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Research Article

The Effects of Fibroblast Growth Factor-2 Blocking on Development of Chick Cervical Vertebra and Relationship with Oxidative Stress and Apoptosis

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Summary

Fibroblast growth factor (FGF) plays a role in the development of bone and cartilage. FGF-2 is a member of this family and blocking of FGF-2 affects the bone development. In this study, effects of FGF-2 blocking on the formation of vertebrae in chick embryos before ossifications and orientation of cervical vertebrae were investigated with histological examinations. In this study, anti-FGF-2 was performed to chick embryos at E4th and E10th days. Samples were taken in E15th days and changes in the cervical spine were evaluated as histochemically (with hematoxylin-eosin, Alizarin red, Masson's trichrome staining) and immunohistochemically (with iNOS, eNOS and TUNEL). Also changings were evaluated by morphometric analysis. In macroscopic examination of the cervical vertebrae significant difference was not detected. But in histochemical staining, defects were observed in cartilage and ossification process. Increasing in oxidative stress was demonstrated by iNOS and eNOS. Also apoptosis which was represented by tunnel was found to be increased. FGF-2 blocking affects the ossification process in the cervical spine by inducing oxidative stress and apoptosis which results with cell death.

Key words: Apoptosis, Cervical vertebra, Chick embryo, Fibroblast growth factor, Morphology, Oxidative stress

Fibroblast Growth Faktör-2 Bloklamasının Tavuk Servikal Omurları Üzerindeki Etkisi ve Oksidatif Stres ve Apoptozis ile İlişkisi

Özet

Fibroblast büyüme faktörü (FGF) kemik, kıkırdak gelişiminde rol oynayan ve bu nedenle kemik oluşumunu yönlendiren bir etkendir. Bu ailenin bir üyesi olan FGF-2'nin bloklaması ile kemik gelişiminin etkilendiği gösterilmiştir. Bu çalışmada kemikleşme öncesi dönemde FGF-2 bloklamasının tavuk embriyolarında vertebra oluşumuna etkisi ve servikal vertebraların bu etki altında nasıl yönlendiği histolojik açıdan incelenmesi amaçlanmıştır. Çalışmamızda E4 ve E10 günlerinde piliç embriyolarına anti FGF-2 uygulanarak E15 günü alınan örneklerde servikal vertebralarda oluşan değişiklikler histokimyasal olarak hematoksilen-eosin, Alizarin kırmızısı, Mason trikrom boyaları ile immünohistokimyasal olarak iNOS, eNOS ve TUNEL ile ayrıca oluşan değişiklikler morfometrik analizler ile değerlendirildi. Servikal vertebraların makroskobik incelemelerinde önemli bir farklılık saptanmazken, histokimyasal boyamalarda kıkırdaklaşma ve kemikleşme süreçlerinde bozukluklar, iNOS ve eNOS ile ortaya konan oksidatif stres artışı, ayrıca TUNEL ile gösterilen apoptoziste fazlalaşma olduğu bulundu.

FGF ailesinin bir üyesi olarak FGF-2 bloklamasının servikal vertebralarda kemikleşme sürecini etkilediği ve bunun serbest oksijen radikallerinden kaynaklanan oksidatif stres ve ilişkili olabileceği apopitozu indükleyerek hücre ölümüne neden olduğunu göstermektedir.

Anahtar Kelimeler: Apoptosis, Servikal omur, Tavuk embriyosu, Fibroblast büyüme faktörü, Morfoloji, Oksidatif Stres

INTRODUCTION

Fibroblast growth factor (FGF) has several different functions. Recently, studies demonstrated that, FGF has effects on ossification. In various studies, different effects of FGF in the development of arms and legs were reported and still interpretations were not fully understood. Blocking studies that were done for understanding the function identified that, blocking was effective and changings were affect the bone tissue.

In this study, to investigate the relationship between FGF and ossification, FGF-2 blocking was done to chick embryos between E4th and E10th days. Sections were taken at E15th days and observations were made with histological and immunohistochemical methods. The relationship between possible changes, oxidative stress and apoptosis were tried to understand.

MATERIAL AND METHODS

Fertile, specific pathogen free eggs of the domestic fowl (White Leghorn, Gallus gallus) were obtained from Celal Bayar University Research Institute of Poultry Disease and Vaccination Manisa, at zero hour of incubation. The eggs were incubated at 37.5º C and 75% relative humidity until the embryos reached desired stage of development according to Hamburger and Hamilton[5,7,29].

