



# Effects of Different Therapeutic Radiation Doses on the Development of Neural Tube Defects in Chick Embryos and the Correlation with Bone Morphogenetic Protein 4 and 7 Expression Levels

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## ABSTRACT

**AIM:** To investigate the effects of different therapeutic radiation doses on the prevalence of neural tube defects (NTDs) in chick embryos and bone morphogenetic protein (BMP) 4 and BMP7 expression levels.

**MATERIAL and METHODS:** The chick embryos (n=143) were derived from fertile, specific pathogen-free eggs of domestic fowl. The presence of NTDs was analyzed using a stereomicroscope, and BMP4 and BMP7 expression levels were assessed by immunohistochemical staining. The chick embryos were divided into five groups: control (no radiation exposure) (n=23), exposure to thorax computerized tomography (CT) (n=30); exposure to abdominopelvic CT (n=30), exposure to cranium CT (n=30), and exposure to brain perfusion CT (n=30).

**RESULTS:** The prevalence of NTDs and BMP4 and BMP7 expression levels in the different groups were compared. In the cranium CT dose group, both the NTD prevalence (20%, p=0.002) and BMP7 (p=0.031) expression levels were significantly higher than those in the other groups. However, none of the medical doses of irradiation altered BMP4 expression levels (p=0.242). No NTDs were detected in the thorax CT and abdominopelvic CT groups.

**CONCLUSION:** Exposure to irradiation at cranium CT doses may induce the development of NTDs and increase BMP7 expression. Dose radiation exposure using thorax CT and abdominopelvic CT protocols does not appear to induce NTDs.

**KEYWORDS:** Chick embryo, BMP4, BMP7, Neural tube defect, Radiation

## INTRODUCTION

Medical imaging and therapeutic radiotherapy are the main sources of human X-ray exposure, with the aforementioned imaging modalities responsible for approximately 40% of annual exposure in the general

population (13). Animal studies have proven that acute or chronic low-dose-ionizing-radiation (i.e.,  $\leq 100$  mSv) and low-dose-rate-ionizing-radiation (i.e.,  $< 6$  mSv/h) are harmful to human health. Radiation exposure may trigger genetic or epigenetic changes and cause abnormal brain growth,

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abnormal embryological development, circulatory problems, early menopause, and tumorigenesis (13). It can also have adverse effects on the immune system and longevity (13).

Bone morphogenetic proteins (BMPs) are extracellular signaling proteins that belong to the transforming growth factor beta family (5). Although they were once thought to play a role only in bone development, it is now known that they play key roles in the development of many organs and systems, especially the central nervous system (12). Oligodendrocytes and glial cells are highly sensitive to the effect of BMPs during embryonic development (12). They direct several functions, including cell proliferation, apoptosis, maturation, and migration. There are 20 structural forms of BMPs. Among these, BMP2, 4, and 7 have effects on glial cells, with most studies focusing on the effect of BMP4 on these cells (12).

During the development of the central nervous system, BMPs are found mostly in the roof plate and dorsal regions. They may also be found in ventral regions in low numbers (8,12). In ventral regions, noggin, an endogenous BMP-specific inhibitor, acts as an antagonist of BMPs. In dorsal regions, BMPs exhibit a dorsalizing effect on neuronal development by enlarging the ventral sides of the neural tube (18). Previous research demonstrated in vitro developmental effects of BMPs in animal studies in which BMPs were overexpressed or inhibited. Exposure of chick embryo neural tubes to the BMP inhibitor noggin induced the formation of dorsal oligodendrocytes (9). Dorsal factors, including BMP4 and 7, induced oligodendrocyte formation in chick embryo spines by inhibiting lineage specification in vivo in middle and dorsal regions (9).

Neural tube defects (NTDs) are serious congenital malformations of the brain and spine. It is estimated that globally 300,000 newborns each year have NTDs (9). Common forms of NTDs are anencephaly and myelomeningocele, a type of spina bifida, which are caused by a closure defect in the brain or spine (7). Most NTD cases are sporadic and nonsyndromic. In terms of NTD prevention, according to previous research, periconceptional folic acid intake can reduce the prevalence of NTDs by 50–70%. However, not all NTDs can be prevented by folic acid supplementation. Other factors that can prevent the development of NTDs need to be identified, and new preventive consultant strategies for NTDs need to be established (4).

The hypothesis of the present study was that different therapeutic radiation doses administered during medical

investigations or treatment would alter the expression levels of BMP4 and 7, which may play a role in NTD progression.

