Morphological Aspects of Diazepam Induced Teratogenicity in Rats

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This study was conducted to determine the adverse effects of diazepam on morphology of rat embryos. Pregnant rats were administered 0.14mg per kg body weight of diazepam, (therapeutic dose) intraperitoneally during different periods of pregnancy and 280mg per kg body weight (high dose) during the period of organogenesis. Adverse effects observed on the fetuses with therapeutic dose were reduction in number (20%), subdermal haemorrhages (20-25%) and omphalocoele (3%). There was significant reduction in weight and crown-rump length ($p<0.05$). With the high dose, overall malformation rate was 9.76%, anomalies of thyroid gland (5.6%), cleft lip (2.46%) and digital defects (1.6%) were noted. Reduction in weight and crown rump length was statistically more significant than the therapeutic dose ($p<0.01$). In addition to increased severity of these adverse effects, maternal mortality (25-50%) was also encountered with high dose in various subgroups. We conclude that as the adverse effects of diazepam were statistically significant, not only with the high dose but also with the therapeutic dose, therefore the possible risk of teratogenesis should be carefully weighed against the therapeutic advantage, when prescribing this drug in women of child bearing age, specially during pregnancy.
Key words: Diazepam, teratology

Teratology is a branch of embryology that deals with the study of congenital anomalies. Its importance in the era of modern medicine cannot be denied.

It is now estimated that one tenth of congenital malformations are due to genetic factors. One tenth due to environmental factors while in 80% of cases both factors are involved[1]. The genes of the mother as well as those of the embryo affect the susceptibility to a teratogen during the development period[2].

Greg[3] stressed the importance of environmental factors in human development and linked congenital cataract to rubella of pregnancy. Later Somers[4] and Lenz[5] independently established causal connection between a sedative termed thalidomide taken during pregnancy and numerous instances of limb defects.

Diazepam is a benzodiazepine marketed for the first time in 1963. Although primarily considered as a tranquilizer it is a muscle relaxant & an analgesic[6,7]. It is used as a muscle relaxant in spastic disorders, tetanus and status epilepticus[8]. Diazepam is widely used in labour to relieve anxiety, to decrease analgesic requirement & to reduce recall of events. It is effective in threatened abortion, pre-eclampsia & eclampsia[9].

Diazepam has been studied to explore its teratogenic potential. Miller & Becker[10] noted decrease in litter size, increased incidence of cleft lip & palate in mice with this drug Guerriero & Fox[11] reported that mean body weight of fetuses of hamster mothers given diazepam, was significantly lower than the controls. Four independent epidemiological studies of human populations have shown an association between cleft lip, cleft palate and prenatal exposure to diazepam[12,13,14]. The teratogenic potential of this drug is however debatable as in few studies it was not found to induce any teratogenicity. It was therefore decided to conduct a study using therapeutic & high dose of diazepam to explore its teratogenic potential, if any.

Methods

Albino Sprague rats (105 female & 35 male) with an average weight of 115-140g, around 10-12 weeks of age were taken for present research. Males and females were kept in separate cages and fed on commercially prepared chick feed number 3. After an adjustment period of two weeks stage of estrus cycle was determined. Mild to moderate swelling of vulva & preponderance of epithelial cells and leukocytes in vaginal smear indicated stage II or III of estrus cycle. Such female rats were kept overnight with the males in a ratio of 3:1. The presence of vaginal plug was counted as day one of pregnancy. The pregnant female rats were divided into various experimental and control groups of eight animals each as given in Table 1.

Diazepam was injected intraperitoneally according to schedule given in Table 1.

On day 21 of pregnancy the rats were weighed and then sacrificed after giving chloroform anesthesia. Midline abdominal incision was made and dissection was carried out to recover uteri along with the embryos.

Following observations were made.

1. Number of resorptions.
2. Number of fetuses.
3. Weight of each fetus determined in grams on an electric balance.
4. Crown rump length of each fetus measured in centimeters.
5. Detailed morphological study was carried out under a dissecting microscope using a magnification of 10x Head, ears, eyes, snout, limb structure, trunk and tail were carefully seen and compared in each fetus in all the groups.

