FREQUENCY OF DIFFERENT CONGENITAL ANOMALIES IN PRENATALLY VALPROIC ACID TREATED CHICK EMBRYOS

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ABSTRACT

Objective: To determine the frequency of different congenital anomalies in surviving chick embryo on hatching after the prenatal administration of valproic acid by comparing with age-matched controls.

Study Design: Experimental study.

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

Material and Methods: Thirty fertilized chicken eggs were injected with valproic acid, incubated and then evaluated for different gross congenital anomalies, on hatching or day 22 of incubation whichever was earlier. Chicks of this group were labeled as experimental group-A. Similarly, another group of thirty fertilized chicken eggs labeled as control group-B, underwent sham treatment using normal saline. The weight and length of alive chicks, the total number of chicks with gross anomalies and the number of different types of gross anomalies in both groups were noted and statistically compared.

Results: In control group-B, 28 chicks hatch out on 21 day of hatching with no visible gross deformities. Whereas in experimental group-A, 23 chicks were alive, out of which, 9 chicks were with delayed hatching on 22 days of hatching. The chicks with gross deformities were 8 (p=0.0008) which included: limb abnormalities (i.e. inverted feet) in 6 chicks (p=0.006), eye abnormality (i.e. closed palpebral fissure of both eyes) in 2 chick (p=0.2), 1 chick showed multiple deformities including gastroschisis, closed palpebral fissures and inverted foot (p=0.45). There were behavioral changes in 10 chicks (p=0.0001). There was statistically significant difference in their weights (p=0.03).

Conclusion: Prenatal exposure of chick embryos to valproic acid increased the incidence of different gross deformities.

Keywords: Chick embryo, Gross deformities, Valproic acid.

INTRODUCTION

Epilepsy is a common neurological disorder and has a prevalence of 5.25 per1000. About one third of epileptic patients are women of reproductive age[1]. Most of the epileptic women need to continue taking medication during pregnancy, since uncontrolled seizures may be harmful to the women as well as to the fetuses. However, medication may still be an issue as some drugs used to treat seizures may contribute to birth defects.

Valproic acid (VPA) is considered to be a drug of first choice for the treatment of generalized and focal epilepsies. It is one of the most frequently-prescribed antiepileptic drugs worldwide due to its broad-spectrum and good tolerability[2,3]. Both the usage and the therapeutic indications of VPA are increasing. Now in addition to epilepsy, it is also employed in the treatment of different pathologic conditions including schizophrenia, bipolar disorders, different forms of headache, as an anticancer drug, retinitis pigmentosa (RP), autoimmune encephalomyelitis and HIV (human immunodeficiency virus) infection[4-10].

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Its human teratogenic effects have been reported since 1980[11]. In 1984, DiLiberti suggested the term 'Fetal Valproate Syndrome' (FVS) for major and minor anomalies caused by teratogenic effects of VPA. These include defects in neural tube (NTDs), heart, craniofacial features, urogenital structures and limbs[12].

Previous studies have shown that the VPA readily crosses the placental barrier to the fetus[13]. Different studies have shown that the risk of major congenital malformations is two to four times as high with the use of valproate as with the use of other antiepileptic drugs, absolute rates of congenital malformations among offspring exposed to valproate in utero have range from 6 to 11%[14].

Many animal studies have been carried out, on the different individual organs in developing embryo, in order to mimic the effects of VPA on human embryo. No trials are available regarding the pattern of incidence of different anomalies in single study and it is still remains to be learned. With this background, this study was undertaken to determine the teratogenic effects of valproic acid in terms of different gross congenital anomalies in chick embryo

Table-1: Comparison of chicks: with gross abnormalities of experimental group-A and control group-B using Fisher exact test.

Number of chicks		Group		Total	*p*-value of difference between A & B
		A	B		
Alive chicks		23	28	51	
Gross deformities	Present	8	0	8	*p*=0.0008*
	Absent	15	28	43	
Limb deformities	Present	6	0	6	*p*= 0.006*
	Absent	17	28	45	
Behavioral changes	Present	10	0	10	*p*<0.0001*
	Absent	13	28	41	
Eye deformities	Present	2	0	2	*p*=0.2
	Absent	21	28	49	
Multiple deformities	Present	1	0	1	*p*=0.45
	Absent	22	28	50	

* = significant

Table-2: Gross comparison of the newly hatched chicks of valproic acid exposed group- A and control group-B using student's t test.

