# 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], Mª S. Martínez-Cruz, A. Martínez-Moreno[b], V. Molina-Hernández[c], J. Pérez[a],**

[a]Department of Anatomy and Comparative Pathology, Faculty of Veterinary Medicine, University of Córdoba, Spain

[b]Department of Animal Health (Parasitology), Faculty of Veterinary Medicine, University of Córdoba, Spain.

[c]School 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

**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) administrated 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 decribed 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 ºC. 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,

93 lymphocytes, macrophages and neutrophils was obtained. Epithelial cells were not
94 included in the cell counting. Extensions were manually performed in Vectabond-
95 treated slides. After air drying, these extensions were fixed using acetone for 5 min and
96 stored at -80 ºC for immunocytochemical studies.

97 *2.3. Immunocytochemistry*

98 A polyclonal rabbit anti-human caspase-3 antibody, (cat. No. RP096, Diagnostic
99 BioSystems, Pleasanton, USA) was used. The primary antibody reacts with cleaved
100 activated caspase-3 but does not recognise full-length caspase-3 or other cleaved
101 caspases. According to the manufacturer, this antibody cross-reacts with activated ovine
102 caspase-3. The avidin-biotin-peroxidase method (Zafra et al., 2013) was used for the
103 immunocytochemical study. Briefly, defrosted peritoneal fluid smears were fixed in
104 acetone for 10 min, and the endogenous peroxidase activity was quenched in $H_2O_2$ in
105 methanol for 15 min, with agitation at room temperature (RT). Smears were washed for
106 10 min in PBS, and then they were incubated in PBS with 5% normal goat serum for 30
107 min at RT. Anti-caspase-3 antibody 1:100 was applied overnight at 4 ºC. After three 10
108 min rinses in PBS-T (PBS buffer with 0.1% Tween-20 detergent), a goat anti-rabbit
109 immunoglobulin serum (Dako, Glostrup, Denmark) diluted 1:200 was applied for 30
110 min as a secondary antibody. As the third reagent, an avidin-biotin peroxidase complex
111 (Vector Laboratories) was applied for one hour. Labelling was visualised by application
112 of the NovaRED$^{TM}$ substrate kit (Vector Laboratories). Sections were counterstained
113 with Mayer's haematoxylin, dehydrated and mounted. In negative control slides,
114 specific primary antibody was substituted with non-immune rabbit serum.

115 *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 $mm^2$ 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 x $10^5$ 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

141 analysed using the Flowing Software (University of Turky, Finland). Ten thousand
142 events were acquired, and the total number of apoptotic cells versus viable cells were
143 counted and expressed as percentages of the total number of peritoneal leucocytes
144 acquired.

145 *2.6. Transmission electron microscopy*

146 For the ultrastructural study, fresh peritoneal cellular pellet samples from UC and 18 dpi
147 groups were used. The remaining groups were not included in the TEM study because
148 the pellet samples were too small for the ICC, flow cytometry and TEM studies.
149 Peritoneal cell pellets were centrifuged at 1500 rpm for 10 min (4 ºC), cut into 1-3 mm
150 cubes, fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded
151 in Epon 812. Thin sections (50 nm) were stained with uranyl acetate and lead citrate and
152 examined and photographed using a Jeol Jem 1400 transmission electron microscope at
153 the Central Research Services (SCAI) of the University of Córdoba.

154 *2.7. Statistical analysis*

155 For the statistical analysis, the GraphPad Prism v6.0 software (GraphPad Software, Inc.
156 San Diego, CA, USA) was used. For each timepoint of the experiment the expression of
157 caspase-3 and Annexin V positive cells was compared using the one-way ANOVA test
158 followed by the Tukey post hoc test for multiple comparisons of means. A value of
159 $P<0.05$ was considered statistically significant.

160 **3. Results**

161 *3.1. Peritoneal leucocyte populations*

162 The differential cell count for peritoneal leucocyte populations is given in Table 1. In
163 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

188 macrophages (1.7%), lymphocytes (0.4%) and granulocytes (1.5%). At 1 dpi the
189 percentage of apoptotic leucocytes suffered a dramatic and significant increase
190 compared to the UC group: 37.8%, 28.2% and 30.7% for macrophages, lymphocytes
191 and granulocytes, respectively. At 3, 9 and 18 dpi the percentage of apoptotic peritoneal
192 macrophages and lymphocytes remained significantly higher with respect to the UC
193 group but it decreased at 18 dpi with respect to previous infection stages (Table 3). In
194 contrast, the percentage of apoptotic granulocytes tended to increase at 3 dpi with
195 respect to 1 dpi, suffered a transient decrease at 9 dpi when migrating larvae reached the
196 liver, and again increased up to 80% at 18 dpi (Table 3).

