Histological Aspects of Diazepam Induced Teratogenicity in Rats

N TAZEEN* R TAFWEEZ* T A RAZA**
*Department of Anatomy, King Edward Medical College, **Postgraduate Medical Institute, Lahore.
Correspondence to Dr. Nadia Tazeen, Assistant Professor Anatomy

This study was conducted to determine the teratogenic potential of therapeutic & high dose of diazepam in rats. Histological study of liver & kidney of fetuses was carried out after exposing them to therapeutic dose (0.14 mg per kg body weight) of diazepam intraperitoneally during different periods of gestation and high dose (280 mg per kg body weight) during the period of organogenesis. Rats 140 in number were divided into various groups & subgroups. Adverse effects were noted and compared with controls.. In the liver, relative tissue weight index (RTWI) was high, histological evidence of inflammation and mild disturbance of architecture to total disarray was noted. Kidney also showed glomerular and tubular damage. in a dose related manner with an increase in RTWI. We conclude that diazepam is teratogenic in a dose related manner, affecting fetal liver and kidney at cellular level, It should be prescribed during period of organogenesis with caution.
Key words: Teratogenesis, RTWI, liver, kidney

It was in 1941, that Gregg[1] pointed out the association of rubella virus infection of the mother during pregnancy, to fetal congenital malformations. Since then extensive research has been conducted in the field of teratology and possible role of various environmental factors including drugs has been explored.

In 1961, Sternbrach and Reeder[2] synthesized a chemical compound 7 chloro-1, 3 dihydryo-1-methyl-5-phenyl-2h-1. It was named diazepam and was the second benzodiazepine to be marketed in 1963. Although primarily considered as a tranquilizer, it is also a muscle relaxant[3], and analgesic[4]. It is used in status epilepticus[5] Its clinical use extends to anxiety, myocardial infarction, cardio-version, cardiac catheterization, angiography, dentistry, bronchoscopy, endoscopy, labor, threatened abortion, pre-eclampsia and eclampsia[6] .The largest dose of diazepam in clinical practice used at times is 480mg daily (approximately 6.6 mg/kg) for the management of tetanus[7]

Widespread use of diazepam has been a source of concern due in large part to its association with problems of abuse & dependence. It is possible that a woman takes it without being aware that she has an early pregnancy. It is also given for the psychiatric complications of pregnancy & is recommended for treating a threatened abortion.

We carried out this research to study the histological changes if any that occur in the liver & kidney of fetuses exposed to therapeutic and high doses of diazepam during different periods of gestations.

Methods

Albino Sprague rats (105 female & 35 male) were taken for present research. Males & females were kept in separate cages & given commercially prepared chick feed number 3 & water ad libitum. After an eclimatization period of two weeks, stage of estrus cycle was determined. Mild to moderate swelling of vulva & preponderance of epithelial cells & leucocytes 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 taken as day one of pregnancy. The pregnant female rats were divided into various experimental & control groups of eight animals each as follows:

Group A

Plain control-(AX) - Given food & water ad libitum throughout pregnancy.

Vehicle Control (A1-A4) Divided into four subgroups & injected solvent intraperitoneally on the days of pregnancy specified.. Solvent was prepared by PDH Labs Its composition, (propylene glycol-40%, Ethyl alcohol 10%, sodium benzoate 5%, & benzyl alcohol 1.5%) was similar to that of vehicle for valium (diazepam) injections.

A1 Day 1-7
A2 Day 8-14
A3 Day 15-21
A4- Day1-21(Entire gestation)

Group B – Given 0.14 mg/kg body weight of diazepam in the form of intraperitoneal injection, on following days.

B1- Day 1 to 7,
B2 Day 8 to 14
B3- Day 15 to 21
B4- Day 1 to 21

Group C: Given 280 mg/kg body weight of diazepam in the form of intraperitoneal injection, on following days.

C1 Day 9
C2 Day10
C3 Day 11
C4 Day 15

Mothers were sacrificed on day 21 of pregnancy Abdominal cavity was opened. Fetuses were dissected out of the two uterine horns. The fetuses obtained were then dissected to obtain their liver & kidneys These organs were separately weighed. Tissues of all these organs were processed in graded alcohol, embedded in paraffin wax. and 4-5 um thick sections were cut. Slides were stained with Harris haematoxylin & eosin by standard procedure[8] The slides were studied under the light microscope using magnification of 40x and 100x Following parameters were noted :

Capsule
General architecture
Parenchymal cells
Status of vasculature

Interstitial tissue

Microscopic picture of liver and kidney of fetuses from different groups were compared with control. Selected sections were photographed. Relative tissue weight index of these two organs was calculated by applying statistical formula.

