



Effects of Dexamethasone Exposure on Neural Crest Cells and Primary and Secondary Neurulation in Chick Embryos

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ABSTRACT

AIM: To evaluate the effects of dexamethasone (Dex) treatment on neural crest cells and primary and secondary neurulation in chick embryos.

MATERIAL and METHODS: Sixty fertilized eggs with an average weight of 65 ± 2 g were incubated in 60%–70% humidity at $37.2^\circ\text{C} \pm 0.1^\circ\text{C}$. After 26 hours of incubation, the control group ($n=12$) received 0.1 mg/kg physiologic saline (S), group 1 ($n=12$) received 0.1 mg/kg Dex, group 2 ($n=12$) received 1 mg/kg Dex, and group 3 ($n=12$) received 5 mg/kg Dex into each embryonic disc. The eggs were incubated until Hamburger–Hamilton stage (HH) 15, HH18, and HH20. Then, the embryos were dissected and evaluated both macroscopically and microscopically.

RESULTS: The mortality rate in the control group, group 1, and groups 2 and 3 was 27%, 48%, and 100%, respectively. The neural tube thicknesses in group 1 significantly increased in HH 15 and HH20 ($p<0.05$). The mitosis number in group 1 significantly decreased in each stage ($p<0.05$). Wnt-1 expression was significantly lower in group 1 in HH15 ($p<0.05$) and HH18 ($p<0.05$), but there was no significant difference in HH20 ($p>0.05$). Fibroblast growth factor (FGF) expression was significantly lower in group 1 in HH15 ($p<0.05$). The expression of N-cadherin was significantly higher in group 1 in HH20 ($p<0.05$). Fibronectin expression decreased in group 1 in HH18 ($p<0.01$).




CONCLUSION: Although the Dex treatment did not result in neural tube closure defect, the mortality rates and neural tube thicknesses increased, whereas mitotic activation and Wnt-1 and FGF signal pathways reduced in some stages.

KEYWORDS: Primary neurulation, Secondary neurulation, Neural crest cells, Dexamethasone, Chick embryo

INTRODUCTION

Neural tube defects (NTDs) are very complicated and multifactorial, with an incidence of 1/1000 across the world. The cellular and molecular disorders causing NTDs are virtually unknown. These anomalies include cranial anencephaly and caudal spina bifida. Comorbidities such

as neural tube closure anomalies, neural tissue losses, and bone anomalies contribute to mortality cases (2,5,12,15,28). In addition, anomalies during secondary neurulation due to caudal bud anomalies become clinically apparent as spinal dysraphism and tense spine syndrome. These are also related to distal bowel and urogenital system anomalies, such as bladder exstrophy (5,7,16,22).

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In vertebrates, neurulation consists of primary and secondary neurulations. In primary neurulation, a neural plaque initially folds and fuses in a craniocaudal fashion (31). Then, secondary neurulation starts with mesenchymal condensation in the caudal bud formed in the caudal part of a neural tube, and neurulation is completed when these structures fused in chicken, rat, and human embryos (5,9,18,21,28,30,40,41). Neural crest cells (NCCs) give rise to the formation of neurons and glia cells of the nervous system, connective tissues of craniofacial structures, and pigment cells of the skin in vertebrates (14,32). Trunk neural crest cells (tNCCs) originated from the caudal region of embryos during the fusion of neural folds (9,13,21,38). NCCs and neural plate border cells (NPBCs) express signaling molecules, such as Wnt, fibroblast growth factor (FGF) and Notch (13,23,27,31,32). Wnt and FGF signaling pathways are required for NCC proliferation, differentiation, and migration during embryogenesis (6,29,38,42) and are essential for the development of the central nervous system and midbrain maturation (6,21,33,34,35,38,39,42). N-cadherin is an important adhesion molecule for neurulation and NCC development (14).

Dexamethasone (Dex) is a glucocorticoid frequently used during prenatal period for the treatment of arthritis, allergic reactions, asthma, autoimmune diseases, chemotherapy, and fetal lung maturation to prevent premature birth. Dex, which pharmacologically falls into class C, has significant side effects if administered during pregnancy, causing encephalocele, meningocele, and minor skeletal defects. Previous studies have shown that these effects are related to the proliferation, migration, and differentiation of NCCs (4,20). Although these side effects of Dex therapy are known, its effects on primary and secondary neurulation are unclear, including the underlying mechanisms. To explain the cellular mechanisms of NTDs that emerge frequently with distinct manifestations, it is necessary to determine the signaling molecules and other proteins released during neurulation that regulate cellular behavior. The aim of this study was to evaluate the effects of Dex treatment on NCCs and primary and secondary neurulation in chick embryos.

■ MATERIAL and METHODS

Experimental and Animal Design

All experiments were approved by the local animal ethics committee of the Acibadem Mehmet Ali Aydinlar University (ACUDEHAM, HDK-2017-48). Sixty fertilized, pathogen-free eggs (*Gallus gallus domesticus*) were used (Bornova Veterinary Control and Research Institute, Izmir, Turkey), with average weight of 65 ± 2 g. Then, these eggs were incubated for 26 hours in 60%–70% humidity at $37.2^\circ\text{C} \pm 0.1^\circ\text{C}$. The position of each egg was changed on its axis every 90 minutes (min). The embryos staged as Hamburger–Hamilton (HH) grade 8 [26–29 hours (h) and four segments] were classified into four groups, with 12 embryos in each group (10). Control group: 0.1 ml saline; group 1: 0.1 mg/kg Dex (Dexamethasone-Water Soluble, D2915 Sigma Chemical, St. Louise, Missouri, USA); group 2: 1 mg/kg Dex; and group 3: 5 mg/kg Dex. Eggshells were opened over the embryonic pole; then, embryonic discs

were identified. For each embryonic disc, 0.1 ml of saline was administered, and the shells were covered with sterile adhesive tapes (18,21). The eggs were incubated until they reached stages HH15 (50–55 h), HH18 (3 days), and HH20 (3–3.5 days) (21,36,41).

