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HISTOLOGICAL ANALYSIS OF THE TEMPORAL INFLUX OF IMMUNE CELLS INTO HEPATIC GRANULOMAS INDUCED BY *TAENIA PISIFORMIS* IN RABBITS

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ABSTRACT

The objective of this study was to evaluate histologically the temporal influx of immune cells into hepatic granulomas in domestic rabbits infected with *Taenia pisiformis* eggs at 28, 77 and 90 days post-infection (dpi). Our study has focused on the histological description of lesions in the liver by *T. pisiformis*, quantification of macrophages, lymphocytes, and eosinophils and measured of the total area of granulomas. Histological differences among days post-infection were found, granulomas area was higher at 77 and 90 dpi compared to 28 dpi. Total lymphocytes increased in relation to the time post-infection. Macrophages were the most numerous cells at 28 and 77 dpi (109.49±5.2 and 56.12±3.69, respectively), though they diminished about 50% at 77 dpi. Eosinophils were scarce and their number decreased with the progress of time post-infection. Foamy macrophages were observed, which had not previously been referred for cestodiasis. It is concluded that the histological characteristics of liver granulomas induced by infection with *T. pisiformis* in rabbits and the influx of immune cells changed at different periods post-infection. This information is fundamental to know more deeply the histological organization of rabbit granulomas in relation to the influx of immune cells in experimental post-infection periods.

Keywords: Granulomas, liver, *Taenia pisiformis*, lymphocytes, macrophages, rabbit.

INTRODUCTION

Infection of wild and domestic rabbits with eggs of *Taenia pisiformis* (*T. pisiformis*) is the most frequent cause of cestodiasis in this species; it starts when a carrier of *T. pisiformis* (dog, fox or some other carnivore) releases gravid proglottids in the faeces that contaminate pastures eaten by the rabbit (intermediate host). Eggs reach the digestive tract of the rabbit, and after hatching, the oncospheres migrate to various organs, resulting in granulomatous lesions or giving rise to the phase of cysticerci or metacestode, that are mainly housed in the peritoneal cavity. Lesions caused in the hepatic tissue are macroscopically observed as white spots with a diameter of 2 to 3 mm and referred as early as 48 hours post-infection. These lesions eventually lead to the formation of granulomas (Flat and Moses, 1975) which are inflammatory nodular formations, mainly comprised by macrophages or histiocytes of dense or loose connective tissue. The typical inflammatory granulomatous response is a tissue chronic reaction that causes targeted lesions as a result of *in situ* persistence of injury by the pathogen agent, where predominantly the mononuclear phagocytic cells form aggregates of macrophages and multinucleated giant cells, which in most cases are surrounded by lymphocytes, and depending on the time of development, they could display fibrosis (Williams and Williams, 1983; Pérez-Torres *et al*. 2002). Liver necrosis and cicatrisation due to the migration of *T. pisiformis* larvae can cause hypoalbuminemia, with increased or normal globulin levels (Melillo, 2007). Furthermore, tissue changes that occur in the liver as a result of granuloma formation are accompanied by changes in the macroscopic appearance of the former, which cause confiscation when after slaughter, being the cause of important economic losses for rabbit breeders (Flatt and Campbell, 1974).

So far, no studies have been performed to quantitatively characterize the process of immune cells temporary influx into liver tissue infected with eggs of *T.*

pisiformis in rabbit. Therefore, the objective of this study was to histologically evaluate the temporary influx of immune cells into hepatic granulomas of domestic rabbits experimentally infected with eggs of *T. pisiformis* at 28, 77 and 90 days post-infection.

MATERIALS AND METHODS

Animals: The study was carried out following the Mexican Law for the Protection of wild and domestic animals, and the Mexican Official Standard NOM-062-ZOO-1999, in accordance with international regulations. New Zealand does were individually housed in wire cages (60 cm L, 90 cm W, 40 cm H) under farm conditions, at room temperature (15-25°C), and fed with Conejina N, Purina® and water ad libitum.

Infection with *Taenia pisiformis* eggs: *Taenia pisiformis* proglottids were obtained from the intestine of naturally infected dogs, which were humanely euthanized at the Canine Control Center in México. After a lengthwise slitting, each intestine was inspected in search for the adult cestodes of *T. pisiformis,* identified by proglottids macroscopic appearance. Proglottids were macerated in a mortar and the presence of eggs was verified with an optical microscope (40X), the eggs were counted by adding 10 µl of the egg suspension in a Neubauer chamber (Betanocurt *et al.*, 2012), resuspended in saline solution and administered p. o. to does with a plastic tube, after tranquilization with Ketamine-Xylazine.

