

The Teratogenic Effects of Antiepileptic Drug, Topiramate, on the Development of Chick Embryos

Jantima Roongruangchai, D.D.S., Ph.D.* , Yadaridee Viravud, D.D.S., M.Sc.* , Vasana Plakornkul, D.V.M., M.Sc.* , Kesorn Sriporaya, M.Sc.* , Sakultra Muangwatana, M.Sc.* , Kosol Roongruangchai, M.Sc., M.D.**

*Department of Anatomy, **Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.

ABSTRACT

Background: Anti-epileptic drugs are known to be the risk of teratogenicity. Topiramate (TPM) is a new kind of such drug, for which no research has confirmed the incidence of producing congenital abnormalities.

Objective: This study was conducted to study the teratogenic effects of TPM by using chick embryos as an animal model and the results can be compared to the human embryo of the same stage.

Methods: Fertilized Leghorn hen eggs were injected in ovo with two concentrations of TPM, which were 10mg, and 20mg, in NSS at a volume of 0.1 ml into the yolk sac at 21 hrs of incubation and repeated injections at 72 hrs at a volume of 0.05 ml. The chick embryos on day 3, 6 and 11 of incubation were sacrificed and all living embryos were processed for total mount and serial section.

Results: The mortality rate increased corresponding to the concentrations of TPM, and the embryonic stage. The total mount of day 3 showed major abnormalities of the eye and heart, such as microphthalmia and looser of heart looping. The serial section of day 3 showed opening of the anterior neuropore, ectopia viscerae and multiple malformations of the eye and heart. Day 6 chick embryos showed ectopia cordis and ectopia viscerae. Moreover, there were retardation and abnormalities of several organs such as eye, heart, liver, mesonephros and gonads. Day 11 chick embryos showed ectopia viscerae and several growth retardations, retardation of ossification of both limb bones and skull bones.

Conclusion: This study showed that TPM might cause embryonic death, growth retardation and abnormalities of the eye, heart, an opening of the anterior neuropore and ectopia viscerae. This might indicate abnormalities to the baby born from mother with gestational epilepsy who was taking this drug continuously, and it might lead to spontaneous abortion or congenital anomalies of the fetus.

Keywords: Topiramate; teratogen; chick embryo (Siriraj Med J 2017;69: 128-136)

INTRODUCTION

Teratogens are any agents that can induce a congenital malformation. They consist of drugs, medications, chemical, radiation, and maternal condition or disease.¹ Topiramate (topamax or TPM) is a new antiepileptic drug for the treatment of epilepsy, mood stabilizer, several psychiatric conditions, and migraine.² It is a sulfamate-substituted analog of fructose-1,6-diphosphate and is identified chemically as 2,3;4,5-bis-O-(1-methylidene)-beta-D-fructopyranosesulfamate which has structural similarity to

acetazolamide and has anticonvulsant effects.⁵ Formula of topiramate is C₁₂H₂₁NO₈S and its molecular weight is 339.363 g/mol. Its mode of actions involves blockade of voltage-dependent sodium channel, potential of GABAergic transmission and inhibition of excitatory pathways at AMPA receptor sites.³ Its mechanisms include: (i) facilitation of inhibitory gamma-aminobutyric acid-A (GABAA)-mediated currents at non-benzodiazepine sites on the GABAA receptor ; (ii) antagonism of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid

Correspondence to: Kosol Roongruangchai

E-mail: parasite52@hotmail.com

Received 16 December 2016 Revised 13 January 2017 Accepted 16 January 2017

doi:10.14456/smj.2017.26

(AMPA) and kainate glutamate receptors; (iii) inhibition of L-type calcium channels and limitation of calcium-dependent second messenger systems; (iv) limitation of activity-dependent depolarization and excitability of voltage-dependent sodium channels; (v) activation of potassium conductance, and (vi) weak inhibition of carbonic anhydrase isoenzymes — CA-II and CA-IV, which are found in both neuronal and peripheral tissues. In addition to epilepsy, topiramate is also used for migraine prophylaxis, bipolar disorder, post-traumatic stress disorder (PTSD), bulimia [nervosa, binge-eating disorder, and obesity].⁴

Zebra fish exposed to topiramate showed multi-malformations included malformations of head, tail, heart, yolk, scoliosis, and growth retardation.⁴ Topiramate is a drug of the D pregnancy category and contraindicated in women who are pregnant according to definitions provided by the U.S. Food and Drug Administration (FDA).⁵

Topiramate was reported to associate with malformations and decreased weights in rodent offspring. Limb defects were seen in rat offspring exposed in utero to high doses of Topiramate (400-500 mg/kg/day).⁶ Rabbit offspring also had malformations (rib and vertebral defects). As with many other AEDs displaying teratogenic effects in animal models, the effects of topiramate in children born of women treated with topiramate are not yet known.⁷ Nowadays, topiramate is used more widely over the world and many of its effects are considered to be teratogenic in human.

Animal studies are very important for the explanation about the mechanism of teratogenesis before new chemicals are introduced.⁶ Mammalian models are commonly used for preclinical evaluation of a new drug because they are closely related to humans. However, the mammalian models are not easy to evaluate and setup, expensive and more time consuming. The chick embryo is an excellent model system for studying the development of higher vertebrate and there are many advantages to use chick embryos as they are available all year, inexpensive, and can be purchased in any specified quantity and also there are plenty of databases of developmental stage which are similar and can be compared to humans.⁸ Chick embryo is an excellent model system for screening the teratogenic effects of new drugs before further studies are carried out in other mammalian models.