Four experimental groups were formed. First group include only sterile phosphate-buffered saline (PBS). Second group was control group (non treated group) and others (group 3 and 4) were FGF-2 blocking groups. FGF-2 blocking was done with Anti FGF-2 (anti-b FGF basic Neutralizing Antibody Purified Rabbit IgG AB-33-NA Minneapolis, MN). FGF-2 blocking was done in 4th and 10th days to 3rd and 4th groups respectively. Every group consisted of 10 eggs. The eggs were washed with 70% alcohol and properly labeled on the outer shell. A hole was made on the blunt pole of the eggs with a sharp and thick needle under laminar flow. To PBS group, 0.1ml volume of sterile PBS was injected with the 28-gauge tuberculin syringe around the embryos' umbilical cord in 4th and 10th days[28]. One mg granulated anti FGF-2 was mixed with 1ml sterile PBS and 1/100 dilutions from this stock solutions prepared and 0.1ml volume of this solution was injected in 4th and 10th days with the tuberculin syringe around the embryos' umbilical cord The holes were sealed with paraffin. The eggs were then placed in an incubator.

The eggs were cracked open at E15th day and the outer shell was chipped out to create a large opening to see the embryo. The viability of the embryos was assessed by the heartbeat. The embryos were transferred to petri dishes by careful dissection among the allantoic stalk and other embryonic structures. All the embryos were fixed with 10% formalin and stained with hematoxylin and eosin (H&E) and examined under stereomicroscope to assess any gross developmental abnormalities. Then, embryos were embedded into paraffin and 5 microns thick paraffin sections were cut and stained with H&E for light microscopic investigation. For immunohistochemical staining, sections were incubated at 60°C overnight and then in xylene for 30 minutes. After washing with a decreasing series of ethanol,

sections were washed with distilled water and PBS for 10 minutes. Sections were then treated with 2% trypsin at 37°C for 15 minutes. After washing with PBS, they were incubated in a solution of 3% H_2O_2 for 15 minutes. to inhibit endogenous peroxidase activity. Then sections were washed with PBS and incubated for 18h at +4°C with primary antibodies: a monoclonal anti-eNOS (rabbit Pab, RB-1711-P1, Neomarkers, Fremont, CA, USA). Afterwards, sections were washed three times for 5 minutes each with PBS, followed by incubation with biotinylated goat IgG, anti-rabbit IgG and then with streptavidin conjugated to horse-radish peroxidase for 30 minutes each (Dako LSAB 2 kit, Peroxidase). After washing, three times for 5 minutes with PBS, sections were incubated DAB (Dako) for 5 minutes to stain immunolabelling and then with Mayer's hematoxylin. Sections were covered with mounting medium and were analyzed light microscopically with a BX 40 microscope (Olympus, Tokyo, Japan). Control samples were processed in an identical manner, but primary antibody was omitted. Two observers blinded to clinical information evaluated the staining scores independently[6]. Detection of the apoptotic cell death in situ using as TUNEL method was used for programmed cell death mechanism. Fragmentation of the DNA in the nucleus is one of the first morphological changes of the apoptotic process and can be detected in histological sections using a terminal deoxynucleotidyltransferase (TdT)-biotin nick end-labeling method (TUNEL) performed with a commercial kit (Dead End Colorimetric TUNEL system, Promega G7130) according to the manufacturer's instructions. Briefly, after proteinase K treatment for 10 minutes, the sections were incubated at 37°C with TdT for 60 minutes. As negative staining control for TUNEL, TdT was omitted during the tailing of reactions[12].

Sections were taken from samples. Morphological integrity, ossification, cartilage formation, oxidative stress and apoptosis were determined with H & E, Alizarin red, Masson's trichrome, i-NOS and e-NOS and TUNEL stainings respectively by a blind observer. Scoring was determined in that; no, at least +1 and mostly +5[26].

Statistical analysis: The data were expressed as mean ± standard deviation (SD). The data were analysed by using repeated measures of variance. Tukey Kramer multiple comparisons test was used to test for differences among means when ANOVA indicated a significant "P"value ($P< 0.05$). Experiments were repeated for three times[6].