Histopathological Analyses

At the end of the incubation period, the embryos were dissected and evaluated macroscopically for mortality rate, embryonic membranes, and vascularity (1,10,21). Then, the embryonic membranes were removed, and the caudal regions of the embryos were fixed in formal acetic acid for 2 h. After the fixation, the embryos were dehydrated for 15 min by passing through an alcohol series (70%, 95%, and 100%). The embryos were cleared in xylene before being embedded in paraffin. Using a microtome (Leica RM2125RT), 5- μm -thick serial sections were cut out of the obtained paraffin blocks. A hematoxylin-eosin dye was stained in one out of every six sections (36,37).

Immunohistochemical Analyses

The embryo sections were incubated overnight at 60°C . When the sections were deparaffinized in xylene, they were rehydrated through a series of ethanol solutions. First, the sections were washed with distilled water and phosphate-buffered saline solution (PBS, P4417; Sigma–Aldrich, St Louis, MO) for 10 min. Then, these were treated with tris-EDTA (pH: 7.6) for 10 min at 90 W and for 15 min at 360 W in a microwave oven. Afterward, the sections were delineated with a Dako PAP pen (Dako, Glostrup, Denmark). The sections were incubated in 3% hydrogen peroxide for 5 min to inhibit endogenous peroxidase activity. After washing with PBS for 5 min, protein blocks were applied into the sections. After washing with PBS, the sections were incubated with primary antibodies FGF (1:200, B-3, Santa Cruz Biotechnology) and fibronectin (1:200, EP5, Santa Cruz Biotechnology) overnight at 40°C in a humidity chamber. The sections were washed with PBS twice for 5 min and were incubated with Post Primary (rabbit anti-mouse IgG) and then with Novolink Polymer (anti-rabbit poly-HRP-IgG; Novocastra, RE715K, Newcastle, UK) for 30 min each. After washing them with PBS twice, the sections were incubated in a NovoLink DAB substrate polymer detection system (RE715K, Novocastra) for 10–15 s. After washing them with distilled water, the sections were counterstained with Mayer's hematoxylin and washed with distilled water. After 3 h of deparaffinization and 25 min of antigen retrieval, the embryo sections were incubated with primary antibodies Wnt-1 (1:100, N/A, Spring Bioscience) and N-cadherin (1:100, monoclonal, Leica) for 30 min using Leica Bond-Max and Leica Refine detection kits (Leica Biosystems, USA). After incubating them with polymers and post-polymers for 15 min each, the DAB substrate applied for 10 min in the sections was counterstained with Mayer's hematoxylin and washed with distilled water. The immunoeexpressions of antibodies were evaluated using an Olympus BX53 bright-field microscope (Olympus, America Inc., USA). The presence of brown staining was considered positive for the antibodies. A semi-quantitative analysis was performed to determine the immune reactivity of the tissue sections. HSCORE was

calculated using the following equation: $HSCORE = \sum Pi (i + 1)$, where *i* is the intensity of labeling, with a value of 1 (weak), 2 (moderate), or 3 (strong), and *Pi* is the percentage of labeled cells for each intensity, varying from 0 to 100% (37).

Histomorphometric Analyses

Five serial sections were used for all histomorphometric analyses. A minimum of four different points in each section were quantified at a magnification of $\times 100$ to determine the thicknesses of the neural tube borders. The rate of mitosis was evaluated using a $\times 400$ objective lens in all neural tube surfaces via semiautomatic image analysis system using the University of Texas Health Science Center At Santorino imaging tool for Windows version 1.28 program. The results were evaluated statistically. The measurements were performed by two independent researchers who were blind to the experimental groups (37,41).

Statistical Analyses

GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. All data are expressed as mean \pm standard error. The data of the groups were compared using non-parametric tests, such as Mann–Whitney U test and Student’s t-test. P values of <0.05 were considered statistically significant.

RESULTS

Histopathological Analyses

A total of 96 fertilized eggs were incubated. In the control group, seven out of 25 eggs applied with saline died. In group 1, 17 out of 35 eggs treated with 0.1 mg/kg Dex died. In groups 2 and 3, 36 out of 36 eggs treated with 1 and 5 mg/kg Dex, respectively, died. Because of these reasons, the mortality rate

in the control group, group 1, and groups 2 and 3 was 27%, 48%, and 100%, respectively. Although vascular structures developed in groups 2 and 3, no embryonic development was observed. There was no difference between the control group and group 1 in terms of embryo size, macroscopic appearance, embryonic membranes, and vascular structures (Figure 1).

Histomorphometric Analyses

When we compared the neural tube border thickness in the control group and group 1, it increased in group 1 in stages HH15 and HH20 ($p < 0.05$), but it decreased in stage HH18 ($p > 0.05$) (Figure 2A, Figure 3–5 a1–a2). The number of mitoses in the neural tube borders in group 1 decreased in stages HH15, HH18, and HH20 compared to the control group ($p < 0.05$), but no NTDs were found (Figure 2B, Figure 3–5 a1–a2).