In 2002, Whetsel et al., studied the systemic and localized teratogenic effects of valproic acid (VPA) in ovo chicken model.⁹ The results showed minor defects of the limb bud, cardiac and eye anomalies. At higher dose it showed neural tube defects, abnormal wing bud,

mild to severe eye defects, microcephaly and enlarged heart tubes. At doses of 20 mg/ml, embryos exhibited 100% mortality.

TPM is a new kind of antiepileptic drug, for which there is still no research on the incidence of producing congenital abnormalities. This study was conducted to study the teratogenic effects of TPM by using chick embryos as animal model and the results can be compared to the human embryo of the same stage by using the Hamberger and Hamilton stage of developing chick embryo which compares to the Carnegie stage of human embryo.¹⁰

MATERIALS AND METHODS

ACUC Guideline

The Use and Euthanasia Procedures of Chicken/Avian Embryos (version: January 19, 2012; ACUC guideline on the use and euthanasia procedures of chicken-avian embryos.doc) (approved by the ACUC February 9, 2012)

Avian embryos are not considered live animals under PHS policy. However, there is a consensus in the scientific community that at a certain point in development, avian embryos can experience pain. Because that exact point is not known for chicken embryos, chicken use and euthanasia guidelines differ across institutions. Cal Poly Pomona has chosen to adopt a guideline with the belief that pain occurs on or after gestation day 13, in anticipation of reviewing protocols including them. The use of chicken embryos at gestation day 12 and younger does not require an ACUC Animal Use. However, the embryos used in this study were all in early stages of embryonic development.

The white leghorn hen (*Gallus gallus domesticus*) eggs from Department of Animal Science, Faculty of Agriculture, Kasetsart University were incubated at 37°C. The eggs were divided into 2 groups, Topiramate injected group (experimental group, n=150) and normal saline injected group (control group, n=25). The experimental group was sub-divided into 2 groups by different dosages of drug, which were 10 and 20 mg/ml TPM, respectively. The eggs were incubated until 21 hrs before injection. All eggs were injected at equal volume of 0.1 ml to yolk sac on 21 hours of incubation. Before injection, the shell was drilled at the blunt end of the egg through the air cell by dental driller and then TPM was injected into the yolk sac. The hole was sealed by paraffin for further incubation until day 3, 6 and 11 of development. All embryos from each stage were recorded for the number of deaths and survivors as well as normal and abnormal which were externally visible. The viability was indicated by the heart beating and the blood circulation. The day

3 embryos were processed for total mount and serial section, the day 6 embryos were processed for the serial section and the day 11 embryos were processed for the cartilage and bone staining.

The total mount of day 3 embryos were stained with Mayer's Camine for 30 minutes and dehydrated with graded series of alcohol for 5 minutes each change. Then the specimens were cleaned and mounted. The serial section of day 3 and day 6 were stained with hematoxylin and eosin. The cartilage and bone staining of day 11 were performed by removing the skin and visceral organs and fixed in 4% paraformaldehyde before washing and dehydration with absolute alcohol then transferred to acetone for 24 hours before staining with alcian blue and alizarin red for 3-4 days each at 37°C and transferred to 1% KOH and glycerol. The crown-rump length (CRL), beak, mandible, and other long bones were measured by the red color of the alizarin red, and then were compared with the control and tested statistically using Statistical

Package for the Social Science (SPSS) by one way analysis of Variance (ANOVA), and the data represented by mean \pm SD with significant difference at $p < 0.05$.

RESULTS

This study was designed to investigate the teratogenic effects of topiramate on different developmental stages of chick embryos compared with the normal development of the corresponding stage.¹¹ Tables 1, 2 and 3 showed the percentage of survival and mortality of days 3, 6 and 11 respectively. Table 1 showed the mortality rate of day 3 embryos were 0%, 5.88% and 17.39% in the control, 10 mg/ml and 20 mg/ml of TPM, respectively. Table 2 showed the mortality rate of day 6 embryos were 0%, 52.94% and 56.52% in the control, 10 mg/ml and 20 mg/ml of TPM, respectively. Table 3 showed the mortality rate of day 11 embryos were 9.09%, 87.50% and 84.21% in the control, 10 mg/ml and 20 mg/ml of TPM, respectively.

TABLE 1. Percentage of survival and mortality of the day 3 chick embryos which were injected by 2 different concentrations of TPM compared with control (Normal saline solution).

Group (Mg/ml)	n	Survival (%)	n	Mortality (%)	n
Control	7	100.00	7	0	0
10	17	94.12	16	5.88	1
20	23	82.61	19	17.39	4

Table 1. showed that the survival rate of the day 3 chick embryos were 100.0%, 94.12% and 82.61% in the control, 10 mg/ml, 20 mg/ml of TPM, respectively. There was slightly increased mortality rate as the dosage of the TPM increased.

TABLE 2. Percentage of survival and mortality of the day 6 chick embryos which were injected by 2 different concentrations of TPM compared with control (Normal saline solution).

