Original Research Article

Acetazolamide induced Skeletal Anomalies in Wistar Rat Fetuses

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Abstract

Aim: To induce skeletal anomalies in wistar rat fetuses by acetazolamide, using double stain technique for fetal rat skeleton with Alizarin Red S and Alcian blue. **Method:**Adult pregnant rats were randomly divided into two groups. Control group received 0.25ml of normal saline and treatment group received 80 &160 mg/kg/b. wt. of acetazolamide on 8th , 9th and 10th day of pregnancy respectively. Pregnancy was terminated on 16th , 18th , 20th day. All fetuses' skeletal deformities were studied by Double staining technique for cartilage and bone. **Results:** severity of defects were linearly related to drug dosage. Most of the defects were observed on right forepaw, as dosage increased the weight of mothers, number and weight of fetuses was reduced. **Conclusion**: increase in dosage of acetazolamide induces limb abnormalities mostly right forepaw.

Keywords: acetazolamide, skeletal deformities, Wistar rats

Introduction

Congenital anomalies occur in skeletal or visceral organs in intrauterine life1 and were classified them according to causes as environmental, genetic and idiopathic[1,2].The causes for abnormal development are explained by teratological studies[3].

Acetazolamide is a carbonic anhydrase inhibitor which is used to treat idiopathic intracranial hypertension in pregnancy, glaucoma and some forms of epilepsy in humans4. In experimental animals the use of acetazolamide showed some teratogenic abnormalities like ectrodactyly, syndactyly, reduction in skeletal ossification, axial skeletal malformation, exencephaly, anophthalmia, microphthalmia, cleft lip and retarded incisor teeth development[5-10].The Embryos are affected by teratogens mostly during the process of organogenesis. In wistar rats this period extends from 6th -10th days of pregnancy. During this period teratogenic agents can lead to significant congenital abnormalities. The peculiar feature of Acetazolamide is lateralization of teratogenic lesion in forelimb with predominant right sidedness. In the present study staining of fetal skeleton is done to know the skeletal abnormalities due to acetazolamide. This technique is useful to observe rapid and detailed observation of bones, to detect ontogeny, evolution, comparative anatomy and in phylogenetic studies[11-14].

Material and methods

Drug: Acetazolamide pre weighed 250mg available from commercial sources.

Animals: Nine adult wistar female rats weighing about 170–250 gm were obtained from animal house Jeeva labs, Hyderabad. Three female rats were mated with one male overnight. The female rats were examined for sperms in the vaginal smear next morning, and sperm positive ones were considered as 0.5 day pregnant. The pregnant rats weredivided into two groups: (control and treatment group). The rats were kept under hygienic conditions, fed libitum and all had free access to water. This study is carried out in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals and protocols are approved by animal ethical committee.

Intervention:

Group-1 (control group): The rats were given 0.25 ml of normal saline orally on the 8th , 9th , and 10th days of the pregnancy. Group-2 (Acetazolamide group): The rats received 80 and 160 mg of acetazolamide orally from 8th , 9th and 10th days of the pregnancy. The eighth day rats which received acetazolamide 8o and 160 mg were terminated on 16th day by ether inhalation. Similarly,9th and 10th day rats were terminated on 18th and 20th day. Fetuses and placentas were removed by transverse abdominal incision.

All the fetuses were weighed and the crown rump lengths were measured by a ruler. The fetuses were sacrificed with ether and examined for gross external malformation. A total of 80 fetuses (27 from the group 1 and 53 from the group 2) were obtained.

Staining technique: Double staining technique for cartilage and bone14,15. Embryos were fixed in 90% ethanol for at least 1 week. Next the embryos were kept in 0.01% Alcian blue 8GX for 3 days. Then rehydration was done through a gradient series of ethanol (70% ethanol, 2 to 3 h, twice; 40% ethanol, 2 to 3 h; 15% ethanol, 2 to 3 h; ddH_2O, until the sample sinks to the bottom of a conical tube). The embryos were further treated with fresh 1% KOH for 1 to 2 days until it becomes clear. Now the embryos are kept in 0.001% Alizarin red for 2 to 3 days until the bone becomes purple. The embryos were rinsed 3 times in 1% KOH, several hours each time. Now the embryos are treated through a gradient series of glycerol-KOH (20% glycerol/ 1% KOH, 24 h;50% glycerol/ 1% KOH, 24 h; 80% glycerol/ 1% KOH, 24 h; 100% glycerol, 24 h x 2). Skeletal abnormalities were noted.

Statistical analysis: All the data were expressed as frequency distribution. The data were analyzed by chi-square test to see the relation between skeletal deformity with duration in acetazolamide induced pregnant rats. For all the statistical analysis SPSSsoftware was used. P value <0.05 was considered as significant.

