EFFECT OF PRENATAL PHENYTOIN EXPOSURE ON THE APICAL ECTODERMAL RIDGES OF CHICK WING BUDS

Hamd Binte Shahab Syed, Muhammad Yunus Khan*

CMH Institute of Medical Sciences (CIMS) Multan/National University of Medical Sciences (NUMS) Pakistan, *College of Physicians and Surgeons Pakistan (CPSP) Islamabad Pakistan

ABSTRACT

Objective: To observe the effect of sodium phenytoin on the development of the apical ectodermal ridges (AER) in chick wing buds.

Study Design: Laboratory based case-control study.

Place and Duration of Study: This study was carried out at the Department of Anatomy located at the Regional Center of College of Physicians and Surgeons Islamabad Pakistan, from Jan 2014 to Jan 2015.

Material and Methods: Sixty fertilized chicken eggs of 'Egyptian fayoumi' breed were selected and divided into two subgroups, experimental and control, each having 30 eggs. A single dose of 3.5 mg sodium phenytoin was injected in each egg of the experimental subgroup while controls were administered same volume of normal saline. The embryos were extracted 96 hours (day 4) after incubation. Histological sections were cut at 5 µm thickness and stained with Feulgen Nuclear and Light Green to measure the maximum thickness of the apical ectodermal ridges. The number of cells and mitoses in each ridges were also counted. SPSS 16 was used for statistical analysis.

Results: There was no significant difference between experimental and control subgroups regarding the maximum thickness and number of cells in the apical ectodermal ridge but there was a statistically significant reduction in the number of mitoses in the experimental ridges.

Conclusion: The study shows that sodium phenytoin exerts an inhibitory effect on the apical ectodermal ridges of developing limbs evident by the decreased number of mitoses in the AER of wing buds of experimental chick embryos making it a potential target site for limb teratogenicity associated with its maternal intake during pregnancy.

Keywords: Apical ectodermal ridge, Chick embryo, Mitoses, Sodium phenytoin.

INTRODUCTION

Women with a history of seizure-related illnesses require antiepileptic medication throughout pregnancy. Phenytoin is a widely used non-sedative antiepileptic drug included in pregnancy category 'D' of teratogenic potential according to the FDA (United States Food and Drug Administration) which justifies the use of this drug if the potential therapeutic benefits outweigh the potential risks[1].

Intrauterine exposure to phenytoin leads to a broad spectrum of fetal anomalies collectively known as the 'fetal hydantoin syndrome'. It comprises a number of birth defects including facial dysmorphism, mental retardation, neurobehavioural disorders, heart defects, abdominal wall defects and limb abnormalities. The birth defect of the appendages is an almost constant feature in the phenytoin babies. It includes hypoplasia of the distal phalanges/nails, a 'finger-like' thumb, syndactyly, polydactyly, absent palmar creases and positional limb defects like club feet. Prospective and case-control studies conducted previously strongly link phenytoin exposure with digital hypoplasia and positional limb defects[2,3].

In comparison with other studies conducted to determine the exact mechanism of phenytoin-mediated limb teratogenicity, the evidence is still inconclusive. The formation of reactive oxygen species (ROS) in response to prenatal phenytoin

Correspondence: Dr Hamd Binte Shahab Syed, H. No. 20/1, Al-Rafiq House, Officer's Colony Chungi No 9 Khanewal Road Multan Pakistan *(Email: hamdsyed@hotmail.com)*

Received: 26 Oct 2016; revised received: 19 Dec 2016; accepted: 22 Dec 2016

exposure can be one mechanism by which there is damage to DNA and other macromolecules resulting in various types of birth defects[4].

In another study, several mechanisms of teratogenicity have been postulated but with uncertainty. These hypotheses include teratogenicity of phenytoin by gene alterations, ischemic-hypoxic damage, apoptosis and folate deficiency[5].

