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Authors: A. Escamilla, R. Pérez-Caballero, R. Zafra, M.J. Bautista, I.L. Pacheco, M.T. Ruiz, M.S. Martínez-Cruz, A. Martínez-Moreno, V. Molina-Hernández, J. Pérez



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**Apoptosis of peritoneal leucocytes during early stages of *Fasciola hepatica* infections
in sheep**

**A. Escamilla^a, R. Pérez-Caballero^b, R. Zafra^b, M.J. Bautista^a, I.L. Pacheco^a, M.T.
Ruiz^a, M^a S. Martínez-Cruz, A. Martínez-Moreno^b, V. Molina-Hernández^c, J.
Pérez^a,**

^aDepartment of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine,
University of Córdoba, Spain

^bDepartment of Animal Health (Parasitology), Faculty of Veterinary Medicine,
University of Córdoba, Spain.

^cSchool of Biological Sciences, Queen's University Belfast, Belfast, UK.

Corresponding author:

José Pérez,

Dep. Anatomía y Anatomía Patológica Comparadas

Edificio de Sanidad Animal, Campus de Rabanales, Ctra. Madrid-Cádiz km 396

14014 Córdoba, Spain

Tel: +34 957218178, Fax: +34 957218682

Highlights

1. *F. hepatica* induces peritoneal leucocytes apoptosis in sheep from 1 dpi onwards
2. Apoptosis of peritoneal leucocytes may allow the larvae to evade the host immune response
3. Peritoneal leucocyte apoptosis is detected earlier with annexin V than caspase-3

Abstract:

Several immunomodulatory properties have been described in *Fasciola hepatica* infections. Apoptosis has been shown to be an effective mechanism to avoid the immune response in helminth infections. The aim of the present work was to study apoptosis in peritoneal leucocytes of sheep experimentally infected with *F. hepatica* during the early stages of infection. Five groups (n=5) of sheep were used. Groups 2–5 were orally infected with 200 metacercariae (mc) and sacrificed at 1, 3, 9 and 18 days post-infection (dpi), respectively. Group 1 was used as the uninfected control (UC). Apoptosis was detected using three different methods: 1) immunocytochemistry (ICC) with a polyclonal antibody anti-active caspase-3; 2) an annexin V flow cytometry assay using the Annexin V-FITC/propidium iodide (PI); and 3) transmission electron microscopy (TEM). The differential leucocyte count revealed that the majority of peritoneal granulocytes were eosinophils, which increased significantly at 9 and 18 dpi with respect to the uninfected controls. The ICC study revealed that the percentage of caspase-3⁺ apoptotic peritoneal leucocytes increased significantly from 3 dpi onwards with respect to the uninfected controls. The flow cytometry annexin V assay detected a very significant (P<0.001) increase of apoptotic peritoneal macrophages, lymphocytes and granulocytes, which remained higher than in the UC until 18 dpi. Transmission electron microscopy studies also confirmed the presence of apoptosis in peritoneal eosinophils at 18 dpi. This is the first report of apoptosis induced by *F. hepatica* in the

peritoneal leucocytes of sheep *in vivo*. The results of this work suggest the importance of apoptosis induction for the survival of the juvenile parasites in the peritoneal migratory stages of infection.

Key words: apoptosis; caspase-3; annexin-V; peritoneal leucocytes; *Fasciola hepatica*; sheep.

Introduction

During the migration of juvenile *Fasciola hepatica* through the peritoneal cavity, until their penetration into the hepatic parenchyma, the parasites secrete different molecules which interact with peritoneal leucocytes in different ways. Since it has been reported that the protective response against *F. hepatica* occurs during the early stages of infection, it would be of interest to study mechanisms of immunomodulation in target species at the peritoneal and early hepatic migratory stages. *In vitro* studies in the rat model demonstrated that secreted excretory products of *F. hepatica* (FhESP) are able to induce apoptosis in eosinophils and peritoneal macrophages, suggesting that apoptosis of effector cells may play a role in the host immune evasion/suppression induced by *F. hepatica* infection (Serradell et al., 2007; Guasconi et al., 2012). *In vivo* studies in sheep have also revealed apoptosis of eosinophils in hepatic lesions both during acute and chronic stages of infection (Escamilla et al., 2016). To date, apoptosis of peritoneal inflammatory cells from sheep infected with *F. hepatica in vivo* has not been reported. Several markers of apoptosis in tissue sections have been investigated, among them activated caspase-3 immunohistochemistry is considered an easy, sensitive and reliable method for detecting and quantifying apoptosis in histological sections (Duan et al.,

2003; Resendes et al., 2004). The annexin V/PI assay has also been used by several authors to evaluate the apoptosis phenomenon (Serradell et al., 2007; Chen et al., 2013).

