1 *Fasciola hepatica* **Induces Eosinophil Apoptosis in the Migratory and Biliary Stages of**

2 **Infection in Sheep**

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Abstract

The aim of the present work was to evaluate the number of apoptotic eosinophils in the livers of sheep experimentally infected with *Fasciola hepatica* during the migratory and biliary stages of infection. Four groups (n = 5) of sheep were used; groups 1-3 were orally infected with 200 metacercariae (mc) and sacrificed at 8 and 28 days post-infection (dpi), and 17 weeks post-infection (wpi), respectively. Group 4 was used as an uninfected control. Apoptosis was detected using immunohistochemistry with a polyclonal antibody against anti-active caspase-3, and transmission electron microscopy (TEM). Eosinophils were identified using the Hansel stain in serial sections for caspase-3, and by ultrastructural features using TEM. At 8 and 28 dpi, numerous caspase-3^{+} eosinophils were mainly found at the periphery of acute hepatic necrotic foci. The percentage of caspase -3^{+} apoptotic eosinophils in the periphery of necrotic foci was high (46.1 to 53.9) at 8 and 28 dpi, respectively, and decreased in granulomas found at 28 dpi (6%). Transmission electron microscopy confirmed the presence of apoptotic eosinophils in hepatic lesions at 8 and 28 dpi. At 17 wpi, apoptotic eosinophils were detected in the infiltrate surrounding some enlarged bile ducts containing adult flukes. This is the first report of apoptosis induced by *F. hepatica* in sheep and the first study reporting apoptosis in eosinophils in hepatic inflammatory infiltrates *in vivo*. The high number of apoptotic eosinophils in acute necrotic tracts during the migratory and biliary stages of infection suggests that eosinophil apoptosis may play a role in *F. hepatica* survival during different stages of infection.

Key words: apoptosis; caspase-3; eosinophil; *Fasciola hepatica*; sheep.

42 **1. Introduction**

43 *Fasciola hepatica* causes liver fluke disease in temperate climates and is responsible for
44 major economic losses in animal production (Spithill et al., 1999). This disease is also
45 considered a serious public health problem in humans (McManus and Dalton, 2006). *F.*
46 *hepatica* often causes chronic infection, which implies the development of strategies by the
47 parasite to modulate/suppress the host immune response (Dalton et al., 2013; Morphew et al.,
48 2013). The parasite modulation of the host immune response is a serious obstacle to obtaining
49 protective vaccines against *F. hepatica* in ruminants (Toet et al., 2014; Molina-Hernández et
50 al., 2015).

51 In helminth infections, eosinophils play important roles either in developing tissue pathology
52 (Cadman et al., 2014) and in the host effector response by releasing cytotoxic granule
53 proteins and various lipid mediators (Klion and Nutman, 2004; Cadman et al., 2014). An
54 effective response in rats infected with *F. hepatica* has been associated with a significant
55 increase in eosinophil infiltration of the gut lamina propria in early post-infection stages (Van
56 Milligen et al., 1998, 1999). In *F. hepatica* infected rats, peritoneal eosinophils producing
57 very high levels of nitric oxide (NO) have been reported (Jedlina et al., 2011). In sheep,
58 peritoneal eosinophils and macrophages, as well as mammary gland eosinophils from *F.*
59 *gigantica* resistant Indonesian thin-tail (ITT) sheep were able to kill juvenile *F. gigantica* in
60 vitro by antibody-dependent cytotoxicity, but they did not kill larvae of *F. hepatica*,
61 suggesting that eosinophils are important effector cells involved in the resistance of sheep to
62 *F. gigantica* (Piedrafita et al., 2007). Previous studies have reported that bovine eosinophils
63 incubated with bovine serum were unable to damage juvenile *F. hepatica*, but major basic
64 protein (MBP) obtained from bovine eosinophils was able to kill juvenile *F. hepatica* at very
65 low concentration (Duffus et al., 1980).

