British Journal of Neurosurgery, 2014; Early Online: 1–7

ISSN: 0268-8697 print / ISSN 1360-046X online
DOI: 10.3109/02688697.2014.976172

informa
healthcare

ORIGINAL ARTICLE

The effect of flurbiprofen on the development of anencephaly in early stage chicken embryos

Ersin Özeren[1], Uygur Er[2], Yahya Güvenç[3], Adnan Demirci[4], Ata Türker Arıkök[5], Engin Şenveli[6] & Rüçhan Behzat Ergün[6]

[1]*Neurosurgery Clinic, Aksaray State Hospital, Aksaray, Turkey,* [2]*Neurosurgery Department, Medical School, Düzce University, Düzce, Turkey,* [3]*Neurosurgery Clinic, Dr. Nafiz Körez State Hospital, Ankara, Turkey,* [4]*Neurosurgery Clinic, Dr. Münif İslamoğlu Kastamonu State Hospital, Kastamonu, Turkey,* [5]*Department of Pathology, Yıldırım Beyazıt Dışkapı Research and Training Hospital, Ankara, Turkey,* [6]*Department of Neurosurgery, Yıldırım Beyazıt Dışkapı Research and Training Hospital, Ankara, Turkey*

Abstract

Objective. **The study investigated the effect of flurbiprofen on the development of anencephaly in early stage chicken embryos.** ***Material and methods.*** **We looked at four groups with a total of 36 embryos. There was a control group, a normal saline group, a normal-dose group and a high-dose group with ten, ten, eight and eight eggs with embryo respectively.** ***Results.*** **Two embryos in the control group, studied with light microscopy at 48 h, were consistent with 28–29 hours' incubation in the Hamburger–Hamilton System. They had open neural tubes. The other embryos in this group were considered normal. One embryo in the normal saline group was on the occlusion stage at 48 h. One embryo showed an open neural tube. They were compatible with 28–29 hours' incubation in the Hamburger–Hamilton system. The remaining eight embryos showed normal development. In the normal dose group, one embryo showed underdevelopment of the embryonic disc and the embryo was dead. In four embryos, the neural tubes were open. One cranial malformation was found that was complicated with anencephaly in one embryo. In two embryos the neural tubes were closed, as they showed normal development, and they reached their expected stages according to the Hamburger–Hamilton classification. There was no malformation or growth retardation. Four experimental embryos were anencephalic in the high dose group, and three embryos had open neural tubes. One embryo exhibited both anencephaly and a neural tube closure defect. None of the embryos in this group showed normal development.** ***Conclusions.*** **Even the usual therapeutic doses of flurbiprofen increased the risk of neural tube defect. Flurbiprofen was found to significantly increase the risk of anencephaly. The provision of improved technical materials and studies with larger sample sizes will reveal the stage of morphological disruption during the development of embryos.**

Keywords: anencephaly; chicken embryo; flurbiprofen; in-vitro fertilization; NTD

Introduction

Congenital anomaly is an abnormal formation of the body structure or deviation from the normal standard development.[1] A congenital malformation is a physical defect present in a baby at birth which can occur as isolated entities affecting one organ system or as multiple defects affecting one or several organ systems. Neural tube defects (NTD)[1] are a major area of congenital malformation. Congenital anomalies of the central nervous system are the second most common congenital anomalies following cardiovascular anomalies.[2] NTDs constitute a heterogeneous and complex group of congenital anomalies.

Anencephaly is one of the most serious cephalic disorders, and is a failure of closing of the neural tube properly during embryonic development. Infants with anencephaly are born without a major part of their brain, skull and scalp. It usually results in the death of the fetus within hours of birth. There is no sufficient explanation, cure or standard treatment for anencephaly. It is thought that a mother's diet and vitamin intake may play a role, but scientists also believe that environmental factors may be involved.[3,4]

The incidence of NTD in the United States and Europe is 0.1%.[5] Say et al. reported that the incidence of spina bifida and anencephaly, the most common form of NTD, was 1.20%[6] in Turkey.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medicines in developed countries.[7] NSAIDs reduce uterine contractions and are given to in-vitro fertilization (IVF) patients.[8–11] Congenital malformations are known to be associated with NSAID use during the neurulation period.[12] This study aimed to assess the impact of flurbiprofen, a non-selective cyclooxygenase-1 (COX-1)/cyclooxygenase-2 (COX-2) inhibitor and an NSAID with analgesic and anti-inflammatory effects, on

Correspondence: Uygur Er, MD, Neurosurgery Clinic, Koru Hospital, Söğütözü C., 4th Sk., No:22/7, 06510, Ankara, Turkey. Tel: + 0090-505-589-23-55. Fax: + 0090-312-284-51-02. E-mail: uygurer@gmail.com

Received for publication17 November 2013; accepted 8 October 2014

the development of anencephaly, the most common type of NTD.

