Effect of Pethidine Hydrochloride on the Development of Neural Tube: A Genetic Analysis Study in a Chick Embryo Model

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■ BACKGROUND: **Neural tube defects are among the most frequent congenital abnormalities of the central nervous system. Progression of neural tube deficits is affected by hereditary predilection and environmental determinants. Pethidine (meperidine) is a fast and powerful opioid analgesic in U.S. Food and Drug Administration category C. There are reports about developmental anomalies due to this medication. The aim of this study was to investigate the effects of different doses of pethidine hydrochloride on neural tube development in a chick embryo model resembling the first month of vertebral growth in mammals.**

■ METHODS: **Seventy-five specific pathogen–free eggs were incubated for 28 hours and divided into 5 groups (including the control group), each consisting of 15 eggs. Pethidine hydrochloride was administered subblastodermically with a Hamilton microinjector in 4 different doses. Incubation was continued until the end of the 48th hour. Subsequently, all eggs were opened, and embryos were cut from the embryonic membranes and evaluated morphologically, genetically, and histopathologically.**

■ RESULTS: **Crown-rump length, somite numbers, and silver-stained nucleolar organizer region (AgNOR) number averages, and total AgNOR/nuclear area ratios decreased in a dose-dependent manner. Examination of neural tube closure revealed statistically significant differences in all experimental groups ($P<0.05$). Messenger RNA levels of the *BRE* gene were decreased in pethidine hydrochloride–exposed embryos compared with the control group. Although this downregulation was not statistically significant, this decrease was striking with a 0.422-fold change in the fifth group.**

■ CONCLUSIONS: **We demonstrated that pethidine hydrochloride affects neuronal development in chicken embryos. The teratogenic mechanism of pethidine hydrochloride is unclear; therefore, further investigation is required.**

INTRODUCTION

Pethidine (meperidine) is a fast and effective analgesic. Analgesia is mainly achieved through the central nervous system. Pethidine binds to specific opioid receptors in the brain and spinal cord, thus inhibiting the transmission of impulses along the polysynaptic pathways of the nociceptive system. Pethidine 70–100 mg corresponds to an equivalent analgesic dose of 10 mg of morphine. The sedative-hypnotic and respiratory depressant effects of pethidine are less pronounced than those of morphine. Pethidine causes only a slight increase in the muscle tone of internal organs and has a spasmolytic effect on the vascular smooth muscle. This leads to a decrease in blood pressure and an increase in heart rate.[1–5]

Meningocele, meningomyelocele, encephalocele, tense cord, and diastematomyelia anomalies are common congenital malformations in neurosurgical practice. The leading etiological factors of these congenital malformations are environmental factors, genetics, and drug use during pregnancy. Preventive medicine is

Key words
- Chicken embryos
- Development
- mRNA
- Neural tube defects
- Pethidine hydrochloride

Abbreviations and Acronyms
AgNOR: Silver-stained nucleolar organizer region
mRNA: Messenger RNA
NOR: Nucleolar organizer region
NT: Neural tube
PCR: Polymerase chain reaction
TAA/NA: Total AgNOR area/nuclear area

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effective for these diseases. Drugs that should be used during pregnancy cause such congenital malformations. However, the pregnancy category of newly developed drugs and the kind of malformations they can cause are not fully known owing to the scarcity of studies.[6-10]

The neural and spinal developmental stages of the chick embryo are very similar to that of the human embryos.[10] Based on our investigation, there is no study in the literature investigating the toxic effects of pethidine on neural tube (NT) development using a chick embryo model. Therefore, this study was designed to fill this knowledge gap and determine the possible toxic effect of different doses of pethidine and the consequences of its use in pregnancy.

The average incubation period in chickens is 22 days; 1 day of incubation is spent in the chicken body, and 21 days are spent in the incubator. Until the egg is placed in the incubator, the embryo in the egg is asleep. After ovulation, an environmental temperature between 15°C and 18°C should be provided to completely stop embryonic development. The optimal temperature required for embryonic development is 37.5°C.

