

# High Dose Cotinine may Induce Neural Tube Defects in a Chick Embryo Model

## Yüksek Doz Kotinin Cıvciv Embriyo Modelinde Nöral Tüp Defektlerine Neden Olabilir

### ABSTRACT

**AIM:** Nicotine is a well-known agent among 4000 chemicals in cigarettes. About 70 to 80% of nicotine is converted to cotinine, a major metabolite. The aim of the present study is to investigate the effect of cotinine on neural tube development in a chick embryo model.

**MATERIAL and METHODS:** Sixty fertile, specific pathogen free eggs were divided into 6 groups for this study. In the first group, a fixed cotinine concentration for each egg was calculated just to simulate the concentration of a smoker's blood level. A second experimental group was designed at a higher cotinine concentration. Embryos that succeeded to reach Hamburger-Hamilton stage 12 from each group were then embedded into paraffin for permanent sections. These two groups were compared with eggs subjected to vehicle (standard alcohol and ten times more alcohol concentration) and control groups (saline and sham groups).

**RESULTS:** Embryos of the cotinine (regular dose), vehicle and control groups were normal, but embryos subjected to higher cotinine concentrations were malformed at the cranial part of the thoracic neural tube.

**CONCLUSION:** Association of cotinine with neural tube defects was demonstrated in the present study. Cigarette smoking may induce hazardous effects on neural tube development.

**KEY WORDS:** Nicotine, Cotinine, Chick embryo, Neural tube defect, Congenital malformation

### ÖZ

**AMAÇ:** Nikotin, sigaranın içerdiği 4000 kimyasal maddeden iyi bilinenlerden biridir. Ortalama %70-80 düzeyinde major metaboliti olan kotinine yıkılır. Bu çalışmada, kotininin nöral tüp gelişimine olan etkisini cıvciv embriyo modelinde araştırılması amaçlanmıştır.

**YÖNTEM ve GEREÇ:** Çalışma için spesifik patojen içermeyen 60 adet döllenmiş yumurta 6 gruba ayrıldı. Birinci gruba, bir sigara içicisinde bulunan ortalama kotinin konsantrasyonuna eşdeğer kotinin verildi. İkinci gruba bunun 10 katı yükseklikte kotinin dozu verildi. Embriyolar Hamburger-Hamilton evrelendirmesine göre 12. evreye ulaşınca çıkarılarak parafin kesitleri alındı. Bu gruplardan elde olunan örnekler ilaç taşıyıcısı olan etil alkol grupları, kontrol grubu ve normal embriyo grubu ile karşılaştırıldı.

**BULGULAR:** Normal doz kotinin verilen grup, etil alkol grupları, serum fizyolojik ve normal kuluçka gruplarında embriyolar normal gelişim gösterirken, yüksek doz kotinin verilen grupta torakal omurganın kranial kesiminde nöral tüpün açık olduğu gözlemlendi.

**SONUÇ:** Bu çalışmada, kotininin nöral tüp defektleri ile ilişkili olduğu gösterilmiştir. Sigara nöral tüp defektlerine neden olabilecek zararlı etkilere yol açabilir.

**ANAHTAR SOZCÜKLER:** Nikotin, Kotinin, Cıvciv embriyosu, Nöral tüp defekti, Doğumsal anomali

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## INTRODUCTION

Cigarette smoke contains over 4,000 chemicals, some of which are well-characterized toxicants and carcinogens (6). Nicotine, tar, nitric oxide, carbon monoxide and aromatic amines, such as the carcinogens o-toluidine, 2-naphthylamine, and 4-aminobiphenyl are well-known chemicals in tobacco. Seventy to 80% of nicotine is converted to cotinine, which is present in smokers' blood at much higher concentrations than that of nicotine (12). Blood concentration of cotinine is about 250 to 900 ng/ml in cigarette smokers (3,9). The half-life of cotinine is about 18 to 24 hours, making it a more reliable marker of recent nicotine exposure (12).

