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A N I N T E R N A T I O N A L J O U R N A L

A Model for the Study of Skeletal Anomalies in Rat Fetuses

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ABSTRACT

The aim of this study was to validate a model of skeletal anomalies in rat fetuses by the administration of ketoconazole (80 mg/kg) to pregnant rats during organogenesis. Bones of the head, trunk and anterior and posterior limbs were examined for detection of anomalies. Statistical differences regarding the number of fetuses and postimplantation resorptions, and fetal and placental weight were significant. The frequency of skeletal anomalies in the head, trunk, and anterior and posterior limbs in the ketoconazole-treated group were also significant when compared to the control group. It could be concluded that the model suggested was valid for study of skeletal anomalies and abnormal bones development in rat fetuse, in spite of the loss of fetuses due to resorptions.

Key words: Azol Antifungals, Ketoconazole, Models, Pregnancy, Skeletal Malformation, Steroids, Teratogenesis, Wistar Rats

INTRODUCTION

The use of ketoconazole during pregnancy is not safe either for humans or for rat fetuses. Ketoconazole, an azol antifungal agent, is embryotoxic and teratogenic when administered in rats during organogenesis at the dose of 80 mg/kg (Briggs et al., 1998), a dose ten times greater than the recommended dose for treatment of mycoses in adults (McGregor and Pont, 1984; King et al., 1998). It causes skeletal malformation in rat fetuses described as syndactyly and oligodactyly (Briggs et al., 1998; Buttar and Moffat, 1989). On the other hand, ketoconazole is a well-known enzymatic inhibitor. It inhibits steroidogenesis in humans (Iranmanesh et al., 1987; Engelhardt et al., 1991), mouse (Kowal, 1983) and in *in vitro* models (Nagai et al., 1986), leading to a reduction of the synthesis of glucocorticoids (Iranmanesh et al., 1987; Engelhardt et al., 1991; Kowal, 1983; Nagai et al., 1986), testosterone (Albertson et al., 1994; Kowal, 1983; Sikka et al., 1985), estrogens (Cummings et al., 1997) and progesterone (Engelhardt et al., 1991; Watanabe and Menzies, 1985). We hypothesized that the ketoconazole-induced skeletal anomalies in rat fetuses might be due to its action in the synthesis of steroids hormone. However, the exact mechanism of teratogenicity induced by ketoconazole has not yet been elucidated. Additionally, we did not find any model in the literature that described a methodology for the study of skeletal anomalies in rat fetuses using ketoconazole. The aim of this study was to validate a model for the study of skeletal malformation in rat fetuses administering ketoconazole at teratogenic doses in pregnant dams during organogenesis.

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MATERIALS AND METHODS

Animals

The approval for the use of animals and for the procedures required for the experiments was obtained by the Ethical Committee for Animals Use in Experimental Studies of the University of Goiás. Female Wistar rats (nulliparous, 2 - 3 month old, 180 - 220 g, sexually mature, n = 28) and males of the same species and age (280 g approximately, n = 6) were obtained from University of Goiás Animal Center. All animals were house-caged for two weeks in a 12 h light/dark cycle room with temperature adjusted to 22 ± 2 ^{o}C before the experiments. The animals had free access to Purina lab chow and water ad libitum during this period. The female rats were then randomly assigned to control group (n=12) and experimental group (n=16).

Mating procedure

After the two weeks of adaptation period, female rats were submitted to daily vaginal smear evaluations every morning. The detection of the pro-estrus in the vaginal smear indicated fertility and one male and female rat were placed together in same cage for 24 h. On the next morning, the microscopically detection of spermatozoa at the vaginal smear indicated that the rats had mated and this was considered the day zero of gestation.

Drugs and treatment

Ketoconazole, gifted by Janssen Cilag Pharmaceutical Inc., was used for the treatment of the pregnant dams of the experimental group. The control group received saline 1ml/kg. A ketoconazole suspension (20mg/ml) was prepared with the help of a few drops of Tween 80. The pregnant dams were treated either with ketoconazole (80 mg/kg) by gavage or with saline (1 ml/kg) from the 6th to the 15th gestational day.

Evaluation of the pregnancy

Once pregnancy was established, weight, and food and water intake was daily recorded, but colonic temperature was recorded on a weekly basis. These measures were taken to avoid any cause of skeletal malformation other than the drug administration. Any clinical sign that might indicate disturbance of pregnancy was also recorded.