Immunohistochemical Analyses

Wnt-1 expression in the neural tube border was significantly lower in group 1 in stages HH15 and HH18 ($p < 0.05$) than in the control group, but it was not significantly different in HH20 ($p > 0.05$) (Figure 3–5 b1–b2, Figure 6A). When we compared the Wnt-1 expression of NCCs in stage HH15, it was significantly lower in group 1 ($p < 0.001$) than in the control group. The decrease Wnt-1 in group 1 in stages HH18 and HH20 was not statistically significant ($p > 0.05$) in NCCs (Figure 3–5 b1–b2, Figure 7A). In stage HH15, the FGF expression was significantly lower in group 1 ($p < 0.05$) than in the control group. When we compared the FGF expression of the control group and group 1 in stage HH18, it was higher in group 1, but the difference was not statistically significant ($p > 0.05$). In stage HH20, the FGF expression in the neural tube borders in the control group and group 1 was not significantly different

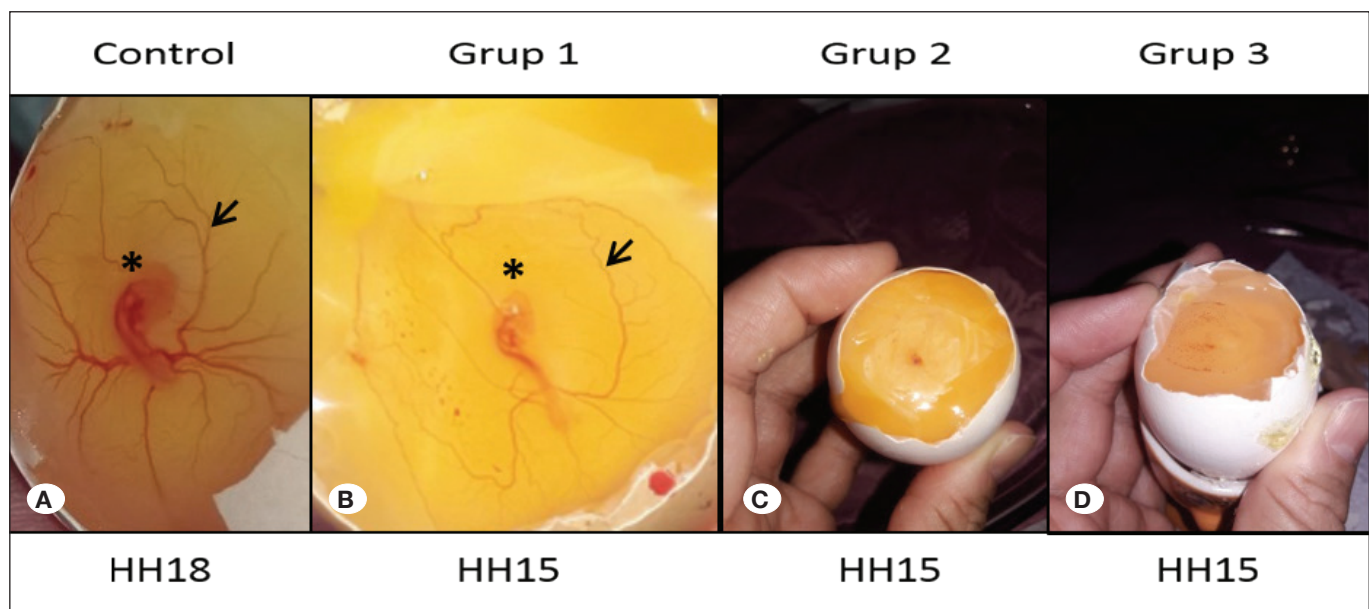


Figure 1: Embryos **A)** Stage HH18 control group; **B)** Stage HH15 group 1 (0,1 mg/kg Dex); **C)** Stage HH15 group 2 (1 mg/kg Dex); **D)** Stage HH15 group 3 (5 mg/kg Dex) **Arrow:** Embryonic vessel, *****Embryo.

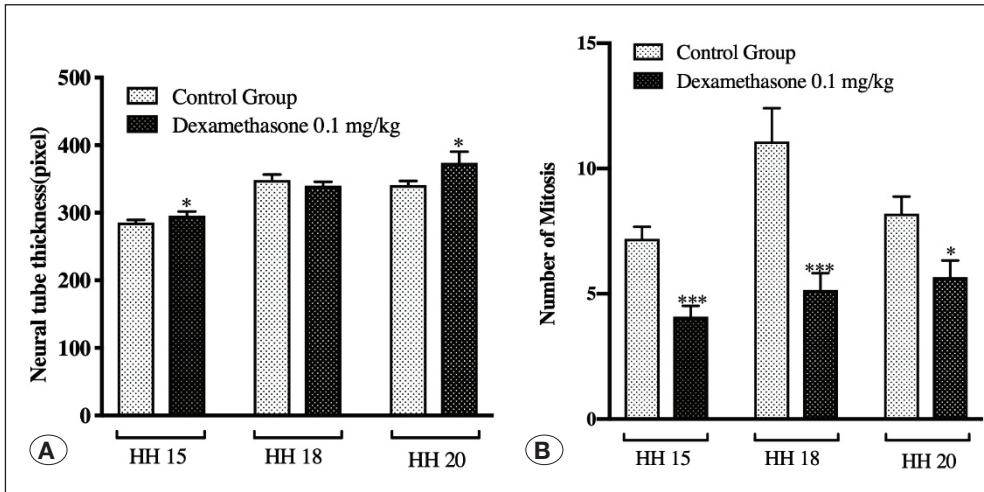


Figure 2: The thicknesses of neural tube border in control group and group 1. The number of mitosis. Compared to control group *p<0.05, **p<0.01, ***p<0.001.