Apoptosis of effector cells such as eosinophils has been reported as a mechanism of immunosuppression during helminth infections (Shin et al., 2000; Yan et al., 2008; Zepeda et al., 2010). It has been reported that *F. hepatica* induces eosinophil apoptosis in the hepatic inflammatory infiltrate in rats *in vivo* (Serradell et al., 2007). *In vitro* studies in the rat model have demonstrated that secreted excretory products of *F. hepatica* (FhESP) are able to induce apoptosis in eosinophils and peritoneal macrophages, suggesting that apoptosis in 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). Although the mechanisms of immune modulation/suppression induced by *F. hepatica* are a serious obstacle to developing protective vaccines in ruminants (Molina-Hernández et al., 2015), to date, apoptosis has not been investigated in ruminants infected with *F. hepatica*.

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). Transmission electron microscopy is also a useful technique to detect apoptotic cells and to identify eosinophils based on the morphological features of their cytoplasmic granules (Duffin et al., 2009) and it allow to evaluate ultrastructure of apoptotic eosinophils (Balic et al., 2006).

The aim of the present work was to evaluate the presence and number of eosinophils undergoing apoptosis in hepatic inflammatory infiltrates from sheep experimentally infected with *F. hepatica* during the migratory (8 and 28 days post-infection -dpi) and biliary stages of infection (17 weeks post-infection –wpi). Activated caspase-3 immunohistochemistry was used for quantifying eosinophil apoptosis and transmission electron microscopy was used to evaluate ultrastructure of apototic eosinophils.

90 **2. Materials and methods**

91 *2.1. Experimental design*

92 Twenty 7-month-old female Merino-breed sheep were used for this study. All animals were
93 obtained from a liver fluke-free farm. Animals were purchased at 1 month of age and housed
94 indoors in the experimental farm of the University of Córdoba until they reached the
95 appropriate age for challenge. All animals were tested monthly for parasite eggs by fecal
96 sedimentation; the results were negative. In addition, all animals were tested for serum IgG
97 specific for *F. hepatica* cathepsin L1 by ELISA prior to challenge and all of them were
98 negative. The sheep were allocated into four groups of five animals each: groups 1, 2 and 3
99 were orally infected with one dose of 200 metarceriae (Ridgeway Research Ltd.
100 Gloucestershire, UK); group 4 was used as the uninfected negative control. Animals were
101 sacrificed by intravenous injection of thiobarbital at different stages of infection. Groups 1
102 and 2 were sacrificed at 8 and 28 days post-infection (dpi) and the animals of group 3 were
103 sacrificed at 17 weeks post-infection (wpi). The experiment was approved by the Bioethics
104 Committee of the University of Cordoba (No. 1118) and was performed taking into account
105 European (2010/63/UE) and Spanish (RD 1201/2005) directives on animal experimentation.

106 *2.2. Histopathology and fluke burdens*

107 At necropsy, the duodenum was ligated proximally and distally to the ductus choledochus (8
108 to 10 cm) and the liver was photographed on the visceral and diaphragmatic aspects for gross
109 evaluation. Liver tissue samples showing hepatic lesions were collected and fixed in 10%
110 neutral buffered formalin for 24 h, then routinely processed and embedded in paraffin wax.
111 Four micron-thick tissue sections were stained with hematoxylin and eosin (H&E) for
112 histopathology. Fluke burden was conducted in animals from group 3 as follows: gallbladders
113 were opened and flukes were recovered, then the bile ducts were carefully opened and flukes

114 were removed with blunt forceps. Finally, the livers were cut into small pieces (1 cm^3) and
115 washed in hot water to collect the remaining flukes.

116 Hansel's stain was used in liver tissue sections to identify the presence of eosinophils. Two 4
117 µm-thick tissue serial sections were obtained. All these tissue sections were deparaffinized
118 and dehydrated through a graded ethanol series. The first section was stained with Hansel's
119 stain, the second by immunohistochemistry for active caspase-3. For Hansel's stain, tissue
120 sections were incubated for 1 min with eosin, washed in distilled water and incubated for 1
121 min in methylene blue, then washed in distilled water, dehydrated and mounted.

