Turk Neurosurg 31(2):268-273, 2021

Original Investigation

DOI: 10.5137/1019-5149.JTN.32092-20.1

Received: 21.08.2020
Accepted: 01.10.2020

Published Online: 03.12.2020

The Evaluation of Survivin and Bcl-2 Expression on the Medical Radiation Doses for Neural Tube Defect Development

Huseyin Utku ADILAY[1], Salim KATAR[1], Bulent GUCLU[2], Mevlut Ozgur TASKAPILIOGLU[3], Eren ALTUN[4], Recep OZDEK[1], Mehmet TIRYAKI[2], Ahmet BEKAR[3]

[1]Balikesir University School of Medicine, Department of Neurosurgery, Balikesir, Turkey
[2]Dr. Lutfi Kirdar Kartal Research and Training Hospital, Neurosurgery Clinic, Istanbul, Turkey
[3]Uludağ University School of Medicine, Department of Neurosurgery, Bursa, Turkey
[4]Balikesir University School of Medicine, Department of Pathology, Balikesir, Turkey

Corresponding author: Salim KATAR ✉ salimkatar@gmail.com

ABSTRACT

AIM: To investigate the effects of different radiation doses on the development of the neural tube defect in chick embryos using computed tomography (CT), and assess its correlation with survivin and Bcl-2 expressions.

MATERIAL and METHODS: A total of 150 chicken eggs were used and grouped into five categories. In Group 1 (n=30), the embryos were not exposed to radiation. In Group 2 (n=30), the embryos were irradiated using lung cancer screening chest CT protocol. In Groups 3 and 4 (n=30 each), the abdominopelvic and adult routine head CT protocols, respectively, were used to irradiate the embryos. In Group 5 (n=30), the embryos were irradiated using adult brain perfusion CT protocol. Subsequently, the embryos were examined under a stereomicroscope to assess the presence of neural tube developmental abnormalities. Moreover, immunohistochemical staining was performed to determine the survivin and Bcl-2 expression levels.

RESULTS: The risk of developing neural tube defect increased with the amount of exposed radiation. Moreover, no significant correlation was observed between the survivin and Bcl-2 expression levels and the radiation dose.

CONCLUSION: Overall, the results of this study indicate that the radiation from CT may cause neural tube defect in chicken embryos.

KEYWORDS: Bcl2, Chicken embryo, Neural tube defect, Radiation, Survivin

ABBREVIATIONS: CNS: Central nervous system, **CT:** Computed tomography, **DAB:** Diaminobenzidine, **mGY:** miliGray, **NTDs:** Neural tube defects, **OSL:** Optically stimulated luminescence, **PBS:** Phosphate-buffered saline

INTRODUCTION

Neural tube defects (NTDs) are common congenital malformations of the central nervous system characterized by incomplete closure of the neural tube during the primary or secondary gestation period. The incidence of NTD ranges from 1 to 10 per 1000 births (13). NTDs account for 7% of all neonatal deaths attributable to congenital malformations; survivors with NTDs typically experience a lifelong disability (3).

In the diagnosis and treatment of many diseases, radiation is commonly preferred. According to the United States Department of Health and Human Services, almost 33,000 females undergo abdominal radiation with diagnostic purposes in the first months of their pregnancy each year in the USA. Ionizing

Huseyin Utku ADILAY : 0000-0003-4867-244X
Salim KATAR : 0000-0002-8311-1122
Bulent GUCLU : 0000-0002-0012-5706
Mevlut Ozgur TASKAPILIOGLU : 0000-0001-5472-9065
Eren ALTUN : 0000-0001-9110-8364
Recep OZDEK : 0000-0002-7625-2269
Mehmet TIRYAKI : 0000-0002-2864-0515
Ahmet BEKAR : 0000-0002-2716-1985

radiation has been known to have an effect on the fetus and embryo, particularly the brain (2). It is a central nervous system (CNS) teratogen and associated with some mental problems such as retardation. According to Schull et al., the lowest dose of gestational radiation exposure at any day that produces significant postnatal behavioral changes is 0.2 Gy (19).

