

The Developmental Gross Morphology of Pancreas in Chick Embryo after Prenatal Administration of Valproic Acid

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ABSTRACT

Objective: To determine the effects of prenatal administration of valproic acid on the developmental gross morphology of pancreas in chick embryo.

Study Design: Experimental study.

Place and Duration of Study: Anatomy Department, Regional Centre, College of Physicians and Surgeons, Islamabad, from February 2010 to February 2011.

Methodology: An experimental group-A and control group-B, comprised of 30 eggs each. Freshly laid fertilized chicken eggs of experimental group were injected with valproic acid, incubated and hatched. Eggs of control group underwent sham treatment using normal saline. The chicks were sacrificed on hatching or day 22 of incubation, whichever was earlier. The pancreata of only alive chicks of both groups were dissected out, and evaluated for gross morphology in terms of length and weight by statistically comparing with control ones. Then pancreata were stained with aldehyde fuchsin and orange-G stain to study other obvious histological effects, if any.

Results: Chicken embryos exposed to valproic acid *in ovo*, showed significant decrease in length and weight of pancreata. The mean of length (cm) of pancreata in group-A was 2.208 ± 0.166 , and group-B was 2.300 ± 0.102 ($p=0.008$). The mean of weight (g) of pancreata in group-A was 0.032 ± 0.009 , and group-B was 0.048 ± 0.005 ($p=0.001$).

Conclusion: Valproic acid exposure showed retarding effect on the gross development of pancreas as depicted by decrease in the length and weight of pancreata.

Key Words: Valproic acid. Chick embryo. Pancreas. Embryology. Development.

INTRODUCTION

Valproic acid is a widely used antiepileptic medication since its first marketing in 1967 in France.¹ Now in addition to epilepsy, it is also used in the treatment of schizophrenia, bipolar disorders, and different forms of headache.²⁻⁴ It is also currently under experimental and clinical investigation as an anticancer medicine.⁵

Valproic acid has been shown to be teratogenic in humans and animals. Its human teratogenic effects have been reported since 1980.⁶ Defects in neural tube, heart, craniofacial features, urogenital structures and limbs of human fetus exposed to valproic acid comprise the fetal valproate syndrome.⁷ Still it is used in increasing number of pregnant patients, not just for epilepsy, but also for psychiatric diseases and migraine headaches.

The exact mechanism of valproic acid induced teratogenicity is not yet fully understood. The embryonic development is a highly coordinated set of processes that depends on the hierarchies of signaling and gene

regulatory networks, and disruption of such network may underlie many cases of chemically induced birth defects. By the experimental observations, one of the proposed mechanisms of valproic acid induced teratogenicity is marked alterations in the expression of multiple genes, many of which are involved in the transcription regulation, cell survival, ion homeostasis, and signal transduction. Some of its teratogenic effects are through inhibition of expression of HOX genes, Pax-1, Pax-2 and Pax-6 genes.⁸⁻¹⁰ Malformation of all the structures that required these genes for their development, have been noted in humans as well as in chicks after exposure to valproic acid during embryogenesis.^{10,11} Pax-6 regulates the normal development of eyes and pancreas, through its key regulating effect on pancreatic islets hormone gene transcription and is required for normal islet development in mice, chick and human.¹²⁻¹⁴ A previous study has shown that the valproic acid decreased the expression of Pax-6 in developing eyes resulted in its anomalies in chicks.¹⁰ Likewise, through its inhibitory effect on Pax-6, which also regulates the normal development of pancreatic islet cells in chick embryo; it may have effect on the development of gross morphology of pancreas.

Keeping in view the involvement of Pax-6 genes in valproic acid induced teratogenicity, this study was designed to determine the gross morphological effects of valproic acid on the developing pancreas of chick embryo.

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METHODOLOGY

This was an experimental study, carried out at the Department of Anatomy, Regional Centre of College of Physicians and Surgeons Pakistan (CPSP), Islamabad. Fertilized chicken eggs belonging to the Rhode Island Red breed of *Gallus domesticus* were obtained from Poultry Research Institute (PRI), Punjab, Rawalpindi. The eggs, which were dirty, damaged and stored for more than 3 days were excluded. Total number of 60 eggs divided into two groups, labeled as experimental group-A and the control group-B, comprised of 30 eggs each. Eggs were thoroughly cleaned with cotton swab soaked in 70% alcohol for sterilization prior to injecting drug/normal saline. Sterilized eggs were placed in the egg racks with blunt end facing above and pointed end below and left in this position for 5 - 15 minutes. This was to let the blastoderm rotate above at the blunt end which protected it from getting damaged during injecting the drug at lower pointed end. To inject the drug in the yolk sac, two holes were drilled in the egg-shell by using sterilized thumb pin. First one was just one finger above the lower pointed end for injecting drug/normal saline and second one was at the top of blunt end to allow escape of air during injection of drug otherwise the drug would not stay inside and it would come out. By using insulin syringe the eggs were injected with 0.4 mg valproic acid in 20 μ l normal saline in the yolk.¹⁰ Eggs of control group were injected with the same volume of normal saline in the same way. After injection, holes in the shell were sealed with molten wax.

