

The Effects of Meloxicam on Neural Tube Development in the Early Stage of Chick Embryos

Meloksikamın Erken Dönem Cıvciv Embriyosunda Nöral Tüp Gelişimine Etkileri

ABSTRACT

AIM: The aim of this study is to demonstrate the effect of meloxicam in early stage chick embryos on neural tube development.

MATERIAL and METHODS: One hundred specific pathogen-free (SPF) chicken eggs were used to investigate the neurulation. SPF eggs were investigated in four groups (n:25). All of the groups were incubated at 37.2 ± 0.1 °C and 60 ± 5 % relative humidity for 30 hours, and an embryological development in the ninth stage as classified by Hamburger and Hamilton was obtained. In the end of the 30th hour, group A (control group) was administered 0.1 ml of saline (0.9% NaCl) in ovo and the other groups were administered meloxicam in increasing doses. At the end of 72 hours, all of the embryos were extracted from eggs and they underwent pathological examination with hematoxylin eosine and immunohistopathological examinations with CD138 and tubulin beta II.

RESULTS: While the groups A and B showed no neural tube defects, totally eight defective embryos were detected in the groups C and D (three in group C and five in group D).

CONCLUSION: Our results suggested that meloxicam, a nonselective COX inhibitor, caused neural tube closure defects when injected at suprathreshold doses. However, further studies with larger numbers of subjects are needed for its use in lower doses.

KEYWORDS: Neural tube defect, Early period, Chick embryo, Neurulation, Meloxicam

ÖZ

AMAÇ: Bu çalışmanın amacı meloksikamın erken dönem cıvciv embriyosunda nöral tüp gelişimine etkilerini ortaya koymaktır.

YÖNTEM ve GEREÇ: Nörülasyonu incelemek için 100 adet özel patojen bulunmayan (SPF) yumurta kullanıldı. SPF yumurtalar 4 grupta incelendi (n:25). Tüm gruplar 30 saat boyunca $37,2 \pm 0,1$ °C sıcaklık ve % 60 ± 5 nem oranında inkübe edildi ve Hamburger – Hamilton sınıflamasına göre 9. evrede bir embriyolojik gelişme elde edildi. 30ncu saati takiben A grubuna (kontrol grubu), in ovo 0,1 ml %0,9 NaCl ve diğer gruplara da artan dozlarda meloksikam enjekte edildi. 72. saatin sonunda tüm embriyolar yumurtadan çıkartıldı ve hematoksilin eozin ile histopatolojik, CD138 ve tubulin beta II ile de immünohistopatolojik olarak incelendi.

BULGULAR: A ve B gruplarında nöral tüp defekti saptanmazken C ve D gruplarında toplam 8 embriyoda defekt izlendi (3'ü C grubunda ve 5'i D grubunda).

SONUÇ: Meloksikamın yüksek dozlarda erken dönem tavuk embriyosunda nöral tüp defekti insidansını arttırdığı gözlemlenmiştir. Ancak düşük dozlarda kullanımı ile ilgili daha geniş denek sayısında ve daha ileri çalışmalara ihtiyaç vardır.

ANAHTAR SÖZCÜKLER: Nöral tüp defekti, Erken dönem, Cıvciv embriyosu, Nörülasyon, Meloksikam

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Received : 25.09.2009

Accepted : 02.12.2009

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INTRODUCTION

Human development involves a series of steps. After precise proliferation and differentiation, adhesion and migration of certain cell groups take place. Thus, the embryo becomes a fetus. Development and differentiation also goes on in the postnatal period (23).

A neural tube defect (NTD) which is a consequence of abnormal neurulation can cause serious medical problems which sometimes even lead to death in the prenatal and postnatal periods. Additionally, this congenital malformation has financial and social effects (28). Maternal drug usage in the first trimester can especially cause embryonic malformations. Several congenital defects result from chromosomal aberrations or translations, and most congenital malformations appear to result from environmental factors. Nutritional deficiency, toxicity, and exposure to radiation carry relatively high risks for embryonic malformation (27).