Materials and methods

This study was carried out at the Microsurgery Laboratory of the Department of Neurosurgery, Ankara Numune Educational and Research Hospital, Ankara, Turkey. The 30 eggs used in the study were Atabey® eggs collected from the Republic of Turkey, Ministry of Agriculture and Rural Affairs, Ankara Poultry Institute, 65 ± 5 g in weight, specific pathogen-free and day 0 fertilized eggs of white chickens. The histopathological analysis was performed at the Pathology Laboratory of Yıldırım Beyazıt Dışkapı Research and Training Hospital Ankara, Turkey.

An egg incubator (Cimuka Egg İncubators, Cimuka, Ankara, Turkey), a light microscope (Opmi Pico, Nikon, Japan), a camera (Canon 60D, Japan; Canon powershot A450, Japan) precision laboratory scale (Densi JW 300, İstanbul, Turkey) and loop (EyeMag Pro S, Carl Zeiss Meditec, AG, Germany) were used in the study.

Forty fertilized eggs were transferred into an egg incubator to ensure continuity of day 0 embryos. The egg incubator had a thermoregulator providing a constant temperature at 37.8 ± 0.2°C, and a fan providing a substantially uniform temperature distribution. Ambient relative humidity was 60–70%. The incubator was initially run empty without eggs to ensure that the conditions were as intended. The eggs were placed in the incubator with sharp ends pointing down. The eggs were automatically rolled every 2 h at a 45° angle to the vertical axis during the incubation period.

Ten eggs were incubated for 48 h without any intervention, whereas 30 eggs were taken from the incubator at 28 h of incubation (Hamburger–Hamilton stage 8). Two eggs were excluded from the study due to eggshell breakage.

The eggshell was sterilized with povidone-iodine (10%) and then ethanol (70%). A small window (1–2 cm) was made aseptically with a specific technique[13–15] in the eggshell (Fig. 1a). The round-shaped embryonic disc became visible after the surrounding membranes were cut open (Fig. 1d and e). Pre-made doses of saline and flurbiprofen were injected as a 10 µL solution into the sub-blastodermic space with Hamilton microsyringe (Fig. 1c). The eggshell window was sealed with a sterile drape after the injection. Then the eggs were hand-turned 180° and placed in the incubator.

One egg from the normal dose group was excluded from the study due to the rupture of embryonic disc during injection. An egg from the high dose group was also excluded from the study at random for standardization. Thus a total of four eggs were excluded from the study.

The embryos in group A were incubated for 48 h without any procedures. The eggs in B, C and D groups were opened by the window technique on the 28th hour of incubation (Hamburger–Hamilton stage 8) and the embryos were treated with control and medication in 10 µL of solution.

Group A (control group, no interference with the embryo).