Although several studies have shown the harmful effects of radiation on brain development and mental status, the impact of radiation on neural tube development has not been investigated. Therefore, the aim of this study is to investigate the effects of different radiation doses on the development of NTD in a chick embryo model using computed tomography (CT) and assess its correlation with survivin and Bcl-2 expressions.

Survivin is a gene that is encoded by a 142-amino acid protein. In humans, it is located in the 17q25 region and is 14.7 kb long. Survivin is a gene that has been pointed out in various cancer types such as lung, kidney, skin, endometrial, stomach, colon, breast, prostate, ovarian, head, and neck cancers and leukemia; that is a member of the inhibitors of apoptosis; and that was studied several times by researchers due to its histopathology and polymorphisms in its promoter region (6). Because of its inhibitory functions against caspase activity, the survivin protein results in a negative selection of apoptosis or a programmed cell death. It has been demonstrated that the frequency of apoptosis increases and tumor growth decreases with the deterioration of the pathways inducing survivin expression (1). Survivin is not expressed in most normal adult tissues but is highly expressed in solid organs and hematologic malignancies linked with increasing angiogenesis and tumorigenesis. However, survivin is expressed anywhere during human and murine embryonic development (14). In a study, it was demonstrated that decreasing the expression of survivin resulted in the reduction of eye and head sizes in embryos (14). On the other hand, survivin's role in embryonic development remains unclear.

Programmed cell death is a common trait of neurodevelopment in all vertebrates. Bcl-2 proto-oncogene is shown to have a role in protecting various cell types from programmed cell death. Bcl-2 protein is expressed extensively in embryonic development when Bcl-2 protein distribution was examined in developing and adult nervous systems (16). Proliferative ventricular regions express postmitotic cells of the cortical plate, cerebellum, hippocampus, and Bcl-2 in spinal cord in addition to neuroepithelial cells (15). In a study, it was demonstrated that p53, Bax, and Caspase-3 genes were active in embryonic cells in early embryonic development stages and Bcl-2 gene in the middle embryonic stage. In the same study, it was also demonstrated that apoptosis-related genes and proteins are organized developmentally in the embryonic cells of *M. olfersii* and result in apoptosis after being exposed to UVB radiation for 12 hours (18). Bcl-2 protein expression is more common in fetal tissues than in adult ones. Therefore, it acts as a supporter for various fetal morphogenesis and for the inducible cell survival as a regulator in normal homeostasis and morphogenesis (11).

In this study, our main hypothesis is that different radiation doses for the purpose of medical diagnosis and treatment can affect apoptosis mechanism by changing the survivin and Bcl-2 expressions and can have a role in the development of neural tube defects by disrupting morphogenesis during neural development.

MATERIAL and METHODS

Chick Embryos and Ionized Radiation Application

In the study, we used specific pathogen-free eggs of a domestic fowl (Has tavuk®, *Gallus gallus*, Bursa, Turkey). Until the embryos rose to Hamburger and Hamilton development stage 6, the eggs were incubated at 37.5°C and 75% relative humidity for 24 hours (8). At this stage, the eggs were grouped into five categories. In Group 1 (n=30), the embryos were not exposed to any radiation. In Group 2 (n=30), the embryos were irradiated using lung cancer screening chest CT protocol. In Groups 3 and 4, abdominopelvic and adult routine head CT protocols, respectively, were used to irradiate the embryos. In Group 5 (n=30), the embryos were irradiated using adult brain perfusion CT protocols. Moreover, all CT scan protocols were calibrated with the reference CT dose values recommended by the American Association of Physicists in Medicine. The embryos were positioned on the CT table in different places for abdominopelvic, lung, and cranial CT protocols to make more precise radiation doses. The embryos under the abdominopelvic CT protocol were placed in the middle of the CT radiation area, those under the lung CT protocol were placed 35 cm away from the middle of the CT radiation area, and those under the cranial CT protocol were placed 70 cm away from the middle of the CT radiation area. 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 (Table I). The optically stimulated luminescence (OSL) dosimetery system was used to measure doses from ionizing radiation. It comprises the InLight nanoDot OSL dosimeters and the MicroStar reader (Landauer Inc., Glenwood, IL, USA). Thirty OSL nanoDot detectors were positioned at opposite poles of each of the 30 eggs in all groups. Prior to irradiation, the background radiation doses of OSL NanoDot were read by the MicroStar reader. After irradiation with CT scan, OSL dosimeters were removed from the eggs and read by the reader. The radiation values in dosimeters were subtracted from the final results. Radiation dose levels were measured in miliGray (mGy) with a ±5% tolerance.