## ■ MATERIAL and METHODS

### Chick Embryos and Ionized Radiation Application

Fertile, specific pathogen-free eggs of domestic fowl (*Gallus gallus domesticus*; Has tavuk, Bursa, Turkey) were used in this study. The eggs were incubated at 37.5°C and 75% relative humidity for 24 hours until the embryos reached Hamburger and Hamilton stage six of development (6).

The eggs were divided into five groups and exposed to different types of CT radiation. In group 1 (n=23), the eggs were not exposed to any irradiation (control group). In group 2 (n=30), the embryos were irradiated using a thorax computerized tomography (CT) protocol. In group 3 (n=30), abdominopelvic CT was applied. In Group 4, routine head (cranium) CT (adult dose) was applied. In group 5 (n=30), a brain perfusion CT protocol (adult dose) was used (Table I).

All CT scan protocols were calibrated using reference CT dose values recommended by the American Association of Physicists in Medicine. A 64-detector multislice CT scanner (SOMATOM Definition; Siemens Medical Solutions, Forchheim, Germany) was used. Dosimetric data and scan parameters were extracted from the Digital Imaging and Communication in Medicine Header of the CT images. An optically stimulated luminescence (OSL) dosimetry system was used to measure the doses of ionizing radiation. The system included an InLight nanoDot OSL dosimeter and a MicroStar reader (Landauer Inc., Glenwood, IL, USA). Thirty OSL nanoDot detectors were positioned at opposite poles of each of the 30 eggs in all the groups. Prior to irradiation, background radiation doses of OSL nanoDot were read using a MicroStar reader (Landauer Inc.). After irradiation by the CT scan, the OSL dosimeter was removed, and the radiation dose was read by the reader. Radiation values in dosimeters were subtracted from the final results. Irradiation dose levels were measured in milligray (mGy) with ±5% tolerance.

### Embryo Collection

After irradiation, the eggs were placed in an incubator for 24 hours until developmental stage 12. At the end of incubation (i.e., 48 hours from the onset of the experiment), the eggs were cracked open. After dissection of the allantoic stalk from other embryonic structures, the embryos were transferred to a petri dish.

**Table I:** Chicken Egg Groups and Their CT Radiation Protocols

Group	n (Eggs)	Radiation Dose
Control	23	Not exposed to radiation
Chest CT	30	Irridiated using lung cancer screening chest CT protocol
Abdominopelvic CT	30	Irridiated using adult routine abdominopelvic CT protocol
Brain CT	30	Irridiated using adult routine head CT protocol
Brain Perfusion CT	30	Irridiated using adult brain perfusion CT protocols

### Examination Under a Stereomicroscope and Analysis of BMP4 and BMP7 Expression by Immunohistochemistry

Tap water was added to the embryos in the petri dish, and they were examined under a stereomicroscope (Olympus, SZX/SZ, Olympus Corporation, Japan) by a pathologist blinded to the protocols. Neural tube closure and neural tube developmental abnormalities were recorded.

The specimens were kept in 10% formalin solution for 48 hours prior to a histopathological assessment. In accordance with standard practice, the specimens were passed through an alcohol (70%, 80%, 90%, 96%, and 100%) and xylol series and then embedded in paraffin blocks. Each block was sliced into 4 µm-thick sections and treated with BMP4 and BMP7 and the following primary antibodies (BMP4: 1:100, rabbit, polyclonal, Biorbyt LLC., San Francisco, CA, USA; BMP7: 1:100, rabbit, polyclonal, Biorbyt LLC). Immunohistochemical staining was performed using a Ventana Benchmark XT autostainer with an XT ultraView DAB kit (Ventana Medical Systems, Roche Diagnostics Co., Mannheim, Germany). Cytoplasmic and nuclear staining were accepted as positive for cellular immunohistochemical expression. A pathologist blinded to the study protocol assessed the clinical findings using a light microscope. (Nikon Eclipse CI; Nikon, Amsterdam, the Netherlands). For automatic scoring, the histogram profile of each image, which indicated the pixel count to a specific intensity level, was evaluated using the program Nis Elements 4.30 (Nikon, Amsterdam, the Netherlands). Pixel density levels for an unspecific stain were excluded. For all tissues, the intensity level was determined after manual selection of the area by the pathologist.

### Statistical Analysis

The conformity of the data (dose amount, BMP4 level, and BMP7 level) to a normal distribution was investigated using the Shapiro–Wilk test. Related variables were expressed as median (minimum: maximum) according to the test results. The Kruskal–Wallis test was used to compare BMP4 and BMP7 levels in the various groups. For binary comparisons between groups, the Dunn–Bonferroni method was used. The Mann–Whitney U test was used to compare NTDs and BMP4 and BMP7 expression levels in the different radiation dose groups as compared with those in the control group. The NTD

variable was expressed as numbers and percentages, and intergroup comparisons of NTDs were investigated using the Fisher–Freeman–Halton test. For subgroup analysis, Fisher's exact chi-square test was used. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, USA). The accepted significance level was  $\alpha=0.05$ .