The eggs were placed in the incubator (manufactured by Memmert Electric Company, Germany). The day when eggs were placed in the incubator was taken as day 0. The eggs were then incubated under standard conditions. The temperature was maintained at $38^{\circ} \pm 0.5^{\circ}\text{C}$, the relative humidity was kept between 60 - 70% and adequate ventilation was maintained. The eggs were manually rotated $\frac{1}{2}$ turn twice daily. The chicks were allowed to hatch by themselves till day 22 of hatching, afterwards the remaining were manually taken out by breaking the shell. Out of 30 eggs in each group, 23 chicks were alive in the experimental group-A and 28 in the control group-B. Only alive chicks were included in study. All the newly hatched chicks were sacrificed by exposing to chloroform in closed glass jar. To dissect out the pancreas, a ventral midline incision was given on the skin, extending from neck to cloaca to approach the abdominal cavity. The duodenal loop was found vertically placed in the ventral side of the abdominal cavity. The pancreas was removed in total along with the duodenal loop and immersed in fixative, the formol saline, for 24 - 36 hours (Figure 1a). After fixation the fixed pancreata were trimmed out of duodenal loop, blotted dry on a piece of tissue paper and measured in terms of length and weight. Length of pancreas was measured by stretching a thread along the whole length,

between the ends of two extremities of pancreas and then distance covered by the thread was measured by scale. Each pancreas was weighed on precision digital balance with 0.001 g readability.

After this, the pancreata of both groups were processed for paraffin embedding and then staining with aldehyde fuchsin and orange G stain, to study any other obvious histological effects on parenchyma and vascular development of pancreas. Statistical analysis of the whole data was done with Statistical Package for Social Sciences (SPSS) computer software program, version 10. Student's t-test was applied to detect any significant difference in means of weight and length of pancreata of chicks of both groups. P-value of equal or less than 0.05 was considered statistically significant.

RESULTS

On gross examination, after dissecting the pancreata out of the duodenal loop, the pancreata of experimental group-A were smaller and thinner as compared to control group-B (Figure 1b). The mean length (cm) and weight (g) of pancreata of group-A were less as compared to mean length (cm) and weight (g) of pancreata of group-B respectively. When these parameters were statistically compared, the p-values showed significant differences (Table I). The mean values of these variables of gross morphology of pancreas (length and weight) are also shown in Table I.

Histological sections were observed to study any other additional obvious histological effects on the parenchyma and the vascular development of pancreas, which may be responsible for the retarded growth of the pancreas. It was noticed that the pancreatic arteries of affected pancreata of experimental group-A showed under-developed tunica media and the laminae of elastic fibers (Figure 2a) as compared to group-B (Figure 2b).

Table I: Parameters of gross comparison of pancreas of newly hatched valproic acid exposed group-A and control group-B.

Parameter	Group				p-value
	A		B		
	N	Mean \pm SD	N	Mean \pm SD	
Length (cm)	23	2.208 \pm 0.166	28	2.300 \pm 0.102	0.008*
Weight (g)	23	0.032 \pm 0.009	28	0.048 \pm 0.005	0.001*

N= Total number of alive chicks, SD = Standard deviation of the mean, * = Significant.

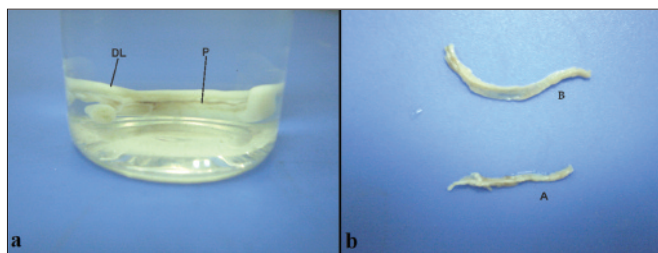


Figure 1: (a) Pancreas (P) dissected out in total along with the Duodenal Loop (DL) for fixation. (b) Gross comparison of pancreas. The pancreas of experimental group-A conspicuously smaller and thinner as compared to control group-B.

