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RESEARCH ARTICLE

Anti-angiogenic and oxidative effects of sodium benzoate at different concentrations in chorioallantoic membrane model

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ABSTRACT

Aim: Due to the increased consumption of packaged foods, exposure to food additives is also increasing. Sodium benzoate (SB), a frequently used food additive, is generally used in alcoholic beverages, fruit, and vegetable juices, carbonated soft drinks, canned food, and various sauces. It is used to inhibit the formation of mold, yeast, and bacteria. This study was carried out to investigate the effects of SB exposure on angiogenesis and oxidant-antioxidant balance.

Materials & methods: Three different concentrations of SB, bevacizumab, and empty pellets were prepared, placed on chorioallantoic membrane (CAM), and examined for anti-angiogenesis. Total antioxidant capacity (TAC) and total oxidant capacity (TOC) measurements were made in the albumen samples, and oxidative stress index (OSI) value was calculated.

Results: The control group had no anti-angiogenic effect, but the bevacizumab group had a strong anti-angiogenic effect.10^{-3} M SB had a weak anti-angiogenic effect, but 10^{-4} M SB and 10^{-5} M SB showed no anti-angiogenic effect. TOC levels increased with SB in a dose-dependent manner. TAC levels decreased depending on the dose in the experimental groups with SB application. OSI levels increased depending on the dose increase in SB.

Conclusions: SB exposure caused a dose-dependent increase in oxidative stress and anti-angiogenic effect in CAM model.

Keywords: sodium benzoate, chorioallantoic membrane, food ingredient, oxidant, anti-oxidant, angiogenesis

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INTRODUCTION

Food additives are added to foods for flavoring, coloring, shelf-life extensions, and standardization. Nowadays, the use of food additives is increasing day by day, and many different chemicals are used as food additives [1]. Sodium benzoate (SB), a food additive, is used in alcoholic beverages, fruit, and vegetable juices, carbonated soft drinks, canned food, various sauces, and cosmetic products to inhibit the growth of mold, yeast, and bacteria [2].

Chicken chorioallantoic membrane (CAM) is an extraembryonic membrane. On the 4th day, the allantois fuses with the chorion and forms CAM. CAM is a tissue rich in blood vessels. Vascularization continues until the 11th day of development [3]. The chicken embryo CAM model is used in many studies, such as tumor angiogenesis, tumor transplantation, angiogenesis, and anti-angiogenesis experiments [4].

Although reactive oxygen species (ROS) perform many physiological functions in the body, even minimal temporary increases in ROS levels cause many effects in cells, tissues, and organs [5]. Antioxidant defense system balance the increase in ROS. Oxidative stress occurs when ROS production increases or when antioxidants cannot balance this increase [5, 6].

Bevacizumab is a monoclonal immunoglobulin G antibody and targets all isoforms of vascular endothelial growth factor-A (VEGF-A) by preventing VEGF-A from binding to endothelial cells.

Bevacizumab inhibits tumor growth by inhibiting the formation of new vessels and plays a role in treating many types of cancer [7]. In our study, we aimed to investigate the effects of SB, one of the commonly used food additives, on angiogenesis and oxidative stress in CAM model.

MATERIALS & METHODS

Every experiment step was carried out by paying attention to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. There is no requirement for animal protocol approval in this model [8]. This study was performed in the multi-disciplinary laboratory of the Alanya Aladdin Keykubat University Faculty of Medicine.

