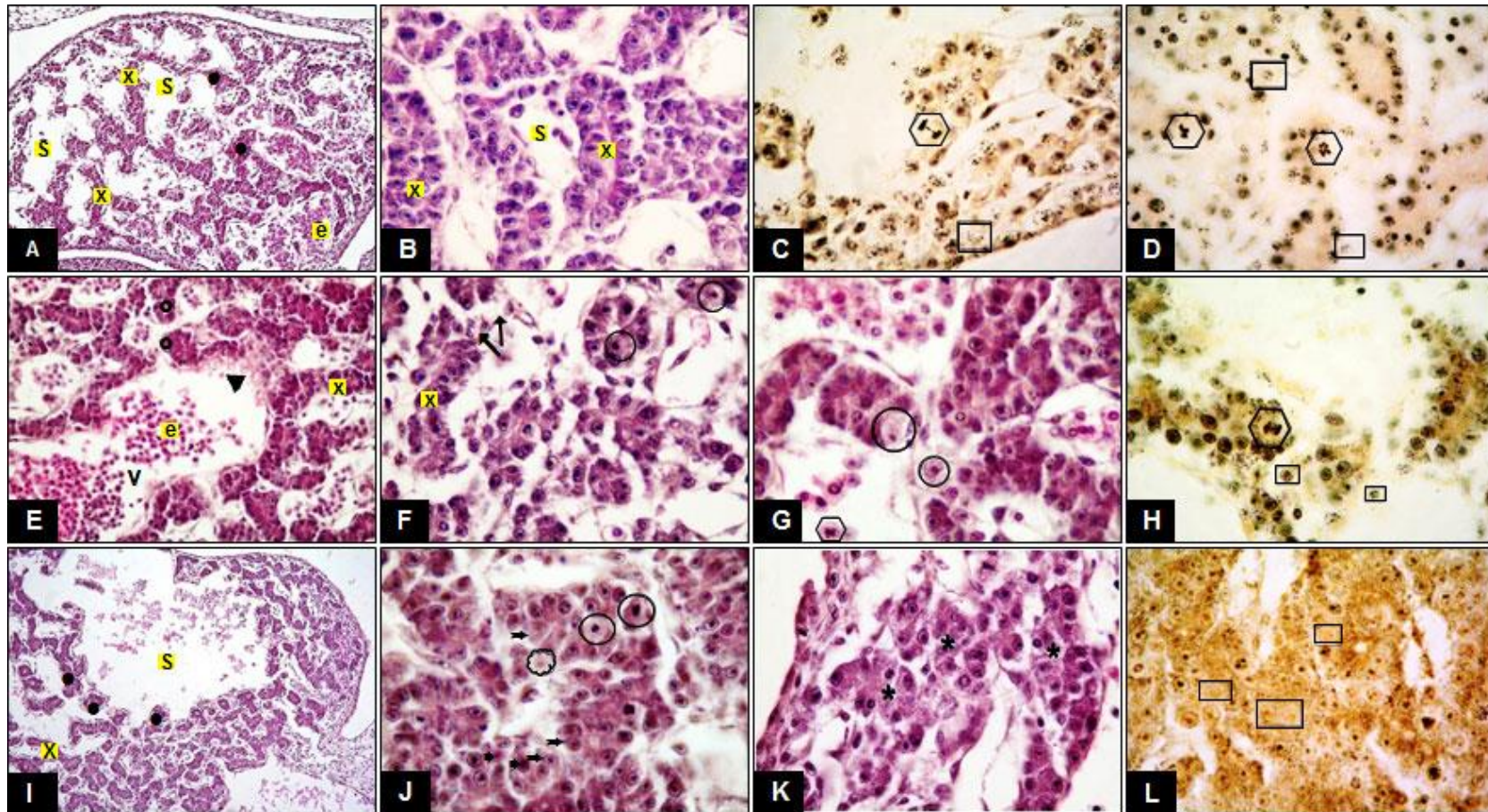
**Figure 3:** Macroscopic photographs of embryos in the eggs. The vitellin vascularisation in the experimental groups is less than control groups. **A.** 7 day control embryo (stage 31), **B.** 10 day control embryo (stage 36), **C.** The group treated with 250 mg/kg of SB (7 day embryo), **D.** The group treated with 250 mg/kg of SB (10 day embryo), **E.** The group treated with 500 mg/kg of SB (7 day embryo), **F.** The group treated with 500 mg/kg of SB (10 day embryo), **G.** The group treated with 1000 mg/kg of sodium benzoate (7 day embryo), **H.** The group treated with 1000 mg/kg of sodium benzoate (10 day embryo).