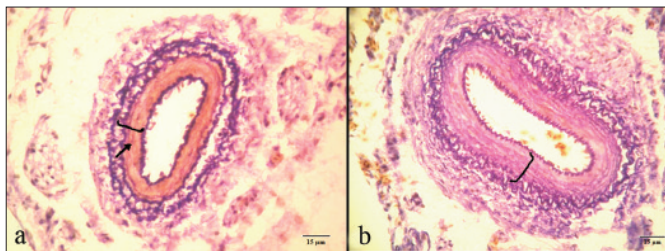


Figure 2: Comparison of the vessels. (a) Cross-section of an artery of pancreas of chick of the experimental group-A showing thinner tunica media containing few elastic laminae (arrow). (b) Cross-section of an artery of pancreas of chick of control group-B showing well-developed tunica media containing several elastic laminae. Aldehyde fuchsin and Orange G-light green. Photomicrograph. Bar, 15µm.

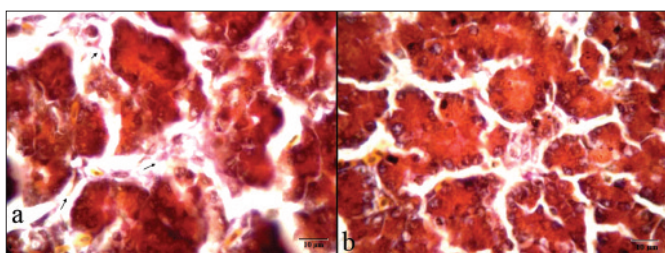


Figure 3: Comparison of the parenchyma: (a) Transverse section of the exocrine pancreas of the chick of experimental group-A showing increase in the amount of connective tissue (arrows) among acini. (b) The parenchyma of pancreas of control group-B showing almost no connective tissue among acini. Aldehyde fuchsin and Orange G-light green. Photomicrograph. Bar, 10 µm.

It was also noticed that the parenchymal mass of affected pancreata showed collagen fibers and fibroblasts among the acini (Figure 3a), depicting the replacement of glandular tissue by fibrous tissue. In control group, the lobes were invested externally by a thin loose collagen capsule. Almost no connective tissue was found within the parenchymal mass of pancreas (Figure 3b).

DISCUSSION

Valproic acid readily crosses the placental barrier to the fetus.¹⁵ It is known that its use in the first trimester is associated with significantly increased risks of several congenital malformations, as compared with no use of antiepileptic agents or with use of other antiepileptic drugs.¹⁶

In the present study, the prenatal administration of valproic acid retarded the growth of pancreas. Previous studies have shown that valproic acid has inhibitory effect on the expression of Pax-6 genes.¹⁰ Pax-6 genes are essential for the normal development of pancreas through its regulatory influence on the development of pancreatic islets.¹²⁻¹⁴ The retarded growth of pancreas in the present study might be due to inhibitory effect of valproic acid on the expression of Pax-6 genes.

The pancreata of the experimental group-A were smaller and less in weight as compared to the control group-B. This is in accordance with the previous study, which has shown that the livers of embryos from VPA-treated mice

were markedly smaller than those of the control embryos. The underlying mechanism was poor vascularity of liver. VPA inhibits angiogenesis by decreasing endothelial Nitric-oxide Synthase (eNOS) expression preceded by Histone Deacetylases (HDAC) inhibition.¹⁷ Another study showed that HDAC inhibition, inhibits angiogenesis partly through inhibition of proliferation and migration of pericyte, which are of key importance in the neoformation of blood vessels, in stabilization of newly formed vessels as well as maintenance of angiostasis in resting tissues.¹⁸ In this study, it was also noticed that the arteries of some of the affected pancreata of experimental group were not as well developed as those of control group which was evident by decrease in the thickness of tunica media and poor development of elastic lamina. So the probable mechanism of under development of pancreas might be poor vascularity.

Previous studies in VPA exposed human fetuses have shown the increased incidence of cardiovascular anomalies.^{11,19} In the experimental study on human umbilical vein endothelial cells, therapeutically relevant concentrations of VPA inhibited proliferation, migration, and tube formation. This was also confirmed in chicken where VPA inhibited angiogenesis *in vivo* in the chorioallantoic membrane. Similarly, embryos from VPA-treated mice showed disturbed vessel formation.¹⁷

It is indicated by all these results that therapeutic plasma levels of VPA inhibit angiogenesis. This poor vascularization might be responsible for distorted structure of pancreatic parenchyma as depicted by the slight increase in the connective tissue within the parenchymal mass of pancreas.

CONCLUSION

Valproic acid exposure showed retarding effect on the gross morphological development of pancreas as depicted by decrease in the length and weight of pancreata. It also showed the inhibitory effects on the development of pancreatic vessels.

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