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ORIGINAL ARTICLE

Evaluation of bone formation in fetal skeletons following prenatal paracetamol administration in single alizarin-stained specimens in Wistar rats

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The study was undertaken to evaluate the effect of paracetamol on skeleton in Wistar rats. The drug was administered orally, once a day, in three doses: P1 — 3.5 mg/kg, P2 — 35.0 mg/kg, P3 — 350.0 mg/kg. The foetuses were delivered by laparotomy on the 21st day of pregnancy and fixed in alcohol. The skeletons were stained with alizarin. Insignificant differences of bone malformations were found.

key words: paracetamol, acetaminophen, congenital-malformation, pregnancy, foetus, rat, teratogen

INTRODUCTION

In reproductive toxicology, as well as in other toxicity studies, various animals and different experimental methods are used instead of humans. However, if a hazard is to be detected and the risk for a human being assessed, then certain experimental conditions must be fulfilled. This is very important especially for widely used xenobiotics, such as the most poplar analgesic and antipyretic drug on market — paracetamol [3].

Several investigators have tested paracetamol for its effects on the developing organism using it in vitro and in vivo methods and have reported that embryotoxicity resulted from the treatment. Neural tube defects, clef palate and limb abnormalities have also been observed following the drug-administration. However, animal data has not been fully confirmed in case of humans. Because of numerous negative findings in humans, paracetamol has been prescribed for pregnant women as a first line painkiller, which is the safest for the mother and the developing baby [1,3,10].

The goal of this experiment is to determine if the congenital malformations caused in rat foetuses are caused by maternal treatment with paracetamol. Particular attention is given to the effects on skeleton malformations that are very sensitive and common markers in teratology.

MATERIAL AND METHODS

The whole experiment was based on an animal experimental model, designed according to WHO technical direction [15] and the guidelines of the Bioethical Committee of the Medical University School of Lublin, Poland.

An experiment was conducted on Wistar breed rats originally obtained from a commercial breeder (Warszawa-Rembertow, Poland) with initial body weight of 180 ± 15 g. The animals were housed in standard laboratory cages (max. 5 pieces per cage) at a room temperature of 20 ± 3°C on a daylight cycle (7 a.m.–7 p.m.). Standard laboratory fodder (Motycz, Poland) and tap water were provided ad libitum. Food and water consumption were monitored daily.

Virgin females after two weeks of acclimatisation period were mated overnight with males of the same stock. The presence of a vaginal plug or sperm in the vaginal smear examined the following morning was taken to indicate successful mating, and the day was considered as the first day of gestation. The insemi-

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nated animals were randomly gathered in experimental or control groups with a minimum of 12 in each group. However, some females were not pregnant despite the presence of spermatozoa in the smear.

Paracetamol (Sigma, Germany; > 99% purity) was ground with Tween 80 (Sigma, Germany) and then diluted in distilled water. The suspension was administered orally with the use of a stomach tube, once a day (between 7.30–8 a.m.), during days 8–14 of gestation. Tested substance was given in three different doses: P1 — 3.5 mg/kg body weight (p = 8 — number of pregnant female; f = 111 — number of foetuses; s = 66 — number of examined alizarin staining specimens), P2 — 35.0 mg/kg b.w. (p = 8, f = 115, s = 75), P3 — 350.0 mg/kg b.w. (p = 7, f = 95, s = 70). The treated volumes were proportionate to the body weight of the animals e.g. 10 ml/kg b.w.

Two control groups were designed: T — the females received the Tween 80 solution during the whole second trimester of pregnancy (p = 10, f = 148, s = 118), K — untreated control (p = 19, f = 275, s = 176). The animals in group T received Tween 80 water suspension in volumes corresponding to those given in the treatment groups.

The body weight gain of the dames was monitored on the days 1, 8, 14, and 21 of pregnancy.

All of the animals were killed, by decapitation, on the 21st day of gestation, and the uterus were delivered by laparotomy. Foetuses were removed, separated from placenta, and examined macroscopically for external malformations. At least 2/3 of the foetuses from each litter were fixed in 95% ethanol for the study of the skeleton by single alizarin red S staining and examined under a stereo-dissection microscope.

Data was statistically analysed using ANOVA [12]. The level of significance was set at $p < 0.05$ ($\alpha = 0.05$).

RESULTS

The treated females consumed as much food and water as the controls and gained comparable weight. There were no signs of maternal toxicity due to paracetamol treatment (data not shown).