### Ethics Committee Approval

All the experimental procedures were reviewed and approved by the animal research ethics committee of Balikesir University (Decision No: 2018/8-7). Animal care and all experiments adhered to European Communities Council Directive (86/609/EEC) on the protection of animals for experimental use.

## RESULTS

Table II provides data on NTDs and BMP4 and BMP7 expression levels in the different radiation dose groups as compared with those in the control group. No NTDs were detected in the thorax CT and abdominopelvic CT groups. NTDs were detected in 1 (3.3%) chick embryo in the perfusion CT group, and 6 (20%) chick embryos in the cranium CT group (Figure 1A, B). In the subgroup analysis, the prevalence of NTDs in the cranium CT group was higher than that in the abdominal CT and thorax CT groups ( $p=0.002$ ).

There was no statistically significant between-group difference in BMP7 levels. In the subgroup analysis, the median BMP7 expression level in the cranium CT group was higher than that in the abdominal CT, thorax CT, and control groups ( $p=0.001$ ) (Figure 2A, B; Table III).

There was no statistically significant between-group difference in BMP4 levels ( $p=0.242$ ).

## DISCUSSION

This study investigated the effect of different therapeutic radiation doses on chick embryos in terms of the prevalence of NTDs as assessed by a stereomicroscope study and BMP4 and BMP7 expression levels, which were determined by an immunohistochemical investigation. In the cranium CT group, both the NTD prevalence and BMP7 expression level were

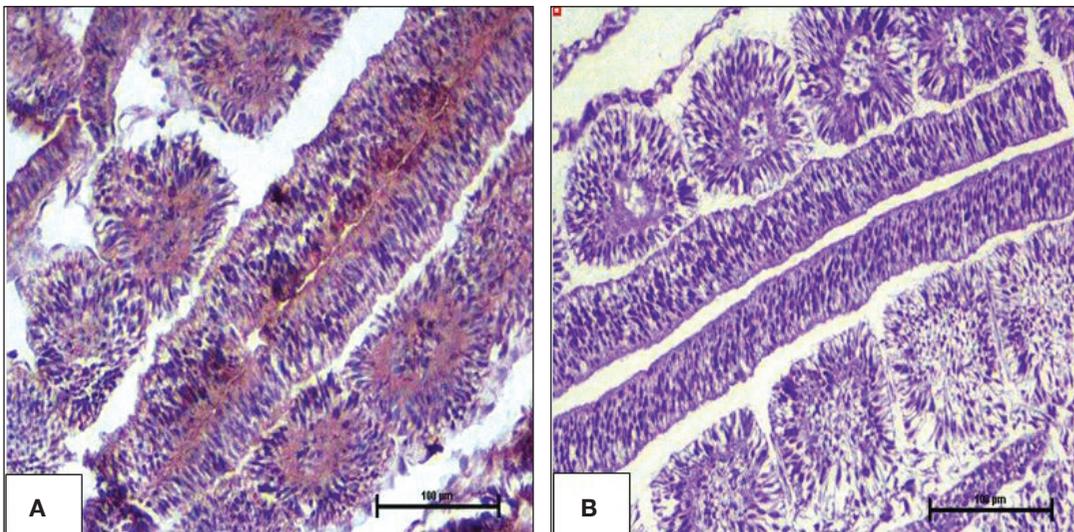
**Table II:** The Details of CT Radiation and the Presence of Neural Tube Defects and the Results of the Expression of BMP4 and BMP7

	Dose median (min-max.)	NTD n (%)	BMP7 median (min-max.)	BMP4 median (min-max.)
Control	0	0 (0)	171.50 (152-202)	133 (80-190)
Chest CT	1.10 (0.61-1.55)	0 (0)	166 (147-187)	135.50 (83-182)
Abdominopelvic CT	22.95 (17-31)	0 (0)	168.50 (143-210)	128 (90-202)
Cranium CBT	47.04 (35.41-58.98)	6 (20)	203 (124-218)	132 (91-205)
Brain Perfusion CT	321.45 (233.30-571.26)	1 (3.3)	183 (159-215)	144.50 (121-194)
<b>p</b>	-	<b>0.002<sup>a</sup></b>	<b>&lt;0.001<sup>b</sup></b>	0.242 <sup>b</sup>

**a:** Fisher-Freeman-Halton Test, **b:** Kruskal Wallis Test.



**Figure 1:** **A)** Closed neural tube of a normal chick embryo in control group. **B)** Neural tube abnormality (pointed by arrow).