If appropriate heat is provided both in its natural environment and in the incubator, the embryonic disc is formed along the long axis of the embryo on the first day. From this disc, cell layers called endoderm, ectoderm, and mesoderm differentiate and begin to develop. All organs and parts of the body are made up of these 3 cell layers. Hamburger and Hamilton[11] examined all developmental stages of the chick embryo in 1951 and divided it into 46 stages. According to these stages, in the 8th stage, a neural plaque is developed and is clear; by the 13th stage, the NT is closed, corresponding to neurulation.

MATERIALS AND METHODS

Study Design

This study was conducted at the Department of Anatomy, Medicine Faculty, Afyonkarahisar Health Sciences University, and approved by the Afyon Kocatepe University Animal Ethics Committee. The experimental eggs were divided into 5 groups of 15 eggs each, as follows: group 1, control group; group 2, 1 mg/kg pethidine hydrochloride; group 3, 2.5 mg/kg pethidine hydrochloride; group 4, 5 mg/kg pethidine hydrochloride; and group 5, 7.5 mg/kg pethidine hydrochloride.

Eggs and Embryos

In this study, 75 white fertilized specific pathogen–free chicken eggs weighing 65 ± 5 g were used. Day 0 eggs were placed in the incubator to point the sharp points. The incubator was maintained at a constant temperature range of 37.8°C ± 0.2°C and humidity of 60%–70%. During the incubation period, the eggs were automatically turned to the vertical axis at a 45° angle every 2 hours. Between the 28th and 30th hour of the study, the fertilized eggs were removed from the device, and a hole of approximately 0.5 cm^2 was opened under sterile conditions. The drugs were injected under the embryo disc using a Hamilton injector according to the previously prepared groups. After drug administration, all the holes in the eggs were closed with a sterile drape, and the eggs were placed back in the incubator. At the end of the 48th hour, the eggs were removed from the incubator and cleaned under sterile conditions. The eggshell was broken, and only the egg yolk was placed in a glass container containing sterile Ringer's lactate solution or saline (0.9% sodium chloride). A watch glass was placed in the container to collect the blastoderm. Then, using fine forceps and fine-tipped scissors, the vitelline membrane was cut from the embryonic membrane over the yolk. The vitelline membrane was separated from the yolk by carefully grasping it at both ends, and the blastoderm attached to the membrane was placed in the watch glass by advancing in liquid. Then the watch glass with the blastoderm was removed from the container. Embryos were examined under a light microscope. At this stage, the crown-rump length of the embryos in all groups, number of somites, and NT closure were determined.

Histologic Examination

After the obtained embryo samples were fixed in 10% neutral formalin, the crown-rump lengths were measured under a microscope (Eclipse E600; Nikon, Tokyo, Japan), and somites were counted, followed by histological tissue analysis. After washing in distilled water for 5 minutes, the sample was passed through the increasing alcohol series. It was kept for 3 minutes in 500, 600, 700, 800, 900, and twice in 1000 degrees of alcohol. The embryo was made visible by adding 1 drop of eosin to 900 mL of alcohol. Embryos that passed through xylene were embedded in paraffin. Sections of 5-μm thickness containing somites and an NT were examined. Deparaffinized and rehydrated sections were subjected to silver staining of nucleolar organizer regions (NORs) and hematoxylin-eosin staining.

Preparation of Silver Staining

Two solutions were required:

- Solution A: 2% gelatin solution dissolved in ultrapure water. Then a 1% gelatin solution was obtained by adding formic acid.
- Solution B: 50% silver nitrate solution in ultrapure water.

The staining solution was freshly prepared. Solution A was combined with solution B in a 1:2 ratio. The mixture was dripped to cover the sections completely, and the sections were stained for 15–30 minutes at room temperature without direct sunlight. After staining, the solution was discarded, and the sections were washed with distilled water. After being kept in a 5% thiosulfate solution for 10 minutes, slides were prepared for microscopic examination.[12]

The silver-stained nucleolar organizer region (AgNOR) samples were evaluated under a light microscope (Eclipse E600) using an image analysis system (NIS-Elements; Nikon); 25 nuclear AgNOR protein images were evaluated per sample. The average AgNOR number and total AgNOR area/nuclear area (TAA/NA) ratio were calculated for each nucleus.