The selected embryos were then photographed. Results were analyzed by applying statistical tests appropriate to experimental design. Significance of difference between the means was tested using the student t test.

Results

General physical condition

Intraperitoneal treatment with diazepam in a dose of 0.14mg/kg body weight produced sedation of pregnant animals of group B for a maximum of 4 hours. The animals showed rapid tolerance to this effect after two to three administrations. The sedated state persisted in animals of group C given 280mg/kg body weight of diazepam, resulting in total lack of food and water intake during this period. Hard nodules appeared at injection site in all the animals after one week of continuous treatment. No behavioural changes were noted in any of the treatment groups.

Maternal mortality

Death occurred in 25-50% of pregnant rats of group C, 15-20 hours after the administration of 280mg/kg body weight of diazepam. Maternal mortality was most pronounced on day 10 (group C2) when 4 out of 8 animals died. No animal died in controls and therapeutic dose groups (Table 2).

Number of fetuses

This litter size of animals given 0.14mg/kg body weight of diazepam during first week (B1) was found to be significantly reduced (Table 3). Fetotoxicity was observed with 280mg/kg body weight of diazepam (Group C) (Table 2) and was maximum in C2 group given drug on day 10 (Fig.1&2).

Table 1. Dose schedule of diazepam in various groups

Days	Days 1-7	Days 8-14	Days 15-21	Days 1-21	Day 9	Day 10	Day 11	Day 15
Food and water only	AX	AX	AX	AX	AX	AX	AX	AX
Vehicle	A1	A2	A3	A4	A2	A2	A2	A2
0.14mg/kg	B1	B2	B3	B4	-	-	-	-
280mg/kg	-	-	-	-	C1	C2	C3	C4

Table 2. The effects of high dose of Diazepam on the maternal mortality and fetotoxicity of rat.

Animal Group	Maternal Mortality	Number of live fetuses	No. of Resorptions	No. of dead features	Malformed fetuses	Fetotoxicity
C1	25%	35	10	12	Nil	22:57=0.385
C2	50%	21	7	9	5	21:41=0.152
C3	37.5%	32	6	8	7	18:52=0.346
C4	37.5%	34	5	3	2	10:54=0.185

Fetotoxicity is determined by the ratio of the total number of resorptions, dead fetuses and malformations to implants.

Table 3. Effect of therapeutic and high dose of Diazepam on the morphometric parameters of developing rat fetuses.

Group	Mean No. of live Fetuses/Animal	Mean Fetal Weight (g)+SD	Mean Crown Rump Length (CMS+SD
AX	10.62	4.4±0.16	3.8±0.14
A1	10.25	4.3±0.13	3.6±0.24
A2	10.50	4.5±0.13	3.7±0.13
A3	10.50	4.4±0.14	3.8±0.13
A4	9.96	4.2±0.242	3.7±0.15
B1	8.5*	4.1±0.18	3.5±0.20
B2	9.75	308± 0.23	3.4±0.48
B3	10.37	3.2*±0.92	3.0*±0.73
B4	10.00	3.4"±0.75	3.2±1.14
C1	5.83	3.0**0.15	2.6**±0.92
C2	5.25**	2.8**±0.18	2.0***±0.74
C3	6.40**	2.7**±0.32	2.5**±0.37
C4	6.80**	2.6**±0.48	2.2***±0.52

SD- Standard deviation

For statistical significance in this table high dose treatment group C had been compared to vehicle control group of second week of pregnancy (A2)* $p<0.05$ **$p<0.01$ ***$p<0.001$

Body weight & C-R length

In animals given 0.14mg/kg body weight of diazepam during first week (B1) and second week (B2) of gestation, decrease in weight and crown-rump length was statistically insignificant. When the same dose was given during third week (B3) and throughout pregnancy (B4) a generalized trend towards decrease in body weight and length was observed which was more marked in later group. In fetuses of animals of group C treated with 280mg/kg body weight of diazepam, a significant reduction of both weight and crown-rump length was observed in all the subgroups (Fig3& Table 3).