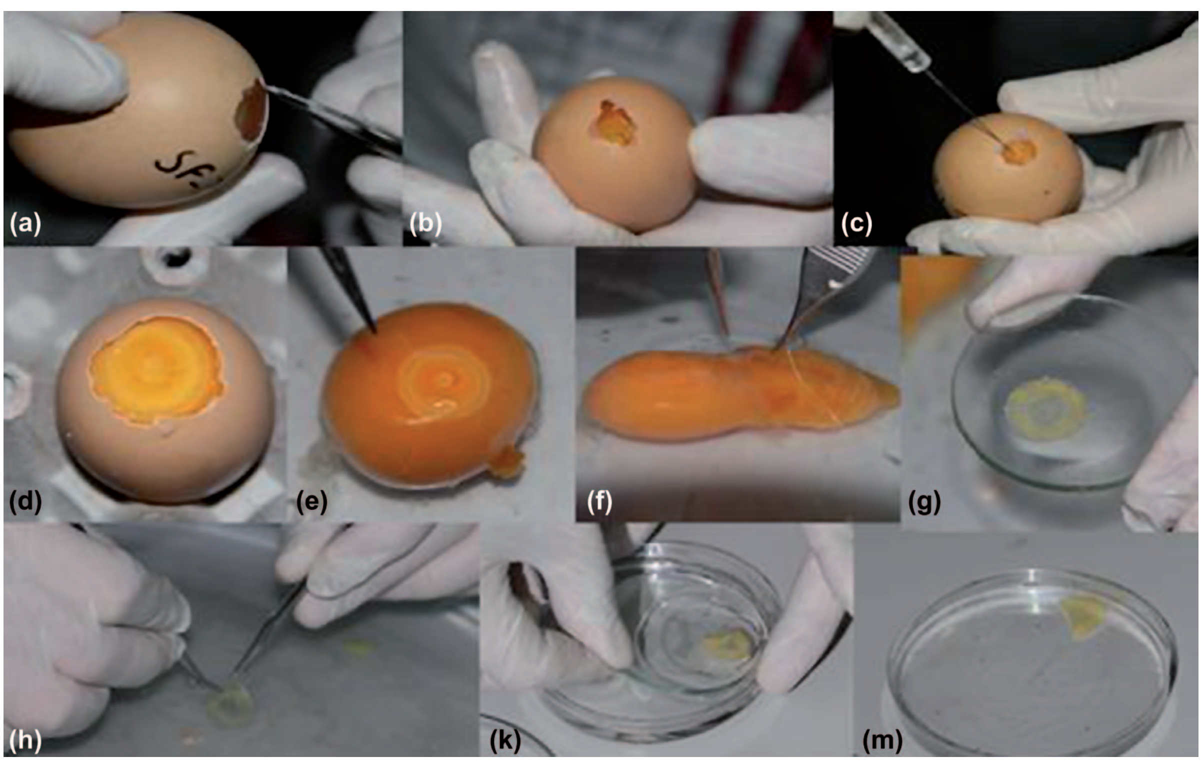

Fig. 1. The egg that is opened using the window method (a,b), micro-syringe injection of drugs to the (into the) embryonic disc (c), macroscopic view of the embryonic disc (d,e), Cutting out vitelline membrane, one of the embryonic membranes over the yolk using microscissors (f), Removal of blastoderm using a watch crystal (g,h), The transfer of the contents from the watch crystal to the Petri dish (k), The embryonic disc material that is transferred to the Petri dish for histopathological examination (m).

Group B embryos were treated only with saline. (saline group, 10 µL 0.9% NaCl was injected).

Group C was given 0.3 mg flurbiprofen in 10 µL saline based on therapeutic equivalent dose in the therapeutic index (300 mg/70 kg).

Group D was given 3 mg flurbiprofen in saline at ten times the therapeutic equivalent dose (3000 mg/70 kg) (high dose group; ten times of adult therapeutic dose of flurbiprofen was given in 10 µL of salin).

The eggs were removed from the incubator after 48 h of incubation. The eggshells were broken and the egg yolks were put in a sterile glass bowl containing saline (0.9% NaCl) (Fig. 1e). The watch glass was then placed into a glass bowl. The vitelline membrane was then removed from the yolk with fine forceps and microscissors (Fig. 1f). The vitelline membrane was then separated from the yolk by holding both ends carefully (Fig. 1h). Blastoderm was floated off into the saline and transferred into a watch glass, and the watch-glass with the blastoderm attached was removed from the bowl (Fig 1g). Then, the embryo morphology was evaluated by a light microscope at 10x mag, and the development of the embryo, NTDs and anencephaly were examined. Then the samples were placed in a Petri dish with formaldehyde for histopathological study.

Embryos were removed from the eggs at 48 h of incubation (Hamburger–Hamilton stage 12). Following incubation, the morphological features of each embryo were evaluated under a light microscope at the Microsurgery Laboratory of the Department of Neurosurgery, Ankara Numune Training and Research Hospital, Ankara, Turkey. The samples were then transferred to Petri dishes containing 10% formaldehyde solutions for histopathological study at the Pathology Laboratory of Yıldırım Beyazıt Dışkapı Research and Training Hospital.

The embryos were fixed in a 10% formaldehyde solution for 24–48 h and processed to be embedded in paraffin. Sequentially they were rinsed with distilled water for 5 min to remove excess fixatives. After washing they were dehydrated for 30 min in a 65%–85%–95% ethanol series. Paraffin wax-embedded and sectioned embryos were stained with eosin for visualization. After that process, the embryos were treated with xylene for clearing, and they were dried at 60°C in an oven for 1 h. A tissue embedding system was used to form paraffin blocks and then the blocks were cooled in the freezer. The paraffin blocks were cut into 3-µ serial sections using a microtome, and these sections mounted on slides for histopathological examination.

Results

Ten embryonated eggs in group A were incubated without any treatment, opened at 48 h, and the embryos were removed from the eggs to be examined under the light microscope. Eight chick embryos reached the appropriate embryonic stage according to the Hamburger–Hamilton method. Cranial neural tube closure was completed in these embryos. Open NTDs were revealed by light microscope in two embryos. They were not at the same developmental stage at a given time according to the Hamburger–Hamilton classification, but they were at that stage of development for 28 or 29 h.