Group (Mg/ml)	n	Survival (%)	n	Mortality (%)	n
Control	7	100.00	7	-	-
10	17	47.06	8	52.94	9
20	23	43.48	10	56.52	13

Table 2. showed that the survival rate of the day 6 chick embryos were 100.0%, 47.06% and 43.48% in the control, 10 mg/ml and 20 mg/ml of TPM, respectively. There was slightly increased mortality rate as the dosage of the TPM increased.

TABLE 3. Percentage of survival and mortality of the day 11 chick embryos which were injected by 2 different concentrations of TPM compared with the control (Normal saline solution).

Group (Mg/ml)	n	Survival		Mortality	
		(%)	n	(%)	n
Control	11	90.91	10	9.09	1
10	32	12.50	4	87.50	28
20	38	15.79	6	84.21	32

Table 3. showed that the survival rate of the day 11 chick embryos were 90.91%, 12.50% and 15.79% in the control, 10 mg/ml and 20 mg/ml of TPM, respectively. There were higher mortality rates as the incubation day increased.

The total mount of the day 3 chick embryo

The survival day 3 chick embryos as indicated by heart beating and the blood circulation were further studied by total mount and serial section techniques to study the detail of the abnormalities in the treated groups compared with the control group.

The day 3 chick embryo was categorized as HH-stage 18 (Hamburger Hammlton, 1951).¹¹ The chick embryo was approximately 36 somites. The secondary brain vesicles were completed, which were telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon. The cephalic flexure and cervical flexure were clearly bent to the right, cephalic flexure was at the mesencephalon and cervical flexure was about 90° at the point between myelencephalon and spinal cord. Mandibular process and the other three arches were distinct characteristics. The otic vesicle located at the level of myelencephalon, optic cup was large horseshoe-shaped located at the diencephalon and the lens situated middle to it. The heart tube was S-shaped looping with the ventricle laid the most ventral and caudal. The neural tube appeared as 2 parallel dense lines extended to the caudal end with the continuous blocks of somites laid adjacent to it. The limb buds enlarged with the posterior pair were larger than the anterior pair.

The total mount of day 3 chick embryo treated with TPM

The effects of TPM 10 mg/ml and 20 mg/ml on day 3 chick embryo were shown in **Fig 1B** and **Fig 1C**, respectively. Both showed growth retardation and were smaller in size when compared with the control group. The embryos also showed retardation of development of several organs such as brain vesicle, anterior and posterior limb buds, branchial arch, neural tube, somite, eye and heart loop. The retardation of brain development caused the absence of cervical flexure. The eye primordia were small and situated at the forebrain. The branchial

arches were lesser in number than the control. The brain vesicles were underdeveloped. The heart loop was looser, heart wall distended and constricted to u-shaped loop. The anterior and posterior limb buds were absent. The somite did not extend to the caudal end. The neural tube appeared as two dense unparallel lines and there was no tail fold.

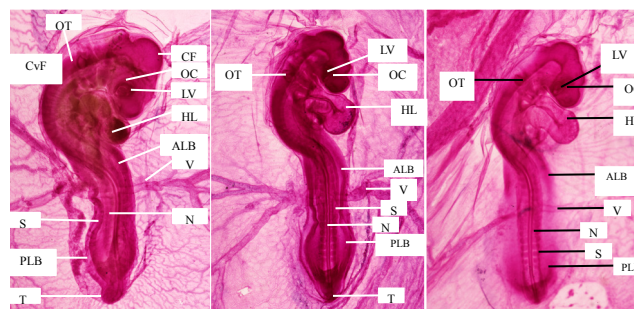


Fig 1. The total mount of day 3 chick embryos, A. control, B. 10 mg/ml, C. 20 mg/ml TPM treated groups. Both treated groups showed growth retardation of brain, eye and flexuring of head, looping of heart, somite, limb bud and tail fold. (CvF, cervical flexure, OT, otocyst, CF, cephalic flexure, OC, optic cup, LV, lens vesicle, HL, heart loop, ALB, anterior limb bud, N, neural tube, S, somite, PLB, posterior limb bud, T, tail fold, V, vitelline vessel).

The serial section of day 3 chick embryo treated with TPM

The effects of TPM on the day 3 chick embryos development were observed by serial section which showed that the chick embryos in experimental groups had growth retardation and abnormalities in several organs such as brain, eye, heart and branchial arches.

Fig 2. illustrated one of the living chick embryos of the group treated with 10 mg/ml of TPM which showed severity of abnormality of brain formation leaving a wide opening of anterior neuropore. The eye and branchial apparatus were normal, so this would eventually bring about the condition known as anencephaly. The eye development showed retardation of the optic cup and

lens vesicle, which originated from the diencephalon. Fig 3. showed small optic cup with equal thickness of both layers, the outer pigment layer and inner nervous layer, and wide separation between them, brought about the wide intraretinal space. The lens was small and retarded with persistent lens pit. Fig 3 also showed that the otic placode did not completely undergo double fusion and the otocyst still attached to the surface ectoderm. The branchial arch showed only mandibular arch.

