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PRELIMINARY EVALUATION OF MATERNOTOXIC EFFECT OF *ASCARIS* EXTRACT IN MICE[1]

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ABSTRACT. Administration intraperitoneally of the *Ascaris suum* extract – ASE-(0.6-1.4 g of *Ascaris* proteins/kg/day) at a late stage of organogenesis (8-12 days of gestation) disturbed course of mouse pregnancy. It has been found that injections of higher doses of ASE to pregnant mice caused the symptoms manifesting maternal toxicity (decreased body weight gain /$p < 0.001$/ as compared to control, intrauterine resorption of litter, vaginal hemorrhages, female mortality and altered behaviour). There is a linear interrelationship between the logarithm of the dose of ASE and mortality of pregnant mice. The DL_{50} value of *Ascaris* proteins for pregnant mice was 1.02 g/kg/day (confidence interval 0.97-1.07 g/kg/day). ASE exerted embryotoxic effects: significantly decreased the number of surviving fetuses per litter and the mean body weight of fetuses, increased the number of fetal resorptions.

Key words: *Ascaris* extract, *Ascaris suum*, maternal toxicity, mortality, disturbed pregnancy, embryotoxicity.

INTRODUCTION

Helminths (including *Ascaris*) and culture fluids of their larvae have been shown to contain various biologically active substances. They most often have toxic, allergenic or immunomodulatory properties. Extract or homogenate produced from *Ascaris suum* induce allergic bronchial asthma, systemic anaphylaxis and shock in experimental animals (Yamatake et al. 1977; Kitoh et al. 1994a, b). The culture fluid of *A. suum* larvae stimulates a specific blastogenic response in lymphocytes obtained from infected swine (Urban and Douvres 1981). Extracts from *Ascaris suum* and other species of helminths contain eosinophil and neutrophil chemotactic factors (Tanaka et al. 1979, Woolley et al. 1995) and inhibit mammalian blood clotting (Crawford et al. 1982; Kitoh et al. 1994a, b). The study reports that *Ascaris* extract contains a mitogenic factor affecting human lymphocytes (Sasagawa et al. 1987). The extract induced an increase in [^{3}H] thymidine incorporation into human lymphocytes at a level similar to that obtained with pokeweed mitogen.

Currently only single reports provide information on helminths metabolites influence on the host somatic and generative cells (Shubber and Salin 1987; Bekish

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2000, 2001). *Ascaris* metabolites have been established to exert a mutagenic effect on somatic cells of bone marrow, and disturb spermatogenesis of invaded mice (Bekish and Bekish 2000, Bekish 2001). On the other hand, different antigens from *Ascaris suum* (whole *Ascaris*, musculocutaneous sac, cavity fluid) have changed the chromosome apparatus of human blood lymphocytes *in vitro*, having provoked the number of aneuploid and aberrant cells (Bekish 1999, Bekish and Bekish 2000).

My previous investigations (Błaszkowska 1999, 2000) showed that homogenate from *Ascaris* tegument injected to mice during early or late organogenesis produced specified types of congenital malformations. On reviewing 476 studies of chemicals tested in mice, rats, rabbits and hamsters, a fairly strong association between maternal toxicity and following phenomena: embryofetal mortality and congenital malformations was observed (Khera 1984, 1987). The objective of this study was to investigate whether substances from *Ascaris suum* extract injected during late organogenesis caused toxic effects in pregnant mice. This experiment was also performed to reveal whether deleterious fetal defects in mice may be associated with maternal toxicity.

MATERIALS AND METHODS

The investigations were carried out on 50 BALB/c pregnant mice and 10 non-pregnant females, weighing 29-31 g, fed with granulated fodder (LKS), water and bovine milk *ad libitum*. The mice were kept in a room with natural lighting and a temperature 18-25°C.