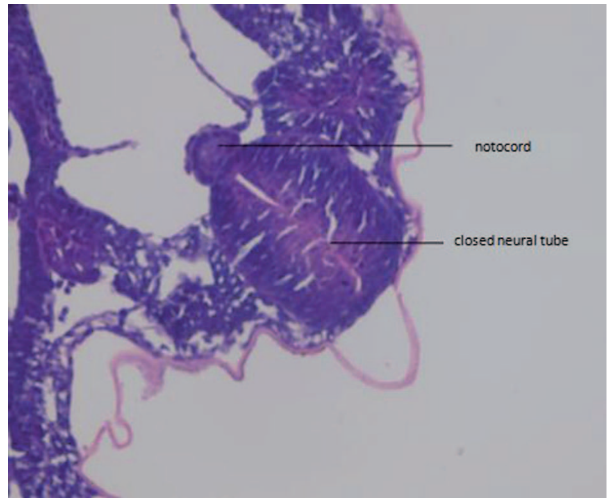

Fig. 2. Internal view of the neural tube.

Ten embryonated eggs in group B were opened at 48 h, and the embryos were removed from the eggs to be examined under the light microscope. One embryo was at the level of neural tube closure. It was at 32 h of development according to Hamburger–Hamilton criteria. The neural tube had not yet closed in one embryo, and it was at that stage of development for 28 or 29 h. The remaining eight embryos showed normal development, and reached the desired stage of development. All these embryos were found to complete neural tube closure (Fig. 2) without any malformation or growth retardation (Fig. 3).

Ten embryonated eggs in group B were injected with flurbiprofen on time, at a dose level equivalent to a therapeutic index dose (300 mg/70 kg/day) at 28 h. Ten embryonated eggs in group B were opened at 48 h, and the embryos were removed from the eggs to be examined under the light microscope. All the eggs were opened at 48 h, and the embryos were removed from the eggs to be examined under the light microscope. In one egg, the embryonic disc was seen to have failed and the embryo was dead. This was attributed to a possible failure of early embryological development within

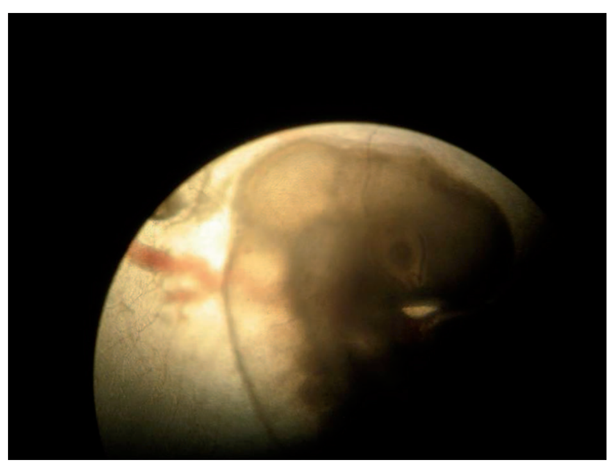

Fig. 3. 48th hour of light microscopic image of normal cerebral development of the embryo continues.

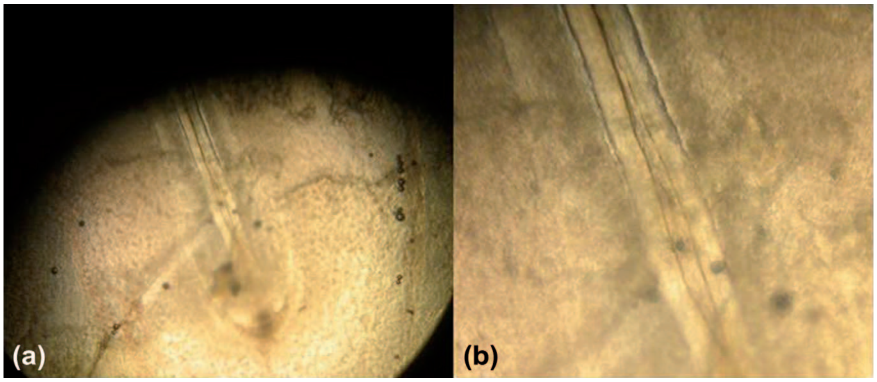

Fig. 4. Section of lumbosacral region at 48th hours stage in chicken embryo is shown in lumbosacral neural tube closure defect (a) magnified photo of picture of a (b).

the incubator. Four embryos failed to complete neural tube closure. In three embryos, the open neural tube was located in the lumbosacral region, (Fig. 4a and b) and in one embryo it was in the thoracic region (Fig. 5a and b). An anencephaly was described in one embryo. The other two embryos were found to complete neural tube closure without any malformation or growth retardation, and they were at the same developmental stage at a given time according to the Hamburger–Hamilton classification.

Eight embryos in group D were given ten times the therapeutic dose of flurbiprofen (3000 mg/70 kg/day) at 28 h. All the eggs were opened at 48 h, and the embryos were examined under light microscope. Four embryos were found to have anencephaly (Fig. 6a). Three embryos displayed an open cranial neural tube, two in the lumbosacral region and one in the multiple regions. One embryo had both anencephaly and other NTD in multiple sites. None of the embryos in this group were found to have normal developmental outcomes. This was confirmed by histopathological examination (Fig. 7a).