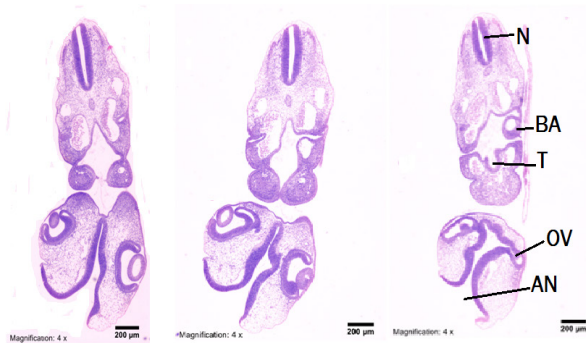


Fig 2. The serial section of day 3 chick embryo of the experiment group of 10 mg/ml showed the opening of the anterior neuropore. AN, anterior neuropore, OV, optic vesicle, T, thyroid diverticulum, BA, branchial arch, N, neural tube.

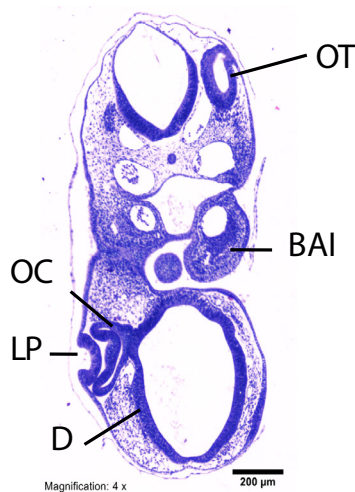


Fig 3. The serial section of day 3 chick embryo of the treatment group of 10 mg/ml showed the retardation of the eye development, optic cup (OC) is very small with the persistent of the intraretinal space (IRS), lens showed the persistent of lens pit (LP), OT, otocyst, BA, branchial arch, D, diencephalon.

The eye development of day 3 chick embryos of the control and the experiment group of 10 mg/ml of TPM, (A, the control group and B, the experiment group) were highly magnified. The normal eye at this stage was illustrated in the control which showed that the optic cup comprised 2 layers, the outer pigment layer and the inner nervous layer. The inner layer was thick and eventually obliterated the intraretinal space. The lens vesicle was an oval vesicle which completely underwent

double fusion and detached from the surface ectoderm. The lens vesicle comprised anterior lens epithelium and elongated posterior lens fiber. The experiment group of 10 mg/ml of TPM showed smaller optic cup with wide separation of the intraretinal space. The lens was incompletely double fused and showed persistence of lens pit, which was severe retardation of the eye development.

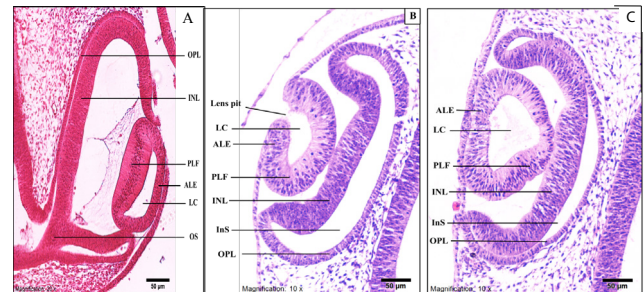


Fig 4. The serial section of the day 3 chick embryos at the level of eye development, A, control group, B, experiment group of 10 mg/ml and C, experiment group of 20 mg/ml, B and C showed retardation of optic cup and lens development, persistent of intraretinal space (InS) and lens pit. LC, lens cavity, ALE, anterior lens epithelium, PLF, posterior lens fiber, INL, inner nervous layer, OPL, outer pigment layer, OS, optic stalk.

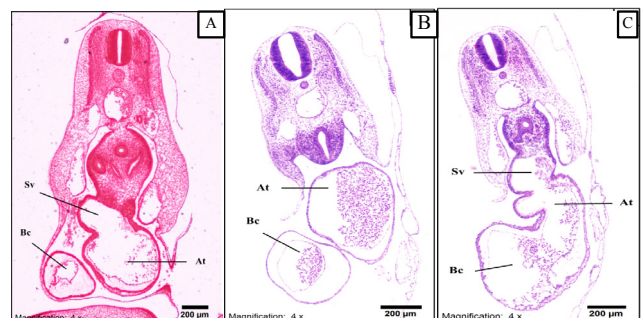


Fig 4.1. The serial section of the day 3 chick embryos at the level of developing heart, A, the control, B, the experiment group of 10 mg/ml and C the experiment group of 20 mg/ml. A showed normal relation of atrium (At), bulbus cordis (Bc) and sinus venosus (Sv) while B and C showed looser of heart looping with very thin heart wall and distended lumen brought about the abnormal relation of heart chambers.

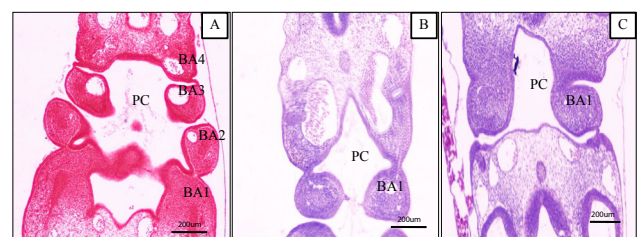


Fig 4.2. The serial section of the day 3 chick embryos at the level of branchial arch development, A, the control, B, the experiment group of 10 mg/ml and C, the experiment group of 20 mg/ml. A showed developing of 4 branchial arches (BA1-4) while B and C showed retardation of development of branchial apparatus which showed only one arch. (PC, pharyngeal cavity).