#### 4. DISCUSSION

According to Minor and Becker <sup>[19]</sup>, sodium benzoate to rats in different stages of pregnancy gave rise to a decrease in fetal live weight and survival rate in the womb. In a short term study with SB changes in body weight of mice and rats were observed <sup>[4]</sup>. It was stated in the study of Kaboğlu and Aktaç <sup>[20]</sup> that the body weight of the mice decreased depending on the dose administered.

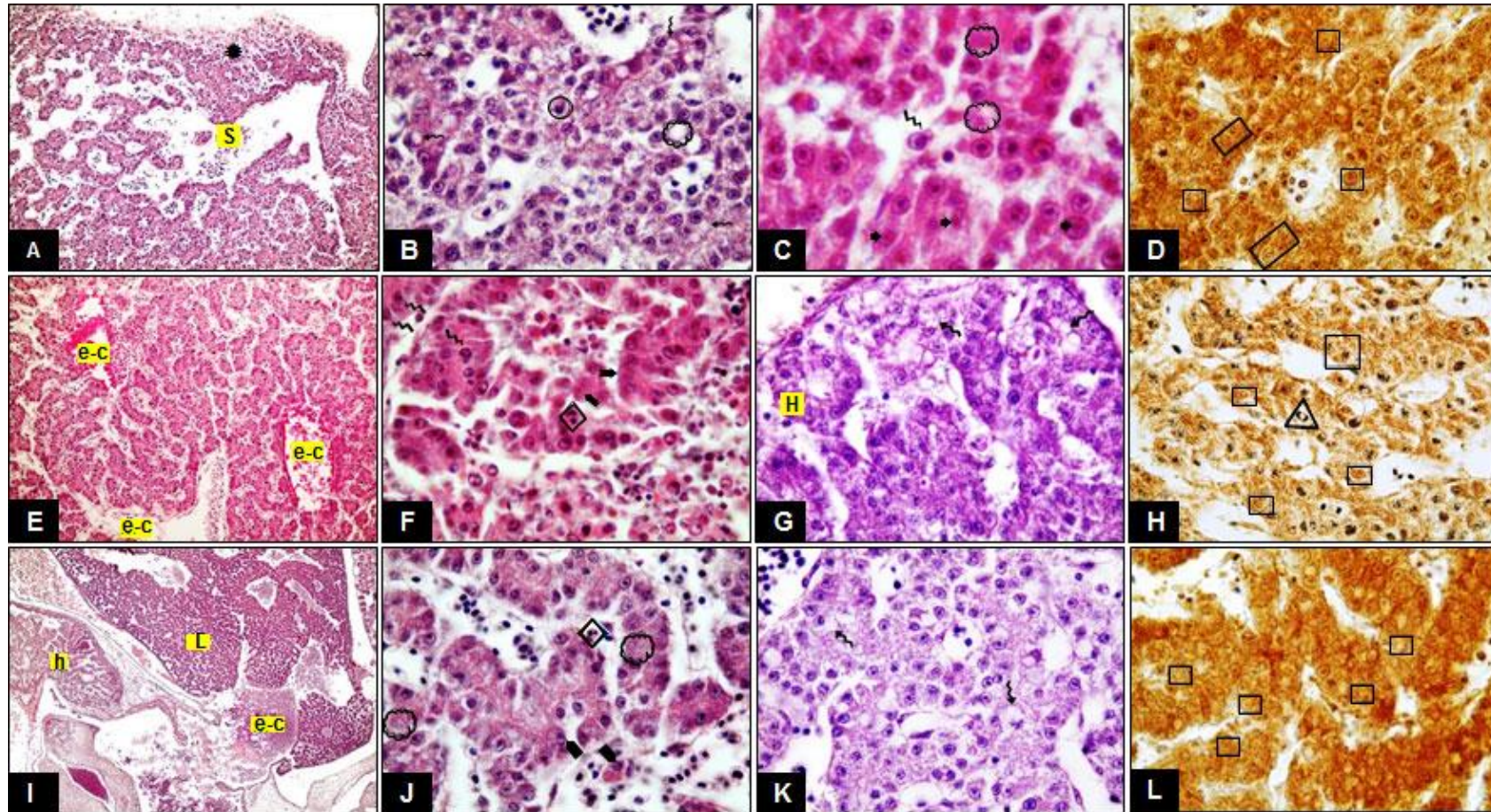
In our study, the embryos in the control and experimental groups were weighed, and it was seen that the average weight of the embryos exposed to SB for 2 and 5 days significantly decreased ( $P < 0,001$ ) compared to the control groups in experimental groups depending on the increasing dose (Fig. 6). This decrease might be related to the individual development process of each embryo as well as being probably related to most undernourishment of the embryos connected with deterioration in the vitellin vein structure due to the effect SB.