There were no statistical differences in foetal parameters between both control groups (T, K). Because of this they were united in one pooled control group (CON) to minimize observation error [4].

The external malformations were not noted in drug-treated and control groups, except for an insignificant number of subcutaneous haematomas which were found on parietal region (K), auricular cochlea (P2), neck (P1, P2, P3), interscapular region (P3), lower limb (K, T, P1) and on the mobile part of the tile (K) (Fig. 1).

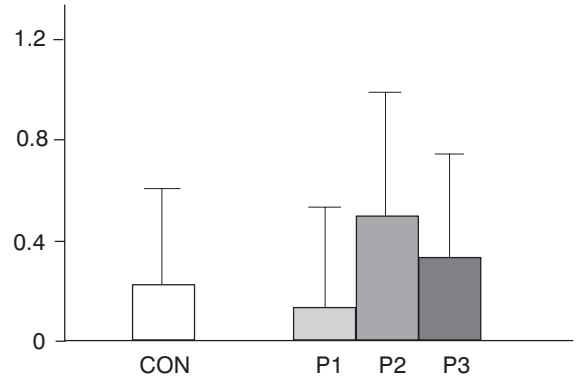

Figure 1. Occurrence of subcutaneous ecchymose in foetuses of rats treated with paracetamol and in the pooled control groups.

The Tables 1 and 2 summarize the frequency and types of bone malformations observed in paracetamol-treated and controls groups.

The examination of 505 skeletons from both control and drug treated groups showed a partial ossification of the nasal, frontal, parietal, interparietal, supraoccipital bones. In one the foetuses from the P2 group, the supraoccipital bone was missing. The other bones of the skull, including basicranial ones, did not show any malformation.

The hyoid bone was not observed in two specimens form P2 group as well as in one from the P3 group. Reduction of alizarin staining of the hyoid bone was found in a single foetus from both control groups and in two foetuses from P3.

The individual cases of the short 13th ribs were noted only in both control groups. The wavy 13th ribs were observed occasionally in all examined groups, except for P3. Bud and short extra unilateral lumbar ribs were observed in single cases in untreated control and in the P3 group. One foetus exposed prenatally to the highest paracetamol doses showed bilateral, well formed short extra lumbar ribs.

The appendicular skeleton did not show any anomalies of the long bones of the upper and lower extremities. The unilateral absence of one metacarpal bone was observed only in control groups. Bilateral absence of the first metacarpal bone was found in all examined groups. One foetus from group P3 had unilateral missing of the 1st and the 4th metacarpal bones. The 5th metatarsal bone was not observed in only a few foetuses form all examined groups.

The sternum, the 5th and/or 6th sternebrae in particular, was the most common malformed part of

Table 1. Occurence of skeletal variations (%) in foetuses of rats treated with paracetamol and in the control groups

	Control groups			Paracetamol-treated groups		
	T	K	CON	P1	P2	P3
Number of examined alizarin staining specimens	118	176	294	66	75	70
Nasal, reduced ossification	—	—	—	—	1 (1.33)	—
Frontal, reduced ossification	—	—	—	1 (1.51)	1 (1.33)	—
Parietal, reduced ossification	10 (8.47)	9 (5.11)	19 (6.46)	2 (3.03)	8 (10.66)	11 (15.71)
Interparietal, reduced ossification	12 (10.17)	10 (5.68)	22 (7.48)	7 (10.60)	11 (14.66)	13 (18.57)
Supraoccipital, missing	—	—	—	—	1 (1.33)	—
Supraoccipital, reduced ossification	5 (4.24)	5 (2.84)	10 (3.40)	2 (3.03)	3 (4.00)	4 (5.71)
Hyoid, missing	—	—	—	—	2 (2.66)	1 (1.43)
Hyoid, reduced ossification	1 (0.85)	1 (0.57)	2 (0.68)	—	—	2 (2.86)
13th rib, unilateral short	1 (0.85)	1 (0.57)	2 (0.68)	—	—	—
13th rib, wavy	1 (0.85)	6 (3.40)	7 (2.38)	2 (3.03)	1 (1.33)	—
Supernumerary rib, bud unilateral (L1)	—	1 (0.57)	1 (0.34)	1 (1.51)	—	3 (4.29)
Supernumerary rib, short unilateral (L1)	—	1 (0.57)	1 (0.34)	—	—	1 (1.43)
Supernumerary rib, short bilateral (L1)	—	—	—	—	—	1 (1.43)
Metacarpal, unilateral missing of one bone	2 (1.70)	1 (0.57)	3 (1.02)	—	—	—
Metacarpal, bilateral missing of one bone	2 (1.70)	2 (1.14)	4 (1.36)	1 (1.51)	2 (2.66)	7 (10.00)
Metacarpal, unilateral missing of two bones	—	—	—	—	—	1 (1.43)
Metatarsal, unilateral missing of two bones	4 (3.40)	6 (3.40)	10 (3.40)	3 (4,54)	3 (4.00)	8 (11.43)