CAM

Fertilized chick eggs free of specific pathogens (SPF) were used in this study. Eggs that had not fully developed or been fertilized were excluded. 50 embryos were divided into five groups, with 10 in each group. The control group as the negative control group, the bevacizumab group as the positive control group, and three different doses of SB (10^{-3} M, 10^{-4} M, and 10^{-5} M) were determined. After disinfecting the fertilized chicken eggs with 70% alcohol on the 1st day, they were placed in the incubator at 37 °C, 60%-80% humidity. On the 3rd day of incubation, 4-5 cc of albumen was taken from the bottom of all eggs. The embryos were placed back in the incubator. On the 5th day of incubation, the window was opened from the opposite side of the hole. The pellets were placed on CAM, where vascular branching could be observed through the windows created. The eggs were placed in the incubator in an upright position. Angiogenesis in CAM was examined on the 6th, 7th, and 8th day of incubation. On the 8th day of incubation, the albumen was taken from each embryo and portioned into Eppendorf tubes. Albumen samples taken for evaluation were kept at -80 °C until they are used.

Anti-Angiogenesis Scoring

The anti-angiogenic effect was evaluated through the window under a stereoscopic microscope. The formation of new vessels from the major branches of the embryo and the expansion of the neovascularity have been assessed. The score showing the anti-angiogenic effect was performed according to the scoring principle determined in previous studies: 0, no change in relation to surrounding capillaries; 0.5, decreased capillary density but not more than pellet; 1, small capillary-free area, which is not bigger than twice the pellet size; 2, capillary-free area around the pellet at least twice the pellet size.

Average score values were obtained with the data obtained by scoring the evaluation of anti-angiogenesis in embryos, and anti-angiogenic effects were evaluated according to this Average score. According to this scoring system, scores less than 0.5 indicate no anti-angiogenic effect, scores between 0.5-1 indicate weak, and scores greater than 1 indicate strong anti-angiogenic effect. Average score values were calculated according to the formula given below [9]:

Average score value=([number of embryos (score 1)×1]+[number of embryos (score 2)×2])/total number of embryos

Measurement of Oxidative Stress Markers

Oxidative stress markers were quantified in albumen samples taken before and after application. Total antioxidant capacity (TAC) measurement was achieved by the automatic measurement method, which resulted in the addition of the liquid sample to the medium and the color change of ABTS radical (3-ethylbenzothiazollin-6-sulfonic acid) with the antioxidant effect in the added sample by a commercial kit (Rel Assay Diagnostic, Gaziantep, Turkey). The results were given in mmoL Trolox equivalent/L. The oxidation reaction was used to measure TOS values with a commercial kit (Rel Assay Diagnostic, Gaziantep, Turkey). The ferric ion formed a colored complex with xylenol orange in an acidic environment. The color intensity, which can be measured spectrophotometrically, is related to the sample's total amount of oxidant molecules. TOS levels are expressed in micromolar hydrogen peroxide equivalents per liter (μol H_2O_2 equivalent/L). Oxidative stress index (OSI) was calculated as the ratio of TOS measurements to TAC measurements [10].

Statistical Analysis

Anti-angiogenesis was evaluated by the average score values according to the scoring system described in previous studies [11]. The raw values were presented as the mean (M) ± standard deviation (SD). Oxidative stress markers were compared using the one-way analysis of variance test. For comparing the groups, Tukey and Duncans post-hoc tests were used. A p-value less than 0.05 was determined as statistically significant.

RESULTS

According to the average score values, no anti-angiogenic effect was observed in the control group, but strong anti-angiogenic effect in the bevacizumab group (average score 1.2) was observed. Moreover, weak anti-angiogenic effect in the 10^{-3} M SB group (average score 0.6), and no anti-angiogenic effect in the 10^{-4} M SB group (mean score 0.4), and 10^{-5} M SB group (mean score 0.1) were observed (**Figure 1**). The scores and the average score of all groups obtained by the calculations were given (**Figure 2**).

TAC values were significantly different between the post-control, 10^{-3} M SB, and 10^{-4} M SB groups compared to the pre-control groups. Although TOS values increased in the post-control, 10^{-3} M SB, 10^{-4} M SB, and 10^{-5} M SB groups compared to the pre-control groups, the highest increase was observed in 10^{-3} M SB group.

