WIADOMOŚCI PARAZYTOLOGICZNE
T. 48 (1) 2002: 55–64

DEVELOPMENTAL ABNORMALITIES IN CHICKEN EMBRYOS AFTER INJECTION PROTEOLYSIS INHIBITORS FROM *ASCARIS SUUM**

JOANNA BŁASZKOWSKA

Chair of Medical Biology and Parasitology, Medical University,
Kościuszki str. 85, 90-436 Łódź, Poland

ABSTRACT. It has been found that trypsin and α-chymotrypsin inhibitors isolated from *Ascaris suum* act embryotoxically and teratogenically on White Leghorn chicken embryos. Mortality rate for the chicken embryos on day 15 of incubation was 45.0 ± 3.5% after injection of trypsin inhibitor and 44.0 ± 3.5% after adminstration α-chymotrypsin inhibitor. Gross examination of surviving embryos and their dissection revealed pathological changes (abdominal dropsy, umbilical hernia, subcutaneous oedema, hemoperitoneum, hemopericardium), symptoms indicating retardation in growth (lack of down, retarded ossification of long bones, decreased mean body weight) as well as malformations (schistocelia, micrognathia, cyclopia, crossed beak, cranial deformities) after injection of inhibitors from *Ascaris*. The highest incidence of embryos with pathological changes and malformations was found after administration of α-chymotrypsin inhibitor. The most commonly occurring abnormality was schistocelia (21.4 ± 3.88%). Growth malformations were not found in the control groups. The trypsin and α-chymotrypsin inhibitors present in *Ascaris* homogenate have a significant disturbing effect on the development of the chicken embryo.

Key words: *Ascaris suum*, inhibitors, chicken embryos, teratogeny.

INTRODUCTION

Parasites secrete various substances (proteins, peptides, fatty acides) while in the host organism (Hammerberg et al. 1980, Clinton et al. 1996, Gimwood and Smith 1996). They often have toxic, immunogenic or inhibitory properties (Kozłowicz 1974, Becker et al. 1995, Devi and Raj 1996). *Ascaris* produces small proteins, between 60 and 150 residues long, which inactivate chymotrypsin and elastase (Huang et al. 1994), trypsin (Grasberger et al. 1994), pepsin (Abuereish and Peanasky 1974), and carboxypeptidase. Trypsin and α-chymotrypsin inhibitors are present in the tegument of *Ascaris suum* in its mature and developmental forms (Rola and Pudles 1966, Grutter 1994). It is thought that these inhibitors are secretory proteins that are released to the environment (Juhasz and Matskasi 1979) and block the activity of the host's proteolytic enzymes absorbed by the parasite (Juhasz 1979, Martzen et al. 1985).

*The paper has been financed by the Medical University of Lodz from statutory fund N° 503-127

The literature contains only a few reports related to the effect of substances coming from parasites on the development of the chicken embryo, a model recommended for the assessment of embryotoxic and teratogenic effects of chemical compounds. Until now, embryotoxic and teratogenic effects of *Toxoplasma gondii* extracts (toxoplasmin) for chickens have been described (Loutz-Ostertag 1996), as well as the toxic effects of *Ascaris* homogenate and proteolysis inhibitors isolated from it on hatched chicks (Kadłubowski and Błaszkowska 1992, Błaszkowska 1998). *Ascaris* proteolysis inhibitors has been demonstrated to produce a deleterious effects on development of mouse fetuses (Kadłubowski and Błaszkowska 1988; Błaszkowska 1999a, b, 2001).

The objective of this study was to investigate whether trypsin and α-chymotrypsin inhibitors present in the tegument of *Ascaris suum* had embryotoxic and teratogenic effects on the organ systems and skeleton of chicken embryos.