Several human studies also suggest that maternal cigarette smoking increases the risk of neural tube defects in offspring (4,21). In a recent study in Norway, the authors correlated the risk of small-for-gestational-age at birth with nicotine concentration in hair of nonsmoking mothers and found an odd ratio equal to 3.4, indicating that maternal passive smoking was strongly associated with small-for-gestational-age at birth (17). Similar findings were also reported by Rabagliato, Ellard and Roquer et al. in offspring studies of smokers (5,18,19). The chemicals in cigarette smoke were shown to induce the developmental neural tube defects such as exencephaly (i.e., cadmium), embryonic apoptosis in mice and malformations (i.e., nicotine) and disruption in the neural tube of chick embryos (i.e., carbon monoxide) (1,2,8,22,25,27)

The aim of this report is to determine whether cotinine, a major metabolite of nicotine, induce adverse effects on the development of the neural tube in an early chick embryo model by light microscopic studies.

## MATERIALS and METHODS

**Chick embryos:** Fertile, specific pathogen free eggs of the domestic fowl (Atabey®, Gallus gallus, Poultry Research Institute, Ankara, Turkey) were selected for the present study. The eggs were incubated at 37.5°C and 75% relative humidity until the embryos reached stage eight of development according to Hamburger and Hamilton (11). At this stage, the eggs were divided into six groups that consisted of 10 eggs per group: Eggs in group 1 and 2 were injected with 10 µL of drug, however the concentration of cotinine was 1 µg/L for group 1 and

10 µg/L for group 2. Group 3 and 4 were injected with 10 µL amounts of vehicle (ethyl alcohol only, the concentration of alcohol was 0.4 mg/dL for group 3 and 4 mg/dL for group 4 at fixed injected volumes). Eggs in group 5 were injected with the same volume of physiological saline (10 µL) and eggs reserved for group 6 were not subjected to any injection and termed as sham group.

**Dosage of cotinine:** The blood cotinine level of a regular smoker is 250 to 900 ng/ml. An equivalent dose for a chick embryo is calculated according to the weight of the egg. The equivalent dose for this blood level of cotinine (Cotinine 98%: (C10 H12 N2O) Sigma – Aldrich Company code: C5923, St.Louis, Missouri, USA) was calculated to be 1 µg/L per egg. The stock solution was prepared to dissolve cotinine in ethyl alcohol and then the calculated dose of cotinine was diluted in 10 µL of physiological saline (0.9% NaCl) for group 1. For the high dose (ten times) cotinine group (group 2), 10 µg/L of cotinine was injected in the same volume of physiological saline.

**Method of injection:** At the eighth stage of development, 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. Using a sterile Hamburger® syringe, 10 µL of fluid was injected from the blunt end under the embryonic disc. The holes were sealed with paraffin. The eggs were then placed in an incubator.

**Embryo collection:** The eggs were opened at 50 hours of incubation. They were cracked open and the outer shell was chipped out to create a wide opening for visualization of the embryo. The viability of the embryos was assessed by the heart beat. The embryos were transferred to a petri dish by careful dissection among the allantoic stalk and other embryonic structures. All the embryos were fixed with 10% formalin and stained with hematoxylin-eosin and examined under stereomicroscope to assess any gross developmental abnormalities. Then, embryos that passed Hamburger Hamilton stage 12 were embedded into paraffin. Sections of five micron thickness were prepared and stained with hematoxylin-eosin for light microscopic examination. Slides were examined with Leica DM 4000 (Germany) photo-light microscopy.