Various agents that cause NTDs have been previously researched by means of different methods (10, 26, 27). Meloxicam is a nonselective cyclooxygenase (COX) inhibitor agent that is widely used as non-steroid analgesic and anti-inflammatory drug (12). There is no study in the literature about the effects of meloxicam on neural tube development to the best of our knowledge.

MATERIALS and METHODS

This study was conducted in cooperation with the neurosurgical unit research laboratory of Celal Bayar University Medical School. Fertilized, specific pathogen-free (SPF) Leghorn chicken eggs were obtained from the Manisa Chicken Research and Vaccination Facility.

1. Incubation and injection

One hundred eggs were weighed (mean \pm s.d.; 65 ± 2 g) and incubated at 37.2 ± 0.1 °C for 30 hours at 60% to 70% humidity. Each egg was repositioned on its axis every 2 hours. After 30 hours of incubation, randomly selected two eggs in each group were sacrificed to ensure that the eggs were in stage 9 according to Hamburger-Hamilton series (4, 9, 11, 21, 22). Each egg was opened under 4X optical magnification and embryonic discs were identified and the same volume of liquid was injected under the discs with a 24-gauge syringe according to New's technique (11, 25).

2. Drug preparation

Considering 7.5 - 15 mg/d dose for a human adult and detected Cmax value of 1.25 ± 0.26 mg/L during administration of 15 mg meloxicam per day, doses were arranged regarding a standard SPF egg weighing 65 ± 5 gr. Meloxicam solutions of three concentrations were prepared as subtherapeutic (group B: 0.75 mg/d or 0.001 mg / 0.1 ml), therapeutic (group C: 7.5 mg/d or 0.01 mg / 0.1 ml) and suprathereapeutic (group D: 15 mg/d or 0.02 mg / 0.1 ml) doses. All solutions and the physiological saline control were titrated to pH 7.2 with sodium bicarbonate due to the acidity of the physiological saline solution (pH 4.4). In all of the groups, 0.1 ml of solution was injected under the each embryonic disc.

3. Groups

The study was designed so as to involve four groups each of which consisted of 25 eggs. Group A (n:25) was the control group. Each subject was injected 0.1 ml of saline (0.9 % NaCl). Subjects in Group B (n:25) were administered 0.001 mg / 0.1 ml of meloxicam in subtherapeutic doses. Therapeutic doses of meloxicam (0.01 mg / 0.1 ml) was administered to the subjects in Group C (n:25) and 0.02 mg / 0.1 ml of meloxicam in suprathereapeutic dose was administered to the subjects in Group D (n:25). However, because of several infertile eggs and severe injury to one of them made differences in the number of the subjects, this study enrolled in that groups (Table I).

One hundred eggs were incubated for 30 hours and then injected with meloxicam solution. The control group consisted of 25 eggs that received 0.1 ml saline (0.9%NaCl). In all groups, the eggs were then closed with sterile adhesive strips and incubation was continued for 72 hours (Figure 1). After 72 hours, all eggs were reopened and all embryos were dissected from embryonic membranes under 4X optical magnification, with adherence to microsurgically held 'water floating technique'. All embryos were then put into a 10% formalin solution for 24 hours and sent for histopathological evaluation.

