Turk Neurosurg, 2024

Original Investigation

DOI: 10.5137/1019-5149.JTN.45237-23.2

Received: 15.08.2023
Accepted: 07.10.2023

Published Online: 25.07.2024

Investigation of the Effect of Tenoxicam on Neural Tube Defect Using Chick Embryo Model

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ABSTRACT

AIM: To evaluate tenoxicam's effects on embryonic neural tube formation to identify potential teratogenicity and determine the underlying mechanisms leading to neural tube defects (NTDs).

MATERIAL and METHODS: This study was conducted at our University's Neuro-embryology Laboratory. A total of 100 fertile chicken eggs were opened using the windowing method after 24 hours of incubation. The embryo models were divided into four groups based on tenoxicam dosage: 0.01, 0.02, 0.10 μg, and control group (0.9% SF was administered). The tenoxicam groups were administered 20 μL volume sub-blastodermally. The eggs were incubated for another 24 hours after being covered with sterile draping. All the eggs were opened at the 48th hour, and the embryos were evaluated.

RESULTS: Each group consisted of 25 chicken embryos. Normal neural tube development was observed in Group 1 (0.01μg) with 23 out of 25 embryos, Group 2 (0.02 μg) with 20 out of 25 embryos, Group 3 (0.10μg) with 16 out of 25 embryos, and Group 4 (control group) with 24 out of 25 embryos. Additionally, the rates of absence of embryo development were 8%, 8%, 12%, and 4% in Groups 1, 2, and 3 and the control group, respectively.

CONCLUSION: We observed that tenoxicam use caused midline closure defects in early chicken embryos in a dose-dependent manner. Further studies are required to determine the mechanisms underlying the embryonic damage and teratogenic effects due to genetic and environmental factors and minimize the development of congenital defects.

KEYWORDS: Tenoxicam, Cyclooxygenase, Prostaglandin, Neural tube defect, Chicken embryo

ABBREVIATIONS: COX: Cyclooxygenase, **Css_{max}:** Maximum steady-state concentration, **Css_{min}:** Minimum steady-state concentration, **H&E:** Hematoxylin and eosin, **NBDPS:** National Birth Defects Prevention Study, **NSAID:** Nonsteroidal anti-inflammatory drug, **NTD:** Neural tube defect, **PG:** Prostaglandin, **TEN:** Tenoxicam

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INTRODUCTION

Neural tube defects (NTDs) are complex congenital anomalies of the central nervous system, affecting 2 in 1,000 births (15,19). Approximately 50% of all fetus' with NTDs are stillborn or electively terminated after being diagnosed prenatally. Live-born infants with NTDs have an elevated risk of mortality before the age of 5 years (4). NTDs are believed to occur because of the interaction of genes with environmental and nutritional factors. Additionally, certain drugs such as thalidomide, antiepileptic drugs, folate antagonists, and androgenic hormones are also implicated in the etiology of NTDs. The use of painkillers and non-steroidal anti-inflammatory drugs (NSAIDs) such as acetaminophen, ibuprofen, and naproxen during the prenatal period is increasing (22). Therefore, examining the teratogenic effects of such drugs during pregnancy is crucial. Tenoxicam (TEN) is a thienothiazine derivative that belongs to the group of chemicals known as NSAIDs (3). TEN not only suppresses prostaglandin synthesis by inhibiting cyclooxygenase but also suppresses leukotrienes (16).

Unlike other NSAIDs, there is no clear study on the teratogenic effect of TEN during pregnancy. Thus, using an embryonic model, we aimed to evaluate the potential impact of TEN on neural tube formation and identify any associations with NTDs. This is an especially relevant study as the pregnancy category for TEN remains to be definitively established.

