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The Effects of *Fasciola hepatica* Infection on the Total Antioxidant Status (TAS) and the Activity of Proteases and Their Inhibitors in Rat Serum

Lidia KOŁODZIEJCZYK, Iwona JAROCKA and Elżbieta SKRZYDLEWSKA

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Fasciola hepatica infection results in increased production of reactive oxygen species (ROS) and changes the activity/ level of antioxidants in the host organism, which leads to oxidative stress formation and oxidative modifications of lipids and proteins. Taking this into account, the aim of this study was to assess the antioxidant potential and the activity of proteases and their inhibitors in the serum of rats infected with *F. hepatica*. Wistar rats were infected *per os* with 30 metacercariae of *F. hepatica*. The total antioxidant status (TAS) and the activity of cathepsin G and elastase and their inhibitors (α_1-antitrypsin and α_2-macroglobulin) were determined at 4, 7, and 10 weeks post infection (wpi). It was confirmed that *F. hepatica* infection leads to a decrease in the antioxidant capacity of serum, which was manifested as a reduction in total antioxidant status by about 24, 39, and 27%, respectively, at 4, 7, and 10 wpi. At the same time, the activity of proteases increased significantly: cathepsin G by about 25, 37, and 30%, and elastase by about 18, 16, and 9% during the course of *F. hepatica* infection, compared with the control group. However, the activity of α_1-antitrypsin was significantly reduced, by 36, 55, and 25%, while α_2-macroglobulin activity was reduced by about 14, 17, and 8% during the same period of fasciolosis. These results indicate that the shift in protease/antiprotease balance towards protease action observed during the course of fasciolosis may result in a decrease in host antioxidant capacity.

Key words: *Fasciola hepatica* cathepsin G; elastase; α_1-antitrypsin; α_2-macroglobulin; proteolytic-anti- proteolytic balance.

Lidia KOŁODZIEJCZYK, Chair and Department of Biology and Medical Parasitology, Pomeranian Medical University, Powstanców Wielkopolskich 72, 70-111 Szczecin, Poland.
E-mail: lkolo@pum.edu.pl
Iwona JAROCKA, Elżbieta SKRZYDLEWSKA, Department of Analytical Chemistry, Medical University of Białystok, Mickiewicza 2, 15-230 Białystok, Poland.

Fasciola hepatica is a parasite of the liver and bile ducts of domestic and wild herbivorous mammals and humans. Fasciolosis can be a serious veterinary problem because of the significant economic losses it causes in cattle and sheep farming (SPITHILL & DALTON 1998). Due to the increase in the number of infections in people worldwide, fasciolosis is now considered an emerging/re-emerging parasitic disease (MAS-COMA 2005).

Many parasitic diseases are accompanied by inflammation processes while inflammatory infiltration cells are the source of reactive oxygen species (ROS). ROS have been found to be involved in the pathogenesis of such diseases as malaria, Chagas disease and schistosomiasis (BECKER *et al*. 2004; MACAO *et al*. 2007; OTHMAN *et al*. 2008). Moreover an increase in the generation of free radicals by peritoneal leukocytes in rats and monocytes in humans during *F. hepatica* infection has also been demonstrated (SMITH *et al*. 1992; ABO-SHOUSHA *et al*. 1999; JEDLINA *et al*. 2011). *F. hepatica* infection in rats has also increasingly been recognized as a cause of mitochondrial dysfunction (LENTON *et al*. 1995). This may lead to a partial inhibition of the respiratory chain, which may in turn enhance autooxidation of a redox carrier, resulting finally in an elevated production of oxygen radicals, especially superoxide anions. An increase in the generation of free radicals can impair cellular metabolism, albeit only when the antioxidant defense system is no longer capable of destroying free radicals (SIES 1997). In the course of fasciolosis a reduced level of non-enzymatic antioxidants and also a decrease in the activity of antioxidant enzymes in rat livers and serum have been observed (KOŁODZIEJCZYK *et al*. 2005, 2006). This is accompanied by the enhancement of lipid peroxidation processes in humans, rats and sheep

(KOŁODZIEJCZYK *et al*. 2005, 2006; KAYA *et al*. 2007; SALEH 2008). Moreover, in the course of fasciolosis in rats increased oxidative modifications of liver proteins have been observed, which are manifested as an increase in the content of carbonyl groups and dityrosine and a reduction in the content of sulfhydryl, amine groups and tryptophan residues (SIEMIENIUK *et al*. 2008). This may lead to changes in the functions of biologically active proteins (DAVIES *et al*. 1987).