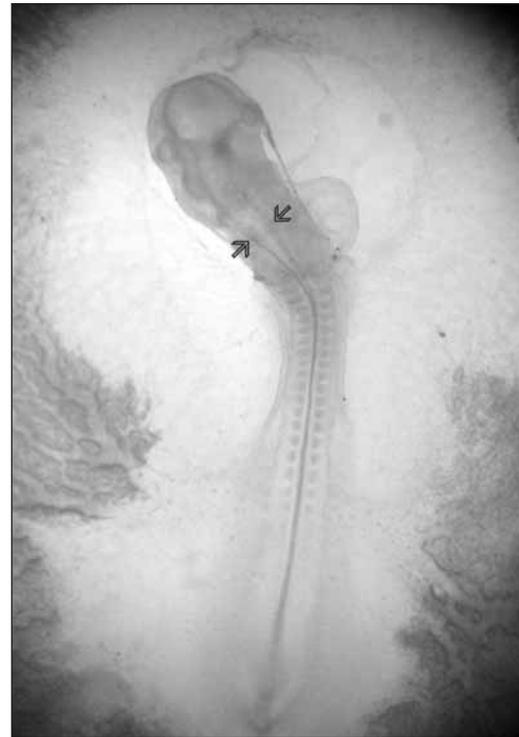
**RESULTS**

At the time of injection, each embryo should have at least four pairs of somite, neural folds of the future midbrain and a portion of the hindbrain, which signifies the criteria of a healthy egg and normal biological development.

After 22 hours of post-injection incubation, 3/60 (5%) of the embryos showed insufficient development. Nine embryos of the group 1 (injected with regular dose of cotinine), 9/10 embryos of group 3 (injected with basic dose ethyl alcohol), all of the embryos of the group 4 (injected with high dose ethyl alcohol), also all embryos of group 5 (injected with physiological saline 0.9% NaCl) and 9/10 embryos of group 6 (only incubated eggs) fulfilled the characteristics of Stage 12 development, however, only 6 embryos of group 2 (injected with high dose of cotinine) reached stage 12. Several characteristics of this stage are: head turning to left side; anterior neuropore closure, identifiable telencephalon; primary optic vesicles and well established optic stalk; auditory pit deep but wide open; slightly S-shaped heart; and head-fold amnion covering the entire forebrain region (11).

In groups 1,3,4,5 and 6, the neural tube was closed in all the embryos that reached Stage 12. However, 4/10 (40%) embryos in group two that reached Stage 12 were malformed (Table I). In these malformed embryos, mesenchyme and development of the organ systems was appropriate for their stage and the rostral part of the neural tube did not differ significantly from other normal embryos. However, the caudal part of the neural tube that corresponds to the thoracic regions of the embryos was defective (Figure 1).

In groups 1,3,4,5 and 6, transverse sections through the spinal cords at the thoracic level showed neural folds in contact with each other. On gross

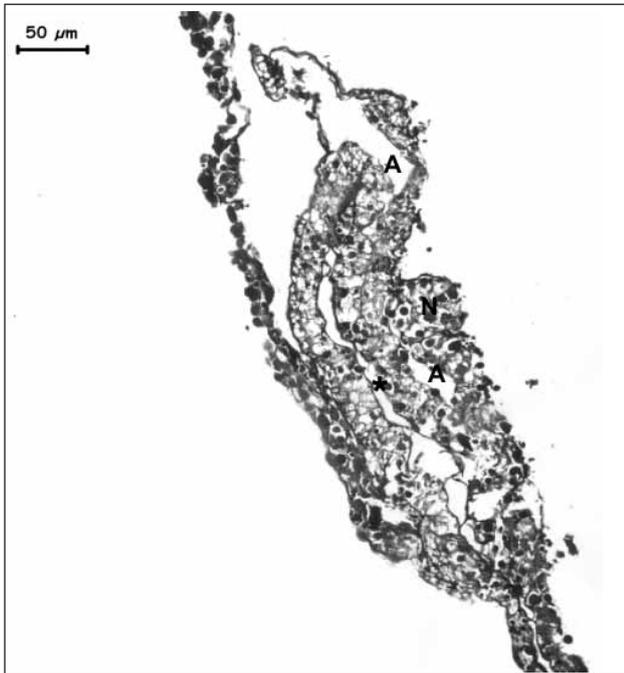


*Figure 1: Morphological examination shows open neural tube (arrows).*

examination, neural tube, superficial ectoderm, notochord and dorsal aorta were prominent in embryos that were not subjected to any experimental procedure. A distinct basal membrane was detected on the dorsal site of the neural tube and this was termed as a marker of complete separation from superficial ectoderm. The notochord was prominent on the transverse sections of the ventral side of neural tube (Figure 2) whereas much of the embryos in group 2 had an open neural tube. The defects were such that the neural folds showed no signs of contact over the neuroepithelium. In the high dose cotinine group, cells demonstrated degenerative findings with pycnotic nuclei and edematous cytoplasm were

