EFFECTS OF DIAZEPAM AND MIDAZOLAM ADMINISTERED TO FEMALE WISTAR RATS DURING THE ORGANOGENIC PERIOD

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ABSTRACT

This study was undertaken to evaluate the teratogenic potential of diazepam (DZ) and midazolam (MD) in rats. Wistar female rats were divided in three groups and received during the organogenic period (6^{th}. to 15^{th}. day of pregnancy) respectively: DZ 10mg/kg/day, MD 5mg/kg/day and control - distillated water 10ml/kg/day. During the pregnancy the dams were observed to ponderal development, food and water intake and signs of systemic toxicity. The dams were sacrificed at term for the evaluation of maternal and fetal toxicity. Number of coporea lutea, post implantation loss, living and death fetuses were recorded. Fetuses were weighted, sexed, examined for external macroscopic and skeletal malformations. The results of ponderal development, food and water intake didn't differ between groups suggesting no maternal toxicity. It was observed a decrease in fetus number of dams from DZ and MD compared to the control group and a decrease in fetus weight. The post-implantations looses were increased in DZ (10.76%) and MD (13.3%) compared to the control (1.47%). The skeletal evaluation demonstrated a retarded development in DZ and MD groups, and higher incidence of malformations. The study suggest teratogenic effects to DZ and MD with the tested doses.

Key words: Diazepam. Midazolam. Organogenisis. Teratogenic

1. INTRODUCTION

Benzodiazepines (BD) are a group of drugs which act upon the central nervous system. BD are the most commonly prescribed tranquilizers and almost 30% of all hospitalized human patients throughout the world receive a prescription for a BD each year [12, 5]. In England, it has been found that about one in three females had taken tranquilizers at some time [8]. In veterinary patients, the reality is not different and diazepam (DZ) and midazolam (MD) are commonly recommended to dogs, cats, horses and pigs [11]. DZ have been shown to induce various malformations in laboratory animals,

including oral cleft [13, 16]. Other BZ such as chlordiazepoxide, nitrazepam and oxazepam have been tested upon the mutagenic and genotoxic effects [5]. Contradictory results have been reported about teratogenic, mutagenic and genotoxic effects of BZ [5, 16, 2, 10]. Thus, further studies are recommended for the evaluation of the teratogenic effects of DZ and MZ, commonly BZ used in human and animal patients.

2. MATERIALS

Animals: Male and female albino Wistar rats were kept under constant conditions: a day/night cycle (lights on: 9:00 – 21:00), a room temperature of 21 ± 1^0 C and 50 ± 5% relative humidity. The animals received a standard pelleted diet (Nivilab CR 1®, Paraná, Brazil) and tape water *ad libitum* during the experiment. All rats were adapted to the conditions of our animal quarters for three weeks before starting the experiment. Breeding, housing and experimental procedures followed guidelines published in the NIH Guide for Care and Use of Laboratory Animals and obeyed current Brazilian laws.

Mating procedure: Males were housed single in a cage with wood shavings as bedding. Three virgin females were placed into a cage of one male for 2 hours each day (7:00 to 9:00 hours) and vaginal smears were evaluated for sperm. The first 24 h period following mating procedure was called day 0 of pregnancy if sperm were detected in the smear. The mating procedure was repeated every day to obtain a minimal of 13 dams per group.

Treatment of animals: The animals were divided in three experimental groups, one control group (n = 13), that received distillated water, a group treated with diazepam (DZ) 10mg/kg/day (Valium®) (n = 15) and a group treated with midazolam (MD) 5mg/kg/day (Dormonid®) (n = 14). All experimental groups were treated by gavage between the 6^{th}. and 15^{th}. of pregnancy (organogenic period).

Evaluation of the animals: All females were evaluated for weight development, mortality, and signs of toxicity.

Cesarean section: On day 21 of pregnancy the female were anaesthetized by ethyl ether inhalation and killed by decapitation. The gravid uterus was weighed with contents. Resorptions as well as living and dead fetuses were counted. The number of implantation sites was determined. All the living fetuses were immediately weighed, numbered with marker pen, examined for externally visible malformations and fixed in a 5% formalin solution. All fetuses were examined for skeletal anomalies after clearing with tripsin and staining with alizarin red [17]. The degree of ossification was evaluated using parameters proposed [4].