MATERIAL and METHODS

Our study was conducted in the Neuro-embryology Laboratory within the Department of Neurosurgery. A total of 100 pathogen-free, fertile, zero-day, white Super Nick eggs were obtained to create this chicken embryo model. The eggs were weighed (average weight 65 ± 2 g) and incubated for 24 hours in an incubator, which rotated automatically every 2 hours, at 37.8°C ± 0.2°C and 65%–75% humidity. At the 24th hour of incubation, all the eggs were opened using the windowing technique. The eggs were divided into four main groups, one of which was the control group (each group, n = 25). Appropriate solutions were prepared by dissolving TEN in water under sterile conditions. The control group was injected with 0.9% saline, while the other groups were injected with increasing doses of TEN (0.01, 0.02, and 0.10 mg) using a Hamilton syringe with a volume of 20 ml, sub-blastodermally (Figures 1A, B).

Drug preparation

We calculated the TEN doses based on a standard SPF egg weighing 65 ± 2 g and considering a human adult dose of 20 mg per day. The minimum (Css_{min}) and maximum (Css_{max}) concentrations after a single 20 mg oral dose of TEN are reportedly 9.7 and 13.6 mg/L, respectively (25).

In Group 1, a sub-therapeutic TEN dose of 0.01 mg was administered, which corresponded to a Css_{min} of 5 mg/L. In Group 2, 0.02 mg of TEN was administered, which corresponded to a therapeutic Css of 10 mg/L. In Group 3, a supra-therapeutic dose of 0.1 mg was administered, corresponding to a Css of 50 mg/L. Subsequently, in groups 1, 2, and 3, 0.01 mg (1mg/2 ml TEN and 20 µL/chick embryo), 0.02 mg (2 mg/2 ml TEN and 20 µL/chick embryo), and 0.10 mg (10 mg/2 ml TEN and 20 µL/chick embryo) of TEN was administered, respectively. In the control group, 0.9% SF was administered.

Subsequently, the opened windows were closed with sterile draping. Thereafter, the eggs were rotated 180° and placed into the incubator for 48 hours. The eggs were opened after 48 hours using the new technique to evaluate embryological development (26). Using the Hamburger–Hamilton Chicken Embryo Classification System, the embryos were evaluated under 40X magnification (Nikon ZMS-20 light microscope). The embryos were classified separately for each group, considering the following parameters: open or closed neural tube and lack of embryological development.

Histopathological analysis

After completing the TEN treatment, the samples were fixed in 10% formaldehyde and placed in Petri dishes. Subsequently, the embryos from each group were dehydrated in graduated alcohols, cleaned in xylene, and embedded in paraffin wax. Four-micron thick serial sections were obtained from the paraffin blocks using a microtome.

The paraffin sections were stained with hematoxylin and eosin (H&E) to visualize the cellular structures and tissue architecture. The histopathological features were observed