RESULTS

To see the effects of FGF-2 blocking, eggs in 3rd and 4th were opened at E15th days examined macroscopically and microscopically. In the comparison of FGF-2 blocking groups (results in 3rd and 4th groups were similar) and PBS group, chick embryos were less developed with weaker limb in FGF-2 blocking groups and there were no structural abnormalities. Whereas in a few egg, shaped curvature was seen in the cervical region but there was no differences if compared with PBS. In microscopic examination under H & E staining, histopathological effects for PBS treated group and FGF-2 blocking groups (Fig. 1) were evaluated. Results in all groups were found to be similar. Difficulties during sectioning cause to fragile tissues and artefacts. While cell morphology and spinal cord was maintained, serious damage, breakage and thinning was observed on surrounding vertebrae. The comparison of PBS and FGF-2 blocking groups were demonstrated that, cartilage was found to be dominance instead of ossification in Alizarin red (Fig. 2), Masson's trichrome (Fig. 3) and Von Kossa (Fig. 4) stainings. PBS and FGF-2 blocking groups were compared with iNOS and eNOS (Fig. 5) and TUNEL (Fig. 6) stainings to investigate the relationship between changings, oxidative stress and

apoptosis. It was found that, increasing in free radicals cause to increase in programmed dying cell in FGF-2 blocking group.

Morphometric analysis of the histopathological observation demonstrated that, similar morphological defectives were determined in FGF-2 blocking groups. There was highly statistically significant in FGF-2 blocking groups when compared with PBS group ($p<0,001$). Cartilage dominance was statistically significant ($p<0.05$) in Alizarin red and Masson's trichrome stainings. Also this changings had relationship with oxidative stress and apoptosis and it was statistically significant ($p<0,05$) in NOS and TUNEL stainings (Table 1).

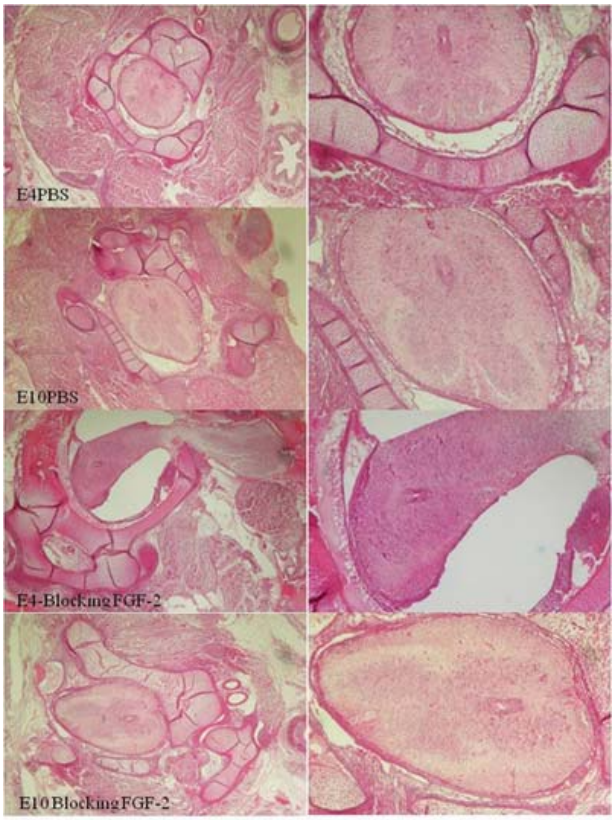


Figure 1: *Sections that taken on E15 days with H&E staining determined morphological abnormalities in FGF-2 blocking group when compared with PBS group. Similar histopathological changes were noted on E4 and E10 days. In impaired morphology, increasing of the tissue fragility, degradation of vertebral ossification and increasing of the cartilage formation were detected.*