Statistical analyses: Data were analyzed by one-way of variance (ANOVA) or, alternatively, by the Kruskal-Wallis test whenever the data did not fit a normal distribution. Using Tukey test tested differences between groups. Proportions were analyzed by Chi-square test or, alternatively, by the Fischer exact test. Difference was considered statiscally significant at $p<0.05$.

3. RESULTS

3.1. Body weight changes and toxicity in female rats

No deaths were induced and no other signs of toxicity were apparent in female rats treated orally during the organogenic period (6^{th}. to 15^{th}. day of pregnancy) with DZ 10mg/kg/day; MD 5mg/kg/day. No statistically significant differences among control, DZ and MD treated groups were found with regard to weight gain, food and water intake.

There were no differences in both absolute and relative organs weight among groups. Table 1 only shows the relative organs weight.

Table 1. Relative organs weight (%) from dams treated during the organogenic period (6th. to 15th. day of pregnancy) with diazepam (DZ) 10mg/kg/day; midazolam (MD) 5mg/kg/day and control (distillated water) 10ml/kg/day. Data are given as means ± SE.

Relative organs weight (%)	Control n = 13	Diazepam n = 15	Midazolam N = 14
Heart	0.37 ± 0.03	0.37 ± 0.04	0.37 ± 0.03
Spleen	0.40 ± 0.15	0.42 ± 0.22	0.41 ± 0.11
Liver	4.69 ± 0.42	4.78 ± 0.42	4.63 ± 0.53
Left kidney	0.35 ± 0.05	0.35 ± 0.04	0.34 ± 0.05
Right kidney	0.33 ± 0.03	0.34 ± 0.04	0.33 ± 0.03

Data were analyzed by ANOVA.

3.2. Reproductive index

As can be seen in Table 2, the number of pups per litter, the pups body weight and the birth index did not differ between groups. Both, DZ and MD elevated significantly the post-implantation loss index.

Table 2. Reproductive index from dams treated during the organogenic period (6^{th}. to 15^{th}. day of pregnancy) with diazepam (DZ) 10mg/kg/day; midazolam (MD) 5mg/kg/day and control (distillated water) 10ml/kg/day. Data are given as means ± SE or percentage.

Reproductive Index	Control	Diazepam	Midazolam
Number of dams	13	15	14
Number of pups	140	138	130
Pups body weight (g)	5.02 ± 0.36	4.73 ± 0.59	4.79 ± 0.64
Pups/litter	10.7 ± 2.62	9.2 ± 3.17	9.3 ± 3.29
Birth live Index (%)	100	99.27	99.23
Post-implantation loss (%)	1.47	10.76*	13.3*

Data were analyzed by ANOVA and Chi-square test.

* Significantly different ($p<0.05$) from control group.

3.3. Evaluation of embryo-fetotoxic effects

The effects of prenatal exposure to DZ (10mg/kg/day) and MD (5mg/kg/day) on occurrence of fetal skeleton abnormalities are shown in Table 3. There were differences between the control and the groups treated with DZ and MD. The frequency of skeleton malformations was increased specially in MD group. Nonetheless, the higher incidence of skeletal abnormalities observed in MD group seems to been due, to a large extent, to an increase in the occurrence of anomalies such as incomplete metatarsale ossification and fontanel enlarged. Anyhow, the higher incidence of skeleton abnormalities as well as the embryolethal effect (post-implantation loss) clearly indicated that diazepam and MD are embryo-fetotoxic to rats.

Table 3. Occurrence of skeleton abnormalities from dams treated during the organogenic period (6^{th}. to 15^{th}. day of pregnancy) with diazepam (DZ) 10 mg/kg/day; midazolam (MD) 5mg/kg/day and control (distillated water) 10 ml/kg/day.