MATERIALS AND METHODS

Ascaris suum obtained from a slaughterhouse were washed thoroughly in physiological saline and destilled water. The trypsin and α-chymotrypsin inhibitors were obtained from *Ascaris* tegument by the modified method of Rola and Pudles (1966). The inhibitory effect of trypsin and α-chymotrypsin inhibitors on the activity of crystalline trypsin and crystalline α-chymotrypsin were evaluated by the photocolorimetric method (briefly: 0.025 mg of crystalline trypsin or 0.025 mg of crystalline α-chymotrypsin, Serva Feinbiochemica Heidelberg, 0.5% casein solution according to Hammarsten-Merck, veronal buffer pH 8.0, evaluation by modified Anson's method, incubation of the sample for 60 min at 37°C, measurement of extinction at 665 nm using Specol-Zeiss).

A total of 680 fertile White Leghorn eggs were used in the present study. Incubation of the eggs was conducted in the physical conditions as described in the earier cited work (Kadłubowski and Błaszkowska 1992). The eggs were first candled on day 4 of incubation. Any unfertilised eggs, with dead or developmentally retarded embryos were removed. Six hundred forty normally developing embryos were randomly divided into 2 groups – trypsin inhibitor, α-chymotrypsin inhibitor (each of 200 eggs), and 2 control groups (each of 120 eggs). On day 4 of incubation in the experimental groups, the inhibitor of 0.9% NaCl solution in volume of 0.1 ml at a dose equal to the LD_{50} value was administered once into the yolk sac. Trypsin inhibitor was injected at a dose of 0.3 mg per egg, α-chymotrypsin inhibitor at a dose of 0.07 mg per egg. LD_{50} values for *Ascaris* inhibitors for the chicken embryo were determined in the experiments conducted earlier (Kadłubowski and Błaszkowska 1992, Błaszkowska 1998). In one control group, eggs were injected (similarly as in the experimental groups) with doses of 0.3 mg of bovine albumin dissolved in 0.1 ml of 0.9% NaCl

solution each (group 1, albumin), whereas eggs in the other group were injected with 0.1 ml of 0.9% NaCl solution (group 2, NaCl).

The incubation of eggs was termined on day 15 of growth. The eggs were candled and the number of surviving and dead embryos determined. Surviving embryos were narcotized by diethyl ether, and on removing from eggs separated from the yolk sac and fetal membrans. The embryos were weighed and grossly examined, after which their internal organs were taken out of abdominal cavities and examined for any abnormalities according by Komarek and Lemez (1986).

After the removal of internal organs embryos were examined for skeletal abnormalities. The skeletal structure of embryos was stained with Peters', method (1977). Bone parts (stained red) and cartilage parts (stained blue) of the skeleton of 15-day chicken embryos were investigated with Romanoff's method (1960). Sections of stained bones from right limbs of embryos were measured: humeral, ulnar, femoral, and tibial. Length was measured with a slide calliper to the nearest accuracy of 0.1 mm.

To evaluate the results achieved, routine methods of medical statistics were applied (including U-test).

RESULTS

Mortality rate for the chicken embryos in the experimental groups on day 15 of incubation was: $45.0 \pm 3.5\%$ following injection of trypsin inhibitor and $44.0 \pm 3.5\%$ following injection of α-chymotrypsin inhibitor. In the control groups mortality rate of the chicken embryos was $5.0 \pm 1.9\%$ after injection of 0.9% NaCl solution and $3.3 \pm 1.6\%$ after administration of 0.3 mg bovine albumin.

Gross examination of surviving embryos and their dissection revealed pathological changes, symptoms of retardation in growth, and defects in development after injection of the inhibitors from *Ascaris* (Table 1). The highest rate of embryos exhibiting pathological changes was found after injection of α-chymotrypsin inhibitor ($50.0 \pm 4.7\%$), after injection of trypsin inhibitor ($36.4 \pm 4.59\%$), *Ascaris* homogenate ($29.5 \pm 4{,}31\%$). Similarly, the rate of embryos with pathological changes was found significantly higher after administration of trypsin inhibitor ($\alpha < 0.001$) than in the control groups (NaCl: $1.8 \pm 1.2\%$, albumin: $3.4 \pm 1.7\%$). The most frequent pathological anomalies to concur were abdominal dropsy and hemoperitoneum. For this reason the sum of the rate of incidence of pathological changes listed in Table 1 is higher than the rate of embryos with pathological anomalies.

