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Evaluation of the Teratogenic Potential of Pyrazinamide in Wistar Rats

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ABSTRACT

We have tested Pyrazinamide (PZA), an essential component of modern short-course tuberculosis treatment regimen, for teratogenicity using Wistar rats. The drug was given by oral intubation from 6-15 days of gestation, at doses of 0, 25, 100 and 500 mg/kg body weight per day. Reduction in body weight and food consumption were observed in the treated dams. On day 20 of gestation, all the dams were killed by cervical dislocation and signs of maternal toxicity, reproductive indices and fetal measurements were recorded. Dams given doses of 100 and 500 mg/kg had significantly higher incidence of reabsorbed fetuses, reduced litter size, and impaired neonatal growth than those given no PZA or only 25 mg/kg dose. External visceral and skeletal examination of all fetuses of PZA-treated dams showed several types of variations which were neither dose related nor having a consistent pattern. However, these variations occurred mostly in the dams treated with the dose of 500 mg/kg.

In conclusion, these data show that in Wistar rats, only high doses of PZA (100 and 500 mg/kg) produced fetotoxicity. No evidence of teratogenic effect of the drug was observed.

INTRODUCTION

Tuberculosis remains a major health problem in most developing countries with an increasing incidence in many developed countries [27-29]. Although pregnant females presenting with tuberculosis are not normally evaluated differently from non-pregnant patients, attention has always been drawn to the use of appropriate chemotherapy, in order to treat the disease in the mother and to reduce the possible adverse effects of the drugs on the fetus [15,29-30].

Tuberculosis cases can be successfully treated with the drugs known as "first-line" agents i.e. isoniazid, rifampicin, and ethambutol [5]. These drugs have been used in pregnant women and were reported to be safe on the fetus, except for few case reports of adverse effects [22,33]. Streptomycin and other "second-line" drugs, e.g. ethionamide, prothionamide and capreomycin have been associated with teratogenic effects [1,11,23].

Pyrazinamide (PZA) has long been used as a second-line drug for tuberculosis and was associated with severe adverse effects particularly hepatotoxicity [14,32]. However, over recent years, and at a lower dose than previously used, this drug has become an essential component of the first-line drugs reducing the duration of treatment from nine months to six months with a much lower incidence of adverse effects than before [24,26]. Despite this change, there is a paucity of reports in the medical literature on the adverse effects of PZA on the fetal and neonatal health [17,20]. Furthermore, no well documented attempts have been made to study the teratogenecity of PZA in experimental animals [19,25]. However, more recently, Davidson and Le [10] have reported their own clinical experience indicating that PZA could be used safely during pregnancy and recommended it for short course anti-tuberculosis treatment.

Because of the lack of teratogenecity data of PZA, recommendations to use this drug in pregnancy is still somewhat uncertain. Therefore we set out to investigate the teratogenic potential for PZA when given during the organogenetic period of pregnancy in normal Wistar rats.

MATERIAL AND METHODS

PZA (Pyrazionic acid, Lot # 51H 2512) was obtained from the Sigma Chemical Company (St. Louis, MO, USA). It is diluted with physiological normal saline to yield the appropriate concentrations required for the experiment. All the other chemicals used in this study, such as Alizarin Red-S, ethyl alcohol, potassium hydroxide and glycerin were obtained from Fisher Scientific Corporation (Fairlawn, NJ, USA).

Untreated sexually mature rats of Wistar strain of both sexes were obtained from the medical college experimental animal house, Riyadh. Animals weighing 180-200 g at the time of mating were acclimated to laboratory conditions for one week, and were fed on commercial lab chow from Ralston Purina Company (St. Louis, MO, USA) and tap water ad libitum. The room environment was controlled with a temperature range of 24°C $\pm$ 1°C, a humidity of 40-70% and a 12 hr light/dark cycle (light from 06:00 to 18:00 hr). All animals were observed for indications of drug toxicity from the beginning of the experiment until being killed.

In each cage, one male rat was housed with two females overnight. The occurrence of copulation was determined by daily inspection for a copulatory plug or a vaginal smear showing sperms. The day in which that evidence of mating was detected was designated as day 1 of gestation and the pregnant females were then placed in an individual cage.

The pregnant dams were divided into four groups (14-15 animals each). Groups 2, 3, 4 were given a suspension of PZA at doses of 25, 100, and 500 mg/kg body weight respectively. The drug was administered daily by the oral route from day 6-15 of gestation. Group 1 dams served as controls and received normal saline by the same route as in the experimental groups.

The drug doses were adjusted daily with the changes of animal weight. The dose levels selected were in the same range as those previously used in other studies, and the highest dose in the present study was reported to be the maximum tolerable dose [4].