Euthanasia: Rabbits were sedated by intramuscular injection of xylazine/ketamine (5/35 mg/kg); fifteen minutes later, rabbits were humanely euthanized using an overdose of sodium pentobarbital (100 mg/kg) (Barbithal®, Holland Laboratories, México). All hepatic lesions and cysticerci in the peritoneal cavity were counted at necropsy, and hepatic samples of lesions detected were recovered, fixed in 10% buffered formalin and processed for histological analysis.

Experimental groups: The tissues used in this study were obtained from retrospective studies. The livers of does were assigned into three groups: Group 1: livers from does infected with 12,000 eggs of *T. pisiformis* and euthanized at 28 days post-infection (dpi) (n=6, body weight 1±0.20 kg); Group 2: livers from does infected with 2,900 eggs and euthanized at 77 dpi (n=4, body weight 3.0±0.20 kg), Group 3: livers from does infected with 1,722 eggs and euthanized at 90 dpi (n=6, body weight 3.5±0.20 klg). In addition, a control group of does the same weight without infection was included for each experimental group.

Histological analysis: From each liver, three samples of granulomatous tissue fragments randomly chosen were analysed. Tissue samples were processed by the paraffin embedding technique, and 6 micron thick sections were obtained. Five different slides representative of each lesion were microscopically analysed, and the number of eosinophils, lymphocytes, and macrophages in 15 microscopic fields was determined according to the technique previously described (Rosas *et al*., 2007), using the 40X objective of a light microscope connected to a digital camera and the image analyser, Motic Image Plus 2.0, moreover, with the objective 10X the total area in the granulomas was measured.

Statistical analysis: Data obtained from the area of granulomas, and the counting of lymphocytes, macrophages, and eosinophils in different dpi, were analysed with Kruskal-Wallis test, and to compare differences between days post-infection a test of Dunn was used.

RESULTS

The number and size of granulomas depend on the time post-infection: The number of hepatic granulomas quantified at 28, 77 and 90 dpi were 8626.3±3241.2, 159.5±40.3 and 230.9±62.11 (mean ± SEM), respectively (Fig. 1). A larger area of hepatic granulomas was observed at 90 and 77 dpi as compared to 28 dpi ($P < 0.001$). It is noteworthy that no significant difference was observed in the area of liver granulomas obtained at 90 and 77 dpi (Figs. 1, 2).

Inflammatory infiltrate in hepatic lesions: The lymphocytes number (mean ± SEM) counted in the granulomas at 28, 77 and 90 dpi were 15.49±1.37, 23.91±2.35 and 66.05±6.84, respectively. In relation to macrophages, the mean ± SEM at 28, 77 and 90 dpi were 109.49±5.2, 56.12±3.69 and 64.58±4.09 respectively, and the eosinophils numbers were 13.86±2.3, 11.52±1.33, 1.92±0.91, respectively (Fig. 3). At 28 and 77 dpi, the number of macrophages was higher as compared to the population of lymphocytes and eosinophils ($P<0.001$), however, at 90 dpi no significant difference between the number of lymphocytes and macrophages was observed, and a drastic decrease in the number of eosinophils was noted. It is striking that the number of lymphocytes increases with infection progression. In contrast, the population of macrophages was higher at 28 dpi (109.49 ± 5.2) with a 50% reduction at 77 dpi (56.12 ± 3.69) (Fig. 3).

Histological aspect of granulomas at 28, 77 and 90 days post-infection: At 28 dpi, liver granulomas had focal lesions with three well-defined areas: an internal zone containing residues of the parasite, a middle zone with the presence of epithelioid macrophages and Langhans giant cells (multinucleated) and an external area with some lymphocytes and few eosinophils (Fig. 4). Granulomas obtained at 77 dpi presented an inner zone

with extensive necrosis and no visible remnants of parasites, the middle area with the moderate presence of macrophages, some of them with foamy appearance, and absence of giant cells, and an outer zone with infiltration of lymphocytes and eosinophils. Is to emphasize that at 77 dpi greater fibrosis appeared compared to day 28 post-infection (Fig. 5). In granulomas obtained at 90 dpi, in the inner zone many epithelioid macrophages were observed, in the middle zone, the presence of abundant lymphocytes was found, and in the outer zone, fibrosis was present (Fig. 6). Although fibrosis was observed in most of the granulomas, there was no presence of calcification.