The aim of this study was to evaluate the effect of *F. hepatica* infection on the total antioxidant status and the activity of neutrophil-derived serine proteases, such as cathepsin G and elastase and their inhibitors, α_2-macroglobulin and α_1-antitrypsin, in rat serum.

Material and Methods

Animal treatment

The experiment was carried out in male Wistar rats aged 5 weeks. The rats were housed in groups with free access to granulated standard feed and water, under a normal light-dark cycle. The study protocol was approved by the Local Bioethics Committee in Szczecin (Poland) in accordance with the Polish Animal Protection Act 1997. The rats were infected *per os* with 30 metacercariae of *F. hepatica* passed through a stomach tube. The metacercariae were obtained from a *Lymnaea truncatula* snail culture according to TAYLOR & MOZLEY (1948) and were classified as viable only if excretory granules were seen under an optical microscope (BORAY 1969). Ten control and ten *F. hepatica*-infected rats were anaesthetized with ketamine at 4, 7, and 10 weeks post-infection (wpi) and blood was collected from the heart. After 30 minutes of incubation the blood was centrifuged at 3000 × g (4°C) and serum was obtained.

Biochemical assays

The activity of cathepsin G (EC 3.4.21.20) and elastase (EC 3.4.21.37) were determined with Suc-Phe-Pro-Phe-pNA (Bachem, Switzerland) at pH 7.6 (WOODBURY & NEURATH 1980) and Suc-Ala-Ala-Val-pNA (Bachem, Switzerland) at pH 8.5 (BIETH *et al*. 1974), respectively. The activity of these proteases was measured by the quantity of p-nitroaniline released after incubation (2h for cathepsin G and 12h for elastase) of the plasma with substrates in concentration 74 mM (1:9; v:v) at 37°C. The protein concentration was determined by biuret assay (GORNALL *et al*. 1949).

The activity of α_1-antitrypsin was determined by an estimation of trypsin activity inhibition measured using hemoglobin as the substrate. 0.1 ml of diluted serum was added to 0.1 ml of porcine trypsin (625U/ml), and this mixture was incubated for 5 min at 37°C. A control treatment for trypsin activity without serum was also prepared. Next, 0.3 ml of 2% hemoglobin was added to the mixture. This solution was incubated at 37°C for 10 min, and the reaction was terminated by the addition of 1.25 ml of 20% trichloroacetic acid. Tyrosine-containing peptides were determined in the filtrate using the Folin-Ciocalteu reagent (BARCLAY & VINQVIST 1994). The amount of tyrosine-containing peptides released by trypsin was inversely related to α_1-antitrypsin activity.

The activity of α_2-macroglobulin was measured with the Unitest™ kit (Unicorn Diagnostics Ltd. UK) (GALLIMORE *et al*. 1983). Plasma was 160-fold diluted with a buffer (0.05M Tris, 0.1M NaCl, pH 8.0) to determine the activity of α_2-macroglobulin. The diluted plasma was incubated with porcine trypsin (50 U/ml) for 2 min at 37°C, and then, following the addition of soy bean trypsin inhibitor, it was incubated for 2 min at 37°C. As soon as trypsin inhibition was completed, the amount of α_2-macroglobulin was determined by measuring the activity of the α_2-macroglobulin-trypsin complex. After 2 min incubation with Bz-Val-Gly-Arg-pNA (1mM) the amount of p-nitroaniline released by trypsin was estimated by measuring absorption at 405 nm.

