The Toxic Effects of Sodium Nitrite on Chick Embryo Second Pharyngeal Arche in *Vitro*

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ABSTRACT

Objective: The food additives, like sodium nitrite and nitrate, are used in many food products to prevent the growth of yeasts and molds. The histopathological effects of sodium nitrite in various tissues such as liver and kidney have been reported, but there has been no report about the histopathological effects on the embryonic organ development. Materials and Methods: 100 *Gallus gallus* fertilized eggs were incubated, then second branchial arches were separated on day 4 at stage 24, and divided into 4 groups which included a control and 3 experimental groups that were treated with 2000, 1000 and 500 µg/ml dosages of sodium nitrite. They were cultured for 7 days. For microscopic studies, the specimens were fixed, and the tissue passage was done. The tissue sections were stained with Hematoxylin-eosin, Toluidine blue, and the histopathological changes (including the number of cells and nucleus diameter) were evaluated. All data were analyzed by using SPSS/16 software ($p<0.05$). Results: The results showed that the mean number of cells from day 2 to day 7 in the experimental group treated with 2000µg/ml sodium nitrite were significantly decreased in comparison with the control ($p<0.05$) and the mean diameter nucleus from day 2 until day 7 in the experimental group (2000µg/ml) was also significantly decreased in comparison with the control ($p<0.05$). Histopathological studies showed the formation of cartilage matrix in the control and experimental groups (1000 and 500 µg/ml sodium nitrite). Conclusion: Considering the results of this study and the other studies, sodium nitrite can cause organ and tissue damages.

Key Words: *Branchial Arch, Mesenchymal Cells, Tissue Modeling, Sodium Nitrite, Toxicity*.

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INTRODUCTION

In the recent decades, the usage of chemicals as food preservatives has been expanded a lot. They can have various benefits. Preservatives can be divided into two categories:

1. Anti-microbial preservatives to prevent the growth of insects, bacteria, and fungi.
2. Antioxidants that prevent the oxidation of food constituents such as; sodium nitrite, sodium nitrate, sodium chloride, sulfates, etc.

Nitrite and nitrate are common additives in processed meat products [1]. When nitrite is added to foods such as cured meats, it can have at least three functions. First, it contributes to the flavor; this may be due to the inhibition of the development of rancid off-flavors. Second, it reacts with myoglobin to give mono nitrosyl hemochrome, which gives the characteristic pink color of cured meat. Third, it hinders the growth of food spoilage bacteria, such as, *Clostridium botulinum*. *C. botulinum* thrives under the anaerobic conditions, and makes neurotoxin which has been known as one of the most lethal natural products. When Nitrite is used while cooking and when salt is added, food poisoning can be prevented by this microorganism [2].

Nitrate and nitrite are used in diet from several various sources. Vegetables are a main source of nitrates, for

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example there exist nitrates about 1000 mg/kg in leaf vegetables like lettuce, and 200 mg/kg in root vegetables such as potatoes. The average levels of nitrite ($NaNO_2$) in cured meat products are in the range 10–40 mg/kg, comparing with the U.S. being in the lower part of the range [3].

In spite of all the technological advantages of nitrite, the creation of nitrosamine carcinogenic substances has caused a lot of concerns about using this additives [4]. Thus, by reducing the amount of sodium nitrite, nitrosamine formation can be significantly reduced [5]. In order to integrate the experimental studies, a model of living tissue (chick embryo second branchial arch) was chosen for continuing the experiments in vitro. Each arch has an external covering of ectoderm and inner covering of endoderm and a mesenchymal filling of a neural crest with a central core of mesoderm [6] between each of these different embryonic populations' components of the arch. The ectoderm forms the epidermis and the sensory neurons of the epibranchial ganglia [7], although, the endoderm rises to the epithelial lining of the pharynx and forms taste buds on the thyroid, parathyroid and thymus [8]. The neural crest cells form the skeletal and connective tissues of the arches [9], the mesoderm musculature, and the endothelial cells [9]. Second branchial arch is called hyoid, and its derivatives are the facial skeleton and laryngeal muscle cells [10].

