WIADOMOŚCI PARAZYTOLOGICZNE
T. 50 (3) 2004: 513–518

EFFECT OF *ASCARIS* CHYMOTRYPSIN INHIBITOR ON FETAL DEVELOPMENT OF MICE[1]

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ABSTRACT. BALB/c mice were given daily doses of 40-80 mg of *Ascaris* chymotrypsin inhibitor /AChI/ per kg body weight from 12th until 15th day of gestation (stage of fetal development). It has been found that injection of AChI disturbed the course of pregnancy (bleeding from uterus, abortions, decreased body weight gain as compared to control /$p < 0.05$/). AChI exhibited embryotoxic effects (a high rate of intrauterine deaths, decreased number of living fetuses and mean fetal weight, delayed skeletal ossification, induced pathological changes of fetal organs and tissues). Congenital malformation (hydronephrosis) was noted in fetuses after injection of higher doses of the chymotrypsin inhibitor from *Ascaris*.

Key words: *Ascaris suum*, chymotrypsin inhibitor, embryotoxicity, maternal toxicity, teratogenicity.

INTRODUCTION

Ascaris lumbricoides, the most frequent human intestinal nematode, has infected over 1,5 billion people worldwide. Human beings can also be infected with *Ascaris suum* (Anderson 1995). Serious complications related to *Ascaris* infection during pregnancy have been well documented in the literature (O'Lorcian and Holland 2000, Devici et al. 2001, Gracia et al. 2002). Ascariosis in pregnant women can cause states of maternal malnutrition, iron deficiency anemia, intestinal obstruction, acute pancreatitis, peritonitis and also genital bleeding. *Ascaris* infection in pregnant women is dangerous for the embryo and fetus (most often intrauterine growth retardation and reduction in fetal weight). Moreover, migrating larvae can also pass through the placenta resulting in a congenital ascariosis (Rathi et al. 1981, da Costa-Macedo and Rey 1990).

Disturbances in the reproduction of the host in the course of ascariosis have not been fully elucidated. In my earlier studies I demonstrated that preparation of chymotrypsin inhibitor from *Ascaris* injected during both implantation and formation of primary organs or organogenesis disturbed the course of pregnancy in mice and pro-

[1] The study has been financed by Medical University of Łódź – research No 502-11-771(5)

duced congenital malformations (Błaszkowska 1999, 2001). The objective of this study was to investigate whether chymotrypsin inhibitor from *Ascaris suum* administered during late stage of pregnancy (period of fetal development) had adverse effects on the course of pregnancy and organ systems as well as skeleton of mouse fetuses.

MATERIALS AND METHODS

The preparation of chymotrypsin inhibitor (proteins of SF_3 fraction with antichymotrypsin properties) was obtained from the cuticle and muscles of *Ascaris suum* by the modified method of Rola and Pudles (1967) as previously described (Błaszkowska 1999, 2001).

The investigations were carried out on 47 BALB/c mice, weighing 25-29 g, fed with granulated fodder /LKS/, water and bovine milk *ad libitum*. The mice were kept in a room with natural lighting and temperature 18-25°C.

The day on which vaginal plugs or sperm in vaginal smears were found in the mice was designated as day 0. The clinical condition of the females was checked throughout pregnancy. After fertilization, mice were weighed daily and the consumed fodder mass per female was measured.

Mice were given chymotrypsin inhibitor intraperitoneally, four times, from day 12 until 15 of gestation in doses from 40 to 80 mg per kg/day. The control group was given 80 mg of bovine albumin/kg/day, respectively. The mice were sacrificed on the 19th day of pregnancy. The uterine contents were examined, and the number of the implantation sites, living and dead fetuses, resorption sites were determined. The live fetuses were weighed and their sex determined.

Percentage fetal loss was calculated by:

$$\frac{(\textit{No. of implantations} - \textit{No. of viable fetuses})}{\textit{No. of implantations}} \times 100$$

For evaluating maternotoxic effects of *Ascaris* preparation the body weight gain in females during gestation, their uteruses were weighed at day 19 of gestation. After macroscopic examination about 50% of the fetuses from each litter were fixed in 70% ethanol in order to evaluate the regularity of skeletal development, and the remainder were fixed in Bouin's solution for macroscopic evaluation of the internal organs. Skeletons of fetuses were stained with alizarin red S according to Dawson's method modified by Jacobsen (1963). Lorke's (1977) criteria were applied for the evaluation of developmental skeletal defects of the fetuses. The development of internal organs in fetuses was evaluated using Wilson razor blade slices (Barrow and Taylor 1969). Cross-section of the head and trunk of fetuses were examined macroscopically under 10x magnification.