The total antioxidant status (TAS) was measured with ABTS reagent (2,2'-azino-di-3-ethylbenzthiazoline sulphonate), which was incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^+$, measured spectrophotometrically at 660 nm. Antioxidants in the added sample caused suppression of colour production. The total antioxidant capacity concentration was compared to the equivalent antioxidant capacity of Trolox and was expressed in nmoles of Trolox/ml. This method was developed by MILLER *et al*. (1993).

A more detailed description of the methodology is presented in the habilitation thesis of KOŁODZIEJCZYK (2010).

Statistics

Data were expressed as means ± SD and analysed with one-way ANOVA and Scheffé F-tests. Differences with $P<0.05$ were considered significant.

Results

In this study an average of 10 mature flukes was recovered from the bile ducts of *F. hepatica*-infected rats at 10 wpi (mean±SD: 10.0±1.01).

The activities of cathepsin G and elastase and their inhibitors (α_1-antitrypsin and α_2-macroglob-

Table 1

Activity of cathepsin G and elastase and their inhibitors (α_1-antitrypsin and α_2-macroglobulin) in the serum of control and *F. hepatica*-infected rats at 4, 7 and 10 wpi (mean±SD

	Weeks post infection					
	4		7		10	
	control rats	infected rats	control rats	infected rats	control rats	infected rats
Cathepsin G pNA, nmol/ml/2h	126 ± 7	158 ± 11[a]	131 ± 6	179 ± 13[a]	129 ± 7	168 ± 14[a]
Elastase pNA, nmol/ml/12h	13.7 ± 0.8	16.1 ± 1.2[a]	13.5 ± 0.7	15.7 ± 1.3[a]	14.0 ± 0.9	15.2 ± 1.3[a]
α_1-antitrypsin pNA, nmol/ml/min	47.2 ± 2.9	64.3 ± 5.3[a]	45.3 ± 3.2	70.1 ± 6.2[a]	47.7 ± 3.3	59.4 ± 4.9[a]
α_2-macroglobulin pNA, nmol/ml/min	32.9 ± 1.8	28.4 ± 2.1[a]	33.4 ± 2.0	27.6 ± 2.3[a]	34.0 ± 2.1	31.4 ± 2.4[a]

[a] – significantly different from control group ($P<0.05$).

ulin) in the serum of rats infected with *F. hepatica* are presented in Table 1. The activity of cathepsin G at 4, 7 and 10 wpi significantly increased by about 25%, 37% and 30% respectively, in comparison with the control. Likewise, the activity of elastase increased by about 18%, 16% and 9% in the course of *F. hepatica* infection (Table 1).

The activity of α_1-antitrypsin significantly decreased by 36%, 55% and 25% at 4, 7 and 10 wpi, respectively. The amount of tyrosine released by the trypsin was inversely related to α_1-antitrypsin activity. During the same period of fasciolosis, the activity of α_2-macroglobulin also decreased by about 14%, 17% and 8% (Table 1).

The serum level of total antioxidant status (TAS) significantly decreased by 24%, 39% and 27% at 4, 7 and 10 wpi, respectively (Fig. 1).