Uterus contents and ovaries examination

On the 21st day of gestation, after complete anesthetization by ethyl ether inhalation, the dams were placed on an experimental table and the peritoneal cavity was opened. The intact uterus with the ovaries attached to it was removed. Ovaries were dissected free and the number of corpora lutea seen under a dissecting microscope in both ovaries was recorded. Uteri were dissected longitudinally and the number of live fetuses, number of dead fetuses (absence of movement when touched with no visible degeneration), number of postimplantation resorptions (degenerating recognizable dead fetuses or implantation site but no recognizable fetus), and total implantation sites were recorded. The placenta and each fetus (live or dead fetuses and resorptions) was then detached, weighed, and recorded. The weight of the live fetuses was recorded as well. The number of preimplantation resorptions was calculated subtracting the total number the corpora lutea in both ovaries by the number of implantation sites (all carcasses of adult females were discarded).

Fetal preparation

At the end of caesarian procedure, the fetuses were prepared either for skeletal or visceral examination. Half of the fetuses were assigned to visceral evaluation using Bouin's solution as fixative but no visceral anomalies were detected but cleft palate in some fetuses. Therefore, data regarding these fetuses were discarded. Regarding skeletal evaluation, right after weighting each fetus, they were placed in a dish containing 95% ethanol solution for dehydration, where they remained for four days and they were subsequently placed in another dish containing ethanol 100% for additional four days to complete dehydration. After dehydration was completed, the fetuses were rinsed in distilled water and eviscerated. Their fat pad between the scapula and the vertebrae was removed through a dorsal midline incision. This incision ran from the base of the skull to the midthorax and penetrated down to the spine column, but not into it.

Alizarin red staining

The dehydrated, eviscerated fetuses were placed in a 2% KOH solution to clear the nonqualified tissues. After twenty-four hours in this solution and when the noncalcified tissues were clear and bones were cream- to buff-colored, they were

placed in a dish filled with 0.5 % KOH solution-containing Alizarin red (enough to obtain the purple color) for another twenty-four hours for staining. At the end of the staining period the fetuses were drained and placed in a 25 % glycerol solution for one week and, subsequently, in a 100% glycerol with a few drops of 0.5 phenol solution for preservation.

Examination of the fetal skeleton

Skeletal examination of all fetuses was made under a dissecting microscope. Anomalies such as absence, abnormal shape and size of the bones were recorded. To facilitate the examination, it was divided into four parts. Examination of the head, which included evaluation of the cranial, facial and palatal bones; examination of the trunk, in which anomalies of the vertebrae, ribs and sternum were recorded; examination of the bones of the forelimbs, including the bones of the metacarpus and phalanges, and examination of the bones of the hindlimbs, which, similarly, included examination of the metatarsal and phalangeal bones. Data recorded were analyzed considering anomalies found in fetuses and litters affected.

STATISTICAL ANALYSIS

Data on maternal body weights, food consumption and water intake, fetal and placental weights and number of pre- and postresorptions were subjected to unpaired Student's t test. Skeletal anomalies regarding the fetuses and litter examinations were evaluated by Fisher's exact test. $P < 0.05$ was set as the acceptable level of significance.

Table 1 - Reproductive performance of female Wistar rats administered saline or ketoconazole 80 mg /kg by gavage during organogenesis.

Parameters	Control	Ketoconazole
Pregnant dams/group	12	16
Dead dams prior to the cesarean	0	4
Dams bearing live fetuses	12	5
Pregnant dams with no fetuses	0	7
Number of fetuses	128	34
Number of fetuses for skeletal examination	62	16
Weight gain during pregnancy (g)		
Implantation (0–5 GD)	17.7 ± 2.1	17.8 ± 2.9 *
Organogenesis (6–15 GD)	26.8 ± 5.8	8.5 ± 10.5 *
Fetal development (16–21 GD)	59.3 ± 16.4	24.8 ± 26.0
Water intake (ml/kg/day)		
Implantation (0–5 GD)	39.4 ± 9.5	39.7 ± 12.3
Organogenesis (6–15 GD)	41.9 ± 11.6	33.6 ± 12.2 *
Fetal development (16–21 GD)	47.9 ± 10.0	43.2 ± 11.0 *
Food intake (g/kg/day)		
Implantation (0–5 GD)	18.3 ± 2.9	17.3 ± 5,0
Organogenesis (6–15 GD)	20.0 ± 4.8	17.4 ± 13.6 *
Fetal development (16–21 GD)	22.2 ± 3.6	18.8 ± 4.8 *
Number of fetuses/litter	10.5 ± 2.1	2.8 ± 4.0 *
Placental weight (g)	0.62 ± 0.09	1.10 ± 0.36 *
Fetal weight (g)	5.00 ± 0.58	4.50 ± 0.80 *
Number of preimplantation resorptions	1.00 ± 1.04	1.41 ± 1.16
Number of postimplantation resorptions	0.75 ± 0.86	6.42 ± 2.46 *