Embryo Collection

We placed the eggs into an incubator for 24 hours again until Hamburger and Hamilton development stage 12 was reached. The incubation lasted 48 hours. Following the incubation process, the eggs were cracked open. Moreover, the allantoic stalk from other embryonic structures were dissected, and the embryos were transferred to a Petri dish. If no embryo was found in the eggs, new eggs were incubated to reach 30 embryos in each group.

Table I: The Details of Computerized Radiation Protocols of the Chicken Egg Groups

	Group 1	Group 2	Group 3	Group 4	Group 5
Scan Mode	-	Spiral	Spiral	Spiral	Spiral
Rotation time	-	0.5/s	0.5/s	1 s	0.33/s
Dedectors configuration	-	64x0,6 mm	64x0.6 mm	64x0.6 mm	64x0.6 mm
Spiral pitch Factor	-	1.2	0.6	0.65	0.74
kVp	-	120	80	120	80
CTDIvol*	-	0.48 mGy(L)	14.6 mGy(S)	59.91 mGy(L)	259.97(x6) (S)
DLP**	-	22 mGy.cm	478 mGy.cm	1843 mGy.cm	13407mGy.cm
Care Dose 4d	-	On	Off	Off	Off

***CTDIvol:** Computed Tomography Dose Index, ****DLP:** Dose Lenght Product.*

Examination under Stereomicroscope and Analysis of Survivin and Bcl-2 Expressions by Immunohistochemistry

Some tap water was added to the embryos, which were transferred to a Petri dish and investigated via the stereomicroscope (Olympus SZX/SZ) by a blinded pathologist in terms of NT closure assessment and the existence of NT developmental abnormalities, if any (Figure 1).

Following the stereomicroscopic examination, 10% buffered formalin was used to fix all embryos, and a graded ethanol series was used for dehydration. The embryos were washed twice before incubating in xylene and were then placed into a paraffin-embedded mixture.

Four-micrometer-thick tissue sections placed on poly-lysine coated slides were incubated for a night at 60°C. To deparaffinize the slides, xylene was used. In addition, graded alcohol/water mixture and antigen retrieval in a microwave oven were used for rehydration. We cooled the tissues to room temperature. A PAP pen (Invitrogen Corporation, CA, USA) was used to draw the limits of the sections. Additionally, to inhibit the endogenous peroxidase activity, the tissues were incubated in 3% hydrogen peroxidase for 15 minutes. Moreover, they were then washed thrice with phosphate-buffered saline (PBS) (5 minutes each) and incubated in a blocking solution.

To perform immunohistochemical staining, primary antibodies including monoclonal Bcl-2 antibody (1:100, Sigma C 3865, Missouri, USA) and rabbit polyclonal survivin antibody (1:100, Abcam- ab16645, Boston, MA, USA) were used for the incubation of the sections for 1 hour at 37°C.

After performing the PBS washing thrice, the secondary antibody (SPlink HRP Broad DAB Bulk Kit for Mouse and Rabbit Antibodies, GBI Labs, Mukilteo, WA, USA) was used for 30 minutes. Then, we added the streptavidin–peroxidase complex for 30 minutes and performed PBS washing thrice. Following the processes mentioned above, fresh 3,3′-diaminobenzidine (DAB) (GBI Labs, Mukilteo, WA, USA) chromogen was used to incubate slides for 1–2 minutes (prepared in a ratio of 1:20). To clean off the DAB, the slides were washed in water, dehydrated, cleared, and mounted. A brown precipitate showed positive results in terms of the primary antibody.