197 *3.4. Apoptosis detected by transmission electron microscopy*

198 The TEM study confirmed apoptosis in peritoneal eosinophils from 18 dpi. The
199 ultrastructural features of eosinophils from the UC group consist of a bilobed nucleus
200 and typical cytoplasmic oval granules ranging from 0.5 to 1 µm in diameter with a
201 moderately electron dense matrix material and a highly electron-dense crystalloid core.
202 Apoptotic TEM changes consisted of margination-condensation of chromatin (early
203 changes) and cytoplasmic and nuclear fragmentation while maintaining cell membranes
204 and organelles (Fig. 2). Macrophages from the 18 dpi group showed morphological
205 changes typical of phagocytic activation such as large cytoplasmic vacuoles containing
206 membrane debris (secondary lysosomes) and enlarged rough endoplasmic reticulum,
207 but nuclear fragmentation indicative of apoptosis was not observed in this cell type.

208

209 **4. Discussion**

210 To study apoptosis in peritoneal leucocytes, it is necessary to first evaluate the
211 differential peritoneal leucocyte count during the course of *F. hepatica* infection. In the

212 UC group, the majority of peritoneal leucocytes consisted of macrophages and
213 lymphocytes with only 1.5% eosinophils. These results agree with those from
214 uninfected control goats (Zafra et al., 2013). The relative increase in the percentage of
215 peritoneal eosinophils and the decrease of the percentage of macrophages and
216 lymphocytes found during the course of the infection was also reported in *F. hepatica*
217 infected goats at 7-9 dpi (Zafra et al., 2013).

218 The results presented in this study show that *F. hepatica* infection induces apoptosis of
219 peritoneal leucocytes in sheep from 1 dpi onwards. The annexin V assay is based on
220 phosphatidylserine (PS) emergence on the outer plasma membrane, an early event in
221 apoptosis, PS binds with high affinity to the anticoagulant annexin V that is visualised
222 by fluorescence microscopy or flow cytometry (Serradell et al., 2009). Activated or
223 cleaved caspase-3 has been used as a marker of apoptosis by immunohistochemistry in
224 healthy tissues (Resendes et al., 2004) and in helminth infected tissue sections (Chen et
225 al., 2008; Escamilla et al., 2016). In the UC group, caspase-3 expression and the
226 annexin V assay showed similar results with a low percentage (0.4-1.7%) of apoptotic
227 peritoneal leucocytes. However, at the very early stages of infection (1 dpi) the annexin
228 V assay detected a higher percentage of apoptosis (37.8%, 28.2% and 30.7% for
229 macrophages, lymphocytes and granulocytes, respectively) whereas caspase-3
230 expression was found only in 1.2% of peritoneal leucocytes. These results suggest that
231 the annexin V assay can detect apoptosis earlier than caspase-3 expression. When the
232 infection progressed (3, 9 and 18), the percentage of apoptotic peritoneal leucocytes
233 detected by caspase-3 and annexin V was similar. In the present study, TEM studies
234 confirmed apoptosis of eosinophils at 18 dpi by the ultrastructural features: cytoplasmic
235 granules with an electron dense core which were similar to that reported in human
236 (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 apoptotis 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.

**Acknowledgments**

This work was supported by EU grants (H2020-635408-PARAGONE) and the Spanish Ministry of Science grant AGL2015-67023-C2-1-R. The TEM studies were carried out by the Central Research Services (SCAI) of the University of Córdoba.

**References**

Balic, A., Cunningham, C.P., Meeusen, E.N., 2006. Eosinophil interactions with *Haemonchus contortus* larvae in the ovine gastrointestinal tract. Parasite Immunol. 28, 107-115.

Chen, K.M., Lee, H.H., Lai, S.C., Hsu, L.S., Wang, C.J., Liu, J.Y. , 2008. Apoptosis in meningoencephalitis of *Angiostrongylus cantonensis*-infected mice. Exp.Parasitol. 119, 385-390.

Chen, X., Li, S., He, L., Wang, X., Liang, P., Chen, W., Bian, M., Ren, M., Lin, J., Liang, C., Xu, J., Wu, Z., Li, X., Huang, Y., Yu, X., 2013. Molecular characterization of severin from *Clonorchis sinensis* excretory/secretory products and its potential anti-apoptotic role in hepatocarcinoma PLC cells. PLoS Negl Trop Dis. 7(12): e2606.

285 Duan, W.R., Garner, D.S., Williams, S.D., Funckes-Shippy, C.L., Spath, I.S., Blomme,
286 E.A. 2003. Comparison of immunohistochemistry for activated caspase-3 and cleaved
287 cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological
288 sections of PC-3 subcutaneous xenografts. J. Pathol. 199, 221-228.