The purpose of the present study was to investigate the effect of sodium nitrite in different densities on the second pharyngeal arch development of the chick embryo in vitro.

MATERIALS AND METHODS

This study was done in a developmental biology lab of Islamic Azad University, Mashhad Branch, Iran. In this experimental study to determine the toxic dosage in vitro, MTT test cell was done on two cell lines, normal L929 and cancerous MCF7. Finally, 2000µg/ml sodium nitrite dosage was confirmed as a toxic dosage. Then, 100 *Gallus Gallus* fertilized eggs were purchased from Agriculture North East of Iran, after that, they were placed in the incubator (37°c, humidity 67%). Then on the 4th day at 24th stage, the second branchial arches were separated in a sterile condition based on the micromanipulation [11]. All samples were washed with normal saline, and cultured in DMEM (Dulbecco's Modified Eagle's Medium) with%15 FBS (fetal bovine serum). The samples were divided into 4 groups as: the control group (I), the group treated with sodium nitrite in concentration of 500 µg/ml (II) , the group treated with sodium nitrite in concentration of 1000 µg/ml (III), and the last group treated with sodium nitrite in the concentration of 2000 µg/ml (IV). Then, all the samples were placed in the 24 house plates, and they were transferred to the co_2 incubator. On 2-7 days of culture, the tissues were removed from the medium. The histological proses were done. The samples were fixed with Bouin, and then were prepared for staining by Hematoxylin and Eosin (H&E), toluidine blue and PAS-Hematoxylin studied by light microscopy. Three slides were selected each day, and the number of cells were counted from different parts of slides randomly by 400X magnification, and also the diameter nucleus was measured. All data were analyzed by using 16th version of spss software. The P value less than 0.05 was reported as statistically significant.

RESULTS

The results illustrated in Figure 1 toluidine staining showed the formation of cartilage matrix during days 3-6 days in the control (I), and experimental groups II, III (1000 and 500 µg/ml sodium nitrite)(1B,1C,2B,2C,3B,3C) and also, the cartilage matrix was not formed in all days in 2000 µg/ml (D). With PAS technique in control group, the matrix glycoproteins were observed purple (PAS^+)(A) and treatment sodium nitric 2000µg/ml (PAS-)(B).