Results

The Liver

Relative Tissue Weight Index (RTWI). The relative tissue weight index of fetuses recovered from group given 0.14mg/kg-body weight of diazepam during first week (B1) was similar to the control groups. There was a slight increase in the RTWI of fetuses of animals receiving the same dose during second week (B2), third week (B3) and throughout pregnancy (B4) & was significant in B3 and B4 groups. (Table-1).

There was a moderately significant increase in the RTWI with 280mg/kg-body weight of diazepam given on 9th (C1), 10th (C2) and 15th days (C4). The increase in RTWI of fetuses from 11th day (C3) treatment group was highly significant (Table-2).

Macroscopic appearance

In all the control groups (A & Ax) fetal liver was dull brown in color, with well developed right, left and middle lobes. It had a pale appearance with areas of reddish brown color and an increased turgor in fetuses of groups treated with 0.14mg/kg body weight of diazepam for 2nd week (B2), third week (B3) and throughout pregnancy (B4). Fetal livers of high dose group C were reddish brown in color and there was serum ooze on handling. All three lobes were well differentiated in all the experimental groups.

Microscopic Appearance

A typical hepatic lobular architecture was seen in the fetal livers of all the control groups and therapeutically treated groups of first week (B1). With the same dose given during second week (B2), third week (subgroup B3) and throughout pregnancy (B4) the hepatocytes were not arranged in a definitive lobular pattern. (Fig-1 and 2). Total disarray of lobualr architecture was seen in all the subgroups of high dose group C given 280mg/kg-body weight of diazepam

Capsule was intact in fetal livers of groups treated with therapeutic dose but disrupted at places in high dose treatment group.

Hepatocytes appeared as rounded cells with homogenous cytoplasm and a rounded vesicular nucleus in fetuses of all the control groups (A & Ax) and in therapeutically treated group of first week (B1). In the treatment group of second week (B2) some cells showed cellular swelling while in the third week (B3) and throughout pregnancy (B4) treated groups, cells with vacuolar degeneration and karyolysis were also seen in addition to the swollen cells. In fetal livers of high dose treatment group,(C) cells in all stages of reversible injury were present. Cellular swelling, vacuolar degeneration and irreversible injury showing coagulative necrosis, nuclei with pyknosis and karyorrhexis were seen. These cells were most abundant along the blood vessels. Some acidophilic anuclear carcasses (ghost cells) were also observed in fetal livers of animals treated on day 11 (C4) of pregnancy. Focal areas of coagulative necrosis 2-3/microscopic field were seen in treated groups of day 15 (C4) of pregnancy (Fig. 3)

Central vein and sinusoids were dilated in therapeutically treated fetal livers of 1st (B1) and 2nd week (B2), while they were congested as well, in 3rd week (B3) and in group treated throughout pregnancy (B4). In the high dose treatment groups, these findings were accompanied by the dilatation and congestion of portal vein and the disruption of its wall at many places The endothelial cells showed signs of injury namely swelling), crenated margins, febrin bridges and RBC adhesiveness (Fig. 4).

Inflammatory cells including neutrophils, lymphocytes and macrophages were scattered all over the field. Predominately around the portal triad, central vein and large blood vessels in subgroups of low dose treatment group B and also around the necrotic areas in high dose treatment group C. Macrophages with engulfed hepatocytes were also observed in these groups Red blood cells were seen interspersed among the hepatocytes in the high dose group.

Kupffer cells lining the peri-sinusoidal space were prominent in all the groups studied (Fig 2).

The Kidney

Relative Tissue Weight Index (RTWI).

RTWI of fetuses from animals treated with 0.14mg/kg body weight of diazepam during first week of pregnancy (B1) was similar to the plain control (Ax) and vehicle (A) groups. It was found to be decreased in fetuses from therapeutically treated groups of second (B2) and third weeks (B3) and throughout pregnancy (B4), (Table 1) but the decrease in RTWI was significant with high dose in subgroup of 9th day (C1). Slight increase was observed with 280mg/kg-body weight of diazepam on 10th (C2) and 11th (C3) days but it was significantly increased in 15th day (C4) treatment group only. (Table 2)

Macroscopic appearance.

The fetal kidneys of all the groups were bean shaped. They had a pale yellow appearance except in the high dose treated subgroups of 10th (C2), 11th (C3) and 15th (C4) days in which they were pink in color. Capsule was intact in all the groups.

Microscopic appearance.