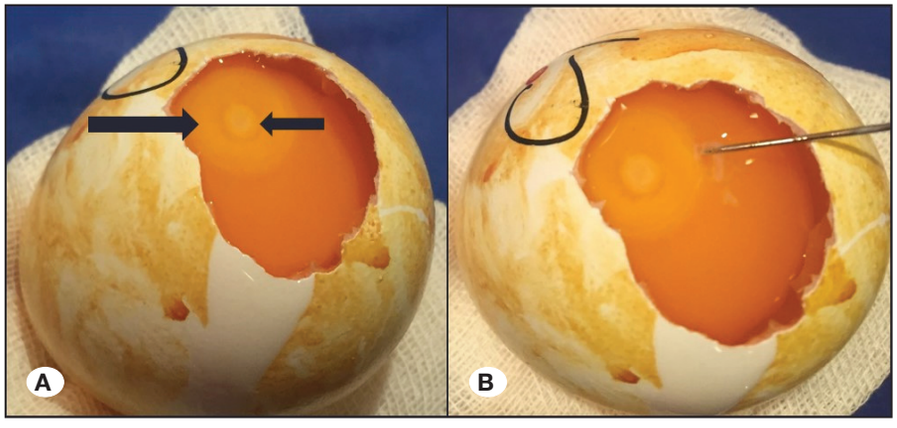
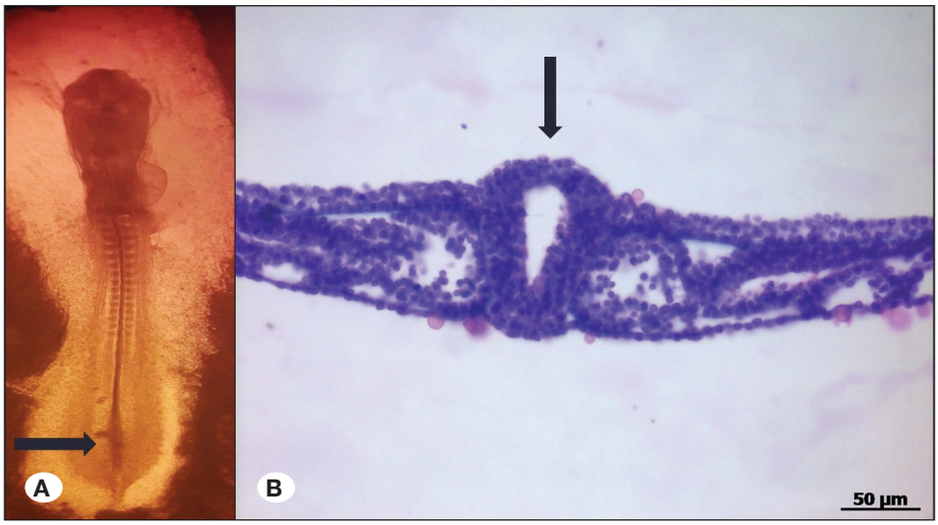


Figure 1: A) Embryonic disc (small arrow) and blastoderm (large arrow) after shell removal. **B)** The solutions being injected under the blastoderm using a Hamilton micro-syringe.



Figures 2: A, B) The normal neural tube of a chicken embryo. Hematoxylin and eosin (H&E) stain was employed to visualize the cellular structures and tissue architecture.

Table I: Descriptive Values And Statistical Analyses of NTD Development of the Four Groups NT, Neural Tube

	Group 1 0.01 µg	Group 2 0.02 µg	Group 3 0.1 µg	Group 4 control	Total	Fisher's exact test
Normal NT	23 92%	20 80%	16 64%	24 96%	83 83%	
No development	2 8%	2 8%	3 12%	1 4%	8 8%	
NTD	0	3 12%	6 24%	0	9 9%	**p=0.019**
Total number	25	25	25	25	100	

NT: *Neural tube,* ***NTD:*** *Neural tube defect.*

and analyzed. The stained sections were studied under a light microscope to identify any potential histopathological alterations (Figures 2A, B).

Statistical Analysis

All statistical analyses were conducted using SPSS version 23.0 (IBM Corp., Armonk, New York). The statistical significance between the groups was assessed using the Fisher's exact test (Table I). A p-value of <0.05 was considered statistically significant.