The aim of the present work was to evaluate the presence and number of inflammatory peritoneal cells undergoing apoptosis in peritoneal fluid from sheep experimentally infected with *F. hepatica* during the earlier peritoneal migratory stages (0, 1, 3, 9 and 18 days post-infection, dpi). Annexin V/PI flow cytometry and activated caspase-3 immunocytochemistry were used to quantify peritoneal inflammatory cell apoptosis, and transmission electron microscopy was used to evaluate the ultrastructure of apoptotic inflammatory cells.

2. Materials and methods

2.1. Experimental design

Forty-five 7-month-old female Merino sheep were used for this study. All animals were obtained from a liver fluke-free farm, and they were tested for *Fasciola* eggs by faecal sedimentation with negative results. They were allocated into five groups of five animals each: group 1 was used as an uninfected control (UC) group and groups 2 to 5 were orally infected with one dose of 200 *F. hepatica* metacercariae (Ridgeway Research Ltd., UK) administered in gelatine capsules with a dosing gun. Sheep in groups 2-5 were sacrificed by an intravenous injection of thiobarbital at 1, 3, 9, and 18 days post-infection (dpi), respectively. The experiment was approved by the Bioethical Committee of the University of Córdoba (No. 1118) and was performed taking into account European (2010/63/UE) and Spanish (RD 1201/2005) directives on animal experimentation.

2.2. Peritoneal cell populations

Peritoneal washing was conducted immediately after the animals were euthanised following the methodology described by Zafra et al., 2013. Briefly, the abdominal cavity was washed with 60 ml sterile phosphate buffer saline (PBS) with 6 mM EDTA (ethylenediaminetetraacetic acid), pre-heated to 37 °C. After a softly massaging for 1 min, 40 ml peritoneal fluid were recovered and centrifuged at 1500 rpm for 10 min and the supernatant was eliminated. Subsequently, cellular pellets were resuspended using the aforementioned medium. Diff-Quick stained smears were used for differential cell count. A total of 600 cells per animal were counted and the percentage of eosinophils, lymphocytes, macrophages and neutrophils was obtained. Epithelial cells were not included in the cell counting. Extensions were manually performed in Vectabond-treated slides. After air drying, these extensions were fixed using acetone for 5 min and stored at -80 °C for immunocytochemical studies.

2.3. Immunocytochemistry

A polyclonal rabbit anti-human caspase-3 antibody, (cat. No. RP096, Diagnostic BioSystems, Pleasanton, USA) was used. The primary antibody reacts with cleaved activated caspase-3 but does not recognise full-length caspase-3 or other cleaved caspases. According to the manufacturer, this antibody cross-reacts with activated ovine caspase-3. The avidin-biotin-peroxidase method (Zafra et al., 2013) was used for the immunocytochemical study. Briefly, defrosted peritoneal fluid smears were fixed in acetone for 10 min, and the endogenous peroxidase activity was quenched in H₂O₂ in methanol for 15 min, with agitation at room temperature (RT). Smears were washed for 10 min in PBS, and then they were incubated in PBS with 5% normal goat serum for 30 min at RT. Anti-caspase-3 antibody 1:100 was applied overnight at 4 °C. After three 10 min rinses in PBS-T (PBS buffer with 0.1% Tween-20 detergent), a goat anti-rabbit immunoglobulin serum (Dako, Glostrup, Denmark) diluted 1:200 was applied for 30

min as a secondary antibody. As the third reagent, an avidin-biotin peroxidase complex (Vector Laboratories) was applied for one hour. Labelling was visualised by application of the NovaRED™ substrate kit (Vector Laboratories). Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. In negative control slides, specific primary antibody was substituted with non-immune rabbit serum.