The recorded results were statistically analysed by means of Fisher's exact test and Student's t-test.

RESULTS AND DISCUSSION

My experiment has showed that four peritoneal injections of *Ascaris* chymotrypsin inhibitor during the late stage of pregnancy produce symptoms of the toxic effect on female: bleeding from uterus, abortions, changes in behaviour of mice after injection, decreased body weight gain compared to control. The above signs are in compliance with criteria of Khera (1987) for existence of maternal toxicity.

Fertilized female mortality was not observed in this test. My previous investigation (Błaszkowska 1999, 2001) demonstrated that identical doses (60, 80 mg/kg/day) of this *Ascaris* inhibitor caused death in a few cases, after its injection during the earlier stage of pregnancy. These observations are in agreement with the variable susceptibility of female depending on gestation stage. It is well known that later injection of the chemical reduces the risk for maternal organism and developing progeny.

Higher doses of the *Ascaris* inhibitor (60-80mg/kg/day) resulted in bleeding from uterus. The antifibrinolytic effect of *Ascaris* chymotrypsin inhibitor was observed in the previous *in vitro* study (Kadłubowski and Ochęcka 1986). The effect of *Ascaris* inhibitor on coagulation phases I and II, the inhibition of thromboplastin and thrombin generation and fibrinogesis retardation of human plasma were found. It was worth to noting, that disturbances in blood coagulation by extracts of *Ascaris suum* were observed in the other *in vitro* study (Crawford et al. 1982). On the other hand, the results of the latest studies (Błaszkowska, unpublished) with protease inhibitor from animal lungs (Traskolan, Polfa) used in comparable doses to *Ascaris* inhibitor (the same ability of the inhibitors to inhibit the activity of crystalline α–chymotrypsin) suggest that the hemorrhage from mouse uteruses which appeared after *Ascaris* inhibitor injection are not connected with its antiproteolytic properties.

In this study, all doses of the inhibitor have been found to reduce the mean body weight gain during pregnancy as compared to control. The mean uterus weight decreased with administration of higher doses of the inhibitor (Table 1).

In this stage of prenatal development, the administration of *Ascaris* chymotrypsin inhibitor provoked a toxic effect on mouse embryo. Embryotoxic effects were suggested by the occurrence of a significantly higher incidence of resorptions and fetal deaths and a lower mean fetal body weight compared to control (Khera 1987). These phenomena were also observed in my present experiment. The mean number of dead fetuses per litter increased with higher inhibitor dose (Table 2). The mean fetal weight showed a tendency to decrease with higher doses of the inhibitor. There was a decrease in the mean number of live fetuses per litter in comparison with the control group (Table 2). The high percentage fetal loss was noted after administration all doses of *Ascaris* inhibitor (18.3-38.0%); in the control group fetal loss was lower (1.7%).

Table 1. Selected features of BALB/c mice following injection of chymotrypsin inhibitor from *Ascaris*. Results are presented as arithmetic means ± SE

Features	Bovine albumin 80 mg [1,2]	Chymotrypsin inhibitor (mg)[2]		
		40	60	80
No. of gravid females	10	10	12	14
No. of females with abortion	0	0	1	4
No. of females at term	10	10	11	10
Mean maternal body weight (g):				
at day 1 of gestation	26.9±0.28	27.9±0.23	26.6±0.29	26.9±0.23
at day 19 of gestation	46.1±0.31	45.9±0.23	42.9±0.22	42.6±0.34
Mean uterus weight	16.1±0.23	15.4±0.26***	14.3±0.20***	13.5±0.22***
Mean body weight[3]	3.1±0.16	2.5±0.19*	2.1±0.13**	2.0±0.29**

[1]control group; [2]dose per kg of body weight per 24 h; [3]mean body weight gain after subtraction of uterus weight; significant differences: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 2. Effects of *Ascaris* chymotrypsin inhibitor on implantation, resorption, live/dead fetuses, fetal weight. Results are presented as arithmetic mean ± SE