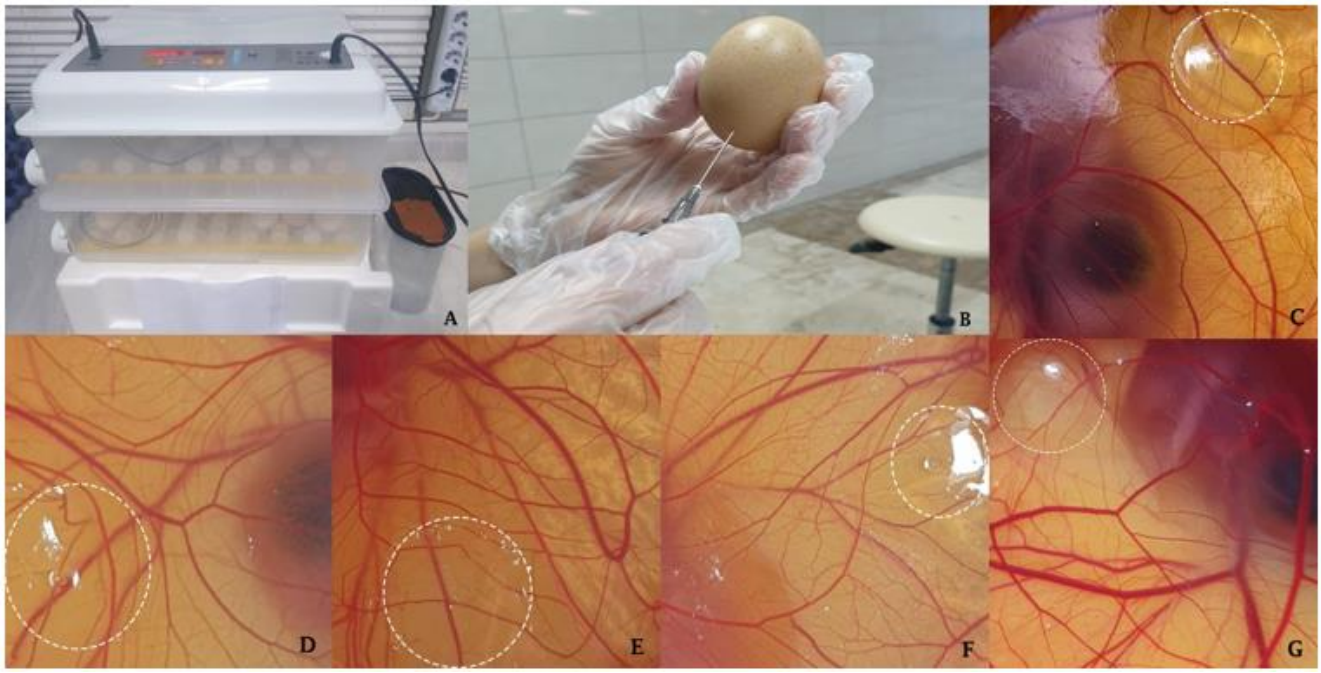

Figure 1. Fertilized chick eggs were placed in incubator (A), albumen was removed with a syringe (B), development of vascular bed after free pellet implantation (well-developed vascularity) (C), score 1 inhibition of vascular bed development after pellet implantation with 10^{-5} M SB (small capillary-free area & decreased capillary density) (D), score 1 inhibition of vascular bed development after pellet implantation with 10^{-4} M SB (small capillary-free area & decreased capillary density) (E), score 2 inhibition of vascular bed development after pellet implantation with 10^{-3} M SB (capillary-free area around pellet) (F), & score 2 inhibition of vascular bed development after pellet implantation with 10^{-6} M SB (capillary-free area around pellet) (Source: Authors' own elaboration)