RNA Extraction and Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from the whole embryo of each chick using PureZOL reagent (Bio-Rad Laboratories, Inc., Hercules, California, USA) according to the manufacturer's protocol. The RNA amount and purity were quantified using a Nanodrop ND-

Table 1. Oligonucleotide Primer Sequences

Gene	Base Sequences 5→3′	Base Longevity	Melting Temperature
Chick-*BRE*-F	GCAGCCTTCCTGAGTCACTT	20	59°C
Chick-*BRE*-R	TGCTCTCTTGGCCATTTCGT	20	57°C
Chick-*GAPDH*-F	GTCAACGGATTTGGCCGTAT	20	57°C
Chick-*GAPDH*-R	AATGCCAAAGTTGTCATGGATG	22	57°C

Chick-*BRE*, chicken brain and reproductive organ-expressed; Chick-*GAPDH*, chicken glyceraldehyde-3-phosphate dehydrogenase.

1000 spectrophotometer V3.7 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). RNA samples were stored at −80°C until further use. All RNA samples were reverse-transcribed into complementary DNA from 1 µg of total RNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Inc.) under the following cycling conditions: 1 cycle at 25°C for 5 minutes, 46°C for 20 minutes, and 95°C for 1 minute each. Real-time polymerase chain reaction (PCR) was performed after reverse transcription. BRE gene expression analysis was performed using Rotor-Gene Q (QIAGEN GmbH, Hilden, Germany). Complementary DNAs that belonged to each embryo were added to SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. Oligonucleotide primers were designed by Oligomer Biotechnology (Ankara, Turkey) as previously stated[13] (Table 1).

We used the following real-time PCR protocol: initial denaturation at 98°C for 30 seconds, followed by 40 cycles at 98°C for 5 seconds and extension at 60°C for 30 seconds. Melting curve analysis was conducted to confirm single product amplification at the end of the PCR, using the following parameters: 65°−95°C, 0.5°C increments at 5 second per step. Each run was performed in triplicate. To calculate relative fold changes in messenger RNA (mRNA) levels of target genes between experimental and control group embryos, the amplification efficiency value and mean threshold cycle values of each gene were entered into REST 2009 Version 2.0.13 (Qiagen, Hilden, Germany).[14]

Statistical Analysis

Analysis of morphohistological findings was performed using IBM SPSS Version 22.0 software (IBM Corporation, Armonk, New York, USA). The data related to NT closure were analyzed using the Chi-square test. Somite number, crown-rump length, and protein content were analyzed using nonparametric Kruskal-Wallis tests. Dunn tests were employed as post hoc tests, and $P < 0.001$ was considered statistically significant. All the genetic data analyses were performed using REST 2009 V2.0.13 software (Qiagen, Hilden, Germany).[14]

RESULTS

When NT closures were evaluated between the groups, there were statistically significant differences in all experimental groups ($P<0.05$). Crown-rump length, somite numbers, TAA/NA ratios, and AgNOR number averages are shown in Table 2. Our results showed that crown-rump length, somite numbers, TAA/NA ratios, and AgNOR number averages decreased in a dose-dependent manner. A statistically significant decrease was detected in the crown-rump length between groups 1 (control) and 5 ($P<0.05$); somite numbers between groups 1 and 5 ($P<0.001$) and groups 2 and 5 ($P<0.05$); AgNOR numbers between groups 1 and 5 ($P<0.05$); and TAA/NA ratios between groups 1 and 3 ($P < 0.05$), groups 1 and 4 ($P<0.05$), and groups 1 and 5 ($P<0.001$) (as shown in Table 2).

Table 2. Statistical Analyses Embryonic Development in Control and Experimental Groups (48-Hour Embryos)

Parameters	Group 1, Control	Group 2, Pethidine HCl 1 mg/kg	Group 3, Pethidine HCl 2.5 mg/kg	Group 4, Pethidine HCl 5 mg/kg	Group 5, Pethidine HCl 7.5 mg/kg	Significant *P* Values
Somite number	22.1 ± 3.0	21.2 ± 3.9	19.0 ± 2.2	18.3 ± 1.5	16.8 ± 1.5	G5-G1 ($P = 0.000$) G5-G2 ($P = 0.005$)
Crown-rump length, µm	764.92 ± 105.20	714.43 ± 104.08	660.06 ± 84.99	651.31 ± 41.23	622.51 ± 32.42	G5-G1 ($P = 0.008$)
AgNOR number	16.5 ± 1.6	15.2 ± 1.5	15.1 ± 0.7	15.1 ± 1.7	14.3 ± 1.1	G5-G1 ($P = 0.015$)
TAA/NA ratio	0.31 ± 0.05	0.26 ± 0.06	0.24 ± 0.01	0.23 ± 0.02	0.21 ± 0.03	G5-G1 ($P = 0.000$) G4-G1 ($P = 0.002$) G3-G1 ($P = 0.040$)
Open NT/closed NT	0/10	3/7	6/4	8/2	9/1	
Stage of embryo	13	12	12	12	11	