122 *2.3. Immunohistochemistry (IHC)*

123 Formalin-fixed, paraffin-embedded sections and the avidin-biotin-peroxidase method (Zafra
124 et al., 2013b) were used for the IHC study. A polyclonal (pAb) rabbit anti-human caspase-3
125 antibody (RP096, Diagnostic BioSystems, Pleasanton, USA) was used as a marker for
126 apoptosis. The primary antibody reacts with cleaved activated caspase-3 but does not
127 recognize full-length caspase-3 or other cleaved caspases; according to the manufacturer, this
128 antibody cross-reacts with activated ovine caspase-3. Liver tissue sections from 8 dpi in
129 which apoptosis had been detected by transmission electron microscopy were used as positive
130 controls.

131 *2.4. Transmission electron microscopy (TEM)*

132 For the ultrastructural study, fresh liver samples from groups 1, 2 and 4 were used. In groups
133 1 and 2, samples were collected from whitish tortuous tracts, cut into 1-mm cubes, fixed in
134 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and embedded in Epon 812. Thin
135 sections (50 nm) were stained with uranyl acetate and lead citrate and examined and
136 photographed using a Jeol Jem 1400 transmission electron microscope.

137 *2.5. Gross lesions evaluation and cell counting*

138 Gross pictures of livers were used to evaluate gross damage (hemorrhages and necrotic tracts
139 at 8 and 28 dpi, and fibrosis and scars at 17 wpi) as reported Zafra et al (2008) using the
140 Image Pro 6.0 software (Media Cybernetics, Silver Spring, USA). The perimeter of the
141 diaphragmatic and visceral aspects of the liver was delineated and the total area obtained,
142 then, the perimeter of the damaged areas was delineated and the area obtained. The
143 percentage of damaged area was then expressed as mean value ± SD per group.

144 Cell counting was carried out in 10 fields of 0.08 mm^2 per animal randomly selected at the
145 periphery of necrotic foci (8 dpi), necrotic foci and granulomas (28 dpi) and inflammatory
146 infiltrate surrounding large bile ducts with erosive cholangitis (17 wpi). Cell counting was
147 carried out in tissue sections stained with Hansel's technique (eosinophils), and IHC
148 (caspase-3^+ eosinophils and apoptotic bodies). Results are given as mean ± SD per group.

149 *2.6. Statistical analyses*

150 The Kolmogorov-Smirnov test was applied to decide whether distributions were parametric.
151 Comparison between pairs of groups was made with the Mann-Whitney U test. $p<0.05$ was
152 considered significant.

153 **3. Results**

154 *3.1. Gross pathology and fluke burdens*

155 All control livers from group 4 showed no gross changes on either the diaphragmatic or
156 visceral surface. Livers from group 1 showed numerous white spots with reddish spots and
157 occasional whitish tortuous tracts ranging from 0.3 to 1 cm in length located on the liver
158 surface, mainly on the diaphragmatic aspect of the left lobe. Livers from group 2 showed
159 numerous whitish tortuous tracts ranging from 0.5 to 3 cm in length, whitish-grey patches

160 ranging up to 1x1x2.5 cm and occasional reddish spots, mainly involving the left lobe. Livers
161 from group 3 showed enlarged, whitish gall bladders and bile ducts, which contained brown
162 fluid and numerous adult flukes. Numerous tortuous greyish scars were found on the liver
163 surface, mainly involving the left lobe. The percentage of damaged hepatic surface was
164 3.5±1.2 at 8 dpi, 8.7±2.3 at 28 dpi and 23.4±6.7 at 17 wpi. The number of flukes in the four
165 animals from group 4 was 54, 40, 34 and 58 (mean 46.5±1.36).