Data are expressed in means ± DP. Statistics: unpaired Student's t test. * $P<0.05$. GD means gestational day.

Table 2 - Prevalence of skeletal alterations found in fetuses of Wistar rats administered saline or ketoconazole 80mg/kg by gavage during organogenesis.

Parameter	Control	Ketoconazole
Head		
Palatal malformation	0% (0/62)	62.5 % (10/16) *
Absence of tympanic bulla	0% (0/62)	31.2 % (5/16) *
Left	0% (0/62)	18.7 % (3/16) *
Right	0% (0/62)	12.5 % (2/16) *
Trunk		
Rudimentary rib in the first lumbar vertebrae [a]	30.64% (19/62)	81.25% (13/16) *
Left	14.51% (9/62)	37.50% (6/16)
Right	16.12% (10/62)	47.75% (7/16) *
Supranumerary rib in the first lumbar vertebrae [b]	1.61% (1/62)	59.37% (19/32) *
Left	1.61% (1/62)	62.50% (10/16) *
Right	0% (0/62)	56.25% (9/16) *
Bones in the sternum		
6 Bones	91.93% (57/62)	50.00% (8/16) *
5 Bones	8.06% (5/62)	37.50% (6/16) *
4 Bones	0% (0/62)	12.50% (2/16) *
Forelimbs		
Phalanges		
8 bones	62.00% (38/62)	0% (0/16) *
6 bones	4.83% (3/62)	25.00% (4/16) *
4 bones	16.12% (10/62)	37.50% (6/16)
Absence	17.74% (11/62)	37.50% (6/16)
Hindlimbs		
Metatarsal bones		
10 bones	69.35% (43/62)	62.50% (10/16)
8 bones	30.64% (19/62)	37.50% (6/16)
Phalangeal bones		
10 l bones	54.83% (35/62)	0% (0/16) *
6 bones	1.61% (1/62)	0% (0/16) *
Absence	43.54% (27/62)	100.00% (16/16) *
Fibula (reduced size)	0% (0/62)	25.00% (4/16) *
Femur (reduced size)	0% (0/62)	12.50% (2/16) *

[a] rudimentary ribs were detected only in one side of the first lumbar vertebrae [b] supranumerary ribs were detected either in both or only in one side of the first lumbar vertebrae. Data are expressed in percentage and the number of fetuses affected/total number of fetuses between parentheses. Statistics: Fischer's exact test. * $P<0.05$

RESULTS

Results showed that ketoconazole induced maternal toxicity, evidenced by maternal death and reduced weight gain in the animals that received ketoconazole during organogenesis (Table 1). Embryotoxicity and fetotoxicity was also observed as shown by the increase of the number of resorptions and fetal weight, respectively. Interestingly, an increase of the placental weight was also observed in the ketoconazole group. Several skeletal anomalies were observed in the animals treated with ketoconazole (Fig. 1). The majority of the parameters evaluated were statistically significant when comparisons between the two groups studies were performed, leading to the conclusion that ketoconazole induced more skeletal anomalies than the control group.

Significant anomalies were found in all parts of the skeleton examined, as head, trunk, forelimbs and hindlimbs, when compared to the control group. The highest prevalence of anomalies in the ketoconazole-administered animals were absence of phalanges in the hindlimbs (100%), rudimentary ribs (81.25%) and supranumerary ribs (59.37%) in the first lumbar vertebrae, palatal malformation (62.50%), reduced number of the six sterna bones (50%), and absence of the forelimbs phalanges (37.5%) (Table 2). Statistical comparisons between litters from the two groups showed that the number of litters affected by skeletal anomalies was higher in the ketoconazole treated group. Skeletal anomalies in the palate and trunk were the major significant anomalies found in the litters of the ketoconazole treated animals (Table 3).