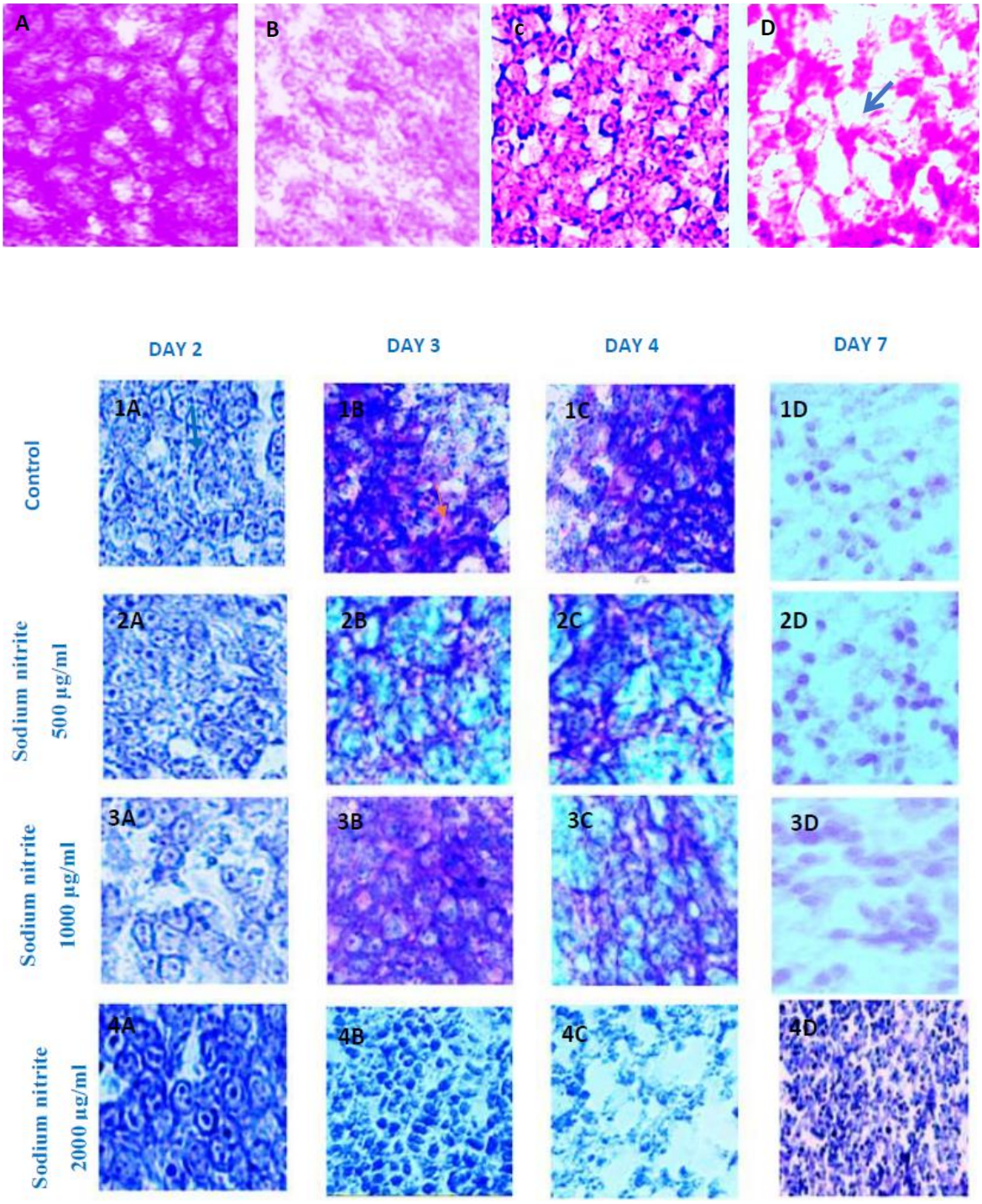

Fig. 1: On the third and fifth days, the treatment with Sodium nitri 500, 1000 µg/ml is similar to the control group of cartilage matrix (2,3B and C). On the second and seventh days, no cartilage matrix was formed in all treatments (A, D).
In 2000 µg/ml, the cartilage matrix was not formed in all days (D).

All data were analyzed by using SPSS/16 software ($p<0.05$).

The result showed that the mean number of cells from day 2 to day 7 in the experimental group treated with 2000µg/ml sodium nitrite were significantly decreased in comparison with the control ($p<0.05$), and the mean diameter nucleus from day 2 until day 7 in the experimental group (2000µg/ml) was also significantly decreased in comparison with the control ($p<0.05$).

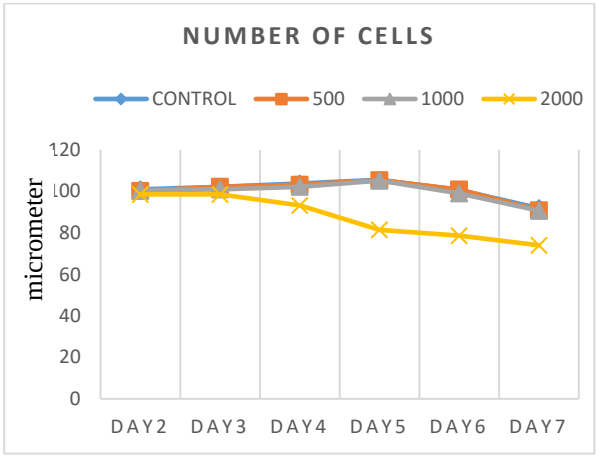

Fig. 2: linear diagram
Comparison of mean number of cells in different days between control and treatment groups