In a recent study[6], it has also been hypothesized that the inhibition of neurotransmission by the anticonvulsants could lead to an alteration of bioelectrically controlled processes in the embryo that result in congenital malformations.

Effect of maternal genotype of functional polymorphisms in genes involved in phenytoin metabolism has been implicated as a predisposing factor for congenital malformations in another research[7].

The apical ectodermal ridge (AER) is one of the main signaling centers during limb development. It is a specialized thickened ectoderm rimming the distal end of the limb bud that controls outgrowth and patterning along the proximodistal axis. The AER secretes a wide variety of molecules involved in cascades of signaling events. The most important of these molecules are the fibroblast growth factors (FGFs). The FGFs, particularly FGF8 and FGF4 represent the principal supply of FGFs from the apical ectodermal ridge. These FGFs provide proliferation factors for the underlying mesoderm that allow normal progression of limb development. They maintain the zone of polarizing activity (ZPA) which is a cluster of cells at the posterior border of the flank. The cells of ZPA produce retinoic acid (RA). Retinoic acid, the active metabolite of vitamin A, is indispensably involved in the normal morphogenesis and organogenesis of most vertebrate species. Its role in limb development has been studied extensively. It initiates the expression of sonic hedgehog (SHH), a secreted factor that regulates limb patterning in the antero-posterior axis. SHH is also responsible for the expression of a gene encoding 'Gremlin' which functions as an apical ectodermal ridge maintenance factor[8-10].

In the chick embryo, at 96 hours (day 4) of development the apical ectodermal ridge reaches a maximum thickness and height after which it regresses[11]. Therefore in this study, duration of exposure of the drug was 4 days.

Experimental work[12] clearly elucidates the requirement of retinoic acid for the initiation of outgrowth in the chick limb bud. Studies have shown that phenytoin causes an altered expression of various genes involved in key morphogenetic events[13].

Since phenytoin can disturb the endogenous metabolic processes, it can also have an adverse effect on the embryological development of the apical ectodermal ridge which may be its primary site of teratogenic action. In the present study, we observed the effects of sodium phenytoin on the apical ectodermal ridge which have not yet been investigated. This may provide a possible explanation for the limb anomalies seen in infants born to mothers exposed to phenytoin during pregnancy.

The purpose of this study was to elucidate the mechanism of the teratogenic effect of phenytoin on limb development which has not yet been determined. For this purpose, the teratogenic dose was adopted. In our laboratory settings, after injecting a series of experimental doses, 3.5 mg of sodium phenytoin per egg was found to be teratogenic in surviving embryos and was therefore, selected as the dose for this study[14].

MATERIAL AND METHODS

The present experimental study was carried out at the Department of Anatomy located in the Regional Centre of College of Physicians and Surgeons, Islamabad, Pakistan from January 2014 to January 2015. Fertilized chicken eggs were injected with sodium phenytoin and compared

with controls to observe the effects of this drug on the development of the AER.

Chicken eggs were obtained by applying pre-fixed inclusion and exclusion criteria. The total number of freshly laid eggs was examined to remove the damaged and dirty eggs. The following inclusion and exclusion criteria were applied.

Eggs belonging to "Egyptian Fayoumi" breed of Gallus domesticus and eggs obtained from Poultry Research Institute (PRI), Rawalpindi were included in the study.

Cracked eggs and eggs stored for more than 03 days were excluded from the study.

Sixty eggs were selected by simple random sampling technique using the random selection table. These eggs were then placed in two main subgroups having 30 eggs each, subgroup B was experimental and A was taken as control. All the eggs were first wiped clean with swabs soaked in 70% alcohol and then placed in racks with the blunt end facing upward for ten minutes. This gave the eggs time to dry and allowed the blastoderm to float upward and settle at the blunt end just beneath the air sac. This prevented damage to the embryo from drug injection at the lower pointed end. Two holes were drilled into each egg with the help of a thumbpin, one at the upper blunt end and the other a fingerbreadth above the pointed lower end. The hole at the upper end allowed air to escape from the egg creating a space for the entrance of the drug or normal saline at the lower end. A sterile insulin syringe (needle length 8 mm, 30 gauge) was used to inject 3.5 mg sodium phenytoin per egg in the experimental group and an equivalent amount of normal saline in the control group directly into the egg albumen[15].