HCl, hydrochloride; G, group; AgNOR, silver-stained nucleolar organizer region; TAA/NA, total AgNOR area/nuclear area; NT, neural tube.

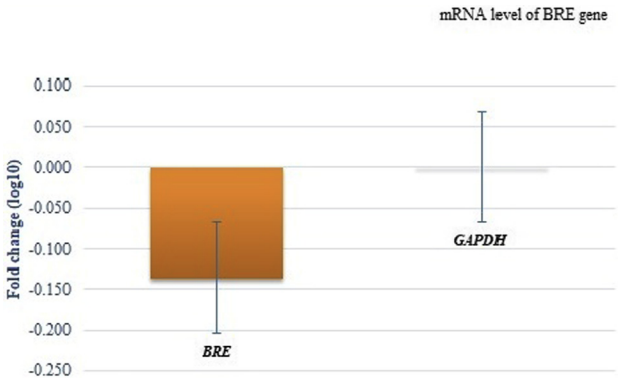

Figure 1. Results of real-time polymerase chain reaction analysis. Relative messenger RNA expression of *BRE* in all chick embryos exposed to pethidine hydrochloride was given as fold regulation level. *GAPDH* is reference gene for normalization. mRNA, messenger RNA.

mRNA Analysis of *BRE* Gene in Chick Embryos

The mRNA levels of the BRE gene in chick embryos of the control and experimental groups were analyzed by real-time PCR. Alteration in BRE mRNA levels of each experimental group embryo was determined according to the mRNA levels of the BRE gene in control embryos. The GAPDH gene was used as a reference gene for normalization. The mRNA levels of the BRE gene were lower in pethidine hydrochloride–exposed embryos than in the control group (0.731; fold regulation value) (Figure 1).

However, this downregulation was not statistically significant. BRE gene expression was downregulated in groups 3 and 5 and upregulated in groups 2 and 4. Additionally, there were no significant differences in group samples compared with the control group in terms of BRE mRNA levels ($P>0.05$). The mRNA levels of the BRE gene in embryos were expressed as fold regulation levels. The fold regulation values were 1.006 for group 2, 0.963 for group 3, 1.153 for group 4, and 0.422 for group 5 (Figure 2).

DISCUSSION

Pethidine, which is frequently used in the practice of neurosurgery, orthopedics, and oncology, is a fast and effective synthetic opioid analgesic drug. It is allocated to pregnancy category C according to the U.S. Food and Drug Administration drug classification.[15] Pethidine is a phenylpiperidine derivative. It has no harmful effect on the respiratory system. In contrast to morphine, it has an analgesic as well as a spasmolytic effect. It is indicated for severe acute pain in the urogenital, gastrointestinal, and biliary tract; skeletal system diseases such as postoperative diseases, fracture, and dislocation; childbirth and similar conditions; and neoplastic diseases.

Pethidine (meperidine) is further metabolized in the liver by CYP2B6, CYP3A4, and CYP2C19, accounting for 57%, 28%, and 15% of its total essential clearance, respectively. The foremost metabolite of pethidine, norpethidine, can accumulate in the brain and lead to important central nervous system toxicities when a high dosage of pethidine is administered. The degree of structure and clearance of norpethidine can be difficult to predict from pethidine owing to the polymorphic and inducible character of CYP2B6. Prolonged expression of CYP2B6 can upregulate the production of