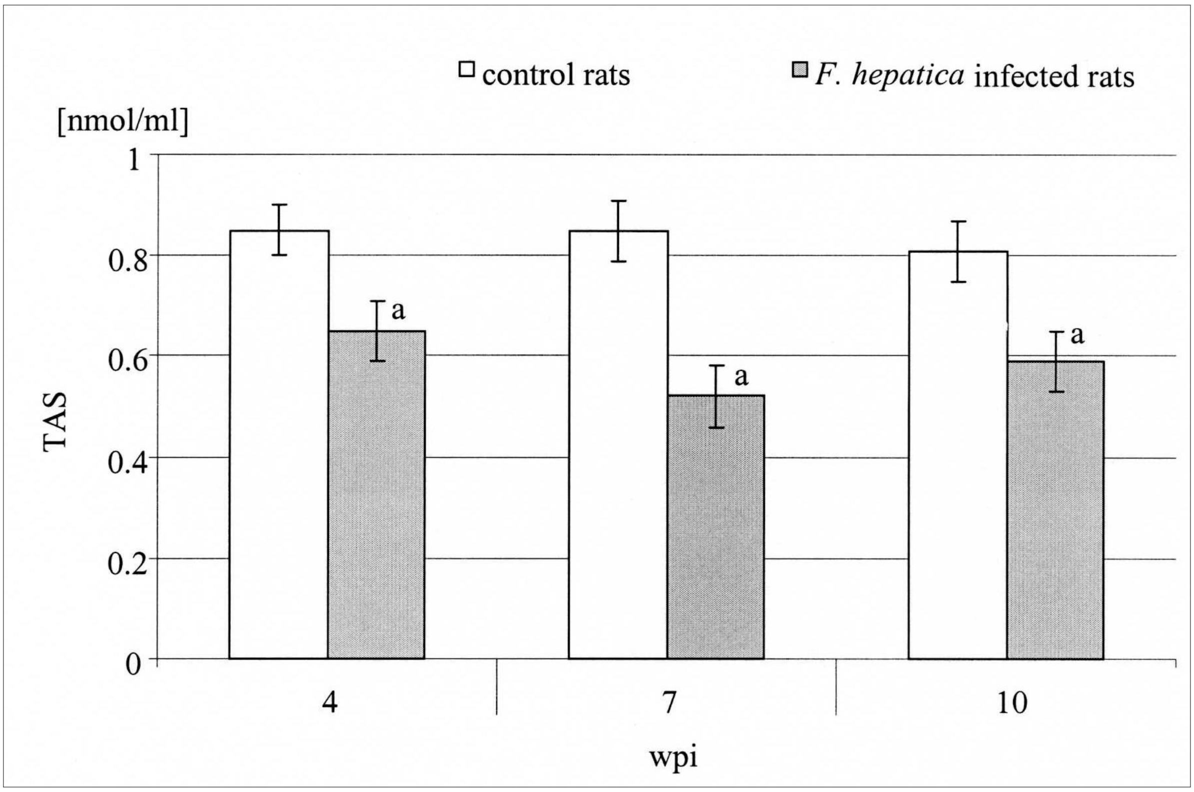

Fig. 1. Total antioxidant status (TAS) in the serum of control and *F. hepatica*-infected rats at 4, 7, and 10 wpi. (a – significantly different from control group at $P<0.05$).

Discussion

A proteolytic-antiproteolytic balance exists in the blood in physiological conditions. The activity of cellular proteases in the blood stream is very low and remains in equilibrium with protease inhibitors (VERCAIGNE-MARKO *et al.* 1985; SOTTRUP-JENSEN 1989). Under such conditions, the inhibitors may effectively regulate the activity of proteases and protect proteins, especially those of the extracellular matrix, against these enzymes. Cathepsin G and elastase are serine proteases present in azurophil granules of neutrophils and monocytes, and also in eosinophils, basophils, mast cells and lymphocytes in the case of elastase (BARRICK *et al.* 1999). The action of these proteases is reduced by endogenous protease inhibitors which constitute about 10% of the total protein in plasma. Inhibitors of serine proteases (serpines) include, among others, α_1-antitrypsin and α_2-macroglobulin synthesized in the liver. The action of a protease is triggered by the inactivation of its inhibitor (MURPHY & REYNOLDS 1993). The inactivation of α_1-antitrypsin and α_2-macroglobulin can occur under the influence of ROS released by activated leukocytes, as well as through proteolytic degradation with participation of different leukocyte proteases (BARRICK *et al.* 1999. This may cause a disturbance in the proteolytic-antiproteolytic balance leading to pathological processes, since cathepsin G and elastase exhibit a wide substrate specificity – they are able to degrade elastin, collagen and proteoglycans, as well as the complement, immunoglobulins, fibrinogen, basic proteins, and other proteins (WATOREK *et al.* 1988). Cathepsin G is particularly aggressive in its action on proteins, since it degrades them not only directly but also by activating procollagenases (CAPODICI & BERG 1989).