The slides were examined and photographed under a light microscope (Nikon Eclipse CI) by a blinded pathologist. The intensity of survivin and Bcl-2 staining was quantified using the Nis Elements 4.30 computer software. To differentiate the pure DAB-stained areas that left a complimentary image, a color deconvolution technique was used. The range of the intensity of pixel differentiated from the DAB images was between 0 and 30. The lightest shade of the color was represented with a value of 30, whereas the darkest shade of the color was represented with a value of 0. The Nis Elements 4.30 standard program was used to create a histogram profile of each image. Moreover, an automated score was given to evaluate the pure DAB staining pattern. The values of pixel intensity which corresponded to non-specified staining values were excluded from the analysis. The area of interest was manually selected under the guidance of an expert pathologists. Then, we calculated the intensity for each chicken embryo. Overall, the histogram intensity showed us that the results were compatible with the four groups defined by the manual scoring (negative, 0; low, 1; moderate, 2; or high, 3).

Statement of Ethics

In this study, all the experimental procedures used in this investigation were reviewed and approved by the Animal Research Ethics Committee of Balikesir University (2018/8). Animal care and all experiments adhered to the European Communities Council Directive of November 24, 1986 (86/609/EEC) related to the protection of animals for experimental use.

Statistical Analysis

Fisher–Freeman–Halton test, chi-squared test, and Fisher's exact chi-squared test were performed to assess between-group differences regarding the categorical variables. Statistical significance was set at $\alpha=0.05$. The SPSS (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY) program was used for statistical analyses.

RESULTS

Our study showed that high-dose CT radiation may cause neural tube developmental abnormalities in chicken embryos (p=0.01) (Figure 2). Survivin intensity score did not change with different CT radiation doses (p>0.05). No significant correlation between the CT radiation-related neural tube developmental abnormalities and survivin expression was observed. No significant change in Bcl-2 intensity score was observed at different CT radiation doses (p>0.05) (Figure 3). Furthermore, no significant correlation between the CT radiation-related neural tube developmental abnormalities and Bcl-2 expression was observed (Table II) (Figure 4).

DISCUSSION

The harmful effects of radiation were well documented. Although the effect of radiation exposure on animals has been extensively studied, its effect on humans is not well characterized yet. Brent mentioned that a radiation dose of as low as 0.5 Gy may be harmful to the human fetus (2). Dekaban retrospectively analyzed the records of 200 pregnant women who were exposed to radiation between the 3rd and 20th week of gestation for diagnostic purposes and found that 22 infants exhibited microcephaly and/or mental retardation (4).

Otake and Schull reviewed the data pertaining to people affected by atomic bomb radiation. They used the absorbed fetal dose estimations and indicated that 8–15 weeks of gestational age was the most critical period in terms of brain damage risk with a nonlinear dose–response relationship (17,19). Granoth found a close relationship between X-ray examination and CNS anomalies in a study conducted in 1979 (7).

CT was developed by Allan McLeod Cormack and Godfrey Hounsfield in 1963 (21). According to estimates, a total of 10.5 million MRI and 12.8 million CT examinations were performed in 2014 in Turkey. Estonia is the world's leading country in terms of the number of CT examinations performed per thousand persons followed by the USA, Luxembourg, France, and Turkey.

Jensh and Brent showed that a fetal exposure of <0.05 Gy does not increase the teratogenic risk (2,9). However, ionizing radiation fulfills Wilson's six principles of teratology (20). As a neurotropic agent, radiation-induced behavioral teratogenic effects can be shown at doses much below those that cause clear structural malformations (20).

In our study, the chicken embryos were examined under a stereomicroscope to assess the presence of neural tube developmental abnormalities. It was found that high-dose CT irradiation (Groups 4 and 5) may cause neural tube developmental abnormalities. At lower CT radiation doses (Groups 2 and 3), we did not observe any neural tube developmental abnormalities or less neural tube developmental abnormalities.