166 *3.2. Histopathological study*

167 Livers from group 4 showed no histopathological changes. Portal spaces showed occasional
168 lymphocytes, but eosinophils were not found in negative control livers. All animals from
169 group 1 presented necrotic foci and focal hemorrhages mainly involving subcapsular areas.
170 Necrotic foci showed abundant cell debris and mild to moderate infiltrates of eosinophils,
171 often with pyknotic nuclei. Necrotic tracts started 0.5-1.5 mm behind migrating larvae.

172 Livers from group 2 presented similar acute necrotic foci and tracts with eosinophil
173 infiltration. In addition, the majority of necrotic foci were surrounded by a core of large
174 activate macrophages and an outer layer of lymphocytes, plasma cells and eosinophils,
175 conforming to the definition of a granuloma.

176 Histopathological changes in the livers from group 3 were typical of chronic fasciolosis Adult
177 flukes were present within severely enlarged bile ducts often showing epithelial erosion,
178 portal fibrosis and a variable amount of inflammatory infiltration, mainly composed of
179 eosinophils, lymphocytes and plasma cells.

180 *3.3. Apoptosis detected by caspase-3 expression*

181 Livers from negative control sheep (group 3) did not show caspase-3$^+$ inflammatory cells. At
182 8 dpi, numerous inflammatory cells located at the periphery of necrotic foci, as well as cell

183 debris (apoptotic bodies), expressed caspase-3, whereas the inflammatory infiltrate not
184 associated with necrotic areas showed only occasional caspase-3^+ inflammatory cells. The
185 pattern of immunostaining was both cytoplasmic and nuclear. Serial sections stained with
186 Hansel's technique allowed us to identify eosinophils (Figs. 1 and 2). The percentage of
187 caspase-3^+ eosinophils with respect to caspase-3^- eosinophils at the periphery of necrotic foci
188 was 46.1% and 53.9% at 8 and 28 dpi, respectively (Table 1), whereas at the periphery of
189 granulomas, the percentage of caspase-3+ eosinophils was only 6% (Table 1). At 17 wpi,
190 eosinophils located the inflammatory infiltrate surrounding some hyperplastic bile ducts,
191 often containing adult flukes, showed numerous caspase-3^+ cells (Fig. 3), whereas the
192 inflammatory infiltrated not associated with enlarged bile ducts only showed occasional
193 caspase-3^+ cells.

194 *3.4. Apoptosis detected by transmission electron microscopy*

195 The TEM study confirmed apoptosis in eosinophils in livers from groups 1 and 2. The
196 ultrastructural features of eosinophils consist of a bilobed nucleus and typical cytoplasmic
197 oval granules ranging from 0.5 to 1 μm in diameter with moderately electron dense matrix
198 material and a highly electron-dense crystalloid core (Fig. 4). Apoptotic TEM changes
199 consisted of margination-condensation of chromatin (early changes) and cytoplasmic and
200 nuclear fragmentation while maintaining cell membranes and organelles (Fig. 4).

201

202 **4. Discussion**

203 Hansel's stain has been used to identifying and quantifying eosinophils in tissues (Nguyen et
204 al., 2012). In the liver tissue sections examined in the present study, eosinophils were
205 identified more easily using Hansel's stain than H&E staining. We used Hansel's stain as a

206 counterstain for caspase-3 immunohistochemistry, but the dark brown color of the
207 immunostaining masked the bright red cytoplasmic granules of eosinophils and did not allow
208 for the identification of this cell type, this is why we used serial sections.

209 Caspase-3 is an effector caspase that plays a crucial role during apoptosis, since it directly
210 activates enzymes that are responsible for DNA fragmentation. Activated or cleaved caspase-
211 3 has been used as a marker of apoptosis via immunohistochemistry in healthy tissues and in
212 helminth-infected tissue sections (Resendes et al., 2004, Chen et al., 2008, Mkupasi et al.,
213 2013). The absence of apoptotic inflammatory cells in non-infected control livers in the
214 present study contrasts with the presence of apoptosis in healthy pig lymphoid tissues, and
215 may be due to the small number of inflammatory cells present in the healthy sheep liver
216 compared with those in lymphoid organs (Resendes et al., 2004).