4. Pathological evaluation

All embryos in four groups were fixed with a 10% buffered formalin solution. Amniotic membranes around the embryos were dissected with a microdissector and photographs were taken through

Table I

Groups	Initially (n)	Infertile (n)	Severe injury (n)	Sacrifice at 30 h exam (n)	Final number examined (n)
A	25	1	-	2	22
B	25	1	1	2	21
C	25	1	-	2	22
D	25	1	-	2	22
Total	100	4	1	8	87



Figure 1: View of an embryo in its 72nd hour (Stage 18) obtained from a light microscope (X40).

the dissection microscope. Formalin-fixed, paraffin-embedded embryo tissue samples were then prepared. Sagittal and axial 5 µm-thick serial sections were cut from paraffin blocks with rotary microtome oriented with an angle of 7° and stained with hematoxylin eosine (HE) and syndecan – I / CD138 (B – A38, Cell Marque, Rocklin, USA) to evaluate the epidermal ectodermal tissue and tubulin Beta II (NCL-TUB-B2, Novocastra, Leica, Newcastle, United Kingdom) to evaluate the paraaxial mesodermal and neural tissue. The structural continuity of neural tubes and somites

was evaluated both macroscopically and microscopically. Any disruption to somite or neural tube continuity was considered a neural tube closure defect.

5. Statistical analysis

Raw data were analyzed in SPSS for Windows 15.0 with Pearson’s chi-square and Fisher’s exact tests, with a value of p < 0.05 indicating statistical significance.

RESULTS

Macroscopic:

Macroscopically, the omphalomesenteric (vitelline) vascularisation quantity was observed. Group A was free of the effects of meloxicam, and omphalomesenteric vascularisation quality and quantity was detected in physiological range. However, under the effect of meloxicam, a decrease in the vascularisation was observed parallel to the dose augmentation from group B to D, as expected (Figure 2A,B,C,D).

HE Stain:

3 NTDs in Group C and 5 NTDs in Group D were detected (Figure 3A,B,C,D).

Immunohistochemical stain:

For further histopathological evaluation, CD138 (syndecan - I) and tubulin beta II stains were applied. The CD138 stained subjects without any NTD,

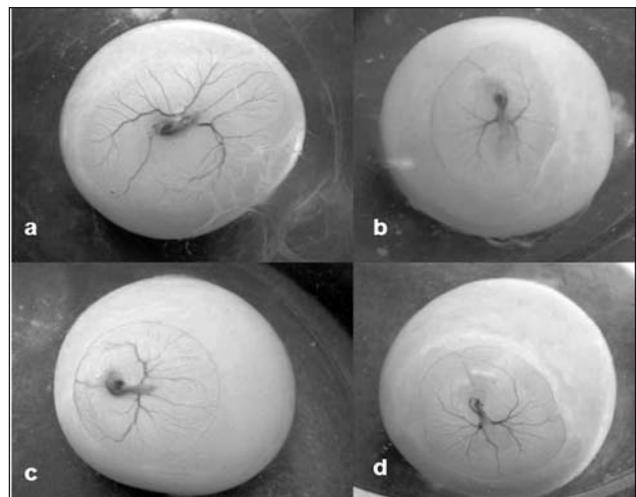


Figure 2A-D: 72nd hour study. Decrease in the omphalomesenteric (vitelline) vascularisation of the embryos can easily be observed from groups a to d. Letters a,b,c,d represent the groups A,B,C,D.

showed CD138 expression of the epithelial cells with histopathological arrangements (Figure 4A). However in the group with NTD it was observed that there was not any significant immunoreactivity in the junction between neural tube and ectoderm (Figure 4B).

In our study, normal histopathological arrangement in the embryos and diffuse, strong membranous and cytoplasmic expression of tubulin beta II in the luminal sides of the neural tube cells were detected. The cells other than neural tube cells, particularly the paraaxial mesoderm and notocord cells showed slight intracytoplasmic expression (Figure 4C). However, immunoreactivity difference between groups with and without NTDs regarding strength of staining and its penetration was insignificant (Figure 4D).

Statistical analyses:

Subjects in the study are summarized in Table I. NTD was not detected in groups A and B. 3 subjects in group C (13%) and 5 subjects in group D (22%) with NTD were histopathologically displayed (see Figure 3). Comparison of group C to A yielded a value of p as 0.23 ($p > 0.05$). But comparison of group D to A yielded p value as 0.048 ($p < 0.05$). Comparison of group C to D revealed p as 0.69 ($p > 0.05$). When all groups were examined in paired matrices using SPSS 15.0 software, p was 0.021 ($p < 0.05$).

