ORİJİNAL ARAŞTIRMA ORIGINAL RESEARCH

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

Tiyopentalin Erken Dönem Civciv Embriyosunda Nöral Tüp Gelişimine Etkileri

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ABSTRACT Objective: The purpose of this study is to investigate the effect of thiopental sodium on neural tube development in a chick embryo model. **Material and Methods:** One hundred specific pathogen-free (SPF) chick eggs were used to investigate the neurulation. SPF eggs were splitted into equal four groups. All of the groups were incubated at 37,2±0,1 °C and 60±5% relative humidity for 30 hours and the eighth stage of the embryonic development as defined by Hamburger and Hamilton, was reached. At the end of 30th hour, group A (control group) was administered in ovo 0,1 ml saline (0,9% NaCl) and the other groups were administered same volume of thiopental solution in saline (0,9% NaCl) calculated for each egg with doses of 2 mg/kg, (Group B) 4 mg/kg, (Group C) 8 mg/kg (Group D) through enjection to under embriyo discs respectively, and then all of them were incubated again. At the end of 72 hours all of embryos were extracted from eggs with kindly dissection and underwent histopathological examination with hematoxilen eosine. **Results:** Groups A and B showed no neural tube defects, 2 defective embryos in group C and 4 defective embryos in group D were detected. **Conclusion:** Although it is not known as a teratogen, in our study on early chick embryo our results suggested that thiopental caused neural tube closure defects when injected at supratherapeutic doses in ovo. As a conclusion supratherapeutic doses of thiopental use in early pregnancy where organogenesis takes place might be responsible for NTD but further studies and different embryo models are needed.

Key Words: Neural tube defects; chick embryo; neurulation; thiopental

ÖZET Amaç: Bu çalışmanın amacı tiyopentalin erken dönem civciv embriyosu modelinde nöral tüp gelişimine etkilerini ortaya koymaktır. **Gereç ve Yöntemler:** Tiyopentalin sinir dokusu oluşumu üzerindeki etkisini incelemek için 100 adet özel patojen bulunmayan (SPF) yumurta kullanıldı. SPF yumurtalar, üç ayrı dozda tiyopental ve kontrol grubu olacak şekilde eşit 4 gruba ayrıldı (n=25). Tüm gruplar 30 saat boyunca 37,2±0,1 °C sıcaklık ve %60±5 nem oranında inkübe edilerek Hamburger-Hamilton sınıflamasına göre 8. evrede embriyolojik gelişim elde edildi. 30. saat sonunda kontrol grubuna (Grup A), in ovo 0,1 mL %0,9 NaCl ve diğer gruplara da 0,1 mL hacimde sırası ile 2 mg/kg (Grup B), 4 mg/kg (Grup C), 8 mg/kg (Grup D) dozlarında yumurta ağırlığına göre hesaplanarak tiyopentalin %0,9 NaCl ile hazırlanan çözeltileri embriyo diskleri altına enjekte edildi ve tekrar inkübasyona bırakıldı. 72. saatin sonunda tüm embriyolar yumurtadan çıkartıldı ve embriyo diskleri dikkatlice diseke edildi. Parafinizasyonu takiben hematoksilen eozin ile boyanarak histopatolojik olarak incelendi. **Bulgular:** A ve B gruplarında nöral tüp defekti saptanmazken C grubunda 2 ve D grubunda 4 adet embriyoda nöral tüp defekti izlendi. **Sonuç:** Tiyopental, teratojen olarak tanımlanmamakla birlikte, in ovo erken dönem civciv embriyosu çalışmamızda supraterapötik dozlarda enjekte edildiğinde nöral tüp defektine sebep olabileceği gösterilmiştir. Sonuç olarak organogenezin yer aldığı erken hamilelik döneminde supraterapötik dozlarda NTD oluşumuna sebep olabilir ancak daha ileri ve farklı embriyo modelleri üzerinde çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Nöral tüp defektleri; civciv embriyosu; sinir dokusunun oluşması; tiyopental