Statistical analysis

Neural tube structures were found to be normal in group A and group B. No cranial malformation was observed.

The detection rates for open neural tube and anencephaly in group C were 50% (4/8) and 12.5% (1/8), respectively.

Fifty percent of four embryos (4/8) in group D had anencephaly, 37.5% (3/8) had NTD, and 12.5% had anencephaly and NTD together.

There was no statistical difference between Groups A and B by the non parametric Mann−Whitney U-test ($p = 0{,}542$), but there was a statistically significant difference between Groups A and C ($p = 0{,}023$), and Groups A and D ($p = 0{,}001$).

There was also a statistically significant difference between Group B and Group C ($p = 0{,}006$) and Group D ($p = 0{,}000$).

There was no statistically significant difference between group C and group D ($p = 0{,}143$). However, given the severity of embryonic developmental retardation in embryos with biological deformities, there was a correlation between the usual dose and high dose groups.

Discussion

NSAIDs are widely used nowadays. They should be used with caution during pregnancy due to limitations and warnings. NSAIDs, used by mothers during an early stage of pregnancy, carry the potential risk of causing congenital anomalies.[16]

Uterine contractions are quite common during embryo transfer in infertility treatment.[17] As a result implantation of the embryo does not occur.[18] Uterine relaxation can be

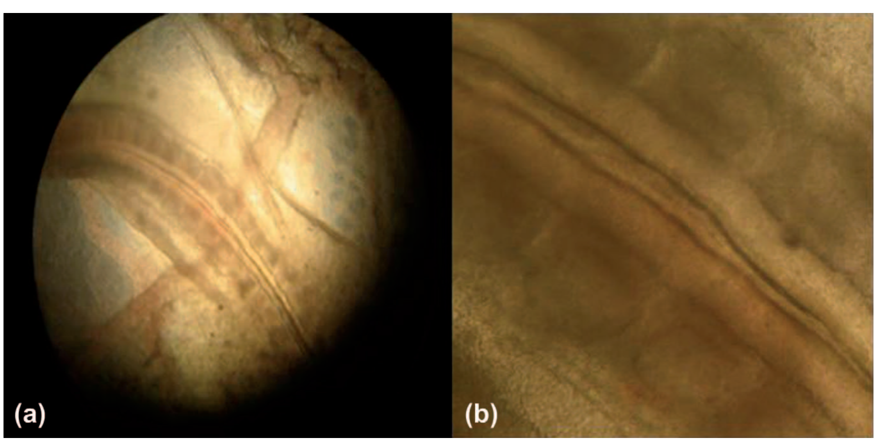

Fig. 5. Section of throcal region at 48th hours stage in chicken embryo is shown in throcal neural tube closure defect (a), magnified photo of picture of a (b).

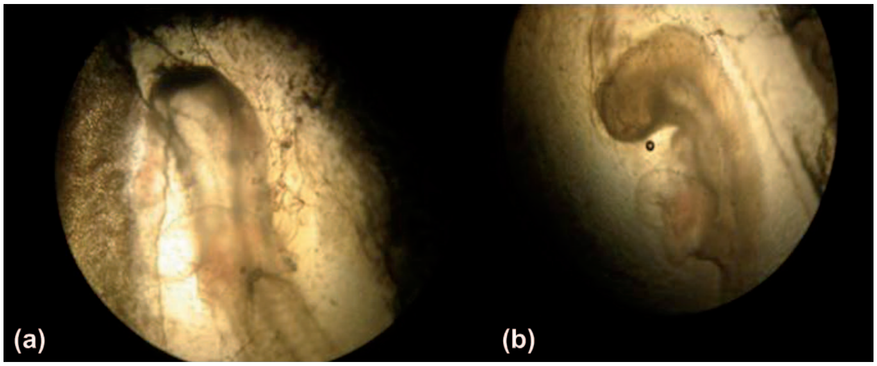

Fig. 6. Views of anencephalic chicken embryos (a) and normal chicken embryos (b) under light microscope.

provided through the administration of NSAIDs. NSAIDs are used in many treatment protocols in daily clinical practice.[8,9]

NTD is the most common congenital malformation of the central nervous system.[19] The etiology is not clear but could be multifactorial.[20] The prevalence of NTD varies between 0.3 and 3 per 1000 birth. Every year around 324 000 children are affected by the disease worldwide.[21,22]

The main modeling methods of NTD include mammalian, poultry, amphibian and computer modeling. These modelings have their advantages and disadvantages when compared with each other. The early chick embryo model that corresponds to the first month of embryonal development in mammals is an ideal modeling.[14]

Recent in-vitro and in-vivo studies have highlighted the molecular mechanisms of neurulation in vertebrates but the morphological development of the human neural tube is poorly understood.[23,24]

It is known, however, that exposure to chemicals and environmental factors on early embryological development can result in developmental defects.[25,26] During this period, differentiation of the distinct cell types of the three primary germ layers results in formation of the tissues and organs of the developing embryo.