2.4. Cell counting from immunocytochemistry

Immunoreactive cells were counted using the Image Pro-plus software 6.0 (Media Cybernetics). Caspase-3⁺ and caspase-3⁻ peritoneal leucocytes were counted in 10 fields of 0.08 mm² per animal randomly selected from the smears. In animals with small numbers of peritoneal leucocytes, at least 300 cells per animal were counted. Results are given as mean ± SD per group.

2.5. Annexin V Flow cytometry assay

Cell density of peritoneal fluid pellets was adjusted to $2-5 \times 10^5$ cells/ml. Annexin V Kit (ANNEX100F, AbD Serotec-Bio-Rad Company- UK) for flow cytometry was used. The assay was performed according to the manufacturer's instructions. Briefly, the cells were suspended in 200 µl of binding buffer. Then, 5 µl of Annexin V-FITC were added to 195 µl of the cell suspension mentioned before. This suspension was mixed and incubated for 10 min in darkness at RT. Subsequently, the suspension was washed in 200 µl of pre-diluted (at 1:4) binding buffer. Next, the cells were resuspended again in 190 µl of pre-diluted binding buffer. Finally, 10 µl of propidium iodide solution were added to this suspension, and the samples were ready to analyse by flow cytometry. For a positive control, the cells were incubated with 3% formaldehyde in buffer for 30 min on ice. Then the formaldehyde was washed away, and the cells were resuspended in cold binding buffer and processed as a normal sample. A CyFlow Cube 6 cytometer

(Sysmex Partec, Germany) and specific protocol based on morphological features (forward scatter/side scatter) were used to determine the leucocyte populations in the peritoneal fluid. The apoptotic cells were identified because of the reactivity of fluorochrome (FL1 for Annex V and FL3 for propidium iodide). Peritoneal leucocytes were differentiated as viable (annexin V-negative and PI-negative) and apoptotic (annexin V-positive, PI-negative). The extent of apoptosis was expressed according to the percentage of each cell population that was apoptotic or viable. The results were analysed using the Flowing Software (University of Turkey, Finland). Ten thousand events were acquired, and the total number of apoptotic cells versus viable cells were counted and expressed as percentages of the total number of peritoneal leucocytes acquired.

2.6. Transmission electron microscopy

For the ultrastructural study, fresh peritoneal cellular pellet samples from UC and 18 dpi groups were used. The remaining groups were not included in the TEM study because the pellet samples were too small for the ICC, flow cytometry and TEM studies.

Peritoneal cell pellets were centrifuged at 1500 rpm for 10 min (4 °C), cut into 1-3 mm cubes, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in Epon 812. Thin sections (50 nm) were stained with uranyl acetate and lead citrate and examined and photographed using a Jeol Jem 1400 transmission electron microscope at the Central Research Services (SCAI) of the University of Córdoba.

2.7. Statistical analysis

For the statistical analysis, the GraphPad Prism v6.0 software (GraphPad Software, Inc. San Diego, CA, USA) was used. For each timepoint of the experiment the expression of caspase-3 and Annexin V positive cells was compared using the one-way ANOVA test

followed by the Tukey post hoc test for multiple comparisons of means. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Peritoneal leucocyte populations

The differential cell count for peritoneal leucocyte populations is given in Table 1. In the UC group and at 1 and 3 dpi, macrophages followed by lymphocytes were the two more abundant peritoneal leucocytes, with occasional neutrophils and eosinophils. At 9 dpi, a significant increase in eosinophils and a significant decrease in macrophages with respect to the UC group were observed, while the percentage of lymphocytes did not change significantly. At 18 dpi, the increase in the percentage of eosinophils and decrease in the percentage of macrophages with respect to the UC group was even more pronounced than at 9 dpi. The percentage of lymphocytes tended to decrease at 18 dpi with respect to the UC group, but the differences were not significant. Neutrophils were occasionally present in all groups and did not change significantly with respect to the UC group.