Results

From six pregnant wistar rats by administrating acetazolamide 53 litters were obtained in which 29 were deformed litters which were used for the morphological study. All control group fetuses(27) were

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observed to be normal without any deformities.Relation of deformity with duration by injecting acetazolamide by 80mg and 160 mg group are given in Table-1 and Table-2. In fore limb on 8th day in 80mg group 2(11), in 160mg group 4(8) fetuses showed deformity and it was not statistically significant (P = 0.140). On 9th day in 80 mg group 4(11), in 160 mg group 4(6) fetuses showed fore limb skeletal deformity and it was not statistically significant (P= 0.231). On 10th day in 80 mg group 4(10), in 160 mg group 5(6) fetuses showed fore limb skeletal deformity respectively. It was not statistically significant (P=0.090). This showed that there was no relation between the duration and deformity in both limbs of 80 and 160 mg group in fore limb.In hind limb on 8th day in 80mg group 1(11), in 160mg group 1(8) fetuses showed deformity and it was not statistically significant (P = 0.811). On 9thday in 80 mg group 1(11), in 160 mg group 1(6) fetuses showed hind limb skeletal deformity and it was not statistically significant (P= 0.643). On 10thday in 80 mg group 1(10), in 160 mg group 1(6) fetuses showed hind limb skeletal deformity. It was not statistically significant (P=0.696). This showed that there was no relation between the duration and deformity in both limbs of 80 and 160 mg group in hind limb.The results show that only high doses of acetazolamide produce effects of limb malformations.As the dosage was increased mothers lost weight and the number of litters produced were decreased. This suggest that acetazolamide in low doses has no adverse effect on prenatal growth.

Discussion

The teratogenic potential of acetazolamide has been found in rats, mice, hamsters, and rabbits, producing limb malformations, such as polydactyly or limb deficiency16. Structurally different type of carbonic anhydrase inhibitors found that dichlorphenamide, ethoxzolamide and others also produced acetazolamide type of forelimb malformation[17].Among four genetically different isoenzyme, CA III, is exceptionally resistant to inhibition by acetazolamide. Immunocytochemical techniques inmice found that CA III was found in salivary glands, large intestine and adipocytes. In rat it was found in skeletal muscle and liver. In liver of male rat concentration of CA-III is 20 times more than female. This effect depends upon pituitary hormones[18].Acetazolamide produces gross limb malformations mainly in the right fore limb. The intensity of deformity depends on dosage, the way of administration of drug and gestation age. The present study is in line with the previous studies were increase in dosage raises right forelimb deformity, left forelimb were less frequent and hind limbs were rarely involved[3,19]. Malformations depends upon site of administration of acetazolamide and vasoconstructiveagentsduring intrauterine life[6,20].In the present study foetal weight, crown rump length was reduced in foetus and food consumption was reduced, resorption rate is increased in pregnant rats these findings are in line with previous Research[5]. The acetazolamide induced malformations are due to regulatory genes that control the embryonic differences in frequency of ectrodactyly[8,21]. Molecular studies found that there was significant difference in protein and DNA content in right limb buds of mouse on administration of acetazolamide. In humans no congenital malformations were observed on administratingcarbonic anhydrase inhibitors during pregnancy[21-24]. Other than acetazolamide, drugs such as adenine, 1,7-dimethylxanthine, aminophylline, retinoic acid, acetoxy-methyl-methyl nitrosamine, aspirin, and cadmium can all cause unilateral defects[25].

Table 1: Effect of different doses of acetazolamide (mg/kg/b. wt.) administrated from days 8-10 days of gestation on fore limb.

Days	Drug 60 mg		Drug 160 mg		Chi square	P-Value
	Present	Absent	Present	Absent		
8th	2	9	4	4	2.170	0.140
9th	4	7	4	2	1.431	0.231
10th	4	6	5	1	2.86	0.090

Table 2: Effect of different doses of acetazolamide (mg/kg/b. wt.) administrated from days 8-10 days of gestation on hind limb.

Days	Drug 60 mg		Drug 160 mg		Chi square	P-Value
	Present	Absent	Present	Absent		
8th	1	10	1	5	0.0572	0.811
9th	1	10	1	5	0.214	0.643
10th	1	9	1	5	0.152	0.696

Conclusion

In conclusion administration of acetazolamide to pregnant wistar rats during organogenesis period caused fetotoxicity only by administrating high doses of the drug. There was no evidence of duration or gestational day related teratogenesis. Detailed genetic and hormonal analysis should be performed, and more animals should be studied to determine the effect of acetazolamide.

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