The parenchymal arrangement in the fetal kidneys of therapeutically treated group B was similar to the control groups A & Ax, being divided into cortex and medulla). In the high dose treatment group of 9th day (C1) it showed a disorganised architecture with cells mostly arranged in the form of clumps

The capsular space was decreased and glomeruli congested in the groups treated with 0.14mg/kg body

weight of diazepam during second week (B2), third week (B3) and throughout pregnancy (B4). With 280mg/kg on day 10 (C2) and day 11 (C3) degenerated glomeruli with increased capsular space were seen (Fig 5).

Tubular cells showed cloudy swelling in the therapeutically treated groups of 2nd week (B2), 3rd week (B3) and throughout pregnancy (B4). While they also showed coagulative necrosis in high dose treatment groups Connective tissue stroma in medulla was increased with fewer tubules in high dose treatment group of 10th (C2), 11th (C3) and 15th (C4) days (Fig. 6). Inflammatory cells and dilated and congested vessels were seen in all the high dose treatment groups.

Fig 1 Photomicrograph of fetal Liver of Subgroup B 2

1. Dilated sinusoids
2. Vesicular nucleus of hepatocyte
3. Neutrophil
4. Kupffer cell

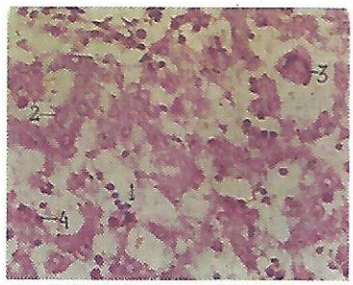
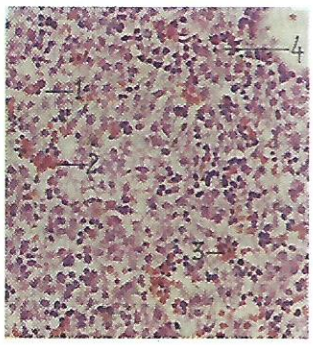


Fig 2 Photomicrograph of fetal Liver of Subgroup B 4 showing loss of lobular architecture

1. Dilated sinusoids
2. RBCs
3. Lymphocyte
4. Kupffer cell



Fige 3 Photomicrograph of fetal liver of subgroup C 4

1. Focal coagulative necrosis
2. Inflam-matory infiltratel

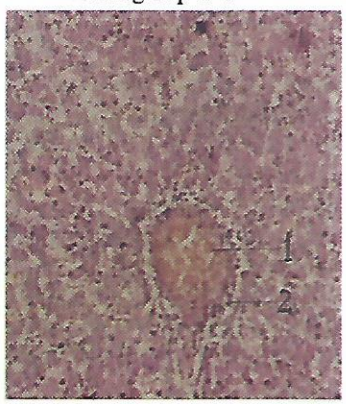


Fig 4 Photomicrograph of fetal liver of subgroup C 4

1. Dilated portal vein
2. Ednothelial cell swelling
3. Fibrin bridge
4. RBCs
5. Damaged bile duct

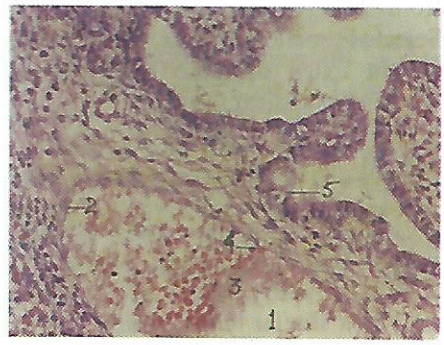


Fig. 5 Photomicrograph of fetal kidney of subgroup C2

1. Degenerated glomerulus with increased capsular space
2. Necrosis of tubular cells

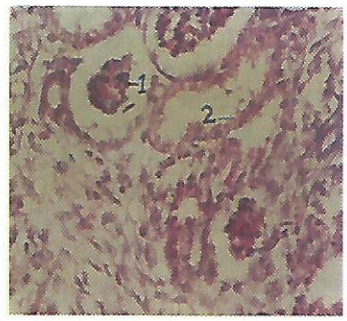
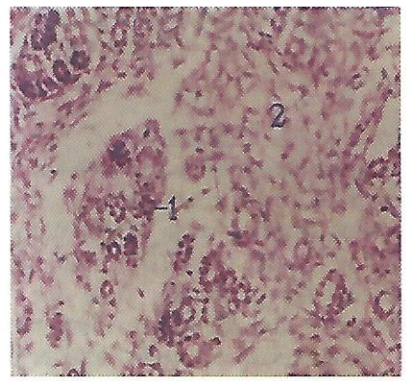


Fig. 6 Photomicrograph of fetal renal medulla subgroupB 4

1. Few medullary tubules
2. Increased Connective tissue stroma



Discussion

The liver

Relative Tissue: Weight Index (RTWI).