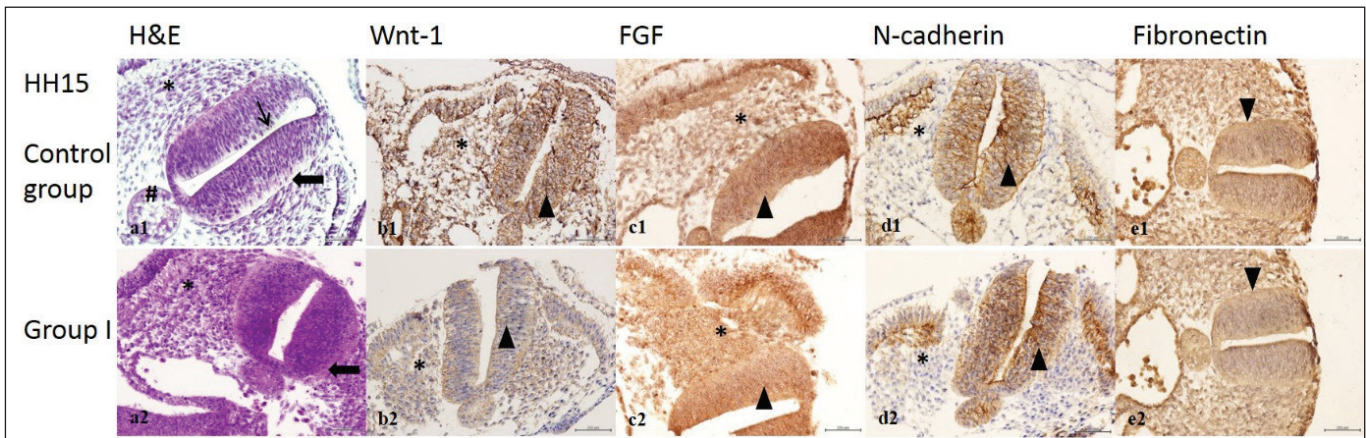


Figure 3: Control group stage HH15 (a1: hematoxyline & eosine; b1: the expressions of Wnt-1; c1: the expressions of FGF; d1: the expressions of N-cadherin; e1: the expressions of Fibronectin) and group I stage HH15 (a2: hematoxyline & eosine; b2: the expressions of Wnt-1; c2: the expressions of FGF; d2: the expressions of N-cadherin; e2: the expressions of Fibronectin). *NCC, thin arrow: mitosis, arrow head: positive immunostaining, #: notochord.

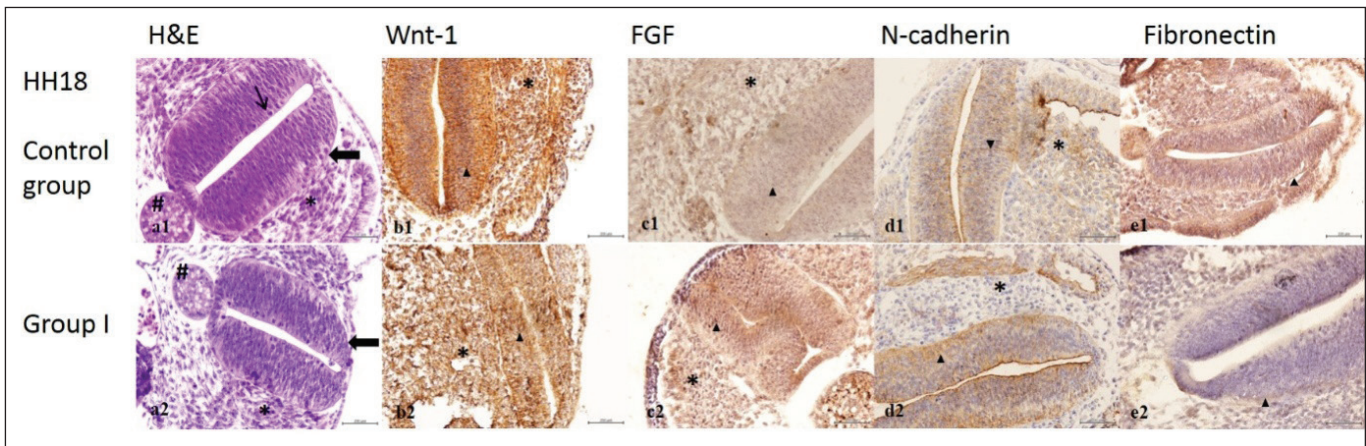


Figure 4: Control group stage HH18 (a1: hematoxyline & eosine; b1: the expressions of Wnt-1; c1: the expressions of FGF; d1: the expressions of N-cadherin; e1: the expressions of Fibronectin) and group I stage HH18 (a2: hematoxyline & eosine; b2: the expressions of Wnt-1; c2: the expressions of FGF; d2: the expressions of N-cadherin; e2: the expressions of Fibronectin). *NCC, thin arrow: mitosis, arrow head: positive immunostaining, #: notochord.

(Figure 3–5 c1–c2, Figure 6B). In stage HH15, the FGF expression was significantly lower in group 1 ($p < 0.001$), but no significant change was detected in stages HH18 and HH20 ($p > 0.05$) in NCCs (Figure 7B). In stage HH20, the expression of N-cadherin was significantly higher in group 1 than in the control group ($p < 0.05$), but there were no significant differences in stages HH15 and HH18 ($p > 0.05$) between the control group and group 1 (Figure 3–5 d1–d2, Figure 6C). In

stage HH18, the fibronectin expression was significantly lower in group 1 ($p < 0.01$). In stage HH15 and HH20, no significant differences in the neural tube border were found between the control group and group 1 (Figure 3–5 e1–e2, Figure 6D).