Ascaris suum obtained from a slaughterhouse (from the intestine of swine) were washed thoroughly in physiological saline and destilled water, and were killed by freezing at -20°C. The homogenate of *Ascaris* tegument (cuticle and muscles) was prepared by disintegrating the parasite tissues in PBS (pH 7.4) using Potter's homogenizer (5000 r.p.m., for 60 min. at 4°C). The homogenate was stirred for 24 h and centrifuged at 7900g for 20 min at 4°C – standard centrifugation in procedures of obtaining extract from *Ascaris*. The supernatant was used as a body wall extract. Using extract in this experiment enabled to eliminate solid and not soluble substances of homogenate (e.g. cuticle), which were making injections difficult. The protein concentration in the extract was determined by Lowry's method (Lowry et al. 1951).

Ten non-pregnant mice (control group A) were injected intraperitoneally, 5 times at 24 h intervals, with 1.4 g of the *Ascaris* proteins per kg of body weight. The clinical status and possible mortality of the animals were monitored throughout 7 days after the extract administration. The mice were killed by intraperitoneal injection of sodium pentobarbitone and their vital organs were examined by dissection.

After fertilization, female mice were weighed daily and consumed fodder mass per female was measured. The day on which vaginal plugs or sperm in vaginal

smears were found in mice was designated as day 0. The maternal condition of mice (possible abortion, death, vaginal hemorrhage, altered behavior) was checked throughout pregnancy.

Mice administered *Ascaris* extract intraperitoneally five times, from 8 until 12 day of gestation, in doses ranging from 0.6 to 1.4 g of extract proteins per kg of body weight every 24 h. Control group was given respectively 1.4 g of bovine albumin per kg/day (group B). The mice were sacrificed on the 19 day of pregnancy. Immediately after death, the uterine contents were examined, and the number and position of the following were recorded: (1) live fetuses, (2) dead fetuses – placenta and fetus at term, but the latter in an advanced stage of autolysis, (3) resorption sites divided into various stages: (a) early – remnants of placenta present but no obvious signs of an embryo, (b) late – remnants of an embryo present.

The mean number of resorptions per litter excludes animals, which had totally resorbed the litters. Percentage fetal loss was calculated by:

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

For evaluating maternotoxic effects of *Ascaris* preparation the body weight gain in females during gestation, their uteruses were weighed at day 19 of gestation.

The significance of the differences between the arithmetical mean from 2 trials were evaluated using the Student's t-test for n_1+n_2-2 degrees of freedom. LD_{50} value and confidence interval for $P = 0.05$ level were calculated according to the Spearman – Kärber method described by Piechocki (1974).

RESULTS

Our experiment on non-pregnant mice showed that five daily peritoneal injection of *Ascaris* extract (1.4 g of *Ascaris* proteins/kg/day – the highest dose injected in pregnant female) did not produce symptoms of toxic effect on females (death, hemorrhage from uterus, pathological changes of organs, changes in behaviour were not observed).

In the present experiment, fertilized female mortality was observed after administration of the highest doses of the extract (0.8-1.4 g of proteins/kg/day – Table 1). There is a linear interrelationship between the logarithm of the *Ascaris* proteins dose and mortality of pregnant mice (Fig.1). The linear correlation coefficient (r) was 0.986 ± 0.063 with the significance level $P < 0.01$. The DL_{50} value of the *Ascaris* proteins for pregnant mice was 1.02 g/kg /day (confidence interval 0.97-1.07 g).

There were traces of bleeding from the uterus in the animals that died. Dissection revealed an accumulation of blood in the uterine cavity. Also in the group injected with the highest doses of the extract, complete resorption of the litter was observed

(Table 1). Some pregnant mice in the groups which received 0.8-1.2 g of *Ascaris* proteins showed signs of vaginal hemorrhage, but they not abort litters.

As can be seen from Table 1, all doses of *Ascaris* substances significantly reduced the mean body weight gain of mice during pregnancy compared to control group. The mean uterus weight decreased with administration of higher doses of the extract.