The serial section of day 6 chick embryo treated with TPM

The results showed that the eye of the control group showed normal development while the experimental groups showed abnormalities of the eye, which were smaller in size when compared with control group and the optic cups and lens were irregularly shaped, and the pigment layer was separated from the nervous layer (retinal detachment) leaving a wide separation of intraretinal space Fig 5. The lens vesicle was small and the posterior lens fiber was less elongated. The lens cavity still persisted.

Fig 6. was the eye of the day 6 embryo in the experiment group of 20 mg/ml of TPM which showed severe abnormality of the eye development, which presented the very small optic cup and lens vesicle which was referred to as the microphthalmia. The optic cup was irregular in shape. The inner nervous layer was thin with a wide separation of the intraretinal space. The lens vesicle was about the day 3 type which showed equal thickness of lens epithelium and the lens cavity persisted.

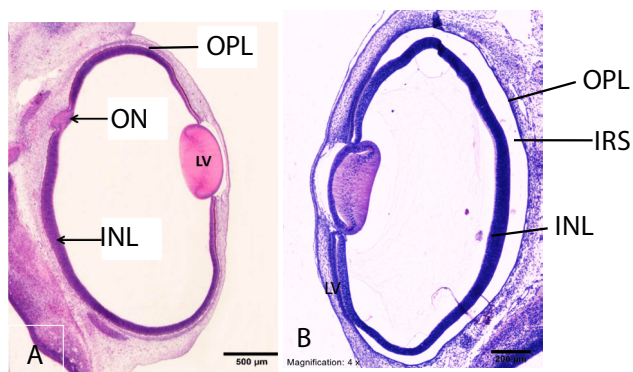


Fig 5. The eye development of the day 6 chick embryo of the control (A) and the experiment group of 10 mg/ml (B), B showed retardation of eye development by exhibited the wide persistent of the intraretinal space (IRS) and slightly underdeveloping lens. OPL, outer pigment layer, INL, inner nervous layer.

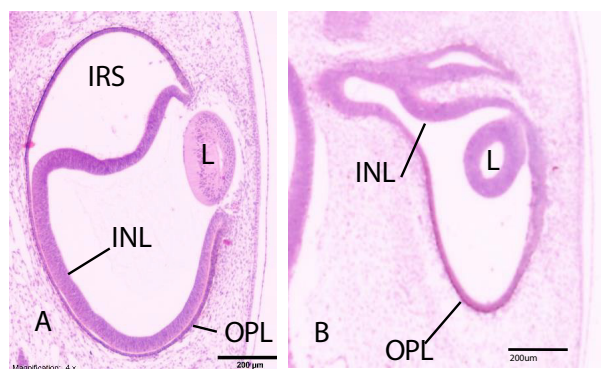


Fig 6. Micrographs showed the severe abnormalities of eye development of day 6 chick embryo, A, the 20 mg/ml, showed the very small optic cup and lens vesicle (microphthalmia) with wide separation of the intraretinal space. Lens (L) was also small and underdeveloped. B, another 20 mg/ml groups showed severe abnormalities of optic primordia represented irregular optic cup and lens. OPL, outer pigment layer, IRS, intraretinal space, INL, inner nervous layer, L, lens.

The heart of the day 6 chick embryo of the control group comprised large and distinctive atrium and ventricle which were separated by the fused endocardial cushion. The atrium laid dorsally to the ventricle which exhibited thicker wall of muscular trabeculae. The septum primum and the interventricular septum were incompletely developed Fig 7A. The heart of the experimental group, which was illustrated in Fig 7B, was from the 10 mg/ml of TPM treated group which showed small and irregular shaped atrium and ventricle. The endocardial cushion was also small and the ventricular wall was thin. This indicated the retardation of heart development.

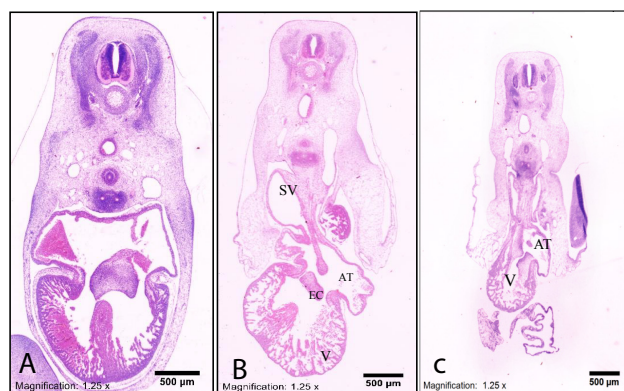


Fig 7. Micrographs of the serial section of day 6 chick embryos at the level of heart development, A, the control, B, 10 mg/ml, C, 20 mg/ml. B showed smaller heart than the control with an irregular shape atrium, smaller endocardial cushion and looser myocardium of the ventricle. C showed severely smaller and abnormal of heart chambers. SV, sinus venosus, AT, atrium, EC, endocardial cushion, V, ventricle.