Although the difference between groups C and D on an individual basis was insignificant, the difference of group D was statistically significant upon total evaluation.

DISCUSSION

NTDs are the consequence of abnormal neurulation in the embryonal period and they are important congenital malformations because they can result in death and medical, financial and social problems (28). NTDs, which range from spina bifida to anencephaly, affect about one in 1,000 neonates in the United States (27). Exposure to chemicals is one of the known reasons for disorders related to nonclosure of the neural tube (1-3, 6, 8, 11, 16, 17, 20, 21). This critical period is the first month of pregnancy that is known as gastrulation. In humans, embryo transforms into blastocyst, thereafter gastrulation phase begins with the implantation (7th - 8th days). This is the critical stage where the three germ cell layers differentiate so as to form the organs.

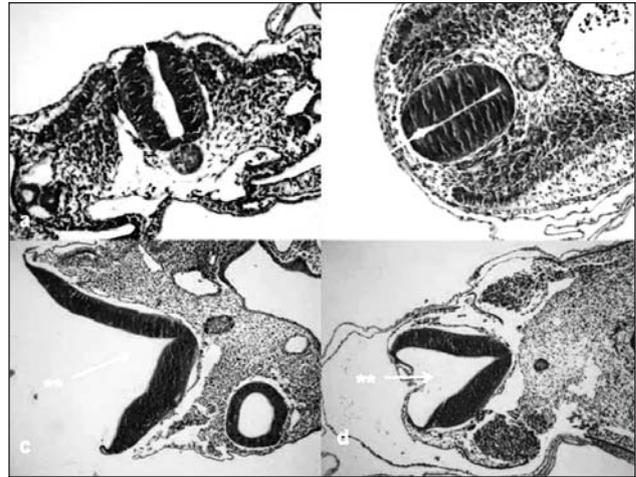


Figure 3A-D: Samples of neural tube sections stained with HE. A. Group A (X200), B. Group B (X200), C. Group C (X100), D. Group D (X100) [(*) Closed neural tube samples from groups A and B. (**) Neural tube closure defects from the groups C and D.]

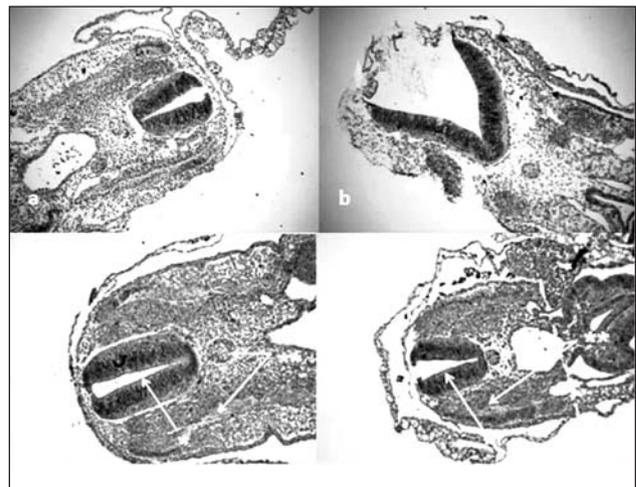


Figure 4A-D: A. CD 138 expression in an embryo without NTD, B. CD138 is not expressed in the embryo with NTD, C. Tubulin beta II expression in an embryo without NTD, D. Tubulin beta II expression in an embryo with NTD (X100).

