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ORIGINAL ARTICLE

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Histo Morphological effects of Valproic Acid on the Development of Pancreatic Islets in Chick

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ABSTRACT

OBJECTIVE: To determine the developmental histo-morphological effects of valproic acid on pancreatic islets by comparing the pancreatic islets of freshly hatched chicks with the pancreatic islets of chicks not receiving this medication intervention.

METHODOLOGY: It was an Experimental study. At department of anatomy CPSP, Regional Centre, Islamabad, extending from February till November 2010. Two groups were made with thirty fertilized chicken eggs each, Group-A (experimental) and Group-B (control). Prior to incubation, Group-A's eggs were injected with valproic acid. Group-B eggs received a sham treatment using regular saline in same volume. After hatching, pancreata were removed and stained with Eosin and hematoxylin, orange G-light green and aldehyde fuchsin stain. (The number of islets and the cells that make them, as well as the area of islets lobe-wise in the pancreatic splenic and third lobes were measured in each section). In the splenic and third lobes of each pancreas, the number of islets along with the cells and area of each islet were measured.

Data were statistically analyzed and compared between experimental and control groups.

RESULTS: The histo-morphological findings demonstrated that valproic acid inhibited the Pancreatic islets development in completely hatched chicks, resulting in a reduction in the quantity and area of the islets as well as in the number of component cells.

CONCLUSION: The formation of pancreatic islets and the cells that make them up is intensely suppressed by VPA. It causes hypotrophy and islet hypoplasia in newborns.

Key words: Pancreatic, valproic acid, chick embryo.

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INTRODUCTION

Entire genetic information, needed to build an individual, is included in the genomes, which controls embryonic development. Exposure of pregnant women to environmental chemicals may cause disruption of these genomes that result in chemically induced birth abnormalities. Preventing birth defects can be achieved by avoiding the use of certain medications and environmental toxins during pregnancy, if we determine which ones are capable of causing birth problems.

In 1967, 2-n-propylpentanoic acid (Valproic acid-VPA) was first made available for sale as an antiepileptic medication (AED) in France. Since then, it has gained recognition as one among the AEDs that is most frequently recommended across all age groups.[1] Presently it's also advised for schizophrenia [2], bipolar illnesses [3], and migraines.[4] Moreover, it is now being studied in clinical and experimental settings as a potent anticancer medication.[5]

VPA has been recognized as a well-known teratogenic agent in humans since 1980.[6] Fetus in utero exposed to valproic acid may have abnormalities in the urogenital system, heart, neural tube (NTDs), limbs, and craniofacial traits. Together, these make

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up the "Fetal Valproate Syndrome".[7]

A study of the literature on the teratogenic effects of VPA from 1978 to 2000 by Kozma[8] revealed that half of the human fetuses exposed to valproic acid had a variety of phenotypic abnormalities. Musculoskeletal, cutaneous, cardiovascular, genital, pulmonary, renal, and central nervous systems (including the neural tube, eyes, and ears) are a few of them. Growth retardations and developmental delays were also discovered.

Prior research has demonstrated that the teratogenicity of VPA is dosage dependent, with doses ranging from 600 mg/d to over 1000 mg/d.[9] Moreover VPA enters the fetus easily through the placental barrier.[10]Despite all of this information, the number of pregnant patients with epilepsy, mental illnesses, and migraine headaches using valproic acid is continually rising.[3]

The exact mechanism by which VPA causes teratogenicity is still unknown. Nevertheless, many experimental findings suggested various pathways. The alterations in different pathways include the following: prevention of shift of WNT signaling and histone deacytylase (HDAC) in human and animal cells[11], changed expression of particular HOX genes[12] and Pax-113, Pax-6 and Pax-2 genes[14], Maternal plasma zinc (Zn) levels reduction due to drug-induced confiscation of Zinc into mother's liver, causing Zn deficiency in embryo[15], Intracellular pH-alteration16, interaction with embryonic zinc metabolism[17], Maternal blood folate levels decline with rising plasma AED levels[18], interfering with embryonic folate metabolism[19] and prevention of liver microsomal epoxide hydrolase[20] resulting in higher exposure of fetus to these substances.

Whitsel et al.[14] observed that those chick embryos which were exposed to valproic acid in ovo resulted in growth delay and higher mortality. Anomalies of the neural tube, circulatory system, craniofacial region, muscles & bones were also noted; these were comparable to those previously found in humans. In addition to reduced qualitative expression of Pax-6 and Pax-2 protein (paired homeodomain transcription factor) in affected eyes, various eye anomalies including microphthalmia, pigment anomalies, aniridia, incomplete choroidal fissure closure, cataract and displaced lens were also discovered during the investigation.