The mesonephros and gonad developed from the intermediate mesoderm. The mesonephros comprised the mesonephric duct, abundant mesonephric tubules and mesonephric corpuscles. The duct differentiated into male genital ducts while the tubules developed into the efferent ductules. Fig 8A showed the normal mesonephros of the control group with abundant tubules and corpuscles, while Fig 8B showed the mesonephros of the experimental group of 10 mg/ml treated group which showed severe retardation of mesonephric development which were represented by small amount of tubules and corpuscles and the appearance of cystic formation within most of the tubules.

The gonad situated medially to the mesonephros represented as a distinctively large ridge of densely packed mesenchyme and presented in Fig 8A was the gonadal ridge of the control. Fig 8B showed the gonadal ridge of the experimental group of 10 mg/ml of TPM treated group which showed very small and underdeveloped gonadal ridge laid ventrally to the abnormal mesonephros.

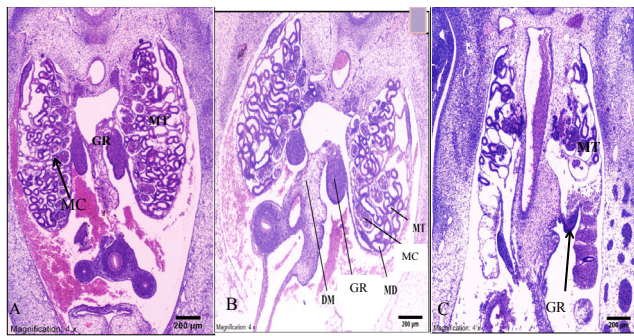


Fig 8. The mesonephros and gonadal ridge (GR) of the control (A) and the experiment groups, (B) the 10 mg/ml treated group and C the 20 mg/ml treated group, B and C showed that the mesonephric tubules (MT) were very few and dilated and the glomeruli were also lesser than the control (A). The gonadal ridges (GR) of B and C were also smaller than the control. MC, mesonephric corpuscle, MD, mesonephric duct, DM, dorsal mesentery.

The effect on day 11 of embryos

The chick embryos on day 11 were collected and investigated for the survived and dead embryos as shown in Table 3. There were 2 different concentrations of topiramate, 10 and 20 mg/ml. All of the experimental groups were studied for the external appearances of embryonic morphology by photograph, and the formation of bone and cartilage by staining with Alcian blue and Alizarin red staining compared with the control group.

External features of the day 11 chick embryo

Normal development of the day 11 chick embryos showed that entire plantar surface of phalanges was covered with well developed papillae and the length of the third toe was approximately 12.7 ± 0.5 mm. Eye lids were opened. The beak length from the anterior edge of nostril to the tip of bill was approximately 4.0 mm. However, the main channel of the auditory meatus was not visible. This study showed normal development of brain, eye, upper limb, lower limb, beak and closure of thoracic and abdominal walls in control group, while the experimental groups showed smaller size of body **Fig 9A and B** and appeared opening of anterior thoracic and abdominal walls (omphalocele) in both concentrations of TPM or the so called ectopia viscerae.

Bone and cartilage staining of the day 11 chick embryos

Fig 10A showed the endoskeleton of the day 11 chick embryo in the control group and **Fig 10B** was the experimental group of 20 mg/ml of TPM. The embryos were stained by alcian blue and alizarin red, in which the alcian blue stained cartilage models while the alizarin red stained bones. **Fig 10** showed the differences between the ossification of the control and experimental groups. The experimental group showed retardation of ossification of

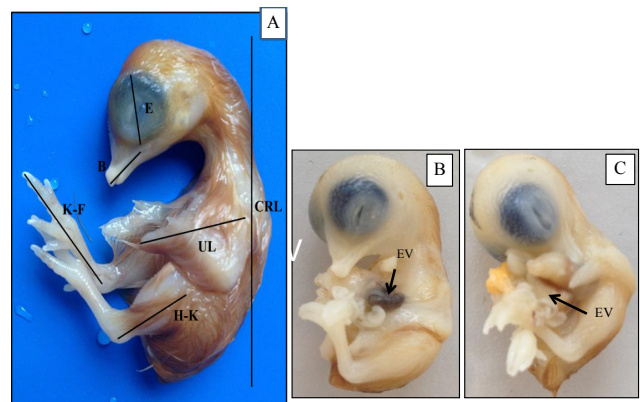


Fig 9. Photographs of the 11 day chick embryos, A, control, B, 10 mg/ml and C, 20 mg/ml, showed the abnormalities of the development of the thoracic and abdominal walls with the ectopia viscerae (EV). E, eye, B, beak, CRL, crown rump length, UL, upper limb, H-K, hip to knee, K-F, knee to foot.

all bones of the body such as skull, beak, cervical vertebrae, upper and lower limb long bones, as indicated by the lack of red color in the center of each bone. **Fig 11A and B** were higher magnification to show the differences in ossification of the skull and the cervical vertebrae.

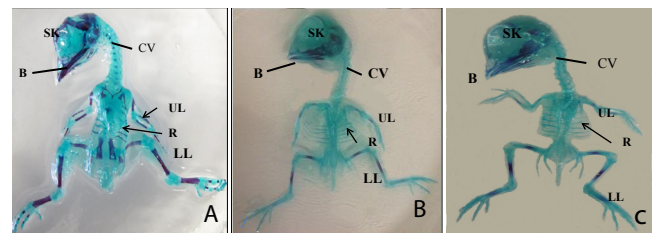


Fig 10. Photographs of the skeletal staining with alcian blue and alizarin red of the day 11 chick embryos, A, the control, B, 10 mg/ml and C, 20 mg/ml experiment groups. The blue represented the cartilage models while the red color (dark blue in these pictures) represented the bones or the ossification centers. There were lesser ossification centers in the experimental groups than the control of the skull (SK), beak (B), cervical vertebrae (CV), upper limb (UL), lower limb (LL) and rib (R).