The holes were immediately sealed with melted wax and eggs were placed in the incubator. The day the eggs were placed in the incubator was taken as day 0. The temperature inside the incubator was kept at 38 ± 0.5ºC and relative humidity was kept between 60 and 70%. Adequate ventilation was also maintained. The eggs were rotated ½ turn twice daily.

At 96 hours (day 4) of development, the eggs were taken out of the incubator and placed horizontally on a table for ten minutes. This allowed the blastoderm to float upward and take a position over the yolk sac just beneath the shell. The eggs were then broken open in a bowl of warm normal saline. This was a delicate procedure. Starting from the broader end, the shell cap was removed exposing the underlying embryo. Survivability was easily determined by observing the pulsatile beating of the heart. The embryos were cleanly dissected out and transferred to a petri dish avoiding unnecessary tractions and hence, trauma[16].

Table: Comparison of histological parameters between control subgroup A and experimental subgroup B.

Parameter	A Mean ± SEM	B Mean ± SEM	*p*-value
Maximum Thickness of AER (µm)	33.296 ± 1.744	31.96 ± 1.565	0.573
Number of cells in AER	52.107 ± 2.281	50.329 ± 2.879	0.628
Number of mitoses in AER	1.009 ± 0.132	0.171 ± 0.050	0.0001*

*Statistically significant ($p<0.05$).

The embryos of both subgroups were labeled and assigned serial numbers. They were fixed in 10% neutral buffered formalin for 72 hours and processing was done following a standardized processing regimen keeping in view the fragility of the embryonal tissue at this delicate age[17]. Each embryo was embedded in paraffin vertically with the head directed downward. Serial sections were cut at 5 µm thickness in the dorsoventral plane (transverse sections) to locate the wing buds.

Sections of these embryos in the region of the wing buds were stained with Feulgen Nuclear stain and counterstained with Light Green[17]. The

slides were mounted in the synthetic resin mountant, Distyrene Plasticizer Xylene (DPX) and studied under light microscope.

The apical ectodermal ridge (AER) was recognized under oil immersion lens (100 X objective) fitted with the linear micrometer in the eyepiece. This ocular micrometer was already calibrated using a stage micrometer. The maximum ectodermal thickness (height) was determined using the linear micrometer along a line which passed through the thickest portion of the apical ectoderm (including periderm) and was normal to the line tangential to the base of the ectoderm. The rest of the parameters of the AER were measured in the selected sections with maximum ridge thickness.

The number of cells and mitoses was counted in AER of each selected section of maximum ridge thickness under oil immersion lens (100 X objective).

Dose Selection

The average weight of one chick egg was 50 grams into which 0.3 mg of sodium phenytoin was injected. This was equivalent to 6 mg/kg (0.3/50 x 1000). This falls into the therapeutic dosage range of phenytoin in humans (5-7 mg/kg)[18,19]. But experiments have shown that this therapeutic dose of 0.3 mg per egg produces no statistically significant defects of neural tube closure in the chick[20]. Preliminary experiments carried out in our laboratory with this dose of 0.3 mg did not produce any teratogenic effects. A reasonable explanation for this is the difference in species between chick and human. As the purpose of this study was to elucidate the mechanism of the teratogenic effect of phenytoin on limb development, therefore, the teratogenic dose of 3.5 mg per egg was adopted.

Data Analysis

The data were analyzed statistically with Statistical Package for Social Sciences (SPSS) computer software program, version 16. The maximum thickness, number of cells and mitoses in the AER were all analyzed by applying t-test for the detection of any significant differences between the means in experimental and control groups. The data were expressed as means ± SEM (standard error of mean). A *p*-value of less than 0.05 is considered as a significant value.