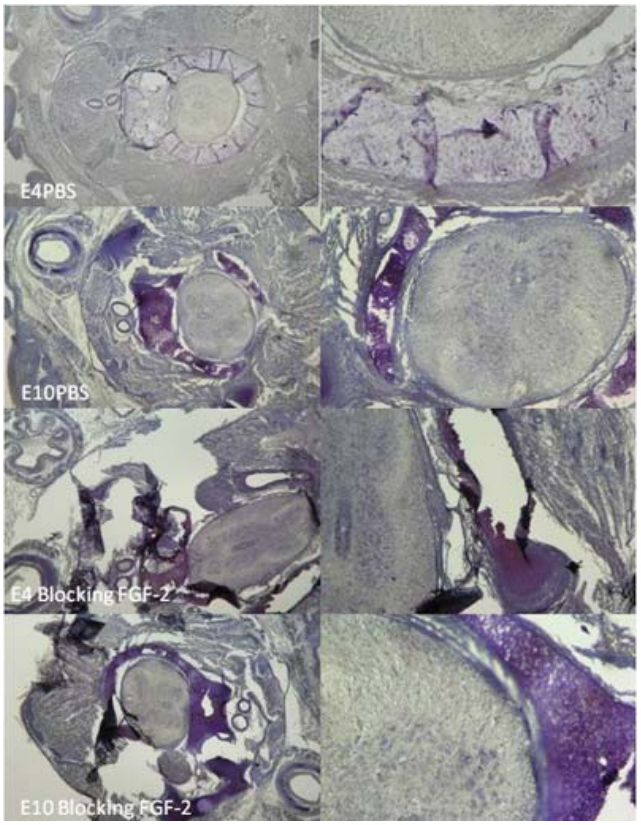


Figure 2: *Sections of E15 days with Alizarin red staining, determined morphological abnormalities in FGF-2 blocking group when compared with PBS group. Similar histopathological changes were noted on E4 and E10 days. In impaired morphology, increasing of the tissue fragility, degradation of vertebral ossification and increasing of the cartilage formation were detected.*

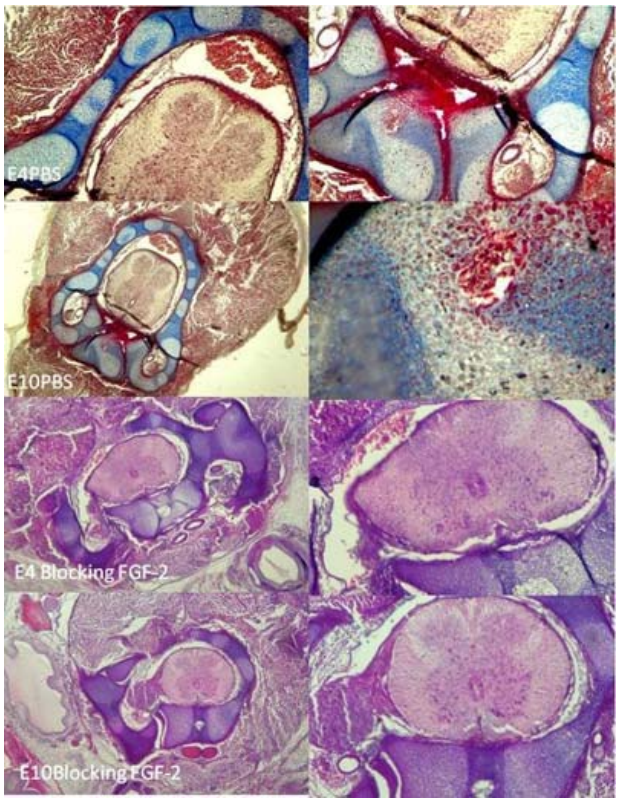


Figure 3: *Sections of E15 days with Masson's trichrome staining, determined morphological abnormalities in FGF-2 blocking group when compared with PBS group. Similar histopathological changes were noted on E4 and E10 days. In impaired morphology, increasing of the tissue fragility, degradation of vertebral ossification and increasing of the cartilage formation were detected.*