Cell division and apoptosis are regulated by survivin, which inhibits the apoptosis protein (5,12). The role of survivin in embryonic development is not known. In our study, no significant correlation between CT radiation-related neural tube developmental abnormalities and survivin expression was observed. Moreover, high-dose CT radiation was observed to induce neural tube developmental abnormalities, but this phenomenon was not associated with an increase in survivin expression. Therefore, we conclude that increased neural tube developmental abnormalities in CT-irradiated chicken embryos were not related with survivin expression.

Table II: The Presence of Neural Tube Defects and the Resulst of the Expression of Survivin and Bcl-2

	n	Group 1 n (%)	n	Group 2 n (%)	n	Group 3 n (%)	n	Group 4 n (%)	n	Group 5 n (%)	p
Neural Tube Defect											
No	30	30 (100.0)	30	30 (100.0)	30	29 (96.6)	30	24 (80.0)	30	23 (76.6)	**0.01***
Yes		0		0		1 (3.4)		6 (20.0)		7 (23.4)	
Survivin Intensity Score											
0		3 (10)		0		2 (6.6)		0		0	
1	30	6 (20)	30	12 (40.0)	30	12 (40.0)	30	11 (36.7)	30	13 (43.3)	>0.05
2		13 (44)		18 (60.0)		16 (54.4)		17 (56.7)		17 (56.7)	
3		8 (26)		0		0		2 (6.6)		0	
BCL2 Intensity Score											
0		0		0		0		0		0	
1		9 (30.0)		14 (46.6)		19 (63.3)		13 (44.3)		7 (23.3)	
2	30	15 (50.0)	30	16 (54.4)	30	11 (36.7)	30	17 (56.7)	30	15 (5.0)	>0.05
3		6 (20.0)		0		0		0		8 (26.7)	

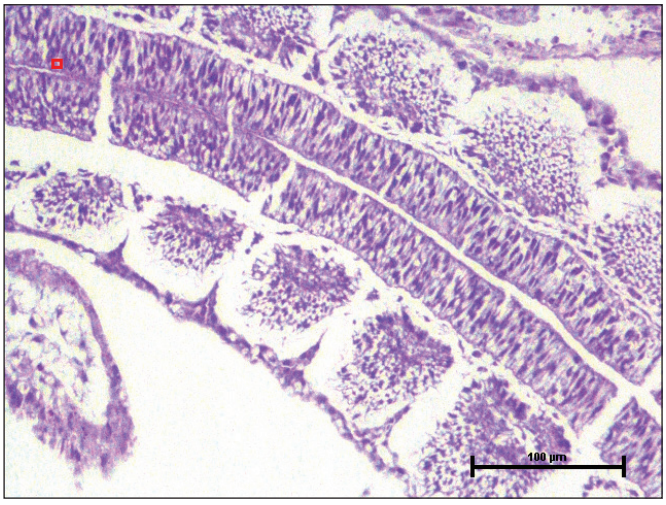

Figure 1: The regular neural tube of a normal chicken embryo in the control group (x10).

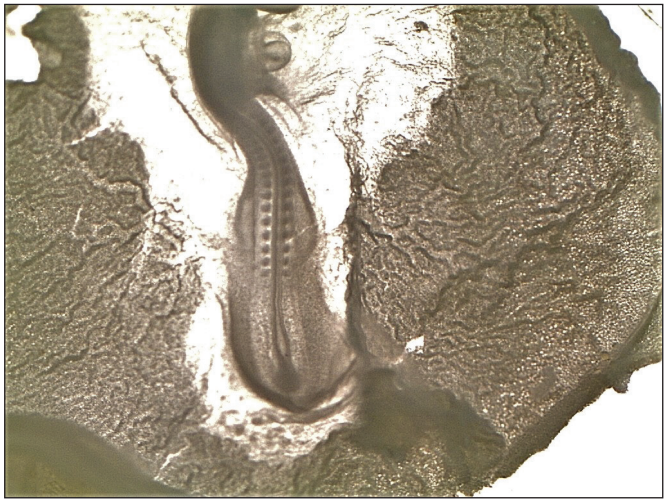

Figure 2: Neural tube abnormality is seen at the caudal segment of chick embryo (x10).