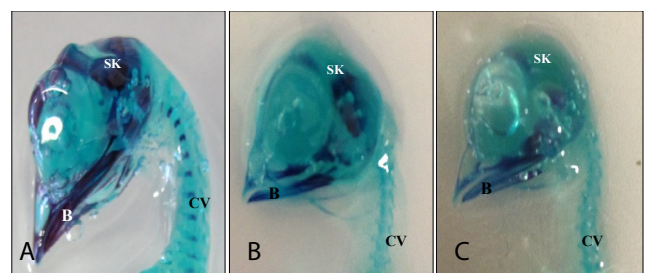


Fig 11. Higher magnification of the head and neck of the day 11 chick embryo stained with the alcian blue and alizarin red to show the skeletal development. A, the control, B, the experiment group of 10 mg/ml and C, the experiment group of 20 mg/ml showed lesser ossification centers (dark blue color) than the control indicated the retardation of bone development of the skull, beak and cervical vertebrae. SK, skull, CV, cervical vertebrae, B, beak.

DISCUSSTON

Topiramate (topamax) is a new antiepileptic drug which was categorized in drug category D⁵ and commonly used for treatment of partial seizures and generalized tonic-clonic seizures.¹² Moreover, topiramate is also used for obesity, as a mood stabilizer, for the treatment of several psychiatric conditions¹³ and migraine.¹⁴ This drug has been reported to cause malformation in animal model such as craniofacial complex in mice, induced various craniofacial and limb anomalies in rats, increased intrauterine fetal death in rabbits¹⁵ and induced malformations of heart, yolk, scoliosis, and growth retardation in zebra fish.⁴ Besides the effect in animal model, topiramate was used to study about efficacy and teratogenicity in pregnancies of those who were treated with topiramate, and the study found that topiramate could produce fetal malformation instance of hypospadias¹⁴ and in first trimester use of topiramate could induce risk of cleft lip and cleft palate.¹⁶

The results of this study showed that the percentage of mortality increased in the older stage and the mortality rate of the treated with topiramate group increased with the increasing concentration. These agreed with other studies in animal model, when the zebra fish embryos were exposed to topiramate at difference concentration including 0.5 mM, 1 mM, 5 mM, 10 mM and 50 mM from initiation of gastrula stage to termination of hatching the result showed that the lethal embryos were increased with the increasing concentration of topiramate. The percentage of mortality increased from 20% to 63% when the zebra fish embryos exposed to topiramate at 5 mM and 10 mM, respectively.⁴

The result of the eye in the day 3 of experimental groups showed growth retardation and was smaller when compared with the control. The optic cup was small and underdeveloped with wide separation of intraretinal space. The lens showed high anterior lens epithelium with the posterior lens fiber which did not elongate and the lens did not separate from surface ectoderm. Moreover, the experimental group of 20 mg/ml of TPM showed the lens with incomplete double fusion process leaving an opening of lens pit. This result agreed with the report in 2002 about the teratogenic effect in stage 14 chick embryos treated with valproic acid which were examined at stage 17. The result showed abnormalities in several organs including the eye. The eye appeared microphthalmic and poorly differentiated, the cornea was thin and the choroid fissure was incompletely closed. Moreover, the expressions of PAX-2 and PAX-6 genes were weak in valproic acid treated embryos which showed that the lens and retina were underdeveloped.¹²

The histological study of the day 6 chick embryos

showed abnormalities of the eye, which were smaller when compared with the control and the optic cups and lens were irregularly shaped, the pigment layer was separated from the nervous layer (retinal detachment) leaving a wide separation of intraretinal space, the posterior lens fiber did not elongate which caused persistent lens cavity, lens laid far from cornea and the corneal wall was thin. Moreover, concentration 20 mg/ml of topiramate showed retardation of eye development in which the optic cup was small when compared with control, with very thin nervous layer and failure to form double-walled optic cup, appearance of wide retinal detachment and lens was small and underdeveloped. The result agreed with Whitsel et.al., in 2002⁹ who studied the effect of valproic acid in chick embryos which showed that chick embryo stage 25 acquired several abnormalities of eye such as microphthalmia of the left eye while the right eye was slightly exophthalmia, abnormalities of craniofacial cartilage, abnormally wide choroid fissure and the expression of PAX-2 and PAX-6 protein was weaker than the control. The effect of heart in all experimental groups showed ectopia cordis, and the heart was smaller with thinner heart wall when compared with the control. The heart situated outside the body cavity (ectopia cordis) as the anterior thoracic wall was absent. Actually, there was no previous study about the effect of topiramate on the heart. The effect on mesonephros and gonad of day 6 chick embryos in all experimental groups showed retardation and smaller mesonephros, and less number of mesonephric tubules. Gonads were also smaller than the control group. The day 11 chick embryos showed higher mortality rate in experimental groups. Moreover, there was an opening of anterior thoracic and abdominal walls (omphalocele) in both concentrations of topiramate. Moreover, the effect of topiramate on development of bone and cartilage on day 11 chick embryos by skeletal staining indicated the retardation of ossification in all experimental groups of both the endochondral (limb bones) and intramembranous (skull bones). This study agreed with recent studies of the effect of valproic in ovo chick embryos in which the results showed that valproic acid induced skeletal anomalies both axial and limb anomalies which major abnormalities were seen at ribs and included irregular shape, bifid ribs, wavy ribs and sometimes there were poorly formed vertebrae with scoliosis.