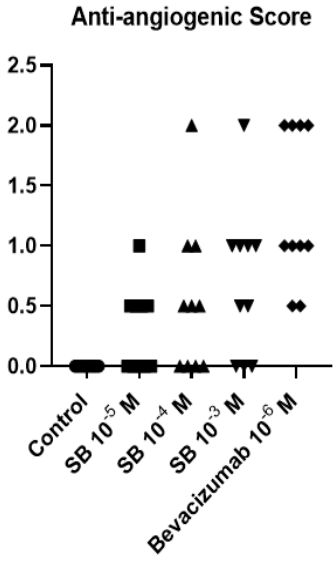

Figure 2. According to anti-angiogenic scoring system previously described anti-angiogenic scores of embryos of control, bevacizumab, & SB at different doses are given (Source: Authors' own elaboration)

Post- and pre-control according to OSI values; there is a significant difference between 10^{-4} M SB and pre-control, post-control, and 10^{-5} M SB groups. It was observed that OSI increased depending on the decrease in TAC level between SB consumption at different concentrations in the experimental groups. The 10^{-3} M SB group has the highest OSI value. The detailed TAC, total oxidant capacity, OSI results, and results of the post-hoc tests are given in **Table 1**.

DISCUSSION

With the increase in the use of packaged foods, food additives, which are used for protecting, coloring, and flavoring, are also increasing. SB is generally used in alcoholic beverages, fruit, and vegetable juices, carbonated soft drinks, canned food, various sauces, and cosmetics. It is a food additive used to inhibit the growth of mold, yeast, and bacteria [1]. Due to the increase in SB use, there have been concerns about whether SB used for food preservation is safe for human health or not [2]. In our study, we aimed to investigate the effects of SB on anti-angiogenic and oxidative stress in CAM model.

CAM model is frequently used to study angiogenesis and anti-angiogenesis [4]. As with any method, it has several advantages and disadvantages. The main disadvantages are non-specific inflammations, sensitivity, difficulty distinguishing between newly formed new vessels and old vessels and distinguishing between remodeling of vessels and new vessel formation [8]. In addition to such disadvantages, many advantages cause CAM model to be preferred. Among these advantages, CAM model provides faster results compared to mammalian models [12], direct access to the vascular system without any metabolic or hormonal effects from the mother, observing the results of the study macroscopically, performing histochemical studies with

Table 1. TAC & SD values of groups (A), TOC & SD values of groups (B), & OSI & SD values of groups (C)

(A) Total antioxidant status				
Groups	**Mean**	**SD**	**SEM**	**p-value**
(A) Total antioxidant status				
Pre-control	0.9097	0.07053	0.02230	
Post-control	0.7116	0.10495	0.03319	
10^{-3} SB	0.6440	0.13642	0.04314	<0.001
10^{-4} SB	0.6232	0.10812	0.03419	
10^{-5} SB	0.8527	0.14900	0.04712	
(B) Total oxidant capacity				
Pre-control	2.3838	0.42310	0.13379	
Post-control	4.4964	0.70808	0.22391	
10^{-3} SB	10.6408	1.19452	0.37774	<0.001
10^{-4} SB	7.0876	0.83312	0.26346	
10^{-5} SB	6.4722	1.62768	0.51472	
(C) Oxidative stress index				
Pre-control	2.6660	0.71458	0.22597	
Post-control	6.4190	1.25730	0.39759	
10^{-3} SB	17.0500	2.96829	0.93866	<0.001
10^{-4} SB	11.5700	1.61058	0.50931	
10^{-5} SB	7.6290	1.40797	0.44524	

different sensitivities such as light or electron microscopy. It is suitable for reverse transcriptase polymerase chain reaction studies, where gene expression is determined for samples, the direct effect of applications such as growth factors can be examined without any effect [9, 13], the cost is low, it is easy to access, and it is easy to follow the developmental processes and simplicity of application [4]. In addition, CAM model does not require ethics committee approval and is the closest model to a whole animal experiment since the embryo and membrane structure are preserved intact. CAM model is used in many studies as one of the most preferred methods, as an in vivo angiogenesis model, due to its many advantages [8].