Parameter	Group				*p*-value
	A		B		
	N	Mean ± SEM	N	Mean ± SEM	
Length (cm)	23	8.68 ± 0.044	28	8.77 ± 0.038	0.12
Weight (g)	23	38.69 ± 0.098	28	38.92 ± 0.049	0.03*

SEM = Standard error of the mean

* = significant

MATERIAL AND METHODS

An experimental study was carried out at the Department of Anatomy, Islamabad, Regional Centre of College of Physicians & Surgeons Pakistan (CPSP) between February 2010 and February 2011. Freshly laid fertilized chicken eggs, belonging to "Rhode Island Red" breed of Gallus domesticus were collected from Poultry Research Institute (PRI), Punjab, Murree Road Rawalpindi. The eggs which were damaged, dirty and stored for more than 03 days were excluded from study. The eggs were randomly

selected by lottery method. For this purpose the eggs were numbered starting from 1 to the total count of the eggs. Then numbers were put on the pieces of paper, placed in a container and thoroughly mixed. Sixty numbers were selected without looking. Randomly selected eggs were divided into two groups, labelled as experimental group-A and control group-B. Each group comprised of 30 eggs. To limit bacterial contamination, the eggs were swabbed with 70% alcohol rapidly and gently, and then given labels. Then eggs were placed in the racks with their blunt ends above and pointed ends below, and left in this position for 5 to 15 minutes to let the blastoderm to rotate and come to lie above at the blunt end. This was done to protect it from getting damaged during injecting the drug at the lower end. A sterilized thumb pin was used to drill two holes in the egg shell to inject the drug in the yolk sac. First hole was just one finger breath above the lower pointed end for the injection and second one was at the top of the blunt end to allow escape of air during injecting drug otherwise the drug would not stay inside, it would come out. The eggs were injected with 0.4 mg VPA in 20µl normal saline in the yolk, following the dose adopted by Whitsel et al[15]. Eggs of control group were injected with the same volume of normal saline in the same way. Holes in the shells were then sealed with melted wax and the eggs were placed in the incubator (Beschickung-loading model 100-800). 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 ± 0.5° C. The relative humidity was kept between 60-70%. The eggs were manually rotated ½ turn twice daily. Candling of eggs was done after every 3 or 4 days to keep track of progress of egg incubation. The chicks were allowed to hatch by themselves till day 22 of incubation, afterwards the remaining were manually taken out by breaking the shell.

The day of hatching and the number of alive and dead chicks was recorded. The newly hatched alive chicks were observed for abnormal posture, gait, behavior, gross abnormalities and their length and weight were taken. The data was analyzed by using Statistical Package for Social Sciences (SPSS) computer software program, version 10. Student's t test was applied to detect any significant difference in means ± SE of gross weight and length of chicks of both groups. Fisher exact test was used to detect any significant difference in total number of chicks with gross anomalies and the number of different types of gross anomalies in both groups. A *p*-value of ≤0.05 was considered statistically significant. The relative frequency of different malformation within the experimental group-A, was measured by taking their percentages

Figure-1: A chick of experimental group (A), which was hatched by manual assistance, showed multiple deformities; inverted foot (1), closed palpebral fissures (2) and gastroschisis (3).

RESULTS

All the hatched chicks of the control group were normal. Chick embryos exposed to VPA *in ovo* showed: increased mortality, increased incidence of gross deformities and delayed hatching. There were number of congenital malformations observed in experimental group, which included limb abnormalities (i.e. inverted feet) and eye abnormality (i.e. closed palpebral fissure of both eyes) as indicated in table-1. One chick showed multiple deformities including gastroschisis, closed palpebral fissures and inverted foot (fig-1). This chick was hatched out by manual assistance out of 23 alive chicks. The probable need for assisted hatching appeared to be decreased mobility due to multiple

deformities. In the control group, all the 28 alive chicks hatched by themselves.