3.2. Apoptosis detected by caspase-3 expression

Immunolabelling with the anti-caspase-3 antibody was detected as a cytoplasmic and nuclear reddish-brownish colour (Fig. 1). The percentage of peritoneal leucocytes expressing caspase-3 is given in Table 2. Cellular pellets from peritoneal leucocytes from the UC group showed occasional caspase-3⁺ cells (0.4 ± 0.6). At 1 dpi the percentage of peritoneal leucocytes caspase-3⁺ was 1.2 ± 1.3 without a significant difference from the UC group. However, at 3, 9 and 18 dpi the percentage of caspase-3⁺ leucocytes was 19.5 ± 5.5 , 19.9 ± 4.0 and 42.6 ± 4.3 , respectively, with statistical

increase with respect to the UC group, and also between the 18 dpi group and the 3 and 9 dpi groups (Table 2).

3.3. Annexin V Flow Cytometry assay

The percentages of apoptotic and viable peritoneal macrophages, lymphocytes and granulocytes are summarised in Table 3. Peritoneal leucocyte populations (macrophages, lymphocytes and granulocytes) were determined according to their morphological features. The UC group showed a very low percentage of apoptotic macrophages (1.7%), lymphocytes (0.4%) and granulocytes (1.5%). At 1 dpi the percentage of apoptotic leucocytes suffered a dramatic and significant increase compared to the UC group: 37.8%, 28.2% and 30.7% for macrophages, lymphocytes and granulocytes, respectively. At 3, 9 and 18 dpi the percentage of apoptotic peritoneal macrophages and lymphocytes remained significantly higher with respect to the UC group but it decreased at 18 dpi with respect to previous infection stages (Table 3). In contrast, the percentage of apoptotic granulocytes tended to increase at 3 dpi with respect to 1 dpi, suffered a transient decrease at 9 dpi when migrating larvae reached the liver, and again increased up to 80% at 18 dpi (Table 3).

3.4. Apoptosis detected by transmission electron microscopy

The TEM study confirmed apoptosis in peritoneal eosinophils from 18 dpi. The ultrastructural features of eosinophils from the UC group consist of a bilobed nucleus and typical cytoplasmic oval granules ranging from 0.5 to 1 μm in diameter with a moderately electron dense matrix material and a highly electron-dense crystalloid core. Apoptotic TEM changes consisted of margination-condensation of chromatin (early changes) and cytoplasmic and nuclear fragmentation while maintaining cell membranes and organelles (Fig. 2). Macrophages from the 18 dpi group showed morphological

changes typical of phagocytic activation such as large cytoplasmic vacuoles containing membrane debris (secondary lysosomes) and enlarged rough endoplasmic reticulum, but nuclear fragmentation indicative of apoptosis was not observed in this cell type.

4. Discussion

To study apoptosis in peritoneal leucocytes, it is necessary to first evaluate the differential peritoneal leucocyte count during the course of *F. hepatica* infection. In the UC group, the majority of peritoneal leucocytes consisted of macrophages and lymphocytes with only 1.5% eosinophils. These results agree with those from uninfected control goats (Zafra et al., 2013). The relative increase in the percentage of peritoneal eosinophils and the decrease of the percentage of macrophages and lymphocytes found during the course of the infection was also reported in *F. hepatica* infected goats at 7-9 dpi (Zafra et al., 2013).

The results presented in this study show that *F. hepatica* infection induces apoptosis of peritoneal leucocytes in sheep from 1 dpi onwards. The annexin V assay is based on phosphatidylserine (PS) emergence on the outer plasma membrane, an early event in apoptosis, PS binds with high affinity to the anticoagulant annexin V that is visualised by fluorescence microscopy or flow cytometry (Serradell et al., 2009). Activated or cleaved caspase-3 has been used as a marker of apoptosis by immunohistochemistry in healthy tissues (Resendes et al., 2004) and in helminth infected tissue sections (Chen et al., 2008; Escamilla et al., 2016). In the UC group, caspase-3 expression and the annexin V assay showed similar results with a low percentage (0.4-1.7%) of apoptotic peritoneal leucocytes. However, at the very early stages of infection (1 dpi) the annexin V assay detected a higher percentage of apoptosis (37.8%, 28.2% and 30.7% for