289 Duffin, R., Leitch, A.E., Sheldrake, T.A., Hallett, J.M., Meyer, C., Fox, S., Alessandri,
290 A.L., Martin, M.C., Brady, H.J., Teixeira, M.M., Dransfield, I., Haslett, C., Rossi, A.G.,
291 2009. The CDK inhibitor, R-roscovitine, promotes eosinophil apoptosis by down-
292 regulation of Mcl-1. FEBS Letters 583, 2540-2546.

293 Escamilla, A., Bautista, M.J., Zafra, R., Pacheco, I.L., Ruiz, M.T., Martinez-Cruz, S.,
294 Méndez, A., Martinez-Moreno, A., Molina-Hernandez, V., Perez, J., 2016. *Fasciola*
295 *hepatica* induces eosinophil apoptosis in the migratory and biliary stages of infection in
296 sheep. Vet. Parasitol. 216, 84-88.

297 Guasconi, L., Serradell, M.C., Masih, D.T., 2012. *Fasciola hepatica* products induce
298 apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148, 359-363.

299 Resendes, A.R., Majo, N., Segales, J., Espadamala, J., Mateu, E., Chianini, F., Nofrarias
300 M., Domingo, M., 2004. Apoptosis in normal lymphoid organs from healthy normal,
301 conventional pigs at different ages detected by tunel and cleaved caspase-3
302 immunohistochemistry in paraffin-embedded tissues. Vet. Immunol. Immunopathol. 99,
303 203-213.

304 Serradell, M.C., Guasconi, L., Cervi, L., Chiapello, L.S., Masih, D.T., 2007. Excretory-
305 secretory products from *Fasciola hepatica* induce eosinophil apoptosis by a caspase-
306 dependent mechanism. Vet. Immunol. Immunopathol. 117, 197-208.

307 Serradell, M.C., Guasconi, L., Masih, D.T., 2009. Involvement of a mitochondrial
308 pathway and key role of hydrogen peroxide during eosinophil apoptosis induced by

309 excretory-secretory products from *Fasciola hepatica.* Mol. Biochem. Parasitol. 163, 95-
310 106.
311 Zafra, R., Perez-Ecija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martinez-Moreno,
312 A., Mulcahy, G., Dalton, J.P., Perez, J. 2013. Early and late peritoneal and hepatic
313 changes in goats immunized with recombinant cathepsin L1 and infected with *Fasciola*
314 *hepatica*. Res. Vet. Sci. 94-602-609.

315

316

317 **Table 1.** Percentage of peritoneal leucocytes (mean±SD) in uninfected control and

318 *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* |

319 UC: uninfected control.

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

321

322

**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 ± SD per group.

| | UC | 1 dpi | 3 dpi | 9 dpi | 18 dpi |
|---|---|---|---|---|---|
| **Caspase 3$^{+}$** | 0.4±0.6 | 1.2±1.3 | 19.5±5.5* | 19.9±4.0* | 42.6±4.3*§ |
| **Caspase 3$^{-}$** | 99.6±0.6 | 98.8±1.3 | 80.5±5.5* | 80.0±4.0* | 57.4±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.

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

| | | | Time post-infection | | | |
|---|---|---|---|---|---|---|
| **Cell population** | **Stage** | **UC** | **1 dpi** | **3 dpi** | **9 dpi** | **18 dpi** |
| Macrophages | NA | 98.5±0.9 | 62.2±11.1* | 76.5±6.7* | 75.0±2.0* | 89.7±2.7* |
| | A | 1.5±0.9 | 37.8±11.1* | 23.5±6.7* | 25.0±2.0 | 10.3±2.7* |
| Lymphocytes | NA | 99.6±0.4 | 71.8±11.4* | 77.4±11.4* | 82.1±1.4* | 95.3±1.4* |
| | A | 0.4±0.4 | 28.3±11.4* | 22,6±11.4* | 17.8±1.4* | 4.7±1.4* |
| Granulocytes | NA | 98.5±1.6 | 69.3±6.7* | 53.6±12.0* | 81.9±4.3* | 20.0±6.0* |
| | A | 1.5±1.6 | 30.7±6.7* | 46.4±12.0* | 18.1±4.3* | 80.0±6.0* |

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

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

351

352

353 **Figure Legends**

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

357 **Figure 2.** 18 dpi. Apoptotic peritoneal eosinophil showing fragmented and condensed
358 nuclei and typical cytoplasmic granules (arrows). Transmission electron microscopy. Bar
359 = 2 um.

360

361

362

363

364