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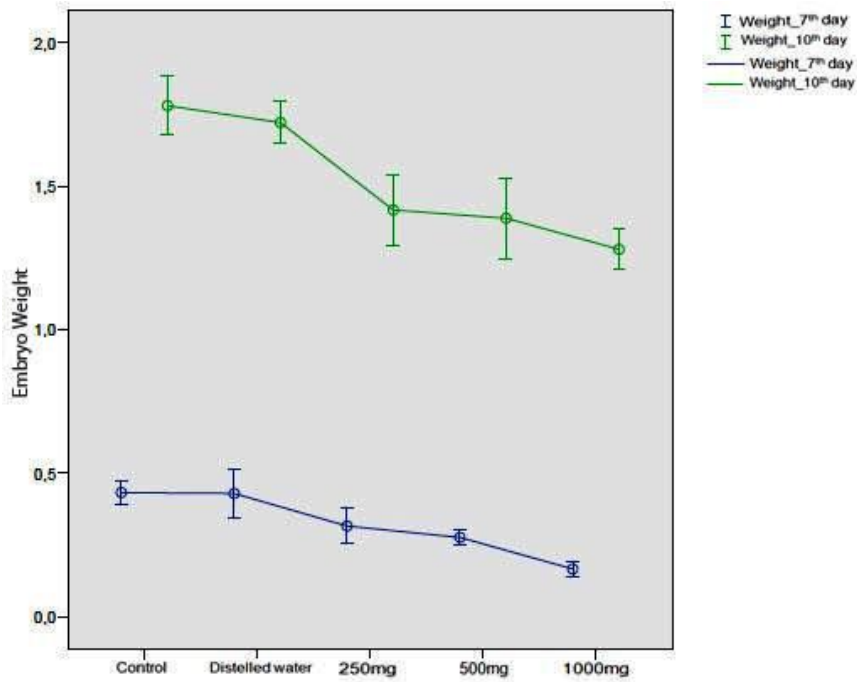


**Figure 4.** The experiment groups exposed to SB for two days (7 day embryos -stage 31). Hepatocytes groups (●) and cords (x), vena centralis (V), dilated sinusoids (S), endothelialrupture (→), erythrocyte (e), degeneration ofveins (▶), degraded forms and scattered cytoplasm hepatocytes (\*), picnotic nucleus in uncertain boundaries hepatocyte ( ), karyolysis ( ), chromatin condensation, chromosetickiness, laggingchromosomes and anaphasebridge ( ), uncertainboundaries nuclei(↗), differentcolorednucleolus (◆), decreased NOR region in hepatocytes ( ). A,B,C,D. 250mg/kg, E,F, G,H. 500mg/kg, I,J,K,L. 1000mg/kg group). **Staining:** A,B, E-G, I-K. H&E, C,D,H,L. Reticulum. **Magnification:** A. 10X, I. 20X, B-H, J-L. 100X.





**Figure 5.** The experiment groups exposed to SB for five day (10 day embryos-stage 36). Parenchymal necrosis ( \* ), dilated sinusoids (S), vacuolization in hepatocytes ( ~ ), pyknotic nucleus ( ● ), karyolysis ( ○ ), deformity and proliferation in hepatocyte nuclei ( ~ ), abnormal boundaries nuclei ( ⇨ ), eccentric nuclei ( ▲ ), micronucleus in hepatocytes ( ◇ ), different colored nucleolus ( ► ), micronucleus in erythrocyte ( ◊ ), NOR region no hepatocytes ( ○ ). Edema and congestion (e-c); L; liver, h; heart. **A, B, C, D.** 250mg/kg, **E, F, G, H.** 500mg/kg, **I, J, K, L.** 1000mg/kg group). **Staining:** A-C, E, F, I, J. H&E, G, K. PAS, D, H, L. Reticulum. **Magnification:** A, E. 20X, C. 160, B, D, F, G, H, J-L. 100X, I. 4X.



**Figure 6.** A graph showing the reduction in the weights of the embryo. This decrease in embryo weights is statistically significant.

Although rarely seen, leukocytoclastic vasculitis disease (inflammation of blood veins), following necrosis in capillary veins after sodium benzoate intake <sup>[21]</sup>. In the present study were macroscopically examined that in the embryos which were exposed to SB for 2 and 5 days a significant decrease in the vitellin vessels was observed especially at the highest dose the decrease was relatively small at lower doses. The negative effect of this decrease is inevitable on organogenesis in the embryonic period. Deterioration in the vein structure in liver tissues, congestion, edema and sinusoidal dilation were encountered on the hispathologic sections.

Related to short term oral intake, it was reported in a study that the serum protein levels were changed by SB and particularly on the hepatocytes on the periportal area, glassy cytoplasm was formed <sup>[4]</sup>. Sodium benzoate administered to the mice was observed to cause vacuolization in the mice liver cells, deterioration of chromatin material. The size of hepatocyte was found to be at least twice as large as that of normal cells while containing bigger and very dark stained nucleus. Also, some of them were found to be without a nucleus <sup>[5]</sup>.