Table 1. Mortality and body weight gain in BALB/c mice at day 19 of gestation following administration of *Ascaris* extract. Results are presented as arithemic mean ± SE

Features	Substance and dose					
		Grams of the extract proteinsb)				
	Bovine albumin 1.4g[a,b)]	0.6	0.8	1.0	1.2	1.4
No. of gravid females	10	10	10	10	10	10
No. of dead females	0	0	2	4	6	10
No.of females with totally resorbed the litter	0	0	1	1	2	–
No. of females at term	10	10	7	5	2	0
Mean maternal body weight[c)] (g)						
– at day 1 of gestation	30.2 ± 0.34	29.1 ± 0.34	29.9 ±0.38	31.0 ± 0.38	30.1 ± 0.32	–
– at day 19 of gestation	48.0 ± 0.41	45.2 ± 0.24	42.6 ± 0.26	42.3 ± 0.42	37.6 ± 0.41	–
Mean uterus weight[c)]	14.8 ± 0.35	12.9 ± 0.33**	10.1 ± 0.37**	9.4 ± 0.26***	6.5 ± 0.50**	–
Mean body weight gain[c,d)]	3.1 ± 0.22	1.9 ± 0.15**	1.6 ± 0.16**	1.4 ± 0.11***	1.2 ± 0.20**	–

[a)] – control group B, [b)] – dose per kg of body weight per 24 h, [c)] – excluding mice with totally resorbed the litters, [d)] – mean body weight gain after substraction of uterus, statistically significant differences: ** – $p < 0.01$, *** – $p < 0.001$