When checking the development of the chicken embryo on day 15 of incubation, malformations were discovered in the experimental groups (Table 1). The most frequent defect was schistocelia (Table 1, Fig.1). Malformations occurring simultaneously were cyclopia and crossed beak (Fig. 2). The

Table 1. Pathological changes and malformations in the chicken embryos on day 15 of incubation after administration of trypsin and α-chymotrypsin inhibitors from *Ascaris*

Feature	0.9% NaCl 0.1 ml [1]	Bovine albumin 0.3 mg [2]	Trypsin inhibitor 0.3 mg	α-chymotrypsin inhibitor 0.07 mg
No. of live embryos	114	116	110	112
Embryos with retardation in ossification %	4.3 ± 1.89	5.2 ± 2.06	45.4 ± 4.74*	51.7 ± 4.72*
Pathological changes %:				
Abdominal dropsy	0.0 ± 0.00	0.0 ± 0.00	10.9 ± 2.97	14.3 ± 3.31
Subcutaneous oedema	0.0 ± 0.00	1.7 ± 1.20	21.8 ± 3.94*	35.7 ± 4.52*
Hemoperitoneum	1.8 ± 1.24	1.7 ± 1.20	10.9 ± 2.97*	16.1 ± 3.47*
Hemopericardium	0.00 ± 0.00	0.0 ± 0.00	4.5 ± 1.98	7.1 ± 2.43
Umbilical hernia	0.0 ± 0.00	0.0 ± 0.00	4.5 ± 1.98	8.0 ± 2.56
Embryos with malformations %	0.00 ± 0.00	0.0 ± 0.00	22.7 ± 3.99	37.5 ± 4.57
Malformations %:				
Cyclopia	0.0 ± 0.00	0.0 ± 0.00	4.5 ± 1.98	8.0 ± 2.56
Crossed beak	0.0 ± 0.00	0.0 ± 0.00	4.5 ± 1.98	8.0 ± 2.56
Micrognathia	0.0 ± 0.00	0.0 ± 0.00	9.1 ± 2.74	14.3 ± 3.31
Schistocelia	0.0 ± 0.00	0.0 ± 0.00	5.5 ± 2.17	21.4 ± 3.88
Cranial deformites	0.0 ± 0.00	0.0 ± 0.00	4.5 ± 1.98	3.6 ± 1.76

* significance $\alpha < 0.001$; [1], [2] – controls groups; dose of substances per egg. Results are presented as frequency ± SD

incidence of these defects was low, from 4.5 ± 1.9% to 8.0 ± 2.6% in embryos from the experimental groups (Table 1). The highest incidence of embryos with malformations was revealed after administration of α-chymotrypsin inhibitor (37.5 ± 4.5%), and the difference was statistically significant ($\alpha < 0.001$) when compared to the rate of embryos with effects after injection of trypsin inhibitor. The embryos in the control groups did not show any malformations.

In the experimental group of embryos some anomalies indicating retardation in growth were noted. One of these anomalies was lack of down in 15-day embryos which occurred at the rate of 12.7 ± 3.2% after injection of trypsin inhibitor and 14.3 ± 3.3% after injection of α-chymotrypsin inhibitor. Another anomaly confirming growth retardation in some embryos was only partial formation of lids, with the lower lid covering the eye only up to the pupil; this occurred after administration of trypsin inhibitor in 9.1 ± 2.7% of embryos, and after injection of α-chymotrypsin inhibitor in 8.0 ± 2.6% of embryos. In the two control groups this anomaly was not observed.