In our study, shape changes on the nucleus, chromatin condensation, vacuolization, swelling and eccentric settlement were encountered due to SB especially at the highest dose. Even if vacuolar degeneration is attributed to a change created to collect damaged cell materials,

\* Corresponding author. Email address: [koca66@yahoo.com](mailto:koca66@yahoo.com)

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cytoplasmic vacuolization seen in hepatocytes might be due to a change in the structure of lipids, disruption of fat metabolism or congestion of gluconeogenesis as a result of adversely affected carbohydrate metabolism, disintegration of protein groups stemming from cell damage or free radical production with respect of SB.

In a study it was stated that abnormal concentrations of nucleus material might cause a decrease in metabolic activity, and in some cases lead to the formation of irregular nucleus membrane <sup>[22]</sup>. In this study we can say that the abnormalities at nuclear level due to SB induced transportation of nucleus material into the cytosol and a decrease in cell activity.

Endoplasmic reticulum which is one of the important organelles of many compound cells gives rise to an increase in activity. This increase stems from additional production of ER related to damage membrane catabolism. Whatever the reason for this increase, it was emphasized in a study that this increase indicated hydroxylation enzymes for the hydrolysis of chemical causing increase and high levels protein synthesis to meet the demand of specific immune defense system and exhibited pathological situation of the cell <sup>[23]</sup>. In a study an increase in the number of mitochondria in the cells of mice and fish livers was attributed to some chemicals (SB and neopybutrin), and this condition was stated to be due to the increasing energy requirement of the cells <sup>[5,24]</sup>.

In our study, depending on the dose administered, in the groups exposed to SB, the cytoplasmic degenerative look of hepatocytes suggested the damage to the cells at the organelle level. Structural deterioration seen in the organelles inevitably cause deterioration in the protein, carbohydrate and lipid mechanisms. As in other cells, the damage in mitochondria, which have an important role in energy production in hepatocytes, such as expansion, loss of cristae, and changing permeability of membranes may be the reason for organelle homeostasis and cellular oxidative process. Indeed, the absence of the glycogen and reticular fibrils which were encountered more in the liver cells in the 36<sup>th</sup> stage compared to the 31<sup>th</sup> stage in both the groups exposed SB for both 2 and 5 days even at the lowest dose can be seen as an indication of deterioration of bio-synthesis activities such as glycogenesis in the hepatocytes. However, as information on the structure of the organelles is limited in the light microscopy studies, we suggest electron microscopy analyses which can provide detailed information on fine structure of cells to reach a definite conclusion on this issue.

SB hasn't any toxic effect on reproduction and development and gave negative results in carcinogenicity and most of the genotoxicity studies <sup>[25]</sup>. Zengin et al. <sup>[26]</sup> put forth that sodium and potassium benzoate decreased mitotic index and increased micronucleus (MN) frequency, and while SB significantly increased DNA damage, potassium benzoate did not. Although Sasaki et

al. <sup>[27]</sup> stated that SB (2000 mg/kg) in mice organs didn't create DNA damage at a statistically significant level, they found, in similar manner to the present study, in genotoxic studies carried out on food additive substances evidence of micronucleus, lagging chromosomes and anaphase bridges <sup>[2,28-30]</sup>.

Nucleolar Organizer Regions (NORs) which are located on the short branches of acrocentric chromosomes are the DNA segments which contain coding genes for rRNA <sup>[31]</sup>. It has been proven that the number of AgNOR in every nucleus is associated to cell proliferation activity <sup>[32]</sup> and it was emphasised that the number of AgNOR in various malignancies may be important in cytopathological diagnosis <sup>[33-35]</sup>.

In this study, it was found that in chicken liver cells during the development period, related to dosage, SB the caused a decrease in NOR regions and mitotic division, chromosome stickness, lagging chromosomes, budding in nuclei and formation of micronucleus. Many studies have found that various chemicals cause damage to chromosomes and micronucleus formation by inhibiting the formation of strands <sup>[36-38]</sup>. SB used in this study is also a chemical substance. Our study suggested that as a result of lagging chromosomes formed in the embryonic liver cells by SB, this substance caused formation of MN and as a result has a genotoxic effect.

Although it is not known how much of SB administrated during the embryonic development period passes to the offspring, it can be suggested according to the results, that the offspring were exposed to SB at a level at which they can be affected and they were affected adversely. We can conclude that the use of SB which is used as food preservative led to embryo growth retardation and histological changes in the liver. Therefore, this study indicates that there is a high risk of SB causing embryotoxicity. This also supported by the findings of other studies. To be able to reach a definitive conclusion, despite the fact that the effects of SB should be comprehensively investigated using different methodologies, we suggest that the use of SB as a food additive, especially during pregnancy, should be controlled, long-term high dosage usage limited and even banning of the addition of SB to human.

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