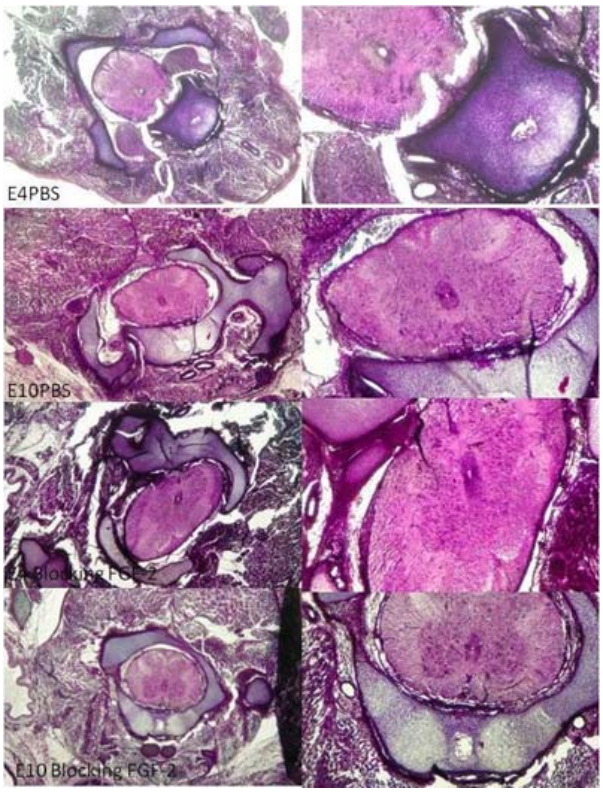


Figure 4: *Sections of E15 days with Von Kossa staining, determined morphological abnormalities in FGF-2 blocking group when compared with PBS group. Similar histopathological changes were noted on E4 and E10 days. In impaired morphology, increasing of the tissue fragility, degradation of vertebral ossification and increasing of the cartilage formation were detected.*

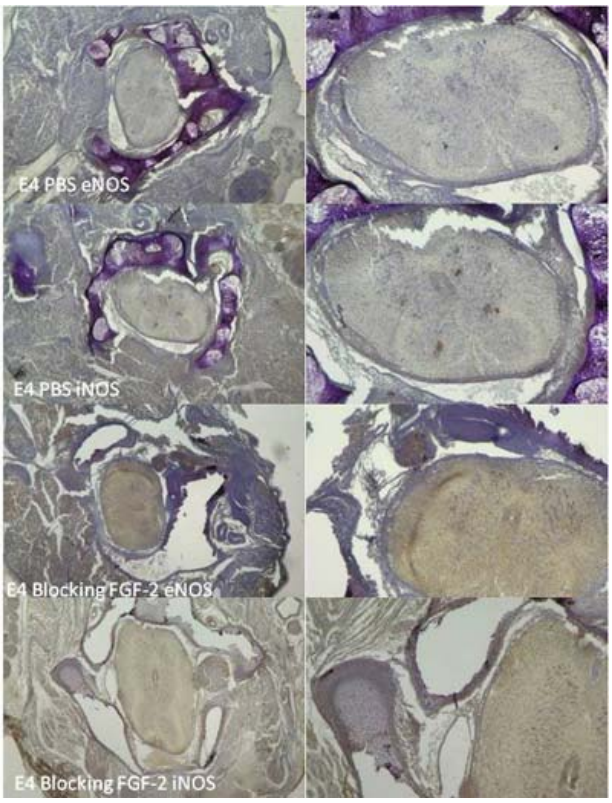


Figure 5: *Sections of E15 days with NOS staining, determined morphological abnormalities in FGF-2 blocking group when compared with PBS group. Similar histopathological changes were noted on E4 and E10 days. In impaired morphology, increasing of the tissue fragility, degradation of vertebral ossification and increasing of the cartilage formation were detected.*

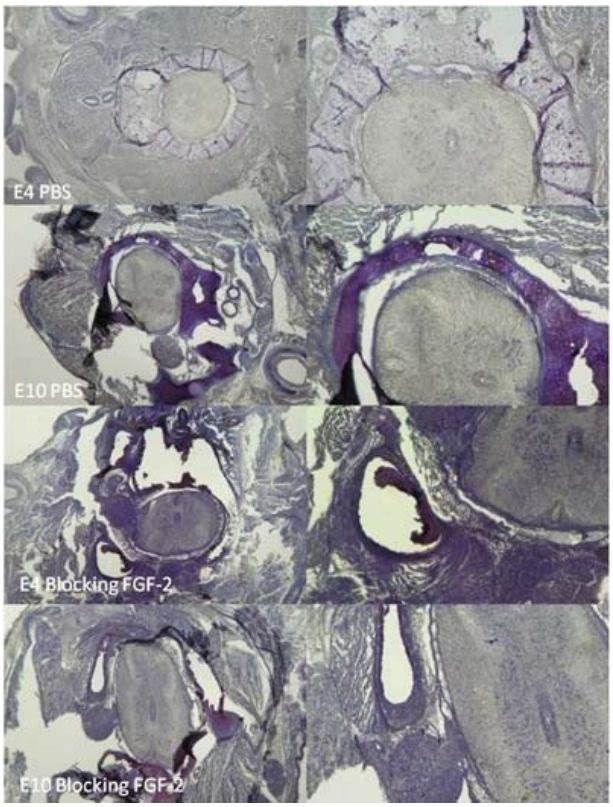
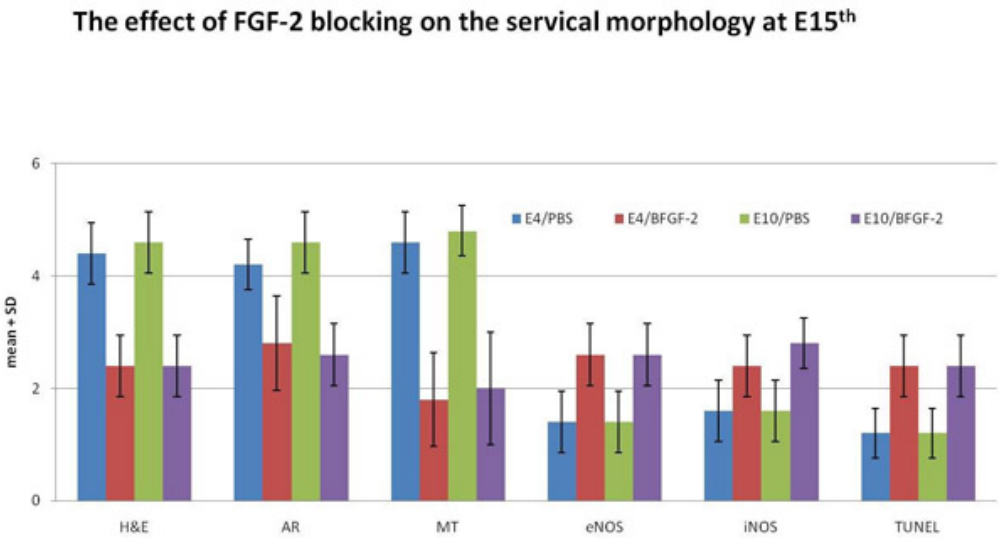