The PAX 2 gene controls how the kidneys, ears, and eyes develop normally.[21] A study of the literature revealed that valproic acid affects all of these structures' proper development in both chicks & human. [8,14]

Similarly, valproic acid caused abnormalities in chicks' eyes by reducing the expression of Pax-6 proteins in developing eyes.[14] Through the transcription of pancreatic islets hormone genes 22–24, the PAX 6 gene also plays a crucial role in normal islet development in mice, chicks, and humans. As Valproic acid inhibits the effects of Pax-6 genes, we can predict that valproic acid may influence the development of pancreatic islet cells in chick embryos. Past studies have revealed Valproic acid teratogenic effects on the development of number of organs which required PAX 2 & 6 genes but there are no studies which addresses its developmental effects on pancreas.

An analysis of the literature revealed both main and slight abnormalities in children born to epileptic mothers taking valproic acid. Furthermore, hypoglycemia was also documented in individuals whose blood glucose levels were assessed.[25] This could suggest that exposure to valproic acid may have impact on the pancreatic islets.

Yasuda et al revealed shared genetic component mutation of PAX6 in aniridia and glucose intolerance in humans. 26 In the light of these findings, the goal of the current investigation was to determine VPA effects on the embryonic histomorphology of pancreatic islets. According to Bacha and Bacha[27], the pancreas of a chick is comparable to that of a mammal. If valproic acid has any teratogenic effects, these may also apply to human.

METHODOLOGY

It was an Experimental study. At department of anatomy CPSP, Regional Centre, Islamabad, extending from February till November 2010. Sixty fertilized chicken eggs of the Gallus domesticus "Rhode Island Red" breed were taken from the Poultry Research Institute (PRI), Punjab, Rawalpindi, for this experimental study. Inclusion criteria included eggs should be freshly laid belonging to “Rhode Island Red” breed of Gallus domesticus obtained from PRI. Cracked and eggs stored for more than 03 days excluded.

The eggs were grouped, A and B groups, each with 30 eggs. Group A received an injection of 0.4 mg of valproic acid in 20µl of normal saline directly into the yolk, in accordance with the dosage recommended by Whitsel et al.[14] Group B received a 20µl injection of normal saline into the yolk as a control. Then eggs were put for incubation into the incubator. That day was taken as day 0.

The eggs were incubated at 38 ± 0.5o C and 60-70% relative humidity, respectively. The chicks hatched on the day 22. Out of 30 eggs in each group, 23 chicks hatched in Group A and, 28 chicks in Group B hatched. Both groups' newly hatched chicks were sacrificed, and the pancreas and duodenum were removed and preserved in formol saline. Following processing, serial transverse slices of the pancreas were cut at a right angle to its long axis for histology, with a thickness of 7µm. Hematoxylin and eosin (H&E) were used to stain serial sections for general histology under a light microscope. Orange G-light green and

Groups	No of islets (Mean ± SE)				Area of islet (µm)2 (Mean ± SE)			
	α	β	mixed	Total	α	β	mixed	Total
Group A	294 ± 9.9	282.8 ± 7.2	45.5 ± 1.6	662.34 ± 17.44	2054665 ± 233648.8	2054665 ± 233648.8	85780.63 ± 9007.11	662.34 ± 17.44
Group B	321.6 ± 2.07	300.5 ± 1.9	43.25 ± 0.27	665.3 ± 4.2	2820331 ± 18176.15	2820331 ± 18176.15	115360.4 ± 743.4	3315558 ± 21367.73
P value	0.004*	0.013*	0.15	0.01*	0.001*	0.001*	0.001*	0.001*

SE = Standard error of the mean * = significant

Table 1: Histological comparison of different types of pancreatic islets in splenic and third lobes among Group (A) and (B) with statistical comparison

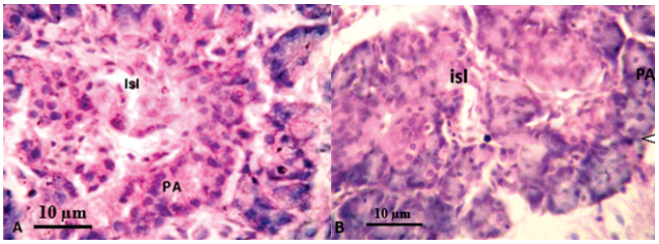

Figure 1: Cross section of splenic lobe of the pancreas showing an islet of Langerhans (isl) encircled by pancreatic acinar (PA) cells in the experimental group (A) and control group (B). Eosin and hematoxylin stain

