



Journal of Medical Sciences

ISSN 1682-4474

science
alert

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JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

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Teratogenic Effects of Diazepam Intake During Pregnancy Leading to Cleft Lip and Palatal Anomalies

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In this research, it has studied the teratogenicity of Diazepam intake during pregnancy and its effects on cleft lip and palatal development. About 30 virgin rats of known age and weight were used. After pregnancy, they were divided in three groups: The first groups were control group which were injected daily with distilled water. The second groups that were injected daily Diazepam 3 mg kg⁻¹ day⁻¹ and the third injected daily with Diazepam 8 mg kg⁻¹ day⁻¹. After embryonic period the pregnant rats were scarified and the embryos were divided in to three groups. The embryos were studied macroscopically for anomalies and then the tissue were fixed and processed, stained and examined microscopically. Anomalies of cleft lip and palatal were evaluated. The findings confirmed that the ratio of normal eye in the control group is 100% and in the experimental groups (1 and 2) were 97.6 and 71.3%, respectively. The statistical results indicate that diazepam intake during pregnancy can lead to cleft lip and palatal anomalies.

Key words: Teratogenicity, diazepam, pregnancy, visual system defects, virgin rats

INTRODUCTION

Diazepam is a sedative and anti-anxiety drug that has been recently increasingly used (Cannizzaro *et al.*, 2005; Tucker, 1995). In the previous studies, it has been known that the physical and chemical properties of diazepam may have some adverse effects on such developmental phenomena as cell division, mitosis and interaction between cells and thus may have some teratogenic effects on embryo (Cannizzaro *et al.*, 2005; McGrath *et al.*, 1999; Fridman *et al.*, 1994; Shader, 1994). It must be cautiously administered for pregnant woman due to possible side effects. Pregnant woman might use it for pregnancy pica, insomnia and physiologic and neurologic disease, because embryonic period is considered to be the most liable to teratogens (Cannizzaro *et al.*, 2005; Ormay *et al.*, 1998; Tucker, 1995; Simmons *et al.*, 1984). Some authors regard diazepam as a possible teratogen inducing oral clefts and nasal septum anomalies but there are disagreements in this matter (Iqbal *et al.*, 2002a; Tucker, 1995; Simmons *et al.*, 1984). After these reports, drug use has increased and side effects have appeared and with the drug use by pregnant woman for insomnia, anxiety or for the necessity of their illness appeared teratogenic effect of this drug such as cleft lips, cleft palate. It was later established that diazepam could cross the placenta readily, appear in fetus, breast milk and may lead to Cleft Lip and Palate (CLP) (Iqbal *et al.*, 2002b; Katz, 1988). In the present study using light microscopy, we investigate the teratogenic effect of diazepam on the palate development and oral cavity.

MATERIALS AND METHODS

Animal's: As the animal model, the Wistar-Albino strain of the laboratory rat inbred for 96 generations by brother sister litter-male mate mating was used. This strain used for many years in laboratory in Tehran University of Medical Science (2005-2006), has never shown a prediction for spontaneous CLP defects. They were kept in individual cages in a controlled room (temperature, 20-25°C humidity, 70 to 80%, exposed to 12 h of daylight). The rats were fed with standard rat food and tap water until experimentation. Twelve hours before the experiment the rats were stopped feeding but allowed free access to tap water. Limitation of food and water was not applied to the animals that were put into their cages after the experiments.

All experiments were conducted in Tehran University according with the recommendations of the ethics

committee on animal's experimentation of medical school. In present study, 30 virgin rats of known age weighing 250-300 g were selected. Two virgin females were kept with one male for 24 h during that time at intervals of two hours the females were examined for the presence of copulation plug. According to our convention the first 24 h period after finding the copulation plug was regarded as the first day of pregnancy.

Drugs: Diazepam was purchased from Sigma Company (USA). Diazepam was dissolved in distilled water. All the above groups were administered IP on daily scale.

Experimental design: After pregnancy, they were divided in three random groups: Control group was including 10 pregnant rats which were injected daily with distilled water. The first group consisted of 10 pregnant rats that were injected daily with Diazepam 3 mg kg⁻¹ day⁻¹ and the second group included 10 pregnant rats that injected daily with Diazepam 8 mg kg⁻¹ day⁻¹. In this study Wistar rats were treated by intraperitoneally injection (7th-15th) of Diazepam during the organogenesis phase (7th-15th days).