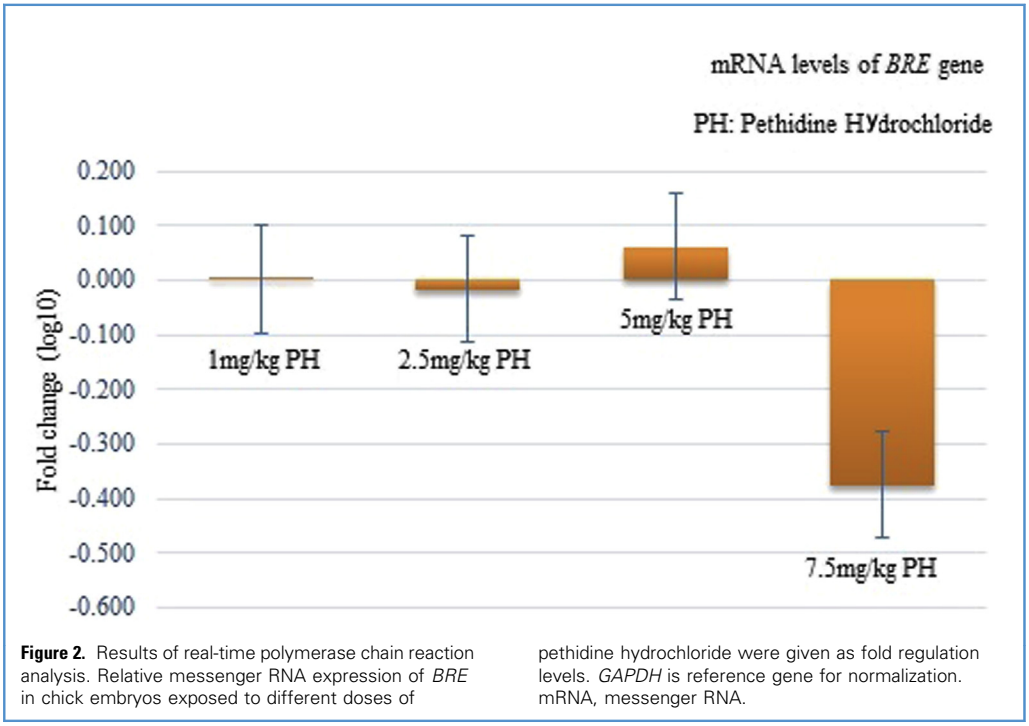

Figure 2. Results of real-time polymerase chain reaction analysis. Relative messenger RNA expression of *BRE* in chick embryos exposed to different doses of pethidine hydrochloride were given as fold regulation levels. *GAPDH* is reference gene for normalization. mRNA, messenger RNA.