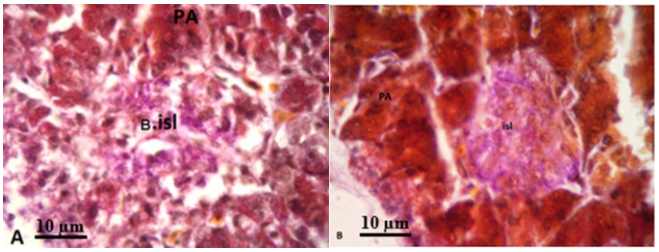

Figure 2: Cross section of splenic lobe: comparing the beta islet (isl) which is encircled by pancreatic acinar cells (PA), in the experimental group (A) and control group (B). Observe how it is malformed in A. Aldehyde fuchsin and Orange G-light green stain

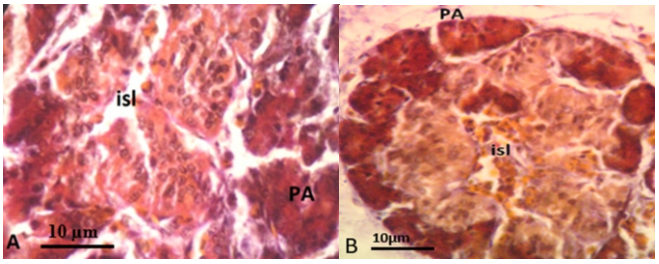

Figure 3: Cross section of the splenic lobe showing centrally populated alpha islet (isl), which is encircled by pancreatic acinar cells (PA). On comparison it can be observed that Experimental Group (A) did not have well developed islet as in B. Aldehyde fuchsin and Orange G-light green stain

aldehyde fuchsin were used in special staining to show the different characteristics of alpha, beta, and delta cells.[28]

For collection of histomorphological data light microscope was used. Using oil immersion lens at 100X the identification and counting of different islets was done in every seventh serial section of splenic and third lobes of pancreas. In addition, different constituent cells including A, B undifferentiated and total cells were counted in different islets. In the end all of these parameters found in splenic and third lobe of pancreas were added to assess their number in each mentioned lobes.

The area of every alpha, beta, and mixed islets was calculated in every seventh section by using "Image J". Then area of each type of islet was added in these sections' lobe-wise to determine the total area of mixed, α, ß and total islets in splenic and third lobes.

Version 10 of the statistical package for social sciences (SPSS) was utilized to do a comprehensive data analysis. The means of all of the above-mentioned parameters in experimental and control groups were compared using Student's t test. The means ± SE were used to express the data. When a p-value was less than 0.05, it was deemed statistically significant.

RESULTS

In the control group B, it was found that large alpha islets filled the whole center region of the splenic lobe. These islets persisted in the third lobe and were encircled by exocrine acini in both lobes. The beta islets were tiny, and there were sporadically mixed islets on the edge. One noteworthy observation was the higher proportion of A cells compared to B cells.

In experimental Group A, the pancreatic histological features and distribution pattern of islets in the splenic and third lobes were similar to those of the control. However, the number of α and β islets per region was reduced (Table 1). Furthermore, the area occupied by the islets in the examined sections, as well as their extension into subsequent serial sections, was comparatively smaller (Table 1).

The observed islets were significantly smaller in size (Fig. 1-3). Histological examination of pancreatic sections revealed distinct differences between control and valproic acid–treated groups. In Group A (control), α, β, and mixed islets were well defined, with uniform cellular arrangement and clear boundaries (Figure 1A). In contrast, Group B (valproic acid–treated) showed a reduction in the number of α and β islets, irregular distribution patterns, and decreased islet area (Figure 1B). The cellular architecture appeared disrupted, with

Groups	α islets (Mean ± SE)			
	A	B	unidentified	Total
Group A	27892.48 ± 2792.73	215.95 ± 26.56	1107.95 ± 109.75	29218.91 ± 2929.37
Group B	37074.61 ± 238.9	302.750 ± 1.9	1468.821 ± 9.4	38850.14 ± 250.3
P value	0.001*	0.001*	0.001*	0.001*
Groups	ß islets (Mean ± SE)			
	A	B	unidentified	Total
Group A	35.47 ± 4.58	7421.60 ± 631.34	236.78 ± 26.18	7693.95 ± 661.9
Group B	50.42 ± 0.32	9503.3 ± 61.2	322.642 ± 2.0	9876.5 ± 63.6
P value	0.001*	0.001*	0.001*	0.001*
Groups	Mixed islet (Mean ± SE)			
	A	B	unidentified	Total
Group A	688.60 ± 39.82	734.13 ± 34.62	52.39 ± 4.05	1475.73 ± 78.37
Group B	818.9 ± 5.2	844.8 ± 5.4	65.71 ± 0.41	1730.3 ± 11.16
P value	0.001*	0.001*	0.001*	0.001*

TABLE-2: Histological comparison of cells present in different types of pancreatic islets of splenic and third lobes. Group (A) versus Group (B)

	Number of A cells (Mean ± SE)	Number of B cells (Mean ± SE)
Group A (N=23)	28616.48 ± 2836.26	8371.73 ± 691.04
Group B (N=28)	37943.96 ± 244.54	10651.18 ± 68.6
P value	0.001*	0.001*

TABLE-3: Histological comparison of Total A & B types of cells-number in splenic and third lobes of pancreatic islets. Group (A) versus Group (B) newly hatched

less compact islet organization compared to controls. These findings are consistent with the quantitative data presented in Table 1.