DISCUSSION

The development of NTDs is a very complex process, which

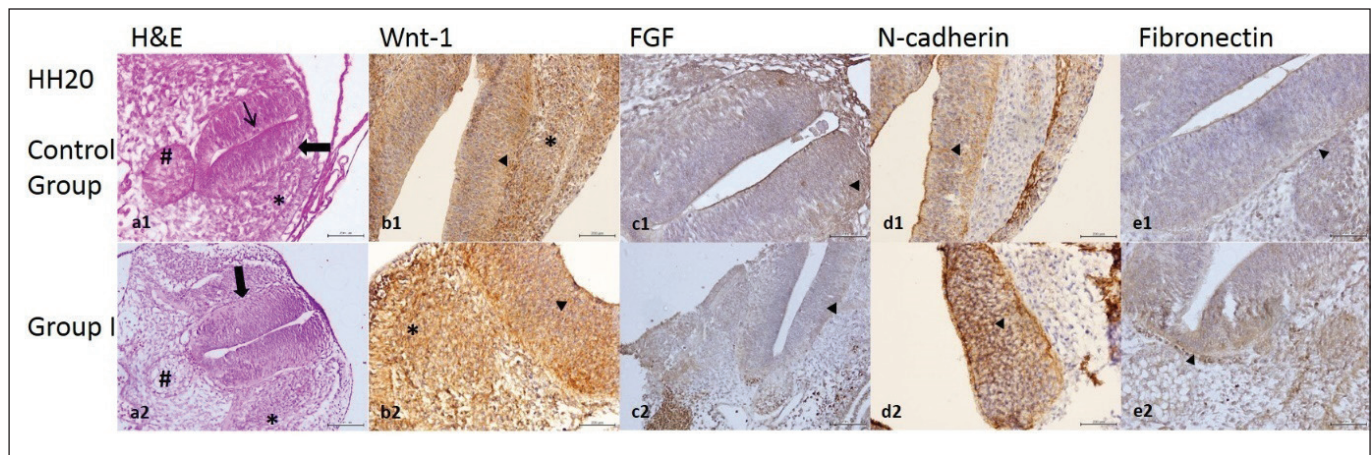


Figure 5: Control group stage HH20 (a1: hematoxyline & eosine; b1: the expressions of Wnt-1; c1: the expressions of FGF; d1: the expressions of N-cadherin; e1: the expressions of Fibronectin) and group I stage HH20 (a2: hematoxyline & eosine; b2: the expressions of Wnt-1; c2: the expressions of FGF; d2: the expressions of N-cadherin; e2: the expressions of Fibronectin). *NCC, thin arrow: mitosis, arrow head: positive immunostaining, #: notochord.

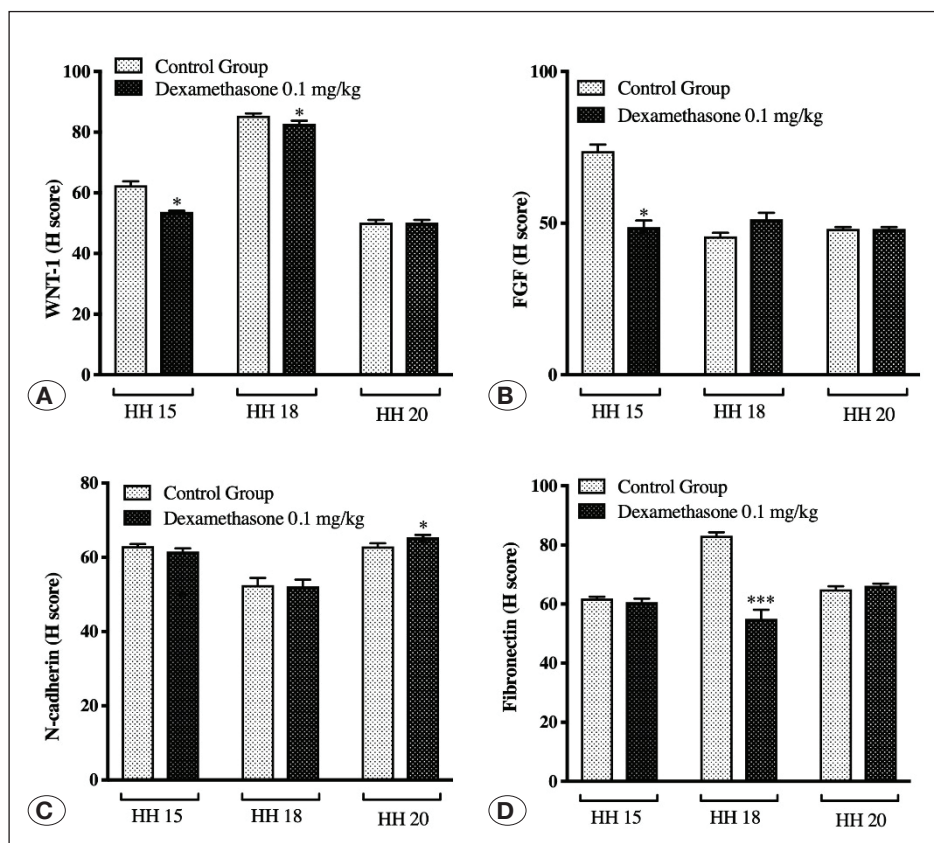


Figure 6: Immunohistochemistry Hscore analyses of Wnt-1, FGF, N-cadherin, Fibronectin in neural tube border control group and group 1 in stages HH15, HH18, HH20. Compared to control group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