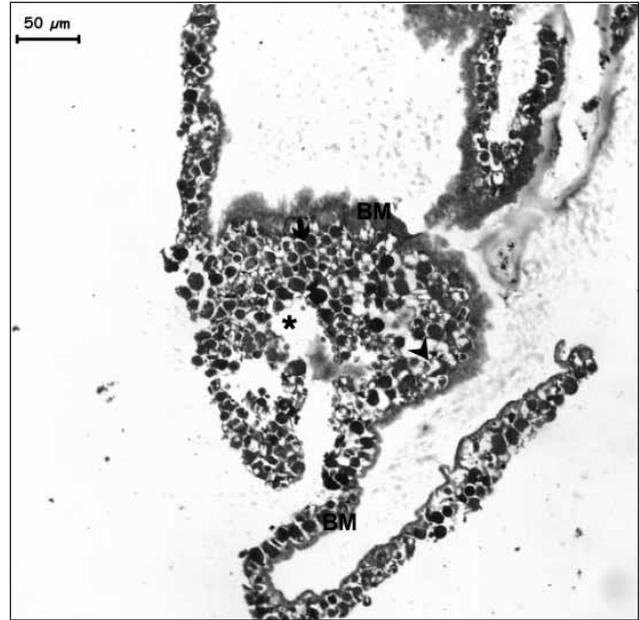
**Table I:** Table demonstrates distribution of the six experimental groups and coincidence of neural tube defects with group 2. Groups 1,3,4,5,6 were associated with normal development except for three undeveloped embryos from each of the group 1,3 and 6.

	Experimental groups (Cotinine)		Vehicle groups (ethyl alcohol)		Control (saline only)	Incubation only
	Regular	Higher (x10)	Regular	Higher (x10)		
Number	10	10	10	10	10	10
Normal Development	9	6	9	10	10	9
Neural tube defect	-	4	-	-	-	-

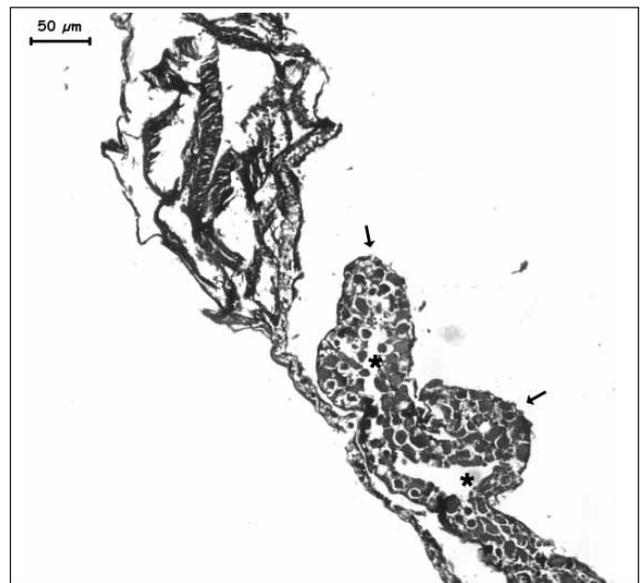


**Figure 2:** Neural tube was entirely closed and normal in appearance in regular dose cotinine group. On histopathological transverse plane sections, neural tube was visible on ventral side of superficial ectoderm with an isolated circular appearance which is almost identical to control group. (A): aorta, (N): Notocord with normal tubular structure in ventral side of neural tube, (\*): Neural tube lumen with normal structure (Hematoxylin-Eosin X400).

prominent on superficial ectoderm and cells of neural ectoderm expected to form the neural tube. Intercellular edema was prominent in cells belonging to the neural fold (Figure 3). The neural tube was not totally closed, particularly at the cranial ending. The superficial ectoderm was also visualized in several regions that would form the future neural tube and did not lose connection with the tube. The basal membrane of the neural tube (originated from superficial ectoderm) was prominent on histological sections but new basal membrane was not visualized on dorsal side of neural tube due to incomplete closure of neural tube and failure of detachment from superficial ectoderm. Clusters of cellular debris that were composed of neural crest like cells in the lumen of caudal site neural tube that was still open and marked detachments from basal membrane were visible to the ventral part of this portion of the neural tube (Figure 3). A bifid neural tube was recognized with findings of degeneration in a chick embryo belonging to same group (Figure 4).