**Figure 2:** **A)** The expression of BMP7; chicken embryo cells showing distinct cytoplasmic apical immunoreactivity (x100). **B)** The chicken embryo cells showing no nuclear immunoreactivity of BMP7 (x100).

significantly higher as compared with the same parameters in the other groups. The medical doses of irradiation administered did not alter BMP4 expression levels. No NTDs were detected in the thorax CT and abdominopelvic CT groups.

Since their first discovery as osteoinductive factors, BMPs have been shown to affect the neurological system, especially in terms of neuroectoderm induction, neural crest cell specification, and neuronal arrangement in the central nervous

system (3). BMP ligands attach to serine/threoninekinase transmembrane receptors and initiate phosphorylation of signal induction molecules SMAD1, SMAD5 and SMAD8 (10). These phosphorylated SMADs bind to SMAD4 and penetrate the cell nucleus to regulate target gene transcription (1,10).

BMP4 plays a critical role in early murine embryological development, with deletion of BMP4 proving lethal 6.5–9.5 days postcoitum (19,20). BMP expression in early

**Table III:** NTD Levels according to BMP7 Expression, Subgroup Analysis

$(p_{\text{group } i \text{ v.s. } \text{group } j})$	Pairwise Comparisons		
	Dose	NTD	BMP7
<b>P</b> <i>Abdominal CT – Thorax CT</i>	-	1.00	1.00
<b>P</b> <i>Abdominal CT – Control</i>	<0.001	1.00	1.00
<b>P</b> <i>Abdominal CT – Cranium CT</i>	-	0.027	0.001
<b>P</b> <i>Abdominal CT – Perfusion CT</i>	-	0.463	0.138
<b>P</b> <i>Thorax CT – Control</i>	<0.001	1.00	1.00
<b>P</b> <i>Thorax CT – Cranium CT</i>	-	0.023	<0.001
<b>P</b> <i>Thorax CT – Perfusion CT</i>	-	0.422	0.068
<b>P</b> <i>Control – Cranium CT</i>	<0.001	0.029	0.031
<b>P</b> <i>Control – Perfusion CT</i>	<0.001	0.475	0.861
<b>P</b> <i>Cranium – Perfusion CT</i>	-	0.213	1.00

embryological development is actively inhibited by the secretion of embryonic factors, including noggin, chordin, and follistatin, to initiate neural induction (3). In vitro treatment of human embryonic stem cells with noggin induces degradation of SMAD4 transcripts via microRNA. This mechanism blocks the BMP4-SMAD signal pathway during neural induction in vivo. Repression or activation of BMP signaling, in conjunction with a corresponding gradient of sonic Hedgehog (shh) expression, specifies whether ectoderm gives rise to neuronal or nonneuronal tissue (3). In neuronal differentiation of chick embryos, fibroblast growth factor 3 (FGF3), BMP4, and BMP7 are expressed in cells destined to become neural cells. FGF signaling is essential for inhibiting BMP expression and for neural cell development. Besides, later inhibition of BMP on epiblast cells may inhibit neural tissue development and induce epidermal-characterized cells (17). BMP7 and FGF8 are expressed in the branchial arches in chick embryos. BMP7 and FGF8 are expressed in the posterior ectodermal margin of the second arch. Thus, inhibitor signals of BMPs influence the activity of FGFs (16). During chick gastrulation, BMP2, 4, and 7 are strongly expressed in the neural plate border, whereas phosphorylated SMAD1, 5, and 8 are moderately expressed in the neural plate border (2,11).

Recent research showed that de novo mutations were a common cause of neurodevelopmental malformations, such as NTDs (14). According to previous research, the prevalence of NTDs was 0.1–0.2% (7).

## CONCLUSIONS

Ionizing radiation is widespread, with potential sources including human-made sources, such as industry, especially the medical industry, and naturally occurring sources (radon and cosmic). Cumulative exposure as a result of repetitive exposure to low-dose medical radiation, such as via CT, may exceed permissible limits (15). Radiation exposure through

inappropriate use of diagnostic modalities is a cause of concern. We believe that the low-dose effects of irradiation, especially in the case of pregnant women and embryos, should be investigated in detail. In our study, there was no significant difference in the BMP4 expression level in any of the groups, irrespective of the irradiation dose. However, in the cranium CT group, BMP7 levels were significantly higher, with a significant increase in NTD development. Our results suggest that exposure to irradiation at cranium CT doses may induce the development of NTDs via increased expression of BMP7.

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