217 In the present study, apoptotic eosinophils were also identified by TEM in liver samples at 8
218 and 28 dpi. Ultrastructurally, eosinophils were identified by the typical cytoplasmic granules
219 with an electron dense core according to the features reported in human (Duffin et al., 2009)
220 and ovine (Balic et al., 2006) eosinophils. The condensation and fragmentation of the nucleus
221 with an intact cytoplasmic membrane was found in apoptotic eosinophils in this work and has
222 also been reported in TEM studies of human (Duffin et al., 2009) and ovine (Balic et al.,
223 2006) eosinophils. Apoptotic eosinophils attached to *Haemonchus contortus* in the gastric tip
224 of sheep were degranulated (Balic et al., 2006); by contrast, in the present study, apoptotic
225 eosinophils found in the liver during the migratory stage of *F. hepatica* contained typical
226 granules. This finding suggests that during the migratory stage of *F. hepatica*, degranulation
227 of eosinophils is not the cause of apoptosis.

228 During the migratory stage (8 and 28 dpi), acute necrotic foci and tracts were found behind
229 migrating larvae, suggesting they induced hepatic necrotic tracts, in agreement with previous

230 reports in sheep (Meeusen et al., 1995, Zafra et al., 2013a,b). The majority of apoptotic
231 eosinophils were found in the periphery of these acute necrotic foci and tracts, while they
232 were occasional in portal infiltrates and granulomas and inflammatory infiltrates not
233 associated with necrotic foci or tracts. Moreover, in the chronic stage of infection (17 wpi),
234 the majority of apoptotic eosinophils were found in the vicinity of large bile ducts, often
235 containing adult flukes inside, and occasionally in inflammatory infiltrates associated with
236 granulomas, fibrotic chronic tracts or portal spaces with smaller bile ducts. This distribution
237 of apoptotic eosinophils suggests that products released by migrating or adult *F. hepatica*
238 may induce the local recruitment of eosinophils. It is possible that products released by *F.*
239 *hepatica* induces eosinophil apoptosis, which is supported by *in vitro* studies reporting that
240 the excretory products (ESP) of *F. hepatica* are able to induce apoptosis in rat eosinophils
241 and macrophages (Serradell et al., 2007, Serradell et al., 2009, Guasconi et al., 2012) and
242 ESP from the trematode *Paragonimus westermani* are able to induce eosinophil apoptosis
243 (Min et al., 2004). Alternatively, enzymes released by eosinophils may cause lytic necrosis
244 and apoptosis, which is supported by the crucial role of eosinophil in mediating tissue
245 damage and granuloma formation in *Schistosoma mansoni* (Silveira-Lemos et al., 2008).
246 Further studies immunolocalizing ESP antigens from *F. hepatica* in hepatic tissue sections
247 from infected sheep are required to confirm if *F. hepatica* ESP are able to induce apoptosis in
248 sheep inflammatory cells.

249 Apoptosis of effector cells of the innate response such as eosinophils has been reported as a
250 mechanism of local immunosuppression during helminth infections such as *P. westermani*
251 (Shin et al., 2000, Min et al., 2004) and the cestode *Taenia solium* (Yan et al., 2008) and
252 *Taenia crassic* (Zepeda et al., 2010). In fasciolosis, eosinophils appear to play a crucial role
253 in the effector host response; thus, an effective response in rats infected with *F. hepatica* has
254 been associated with a significant increase in eosinophil infiltration of the gut lamina propria