**Figure 3:** In high dose cotinine group, transverse section revealed open neural tube and cells demonstrated degenerative findings with pycnotic nuclei and edematous cytoplasm (↑) were prominent on superficial ectoderm and cells of neural ectoderm which was expected to form neural tube. Intercellular edema was prominent (➤), BM: Basement membrane, (\*): Lumen of degenerative neural tube (Hematoxylin-Eosin X400).



**Figure 4:** A bifid neural tube (↑) was recognized with findings of degeneration in a chick embryo belonging to higher dose cotinine group. (\*): Lumen's of bifid neural tube (Hematoxylin-Eosin X400).

## DISCUSSION

Genetic predisposition and environmental trigger factors play a major role in the etiology of neural tube defects (NTD). Although few of the

environmental causes and none of the genetic factors have been identified in humans so far, numerous teratogens and nutritional deficiencies have been suggested as possible factors. Studies on the teratogenic effects of smoking have focused on possible cardiac malformations but few studies addressed central nervous system malformations such as neural tube defects (4, 15, 21). The present study demonstrated the contributory effect of cotinine at higher doses on neural tube defects in an early chick embryo model.

The early chick embryo model is an ideal model that corresponds to the first month of embryonic development in mammals and it is well suited for the investigation of chemicals on the development of embryos. Numerous chemical agents such as caffeine, phenytoin, diazepam and local anesthetics are known to cause neural tube defects in chick embryos (7, 10, 13). Stage eight embryos were generally chosen for these investigations since developing neural tissues exhibit a gradual variation on the degree of opening along its length that provides an excellent opportunity to study the effect of chemical agents on neural tube closure.

Smoking is well known to induce lung and heart diseases, probably in correlation with its adverse effects on microvasculature. Smoking may also delay wound healing due to the failure in migration of fibroblasts to the wound area and thus prevent the formation of healing (26). Cigarette smoke contains chemicals that inhibit growth, major vessel development, capillary plexus formation, and cell proliferation in the chorioallantoic membrane (CAM) of chick embryo (16). Contents of tobacco like 3-ethyl pyridine have significant effects on tissue growth compounds; in addition, pyrazine and 2-ethylpyrazine induces significant growth retardation in the CAM. On the other hand, Lee and colleagues suggested that nicotine contributed to neuroprotection due to attenuation of nitric oxide expression and arachidonic acid derivatives on a spinal cord injury model (14), nevertheless studies on embryo models demonstrated significant central nervous system damage through nicotine-induced toxicity. Zhao and Reece suggested that nicotine increases calcium and influence embryonic development in a concentration-dependent manner (27). Roy et al. reported that their study results support the idea that nicotine is a neuroteratogen, particularly targeting brain development at

concentrations below the threshold for dysmorphogenesis (20).

Cotinine is the principal metabolite of nicotine with an elimination half-life of 17 hours and it is eliminated over a much longer period of time when compared to nicotine (23). Cotinine has nicotine-like biological activity, but its potency is lower (24). Thus, cotinine is an acceptable prototype among tobacco alkaloids to investigate the hazardous effects of smoking on neural tube development. Although cotinine has a lower biological effectiveness when compared to nicotine, it is certainly associated with the formation of neural tube defects at the thoracolumbar region, particularly at higher doses.

In conclusion, nicotine is mainly metabolized to cotinine, which leads to open neural tube defects. Apart from nicotine or cotinine, tobacco contains more than 4,000 chemicals some of which are well-defined toxicants and carcinogens that may induce a summative effect with cotinine or initiate other central nervous system malformations.

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