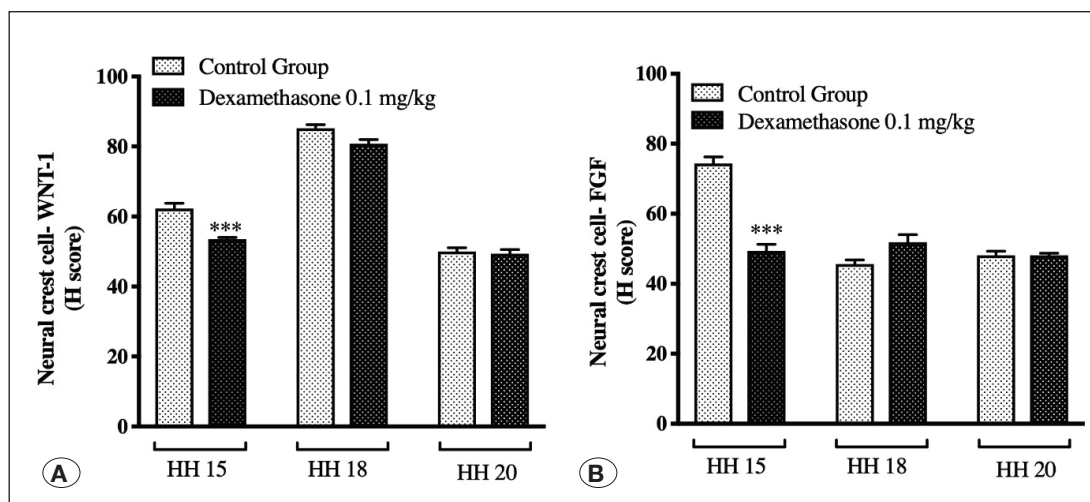


Figure 7: Immunohistochemistry Hscore analyses of Wnt-1 and FGF in neural crest cells control group and group 1 in stages HH15, HH18, HH20. Compared to control group * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

includes cell proliferation and migration from the third week onwards of the development and steer signaling pathways. It is also a process vulnerable to the effects of drugs, chemicals, and viruses, and it is a very common health problem worldwide. Negative results can be fatal due to an open or closed neural tube defect, with damages in soft and bone tissues and different organs. Abnormalities in primary and secondary neurulation process can cause NTDs with destructive effects on the central nervous system (32). Chick embryos, which are cheaper and easier to manipulate than other species' eggs, are very appropriate models for the investigation of NTDs. Moreover, NTDs are seen in the lower spinal level of chicks; thus, chick embryos are the closest to human models (5). Because of these reasons, we investigated the effects of Dex treatment on NCCs and neurulation in stages HH15, HH18, and HH20 in the caudal part of chick embryos. In this study, there were no significant differences between the group exposed to low-dose Dex and the control group in terms of embryo sizes, embryonic membranes, vascular structures, and neural tube effects. Aden et al. have shown that a single dose of Dex in embryonic day (E) 16 leads to an increased apoptosis in cerebellar granule neurons and other parts of the brain (1). Glucocorticoids have hazardous side effects on lung and brain development because glucocorticoid receptors start expressing during the embryonic period (3,8,11). Gulino et al. claim that many regions, including the hippocampus and cerebellar cortex, are affected by the harmful effects of glucocorticoids in prenatal and neonatal periods. These harmful effects are at the level of cell proliferation, growth, and differentiation of neurons in the cranial region (8). In our study, a single dose of Dex in E0 leads to a high mortality rate. Mortality rates are increasing in a dose-dependent manner, so embryos were not obtained after a high-dose Dex treatment in groups 2 and 3. Although mitotic rates were reduced between the groups, the thickening of the neural tube wall was higher than that in the control group.

Dexamethasone, a synthetic glucocorticoid, can pass through the placental barrier and affect the fetal development. Manojlavic-Stojanoski et al. have shown that the administration

of Dex at 16 days of gestation reduced the thyroid follicular cell proliferation, and at 21 days of gestation, all thyroid components, such as follicular cells, C cells, and colloids, were reduced depending on antiproliferative effects in rats. Moreover, the volume of the adrenal glands decreased after the administration of Dex during the intrauterine period (20). Although Smith and Schoenwolf showed that neuroepithelial cells divide regularly to shape the neural tube of chick embryos (31), the mitotic rate in the neural tube decreased after the Dex administration in every embryonic stage compared to the control group.

tNCCs primarily prefer the inter-somitic field, so they migrate first ventrally between the NT and somites in chick embryos. When somites completely matured, tNCCs pass through the anterior region of the sclerotome and basement membrane of the dermomyotome. The third part is to migrate in dorsolateral space (2). There is a 48-h difference between the first and third wave of tNCC migration in chick embryos (13,21,38).

The Wnt signaling pathway, especially Wnt-1, Wnt-3a, Wnt-6, Wnt-7, and Wnt-8a, is critically important in NPBC and NCC migration during embryonic development (13,21,26,29,33,34,42). Prasad et al. have shown that the expressions of the Wnt pathway induces neural plate border formation and NCC induction. When the Wnt signaling pathway was inhibited experimentally, the development of NCCs was damaged in chick and frog embryos (26). There is a significant reduction in stages HH15 and HH18, depending on the dose of Dex in our study. Moreover, the expressions of Wnt-1 in NCCs were decreased after the Dex treatment, but this decrease was only statistically significant in HH15. Previous studies have shown that NCC development and maintenance and neural fold fusion require continuous activities of Wnt from NPB (15,25). Wnt expression is important for the development and continuation of NCCs (25,42).