Features	Bovine albumin 80 mg [1,2]	Chymotrypsin inhibitor (mg)[2]		
		40	60	80
Number of litters	10	10	11	10
Number of implantations per litter	11.7±0.22	10.9±0.23	10.8±0.23	10.8±0.25
Number of resorptions per litter	0.1±0.10	1.4±0.22***	1.2±0.19***	1.9±0.23***
Number of fetuses per litter:				
– live	11.5±0.22	8.9±0.23***	8.0±0.25***	6.7±0.26***
– dead	0.0±0.00	0.8±0.25	1.7±0.29	2.3±0.30
Fetal body weight (g)	1.19±0.09	1.07±0.016*	1.05±0.013*	1.05±0.012*
Fetal loss (%)	1.7	18.3	25.9	38.0

For explanations see Table 1. Significant differences: *$p < 0.05$, ***$p < 0.001$

This research demonstrates that the *Ascaris* inhibitor injected during pregnancy (12-15th days) produced anomalies of fetuses (Table 3). The examination of the fetuses revealed pathological changes of organs and retarded ossification of skeleton which increased in parallel to the increase in the dose of chymotrypsin inhibitor (Table 3). The most common retardations in ossification were: missing 13th rib, absence of ossification centres from the sternum, fewer than 3 ossification centres for the metatarsus, visible enlargement in the size of fontanelles (as compared to control). The frequency of fetuses with delayed ossification was much lower in the control group (Table 3).

The congenital malformation (hydronephrosis) was detected in fetuses after administration of the higher doses of this inhibitor (Table 3). My previous investigations showed that the *Ascaris* chymotrypsin inhibitor injected during organogenesis (8-12th days of pregnancy) induced many types of fetal defects e.g. cleft palate, fusion of ribs, micrognathia (Błaszkowska 1999). When the inhibitor was

injected with the same doses in the earlier period of pregnancy it caused other congenital malformations i.e. exencephaly and hydrocephalus (Błaszkowska 2001). The types of defects seen in mouse fetuses after the *Ascaris* inhibitor injection were similar to the mouse malformations listed by Khera (1984) after the administration of chemicals causing maternotoxic effects. In general, each organ of the embryo passes through a period of development during which it is particularly susceptible to teratogenic agents. The highest sensitivity, at least for anatomical defects, occurs during formation of primary and final organs (Wilson 1975).

Table 3. Anomalies of mice fetuses following injection of *Ascaris* chymotrypsin inhibitor. In parentheses, the percent of affected fetuses

Features	Bovine albumin 80 mg [1,2]	Chymotrypsin inhibitor (mg)[2]		
		40	60	80
No. of live fetuses	112	89	88	67
No. of female/male	58/54	46/43	43/45	32/35
No. of fetuses examined for evaluation internal organs/skeleton	56/56	44/45	44/44	33/34
Anomalies noted in gross fetal observations:				
– subcutaneous oedema	0(0.0)	1(1.1)	9(10.2)	7(10.4)
– hemorrhage in extremities	0(0.0)	1(1.1)	8(9.1)	9(13.4)
Anomalies noted at Wilson's razor blade section:				
– hydronephrosis	0(0.0)	0(0.0)	4(10.3)	5(15.2)
– hemorrhage in cranium	0(0.0)	0(0.0)	1(2.7)	6(18.2)
– hemorrhage in abdominal cavity	1(1.8)	5(11.4)	4(10.3)	5(15.2)
– hemorrhage in thorax	0(0.0)	2(4.5)	0(0.0)	6(18.2)
Anomalies noted at studies of skeleton:				
Fetuses with delayed ossification[4]	4(7.1)	14(31.1)**	18(40.0)***	16(47.1)***
– retarded cranial ossification	1(1.8)	9(20.0)**	10(22.7)**	8(23.5)**
– absence of 13th rib	1(1.8)	8(17.8)**	8(18.2)**	10(29.4)***
– reduced sternal ossification centres	2(3.6)	8(17.8)*	9(20.5)**	9(26.4)**
– reduced ossification of phalanges	2(3.6)	9(20.0)*	9(20.5)*	10(29.4)***

For explanations see Table 1; [4]These fetuses had simultaneously few signs of retardation of ossification

Previous and present researches indicate that the nature and intensity of prenatal disturbances are determined by the *Ascaris* inhibitor dose and time of the injection. The chymotrypsin inhibitor always produced fetal defects independently of the time of administration during gestation. This protease inhibitor from *Ascaris suum* predominantly caused the higher number of different types of malformations and also embryolethal effect when given during organogenesis.

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Zaakceptowano do druku 14 czerwca 2004