Table 2. Occurrence of sternebral variations (%) in foetuses of rats treated with paracetamol and in the control groups

	Control groups			Paracetamol-treated groups		
	T	K	CON	P1	P2	P3
Number of examined alizarin staining specimens	118	176	294	66	75	70
6th sternebrae, missing	1 (0.85)	1 (0.57)	2 (0.68)	2 (3.03)	—	1 (1.43)
6th sternebrae, rudimentary	3 (2.54)	1 (0.57)	4 (1.36)	—	1 (1.33)	4 (5.71)
6th sternebrae, cleaved	—	1 (0.57)	1 (0.34)	—	—	—
6th sternebrae, bifurcated at distal end	1 (0.85)	—	1 (0.34)	1 (1.51)	—	—
6th sternebrae, reduced ossification	8 (6.78)	12 (6.81)	20 (6.80)	4 (6.06)	10 (13.33)	6 (8.57)
5th sternebrae, missing	1 (0.85)	—	1 (0.34)	—	1 (1.33)	2 (2.86)
5th sternebrae, rudimentary	8 (6.78)	13 (7.39)	21 (7.14)	—	1 (1.33)	2 (2.86)
5th sternebrae, dumbbell-shaped	5 (4.24)	9 (5.11)	14 (4.76)	3 (4.54)	2 (2.66)	3 (4.29)
5th sternebrae, reduced ossification	2 (1.70)	1 (0.57)	3 (1.02)	1 (1.51)	2 (2.66)	3 (4.29)
4th sternebrae, rudimentary	1 (0.85)	1 (0.57)	2 (0.68)	1 (1.51)	—	—
4th sternebrae, dumbbell-shaped	—	—	—	—	1 (1.33)	2 (2.86)
3rd sternebrae, dumbbell-shaped	—	—	—	—	—	1 (1.43)
1st sternebrae, reduced ossification	1 (0.85)	—	1 (0.34)	—	—	—

the skeleton (Fig. 2, 3). The missing, rudimentary and poorly ossified sternebrae were the most common observed anomalies. The others malformations such as bifureated and dumbbell-shaped sternebraes were seen in control as well as in paracetamol-treatment groups. Only in one case from the untreated control group the cleavage of the 6th sternebrae was found.

The mean increment of all bone anomalies was not statistically different when compared with both control groups and between drug-treated groups.

DISCUSSION

The result presented above showed that paracetamol did not elevate the skeleton malformations, and it is well tolerated by foetal and maternal organism. My previous paper showed that the embryotoxic effect of a decrease in the foetal body weight was observed only in group P3 where paracetamol was administered in the highest dose [2]. However, Lubawy and Garrett [6] who treated rats with paracetamol once a day at doses 125 or 250 mg/kg/day between days 8 and 14 of the rat pregnancy did not observe any symptoms of the embryotoxicity. The lower foetal weight and length were reported after paracetamol being administered three times a day at doses 50, 100, 200 mg/kg by Wyskiel [16]. The placental weight and tail length were increased in the highest drug-treatment group. The pre- and postimplantation mortality factors did not differ from the levels observed in control groups, similarly to the present study. The spectrum of the bone malformation was the same as presented in this paper. The sternum and calvaria bones of the skull were the most often malformed part of the rat skeleton, but like in this study the differences were not significant. The premature closure of the arterial duct, 1.4-hours after paracetamol administration on the last day of rats' pregnancy and the 40% increase of the cardiac ventricles volume was also reported [8].

Reproductive, embryo- and foetaltoxicology studies were also carried out on mice. Reel et al. [11] reported paracetamol-related decrease of the birthweight of the CD mouse. In another study the decrease of glutathione concentration in foetal mouse liver without any malformations related to the paracetamol administration between days 6 and 13 of mouse gestation was distinguished [5].