Figure 6: *Sections of E15 days with TUNEL staining, determined apoptosis in FGF-2 blocking group when compared with PBS group.*

Table 1: Morphometric analyses of staining for histochemistry and immunocytochemistry from E15 cervical sections. There were significant alterations in the staining intensity and quantity with FGF-2 blocking compared to that of PBS.



DISCUSSION

This study demonstrated that, FGF-2 blocking at E4th and E10th days on chick eggs which were opened on E15th days cause to morphological changings. Histopathological observations demonstrated that, morphological changings were related with oxidative stress and apoptosis and cause to cartilage dominance.

Vertebral development of chick embryos (development of cartilage and bone tissue) were examined with Alcian blue and Alizarin red staining. Cartilage tissue formation was started at 5th day, whereas ossification tissue formation was extended from 13th day to 16th day. Intramembranous periosteal ossification is seen around the chondrocyte hypertrophy and vascularization is occurred in different way than mammals. The proliferation of undifferentiated mesenchymal cells and chondrocytes were observed to increase from 5th day. This proliferation was also occurred in the spinal cord and the labeled with 3H-thymidine autoradiography[24]. Many studies have been done to investigate the development stages. Heidweiller[8] examined development of ossification; muscle and tendon in postnatal period. In a study Turgut et al. applied pinealectomy in postnatal 3rd day and detected the ossification and scoliotic changes on 4th and 7th cervical vertebra at 8th week on computed tomography scan. In the same study, radiological investigations determined heterogeneous bone density with reduced width and length of the cervical vertebrae. Histopathologic finding was reduction in the number of osteocytes[27]. FGF family plays important roles during embryonic development and affected bones and all organs' development[10,11]. It is known that both FGF and FGF receptors have effects on bone and neural tissue development[22,23,30] but mechanism is still unclear. Their effects on osteoprogenitor cell replication, osteoblast differentiation, apoptosis and relation with gene expression are analyzed and these studies are expected to be target for treatment[14]. FGF-2 is particularly important in bone development, maturation and diseases. Mutations on receptors are thought to be important for the ossification. However, the presence of pleiotropic effects is an important feature for FGF-2 and description of the mechanism is quite difficult. Here, FGF-2 isoforms appear to be an answer and is thought to organize the osteoblast function in membranous ossification[13]. This function was shown particularly in calvarial bone sutures and has been found to affect proliferation, differentiation and cell death with bone morphogenetic protein (BMP). Thus, the organization of membranous ossification is performed[4].