RESULTS

The total number of eggs was 60. The histological picture of the experimental specimens resembled the controls (fig-1). In the controls, the AER was well demarcated in all the specimens with prominently stained cells. The ectodermal-mesodermal interface was clearly visible due to staining by Light Green. The number of mitoses in the AER of the

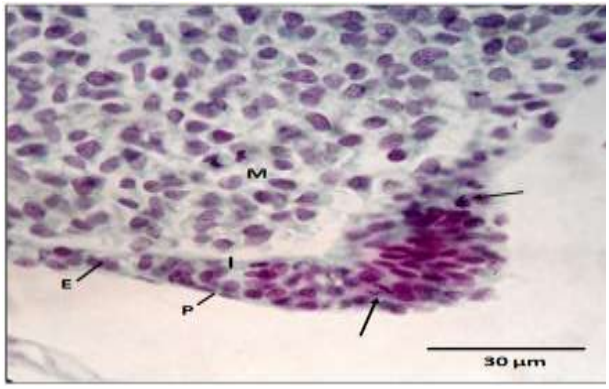

Figure-1: High-power photomicrograph showing the apical ectodermal ridge of 96 hours old chick embryo belonging to control subgroup A with arrows pointing toward mitoses. There is a distinct ectodermal-mesodermal interface (I). Mesoderm (M), Ectoderm (E), Periderm(P). Feulgen Nuclear and Light Green staining. (Scale bar=30 µm).

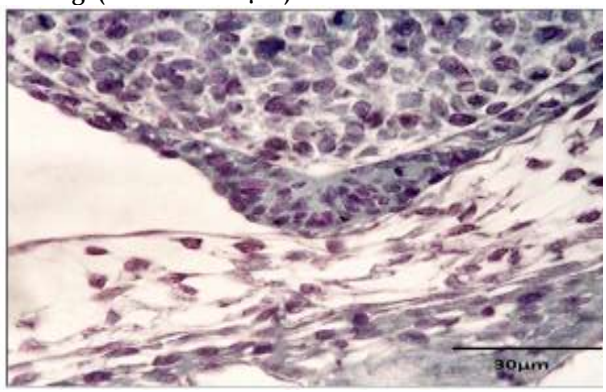

Figure-2: High-power photomicrograph of apical ectodermal ridge of a 96 hours old chick embryo belonging to experimental subgroup B. There is a distinct ectodermal-mesodermal interface. No prominent mitotic figure is visible. Feulgen Nuclear and Light Green staining. (Scale bar=30 µm).

experimental groups (fig-2) appeared to be less as compared to the controls.

The maximum thickness of AER in the experimental subgroup had a mean value ± SEM of 31.96 ± 1.565 µm. This was slightly lower than the control subgroup. The difference of mean values was not statistically significant (table).

The total number of cells in the AER of experimental group had a mean value ± SEM of 50.329 ± 2.879 which was slightly lower as compared to the control mean value. The difference between the mean values of control and experimental subgroups was not statistically significant (table).

The number of mitoses in AER of experimental subgroup had a mean value ± SEM of 0.171 ± 0.050. The difference of mean values of control and experimental groups was found to be statistically significant as indicated by a *p*-value of 0.0001 (table).

DISCUSSION

Observation of the AER was the main objective of this study. As evident from the latest international research[21], exogenous factors that lead to disruption of the expression of fibroblast growth factors by the apical ectodermal ridge can lead to congenital limb malformations. Increased cell death in the ZPA is the most pronounced result. The ZPA and AER are closely interdependent on one another by feedback loops.

Experiments[22] conducted on developing chick limbs have established an inductive role of the AER in limb formation. These studies elucidate the importance of the AER as well as non-ridge ectoderm for normal development of the limbs. An abnormality in ectoderm adversely affects the survival of the mesoderm accompanied by disturbed SHH levels.