Environmental factors are known to cause neuronal migration disorders before the 12th week of gestation. Intrauterine acquired infections in particular, such as CMV, exposure to ionizing radiation and methylmercury, placental ischemia with abnormalities in the maternal circulation due to maternal abdominal trauma, carbon monoxide intoxication and vaginal bleeding in early pregnancy may account for abnormal neuronal migration. Twin–twin transfusion syndrome leading to hypoxic brain injury and feto-maternal bleeding, chronic maternal diseases, maternal hyperthermia, diabetes mellitus, alcohol consumption during pregnancy (fetal alcohol syndrome), and injuries from exposure to toxins including vitamin A, cocaine and organic mercury and ergotamine have been shown to play an important role in the development of cortical dysplasia. Geelen et al. released a study in 1980 showing that anencephaly can be produced by hypervitaminosis A in pregnant rats.[27]

In addition, experimental studies concerning the development of the chicken embryo have shown that cytochalasins, ionofor A23187, papaverine, diazepam, caffeine, ethanol, folic acid antagonists such as methotrexate and aminopterin, antiepileptic drugs such as phenytoin and valproic acid, and local anesthetics cause neural tube closure defects.[24,28–32]

Lee et al. reported that 500 μg/ml of caffeine significantly increases the incidence of NTD in early stage chicken embryos.[29,30] Moreover, Ünlü et al. showed that verapamil-HCl can cause NTD in early chick embryo model.

Barutçuoğlu et al. in their study showed that even low-dose ethanol exposure is associated with growth retardation of the embryo and increased risk of NTD compared to a control group.[24]

Nagele et al. conducted a study using 400 μg/ml diazepam in an in-vitro organ culture model, and detected NTDs in almost 80% of chicken embryos in the middle brain area by electron microscope.[33] In a similar study, Cetinkal et al. reported that high doses of meloxicam increase the incidence of NTD in early stage chicken embryos.[34]

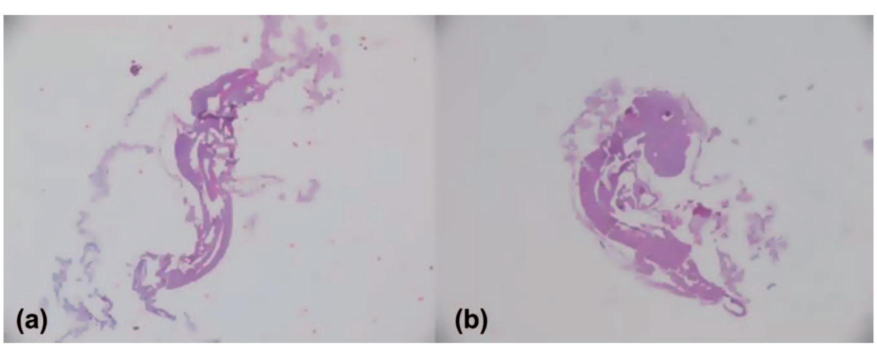

Fig. 7. Histopahtologic views of anencephalic chicken embryos (a) and normal chicken embryos (b) under light microscope.

Anencephaly is the most serious and invariably fatal paradigm of the various cerebral dysraphic disorders. It is characterized by the total or partial absence of the cranial vault, the covering skin and the brain tissue.[35–39] Chandler et al. showed in their study that increased intake of folate, thiamine, betaine, iron or vitamin A contributes to a decrease in the incidence of anencephaly.[40] The defects are classified into six categories in the study of Nakatsu et al. which was conducted on 47 human embryos in 2000. NTD was found to affect the level of severity of disease and intrauterine survival in this study. According to the Carnegie classification, the total dysraphic group (those with both spinal and cranial NTD) has the shortest intrauterine life span.[41]

In this study, in the usual therapeutic dose group, the incidence of NTD was 63%, of which anencephaly comprised 20%. All embryos had NTD in the high dose group, of which 63% were anencephalic.

The embryos that were monitored under the microscope after 48 h had reached the appropriate embryonic stage according to the Hamburger–Hamilton method. Although the cranial slope was expected to be clearly visible at 33–38 h (Hamburger–Hamilton Stage 10), none of the embryos had normal head shape in the anencephalic group. All anencephalic embryos displayed normal development of the heart, a two-chambered heart showing normal directional flow of blood. At 25–30 h of incubation, paired heart vesicles fuse posteriorly to form a continuous tube. At approximately 33 h, the heart tube bends to form an "S" shape, and blood circulation starts.[42]

In this study, the lack of a statistically significant difference between the control group and saline group is an expected result. That an invasive procedure has less or no effect on the development of the embryo shows that the applied procedures have a minimal effect on the embryo. On the contrary, the drug can be said to be effective in the development of the embryo. Although this study does not explain it explicitly, there is a direct relationship between anencephaly and the increase in dosage. However, an obvious limitation of the present study is the lack of statistical analysis of the effect of normal and high dose of drug on embryonal development.