Histological examination: After embryonic period, on the 17th of gestation period all the pregnant rats were scarified and the embryos were divided into three groups. Embryos were treated with fixation solution (Bouin's) for 24 h. Samples were put in paraffin after fixation and undergone tissue passage with H and E. They were then studied under light microscopy for anomalies. Then the embryos were decapped and after decalcification, tissue sections were prepared (Simmons *et al.*, 1984; Iqbal *et al.*, 2002a, b). The sections were haematoxylin-eosin (H & E) For morphometric study stained. A×100 objective was used with ×10 eyepieces.

Statistical analysis: All values were expressed as number and percentage. Statistical significance was determined by using fisher's exact test for paired data. p-value <0.05 was considered significant.

RESULTS

In the present study, the total number of embryos obtained through cesarean section including both live and dead ones were counted. The total number of embryos obtained from 10 pregnant rat in every group were 91 for control, 89 for first group and 87 for second group. From these group, the number of living embryos

were 90, 85, 80, respectively. At the morphological and histological levels, organogenesis and development of the cleft lip feature was noted. Microscopic studies of the frontal sections of the head were performed to investigate if the fusion of palate has occurred. The macroscopic and microscopic results are shown in Table 1. the results were tested the fisher's exact test. Present results show that the frequency of normal lip and cleft was 100, 97.6 and 71.3% in the control, the first and second test groups, respectively. At the significance level of $\alpha = 0.05$ a direct association is seen between drug use and CLP in the embryos. Based on microscopic studies performed on the 17 day embryos obtained through cesarean section, the apparent morphology was seen to be normal. C curvature of the body was maintained and limbs were normal in position and size, head and face, eyes and ears, lips and mouth were normal in position as well. The first test group which were administrated a permitted dose of the drug, showed no aberration form normal and hence no significant difference from the control group. The microscopic studies on the embryos of the second test group showed that the shapes of the body and C curvature were abnormal and deformity was detectable on the face. In addition to the change in the shapes of face, eyes and mouth, some of the subjects were found to have cleft lip. Microscopic and histologic studies of the control and the first test groups that appendices were normally fused and nasal cavity was separated totally form the oral cavity. The mesenchyma was condensed in the middle area and the fusion site and the tongue position was normal too (Fig 1), while microscopic studies of the second test group revealed a number of malformities. A number of the embryos were of cleft lips and palate. In the letter, palate appendices were not normally fused and nasal cavity was abnormal. Many samples of this sort had their primary plate infused with the appendices. The clefts were lateral or bilateral (Fig. 2a and b). The results of the detailed microscopic analyses on the frontal sections of the embryos especially those of oral and nasal cavities are shown in Table 1. The means of the results are plotted (Table 1). the average number of an anomalous samples bearing CLP were drastically different in the second test group, from the other two groups.

Table 1: Perc entage of anomalies from using diazepam administration on 17 days old embryos obtained through cesarean section

Tests groups	Administration	Total of live birth embryos (n)	No. of normal lip and palate	Normal lip and palate percentage
Control group	Distilled Water	90	90	100
First test group	Diazepam 3 mg kg ⁻¹ day ⁻¹	85	83	97.6
Secondary test group	Diazepam 8 mg kg ⁻¹ day ⁻¹	80	57	71.3

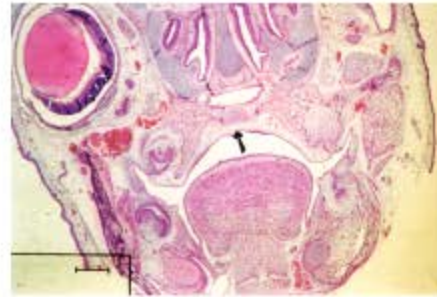
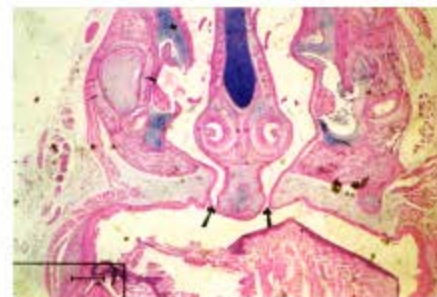
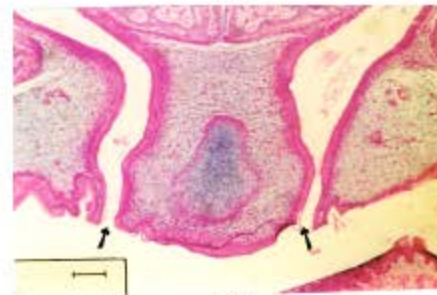


Fig 1: Frontal section of the heads in the 17 day embryos of the control group. Normal fusion of the palate and separation of the oral and nasal cavities are seen. H & E staining, x100 magnitude