macrophages, lymphocytes and granulocytes, respectively) whereas caspase-3 expression was found only in 1.2% of peritoneal leucocytes. These results suggest that the annexin V assay can detect apoptosis earlier than caspase-3 expression. When the infection progressed (3, 9 and 18), the percentage of apoptotic peritoneal leucocytes detected by caspase-3 and annexin V was similar. In the present study, TEM studies confirmed apoptosis of eosinophils at 18 dpi by the ultrastructural features: cytoplasmic granules with an electron dense core which were similar to that reported in human (Duffin et al., 2009) and ovine (Balic et al., 2006) eosinophils. The condensation and fragmentation of the nucleus with an intact cytoplasmic membrane found in apoptotic eosinophils in this work has also been reported in TEM studies of human (Duffin et al., 2009) and ovine (Balic et al., 2006; Escamilla et al., 2016) eosinophils. Compared to caspase-3 and annexin V, TEM studies were less useful to quantify the percentage of apoptotic leucocytes due to the small amount of sample examined.

It has been reported that excretory secretory products of *F. hepatica* (FhESP) are able to induce apoptosis of rat eosinophils and macrophages *in vitro* (Serradell et al., 2007; 2009; Guasconi et al., 2012). *In vivo* studies in sheep infected with *F. hepatica* have also revealed apoptosis of eosinophils in hepatic lesions during early stages of infection (9 dpi) and in chronic hepatic lesions (Escamilla et al., 2016). The evaluation of the percentage of leucocytes suffering apoptosis in areas where the migratory larvae of *F. hepatica* are located is necessary to understand the importance of this phenomenon as a modulatory mechanism to avoid the host response against migrating larvae. In a previous study in the liver, 46.1% and 53.9% of eosinophils located in the periphery of necrotic tracts and foci at 8 and 28 dpi, respectively, were apoptotic (Escamilla et al., 2016). Similarly in the present study, a dramatic increase of apoptotic macrophages, lymphocytes and granulocytes were found at 1 dpi with respect to the UC group. These

results suggest that *F. hepatica* migratory larvae are able to induce a rapid apoptosis of peritoneal leucocytes which may allow the larvae to migrate through the peritoneum evading the host response.

In summary, the present work is the first report of apoptosis in peritoneal leucocytes during the peritoneal migratory stages of *F. hepatica* infection in sheep *in vivo*. The dramatic increase in apoptotic peritoneal macrophages, lymphocytes and granulocytes found at 1 dpi with respect to the UC group suggests that *F. hepatica* migratory larvae are able to induce apoptosis of important percentage of peritoneal effector cells such as macrophages and eosinophils, as well as lymphocytes, allowing the larvae to migrate through the peritoneal cavity evading the host response. The knowledge of apoptotic mechanisms induced by *F. hepatica*, and the parasite molecules involved in apoptosis induction, are important in order to potentially include them in vaccine candidates to improve vaccine efficacy against this parasite.

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Figure Legends

Figure 1. Peritoneal smear, 3 dpi. Caspase 3 expression is observed as brown cytoplasmic and nuclear colour in peritoneal leucocytes (arrow) whereas other are negative (arrowheads). E: epithelial cell. ABC method-haematoxylin counterstain, x400.