FGF signaling is also important for the development of NCCs in chick and human embryonic stem cell-derived neural crest model system (17,39,42). A decline in FGF levels leads to a deterioration in neural differentiation in a newly formed spinal

cord (19). Prasad et al. have shown that when FGF signaling is inhibited, NCC markers disappear in chick embryos. In addition to the close relationship between FGF and Wnt, it also affects the NCCs gene network and NCC development (25,26). Wnt signaling is upregulated in the neural tube borders related to FGF signaling and induces the fate of NCCs (29). FGF expression in stage HH15 has been significantly reduced as a result of Dex application, and no significant difference was detected in stages HH18 and HH20 in this study. The expression of FGF in group 1 was decreased significantly in stage HH15, and no significant difference was found in stages HH18 and HH20.

When NCC migration starts, the expression of N-cadherin is rapidly reduced. The downregulation of N-cadherin is a fundamental factor to start NCC migration (38). The level of N-cadherin should be reduced in order for NCCs to transform from nonmotile to motile and migrate (24). Osorio et al. claimed the expressions of N-cadherin in dorsal cells of the secondary neural tube in the posterior region. Although the expression of N-cadherin was significantly higher in stage HH20 after the treatment with Dex, no significant differences were found between stages HH15 and HH18 in the neural tube border. The neural tube basal lamina contains extracellular matrix components, such as fibronectin and tenascin-C during neurulation (21). Fibronectin expression only decreased in stage HH18 after the Dex treatment, and no significant difference in neural tube border was found between stages HH15 and HH20.

CONCLUSION

Dex is given to patients due to chronic diseases and problems that may develop during pregnancy. Studies have found that Dex impairs neurogenesis and causes intrauterine growth disorders. In this study, it was evaluated whether it has an effect on neural tube closure defects. Based on the results, although Dex treatment did not result in a neural tube closure defect, the mortality rates increased, whereas mitotic activation and Wnt-1 and FGF signal pathways reduced in some stages. Therefore, this study should be repeated in rat embryos to evaluate neural tube development. In addition, the spinal cord, peripheral nervous system, urogenital system, and other structures developing from NCCs and anomalies should be evaluated with this model in newborn mammals.

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AUTHORSHIP CONTRIBUTION

Study conception and design: SU, STE

Data collection: SU, STE, NA, US

Analysis and interpretation of results: SU, NA, DCK, AU

Draft manuscript preparation: SU, STE, NA, UU

Critical revision of the article: SU, STE, NA

Other (study supervision, fundings, materials, etc...): SU, STE, AU

All authors (SU, STE, NA, DCK, UU, AU) reviewed the results and approved the final version of the manuscript.

REFERENCES

- Aden P, Paulsen RE, Moehlen J, Loberg EM, Goverud IL, Liestol K, Lomo J: Glucocorticoids dexamethasone and hydrocortisone inhibit and accelerate maturation of chick cerebellar granule neurons. *Brain Research* 1418: 32-41, 2011
- Broom ER, Gilthorpe JD, Butts T, Campo-Paysaa F and Wingate RJT: The roof plate boundary is a bi-directional organiser of dorsal neural tube and choroid plexus development. *Development* 139:4261-4270, 2012
- Busada JT, Cidlowski JA: Mechanisms of glucocorticoid action during development. *Current Topics in Developmental Biology* 125:147-170, 2016
- Cheng X, Li H, Yan Y, Wang G, Berman Z, Chuai M, Yang X: Usage of dexamethasone increases the risk of cranial neural crest dysplasia in the chick embryo. *Toxicological Sciences* 158(1):1-12, 2017
- Dady A, Havis E, Esciou V, Catala M, Duband JL: Junctional neurulation: A unique developmental program shaping a discrete region of the spinal cord highly susceptible to neural tube defects. *J Neuroscience* 34(39):13208-13221, 2014
- Deardorff MA, Tan C, Conrad LJ, Klein PS: Frizzled-8 is expressed in the Spemann organizer and plays a role in early morphogenesis. *Development* 125:2687-2700, 1998
- Greene NDE, Copp AJ: Development of the vertebrate central nervous system: Formation of the neural tube. *Prenat Diagn* 29:303-311, 2009
- Gulino A, De Smaele E, Ferretti E: Glucocorticoids and neonatal brain injury: The hedgehog connection. *J Clin Invest* 119(2):243-246, 2009
- Hall BK: The neural crest and neural crest cells: Discovery and significance for theories of embryonic organization. *J Biosci* 33(5):781-793, 2008
- Hamburger V, Hamilton HL: A series of normal stages in the development of the chick embryo. *Developmental Dynamics* 195(4):231-272, 1992
- Heine VM, Rowitch DH: Hedgehog signaling has a protective effect in glucocorticoid-induced mouse neonatal brain injury through an 11betaHSD2-dependent mechanism. *J ClinInves* 119(2):267-277, 2009
- Heuer GG, Moldenhauer JS, Adzick NS: Prenatal surgery for myelomeningocele: Review of the literature and future directions. *Childs Nerv Syst* 33(7):1149-1155, 2017
- Kalcheim C, Burstyn-Cohen T: Early stages of neural crest ontogeny: Formation and regulation of cell delamination. *Int J DevBiol* 49:105-116, 2005
- Kerosuo L, Bronner ME: Biphasic influence of Miz1 on neural crest development by regulating cell survival and apical adhesion complex formation in the developing neural tube. *MolBiol Cell* 25(3):347-355, 2014