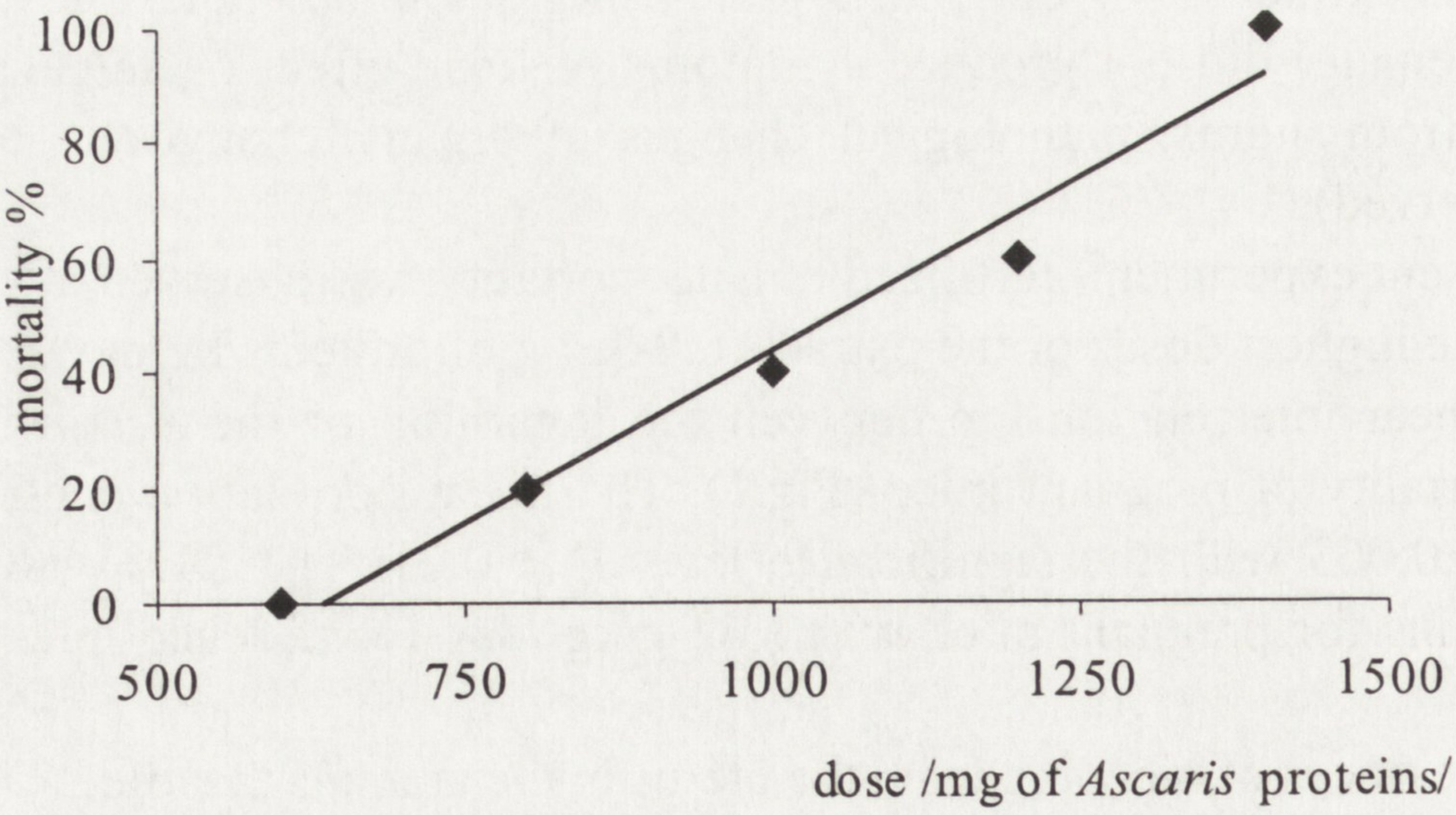

Fig. 1. The mortality curve of pregnant mice after injection of *Ascaris* extract

It was found that the mean weight of fodder consumed by females in 24 h was lower by 7-12% in all the groups of mice receiving the extract compared to control. Reduced movement activity was observed in mice after injection of *Ascaris* extract (mice remained motionless up to 2-3 h following injection).

There was a decrease in the mean number of live fetuses per litter in comparison with control group (Table 2). The mean number of dead fetuses per litter also increased with the higher proteins dose. High percentage fetal loss was noted after administration all doses of *Ascaris* extract (27.0-52.9%); in the control group fetal loss was only 1.83%.

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

Features	Substance and dose				
		Grams of the extract proteinsb)			
	Bovine albumin 1.4g[a,b)]	0.6	0.8	1.0	1.2
Numbers of litters	10	10	7	5	2
Number of implantations per litter	10.9 ± 0.23	9.6 ± 0.22**	9.1 ± 0.26***	8.8 ± 0.37***	8.5 ±0.50***
Number of resorptions per litter:					
– early	0.2 ± 0.13	1.4 ± 0.17***	1.7 ± 0.18***	2.2 ± 0.20***	3.0 ± 0.00***
– late	0.0 ± 0.00	0.2 ± 0.13	0.3 ± 0.18	0.4 ± 0.24	0.5 ± 0.50
Number of featuses per litter:					
– live	10.7 ± 0.21	7.0 ± 0.21***	6.4 ± 0.28***	5.4 ± 0.24***	4.0 ± 0.00***
– dead	0.1 ± 0.10	0.9 ± 0.22**	1.3 ± 0.19***	1.4 ± 0.24***	1.5 ± 0.49***
Fetal body weight (g)	1.24 ± 0.022	1.21 ± 0.032	1.13 ± 0.036*	1.11 ± 0.031*	1.02 ± 0.020
Fetal loss (%)	1.83	27.00	29.7	38.6	52.9

For explanation see Table 1. Statistically significant differences: * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$

DISCUSSION

Data from animal teratology studies were surveyed to determine whether embryo-fetal mortality and fetal malformations result from a primary action of the agent on conceptus or if they are secondary to maternal toxicity – a consequence of high dose administration of various substances. A fairly strong association between embryo-fetal mortality and maternal toxicity was revealed by analysis of data from hamsters, mice, rats and rabbits in 234 studies of chemical agents (Khera 1984, 1985). Further, a consistent pattern of fetal malformations associated with maternotoxic effects was discovered in a survey of 476 studies of chemical agents tested in rodent species (Khera 1987). In these reviews, it was postulated that maternal toxicity *per se* could possibly caused deleterious fetal effects. For evaluating mater-

notoxic effects in experimental studies, the minimum maternal data required would be frequent measurements of maternal body weight and food consumption, signs of altered behavior, death, and gross lesions in necropsy. From the experiments presented, it is apparent that the *Ascaris* extract provoked the symptoms of toxicity in the pregnant females mice.