Regarding the behavior, Out of 23 alive chicks of the experimental group, 10 chicks showed behavioral changes. Some of them were listless, slow and sluggish in behavior, could not stand erect, showed diminished mobility and did not follow the voices of other chicks when taken away from them (fig-2a). The chicks of control group were active, tried to remain together in group and followed the voices of other chicks when taken away from them (fig-2b).

On gross examination many of the chicks of experimental group A were weak. When means of the weights of newly hatched chicks of two groups were compared, there was significant increase in low birth weights as indicated by *p*-value of 0.03. There was no statistically significant difference between the means of the lengths of chicks of two groups as indicated by *p*-value of 0.12 (table-2).

Fig-1 a chick of experimental group (A), which was hatched by manual assistance, showed multiple deformities; inverted foot (1), closed palpebral fissures (2) and gastroschisis (3).

There was significant increase in the number of congenital malformations in the experimental group as indicated by *p*-value of 0.0008 (table-1). The comparison of percentages of chicks with gross abnormalities, within the experimental group-A, and with the control group- B has been shown in fig-3

DISCUSSION

Embryonic development is regulated by the hierarchies of signaling and gene regulatory networks. During pregnancy, maternal exposure to exogenous agents induces disruption of such networks resulting in chemically induced birth defects.

VPA is a known teratogen. It has now become evident from systematic review of all cohort studies that use of VPA monotherapy in the first trimester resulted in significantly higher rates of major congenital malformations, as compared with no use of antiepileptic drugs or with use of other antiepileptic drugs[16]. This is in accordance with our study. In the present study, there were number of congenital malformations observed in the experimental group. Previous studies have shown that exposure to VPA during embryogenesis may result in the multiple birth defects. The most commonly reported anomalies in VPA exposed embryos, in both human and animals are musculoskeletal deformities. In human patients exposed to VPA in utero, 63% had musculoskeletal deformities[17]. This is in accordance with findings in our study. The most commonly observed deformities were limb deformities. In the previous studies it has been shown that the mechanism underlying limb

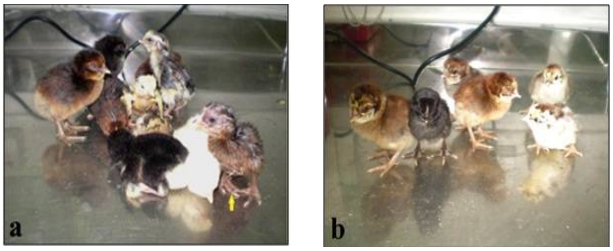

Figure-2: (a): Chicks of experimental group (A). Listless, slow and sluggish in behavior, could not stand erect, showed diminished mobility and limb deformities (yellow arrow). (b): Chicks of control group (B). Active, could stand erect and no visible gross deformities.

deformities was disruption of genes that regulate pattern formation of somities in embryo. Barnes et al have showed that the VPA induced somite teratogenesis by inhibiting the expression of *Pax-1* genes[18].

The limb abnormalities after VPA exposure may include pre- and postaxial polydactyly, overlapping digits, talipes (clubfoot), clinodactyly, arachnodactyly, hip dislocation, limb deficiencies, preaxial and postaxial polydactyly, reduction malformations of the arms and hands and radial ray defects[19,20] . Contractures of the small joints of the hands are also common musculoskeletal manifestation in children with VPA exposure[17].

Likewise eye abnormalities have also been observed previously, in chicks exposed to VPA. The underlying mechanism was found to be disruption of genes; *Pax-2* and *Pax-6* genes[15]. Abnormal ophthalmic findings are common in children with confirmed FVS syndrome, which include myopia, strabismus, astigmatism, anisometropia, epicanthus, color vision deficiency and bilateral congenital cataract[21].