In vitro studies showed that paracetamol caused low-level morphological transformations of mouse embryo cells, but had no adverse effect on the development of foetal cultured salivary glands [7,9]. The malformations were observed when paracetamol was added to the cultured rat embryos at doses

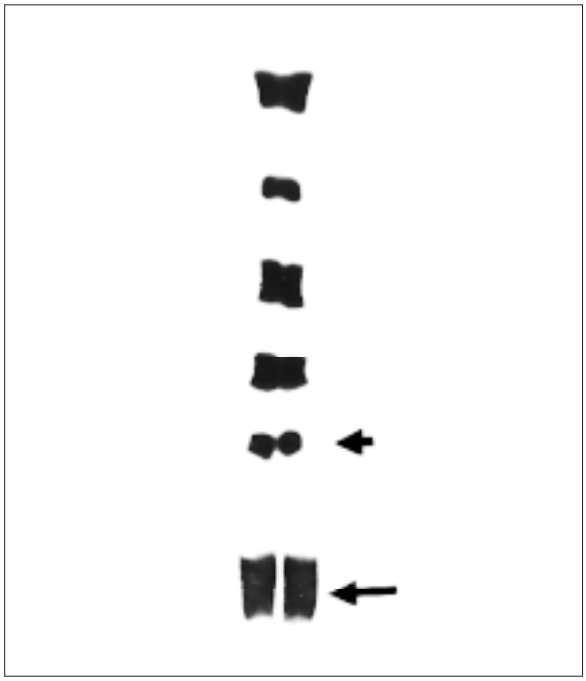

Figure 2. Sternum of rat foetus on gestation day 21 in alizarin red-S staining. Cleaved 6th sternebrae (long arrow) and symmetrically dumbbell-shaped 5th sternebrae (short arrow).

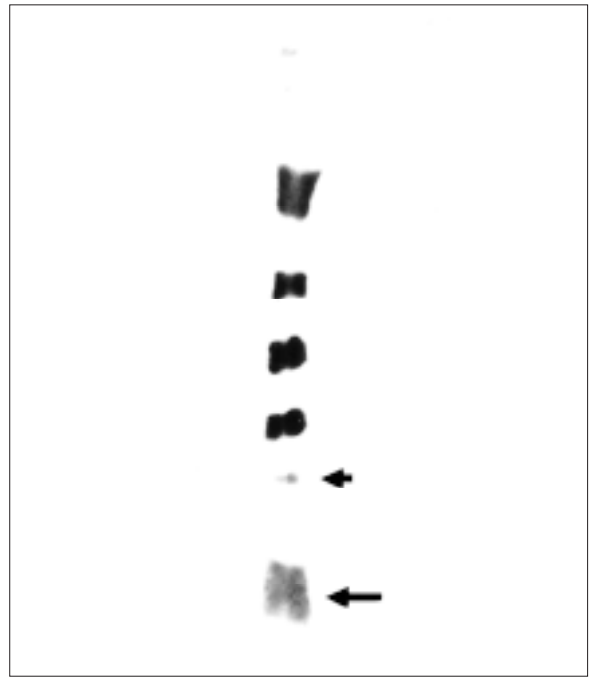

Figure 3. Sternum of rat foetus on gestation day 21 in alizarin red-S staining. Poorly ossified, bifurcated at distal end 6th sternebrae (long arrow) and asymmetrically dumbbell-shaped 5th sternebrae (short arrow).

50 mg/l. Neural defects, such as abnormal closure of the anterior neuropores were increased when paracetamol or its mixture with 3-hydroxy metabolite was added to the medium but not when paracetamol was administered by an intra-amniotic injection [13]. The principal role in paracetamol embryotoxicity is related to the toxic effect of its metabolites. Its embryotoxicity increased when glutathione synthesis was inhibited with buthionine sulphoximine and decreased when glutatione synthesis was stimulated with N-acetylcysteine.

The big, epidemiological, human studies showed good paracetamol tolerability [1,3]. However, a few case reports presented a possible association with gastroshisis and congenital dislocations of the hip [14]. Generally, paracetamol is considered a safe drug during pregnancy for both mother and child.

Based on the results of this study, I confirm that paracetamol did not delay the ossificaion of foetal bones. However, other studies, especially neurotoxicological and behavioural ones, are needed to proove good prenatal tolerability of this drug.

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