One of the other important mechanisms for bone development, maturation, and diseases is oxidative stress and its relationship with osteoblasts. The free radicals cause to cell apoptosis by using factors such as the c-Jun N-terminal kinase (which used in the signaling mechanisms) and mitochondrial ways[25]. Nitric oxide (NO) is a double-acting agent and its effects in low and high dose are realized in different ways. This dual effect can be seen in apoptotic mechanisms too and on the one hand can cause to cell death on the other hand can be saved from the death[2,25]. Knock out studies demonstrated that, nitric oxide synthase (NOS) was effective in the ossification[1,21]. For ossification, apoptotic mechanisms have been used to provide balance between bone formation and resorption during embryonic and regeneration period[20].

Opperman et al., reported that, FGF-2 is a contributory factor on membranous ossification of the craniofacial skeleton[18,19]. In an another study Diez del Corral et al.[3] reported that, FGF factors had a role in differentiation of ossification during the axis expansion. Heywood et

al.[9] identified that chick embryos' development was affected by 4-aminopyridine. Body mass, muscle mass length and width of the tibia and femur were increased. These studies showed that FGF family may play a role in the development of the somites and spinal cord. Ohuchi and Noji[17], suggested that, the effects of FGF on development can occur over the factors such as HOX genes, transforming growth factors, and also the cells through interaction with each other and matrix. In our study, after FGF-2 blocking, growth retardation, reduction in thickness and width of the cervical bone, histopathological morphological abnormalities and cartilage domination in the cervical bone were seen and these results were consistent with literature.

Naganawa et al. reported that, FGF-2 genes disruption caues to decrease in bone mass and improvement was provided by BMP-2. These two factors, organize the mesenchymal, preosteoblast and osteoblast apoptosis[16]. Removing of these factors increase the osteogenic cells, decrease the preosteoblast apoptosis and prevent the coalescence of adjacent limbs. Therefore, these factors have a role in organizing the membranous ossification on both limbs and cranial bones[4,15]. These observations and results of our study were thought, there is relation between FGF-2 blocking and oxidative stress and apoptotic cell death.

INTERPRETATION:

FGF family and particularly FGF-2 plays a decisive role in bone development. In our study, FGF-2 played a role in organizing the development of cervical vertebrae by increasing the oxidative stress and apoptotic cell death. This relationship should be established with double staining and relation between genes, cellular adhesion and matrix should be detailed by the molecular mechanisms. These effects were thought that, FGF-2 can be used to prevent cervical vertebra development abnormalities even as a therapeutic agent in cervical vertebra damages in future therefore further studies required.

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REFERENCES

1. *Afzal F, Polak J, Buttery L. Endothelial nitric oxide synthase in the control of osteoblastic mineralizing activity and bone integrity. J Pathol. 2004;202(4):503-10.*
2. *Chang CC, et al., 2006, Chang CC, Liao YS, Lin YL, Chen RM. Nitric oxide protects osteoblasts from oxidative stress-induced apoptotic insults via a mitochondria-dependent mechanism. J Orthop Res. 2006;24(10):1917-25.*
3. *Diez del Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron. 2003;40(1):65-79.*
4. *Fromigue O, Modrowski D, Marie PJ. Apoptosis in membranous bone formation: role of fibroblast growth factor and bone morphogenetic protein signaling. Crit Rev Eukaryot Gene Expr. 2005;15(1):75-92.*