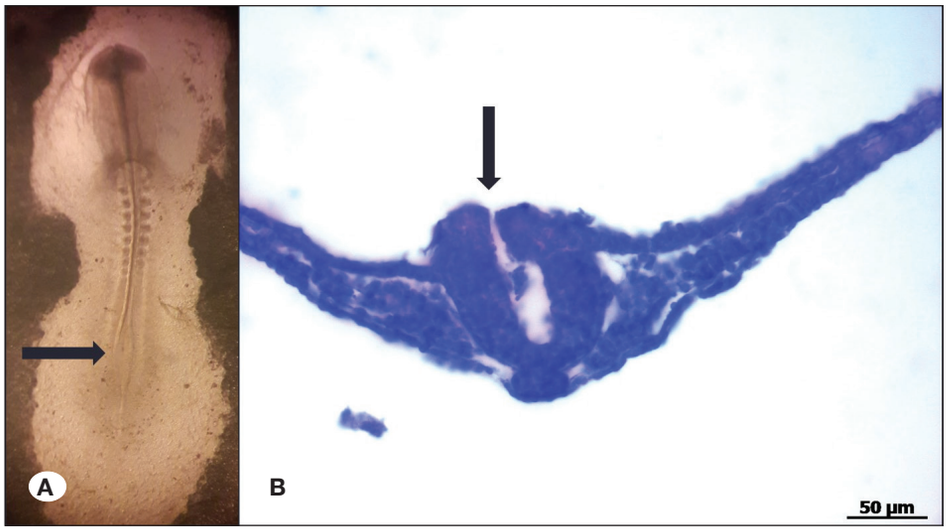
RESULTS

In Group 1, 92% (n=23) of the embryos exhibited closed neural tubes, while 8% (n=2) of the embryos showed no development. None of the embryos in Group 1 displayed neural tube patency. In Group 2, 80% (n=20) of the embryos exhibited closed neural tubes, 8% (n=2) did not demonstrate any development, and 12% (n=3) exhibited open neural tubes. In Group 3, 64% (n=16) of the embryos exhibited closed neural tubes, 12% (n=3) did not demonstrate any development, and 24% (n=6) exhibited open neural tubes (Figures 3A, B). In the control group (Group 4), 96% (n=24) of the embryos exhibited closed neural tubes and 4% (n=1) did not demonstrate any development. No instances of neural tube patency were observed in any of the embryos (Table I). Microscopic examination of the stained tissue samples from the control group confirmed closed neural tubes, which were consistent with the stereomicroscopic findings (Figures 2A, B).

Comparison between the groups indicated no statistically significant differences in NTDs between Groups 1 and 4 and between Groups 2 and 4 ($p>0.05$). However, a statistically significant difference in the presence of NTDs was observed between Groups 3 and 4 ($p<0.05$). Embryos displaying no development were also considered abnormal. Statistical analyses revealed 8% (n=2) of the embryos in Groups 1 and 2, 12% (n=3) in Group 3, and 4% (n=1) in Group 4 did not show any development. There was no statistically significant difference between Groups 1, 2 and 3 (Fisher's exact test; p=0.019).

DISCUSSION

Pain, due to several causes, is a common problem during pregnancy, and the use of painkillers is often necessary.



Figures 3: A, B) Embryo with a neural tube defect (serial tissue sections stained with hematoxylin and eosin [H&E]).

According to a multinational study conducted in 2014, approximately 50% of pregnant women use painkillers, especially paracetamol and NSAID, during their pregnancy (18). Therefore, examining and understanding the effects of NSAIDs on the fetus is essential.

NSAIDs constitute a class of medications used for pain relief and reduction in fever and inflammation. These analgesic drugs inhibit cyclooxygenase (COX) isoenzymes to achieve their effects. Furthermore, NSAIDs bind to the COX enzymes selectively or non-selectively. Inhibition of the COX enzyme leads to a decrease in the formation of thromboxane, prostaglandin, and prostacyclin from arachidonic acid. The COX enzyme has two isotypes, COX-1 and COX-2, which are expressed in numerous tissues such as the endothelium and blood cells whereas, COX-2 is induced by proinflammatory cytokines (5,11,23).

All NSAIDs can cross the placenta, underscoring the significance of comprehending their influence on early fetal development (24). Thus, NSAIDs could have harmful effects on the fetus by inhibiting prostaglandin synthesis (1). In our study, we evaluated the effects of TEN, which is related to nonselective NSAIDs, on embryonic development.

The neural tube develops and completely closes during the first four weeks of pregnancy. During this process, failure to close anywhere along the neuroaxis is called an NTD. NTDs are congenital malformations that usually lead to permanent deficits; however, some fetus' die (7). An NTD is a multi-step event influenced by genetic and environmental factors. The environmental factors include maternal diabetes mellitus, obesity, use of antiepileptic drugs, alcohol consumption, maternal age, and nutritional habits (2,9,12,27). In our study, high doses of tenoxicam was found to cause NTDs in chicken embryos.