The experiment revealed a decrease in the mean body weight of embryos in the experimental groups after administration of *Ascaris* inhibitors in comparison to the control groups (Table 2). The differences were statistically significant

Fig. 1. Schistocelia in chicken embryo on day 15 of incubation after *Ascaris* trypsin inhibitor injection

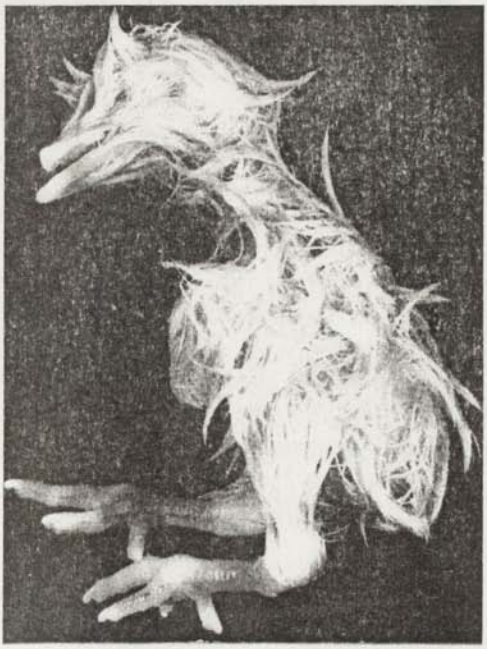

Fig. 2. Cyclopia and crossed beak in chicken embryo on day 15 of incubation after *Ascaris* α-chymotrypsin inhibitor injection

($\alpha < 0.001$) for both *Ascaris* inhibitors used in the experiment, whereas no significant difference between the mean body weights of embryos from the two control groups (NaCl, albumin) was noted.

Table 2. The effect of trypsin and α-chymotrypsin inhibitors from *Ascaris* on selected qualities of the chicken embryos on day 15 of incubation

Feature	0.9% NaCl 0.1 ml [1)]	Bovine albumin 0.3 mg [2)]	Trypsin inhibitor 0.3 mg	α-chymotrypsin inhibitor 0.07 mg
No. of live embryos	114	116	110	112
Body weight (g)	10.6 ± 0.26	10.4 ± 0.19	8.36 ± 0.23*	7.22 ± 0.21*
Bone's length: (mm)				
humerus	6.44 ± 0.16	6.64 ± 0.15	4.57 ± 0.11*	4.07 ± 0.13*
ulna	6.04 ± 0.16	6.24 ± 0.14	4.47 ± 0.11*	3.89 ± 0.13*
femur	9.23 ± 0.17	9.02 ± 0.18	6.86 ± 0.16*	6.16 ± 0.17*
tibia	12.4 ± 0.24	12.0 ± 0.25	8.22 ± 0.17*	7.86 ± 0.28*

* significance $\alpha < 0.001$; [1)], [2)] – control groups; dose of substances per egg. Results are presented as arithmetic mean ± SE

Analysis of the stained skeletons (both bone and cartilage parts) of the chicken embryos on day 15 of incubation revealed considerable retardation in ossification after administration of *Ascaris* inhibitors in the experimental groups as compared to the control groups. Ossification was found to be retarded for skull covering bones, especially parietal, occipital and temporal bones; for pectoral girdle bones: collar, scapular, and coracoid; for ribs and for pelvic bones. The highest incidence of retardation in ossification was noted after administration of α-chymotrypsin inhibitor (51.7 ± 4.7%; Table 1).

When evaluating the process of skeleton ossification in the chicken embryos in the control and experimental groups, the length of selected long bones of limb was measured (Table 2). Injection of *Ascaris* inhibitors was found to result in lower mean length of the measured bones as compared to the control groups. It is worth noting that the lowest values of the mean length of particular bones were found in the group of embryos that received α-chymotrypsin inhibitor. Significant difference ($\alpha < 0.001$) was shown between the mean length of the long bones in the embryos from the control groups and the analogous value from the experimental groups. No significant difference was found between the mean length of particular bones in the control groups.

Gross examination of the skeletal system in embryos from the experimental groups revealed not only shorter bones of the hind limb but also their apparent thickening and deformation of joints. This was the case in 14.5 ± 3.4% (trypsin inhibitor) and 17.9 ± 3.6% (α-chymotrypsin inhibitor) of chicken embryos.