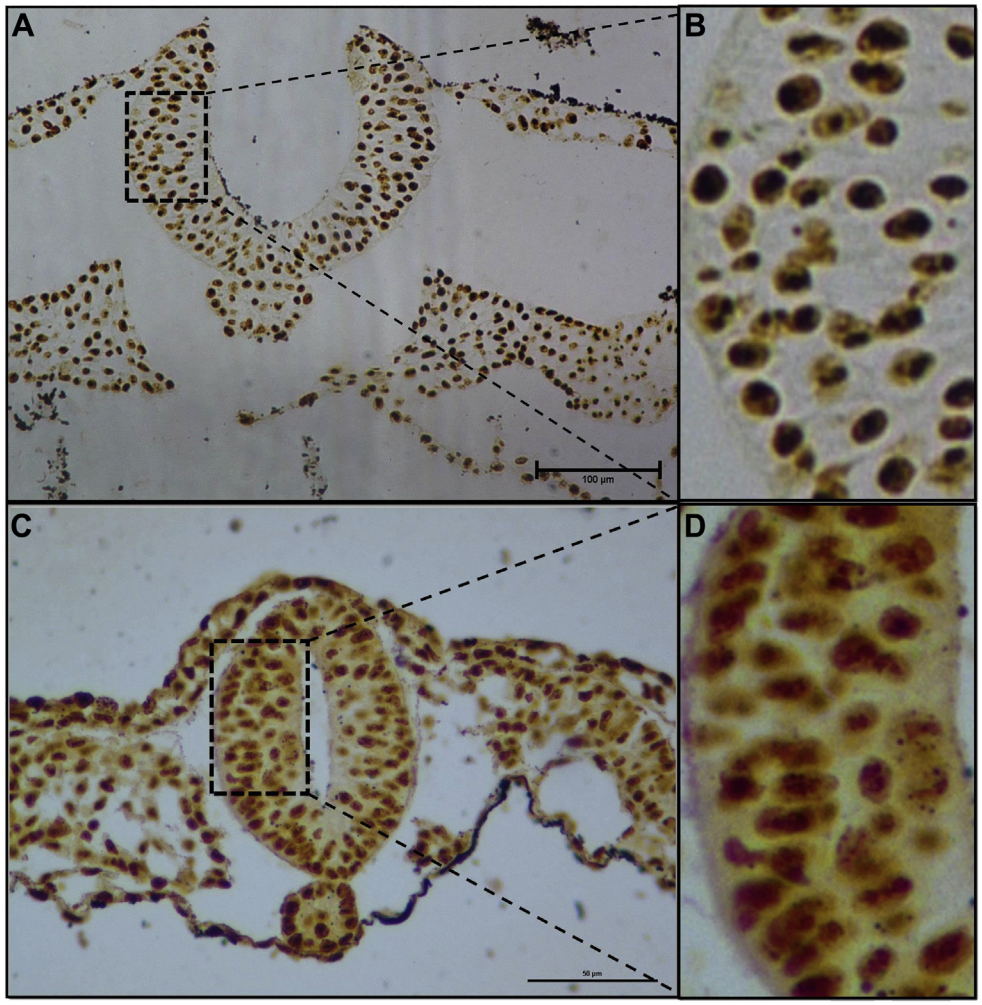

Figure 3. Demonstration of the silver-stained nucleolar organizer region staining cells. (**A**) Control group (×20). (**B**) Control group (×100). (**C**) Experimental group (×20). (**D**) Experimental group (×100).

norpethidine and result in the rise of unfavorable circumstances, mostly including convulsions. Owing to the random constitution of pethidine metabolism and distribution, it is usually not administered to elderly patients with compromised liver or kidney function.[16]

In the study conducted by Geber and Schramm,[4] pethidine was administered to 6 groups of rats at different doses. It was reported that the rate of both fetal death and congenital malformations increased as the dose of pethidine increased.

Congenital central nervous system anomalies are the most common, followed by congenital cardiovascular abnormalities.[8,17] NT defects occur as a result of improper or incomplete closure of the NT in the 3rd and 4th weeks of embryonic development. It is the most common congenital anomaly of the central nervous system that can lead to death or lifelong disability. NT defects occur in approximately 6 in 10,000 pregnancies.[18] There are many studies on the development of the NT using the chicken embryo model.[8-10,19,20] The early development of the chicken embryo, especially the first 48 hours, is similar to the first month of embryonic development of the mammalian spine. Therefore, chick embryos are suitable models for research on the neurulation stage. Using this model, substances thought to be toxic to the NT during pregnancy were investigated.[9,21,22] In some studies, the effect of the most commonly used teratogenic substances on the open or closed state of the NT was examined.[6,7] One of the 6 parameters of the current study was the NT on/off examination. Our results showed that the mean number of NT openings increased depending on the dose (Figure 3). While the NT was 100% closed in group 1 (control), the NT was 30%, 60%, 80%, and 90% open in groups 2, 3, 4, and 5 (Figures 4 and 5).

Nucleolar organizer regions (NORs) are fragments of metaphase chromosomes where ribosomal genes are established and that match with auxiliary bindings. Throughout the interphase,

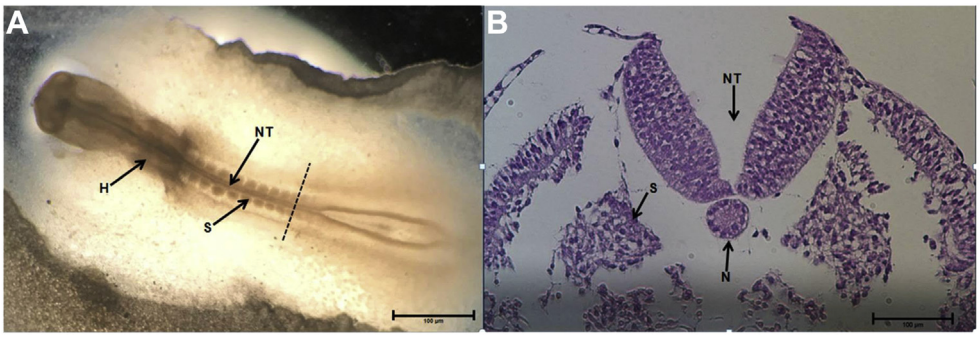

Figure 4. (**A**) The opening in the neural tube is seen in the light microscopic view of experimental groups (48 hours). (**B**) Histopathological view of the embryo with open neural tube in lumbar segment under light microscope (hematoxylin-eosin, ×20). NT, neural tube; H, heart; S, somites; N, notochord.