The chicks of experimental group showed behavioral changes and delayed hatching. The result of our study corresponds with the previous observations made by Moore et al who noticed that the anticonvulsants, especially VPA taken during pregnancy were associated with an increased risk of developmental delay and different behavioral problems in the children. The reported behavioral problems included autistic features or autism, Asperger's syndrome, learning difficulties, speech delay, gross and fine motor delay[22]. Different studies identified valproate as a drug carrying potential risks for developmental delay, growth restrictions and cognitive impairment[23]. The experimental work on the pregnant rats has shown that disruption of the early embryonic serotonergic neuronal development might be involved in the etiology of these behavioral changes[24]. Another possible cause might be altered folate metabolism because it has been implicated in the metabolism of neurotransmitter molecules[25]. In 2015, Baker et al reported that in utero exposure to valproate as compared with other antiepileptic agents was associated with a lower IQ in children[26].

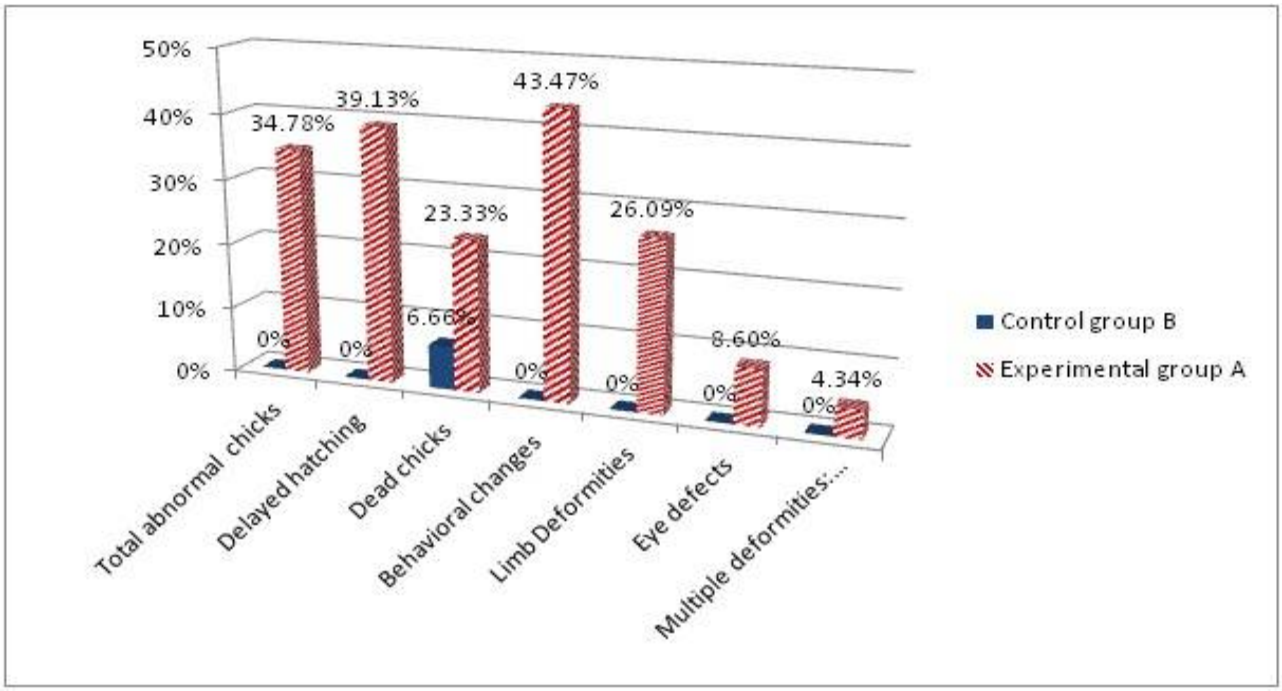

Figure-3: Comparison of percentages of chicks with abnormalities of experimental group (A) and control group (B).

The chicks of experimental group (A) were weak as compared to control group (B). This was in accordance with the previous human study in which 10% of babies are small for gestational age after VPA exposure during pregnancy[27]. Another study showed that exposure of Sprague-Dawley rats to VPA during pregnancy resulted in intra uterine growth retardation of pups[28].

CONCLUSION

Prenatal exposure of chick embryos to VPA increased the incidence of different gross deformities. The most commonly observed deformity was limb deformity.

CONFLICT OF INTEREST

This study has no conflict of interest to declare by any author.

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