255 during early post-infection stages (Van Milligen et al., 1998, 1999). In *F. gigantica* resistant
256 Indonesian thin-tail (ITT) sheep, peritoneal eosinophils and macrophages are able to kill
257 juvenile *F. gigantica in vitro* by an antibody-dependent cytotoxicity mechanism dependent on
258 NO production, but they do not kill the larvae of *F. hepatica*, suggesting that eosinophils are
259 important effector cells involved in the resistance of sheep to *F. gigantica* (Piedrafita et al.,
260 2007). The results of the present work revealed a high number of apoptotic eosinophils in
261 acute necrotic foci at 8 and 28 dpi, while the number of apoptotic eosinophils decreased
262 significantly in necrotic granulomas at 28 dpi. Since acute necrotic foci occur behind
263 migrating larvae, and eosinophils are crucial effector of the effective host innate response,
264 apoptosis of this effector cell may be an important mechanism used by *F. hepatica* larvae to
265 evade the host innate response during the hepatic migratory stage in which the parasites are
266 more exposed to the host immune response than when they reach the biliary system.

267 In summary, the present work is the first report of eosinophil apoptosis during the migratory
268 and biliary stages of *F. hepatica* infection in sheep. Eosinophil apoptosis was detected by
269 active caspase-3 tissue expression and transmission electron microscopy, and it was found in
270 a high number of eosinophils mainly located at the periphery of acute necrotic tracts during
271 the migratory stage of infection, suggesting that this may be a mechanism by which the
272 parasite evades the host response. Further studies are required to investigate the presence of
273 apoptosis in other inflammatory cells such as macrophages and lymphocytes as well as the
274 presence of apoptosis in peritoneal inflammatory cells.

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413 **Figure Legends**

414 **Figure 1**. Liver, 8 days post-infection. Caspase-3. Necrotic focus (N) showing cell debris and
415 numerous inflammatory cells, many of them expressing caspase-3 (brown, arrow). Note a
416 bile duct (B) with severe inflammatory infiltrate in the portal space negative for caspase-3
417 except for one cell (brown). ABC method, x400.

418 **Figure 2.** Liver, 8 days post-infection. Serial section of same area observed in Figure 6. Note
419 the bile duct (B) surrounded by a severe inflammatory infiltrate and a necrotic focus (N) with
420 abundant cellular debris and moderate inflammatory infiltrate, the majority of cells with a red
421 cytoplasm are eosinophils. Hansel staining, x400.

422 **Figure 3.** Liver, 17 weeks post-infection. Detail of a bile duct showing the lumen (L) with a
423 lack of epithelium (erosive cholangitis) and numerous caspase-3^+ cells in the associated
424 inflammatory infiltrate. ABC method, x400.

425 **Figure 4**. Liver, 8 days post-infection. Eosinophils showing normal ultrastructural features
426 (E) and apoptotic eosinophils (AE) showing condensed, electron dense nuclei and typical
427 granules with electron dense structures. An apoptotic neutrophil (AN) with a multilobed
428 electron dense nucleus and typical round to oval electron dense cytoplasmic granules is also
429 observed. Transmission electron microscopy. Bar = 2 µm.

430

431 **Table 1.** Number of caspase-3$^+$ eosinophils and apoptotic bodies in *F. hepatica* infected
432 sheep at 8 days post-infection (dpi), 28 dpi and 17 weeks post-infection (wpi) and in negative
433 control sheep. Results are expressed as mean±SD per field of 0.08 mm^2.

	8 dpi	**28 dpi**		**17 wpi**	**controls**
	NF	**NF**	**Granulomas**		
caspase-3$^+$ eosinophils	10.6±1.8	13.7±5.7	1.3±1,0	17.3±7.8	0
caspase-3$^-$ eosinophils	12.4±1.9	11.7±3.7	20.3± 8.2	29.3±12.8	0
% caspase-3$^+$ eosinophils	46.1	53.9	6.0	37.1	0
caspase-3$^+$ A.B.	18.5±0.9	32.6*±2.2	6.7‡±3.5	11.3±5.7	0

434 NF: necrotic foci; AB: apoptotic bodies.

435 *Significant differences (P<0.05) respect to 8 dpi.

436 ‡ Significant differences (P<0.05) respect to NF at 8 and 28 dpi.