(a)



(b)

Fig 2: Frontal section of the heads of the 17 day embryos of the second group. Bilateral cleft palate is shown. H & E staining Fig. 2a. x40 magnitude. Fig. 2b: x100 magnitude

DISCUSSION

Diazepam is a sedative and anti-anxiety drug (Canrizzaro *et al.*, 2005; Toker, 1995; Fridman *et al.*, 1994). Studies show the increased rate of embryonic abnormalities secondary to its high dose administration (Simmons *et al.*, 1984; Cilman, 1990). Both retrospective and longitudinal

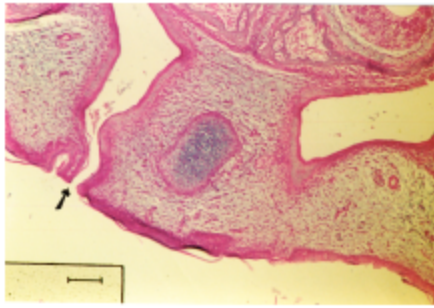


Fig. 3: Lateral cleft palate of second test group. H & E staining, x400 magnitude

studies prove the increased incidence of the neural system disorders, CLP and blindness due to its increased use (Tucker, 1995; Shader, 1994; Simmons *et al.*, 1984; Cilman, 1990; McElhatton, 1994). On the other hand, there are a number of pregnant women that are to use it to counter with anxiety, stress or epilepsy. Occasionally, the drug is used to treat pregnancy pica (Iqbal *et al.*, 2002a, b; Shader, 1994; Cannizzaro *et al.*, 2005; Simmons *et al.*, 1984; Cilman, 1990; Katz, 1988; Salzman, 1999). The drug use could lead to a series of abnormalities at the different dosages. The previous study focuses on the drug effect on the development of lip and mouth. The development of the head and face in human starts at the 4th week of pregnancy equivalent with the 7th to 15th day of the mouse embryogenesis (Ornoy *et al.*, 1998). The period is known to be susceptible to the teratogenic agents. In the embryonic stage, a primary oral-nasal cavity first appears. Thence, fusion of the palate appendices of the palate will separate the nasal and oral cavities. Prominences of the maxillary arise at the lateral region of the primary mouth and those of the mandibles at the caudal region. As the prominences of the mandibles grow, inter jaw segment forms and each part of it forms another one. The edge forms the upper lip, the maxillae encounters the front teethes and the palate constitutes the triangle part of the primary palate. The main part of palate is formed by two protruding from maxillary prominences. These protruding, so called palatal ceiling are deflected downward both sides of the tongue. The palatal ceilings are then pulled upward and finally fused. In present study, diazepam effect on the development of the head and face was investigated by injection of the various doses of diazepam (McGrath *et al.*, 1999; Iqbal *et al.*, 1994a; Ornoy *et al.*, 1998; Johnson *et al.*, 1995). The results of the statistical analyses showed that the frequency of the normal lip in control group was 100%. In the second test group which was administrated $8 \text{ mg kg}^{-1} \text{ day}^{-1}$ of diazepam, the frequency of the normal lip and palate was 71.3%. This

data shows a direct association between the diazepam use and the onset of CLP ($p < 0.001$). Thus the administrated dose of $8 \text{ mg kg}^{-1} \text{ day}^{-1}$ induces lip and palate abnormalities. As a result, use of diazepam over the permitted dose in the liable period of pregnancy would stimulate the embryonic tissues and disturb their normal development failure in the fusion of palate ceiling leads to the cleft palate (Fig 2a, b and 3). Cleft palate seems to be due to the decrease in the palatal ceiling sizes. In the subjects with cleft palate, tongue is not properly positioned and isn't lowered suitably. On the other hand, the growth of the middle nasal septum is among the most important factors in the junction of the palate appendices (Soumerai *et al.*, 2003; Johnson *et al.*, 1995). A statistically significant different is seen between the second test group and the control group. Following the increase in the dose of drug, the increase incidence of CLP is observed. This proves a dose-dependent onset of the abnormally. The comparisons of the mean difference between second test group and other groups reveals the high-dose administration of diazepam in the vulnerable stage of pregnancy at the permitted dose had no observable adverse effect on the first test group. As a conclusion, it appears that its administration for the pregnant mothers obliged to use it at permitted doses and considering the logical consumption pattern wouldn't cause any problem to the pregnant mothers and their embryos. It must be noted, however, it may be used as soon as its advantages outweigh disadvantages.

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