As shown in this study, NSAIDs should be used with caution prior to or during the early stages of pregnancy. No study has been found in the literature that investigated subgroups of NTD caused by flurbiprofen. This study concluded that even a low dose of flubriprofen increases the frequency of NTD. It has been shown that flurbiprofen significantly increases the rate of anencephaly, a cranial NTD. Anencephaly and other dysraphic disorders may primarily affect the early production of mesenchyme resulting in failure of the whole neural folds to elevate, thus causing the loss of a strengthening effect to the neuroectoderm,which leads to failure of the neural tube to close. During the embryonic development, flubriprofen may affect the proximal closing sites of the neural tube and specifically cause anencephaly rather than spina bifida. Sustaining some substances during pregnancy may affect closure at specific sites of the neural tube. Thus it can be mentioned some substances causes a specific type of NTD. At the same time, some substances may cause overdistension of neural tube and some anencephaly cases may occur due to a rupture of an overdistended neural tube rather than to absence of neural tube closure . But the exact mechanism is unknown today.

The provision of improved technical materials and studies with larger sample sizes will reveal the stage of morphological disruption during the development of embryo, especially in cranial NTD.

Conclusion

This study showed that flurbiprofen can induce a significant increase in the incidence of NTD even at usual therapeutic dose levels. It has also been shown that flurbiprofen significantly increases the rate of anencephaly, a cranial NTD. We believe that this study will be a step towards a comprehensive understanding of the biochemical processes relevant to morphologic development of embryo.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

1. Opitz JM, Wilson GN. Causes and pathogenesis of birth defects. In: Gilbert-Barness EG, ed. *Potter's Pathology of the Fetus and Infant*. St. Louis: Mosby 1997:44–51.
2. Tunçbilek E, Boduroğlu K, Alikaşifoğlu M. Neural tube defects in Turkey: prevalence, distribution and risk factors. *Turk J Pediatr* 1999;41:299–305.
3. Al-Gazali LI, Sztriha L, Dawodu A, *et al*. Pattern of central nervous system anomalies in a population with a high rate of consanguineous marriages. *Clin Genet* 1999;55:95–102.
4. Zlotogora J. Genetic disorders among Palestinian Arabs: 1. Effects of consanguinity. *Am J Med Genet* 1997;68:472–5.
5. Donkelaar HJ, Lammens M, Hori A. *Clinical Neuroembryology Development and Developmental Disorders of the Human Central Nervous System*. Berlin: Springer, 2006:161.
6. Say B, Tunçbilek E, Balcı S, *et al*. Incidence of congenital malformations in a sample of the Turkish population. *Hum Hered* 1973;23:434–41.
7. Brooks P. Use and benefits of nonsteroidal anti-inflammatory drugs. *Am J Med* 1998;104:9–13.
8. Bernabeu R, Roca M, Torres A, Ten J. Indomethacine effect on implantation rates in oocyte recipients. *Hum Reprod* 2006;21:364–9.
9. Fanchin RC, Righini F, Olivennes S, Taylor D, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer alter pregnancy rates after in vitro fertilization. *Hum Reprod* 1998;13:1968–74.
10. Firouzabadi RD, Ghandi S, Tayebi N. Effect of administration of single dose piroxicam before embryo transfer on implantation and pregnancy rates in IVF cycles. *J Biol Sci* 2007;7:123–6.
11. Moon HS, Park SH, Lee JO, Kim KS, Joo BS. Treatment with piroxicam before embryo transfer increases the pregnancy rates after in vitro fertilization and embryo transfer. *Fertil Steril* 2004;82:816–20.
12. Ofori B, Oraichi D, Blais L, Rey E, Berard A. Risk of congenital anomalies in pregnant users of non-steroidal anti-inflammatory drugs: A nested case-control study. Birth Defects Res B Dev Reprod Toxico*l* 2006;77:268–79.
13. Andacht T, Hu W, Ivarie R. Rapid and improved method for window in eggs accessing the stage X chicken embryo. *Mol Reprod Dev* 2004;69:31–4.
14. Drake VJ, Koprowski SL, Lough J, Hu N, Smith SM. Gastrulating chick embryo as a model for evaluating teratogenicity: A comparison of three approaches. *Birth Defects Res A Clin Mol Teratol* 2006;76:66–71.
15. Ünlü A. Methods of developmental research. *Acta Neurochir Suppl* 2002;83:71–8.