The results of this study have shown that *F. hepatica* infection causes a significant, though variable, increase in the activity of cellular serine proteolytic enzymes (cathepsin G and elastase) in rat serum. It is known that serine proteases activate NAD(P)H oxidase, which is synthesized as a proenzyme (BUCURENCI *et al.* 1992) and that this enzyme is responsible for ROS generation in neutrophils (WEISS 1989). Enhanced ROS generation has been found in the course of *F. hepatica* infection (SMITH *et al.* 1992; SIBILLE *et al.* 2004; JEDLINA *et al.* 2011). However, our study indicates that the level of total antioxidant status (TAS) of rat serum decreases significantly in the course of *F. hepatica* infection (by about 24%, 39% and 27% at 4, 7 and 10 wpi, respectively). A decrease in total antioxidant status was also observed in the liver of rats during *F. hepatica* infection. Such a situation leads to oxidative stress formation that results in oxidative modifications of cellular lipids and proteins (SIEMIENIUK *et al.* 2008). Previous studies indicate an increase in lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in the course of rat fasciolosis as well as changes in protein structure (KOŁODZIEJCZYK *et al.* 2005, 2006; SIEMIENIUK *et al.* 2008). Oxidative modifications of cell membrane lipids and proteins cause changes in their structure and function and in consequence may modify membrane permeability for cellular components (PETIT *et al.* 1995). This may imply a possible release of enzymes from cells (neutrophils) into the extracellular space and the blood stream. The increase in the activity of cathepsin G and elastase in the blood serum confirms this suggestion. Independently from the above, another mechanism of enhanced activity of proteases may be proposed. Serine proteases are synthesized as inactive proenzymes and are activated also via oxidative modifications caused by free radicals or low molecular aldehydes (WEISS 1989).

Protease activity is balanced by the action of their inhibitors, but during *F. hepatica* infection an increase in protease activity is accompanied by a decrease in the activity of their inhibitors, α_2-macroglobulin and α_1-antitrypsin. This may be caused by ROS reaction and inhibitor molecules (BUCURENCI *et al.* 1992). It is known that the structure and function of α_2-macroglobulin depends on the presence of disulphide bridges between cysteine residues (Cys^{225}-Cys^{408} and Cys^{447}-Cys^{540}) (SOTTRUP-JENSEN 1989), and also that cysteine and methionine are amino acids extremely sensitive to ROS (DAVIES *et al.* 1987). It has been demonstrated that the cysteine content in oxidized protein molecules is altered (SCHWARTZ *et al.* 1987) which may lead to the inhibition of α_2-macroglobulin activity. Moreover, oxidants disturb the structure and function of α_1-antitrypsin through modification of methionyl residues of the protein to dimethyl sulphoxide (SHECHTER 1986). It has also been demonstrated that hydroxyl radicals cause oxidative modification in two out of the eight methionine residues, of which one is in the reactive centre of α_1-antitrypsin (JOHNSON & TRAVIS 1979). Moreover, the methionyl residue found in the enzyme-binding site is probably included in this process as well. Other amino acid residues are also included in ROS-induced changes in the protein structure (DAVIES *et al.* 1987; GEBICKI & GEBICKI 1993). As a consequence of amino acid modifications, the secondary and tertiary structure of protein can be changed, leading to a loss of enzymatic activity (STADTMAN & BERLETT 1991). Independently, the activity of inhibitors may be also decreased by hydrolysis by proteases (VERCAIGNE-MARKO *et al.* 1985; VISSER *et al.* 1988), the activity of which is enhanced. α_1-antitrypsin, as a polyvalent inhibitor, suppresses the activity of leukocyte cathepsin G and of elastase, forming inactive, stable complexes of 1:1 stechiometry (HUBER & CARRELL 1989). In this way, it prevents possible tissue damage caused by these proteases (WEISS 1989), since proteases can penetrate into the extracellular space from the blood and uncontrolled proteolysis may occur. The participation of ROS in the proteolytic-antiproteolytic balance shift has also been demonstrated in other disorders such as cancer (SKRZYDLEWSKA *et al.* 2005).

In conclusion, our results imply that the fasciolosis-reduced antioxidative abilities of the host organism may lead to changes in the activity of proteases and their inhibitors, which may cause a proteolytic-antiproteolytic imbalance.

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