Transplantation experiments conducted on chick limb buds of various stages highlight the regulative influence of the chick limb bud tip in response to different graft sites. The results of this study show how the AER plays a key role in proximodistal specification during limb patterning[23].

There is international research providing evidence of proliferation activity in both the mesenchyme and ectoderm of the developing limbs in human embryos (4th-8th week old) using electron microscope and immunohistochemistry. Observation of mitotic and apoptotic figures at the ultrastructural level mark the degree of activity in the AER[24].

In comparison with this study, in our laboratory settings, the mitotic spindles could be easily identified on the light microscope after staining of the AER sections with Feulgen Nuclear and Light Green stains (fig-1). Therefore, in our study, this histological feature was used as one of the parameters for assessment of the activity level in the AER.

These results clearly show that prenatal exposure to sodium phenytoin has an inhibitory effect on the development of the limbs. Although phenytoin did not have much effect on the maximum thickness and total number of cells in the AER, it had a substantial effect on the number of mitotic figures in the AER which was significantly reduced. Since the AER is an important signaling center for limb development, it is highly likely that this disturbance of mitotic activity in the AER was responsible for the limb anomalies observed in experimental chicks. So, this could be an important pathway by which the antiepileptic drug sodium phenytoin exerts its teratogenic influence on limb development.

This study has significance in Pakistan where nearly 50% of the patients prescribed with AEDs including phenytoin are females. Understanding the mechanism of drug teratogenicity could educate and also provide a foundation for prevention of fetal malformations in epileptic and non-epileptic women who are taking this drug during pregnancy[25].

On the basis of this conclusive evidence, further research could be carried out to prevent limb teratogenicity in mothers taking phenytoin by preventing its inhibitory effects on the AER.

One experimental approach could be the application of retinoic acid to the chick wing bud in order to rescue the AER from the effects of phenytoin because retinoic acid initiates the expression of sonic hedgehog which in turn is responsible for the production of 'AER maintenance factor'.

CONCLUSION

The study shows that sodium phenytoin exerts an inhibitory effect on the apical ectodermal ridges of developing limbs evident by the decreased number of mitoses in the AER of wing buds of experimental chick embryos making it a potential target site for limb teratogenicity associated with its maternal intake during pregnancy.

CONFLICT OF INTEREST

There is no conflict of interest to declare by any of the authors.