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Central nervous system (CNS) malformations are some of the most common of all congenital abnormalities. Neural tube defects (NTDs) are the most frequent malformations of central nervous system ranging from spina bifida to anencephaly affecting one in 1,000 neonates in the United States.[1,2] Genetic predisposition and environmental factors play a major role in the etiology of NTDs. Nutritional deficiency, toxicity, and exposure to radiation and chemicals carry relatively high risks for embryonic malformation.[3,4] Embryo is vulnerable to teratogens especially in organogenesis stage and maternal drug usage in the first trimester can cause embryonic malformations. This time period is very important, since central nervous system development takes place and a woman is frequently uncertain or unaware of her pregnancy at the time of exposure.[5] Early chick embryo model corresponds to the first month of embryonic development in mammals.[6]

Thiopental is one of the induction agents used widely in anesthesia also used in pregnants. Although it is not known as a teratogen, some studies suggest that thiopental may induce abnormal embryogenesis.[7] The aim of this study is to demonstrate the effect of thiopental in early chick embryo model.

MATERIAL AND METHODS

CHICK EMBRYOS

One hundred fertile, specific pathogen free eggs weighed (mean±s.d.; 65±2 g) of the domestic fowl (White Leghorn, Gallus gallus) were obtained from Manisa Research Institute of Poultry Disease and Vaccination Centre, were used for this study. The eggs were incubated at 37.5°C and 60%-70% relative humidity and repositioned every 4 hours for 30 hours until the embryos reached stage eight of Hamburger and Hamilton.[8] At this stage the eggs were randomly divided into four groups that consist of 25 eggs per group. Two eggs in each group was opened under 4X optical magnification to ensure that the eggs were in stage 8 according to Hamburger-Hamilton series.

DRUG PREPARATION

As compared to regular human dose of thiopental for induction of anesthesia ranging 3-5 mg/kg, study was designed for three different doses of drug and a control group. 2 mg/kg, 4 mg/kg, 8 mg/kg doses were chosen for this purpose representing subtherapeutic, therapeutic and supratherapeutic doses for groups B, C and D respectively. Doses were arranged regarding a standard SPF egg weighing 65±2 g and calculated for each egg. As total amount of volume planned for injection under embryonic disk is 0,1 ml three different concentrations of thiopental sodium dissolved in sterile saline (%0,9 NaCl) solutions (0,14 mg/0,1 mL Group B, 0,28 mg/0,1 mL Group C, 0,58 mg/0,1 mL Group D) were prepared. Group A was the control group and each subject was injected 0.1 mL of sterile saline (0,9% NaCl).

INJECTION METHOD

The eggs were washed with 70% alcohol and labeled with numbers on the outer shell. A hole was made on the blunt pole of the eggs with a sharp and thick needle and embryonic discs were identified under 4X optical magnification. Using a sterile 28-gauge needle mounted on tuberculin syringe, 0.1 mL of the fluid was injected from the blunt end under the embryonic disc according to New's technique.[9] Embryonic disc was not formed during incubation in one egg in each group suggesting absence of fertilization and excluded from the study. After careful injection the holes were sealed. The eggs were then placed into the incubator for next 42 hours and repositioned every 4 hours.

EMBRYO COLLECTION

The eggs were opened at 72 hours of incubation and outer shell was chipped out to create a wide opening for visualization of the embryo and heart beat for viability. The embryos were transferred to a petri dish carefully in a water filled container by floating technique. Careful dissection was made for embryonic discs under 4X magnification. In group B one egg was injured during repositioning and one egg from group D was injured during dissection as

the yolk adhesion to the hole made on the outer shell during injection. Totally 86 embryos was collected and were fixed with 10% formalin solution for 24 hours and embedded into paraffin. Sections of 5 micron thick serial sections were cut from paraffin blocks with rotary microtome oriented with an angle of 7° and stained with hematoxylin-eosin (HE) for light microscopic examination. Slides were examined with Leica DM 4000 (Germany) photo-light microscopy by a pathologist blinded to group assignment. Any disruption to somite or neural tube continuity was considered as a neural tube closure defect.