Higher doses of *Ascaris* preparation (0.8-1.2 g of proteins/kg/day) resulted in total resorption of the litter, which were always accompanied by hemorrhage in uterus. In contrast to the lack of hemorrhagic effect in non-pregnant mice, the use of the *Ascaris* extract in pregnancy was associated with hemorrhagic appearance of placenta in these animals. The absence of hemorrhage from elsewhere in the uterine wall, confirmed by autopsy, suggests that the placenta may be the site of primary damage. 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). It has been found that extracts from *Ascaris* prolonged the whole blood clotting time and the kaolin-activated, partial thromboplastin time but it did not alter the protrombin time. The maximal concentration of anticoagulant activity was found in the pseudocoelomic fluid of the worm. High activity was also noted in the cuticle and secretory/excretory products. In experiment *in vivo*, blood coagulopathy in dogs with shock induced by intravenous injection of heartworm extract was observed (Kitoh et al. 1994a, b). The precise mechanisms by which helminthes inhibit coagulation and the role of these phenomena in disturbances of pregnancy in host organism merit further investigation.

Embryofetal deaths were present in association with maternal toxicity in 75% of the studies conducted at maternotoxic doses of chemicals in mice (Khera 1984, 1987). This phenomenon was also observed in my experiments. The mean number of resorptions per litter increased with the increase of the *Ascaris* proteins dose.

In my previous studies (Błaszkowska 1999, 2000), it was observed that *Ascaris* tegumental homogenate injected into pregnant mice had exerted a teratogenic effect. The most frequent congenital malformations were: cleft palate, fusion rib. Doses of *Ascaris* proteins per kg/day, which caused congenital malformations in mice, were in the range between 3/4 and 1 of the mean lethal dose – LD_{50} for pregnant BALB/c mice. The types of fetal defects seen in mouse fetuses after *Ascaris* preparation injection were similar to the mouse malformations listed by Khera (1987), which were noticed after administration of the toxic substances to gravid mice. The mechanism by which extract of *Ascaris suum* exerts its embryotoxic and teratogenic effects is not clear. The phenomena may be accounted for indirect action of the substances from *Ascaris* tegument on the pregnant organism causing maternotoxic effect. Disturbances in maternal health can produce deleterious consequences for fetal development, what was proved during previous studies reported by Khera (1984, 1987).

It has been commonly accepted that mutagenic chemicals administered into experimental animals may induce preimplantation losses of nonviable zygotes, early fetal deaths, sterility, abortion and congenital malformation (Epstein et al. 1972). It is worth to stress that mutagenic activity of antigens from *Ascaris suum* (extract from whole *Ascaris*, musculocutaneous sac, cavity fluid) was observed by Bekish (1999). It has been determined that the above-mentioned antigens possessed mutagenic effect resulting in a increase of the number of micronuclear containing poly and normochromophilic erythrocytes the largest being that of the *Ascaris suum* musculocutaneous sac. Extracts from *Ascaris suum* have changed the chromosome apparatus of human blood lymphocytes *in vitro*, having provoked the number of aneuploid and aberrant cells. On the other hand, it has been found that the nematode metabolites of *Trichinella spiralis*, migrate larvae of *Toxocara canis* and *Ascaris suum* have possessed the mutagenic influence on the somatic cells of experimental animals, which is characterized by the growth in the bone marrow the number of micronuclei erythrocytes, the cells with the change of the structure and the number of chromosomes (Bekish and Bekish 2000).

The signs observed in pregnant mice suggest that *Ascaris* extract injected during late organogenesis caused maternal toxicity. The detection of such biological activity of *Ascaris* substances from tegumental extract may contribute to the explanation of some mechanism of reproduction disturbances of host in this helminthosis.

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