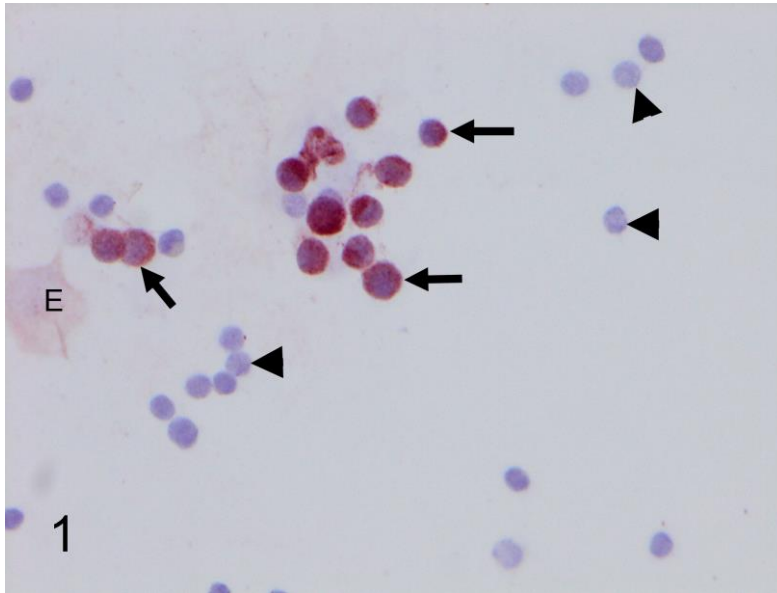


Figure 2. 18 dpi. Apoptotic peritoneal eosinophil showing fragmented and condensed nuclei and typical cytoplasmic granules (arrows). Transmission electron microscopy. Bar = 2 μ m.



Table 1. Percentage of peritoneal leucocytes (mean±SD) in uninfected control and *Fasciola hepatica* infected sheep during early stages of infection.

Percentage of peritoneal leucocytes (%)				
Group	Lymphocytes	Macrophages	Neutrophils	Eosinophils
UC	36.4±14.0	60.0±14.7	1.1±2.0	2.7±1.1
1 dpi	35.4±15.8	61.4±28.0	1.9±2.7	1.4±2.2
3 dpi	44.2±11.8	53.3±11.6	0.3±0.5	2.3±2.7
9 dpi	40.6±18.2	32.1±5.6*	2.9±2.7	24.3±11.7*
18 dpi	24.1±9.5	25.8±8.9*	0.5±1.1	49.3±14.1*

UC: uninfected control.

* Significant difference ($P < 0.05$) compared to the uninfected control group.

Table 2. Results from immunocytochemistry of peritoneal leucocytes expressed in percentage of caspase 3 positive and negative cells in each day post-infection (dpi). Results expressed as mean \pm SD per group.

	UC	1 dpi	3 dpi	9 dpi	18 dpi
Caspase 3⁺	0.4 \pm 0.6	1.2 \pm 1.3	19.5 \pm 5.5*	19.9 \pm 4.0*	42.6 \pm 4.3*§
Caspase 3⁻	99.6 \pm 0.6	98.8 \pm 1.3	80.5 \pm 5.5*	80.0 \pm 4.0*	57.4 \pm 4.3*§

UC: uninfected control.

* Significant difference ($P < 0.05$) compared to the uninfected control group.

§ Significant difference ($P < 0.05$) compared to the uninfected control, 1, 3 and 9 dpi groups.

Table 3. Percentage of non-apoptotic and apoptotic peritoneal macrophages, lymphocytes and granulocytes according to the annexin V flow cytometry assay. Results are expressed as mean \pm SD per group.

Cell population	Stage	UC	Time post-infection			
			1 dpi	3 dpi	9 dpi	18 dpi
Macrophages	NA	98.5 \pm 0.9	62.2 \pm 11.1*	76.5 \pm 6.7*	75.0 \pm 2.0*	89.7 \pm 2.7*
	A	1.5 \pm 0.9	37.8 \pm 11.1*	23.5 \pm 6.7*	25.0 \pm 2.0	10.3 \pm 2.7*
Lymphocytes	NA	99.6 \pm 0.4	71.8 \pm 11.4*	77.4 \pm 11.4*	82.1 \pm 1.4*	95.3 \pm 1.4*
	A	0.4 \pm 0.4	28.3 \pm 11.4*	22.6 \pm 11.4*	17.8 \pm 1.4*	4.7 \pm 1.4*
Granulocytes	NA	98.5 \pm 1.6	69.3 \pm 6.7*	53.6 \pm 12.0*	81.9 \pm 4.3*	20.0 \pm 6.0*
	A	1.5 \pm 1.6	30.7 \pm 6.7*	46.4 \pm 12.0*	18.1 \pm 4.3*	80.0 \pm 6.0*

UC: Uninfected control; N: Non-apoptotic cells; A: Apoptotic cells.

* Significant difference ($P < .05$) compared to the uninfected control group.