STATISTICAL ANALYSIS

Subjects in the study are summarized in Table 1. Totally 86 embryos evaluated histopathologically. 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

Totally 86 embryos were examined as two eggs were sacrified at 30th hour from each group for determination of stage 8 development, one egg from each group identified as unfertilized, one egg from group B and another from Group D were injured and excluded from the study (Table 2). While the groups A and B showed no neural tube defects (Figure 1), in group C neural tube closure defect detected histopathologically in 2 embryos however it was statistically insignificant. In group D, 4 embryos had neural tube closure defect histopathologically (Figure 2). Subjects and statistical analyses are summarized in Table 1. Comparison of group D with control group yielded a value of p as 0.048 was statistically significant ($p<0.05$). When all groups examined in paired matrices p were 0.044 (Table 1).

DISCUSSION

Every drug if administered adequate and in certain gestational period has teratogenic potential if could pass through the placenta.[10] This condition is also true for anesthetic drugs even for single and short term of exposion.[11,12] None of the anesthetic drugs, opioid analgesic, sedative and hypnotics used recently has defined as teratogen and safer than another.[13,14] Most human studies have not found a significant difference in the overall rate of congenital anomalies among women receiving general anesthesia while undergoing surgery.[15]

The chick embryo is a useful model system for studying many aspects of vertebrate embryogenesis. It is well suited for the investigation of chemicals on the development of embryos.[16] Stage 8 embryos were generally chosen for these investigations since developing neural tissues exhibit a gradual variation on the degree of opening along its length that provides an excellent opportunity to study the

TABLE 1: Subjects and statistical analysis of groups.

Groups	Neural Tube Defect (+)	Neural Tube Defect (-)	Statistical Analysis (p value) [Groups]
A (n=22)	0	22	
B (n=21)	0	21	
C (n=22)	2	20	0.244 [A-C]
D (n=21)	4	17	0.048 [A-D]
Total (n=86)	6	80	0.044 [A-B-C-D]

TABLE 2: Detailed assessment of subjects in each group

Groups	Initial Number of eggs	Exculed from study: Sacrified at 30th hour	Exculed from study: Unfertilized	Exculed from study: Injured	Final number evaluated
A (Control)	25	2	1	0	22
B (2 mg/kg)	25	2	1	1	21
C (4 mg/kg)	25	2	1	0	22
D (8 mg/kg)	25	2	1	1	21
Total	100	8	4	2	86

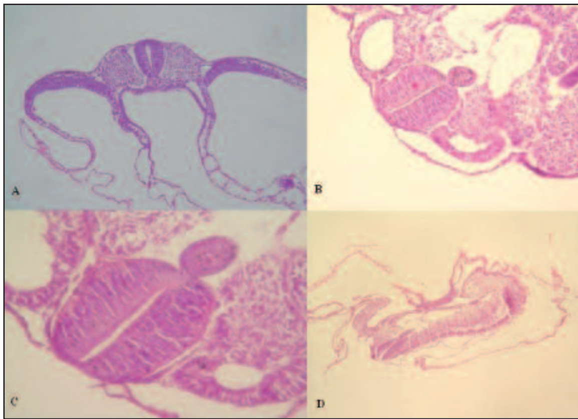

FIGURE 1: Embryo sections from group A (Control) painted with HE **A.** X40 objectives **B.** X100 objectives **C.** X200 objectives **D.** X10 objectives in sagittal plane.
(See for colored form http://anestezi.turkiyeklinikleri.com/)

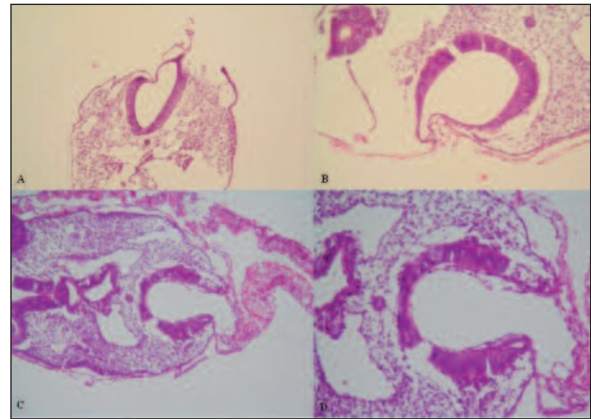

FIGURE 2: Embryo sections from NTD **A.** Group C X40 objectives **B.** Group C X100 objectives **C.** Group D X40 objectives **D.** Group D X100 objectives.
(See for colored form http://anestezi.turkiyeklinikleri.com/)

effects of chemical agents on neural tube closure. Previous studies on early period chick embryos have demonstrated the important role of apoptosis and contractile activity of microflaments in the neural tube closure which are also modulated by intracellular calcium levels.[17-20]