REFERENCES

1. Tomson T, Battino D. Teratogenic effects of antiepileptic drugs. The Lancet Neurol 2012; 11(9): 803-13.
2. Singh R, Kumar N, Arora S, Bhandari R, Jain A. Fetal hydantoin syndrome and its anaesthetic implications: a case report. Case Rep Anesthesiol 2012.
3. Ban L, Fleming KM, Doyle P, Smeeth L, Hubbard RB, Fiaschi L, et al. Congenital anomalies in children of mothers taking antiepileptic drugs with and without periconceptional high dose folic acid use: a population-based cohort study. PloS one 2015; 10(7): e0131130.
4. Wells PG, McCallum GP, Lam KC, Henderson JT, Ondovcik SL. Oxidative DNA damage and repair in teratogenesis and neurodevelopmental deficits. Birth Defects Res C Embryo Today 2010; 90(2): 103-9.
5. Etemad L, Moshiri M, Moallem SA. Epilepsy drugs and effects on fetal development: Potential mechanisms. J Res Med Sci 2012; 17(9): 876–81.
6. Hernandez-Diaz M, Levin M. Alteration of bioelectrically-controlled processes in the embryo: a teratogenic mechanism for anticonvulsants. Reprod Toxicol 2014; 47: 111-4.
7. Azzato EM, Chen RA, Wacholder S, Chanock SJ, Klebanoff MA, Caporaso NE. Maternal EPHX1 polymorphisms and risk of phenytoin-induced congenital malformations. Pharmacogenet Genom. 2010; 20(1): 58-63.
8. Sadler TW. Langman's Medical Embryology. 12th ed. Philadelphia: Lippencott William and Wilkins 2010; 155-57.
9. Johnson D. Pectoral girdle and upper limb. In: Standring S (editor). Gray's Anatomy: The Anatomical Basis of Clinical Practice. 40th ed. Spain: Churchill Livingstone 2008; 775-906.
10. Tickle C. How the embryo makes a limb: determination, polarity and identity. J Anat 2015; 227(4): 418-30.
11. Rodriguez-Leon J, Tomas AR, Johnson A, Kawakami Y. Recent advances in the study of limb development: the emergence and function of the apical ectodermal ridge. J Stem Cells 2013; 8(2): 79-98.
12. Cunningham TJ, Duester G. Mechanisms of retinoic acid signalling and its roles in organ and limb development. Nat Rev Mol Cell Biol 2015; 16(2): 110-23.
13. Rajkumar R, Venkatesan V, Thanigai S. Tesicular gene expression profiling of phenytoin treated albino rats using cDNA microarray. IJRPB 2013; 1(5): 748.
14. Madhale NR, Kudalkar U. Study of an epileptic drug on growing chick embryo of white leghorn chicken to observe gross malformations. JCHM 2015; 2(4): 208-11.
15. Ahangari YJ, Hashemi SR, Akhlaghi A, Atashi H, Esmaili Z, Ghorbani M, et al. Effect of in ovo injection of royal jelly on post-hatch growth performance and immune response in broiler chickens challenged with newcastle disease virus. Iran J Appl Anim Sci 2013; 3(1): 201-06.
16. Zareen N, Khan MY. A shell-less chick embryo culturing technique, reproduced successfully under local circumstances. J Coll Physicians Surg Pak 2008; 18(9): 595-6.
17. Bancroft JD, Gamble M. Ed's. Theory and practice of histological techniques. 5th ed. London: Churchill Livingstone 2002; 90, 224-5.
18. Fauci AS, Braunwald E, Kasper DL, Hauser SL, Longo DL, Jameson JL, et al. Harrison's Manual of Medicine. 17th ed. New Delhi: McGraw-Hill 2009.
19. Clarke CRA. Neurological disease. In: Kumar P, Clark M (editors). Kumar & Clark's Clinical Medicine. 7th ed. Spain: Saunders 2009; 1095-84.
20. Temiz C, Temiz P, Demirel A, Sayin M, Umur AS, Ozer FD. Effect of sodium phenytoin concentration on neural tube development in the early stages of chicken embryo development. J Clin Neurosci 2009; 16(2): 307-11.
21. Molineaux AC, Maier JA, Schecker T, Sears KE. Exogenous retinoic acid induces digit reduction in opossums (Monodelphis domestica) by disrupting cell death and proliferation, and apical ectodermal ridge ands zone of polarizing activity function. Birth Defects Res A Clin Mol Teratol 2015; 103(3): 225-34.
22. Fernandez-Teran M, Ros MA, Mariani FV. Evidence that the limb ectoderm is required for survival of the underlying mesoderm. Dev Biol 2013; 381(2): 341-52.
23. Rosello-Diez A, Torres M. Regulative patterning in limb bud transplants is induced by distalizing activity of apical ectodermal ridge signals on host limb cells. Dev Dyn 2011; 240(5): 1203-11.
24. Bečić T, Bilan K, Mardešić S, Vukojević K, Saraga-Babić M. Spatiotemporal distribution of proliferation, proapoptotic and antiapoptotic factors in the early human limb development. Acta Histochemica 2016; 118(5): 527-36.
25. Mazhar F, Shamim S, Malhi SM. Drug utilization evaluation of antiepileptics in three selected multidisciplinary teaching hospitals of Pakistan. Int J Pharmacy & Pharma Sci 2014; 6(5): 59-66.