15. Kimura-Yoshida C, Mochida K, Ellwanger K, Niehrs C, Matsuo I: Fate specification of neural plate border by canonical Wnt signaling and Grhl3 is crucial for neural tube closure. *E Bio Medicine* 2(6):513-527, 2015
16. Lee JY, Kim SP, Kim SW, Park SH, Choi JW, Phi JH, Kim SK, Pang D, Wang KC: Pathoembryogenesis of terminal myelocystocele: Terminal balloon in secondary neurulation of the chick embryo. *Childs Nerv Syst* 29:1683-1688, 2013
17. Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, García-Castro MI: WNT/ β -catenin signaling mediates human neural crest induction via a pre-neural border intermediate. *Development* 143(3):398-410, 2016
18. Lowery LA, Sive H: Strategies of vertebrate neurulation and a re-evaluation of teleost neural tube formation. *Mechanisms of Development* 121:1189-1197, 2004
19. Lunn JS, Fishwick KJ, Halley PA, Storey KG: A spatial and temporal map of FGF/Erk1/2 activity and response repertoires in the early chick embryo. *Developmental Biology* 302:536-552, 2007
20. Manojlović-Stojanoski MN, Filipović BR, Nestorović NM, Šošić-Jurjević BT, Ristić NM, Trifunović SL, Milošević VL: Morpho-functional characteristics of rat fetal thyroid gland are affected by prenatal dexamethasone exposure. *Steroids* 84:22-29, 2014
21. Osório L, Teillet MA, Palmeirim I, Catala M: Neural crest ontogeny during secondary neurulation: A gene expression pattern study in the chick embryo. *Int J Dev Biol* 53:641-648, 2009
22. Pang D: Sacral agenesis and caudal spinal cord malformations. *Neurosurgery* 32(5):755-778, 1993
23. Patthey C, Edlund T, Gunhaga L: Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate. *Development* 136(1):73-83, 2009
24. Perris R, Perissinotto D: Role of the extracellular matrix during neural crest cell migration. *MechDev* 95(1-2):3-21, 2009
25. Pla P, Monsoro-Burq AH: The neural border: Induction, specification and maturation of the territory that generates neural crest cells. *Dev Biol* 1:36-46, 2018
26. Prasad MS, Charney RM, García-Castro MI: Specification and formation of the neural crest: Perspectives on lineage segregation. *Genesis* 57(1):e23276, 2019
27. Saad K, Otto A, Theis S, Kennerley N, Munsterberg A, Luke G, Patel K: Detailed expression profile of all six Glypicans and their modifying enzyme Notum during chick embryogenesis and their role in dorsal-ventral patterning of the neural tube. *Gene* 609:38-51, 2017
28. Sadler TW: Embryology of neural tube development. *Am J Med Genet Part C* 135C:2-8, 2005
29. Schille C, Schambony A: Signaling pathways and tissue interactions in neural plate border formation. *Neurogenesis* 23;4(1):e1292783, 2017
30. Shimokita E, Takahashi Y: Secondary neurulation: Fate-mapping and gene manipulation of the neural tube in tail bud. *Develop Growth Differ* 53:401-410, 2011
31. Smith JL, Schoenwolf GC: Neurulation: Coming to closure. *TINS* 20:510-17, 1997
32. Steventon B, Araya C, Linker C, Kuriyama S, Mayor R: Differential requirements of BMP and Wnt signaling during gastrulation and neurulation define two steps in neural crest induction. *Development* 136:771-779, 2009
33. Stuhlmiller TJ, Garcia-Castro MI: Current perspectives of the signaling pathways directing neural crest induction. *Cell Mol Life Sci* 69:3715-3737, 2012
34. Stuhlmiller TJ, García-Castro MI: FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction. *Development* 139(2):289-300, 2012
35. Takada S, Stark KL, Shea MJ, Vassileva G, McMahon JA, McMahon AP: Wnt-3a regulates somite and tailbud formation in the Mouse embryo. *Genes&Development* 8:174-189, 1994
36. Tural Emon S, Uslu S, Orakdogan M, Somay H: Effects of the popular food additive sodium benzoate on neural tube development in chicken embryo. *Turk Neurosurg* 25(2):294-297, 2015
37. Uslu S, Oktem G, Uysal A, Soner BC, Arbak S, Ince U: Stem cell and extracellular matrix-related molecules increase following melatonin treatment in the skin of postmenopausal rats. *Cell Biol Int* 38(8):924-932, 2014
38. Vega-Lopez GA, Cerrizuela S, Aybar MJ: Trunk neural crest cells: Formation, migration and beyond. *Int J Dev Biol* 61(1-2):5-15, 2017
39. Vieira C, Pombero A, Garcia-Lopez R, Gimeno L, Echevarria D, Martinez S: Molecular mechanisms controlling brain development: An overview of neuroepithelial secondary organizers. *Int J Dev Biol* 54:7-20, 2010
40. Yang HJ, Wang KC, Chi JG, Lee MS, Lee YJ, Kim SK, Cho BK: Neural differentiation of caudal cell mass (secondary neurulation) in chick embryos: Hamburger and Hamilton Stages 16-45. *Developmental Brain Research* 142:31-36, 2003
41. Yang HJ, Wang KC, Chi JG, Lee MS, Lee YJ, Kim SK, Lee CS, Cho BK: Cytokinetics of secondary neurulation in chick embryos: Hamburger and Hamilton stages 16-45. *Childs Nerv Syst* 22:567-571, 2006
42. Yardley N, Garcia-Castro MI: FGF signaling transforms non-neural ectoderm into neural crest. *Developmental Biology* 372:166-177, 2012