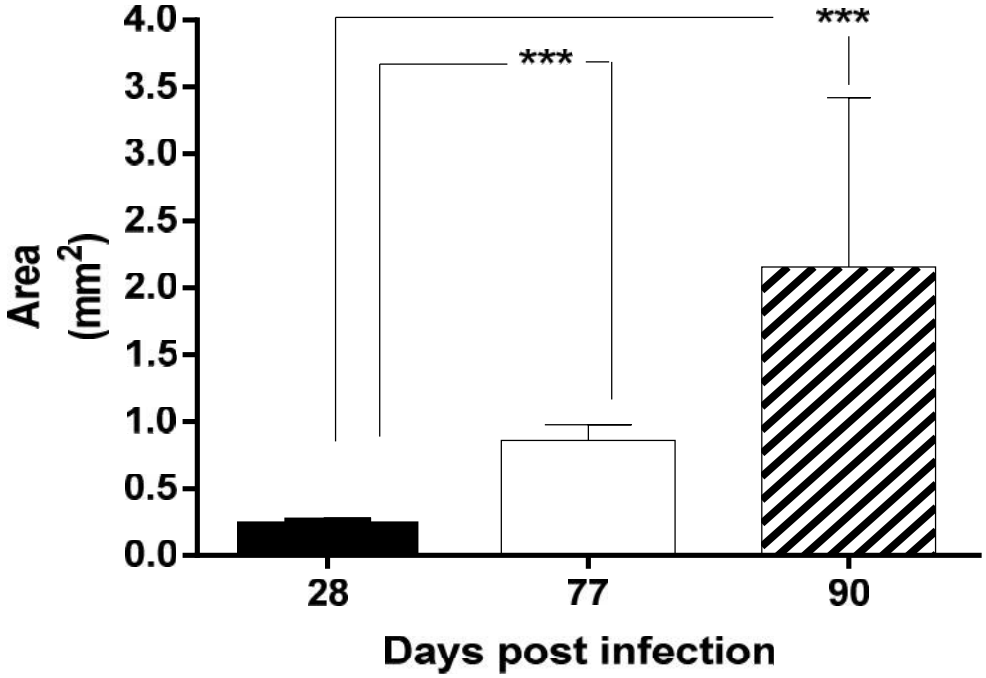

Fig. 1. Areas of hepatic granulomas. Granulomas in rabbit livers were measured (mean±SEM) at 28, 77 and 90 days post-infection with *T. pisiformis*. Kruskal-Wallis and Dunn test (* P = 0.0001).**