	CONTROL	DIAZEPAM	MIDAZOLAM
FETUSES EXAMINED (n)	**140**	**138**	**130**
FETUSES WITH SKELETON ABNORMALITIES (%)	**16.42**	**22.46**	**49.21***
INCOMPLETE OSSIFICATION			
ALL SKELETON	**0.71**	**0.72**	**0**
SKULL	**3.57**	**5.1**	**30***
OS PARIETALE	**0**	**0**	**8.46***
OS INTERPARIETALE	**4.28**	**2.9**	**3.84**
OS SUPRAOCCIPITALE	**2.85**	**0.72**	**5.38**
OS ZYGOMATICUM	**0**	**0.72**	**0**
SCAPULA	**0.71**	**0**	**0**
HUMERUS	**0.71**	**0**	**0**
STERNEBRA	**0**	**0.72**	**3.85***
OS PUBIS	**0.71**	**0**	**0**
RIBS	**0**	**0**	**0.77**
OS METACARPALE	**0**	**0.72**	**0**
OS METATARSALE	**7.14**	**18.84***	**33.1***
OTHER ANOMALIES			
FONTANEL ENLARGED	**4.48**	**18.84***	**33.1***
OS INTERPARIETALE DISCONNECTED	**1.43**	**0**	**6.15***
OS SUPRAOCCIPITALE BIFURCATED	**0**	**0**	**2.31**
OS BASISPHENOID OPEN	**0**	**0.72**	**0**
ADDITIONAL RIB	**40.7**	**31.1**	**34.6**
RIBS WAVY	**1.43**	**1.45**	**3.07**
STERNEBRA WITH ADDITIONAL OSSIF. CENTER	**1.43**	**0**	**0**
RADIUS ULNA BENT	**0**	**2.17**	**0**
VERTEBRAE IRREGULAR SHAPED	**0**	**0**	**2.31**

Data were analyzed by Chi-square test. * Significantly different ($p<0.05$) from control.

4. DISCUSSION

The dams did not present any sign of toxicity and neither decrease in maternal weight gain, which is an indirect evaluation of toxicity. Data shows that the relative organs weight didn't differ among the groups. The maternal deaths as well as the decrease in overall weight gain during the pregnancy, are indications of maternal toxicity. These maternally toxic doses of any substance also proved to be embryofeto-toxic as revealed by three outcomes evaluated: embryolethality, prenatal growth retardation and fetal malformations [14].

According to this study, the increase of post-implantation loss index was statistically different among DZ and MD groups compared to the control, without any sign of maternal toxicity. The increase in the post-implantation loss reported here, may have occurred due to a toxic effect of DZ and MD on the embryo.

DZ caused cleft palate in mice at 100mg/kg, which is highly sedative to mothers and is much higher than the human clinical dose [3]. DZ (20 and 80mg/kg/day) administered to female rats during the organogenic period and didn't cause any sign of toxicity [1]. Implantation sites number and reabsorptions were not modified. Fertilization and embryonic development were not affected with the administration of MD to female mouse [15].

The role of maternal toxicity in causing fetal malformations is still a matter of controversy. Khera [6, 7] reviewed the published data and examined the relationship between maternal toxicity, malformation and embryotoxicity. According the author, in the mouse, even malformations as severe as neural tube defects, fused or missing ribs, and fused or scrambled sternebrae could be caused by maternal toxicity [7]. On the other hand, in rats and rabbits, the author found that maternal toxicity was associated with gross structural anomalies such as fused, supernumerary, missing or wavy ribs; fused, missing or split vertebrae, and fused, missing or non-aligned sternebrae [6]. Although most authors do not agree with Khera's conclusions that major malformations (exencephaly and open eyes) can be secondary to maternal toxicity, it is generally accepted that some variations and reversible minor structural anomalies (extra or wavy ribs) could result from maternal toxic

effects. An increased frequency of variations and minor malformations found only at maternally toxic doses does not necessarily reflect the tetarogenic potential of the substance [14].

The groups that received DZ and MD showed a significant increase in number of malformations or anomalies.

The examinations of the skeleton of fetuses are carried out within the terms of reference of the test of chemical substances for embryotoxicity [9]. The author classify deviations from normal found on skeleton as individual variations of normal, developmental retardation of the skeleton and malformations. The transition between these three groups are fleeting and extremely difficult to establish in the particular case whether a certain bone finding is still an individual variation or already a developmental retardation [9].

Most of the skeleton alterations found on DZ and DZ treated groups are developmental retardations of skeleton. According to the results, it's possible to suggest that DZ and MD in the tested doses are responsible to cause these alterations on the fetuses skeleton. The importance of these findings are relevant because the extensive use of the BZ agents in veterinary and human patients.

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