Additionally, it was noted that the formed islets and exocrine pancreatic acini had altered structures, and there had been a decrease in the number of islet constituent cells (Tables 2 and 3). Additionally, a minor increase in connective tissue was observed within the pancreatic parenchymal mass.

DISCUSSION

The purpose of this study was to evaluate the histomorphological effects of valproic acid (VPA) on the development of chick embryonic pancreatic islets, with a focus on the A and B cells.

The development of alpha, beta, and mixed islets was examined in this study, and the findings revealed hypoplastic and hypotrophic alterations that led to a reduction in the areas of all three islet types as well as a drop in the number of alpha and beta islets. Regarding mean of total number of mixed islets overall, there was no discernible difference between the experimental and control groups. However these islets were extremely small in size in treated group. The cause of the negligible difference in the number of mixed islets might be their fewer occurrences in normal pancreas. Furthermore, it was also observed that developed island were deformed. The outcomes and dysmorphogenetic pattern matched those found in homozygous mutant mice for Pax-6, the research conducted by Dames and colleagues.29 So, it can be assumed that mutation of Pax6 genes may be responsible for these retarding changes in the normal the development of islets.

Pax6 has been linked to the formation of pancreatic islets in several studies. The pups, that Ashery-Padan et al. generated to have a Pax6 mutation, died a few days after birth due to a lack of insulin-producing cells and an overt diabetic phenotype (hypoinsulinemia, hyperglycemia, weight loss, and ketosis).30 In another study by St-Onge et al. they also revealed that the pancreas of Pax6 homozygous mutant mice lacks glucagon-producing cells.31These findings imply that Pax6 plays a crucial role in controlling the specification and upkeep of the distinct lineages found in the pancreatic islet. Whitsel et al. have demonstrated that exposure to VPA significantly reduced Pax-6 expression in chick embryos. The incidence of ocular abnormalities was higher in the treated embryos.15

Additionally, it is known that Pax-6 plays a role in the development of the pancreatic islets, ears, eyes, and several other tissues. When these two results are combined, it can be said that Pax-6 is necessary for the correct growth of islets and that VPA inhibits its expression. It can be assumed that interruption of Pax6 genes may have affected the formation of pancreatic islets in our investigation.

Clinical research has now revealed a number of examples where the relationship between various structural malformations caused by the pax6 mutation has been highlighted. A trisomy 21 patient with microophthalmia and hypopituitarism, as well as neonatal diabetes mellitus, was recently reported by Solomon et al. It was discovered that he received a distinct PAX6 mutation from each parent.32

CONCLUSION

Therefore, the findings from the aforementioned research can be used to support the inhibitory effects of VPA on the formation of pancreatic islets.

Limitations of the study: This study shows that VPA may have an inherent ability to deregulate the expression of genes that are known to have critical functions in islets formation. Further genetic studies are needed to detect which of the several genes may be primary VPA targets; moreover, effects on pancreatic islets of human embryo are yet to be determined

Recommendations: In this study the effect of valproic acid on the development of pancreatic islets and its constituent cells was evaluated quantitatively. The hormonal assay of glucagon and insulin can be done to assess the effects on the functions of A and B cells of islets respectively. This will provide important supplementary information.

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CONFLICT OF INTEREST

Author declared no conflict of interest

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AUTHORS CONTRIBUTIONS

LA: Conception, Design of the work, Data collection on, and Drawing, Reviewed, Final approval, Agreement to be accountable

NR: Conception, Design of the work, Acquistion on, Data Analysis, and Drawing, Reviewed, Final approval, Agreement to be accountable.

SMAH: Conception, Design of the work, Interpretation on of data for the work, and Drawing, Reviewed, Final approval, Agreement to be accountable.

SZH: Conception, Design of the work, Data collection on, and Drawing, Reviewed, Final approval, Agreement to be accountable .

NJ: Conception, Design of the work, Data analysis, and Drawing, Reviewed, Final approval, Agreement to be accountable

RSM: Conception, Design of the work, Data analysis, Drafting and Drawing, Reviewed, Final approval, Agreement to be accountable

DATA SHARING POLICY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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