Due to the number of new substances, chemicals and drugs coming into use in everyday life with increasing amount, there is a high demand for a rapid, reliable and cost-effective method for detection the developmental toxicity. The *in-vitro* teratogenicity test systems based on

a vast range of cells and tissues are good for detecting the embryotoxic hazards of chemicals. However, for teratogen testing, it is necessary to perform in animal models, as the target organs of specificity and age related to human are also able to predict. The preclinical evaluation of drugs traditionally involves large numbers of animal to predict possible drug side-effects and teratogenicity screening. Rats, mice, rabbits and other non human primates require more complicated procedures, time consumption, expense and number of animals is restricted. This is in contrast to *in ovo* chick embryos which are available in large number with easier method of inducing substances especially in the period of organogenesis. There are also databases of human age related organogenesis.

TPM leads to growth retardation and malformation of eye, heart, mesonephros and gonad, ectopia viscerae and also retardation of ossification of bones of the head and trunk which can be compare to the 4th -7th weeks of human embryo. This was the significant data to predict the abnormalities which could occur in human embryos especially in the 1st trimester of pregnancy in which pregnant women should avoid consuming the drug.

CONCLUSION

TPM, an antiepileptic drug, was illustrated in this study to cause embryonic death and congenital abnormalities in chick embryos which indicated the risk to cause the same effects when TPM was used in pregnant women. Further study should be carried out in other animal models to confirm its teratogenic effects and its use in pregnant woman should be highly cautioned.

REFERENCES

1. Barness EG. Review: Teratogenic cause of malformations. *Ann Clin Lab Sci* 2010;40(2):99-114.
2. Ornoy A, Zvi N, Arnon J, Wajnberg R, Shechtman S, Diav-Citrin O. The outcome of pregnancy following topiramate treatment: a study on 52 pregnancies. *Reprod Toxicol* 2008;25(3): 388-9.
3. Perucca EA. Pharmacological and clinical review on topiramate, a new antiepileptic drug. *Pharmacol Res* 1997;35(4):241-56.
4. Lee SH, Kang JW, Lin T, Lee JE, Jin DI. Teratogenic potential of antiepileptic drugs in the zebrafish model. *Biomed Res Int* 2013;2013:726478.
5. Taketomo CK, Hodding JH, Kraus DM. *Pediatric dosage handbook*. 6th ed. Hudson (Ohio): Lexi-Comp; 1999.p.27.
6. Glauser TA. Topiramate. *Semin Pediatr Neurol* 1997;4(1):34-42.
7. Natekar PE. Methotrexate Induced Gross Malformations in Chick Embryos. *J Hum Ecol* 2007; 21(3):223-26.
8. Darnell D, Schoenwolf G. The chick embryo as a model system for analyzing mechanisms of development. In: Walker J, Tuan R, Lo C, editors. *Developmental Biology Protocols. Methods in Molecular Biology™*. 135: Humana Press; 2000. p. 25-9.
9. Whitsel AI, Johnson CB, Forehand CJ. An *in ovo* chicken model to study the systemic and localized teratogenic effects of valproic acid. *Teratology* 2002;66(4):153-63.
10. Ahir BK, Pratten MK. Developmental cardiotoxicity effects of four commonly used antiepileptic drugs in embryonic chick heart micromass culture and embryonic stem cell culture systems. *Toxicol In Vitro* 2014;28(5):948-60.
11. Hamburger V, Hamilton HL. A series of normal stage in the development of the development of the chick embryo. 1951. *Dev Dyn* 1992;195(4):231-72.
12. Jackson MJ. Choice of antiepileptic drug, which one to try first and what to do if it fails. *Practical Neurology* 2005;5:6-17.
13. Gordon A, Price LH. Mood stabilization and weight loss with topiramate. *Am J Psychiatry* 1999; 156:968-9.
14. Hunt S, Russell A, Smithson WH, Parsons L, Robertson I, Waddell R, et al. Topiramate in pregnancy: preliminary experience from the UK Epilepsy and Pregnancy Register. *Neurology* 2008;71:272-6.
15. Palmieri C, Canger R. Teratogenic potential of the newer antiepileptic drugs and how should this influence prescribing. *CNS Drugs* 2002;16:755-64.
16. Vajda FJ, Graham J, Roten A, Lander CM, O'Brien TJ, Eadie M. Teratogenicity of the newer antiepileptic drugs, the Australian experience. *J Clin Neurosci* 2012;19(1):57-9.
17. Margulis AV, Mitchell AA, Gilboa SM, Werler MM, Mittleman MA, Glynn RJ, et al. Use of topiramate in pregnancy and risk of oral clefts. *Am J Obstet Gynecol* 2012;207(5):405.e1-7.