NORs are linked with a large number of regulative proteins and comprise parts of operative subunits of the nucleolus. These compositions include all the essential elements for ribosomal RNA organization and are the sites where ribosomal gene transcription occurs. A unique group of extremely argyrophilic acidic proteins is present in the NORs, hence enabling NORs to be precisely and selectively envisioned at the light microscopic level by silver nitrate staining methods (i.e., AgNORs). Changes in AgNOR protein levels also show the metabolic actions of the cells. Originally practiced as a parameter of malignancy, the AgNOR parameter is beneficial for evaluating the prognosis of cancer (Figure 3).[23-27]

Ertekin et al.[23] used the TAA/NA measurement as a novel approach that can be used in routine cytopathology to determine the proliferation activity of cells. Accordingly, we evaluated the usability of the low AgNOR and TAA/NA ratios as regression criteria for cell growth. Our results showed that the average number of AgNORs decreased in a dose-dependent manner. This difference was statistically significant between the control group and group 3 ($P < 0.05$), the control group and group 4 ($P < 0.05$), and the control group and group 5 ($P<0.001$). Although the difference in the ratio of TAA/NA was negligible between the control group and all experimental groups, a significant difference was found between the control group and group 5 ($P<0.05$) (Table 2).

Another parameter examined was the embryonic stage average. The embryonic stage average was 13 in the control group (group 1) and 11 in group 5. The embryonic stage average was 12 in all other groups. Our results support the theory of Ertekin et al.[10]

There are no previous reports linking or investigating pethidine hydrochloride or any synthetic opioid analgesic drug with neural

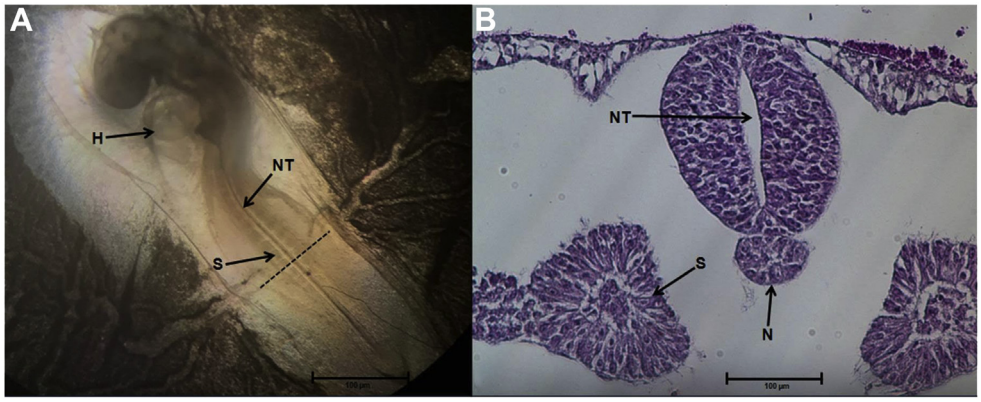

Figure 5. (**A**) Normal appearance of 48 hours postincubation chick embryo of control group stage 13. (**B**) Histopathological views of normal chicken embryos in thoracic segment under light microscope (hematoxylin-eosin, ×20). NT, neural tube; H, heart; S, somites; N, notochord.