DISCUSSION

The chicken embryo has been used to assess embryotoxicity and teratogenic effects of chemicals (Rouyan et al. 1994, Edrington et al. 1995, Illanes et al. 1995). Wilson (1975) recommended the use of a chick embryo for the detection of teratogenic effects of compounds besides those of various mammalian species, for four reasons: (1) it is readily available, (2) large numbers of test animals may be used with relatively small expenditures for equipment and personnel, (3) it is highly sensitive to a broad range of chemical and physical agents, (4) it closely parallels the mammal in morphological development.

The experiments conducted earlier (Kadłubowski and Błaszkowska 1992, Błaszkowska 1998) demonstrated toxic effects of homogenate of *Ascaris* tegument and proteolysis inhibitors present in it on the chicken embryos. In the course of those investigations, the LD_{50} value for both *Ascaris* inhibitors was estimated at different stages of incubation until hatching. Nonviable embryos with severe abnormalities were removed which precluded the finding of majority of the induced developmental defects in the hatched chickens. In the hereby presented study, in order to have a better picture of the effect of inhibitors from *Ascaris* on the developing chicken embryos, incubation was conducted only up to the 15th day of development of the embryo. On day 15 of incubation the chicken embryo has fully formed viscera and cartilage skeleton, the elements of which exhibit ossification (Romanoff 1960).

The 4th day of incubation is critical in the development of the chicken embryo as a starting point of formation of primary organs and cartilage skeleton, and therefore many investigators (Karnofsky and Lacon 1966) recommend injecting the tested chemicals at this very stage. In the studies carried out by the present author, *Ascaris* inhibitors (trypsin inhibitor, α-chymotrypsin inhibitor) were injected into the yolk sac on day 4 of incubation.

The decrease in the body weight of embryos injected with substances, as compared to the control group, may be an indication of toxic effect of a given chemical on the embryo (McLaughlin and Morliac 1963). The conducted experiment demonstrated that administration of *Ascaris* inhibitors into embryos resulted in the mean body weight lower than in the embryos from the both control groups. It is worth noting that the mean body weight of embryos from the both control groups (10.6g, NaCl; 10.4g, albumin) was close to the analogous values reported by Romanoff (1960), which proves that the same routine incubation conditions were employed.

To stain the skeletal system of chicken embryos, a modified Peters' method (1977) for staining the rodent embryo skeleton was used. By using this 2-step method of skeleton staining, a thorough observation of the whole process of ossification of primary cartilage skeleton is possible. Other investigators (Verrett and Scot 1980) used Dawson method (1976) to examine the growth of

skeleton in the chicken embryo, the method in which staining of only osseous parts of skeleton is possible. It should be noted that with some skeletal parts (e.g. sternum, scapula, coracoid bone) the process of ossification is complete only after chickens are hatched (Romanoff 1960). Considering this, the use of Peters' method in the present study allowed me to assess the process of skeletal formation of the chicken embryo with higher accuracy. The measurements of sections of selected long legs bones showed a significant retardation in ossification in the group of embryos injected with *Ascaris* inhibitors, as compared to the control groups. On examining the skeleton, significant retardation in ossification of skull covering bones, sternum, scapula as well as deformation of joints was found in the group of embryos injected with *Ascaris* inhibitors.

In the course of the present study, incidences of malformations in the chicken embryo after administration of trypsin and α-chymotrypsin inhibitors were stated. Thus, apart from toxic effects (numerous pathological changes, decreased mean body weight), *Ascaris* proteolysis inhibitors, and especially α-chymotrypsin inhibitor, exert also teratogenic effects in the chicken. Moreover, the results of this investigation of the chicken embryo confirm that incubation of the chicken embryo for only 15 days enables detection of some developmental abnormalities in surviving embryos, which in the further course of incubation lead to death, precluding such embryos from the assessment of surviving hatched chickens. For example, on day 15 of incubation some embryos were found to have schistocelia, whereas this defect was not stated in the hatched chickens assessed in the earlier investigations (Kadłubowski and Błaszkowska 1992, Błaszkowska 1998).