Maternal body weights and food consumption (g/day) were measured daily throughout the duration of the pregnancy. At the end of the experiment, the food efficiency index (FEI) which is the ratio of body weight gain to the amount of food consumed during the same period, was calculated.

On day 20 of gestation, the dams were killed by cervical dislocation. The ovaries and uteri were removed by a cesarean section and fetal toxicity was assessed as described in previous studies [3,18]. The number of viable and non-viable fetuses, resorptions, implantations, and corpora lutea were recorded. After separating the live fetuses from their amniotic and yolk sac membranes, they were individually weighed, sexed externally, and measured for crown-rump length (CRL), and tail length (TL). Each fetus was examined under an illuminating glass for external malformations and abnormal variations. Fetal wastage rate (FWR) was calculated as: (Number of resorptions + Number of dead fetuses)/Number of implants x 100.

Ten pups from each group were randomly taken for organ weight change study. The brain, liver, spleen, heart and kidneys of each pup were weighed at necropsy. Two thirds of the remaining fetuses from each litter, which were randomly selected, were fixed in Boin's solution and examined by the Wilson razor sectioning technique [35]. The one-third remaining of the fetuses were stained with Alizarin Red-S and Alcian Blue, in order to examine skeletal development and fetal ossification as indicated by differential staining methods [2].

The results of food consumption and maternal weight gain were tested by ANOVA. Data were statistically analysed using the litter as a unit. Fetal growth and developmental data including body weight, CRL, TL, growth index and fetal organ weight changes were analyzed by Student's t-test. Resorptions, intra-uterine deaths, fetal wastage and external visceral and skeletal variations were evaluated using Chi-Square with Yate's correction. Results were expressed as mean $\pm$ SEM and significance was set at $P < 0.05$.

RESULTS

Table 1 shows that the food efficiency index (FEI) was significantly reduced in the PZA-treated groups 3 and 4 compared to group 2 and the control group (group 1). During days 6-15 of gestation, at doses of 100 and 500 mg/kg, the FEI decreased by 55.4% and 70.3% respectively as compared to the control group. However, during the same period, the dose of 25 mg/kg dose did not affect this index. At the advanced stage of gestation (days 15-20) only the high dose of PZA (500 mg/kg) led to a significant decrease in the FEI compared to the control group.

Table 1. Effect of different doses of PZA (in mg/kg body weight) adminstered daily from days 6-15 of gestation on weight gain and food consumption of the dams (mean ± SEM).

Observations	0 (Group 1)	25 (Group 2)	100 (Group 3)	500 (Group 4)
No. of dams used	15	15	15	15
Rate of pregnancy (%) ♣	12/15 (80)	11/15 (73)	12/15 (80)	11/15 (73)
Maternal weight gain (g)				
Day 6-15	41.25 ± 6.17	41.25 ± 10.54	21.87 ± 1.12**	12.87 ± 2.91**
Day 15-20	22.37 ± 4.61	27.84 ± 2.65	36.42 ± 4.34	16.87 ± 3.17
Day 1-20	81.00 ± 9.31	85.87 ± 12.31	102.12 ± 6.00	54.17 ± 3.40*
Food consumption(g/rat/day)				
Day 6-15	3.37 ± 1.05	3.00 ± 0.53	1.50 ± 0.42	1.62 ± 0.56
Day 15-20	1.87 ± 0.22	4.37 ± 1.01*	6.00 ± 1.14**	5.12 ± 1.32*
Day 1-20	5.37 ± 1.60	10.42 ± 4.92	3.50 ± 1.00	4.37 ± 0.67
Food efficiency index (FEI) ♣♣				
Day 6-15	2.96 ± 0.44	2.91 ± 0.74	1.32 ± 0.06**	0.88 ± 0.10**
Day 15-20	1.54 ± 0.30	1.96 ± 0.18	3.17 ± 0.20	1.28 ± 0.27*
Day 1-20	6.22 ± 0.71	7.93 ± 1.14	7.65 ± 0.51	4.93 ±1.29

♣ Rate of pregnancy = No. pregnant /No. bred.

♣♣ FEI body weight increase /amount of food consumed during the same period of time.

* As compared with the control group 1, $p < 0.05$, ** As compared with the control group 1, $p < 0.01$.

Table 2 summarises the reproduction status of the female rats. Neither mortality nor clinical signs indicating maternal toxicity were observed. The number of live fetuses per litter was significantly decreased in group 4 compared to the control group. The toxicity of PZA is further confirmed by its effect on fetal resorptions and intra-uterine deaths. Compared to all other groups, fetuses of group 4 dams had much higher percentage of resorptions (26.44%) $P < 0.01$. Furthermore, Groups 3 and 4 had significantly higher percentage of intra-uterine deaths (30.4% and 62.8% respectively) when compared to groups 1 and 2 (6.45% and 7.43% respectively). Subsequently the calculated fetal wastage rate (FWR) is shown to be increased in groups 3 and 4 compared to groups 1 and 2 ($P < 0.001$). Doses of 25 mg/kg and 100 mg/kg had similar effect on the percentage of resorption as the control group. The 25 mg/kg dose had no statistically significant effect on the percentage of intra-uterine-death or FWR when compared to the control group.