The increase in RTWI in fetuses from groups given therapeutic as well as high dose could be attributed to increased congestion, swelling of the cellular element and inflammatory reaction in the liver. These changes were more intense with the high dose, so the increase in weight was also more marked in these groups.

Macroscopic appearance.

The pale appearance and increased turgor of fetal liver in therapeutically treated groups could be due to cellular swelling produced by diazepam. With high dose, increased

congestion and damage to the vasculature was responsible for the reddish brown appearance of treated livers.

Microscopic appearance.

Hepatotoxicity was exhibited by diazepam administraion with the high dose given on 9th, 10th, and 15th day and with the therapeutic dose in second week, third week and throughout pregnancy but not in the first week. This is because the liver primordium appears on the 10th day of rat development[9]. The disorganized architecture of fetal liver could be explained on the basis of harmful effects of diazepam on the structure and function of fibroblasts[10] thus affecting the synthesis of connective tissue fibers. The stromal tissue is laid down earlier forming basis for the organized parenchymal arrangement specific to an organ, thus any delay in the process results in disorganized architecture.

The dilatation and congestion of vessels with the therapeutic dose which became marked with the high dose treatment could be due to increased blood supply to an inflamed organ whereas congestion could be due to the effects of diazepam on the heart, impairing the venous drainage[11,12]. Damage to vasculature with 30-100mg dose has also been reported[13]

Report of Mirando et al[14] regarding raised liver enzyme in patients receiving diazepam in therapeutic dose, agrees with the changes observed in this experiment. Diazepam interferes with the coupling of mitochondrial respiration with ATP synthesis leading to impairment of sodium pump mechanism, resulting in retention of sodium and thus water. When the stress to hepatocytes was acute and severe, the injury progressed to irreversibility resulting into coagulative necrosis of cells, focal necrotic areas, pyknosis and karyorrhexis of nuclei and appearance of ghost cells. The effects of diazepam on chromosome breakage[15] and consequently on cytoplasmic organelles of hepatocytes[16] could explain these findings. Inflammatory cells and prominent Kupffer cells suggest injury to the parenchyma.

The kidney

Relative Tissue Weight Index (RTWI)

Kidney starts developing on the 10th day, so decrease in RTWI was observed in therapeutically treated groups of second week, third week and throughout pregnancy. With high dose, the decrease in RTWI noted on 9th, 10th and 11th day treatment groups resulted from reduced number of cells and presence of degenerated cells. On 15th day treatment group the much greater congestion could account for the increase in the RTWI. Hypoplasia of kidney was also documented by Sherif et al.[17] Diazepam also interferes with the cellular growth by causing a delay in the differentiation of cells[18]

Microscopic appearance

When fetal kidney was subjected to biochemical insult with high dose of diazepam during the period of organogenesis it developed only a few nephrons and cells exhibited signs of injury. These findings could be explained due to the suppressing effects of diazepam on the cellular growth rate[10], a delay in the differentiation of cells[18], the chromosome breakage and consequent damage to the cellular organelles as was also observed by Marquez et al.[19] and Breen and Stenchever[15]

Table 1 Effect of Therapeutic Dose of Diazepam on the Relative Tissue Weight Index (R.T.W.I) of Rat Fetuses

	VEHICLE GIVEN ON DAYS				DIAZEPAM (0.14 mg/kg) GIVEN ON DAYS			
	1-7	8-14	15-21	1-21	1-7	8-14	15-21	1-21
GROUPS	**A1**	**A2**	**A3**	**A4**	**B1**	**B2**	**B3**	**B4**
Mean Body Weight (Mg)	4380	4500	4410	4250	4100	3800	3200	3420
Liver Weight (Mg)	216.6	220.2	213.3	209.5	200.5	195.3	166.2	178.3
R.T.W.I Of Liver	4.93	4.89	4.836	4.92	4.89	5.13	**5.19*** $p<0.05$	**5.21*** $p<0.05$
Kidney Weight (Mg)	34.20	35.960	35.12	33.10	31.15	27.92	23.65	25.04
R.T.W.I Of Kidney	0.786	0.799	0.796	0.778	0.759	0.734	0.739	0.732

Table 2 Effect of High Dose of Diazepam on the Relative Tissue Weight Index (R.T.W.I) of Rat Fetuses