Data from the National Birth Defects Prevention Study (NBDPS), which included women who reported using aspirin, ibuprofen, or naproxen during their first trimester, revealed an elevated risk of NTDs in the offsprings exposed to NSAIDs (14). The adjusted odds ratio indicated a higher likelihood of encountering conditions such as spina bifida, anencephaly/craniorachischisis, and encephalocele following exposure to aspirin and ibuprofen than following exposure to naproxen.

Recent avian studies have demonstrated that exposure to celecoxib during embryonic development could cause dose-dependent impairments in brain development. Additionally, exposure to celecoxib inhibits the migration and differentiation of pericytes in vitro, a process crucial for the formation of blood vessels in the central nervous system (17). Similarly, a study by Ertekin (10) on chick embryos revealed that diclofenac sodium had a direct teratogenic effect on neural tube formation in a dose-dependent manner.

In a study on mouse embryos that were cultured and subjected to NSAIDs, there were no abnormalities in neural tube closure (20). Additionally, a study by Dathe (8), which included 1,117 pregnant women who had used ibuprofen in the first trimester, did not identify significantly elevated risks of spontaneous abortion and birth defects. Conversely, in the NBDPS cohort, the use of ibuprofen in the first trimester was associated with a minor-to-moderate increased risk of congenital anomalies such as spina bifida and defects of various systems (14). These results imply that although these NSAIDs lack specificity, COX-2 might substantially influence, but not exclusively impact, the neurulation regulation. Thus, future studies should explore the involvement of COX-2 in the development of the central nervous system.

Our early chicken embryo model demonstrated that TEN causes midline closure defects in a dose-dependent manner. Our findings are consistent with those of previous studies that evaluated the effects of different NSAIDs on NTDs. Meloxicam, diclofenac sodium, and metamizole have been shown to cause NTDs in chicken embryos, mainly when administered at high doses (6,10,13). Additionally, Özeren et al. (21) demonstrated that flurbiprofen, a NSAID derived from phenyl alkanoic acid and having analgesic, antipyretic, and anti-inflammatory properties, can cause NTDs even at regular therapeutic doses in chicken embryos. To the best of

our knowledge, most of the previous studies have focused on antiepileptic drugs and other NSAIDs. There are no reports on the relationship between TEN and developing chick embryos, specifically the development of the central nervous system.

Considering similar studies, the exact role of TEN, which inhibits the COX enzyme and blocks prostaglandin synthesis, in neural tube development remains unknown under current conditions. We aim to elucidate these mechanisms through more detailed studies in the future.

CONCLUSION

We found that TEN, which is thought to block the prostaglandin production, causes midline closure defects in early chicken embryos in a dose-dependent manner by inhibiting the COX enzyme. Especially in early pregnancy, the effect of this drug should be considered, and different treatment methods should be evaluated. To the best of our knowledge, there are no reports on the effects of TEN on developing chick embryos, especially regarding neural tube development.

ACKNOWLEDGEMENTS

This study was abstracted from the data of a specialty thesis in neurosurgery given by the first author (BB) under the supervision of the senior author (MAU) at Ankara University School of Medicine (Thesis Number: 525409, 2018. URL: https://tez.yok.gov.tr/UlusalTezMerkezi/tezDetay.jsp?id=myK65GX9XneyfsjBu8Q8zQ&no=l9NqMRDi-BolUw31aL_zKw, last accessed on October 2023).

Preparation for publication of this article is partly supported by Turkish Neurosurgical Society. The authors wish to thank ENAGO for its English editing and manuscript proofreading services.

AUTHORSHIP CONTRIBUTION

Study conception and design: BB
Data collection: ESA
Analysis and interpretation of results: DOT
Draft manuscript preparation: BB, OM, EAB
Critical revision of the article: OO, OM
Other (study supervision, fundings, materials, etc...): BB, MAU
All authors (BB, OO, OM, ESA, DOT, EAB, MAU) reviewed the results and approved the final version of the manuscript.

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