The effect of PZA on somatometric measurements at birth is presented in table 3. There was no significant difference in the male/female ratio, CRL or TL measurements of the pups when the control and treated groups were compared. Only the high dose of PZA (500 mg/kg) led to 32.4% reduction in the mean body weight of the pups ($P <0.05$) compared to the control group.

This dose (500 mg/kg) also caused significant ($P < 0.05$) reduction (88.8%) in the growth index which is calculated as the mean weight/pup/litter X mean CRL/pup/litter X litter size divided by 100.

Table 4 shows the incidence of external visceral and skeletal malformations among the offspring of the PZA - treated dams. The incidence of microcephaly and runts was significantly higher in group 3 compared to all other groups including group 4, and the incidence of dilated renal pelvis was significantly higher in group 3 and 4 compared to the control group. Only the PZA dose of 500 mg/kg led to higher incidence of haematoma, incompletely ossified or shortened premaxilla and maxilla, missing 4th and 5th sternal bones, 14th unilateral and fused ribs, and mispositioned clavicle compared to the control group.

Table 2. Effect of different doses of PZA (in mg/kg body weight) adminstered daily from days 6-15 of gestation on fetuses in the dams (mean ± SEM).

Observations	0 (Group 1)	25 (Group 2)	100 (Group 3)	500 (Group 4)
No. of implants per litter	10.33 ± 0.58	9.90 ± 1.74	11.75 ± 0.98	13.63 ± 1.18*
No. of live fetuses per litter	9.41 ± 0.95	7.90 ± 0.98	8.16 ± 1.13	5.09 ± 0.30**
Percentage of resorptions	2.41	0.91	1.41	26.44***
Percentage of intra-uterine deaths	6.45	7.43	30.4***	62.80***
Fetal wastage rate (FWR) ♣	5.64	8.44	29.78***	62.83***

* As compared to group 1, ($p < 0.05$), ** As compared to group 1, ($p < 0.01$), *** Each compared to group 1, ($p < 0.001$),
♣ FWR = (No. of resorptions + no. of dead fetuses)/No. of implants x 100.

Table 3. Effect of different doses of PZA (in mg/kg body weight) adminstered daily from days 6-15 of gestation on growth and development of the rat pups (means ± SEM).

Observations	0 (Group 1)	25 (Group 2)	100 (Group 3)	500 (Group 4)
No. of pups born	119	90	98	56
Male/female ratio of live pups	54/65 (0.83)	48/42 (1.14)	54/44 (1.22)	34/22 (1.54)
Body weight (g)	2.96 ± 0.16	3.32 ± 0.90	2.79 ± 0.17	2.00 ± 0.31*
Crown-rump-length (cm)	2.50 ± 0.27	2.82 ± 0.31	2.27 ± 0.32	1.52 ± 0.52
Tail length (cm)	1.41 ± 0.03	1.39 ± 0.02	1.45 ± 0.04	1.32 ± 0.03
Growth index	0.63 ± 0.05	0.76 ± 0.12	0.55 ± 0.09	0.07 ± 0.02*

* As compared to group 1, $p < 0.05$.

Table 4. Effect of PZA (in mg/kg body weight) adminstered daily from 6-15 of gestation on external, visceral and skeletal development of pups (mean±SEM)

Observations	0 (Group 1)	25 (Group 2)	100 (Group 3)	500 (Group 4)
External Examination:				
No. of fetuses examined	119	90	98	56
No. of fetuses with anomaly	7	16	17	19
No. of fetuses and (%) with:				
Hematoma	3 (2.56)	0	10 (10.20)	10 (17.85)**
Runt	1 (0.85)	3(3.26)	6 (6.12)*	3 (5.35)
Microcephaly	2 (1.70)	3 (3.26)	10 (10.26)*	3 (5.36)
Crooked tail	2 (1.70)	5 (5.43)	5 (5.10)	7 (12.50)*
Short tail	7 (7.60)	5 (5.43)	5 (5.10)	7 (12.50)
Visceral Examination:				
No. of fetuses examined	67	62	62	34
No. fetuses with anomally	5	10	16	20
No. of fetuses and (%) with:				
Dilated renal palvis	5 (7.46)	6 (9.83)	12 (19.35)*	10 (29.41)***
Distended pelvis	5 (7.46)	4 (6.55)	4 (6.45)	10 (24.41)***
Skelatal Examination:				
No. of fetuses examined	50	30	30	16
No. of fetuses with anomaly	5	8	8	11
No. of fetuses and (%) with:				
Incompletely ossified				
Premaxilla	0	0	0	3 (18.75)***
Maxilla	0	0	0	3 (18.75)***
Shortened				
Premaxilla	0	0	0	3 (18/75)***
Maxilla	0	0	0	3 (18.75)***
Sternbrae				
Missing 4th	3 (6.0)	5 (16.67)	5 (16.67)	5 (31.25)***
Missing 5th	3 (6.0)	5 (16.67)	6 (16.67)	6 (37.50)***
Ribs				
14th unilateral	2 (4.0)	3 (10.0)	2 (6.67)	4 (25.00)***
Fused	0	0	0	2 (12.50)***
Clavicle mispositioned	0	0	0	2 (12.50)***