Morphological observations

Twenty one-day-old fetuses recovered from plain and vehicle control groups were all normal and were quite similar in general appearance & morphological details.

Diazepam treatment in therapeutic dose (Group B) caused a 3% malformation rate. Subdermal haemorrhages in the form of petechae and bruises, edema of face and body, was found in 20-25% of fetuses (B2, B3, B4 groups). In addition to subdermal haemorrhages the anomaly observed was the herniation of the gut (Fig.4), in fetuses from second week treatment group (B2) and those treated throughout pregnancy (B4).

With 280mg/kg body weight diazepam, (Group C) the malformation rate came out to be 9.76% which was most significant on day 9 (C1) and day 10 (C2). The malformations exhibited were: Incomplete cleft palate (2.64%) was found in fetuses of groups given a single dose diazepam on day 11 (C3).

Digital defects (1.6% in the form of absence of one or more digits of hindlimbs) were found in fetuses recovered from animals given diazepam on day 10 (C2) and day 11(C3) of gestation.

Anomalies of thyroid gland (5.6%) in the form of total absence and hypogenesis was seen in fetuses of pregnant rats given 280mg/kg body weight of diazepam on day 10(C2) and day 11(C3) pregnancy (Fig.5).

Ninety percent fetuses of all the subgroups of high dose (Groups C given 280mg/kg body weight of diazepam) showed subdermal haemorrhages in the form of petechae, bruises, edema of face and body.

Fig.1. Control subgroup A2 showing fetuses
F1-3 fetuses in right horn. F4-8 fetuses in left horn of uterus.

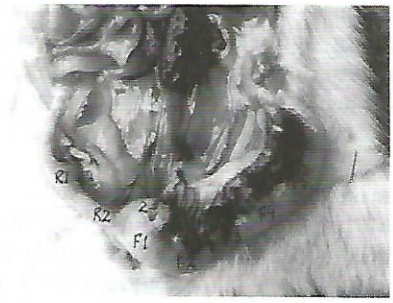

Fig. 2 Subgroup C2 showing resorption
R1-2: Resorption sites of fetuses. F1-5: small sized fetuses. No.2 indicates placenta

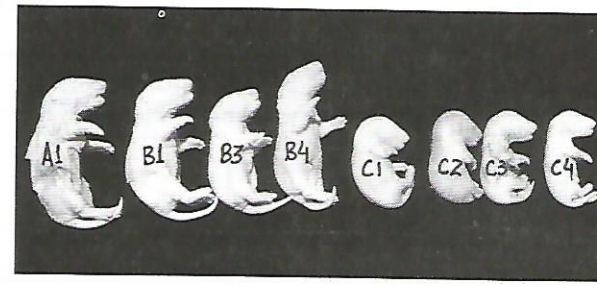

Fig. 3. Fetuses from animals of various subgroups.

Fig.4. Fetus from animal subgroup B4 showing ompalocoele
No.1 indicates omphalocoele

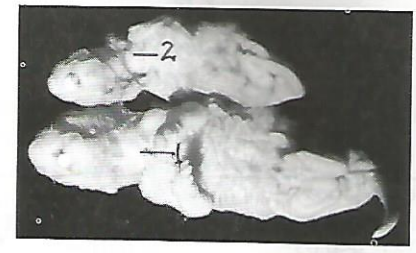

Fig. 5. Fetuses from subgroup A2 (control) & C3 (high dose group) showing thyroid gland
No.1 well developed gland. No.2.Hypoplastic thyroid gland.

Discussion

A study carried out by Bolbus[15] revealed that only 20% of pregnant women used no drugs during pregnancy while 900 different drugs excluding nutritional supplements were taken by other pregnant women with an average of four per individual. 40% of these women take the drugs during critical period of human development. Diazepam is also one of the drugs frequently used by women during pregnancy.