	Vehicle Control Group Vehicle Given On Days 8-14	Diazepam 280 Mg/Kg Given On Day 9	Day 10	Day 11	Day 15
GROUPS	**A2**	**C1**	**C2**	**C3**	**C4**
Mean body weight (mg)	4500	3000	2815	2762	2636
Liver weight (mg)	220.2.2	174.7	160.2	162.3	145.5
R.t.w.i of liver	4.89	**5.56** $p<0.01$	**5.69** $p<0.01$	**5.87** $p<0.01$	**5.51** $p<0.01$
Kidney weight (mg)	35.960	19.23	20.85	21.215	21.10
R.t.w.i of kidney	0.799	**0.641** $p<0.05$	0.740	0.767	**0.811** $p<0.01$

Conclusion

Diazepam appears to have damaging effects on liver & kidney of rat fetus both in therapeutic as well as high doses. This aspect should be borne in mind while prescribing this drug in pregnancy. Possible risks and benefits should be carefully weighed.

Acknowledgement

We are grateful to Mr. Fazl-e-Azim Statistician for the statistical work and the laboratory staff of PGMI & KEMC for their technical assistance.

References

1. Gregg NM, 1941. Congenital cataract following german measles in mothers. Trans ophthalmol Soc. Aust., 3:35-39
2. Sternbach, L and Reeder, 1961.Quinazolines and 1,4-benzodiazepenes. Transformation of 7-chloro-2methyl-amino-5-phenyl-3H –1,4 benzodiazepine 4 oxide. J. Org. Chem., 26:4936-41.
3. Katz RA, Aldes JH & Rector M, 1962. A new drug approach to muscle relaxation. J Neuropsychiatry, 3:91-95
4. Dundee JW & Haslett WHK, 1970. The benzodiazepines. A review of their actions and uses relative to anesthetic practice. Br. J. Anaesthesia,42:217-234.
5. Young RR and Delwaide PJ, 1981. Drug therapy, spasticity. New Engl. J. Med., 304:96-99.
6. Leon TH, Ratman S and Sivasamboo R, 1968. Use of benzodiazepines in the management of eclampcsia. J. Obstet. Gynaecol. Br. Common., 75:856-62.
7. Odusote TM, 1976. Drug therapy in tetanus patients. Trop. Geogr.Med., 28:194-8.
8. Disbrey BD and Rack JH, 1970. Practical basis of staining. In : Histological laboratory methods. Livingstone, London pp 82-92
9. Rugh R, 1964. In: Vertebrate Embryology. Hartcourt Brance and World, Newyork/ Burlingame, pp233-302.
10. Stenchever MA and Frankel, 1969. Some effects of diazepam in human cells in vitro. Am J Obst Gynecol.,103:836-42
11. Berry DG, 1975. Effects of diazepam on the isolated chick embryo heart. Proc Soc Exp Biol Med., 150: 240-43.
12. Marquez OMC, Ramirez MVG and Orozco AN, 1983. Ultrastructural alterations of fetal mice heart produced by treatmen twith Diazepam during gestation. Proc West Pharmacol Soc, 26:83-4.
13. Shah RM, Donaldson D and Burdett D, 1979. Teratogenic effects of diazepam in the hamsters. Can J Physiol Pharmacol., 57:556-61.
14. Miranda R, Ceckler T, Gullit R and Kellog C, 1990. Early developmental exposure to benzodiazepines ligands alters brain 31P- NMR Spectra in young adult rats. Brain Research, 506: 85-92.
15. Breen PC and Stenchever MA, 1970. Some effects of diazepam on the fine stucture of human fibroblasts in tissue culture. Am J Obst Gynecol., 108: 520-27.
16. Marquez OMC, Ramirez MVG and Orozco AN, 1985. Ultrastructural changes in hepatocytes of the mouse fetus exposed to diazepam. Proc West Pharmacol Soc, 32:53-6.
17. Sherif ZM, Abdel-Rahman NS and Ahmed YY, 1991. Teratogenic effect of cocaine and diazepam in CFI mice. Teratology, 43:11-17.
18. Marquez OMC, Ramirez MVG and Orozco AN, 1992. Ultrastructural changes in photoreceptors and retinal layers of the mouse fetus exposed to diazepam. Proc West Pharmacol Soc, 35:43-6.
19. Marquez OMC, Orozco AN, Bouchot-Ggamus E and Fernandez-Gabarron F 1991. Increase of bone calcium by prenatal exposure to Diazepam Proc West Pharmacol Soc, 34:383-5