VEGF is an important molecule involved in regulating neovascularization by inducing angiogenesis. VEGF expression is also involved in tumor formation and development by triggering tumor angiogenesis. Accordingly, VEGF inhibition is used in the treatment of many types of cancer [14]. Bevacizumab, a humanized monoclonal antibody obtained from Chinese hamsters, binds to VEGF-A isoforms and exerts its effects [7]. Bevacizumab is the first VEGF inhibitory agent used and approved for cancer treatment [15]. It has been shown in previous studies that bevacizumab causes anti-angiogenic effects in CAM model [16]. In our study, it was determined that bevacizumab caused a strong anti-angiogenic effect in accordance with these data. In this study, it is found that SB caused an increased anti-angiogenic effect at higher doses, but this effects was lower than the effects of bevacizumab.

Studies in zebrafish embryos have shown that SB causes developmental anomalies in the embryonic period and that SB reduces the hatching of zebrafish embryos depending on the dose [17]. Another study showed that SB caused deterioration of semen quality, and inflammation, increased p53 expression, and induced apoptosis. In addition, low-dose SB caused apoptosis and cell damage in testicles with long-term administration [18]. In our study, it was found that SB caused an anti-angiogenic effect at high doses, depending on the dose, which was consistent with these data.

It has been shown that SB causes damage by causing oxidative stress in the testes, kidneys, liver, embryos, and different organs in rats and zebrafish [19]. Free radicals can be formed from the metabolic products of SB [20]. Another study using chicken embryos showed that SB applied during the embryonic development period caused growth retardation in the liver of the embryo, deterioration in the vein structure, and histological changes causing embryonic toxicity. The significant decrease observed in the vitelline vessels of embryos after exposure was also detected macroscopically, and it was shown that harmful effects cause adverse consequences on organogenesis [21, 22]. In our study, consistent with these data, it was found that SB caused an anti-angiogenic effect at high doses, depending on dose.

Angiogenesis and oxidative stress are two closely related concepts. There is ample evidence in the literature that increased oxidative stress inhibits angiogenesis, and if the antioxidant system balances oxidative stress, angiogenesis and, thus tissue repair will be promoted. Angiogenesis is regulated by complex mechanisms. Many different factors activate or inhibit angiogenesis [2, 23]. In our research, it was determined that SB increases oxidative stress in a dose-dependent manner in CAM model, which is consistent with the studies mentioned. This study is one of the limited studies in the literature evaluating oxidative stress in CAM model and makes an essential contribution to the literature in this respect. In addition, SB induced increase in oxidative stress and OSI can trigger oxidative damage, which is a mechanism that can increase the damage in cells, tissues, and organs [6, 25]. For this reason, it may be recommended to be careful against SB exposure during pregnancy, which includes the formation and development stages of tissues and organs. In addition, an increase in oxidative stress can be shown as one of the possible mechanisms leading to the anti-angiogenic effect of SB.

For this reason, one of the possible causes for the anti-angiogenic effect of SB, which we have also shown as a result of our research, is the oxidant effect we found. In addition, it may be recommended to increase antioxidants that may be protective against such oxidative stress-increasing factors in pregnant women. In this way, OSI can be reduced. For antioxidant supplementation, a natural, healthy, and

balanced diet mainly based on fresh fruits and vegetables, avoidance of cigarettes, alcohol, and similar toxic chemicals, and regular exercise can be recommended. There are many determining factors in ensuring the oxidant-antioxidant balance. For this reason, TAC, TOS, and OSI measurement, which can show all the components of the relevant balance, is one of the superior aspects of our study [10].

It was determined that SB caused oxidative stress and anti-angiogenic effects during embryo development. Keeping in mind that embryo development is affected by SB exposure, the exposure of SB during pregnancy should be inspected. People especially pregnant woman must avoid from long-term and high-dose SB exposure.

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Ethics declaration: The authors stated that this study does not require animal protocol approval. Every experiment step was carried out by paying attention to the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Written informed consents were obtained from the participants.

Declaration of interest: No conflict of interest is declared by authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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