General physical condition

Diazepam is a depressent of central nervous system, which produces sedation in small doses; hypnosis in moderate doses & stupor with further increase in dose[16]. Diazepam 0.14mg/kg body weight (equivalent to 10mg dose, used as a sedative in clinical practice[17]), produces sedation of pregnant rats that progresses to stupor and even death in high doses. Similar findings were seen in our study (Group C).

Maternal mortality

With high dose of diazepam maternal mortality was 25% on the 9th day, 50% on the 10th day and 37.5% each on the 11th & 15th day. This observation correlates with the findings of others[18,19,20].

Number of fetuses

When low dose diazepam (equivalent to human therapeutic dose) was given to pregnant rats during first week, it produced 20% reduction in the number of fetuses when compared to the control group. If a teratogen acts on the rapidly dividing cells during pre-differentiation stage, either it damages almost all the cells of the embryo, ensuing death or only a few cells are affected. Thus the regulative potentialities of the embryo compensate for the loss and no abnormalities become apparent[1].

Diazepam produces chromosomal breakage of chromatid and iso-chromatid variety[21], and dose-related inhibition of meiosis in oocytes[23]. Ova that are degenerative or arrested at metaphase-I would not progress to a zygote after fertilization so reduction in the number of fetuses compared to the control was observed when diazepam was given post conception.

Fetotoxicity that is estimated by the ratio of the total number of dead fetuses, resorptions and malformations to implants were significant with 280mg/kg body weight of diazepam. This finding agrees with the reports of Miller and Becker[10] and Tarlok et al[19] who also documented resorptions with high dose of diazepam.

Body weight and C-R length

In groups treated with therapeutic dose in third week and throughout pregnancy, significant reduction in both weight and crown rump length of fetuses was noticed. This could be attributed to the sedative effect of diazepam thereby decreasing the food and water intake of mothers. Animals became tolerant to this effect after four to five days. The decrease in weight and length was more marked in group treated with therapeutic dose only in the third week. Runted fetuses (50% the size of normal fetuses) were recovered from groups given high dose of diazepam. This finding is also documented by Tarlok et al[19].

Morphological observations

Three percent incidence of omphalocoele was observed when therapeutic dose was given in second week and throughout pregnancy. This finding has also been reported by Shah et al[18], but with a higher dose. It can be explained on the basis of effects of diazepam, which cause an increase in the size of liver & decrease in the size of fetuses, thereby decreasing the abdominal capacity. Hence the intestines could not be accommodated.

The development of thyroid gland, whose diverticulum arises in second week and reaches its final position on the 14th day[24] was also affected by high dose of diazepam given on the 10th and 11th day of gestation. This anomaly has not been previously reported. The harmful effects of diazepam on cytoplasmic organelles and chromosomes of cells added by suppression of neuro axis as reported by Kellog et al[25] thereby decreasing the TSH secretion may be responsible for the observed anomaly in this study.

Subdermal haemorrhage, oedema of face and body found in 20-25% of fetuses of therapeutic dose group and 90% fetuses of high dose group were also reported by Shah et al[18,26] could be attributed to the venous engorgement resulting from decrease in cardiac output[27] and damage to the vessel wall musculature[28].

The cleft palate was observed but its occurrence was not significant in present study. On the contrary Miller and Becker[10] noted a significant incidence of complete and incomplete types of cleft palate in mice and hamsters given dizepam on day 14 of pregnancy. Epidemiological studies in humans give controversial relationship between cleft lip, plate and diazepam.

Sherif et al[20] noted anomalies of skull bones, vertebrae and sternum with 20-40mg/kg body weight of diazepam, contrary to the absence of these findings in present study.

Conclusions

We conclude that as the adverse effects of diazepam were statistically significant, not only with the high dose but also with the therapeutic dose therefore the possible risk of teratogenesis should be carefully weighed against the therapeutic advantage, when prescribing this drug in women of child bearing age, specially during pregnancy.

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