* As compared to group 1, $p < 0.5$, ** As compared to group 1, $p < 0.01$, *** As compared to group 1, $p < 0.001$.

DISCUSSION

Compared to the non-treated group, Wistar-rat dams treated with PZA had similar rate of pregnancy and no excess fetal mortality. However, maternal toxicity was evident by significant lower food consumption and smaller body weight during pregnancy than the control group. Whether the reduced food intake in the PZA-treated animals contributed to the fetotoxicity is unclear. The role of maternal nutrition was assessed by calculating the food efficiency index (FEI), a measure of food utilization [7]. The significant alteration in FEI during gestation suggests that maternal nutrition may have been a factor in reducing maternal weight gain, and it is possible that the reduced food intake in the treated dams was further aggravated by anorexia, a well known side effect of PZA [9,16]. Our data corroborate a previous study on the deleterious effect of PZA on food intake and weight gain in PZA-treated rats which was found to be due to its hepatotoxic and nephrotoxic effects [38].

The significant increase in the number of implantations per litter in the animals given the high dose of 500 mg/kg (Table 2) compared to the control group had probably not resulted from the administration of PZA, because the drug was started from day 6 of pregnancy, after the completion of implantation. Similarly, the observed fetal resorptions were generally complete or only with small amount of macerated tissues indicating that they probably occurred during early stages of pregnancy. These results suggest that only high doses of PZA (i.e. 500 mg/kg and to a lesser degree 100 mg/kg) may produce recognisable deleterious effects on fetal development.

With regard to the somatometric measurements of the offspring, PZA did not produce any significant effect on the sex ratio, length of the pups, or tail length. However, body weight and growth index were reduced only with the dose of 500 mg/kg (Table 3) suggesting that PZA in low doses has no adverse effects on pre-natal growth. Even if high PZA dose of 500 mg/kg appears to be associated with adverse effects, other factors have been shown to contribute to intra-uterine growth retardation and reduced litter size including low calorie intake, decreased food consumption and reduced protein synthesis [8,12,37].

Among the external features noticed on fetal examination, the incidence of runts, haematomas, and crocked tails was increased in groups 3 and 4 compared to group 1. As to visceral examination, dilated renal pelvis and distended pelvis were also observed to be higher in groups 3 and 4. However, these anomalies, have been considered to be spontaneous and are commonly seen in term rats [20]. In fact, the developing renal parenchyma has been shown to dilate the renal pelvis causing "apparent hydronephrosis" [36].

The incidence of several skeletal anomalies (Table 4) was found to be significantly higher in the group given PZA dose of 500 mg/kg than in other groups. Although the appearance of

skeletal variations and delayed ossifications are not uncommon in teratological studies [21], a consistent effect of PZA occurred only with the highest dose used in this study (500 mg/kg), indicating that this dose is probably truly fetotoxic. The presence of 14th rib at the thoracolumbar border is one of the most common anatomical variations in rats and mice [13]. Shortened and mispositioned bone anomalies have been described as indicative of an inhibitory effect of PZA rather than teratogenesis [21].

Indications of fetotoxicity were mostly observed in the group of animals given the dose of 500 mg/kg which is regarded to be 20 times the therapeutic dose [4]. The mechanism of this inhibitory effect of PZA on the fetus is not fully understood but factors affecting fetal health and growth retardation are well described by Broughton-Pipkin et al [6].

The precise mechanism of action of PZA on the mycobacteria is unknown, but the drug is apparently hydrolyzed within the mycobacteria by pyrazinamidase to its active form, pyrazionic acid and then to 5-hydroxypyrazionic acid [34]. Urate retention occurring after PZA administration is attributed to the formation of pyrazionic acid. To ascertain whether the PZA metabolites are related to neonatal and fetal complications, more studies are required.

In conclusion, administration of PZA to pregnant Wistar rats during the organo-genetic period caused fetotoxicity, following only high doses of the drug. There was no evidence of drug-related teratogenesis.

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