5. *Guney O, Canbilen A, Konak A, Acar O. The effects of folic acid in the prevention of neural tube development defects caused by phenytoin in early chick embryos. Spine 2003;28:442-5.*
6. *Gürpınar T, Ekerbiçer N, Uysal N, Barut T, Tarakçı F, Tuglu MI. The effects of the melatonin treatment on the oxidative stress and apoptosis in diabetic eye and brain. ScientificWorld Journal. 2012;2012:498489. doi: 10.1100/2012/498489. Epub 2012 May 1.*
7. *Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Dev Dyn 1992;195:231-72.*
8. *Heidweiller J. Post natal development of the neck system in the chicken (Gallus domesticus). Am J Anat. 1989;186(3):258-70.*
9. *Heywood JL, McEntee GM, Stickland NC. In ovo neuromuscular stimulation alters the skeletal muscle phenotype of the chick. J Muscle Res Cell Motil. 2005;26(1):49-56. Epub 2005 Oct 14.*
10. *Karabagli H, Karabagli P, Ladher RK, Schoenwolf GC. Survey of fibroblast growth factor expression during chick organogenesis. Anat Rec. 2002 Sep 1;268(1):1-6.*
11. *Karabagli H, Karabagli P, Ladher RK, Schoenwolf GC. Comparison of the expression patterns of several fibroblast growth factors during chick gastrulation and neurulation. Anat Embryol (Berl). 2002 Oct;205(5-6):365-70.*
12. *Mammadov R, Simsir A, Tuglu I, Evren V, Gurer E, Özyurt C. The effect of testosterone treatment on urodynamic findings and histopathomorphology of pelvic floor muscles in female rats with experimentally induced stress urinary incontinence. Int Urol Nephrol. 2011;43(4):1003-8.*
13. *Marie PJ, Coffin JD, Hurley MM. FGF and FGFR signaling in chondrodysplasias and craniosynostosis. J Cell Biochem. 2005;96(5):888-96.*
14. *Marie PJ. Fibroblast growth factor signaling controlling osteoblast differentiation. Gene. 2003;316:23-32.*
15. *Modrowski D, Orosco A, Thévenard J, Fromigué O, Marie PJ. Syndecan-2 overexpression induces osteosarcoma cell apoptosis: Implication of syndecan-2 cytoplasmic domain and JNK signaling. Bone. 2005;37(2):180-9.*
16. *Naganawa T, Xiao L, Coffin JD, Doetschman T, Sabbieti MG, Agas D, Hurley MM. Reduced expression and function of bone morphogenetic protein-2 in bones of Fgf2 null mice. J Cell Biochem. 2008;103(6):1975-88.*
17. *Ohuchi H, Noji S. Fibroblast-growth-factor-induced additional limbs in the study of initiation of limb formation, limb identity, myogenesis, and innervation. Cell Tissue Res. 1999;296(1):45-56.*
18. *Opperman LA. Cranial sutures as intramembranous bone growth sites. Dev Dyn. 2000;219(4):472-85.*
19. *Opperman LA, Adab K, Gakunga PT. Transforming growth factor-beta 2 and TGF-beta 3 regulate fetal rat cranial suture morphogenesis by regulating rates of cell proliferation and apoptosis. Dev Dyn. 2000;219(2):237-47.*
20. *Rice DP, Kim HJ, Thesleff I. Apoptosis in murine calvarial bone and suture development. Eur J Oral Sci. 1999;107(4):265-75.*
21. *Saura M, Tarin C, Zaragoza C. Recent insights into the implication of nitric oxide in osteoblast differentiation and proliferation during bone development. ScientificWorld Journal. 2010;10:624-32. doi: 10.1100/tsw.2010.58.*
22. *Shapiro F. Light and electron microscopic abnormalities in diastrophic dysplasia growth cartilage. Calcif Tissue Int. 1992;51(4):324-31.*
23. *Shapiro F. Vertebral development of the chick embryo during days 3-19 of incubation. J Morphol. 1992;213(3):317-33.)*
24. *Su N, Du X, Chen L. FGF signaling: its role in bone development and human skeleton diseases. Front Biosci. 2008;13:2842-65.*
25. *Tai YT, et al., 2007 Tai YT, Cherng YG, Chang CC, Hwang YP, Chen JT, Chen RM. Pretreatment with low nitric oxide protects osteoblasts from high nitric oxide-induced apoptotic insults through regulation of c-Jun N-terminal kinase/c-Jun-mediated Bcl-2 gene expression and protein translocation. J Orthop Res. 2007;25(5):625-35.*
26. *Toprak AB, Ozbilgin K, Toprak V, Tuglu I, Guler C. A histological analysis of the protective effect of ischemic preconditioning in the rat retina. Curr Eye Res. 2002;24(3):234-9.*
27. *Turgut M, Kaplan S, Turgut AT, Aslan H, Güvenç T, Cullu E, Erdogan S. Morphological, stereological and radiological changes in pinealectomized chicken cervical vertebrae. J Pineal Res. 2005;39(4):392-9.*
28. *Umur AS, Selcuki M, Bursali A, Umur N, Kara B, Vatansever HS, Duransoy YK. Simultaneous folate intake may prevent adverse effect of valproic acid on neurulating nervous system. Childs Nerv Syst. 2012;28(5):729-37.*
29. *Ureci E, Asan Z, Eser M, Tanriverdi T, Alkan F, Erdincler P. The effects of valproic acid and levetiracetam on chicken embryos. J Clin Neurosci. 2011;18(6):816-20.*
30. *Walshe J, Mason I. Expression of FGFR1, FGFR2 and FGFR3 during early neural development in the chick embryo. Mech Dev. 2000;90(1):103-10.*