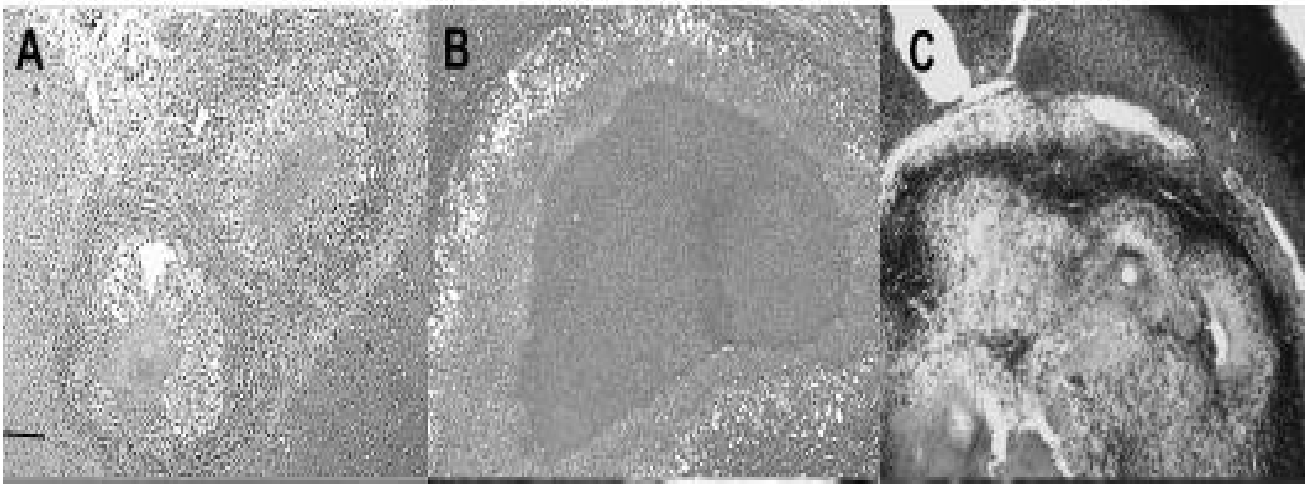

Fig. 2. Representative photomicrographs of granulomas in the infected liver with *T. pisiformis* eggs at different days post infection in rabbits. A) 28 dpi, B) 77 dpi, C) 90 dpi. (H & E, 40X).