The studies conducted have shown for the first time that both proteolysis inhibitors isolated from *Ascaris suum* affect the chicken embryo disturbing its growth processes. These results are confirmed by the previously conducted experiments (Kadłubowski and Błaszkowska 1988, Błaszkowska 1999a, b; 2001) that revealed embryotoxic, fetotoxic, and teratogenic effects of these inhibitors derived from *Ascaris* on white mice. Worthy of note are also observations made by Gebhardt (1972) who, while analyzing the estimates of teratogenic activity of various substances on several species of animals, noticed a great similarity in the reaction of rodent and chicken embryos to the same agents. In most cases, substances teratogenic to the chicken induced also developmental abnormalities in the rodent.

The demonstration of embryotoxic and teratogenic activity of *Ascaris* proteolysis inhibitors may contribute to the explanation of some regulatory mechanisms in the host-parasite relationship, and especially of the influence of helminth infections on the host's reproductive performance.

CONCLUSIONS

1. The trypsin and α-chymotrypsin inhibitors from *Ascaris suum* administration into yolk sac act embryotoxically, significantly increasing mortality rate of White Leghorn chicken embryos.

2. The *Ascaris* proteolysis inhibitors reduce average mass of embryos, produce delay of bone formation and pathological changes in chicken embryos.

3. The *Ascaris* inhibitors reveal teratogenic action for chicken embryos.

REFERENCES

Abuereish G.M., Peanasky R.J. 1974. Pepsin inhibitors from *Ascaris lumbricoides*: isolation, purification and some properties. *Journal of Biological Chemistry* 249: 1558–1565.

Becker M.M., Harrop S.A., Dalton J.P., Kalina B.H., Mcmanus D.P., Brindley P.J. 1995. Cloning and characterization of the *Schistosoma japonicum* aspartic proteinase involved in hemoglobin degradation. *Journal of Biological Chemistry* 270: 2449–34501.

Błaszkowska J. 1998. The effect of alpha-chymotrypsin inhibitor from *Ascaris lumbricoides suum* on chick embryo. *Wiadomości Parazytologiczne* 44: 63–71.

Błaszkowska J. 1999a. Disturbances of mouse pregnancy after injection of *Ascaris* trypsin inhibitor during early organogenesis. *Zoologica Poloniae* 44: 23–36.

Błaszkowska J. 1999b. The effect of *Ascaris* tegumental homogenate and α-chymotrypsin inhibitor isolated from the nematode on mouse pregnancy. *Helminthologia* 36: 225–234.

Błaszkowska J. 2001. Disturbances of mouse pregnancy after injection of *Ascaris* α-chymotrypsin inhibitor during early organogenesis. *Helminthologia* 38: 15–21.

Clinton W.A., Baig J.S., Robinson P. 1996. *Taenia saginata* oncospher excretory/secretory peptidases. *Journal of Parasitology* 82: 7–10.

Devi C.M., Raj R.K., 1996. Histopathological changes in the spleen of BALB/c mice caused by excretory-secretory protein/s/ of *Setaria digitata* (Von Listow). *Indian Journal of Experimental Biology* 34: 32–36.

Dawson A.A. 1976. A note on the staining of the skeleton of cleared specimens with alizarin red S. *Stain Technology* 1: 123–124.

Edrington T.S., Harvey R.B., Kubena L.F. 1995. Toxic effects of aflatoxin B1 and ochratoxin A, alone and in combination, on chicken embryos. *Bulletin Environmental Contamination and Toxicology* 54: 331–336.

Gebhardt D.O. 1972. The use of chick embryo in applied teratology. *Advances of Teratology* 5: 97–111.

Gimwood J., Smith J.E. 1966. *Toxoplasma gondii*: the role of parasite surface and secreted proteins in host cell invasion. *International Journal of Parasitology* 26: 169–173.

Grasberger D.L., Clore G.M., Gronenborn A.M. 1994. High-resolution structure of *Ascaris* trypsin inhibitor in solution: direct evidence for a pH-induced conformational transtition in the reactive site. *Structure* 2: 669–678.

Grutter M.G. 1994. Proteinase inhibitors: another new fold. Structure 2: 575–576.

Hammerberg B.C., Dangler C., Williams J.F. 1980. *Taenia taeniaeformis* – chemical composition of parasite factory affecting coagulation and complement cascades. *Journal of Parasitology* 66: 569–576.