16. Ericson A, Källén BA. Nonsteroidal anti-inflammatory drugs in early pregnancy. *Reprod Toxicol* 2001;15:371–5.
17. Bulletti C, De Ziegler D, Polli V, *et al*. Uterine contractility during the menstrual cycle. *Hum Reprod* 2000;15:81–9.
18. Poindexter AN, Thompson DJ, Gibbons WE, *et al*. Residual embryos in failed embryo transfer. *Fertil Steril* 1986;46:262–7.
19. Manning N, Archer N. Treatment and outcome of serious structural congenital heart disease. *Semin Neonatol* 2001;6:37–47.
20. Copp AJ, Bernfield M. Etiology and pathogenesis of human neural tube defects: insights from mouse models. *Curr Opin Pediatr* 1994;6:624–31.
21. Kondo A, Kamihira O, Ozawa H. Neural tube defects: prevalence, etiology and prevention. Int J Urol 2009;16:49–57.
22. Mastroiacovo P, Leoncini E. More folic acid, the five questions: why, who, when, how much, and how. Biofactors 2011;37:272–9.
23. Padmanabhan R. Etiology, pathogenesis and prevention of neural tube defects. *Congenit Anom Kyoto* 2006;46:55–67.
24. Barutçuoğlu M, Selçuki M, Vatansever S, Umur AŞ, İnan S. Erken dönem tavuk embryonal tüp gelişiminde etanolün etkisi. *Türk Nöroşirürji Dergisi* 2001;11:32–6.
25. Bupp Becker SR, Shibley IA Jr. Teratogenicity of ethanol in different chicken strains. *Alcohol Alcohol* 1998;33:457–64.
26. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *Dev Dyn* 1992;195;231–72.
27. Geelen JA, Langman J, Lowdon JD. The influence of excess vitamin A on neural tube closure in the mouse embryo. Anat Embryol (Berl) 1980;159:223–34.
28. Güney Ö, Canbilen A, Konak A, Acar O. The effects of folic acid in the prevention of neural tube development defects caused by phenytoin in early chick embryos. *Spine* 2003;28:442–5.
29. Lee H, Nagele RG, Pietrolungo JF. Toxic and teratologic effects of caffeine on explanted early chick embryos. *Teratology* 1982;25: 19–25.
30. Lee H, Bush KT, Nagele RG. Time-lapse photographic study of neural tube closure defects caused by xylocaine in the chick. *Teratology* 1988;37:263–9.
31. Schmitt S. From eggs to fossils: epigenesis and transformation of species in Pander's biology. *Int J Dev Biol* 2005;49:1–8.
32. Vatansever HS, Umur AŞ, İnan VS, Selçuki M. The effects of methotrexate on the development of neural tube defects in the chick embryo. *Turk J Vet Anim Sci* 2003;27:1119–25.
33. Nagele RG, Bush KT, Hunter ET, Kosciuk MC, Lee H. Biomechanical basis of diazepam-induced neural tube defects in early chick embryos: a morphometric study. Teratology 1989;40:29–36.
34. Cetinkal A, Colak A, Topuz K, *et al*. The effects of meloxicam on neural tube development in the early stage of chick embryos. *Turk Neurosurg* 2010;20:111–16.
35. Giroud A. Anencephaly. *Handb Clin Neurol* 1977;30:173–208.
36. Lemire RJ, Beckwith JB, Warkany J. *Anencephaly*. 1978. New York: Raven.
37. Norman MG, McGillivray BC, Kalousek DK, Hill A, Poskitt KJ. *Congenital Malformations of the Brain. Pathological, Embryological, Clinical, Radiological and Genetic Aspects*. New York: Oxford University Press, 1995.
38. Larroche JC, Encha-Razavi F, de Vries F. Central nervous system. In: Gilbert-Barness, ed. *Potter's Pathology of the Fetus and Infant*. St. Louis, MI: Mosby, 1997:1045–56.
39. Potter EL, Craig JM. *Pathology of the Fetus and the Infant*, 3 ed. Chicago, IL: Year Book, 1975:522–547.
40. Chandler AL, Hobbs CA, Mosley BS, *et al*. National Birth Defects Prevention Study. Neural tube defects and maternal intake of micronutrients related to one-carbon metabolism or antioxidant activity. Birth Defects Res A Clin Mol Teratol 2012;94:864–74.
41. Nakatsu T, Uwabe C, Shiota K. Neural tube closure in humans initiates at multiple sites: Evidence from human embryos and implications for the pathogenesis of neural tube defects. *Anat Embryol (Berl)* 2000;201:455–66.
42. McLaughlin SJ, McCain ER. In vivo and in vitro development of the chicken heart. In: Karcher SJ, ed. *Tested Studies for Laboratory Teaching, vol. 19*. Proceedings of the 19th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). 1998:331–332.