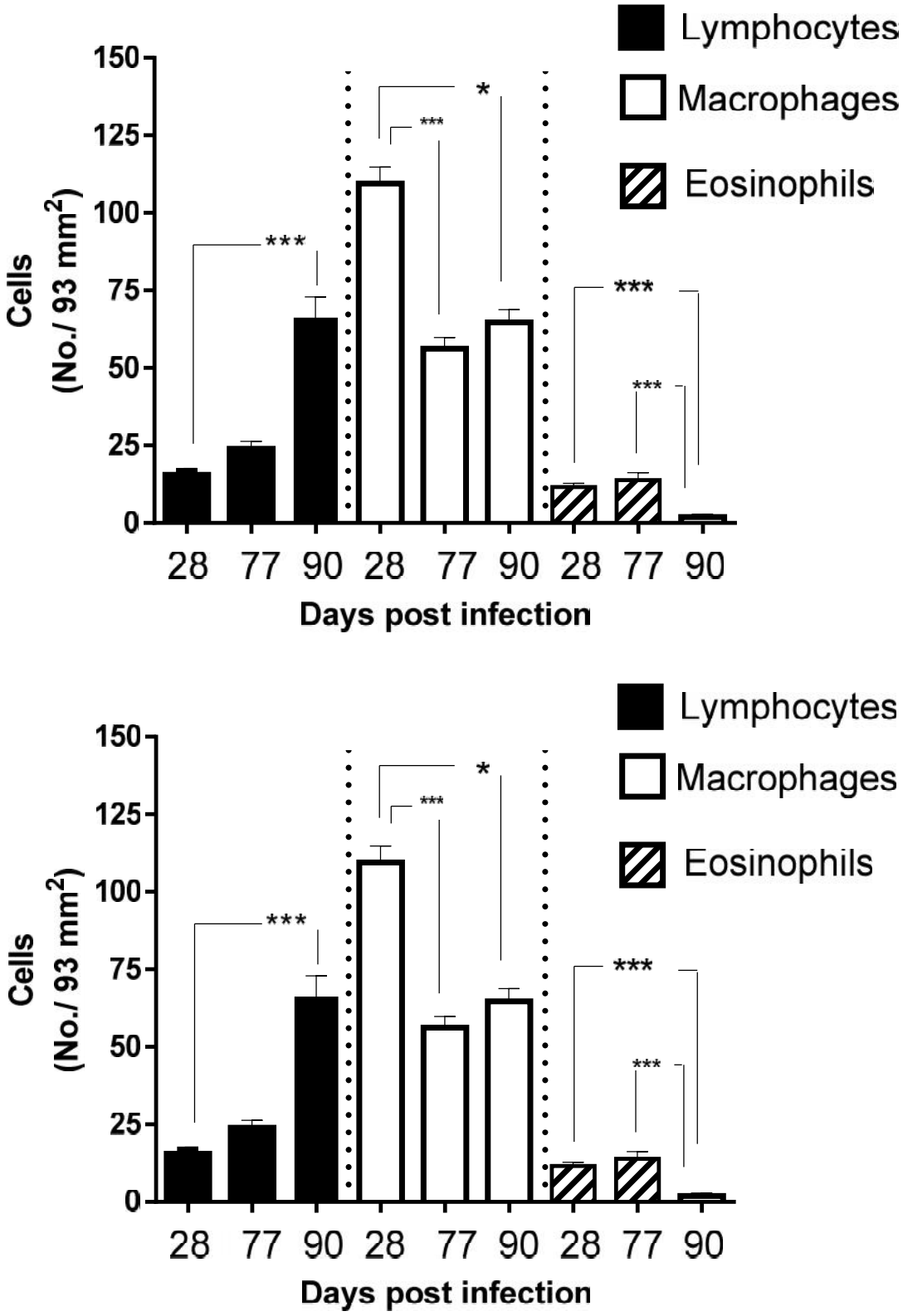

Fig. 3. Number (mean±SE) of lymphocytes, macrophages and eosinophils in granulomas of rabbit livers at 28, 77 and 90 days post-infection with T. pisiformis. Kruskal-Wallis and Dunn (* P= 0.0001, P= 0.05 *).**

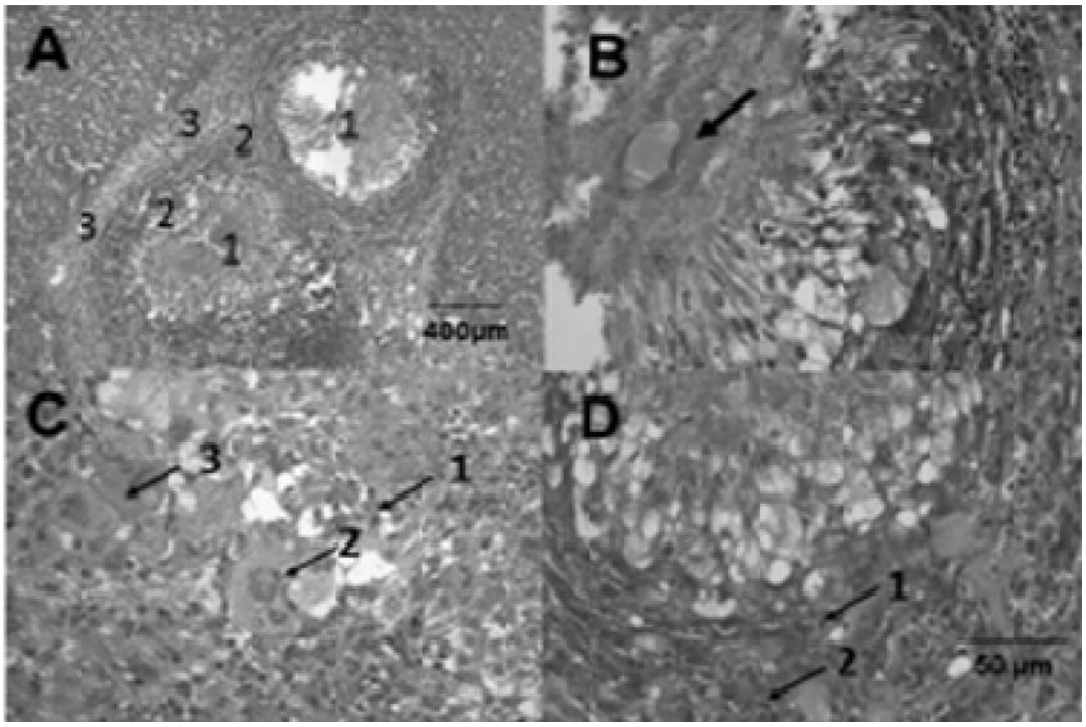

Fig. 4. Microscopic aspect of granulomas at 28 days post-infection. A) Three well-defined areas are observed: 1) internal; 2) middle; 3) external (100X). B) In the middle zone, necrosis is observed with traces of parasite (arrow). C) Middle zone with the presence of macrophages (1), the presence of Langhans giant cells (2) and multinucleated cells (3). D) External zone with lymphocytes (1) and eosinophils (2). (H & E, 400X).