Nagele et al. investigated the diazepam induced neural tube defects in chick embryos that were explanted at Stage 8 of development and cultured for 6 hours.[21] Nearly 80% of these embryos showed neural tube closure defects, especially in the midbrain region, diazepam is shown to specifically inhibit the contractile activity of microfilament bundles.[21-24] Calcium antagonists administered on chick embryo cultures causes latency in neural fold formation and calcium agonists causes early neural fold formation.[19] Drugs like diazepam, papaverine and cytochalasin B effects on microfilaments and cause experimental neural tube defects supports the importance of microfilaments on neurulation.[25-27]

Local anesthetics inhibit elevation of the chick neural folds by disturbing the organization and calcium dependent function of microfilaments in neuroepithelial cells.[28] Another agent studied on early chick embryo, ethanol has an importance on embryonal development step. Enhart et al. showed that embryonal defect incidence increases with dosage.[29] Major effect of ethanol on embryos during gastrulation period is aborting neural cell growth and causing apoptosis on cells. It is not clear but ethanol can lack the signal between cells that is essential for neural tissue growth or the apoptosis mechanisms that causes neurulation. It is showed that ethanol has both N-methyl-D-aspartate (NMDA) antagonist and γ-aminobutiric acid type A ($GABA_A$) agonist properties.[30]

Thiopental has been used since 1930s. In studies with rats and mice treated with 1.5-3 times the human dose, thiopental was not found to be teratogenic.[31] In a retrospective study women treated with thiopental during first months of their pregnancy, there was no increase in congenital anomalies.[32] The main effect of thiopental is to suppress transmission of excitatory neurotransmitters and enhance transmission of inhibitory neurotransmitters GABA and benzodiazepine. Action mechanisms of thiopental is similar with diazepam and other benzodiazepines which are known to be teratogen. Ikonomidou et al. reported that infant rats exposed with drugs that block NMDA glutamate receptors causes apoptotic neurodegeneration in the developing brain.[33] And follow-up studies determined that similar neuroapoptotic reaction is induced in infant rat or mouse brain by drugs that activate $GABA_A$ receptors, ethanol which has both NMDA antagonist and $GABA_A$ agonist properties.[34,35]

Progesterone also acts in the brain by stimulating GABA signaling pathways in specific areas of the brain and may contribute to the formation of neural tube defects.[36]

Knowledge of drug usage in pregnancy is limited because prospective studies are not ethical. Drugs with high molecular weight, low lipid solubility and high degree of ionisation and high protein binding, can hardly pass through placenta and more time for diffusion equilibrium is needed especially for single dose is given. Medications used in anesthesia practice with low molecular weight, high lipid solubility, low degree of ionization and low protein binding, making these drugs to cross placenta easily.[37] When a drug crosses the placenta, its teratogenicity depends on the stage of fetal development. Between the stages conception and implantation, the embryo is undifferentiated and repair is possible through multiplication of the still omnipotential cells so can result in death or in intact survival.[38] Organogenesis stage, is the period of maximum sensitivity to teratogenicity because of tissue differentiation and teratogens may cause structural anomalies in this period. When organogenesis is completed, teratogen exposure will affect fetal growth, organ size or organ function.[39]

Nearly 2% of pregnant women undergo surgery during pregnancy. Thiopental is one of the induction agents used widely in anesthesia also used in pregnants. Although it is not known as a teratogen, activation of GABA system with supratherapeutic doses of thiopental in early pregnancy where organogenesis takes place might be responsible for NTD or may cause adverse consequences on central nervous system development but further studies and different embryo models are needed.

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