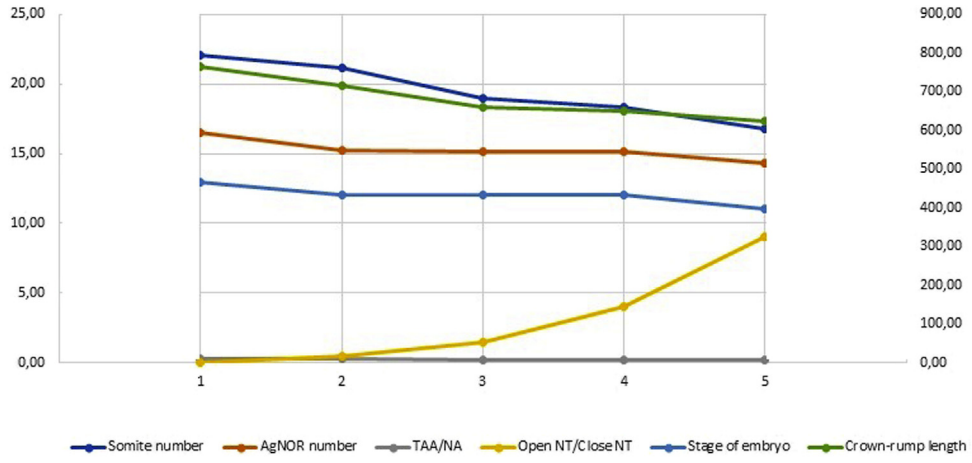

Figure 6. Graph of the results of all parameters of the control and experimental groups. AgNOR, silver-stained nucleolar organizer region; TAA/NA, total AgNOR area/nuclear area; NT, neural tube.

tube defects. However, Ertekin et al.[10] found that as the dosage of diclofenac sodium increased, midline closure caused defects in early chicken embryos, and the crown-rump length and the somite count decreased significantly. In our study, the mean crown-rump length and somite numbers decreased in a dose-dependent manner, and this decrease was determined to be statistically significant between the control group and group 5, especially in the crown-rump length parameter ($P < 0.05$). A significant difference was detected in the median number of somites between the control group and group 5 ($P<0.001$) and between groups 2 and 5 ($P<0.05$) (**Figure 6**).

The *BRE* gene is most highly expressed in the nervous and reproductive systems. Overexpression of the *BRE* gene can promote the formation and differentiation of somites. *BRE* is mainly expressed in the NT, neural crest cells, somites, and neurites during early embryonic development.[13] In our study, *BRE* expression was significantly downregulated in group 5 compared with the control group and group 2. *BRE* mRNA levels in group 2 and the control group were almost the same. Following this result, a statistically significant decrease in somite numbers was observed between the control group and group 5 and between the control group and group 2. We suggest that *BRE* expression is affected by the high dosage of pethidine hydrochloride. When the *BRE* mRNA level decreased, somite development was delayed. Wang et al.[13] reported that *BRE* expression was silenced in half of the NT, which inhibited somite growth, and suggested that reduced *BRE* expression in the NT indirectly interfered with somite growth and differentiation. Additionally, these authors suggested that *BRE* is also involved in NT, neural crest cell, and somite development by modulating the cell cycle. Our results support this suggestion, as in our study, the lowering of the mitotic index was directly proportional to the downregulation of *BRE* in pethidine hydrochloride–exposed embryos. Furthermore, this gene was found to be downregulated after DNA damage. The *BRE* gene was first reported in 1995 when screening complementary DNA libraries for genes responding to DNA damage.[28]

CONCLUSIONS

Our study showed a direct teratogenic effect of pethidine hydrochloride on the NT formation process in chick embryos in a dose-dependent manner. Pethidine hydrochloride significantly reduced the AgNOR number, TAA/NA ratio, crown-rump length, and somite number. The mRNA levels of the *BRE* gene were lower in pethidine hydrochloride–exposed embryos than in control embryos. Although this downregulation was not at a statistically significant level, this decrease was striking with a 0.422-fold change in group 5. However, the model chosen does not directly reflect the context and conditions of a developing human embryo. Therefore, our results cannot be directly extended to humans. However, the chick embryo model has the advantage of allowing potentially hazardous substances to be investigated directly on the embryo. In our study, specific neuronal markers were not used to determine the toxicity induced by pethidine. We interpreted our results based on genetic and histopathological findings. Improved technical materials and studies with larger sample sizes will help to demonstrate the possible toxic effects of pethidine hydrochloride in the prenatal period. Further research would be valuable to explain the exact mechanism of pethidine toxicity. The current findings cannot serve as conclusive evidence of the use of pethidine hydrochloride as embryotoxic, but they provide an experimental basis for warning about the use of this drug in pregnancy.

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