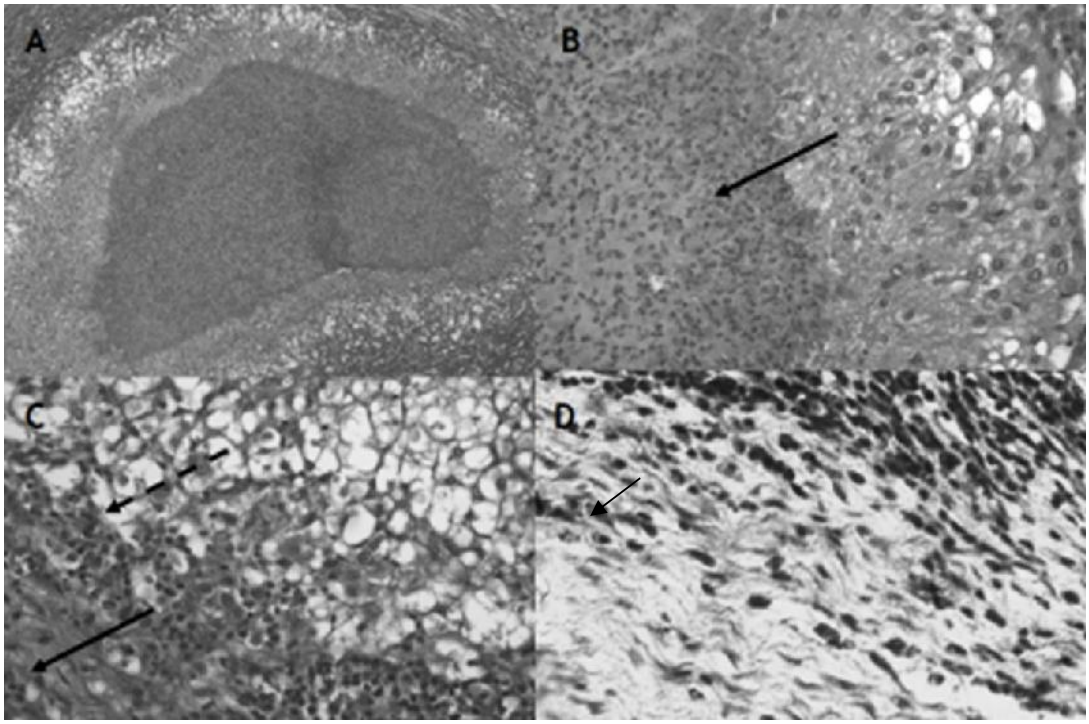

Fig. 5. Histological aspect of a granuloma at 77 days post-infection. A) Three histological zones (H & E, 100X) are distinguished. B and C) The middle area with extensive necrosis (arrow) and moderate presence of macrophages (arrow) and presence of foamy cells (dashed arrow). D) External zone with lymphocytes (solid arrow), eosinophils (dashed arrow) and presence of fibrosis (dotted arrow). (H & E, 400X).