Huang K., Strynadka C.J., Bernard V.B., Peanasky R.J., James N.G. 1994. The molecular structure of the complex of *Ascaris* chymotrypsin/elastase inhibitor with porcine elastase. Structure 2: 679–689.

Illanes J., Blanquez M.J., Gonzuale M.E., Rojo C. 1995. Induccion experimental a malformaciones por instilacion de etanol en huevos embrionados de Pollo *(Gallus gallus)*. *Anatomy, Histology, Embryology* 24: 217–221.

Juhasz S. 1979. Proteolytic enzymes and enzymes inhibitors in *Ascaris suum*. II. Protease inhibitors produced *in vivo* in enzyme-free medium. *Acta Veterinaria Academiae Scientiarum Hungaricae* 27: 89–91.

Juhasz S., Matskasi J. 1979. Proteolytic enzymes and enzymes inhibitors in *Ascaris suum*. III. *In vitro* study of proteinase inactivation by the parasite. *Acta Veterinaria Academiae Scientiarum Hungaricae* 27: 93–96.

Kadłubowski R., Błaszkowska J. 1988. Proteases and their inhibitors in the host-parasite system. VIII. The effect of *Ascaris* tegumental homogenate and trypsin inhibitor isolated from the nematode on the number of foetuses per litter in mice. *Acta Parasitologica Polonica* 33: 285–293.

Kadłubowski R., Błaszkowska J. 1992. Działanie homogenatu *Ascaris lumbricoides suis* oraz jej inhibitora trypsyny na zarodek kury. *Wiadomości Parazytologiczne* 38: 31–41.

Karnofsky D.A., Lacon C.R. 1966. The effecf of 1-β-arabino-furano-sylcytosine on the developing chick embryo. *Biochemical Pharmacology* 15: 1435–1442.

Komarek V., Lemez L. 1986. *Anatomia avium domestcarum et embryologia galli*. Bratislavia, Priroda, 1–288.

Kozłowicz S. 1974. Zmiany patomorfologiczne w trzustce w przebiegu glistnicy jelita u świni domowej (*Sus domestica*). *Wiadomości Parazytologiczne* 22: 524–533.

Loutz-Ostertag I. 1966. Action de l'extrait de *Toxoplasma* (toxoplasmine) sur le developpment embryonnaire du poluet. *Archives d' Anatomie Macroscopique* 55: 363–386.

Martzen M.R., Geise G.L., Hogan R.J., Peanasky R.J. 1985. *Ascaris suum*: localization of the host proteases and parasite proteinase inhibitors in cross section using immunochemical and fluorescent probe technique. *Experimental Parasitology* 60: 139–149.

Mclaughlin J., Morliac I. P. 1963. The injection of chemicals into the yolk sac of fertile eggs prior to incubation as a toxicity test. *Toxicology and Applied Pharmacology* 5: 760–771.

Peters P.W. 1977. Duble staining of fetal skeletons for cartilage and bone, In: Neubert, D., Merker, H.J. and Kwasigroch, T. E (Eds) *Methods in Prenatal Toxicology*. Stuttgart, Georg Thieme Publisher, 153–154.

Rola F.H., Pudles J. 1966. Studies on the chymotryptic inhibitor from *Ascaris lumbricoides suum*, purification and properties. *Archives of Biochemistry and Biophysics* 13: 134–142.

Romanoff A.L. 1960. The Avian Embryo. New-York, The Macmillan Company, pp. 1–450.

Rouyan G.S., Meisel-Mikolajczyk F., Rumin W. 1994. The toxicity of antigenes extracted from strains of *Bacteroides vulgatus* from different origin to chick embryos. *Acta Microbiologica Polonica* 43: 97–103.

Verrett N., Scot W.F. 1980. Toxicity and teratogenicity of food additive chemicals in the developing chicken embryo. *Toxicology and Applied Pharmacology* 56: 265–273.

Wilson J.G. 1975. Reproduction and teratogenesis: current method and suggested improvements. *Journal of the Association Analytical Chemistry* 58: 657–667.