The early chick embryo model corresponds to the first month of embryonic development in mammals (28). It is also well suited to investigation of the effects of chemicals on the development of embryos. Neurulation in avians can also be examined in two stages as in most of the other animals. In the primary neurulation period, neural plate is shaped. It is elongated and bent to form a tube that extends the entire length of the anterior-posterior axis. To form the neural tube, the neural plate undergoes a bending process by which the lateral edges, or neural folds, elevate, rotate around the actin-rich dorsal-

lateral hinge points, and meet at the dorsal midline and then they fuse. This happens in the secondary neurulation stage. In the cranial region, closure is initiated at defined fusion points located at the anterior point of the forebrain, the forebrain/midbrain boundary, and the hindbrain / spinal cord boundary. Following fusion, closure along the remainder of the neural tube, proceeds. There are a number of intrinsic and extrinsic genetic, molecular and environmental factors that regulate neural tube morphogenesis and the lack of these factors can cause neural tube closure defects.

Generally, genetic factors (trisomy 13, 18, 21), geographical conditions, maternal age, socioeconomic factors, disease related to zinc and folic acid metabolism, diabetes mellitus, an elevated body temperature of the mother in the first trimester, maternal alcohol abuse, and exposure to valproate are known as the factors attributable to neural tube closure defects (1, 3, 7, 13).

It has been shown in experimental studies that folic acid antagonists like cytochalasins, ionophore A23187, papaverin, diazepam, verapamil, caffeine, ethanol, methotrexate, and aminopterin; antiepileptics such as phenitoin, valproic acid; and local anesthetics cause neural tube closure defects in the early period chicken embryos (9, 10, 14-21, 24, 26, 27, 30, 31).

Previous studies on early period chicken embryos have demonstrated the important role of apoptosis in the neural tube closure (26). Neurulation is the first and the most important step in the formation of the main structure of the central nervous system. Four consequent steps are; formation of the neural plate, its shaping, and bending of the neural plate, closure of the neural growth plate (5). Interruption of the development process in any of these stages causes different NTDs. Closure of the neural growth plate consists of two sub-stages. First is the fusion of the neural folds, second is the separation of the neural and surface ectoderm and differentiation of the surface ectoderm cells to form the skin layer in future. The cells between surface ectoderm and neural layer transform into neural crest cells. They later on are shaped to form the peripheral nerves. In the end of neurulation, neural layer and surface ectoderm layers should detach. Weil et al. reported the necessity of the apoptosis mechanism for the closure of neural tube (29).

Meloxicam is a nonsteroidal anti-inflammatory drug (NSAID) of the oxicam class. It is a preferential COX-2 inhibitor, which also dose dependently inhibits COX-1 (12). It is widely used in the treatment of osteoarthritis, rheumatoid arthritis, ankylosing spondylitis, and other rheumatological conditions. Meloxicam has an oral bioavailability of 89%, and its maximum plasma concentrations (C_{max}) are achieved within 4 to 11 h. It is extensively bound to plasma proteins (99%), mainly to albumin. The elimination half-life (t_{1/2}) of meloxicam ranges from 13 to 20 h, and it is suitable for once-daily dosing. Meloxicam is extensively metabolized in the liver, primarily by the polymorphic cytochrome P450 2C9 (CYP2C9) enzyme, and to a minor extent by CYP3A4 enzyme, to four pharmacologically inactive metabolites. Only negligible amounts of the parent drug are found in urine and in feces. The effect of different genotypes on the pharmacokinetics of meloxicam is not known (12).

To our knowledge, there are no reports on the relation of meloxicam and developing chick embryos as far as the development of the central nervous system is concerned.

That the drug subject to our study was in group B in the first and second trimesters and in group D in the third trimester regarding its safe administration, aroused our interest and compelled us to study this material, because it was a frequently used non steroid, non-selective COX inhibitor, analgesic and anti-inflammatory drug.

CONCLUSION

The results of our investigation on early stage chicken embryo disclosed that the application of meloxicam in high doses causes retardation in general embryonic progress and further retardation in the development of the neural system which ends in an open neural tube. Supratherapeutic doses of meloxicam may be responsible for a neonate with NTD but further studies enrolling a larger number of subjects are needed to determine the safety of meloxicam in therapeutic doses.

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