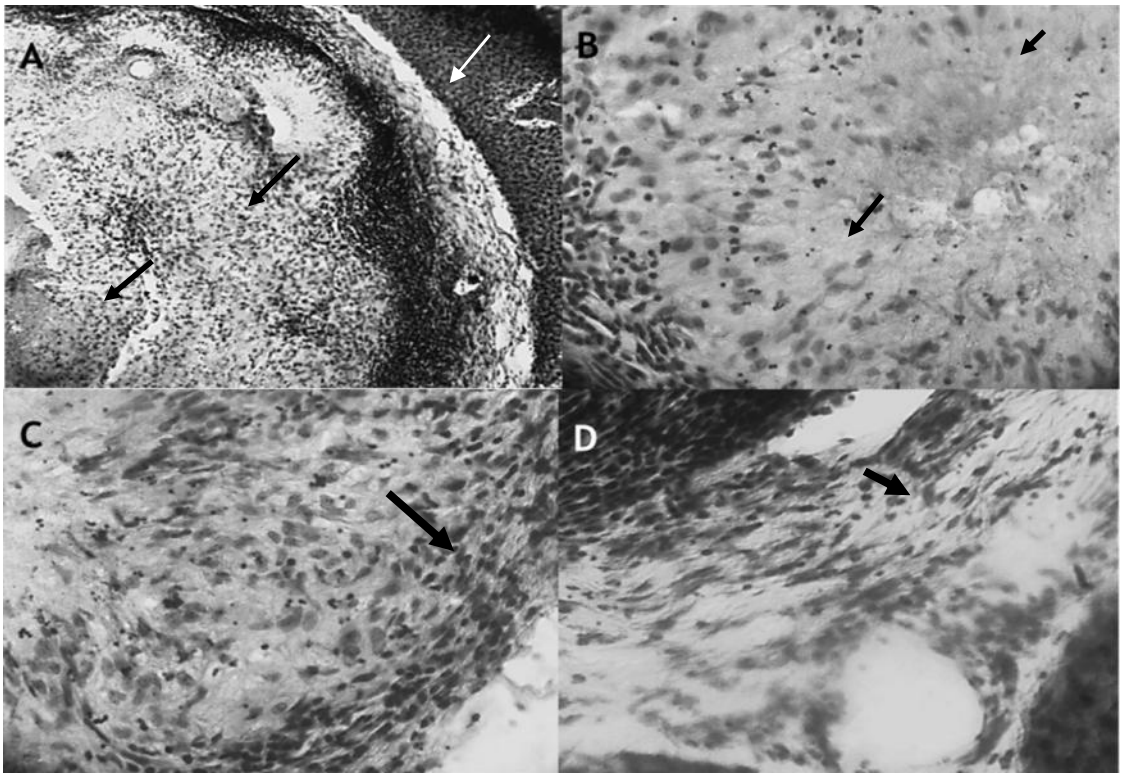

Fig. 6. Microscopic aspect of granulomas at 90 dpi. A) Three distinct zones are observed. B) The medullary area with necrosis and high amount of epithelioid macrophages. C) The middle area with a large number of cells. D) Cortical area with fibrosis.

DISCUSSION

Our results show that hepatic granulomas induced by *Taenia pisiformis* in rabbits, differ in size and cellular composition depending on the dpi. At 28 dpi, necrosis was observed and macrophages were the most abundant leucocytes with a moderate presence of lymphocytes and eosinophils cell population, however, they showed little fibrous tissue and no presence of calcifications. These results differ from a previous study in granulomas at 30 dpi, where they observed fibrosis and calcification (Flatt and Moses, 1975), this difference could be due to infective doses or differences between strains of *T. pisiformis* used to infect, it is possible parasites could have genetic differences which could affect their virulence/pathogenicity, and consequently, differently affect the responsiveness induced in the host.

The presence of lymphocytes, macrophages, and in some cases eosinophil, coincides with a previous study, in which they report the existence of an accumulation phase in which participate these three types of effector cells (Co *et al*., 2004).

The mean of macrophages and lymphocytes at 28 dpi was 109.49 and 15.49. These results differ from those described by Betancourt-Alonso *et al.* (2012), whom evaluated granulomas at 21 dpi founding 38 and 37 macrophages and lymphocyte, respectively. However, these differences can be attributed to the infective dose of 3,000 eggs from *T. pisiformis*, whereas in our study was 12,000 eggs per rabbit. The abundance of epithelioid macrophages and lymphocytes in the granulomas at 28 dpi, may indicate that at this stage the triggered immune response is TH1-type, as in this type of response occurs a strong activation of T lymphocytes, inducers of an intense macrophage activation.

In a murine model of *Taenia crassiceps* infection, TH1 response has been referred as a generator of a non-permissive environment for the development of metacestodes (Terrazas *et al*., 1999). Although in our study we did not evaluate cytokines, in granulomas induced by *M. tuberculosis* it has been reported a proinflammatory response, with IFN-γ, TNF-α and IL-1 (Silva *et al*., 2012). In our work, foamy macrophages were observed, which had not previously been referred to this parasitosis. The presence of such cells has been considered characteristic of granulomas induced by *Mycobacterium tuberculosis* (Silva *et al*., 2012).

Histological characteristics observed in the granulomas at 77 dpi, as extensive necrosis with no visible traces of parasites, moderate presence of macrophages (some foamy macrophages), absence of giant cells, significant infiltration of lymphocytes and eosinophils, and increased fibrosis, suggest that the response is at the end of the effector phase, which

coincides with that reported in another study (Co *et al*., 2004).

This study is a pioneer in evaluating liver granulomas in domestic rabbits infected with *T. pisiformis* eggs at 90 dpi, where we found that the number of macrophages and lymphocytes decreased, and the observed fibrosis is higher than in others dpi, suggesting a marked repair phase (Co *et al*., 2004). Unlike previous studies that have focused on the histological description of lesions in the liver by *T. pisiformis*, in our study, we performed quantification of macrophages, lymphocytes, and eosinophils and we measured the total area of granulomas. This information allows us to know more deeply the histological organization of granulomas in relation to the influx of immune cells in experimental post-infection periods. Derived from these results, further studies are necessary to characterize subpopulations of CD4+ and CD8+ lymphocytes in the granulomas at different times post infection with *T. pisiformis* and assess the expression of proinflammatory cytokines.

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