DISCUSSION

The high prevalence of skeletal malformation in the ketoconazole-treated group made this model appropriate for the study of skeletal malformation. The types of skeletal anomalies that could be studied using this model were palate malformation, absence of bones in both limbs and bones of the hindlimbs with reduced size. As the number of bones in the sternum, forelimbs and hindlimbs were reduced in the animals of ketoconazole-treated group, wich suggests delayed development of the trunk and limbs, suitability for the study of the mechanisms involved in the development of fetal bones could also be considered for this model. However, the mechanisms of skeletal malformation are not yet clear.

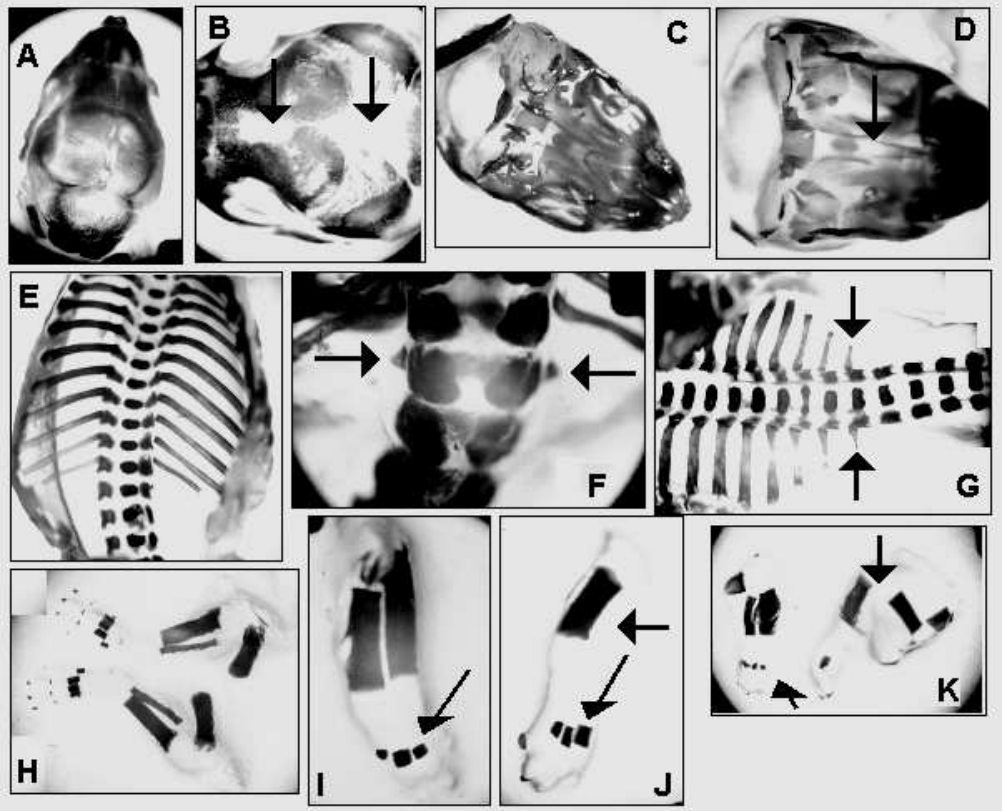

Figure 1 - Examples of skeletal anomalies in rat fetuses of dams administered ketoconazole 80 mg/kg during organogenesis.
The bones were stained with Alizarin red and photographed under a dissecting microscope. A normal skull bones; B unossified bones of the skull; C normal palate; D cleft palate, E normal ribs, F and G supranumerary ribs; H normal hind limbs; I only three metatarsal bones; J and K absence of fibula and absence of phalangeal bone.

Azol antifungals, like ketoconazole, in spite of their antifungal properties, are also inhibitors of the steroidogenesis enzymes. Glucocorticoids (Iranmanesh et al., 1987; Engelhardt et al., 1991; Kowal, 1983; Nagai et al., 1986), progesterone (Cummings et al., 1997), testosterone (Sikka et al., 1985) and estrogens (Watanabe and Menzies, 1985) are steroids inhibited by ketoconazole. The skeletal malformation found might be related to the ketoconazole-induced inhibition of steroidogenesis enzymes.

The model proposed here would be appropriate for the study of the mechanisms involved in the skeletal malformation induced by ketoconazole, as well as the role of steroids hormones in skeletal anomalies. The maternal toxicity observed in this experiment could be a disadvantage of the model proposed because it implied in difficulty in achieving the number of fetus necessary for statiscal analysis.