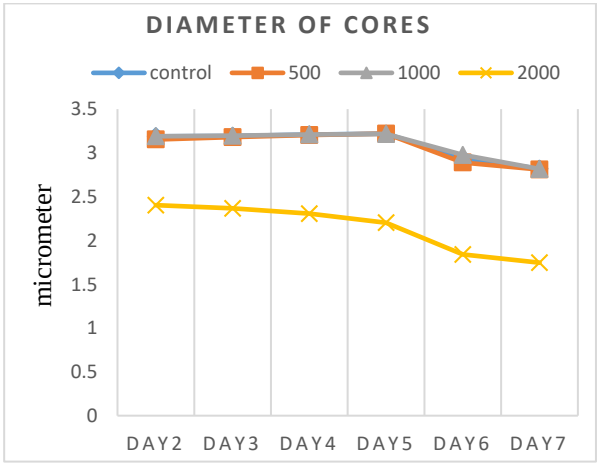

Fig. 3: linear diagram
Comparison of the average diameter of the nuclei in different days between the control and treatment groups

DISCUSSION

Nitrite and sodium nitrates as food preservatives have histopathological effects and are carcinogenic [12]. They can cause necrosis in liver cells, hemosiderin deposition in the liver, spleen and lymph nodes, testicular degeneration, they can also reduce the sperm motility and increase the female sex [13], decrease the levels of progesterone in cows fed with high levels of nitrate [14] and lead to the death of mouse spleen cells [15]. It has been indicated that sodium nitrite causes DNA double-strand breaks and sister chromatid union which is the breaking followed by a reunion of both sister chromatids at an identical site [16]. But there aren't any report of the histopathological effect of sodium nitrite in the second pharyngeal arch. This study showed that mean number of cells from 2-7days in the experimental group IV (2000 µg/ml sodium nitrite) was significantly declined in comparison with the control (Figure 2) and also, the mean diameter of nucleus from 2-7days in the experimental group IV (2000 µg/ml sodium nitrite) was extremely decreased in comparison with the control (Figure 3). It seemed that sodium nitrite treatment plan in this study probably has been effective to cell death, reducing the number of cells and the diameter of the nucleus.

The nitrite anion (NO_2^-) was considered physiologically neutral and as a source of nitric oxide (NO) metabolism. But, today with increasing evidence, it has been shown that nitrite lies at the center of the sensitive hypoxia and transfer chemistry signaling [17]. The most important nitrogen free radicals have been proxy nitrite and nitric oxide. The overproduction of these radicals leads to the oxidative stress that has a destructive effect on the cell physiology such as DNA strand break and the peroxidation of cell membranes, damages the transporter proteins in the membrane, damages the enzymes in the cell, changes the process of cell signaling, and changes the transcription and gene expression [18].

[16] studied the effects of different dosages of SN (sodium nitrite) on Swiss mice, and they observed mild to moderate degenerative changes of the liver and kidney. SN caused synthesis of nitric oxide and increased the activity of nitrotyrosine in the liver, and also cell death was observed in the treated groups with high concentration. The reduction of cell numbers and nucleus diameter in treated group IV (2000 µg/ml sodium nitrite) might be because of the oxidative stress. Studies by [19] on Methylnitrosourea (MNU) and marine foods indicated that this nitrogenous compounds with the effect of the cell cycle, apoptosis, DNA strand break, creating reactive oxygen species (ROS) could lead to the fetal malformation and retinal abnormalities. In a study by [20], the oral medication by SB (sodium benzoate) in zebra fish larvae was reported to cause the disarrangement of the muscular fibers, disorder in the neurotransmission of motional neurons, the intestinal abnormalities and kidney deformation as the administered dose increased; in fact, the effects on gene expression and the dysfunction of enzymes were caused by SB.

Also, in a study by [21], the oral administration of various concentrations of 30 to 120 mg / kg sodium benzoate at intervals of 48 hours for 14 days at high and low doses in white blood cell counts in rats were associated with a decrease in the cellular immunity and increased mortality.

Contrary to the results of the present study, the use of sodium nitrite, especially its high concentration, resulted in no formation of the cartilage matrix. Therefore, many antimicrobial agents appeared to have dose-dependent and high-dose histopathologic effects, and even mortality.

In conclusion, SN is an additive with the histopathological effects on the second branchial arches in

the chick embryo. This might be due to its effects on gene expression and cell cycle, and releasing free radicals in tissue. However, the mechanism of histopathological effect of this material needs to be clarified by more detailed studies.

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