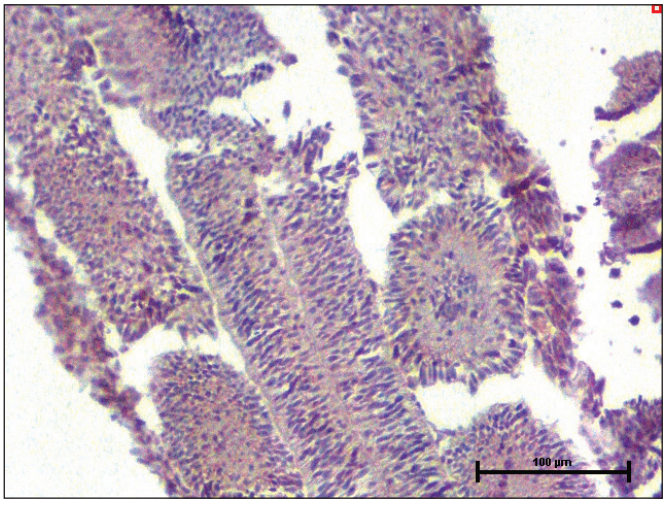

Figure 3: The expression of Bcl-2, chicken embryo cells showing cytoplasmic and nuclear immunoreactivity (x100).

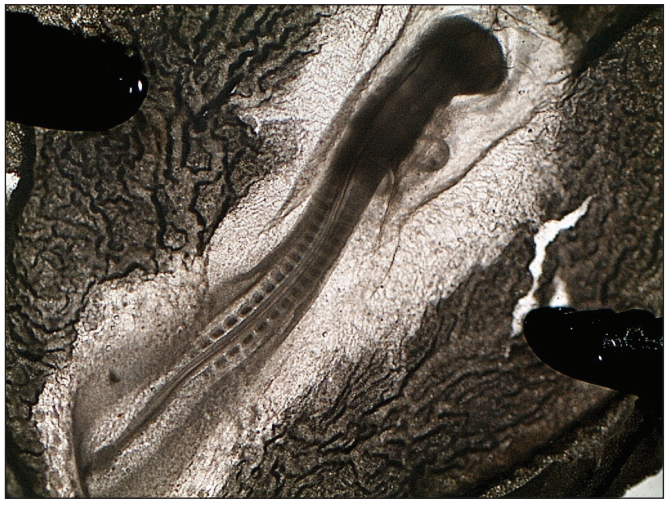

Figure 4: The chicken embryo cells showing no cytoplasmic and nuclear immunoreactivity of Bcl-2 (x100).

Bcl-2 is a proto-oncogene that functions as a repressor of programmed cell death (10). Bcl-2 is more commonly expressed in the neurons of the developing brain as compared with that of adults (15). Bcl-2 expression in many brain regions is maintained at a high level throughout the period of cell death (15). In our study, since no significant correlation between CT radiation-related neural tube developmental abnormalities and Bcl-2 expression was observed, we conclude that the increased incidence of neural tube developmental abnormalities in CT-irradiated chicken embryos were not related with Bcl-2 expression.

Moreover, we conclude that high-dose CT radiation may cause neural tube developmental abnormalities in chicken embryos and this effect was not related with the survivin and Bcl-2 expressions. Many other factors were related with the neural tube abnormalities, with CT radiation being one of the possible factors. The correlation between CT radiation exposure and neural tube abnormalities and the relation between this kind of neural tube abnormalities and the survivin and Bcl-2 expressions were investigated. Based on the results of this study, we could not find any correlation between this kind of neural tube abnormalities and the survivin and Bcl-2 expressions. Further studies are needed in order to investigate the correlation between CT radiation exposure and neural tube abnormalities as well as the pathogenesis causing this abnormality.

CONCLUSION

Overall, our study showed that high-dose CT radiation may cause neural tube developmental abnormalities in chicken embryos. However, we found no significant correlation between the CT radiation dose and the survivin and Bcl-2 expressions as well as the neural tube developmental abnormalities and the survivin and Bcl-2 expressions.