Although this maternal toxicity might be related to the decrease of food and water intake during organogenesis, it seemed more suitable to attribute this toxicity to the ketoconazole itself, once ketoconazole also provoked embryotoxicity, as observed in our experiment. In fact, there are some reports in the literature that show ketoconazole-induced embryotoxicity (Briggs et al., 1998; Buttar and Moffat, 1989). We concluded that in spite of the maternal toxicity of ketoconazole, which might be related to the effects of ketoconazole in skeletal malformation in fetuses from dams treated with ketoconazole, the administration of ketoconazole 80 mg/kg by gavage in pregnant dams during organogenesis, would be an appropriate model for the study of the role of steroids hormones in skeletal malformation in fetuses, and for the study of skeletal malformation itself in rat fetuses as well.

Table 3 - Prevalence of skeletal alterations found in litters of Wistar rats administered saline or ketoconazole 80mg/kg by gavage during organogenesis.

Parameter	Control		Ketoconazole	
Head				
Palatal malformation	0%	(0/12)	100.0 %	(5/5) *
Absence of tympanic bulla	0%	(0/12)	20.0 %	(1/5)
Left	0%	(0/12)	20.0 %	(1/5)
Right	0%	(0/12)	20.0 %	(1/5)
Trunk				
Rudimentary rib in the first lumbar vertebrae [a]	58.4%	(7/12)	80.0%	(4/5)
Left	58.4%	(7/12)	60.0%	(3/5)
Right	58.4%	(7/12)	80.0%	(4/5)
Supranumerary rib in the first lumbar vertebrae [b]	8.4%	(1/12)	100.0%	(5/5) *
Left	8.4%	(1/12)	100.0%	(5/5) *
Right	0%	(0/12)	100.0%	(5/5) *
Numbers of bones in the sternum				
6 bones	100.0%	(12/12)	20.0%	(1/5) *
5 bones	25%	(3/12)	60.0%	(3/5)
4 bones	0%	(0/12)	20.0 %	(1/5)
Forelimbs				
Phalanges				
8 bones	91.6%	(11/12)	0%	(0/5) *
6 bones	25.0%	(3/12)	40.0%	(2/5)
4 bones	25.0%	(3/12)	40.0%	(2/5)
Absence bones	41.7%	(5/12)	60.0%	(3/5)
Hindlimbs				
Metatarsal bones				
10 bones	91.6%	(11/12)	80.0%	(4/5)
8 bones	66.7%	(8/12)	20.0 %	(1/5)
Phalangeal bones				
10 bones	75.0%	(9/12)	0%	(0/5) *
6 bones	8.4%	(1/12)	0%	(0/5)
Absence of bones	75.0%	(9/12)	100.0%	(5/5)
Fibula (reduced size)	0%	(0/12)	40.0%	(2/5)
Femur (reduced size)	0%	(0/12)	20.0%	(1/5)

[a] rudimentary ribs were detected only in one side of the first lumbar vertebrae [b] supranumerary ribs were detected either in both or only in one side of the first lumbar vertebrae. Data are expressed in percentage and the number of litters affected/total number of fetuses between parentheses. Statistics: Fischer's exact test. * $P<0.05$.

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RESUMO

O cetoconazol, uma droga que inibe a esteroidogênese, provoca teratogênese em fetos de ratos quando administrado em altas doses durante a organogênese. Entretanto, o mecanismo das

malformações esqueléticas induzidas pelo cetoconazol não é claro e não existe nenhum método descrito na literatura que permita estudar os mecanismos envolvidos nas malformações esqueléticas induzidas pelo cetoconazol. O objetivo deste estudo é validar um modelo de malformações esqueléticas em fetos de ratos através da administração de cetoconazol 80 mg/kg durante a organogênses a ratas prenhes. Os ossos da cabeça, do tronco e dos membros anteriores e posteriores foram examinados para a detecção de anomalias. Foram encontradas diferenças estatísticas no número de fetos, de reabsorções pós-implantação e nos pesos dos fetos e das placentas. As freqüências de anormalidades esqueléticas na cabeça, no tronco e nos membros anteriores e posteriores no grupo tratado com cetoconazol também foram significantes quando comparadas às do grupo controle. Concluímos que o método sugerido é válido para o estudo de malformações esqueléticas e desenvolvimento anormal dos ossos em fetos de ratos, apesar da grande perda de fetos decorrente das reabsorções.

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