ACKNOWLEDGEMENTS

The financing of the article provided by Balikesir University (BAP. 2017/203).

REFERENCES

1. Adida C, Crotty PL, McGrath J, Berrebi D, Diebold J, Altieri DC: Developmentally regulated expression of the novel cancer anti-apoptosis gene survivin in human and mouse differentiation. Am J Pathol 152(1):43-49, 1998
2. Brent RL: Radiation teratogenesis.Teratology 21:281-298,1980
3. Cragan JD, Roberts HE, Edmonds LD, Khoury MJ, Kirby RS, Shaw GM, Velie EM, Merz RD, Forrester MB, Williamson RA, Krishnamurti DS, Stevenson RE, Dean JH: Surveillance for anencephaly and spina bifida and the impact of prenatal diagnosis-United States, 1985-1994. MMWR CDC Surveill Summ 44:1-13, 1995
4. Dekaban AS: Abnormalities in children exposed to x-radiation during various stages of gestation: Tentative timetable of radiation injury to the human fetus. J Nucl Med 9:412-477, 1968
5. Deveraux QL, Reed JC: IAP family proteins-suppressors of apoptosis. Genes Dev 13:1253-1262, 1999
6. Eroz R, Bircan D, Yuce H, Ozmerdivenli R: Survivin hakkında bilinenler: Survivin ile ilgili türkiye'de yapılmış olan çalışmalar. Düzce Üniversitesi Sağlık Bilimleri Enstitüsü Dergisi 5(3):39-58, 2015
7. Granoth G: Defects of the central nervous system in Finland. IV Associations with diagnostic X-ray examinations. Am J Obstet Gynecol 133:191-194, 1979
8. Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. J Morphol 88:49-92, 1951
9. Jensh RP, Brent RL: The effect of low level prenatal X-irradiation on postnatal development in the Wistar rat. Proc Soc Exp Biol Med 184:256–263, 1987
10. Korsmeyer SJ: Bcl-2: An antidote to programmed cell death. Cancer Surv 15:105-118, 1992
11. LeBrun DP, Warnke RA, Cleary ML: Expression of bcl-2 in fetal tissues suggests a role in morphogenesis. Am J Pathol 142(3):743-753, 1993
12. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, Altieri DC: Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 396:580–584, 1998
13. Lo A, Polšek D, Sidhu S: Estimating the burden of neural tube defects in low- and middle-income countries. J Glob Health 4:010402, 2014
14. Ma AC, Lin R, Chan PK, Leung JCK, Chan LYY, Meng A, Verfaillie CM, Liang R, Leung AYH: The role of survivin in angiogenesis during zebrafish embryonic development. BMC Dev Biol 7(1):50, 2007
15. Merry DE, Veis DJ, Hickey WF, Korsmeyer SJ: bcl-2 protein expression is widespread in the developing nervous system and retained in the adult PNS. Development 120(2):301-311, 1994
16. Opferman JT, Kothari A: Anti-apoptotic BCL-2 family members in development. Cell Death Differ 25(1):37-45, 2018
17. Otake M, Schull WJ: In utero exposure to A-bomb radiation and mental retardation: A reassessment. Br J Radiol 57:409-414, 1984
18. Schramm H, Jaramillo ML, Quadros T de, Zeni EC, Müller YMR, Ammar D, Nazari EM: Effect of UVB radiation exposure in the expression of genes and proteins related to apoptosis in freshwater prawn embryos. Aquat Toxicol 191:25-33, 2017
19. Schull WJ, Norton S, Jensh RP: Ionizing radiation and the developing brain. Neurotoxicol Teratol 12:249-260, 1990
20. Vorhees CV: Principles of behavioural teratology. In: Riley EP, Vorhees CV, (eds). Handbook of Behavioural Teratology. New York: Plenum Press, 1986:23-48
21. Wolpert SM: Neuroradiology classics. American J Neuroradiol 21(3):605-606, 2000