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Gender as a factor of susceptibility to infection in experimental hydatidosis

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ABSTRACT. We developed an experimental model of hydatidosis in BALB/c mice with six groups, a group of females and another group of males was infected with PSC of *Echinococcus granulosus*. Another two groups were gonadectomised and infected with PSC, and another two groups were healthy controls. They were all bled and sacrificed after sixteen weeks post-infection. The cysts in the abdominal cavity were count, and samples were taken from liver. A microscopic study was made of the tissue around the cyst to evaluate the chronic inflammatory response. In addition the seric levels of estradiol and testosterone by means of radioimmunoassay (RIA) were determined. The results were that the females presented a greater number of cysts in liver than the males, in addition the levels to estradiol almost rose to the double in males and females after 16 weeks post-infection, and the testosterone diminished. The granulomatous response around the cysts was greater in the females than in the males. The gonadectomization affected the susceptibility to the infection in females, diminishing in number of hepatic cysts found. One concluded that the females are more susceptible to the infection by metacestode of *E. granulosus*, than the males. The female displayed one better granulomatous answer than the males. Nevertheless this was not sufficient to eliminate the parasite or to inhibit its growth. The levels of estradiol and testosterone undergo an imbalance, observing that estradiol increased in chronic stages of the infection whereas the testosterone diminishes, which would indicate to us that probably the parasite causes hormonal imbalance in chronic stages, to be able to remain by long periods in its host.

Key words: Murine hydatidosis, testosterone, estradiol, sexual dimorphism.

RESUMEN. Se desarrolló un modelo experimental de hidatidosis en ratones BALB/c con seis grupos, un grupo de hembras y otro de machos fueron infectados con PSC de *E. granulosus*. Otros dos grupos fueron gonadectomizados e infectados con PSC, los dos últimos grupos son controles sanos. Todos fueron sangrados y sacrificados a las dieciséis semanas post-infección. Se contaron los quistes en la cavidad abdominal y se tomaron muestras de hígado. Se hizo un estudio microscópico del tejido alrededor del quiste para evaluar la respuesta inflamatoria crónica, Además se determinaron los niveles séricos de estradiol y testosterona por medio de radioinmunoanálisis (RIA). Los resultados fueron que las hembras presentaron un mayor número de quistes en hígado que los machos, los niveles de estradiol se elevaron casi al doble en machos y hembras a las 16 semanas post-infección, y la testosterona disminuyó. La respuesta granulomatosa alrededor del quiste fue mayor en las hembras que en los machos. La gonadectomización afectó la susceptibilidad a la infección en hembras, disminuyendo en número de quistes hepáticos encontrados. Se concluyó que las hembras son más susceptibles a la infección por el metacestodo de *E. granulosus*, que los machos. Las hembras presentaron una mejor respuesta granulomatosa que los machos, sin embargo ésta no fue suficiente para eliminar al parásito o inhibir su crecimiento. Los niveles de estradiol y testosterona sufren un desequilibrio, viéndose que el estradiol se incrementa en etapas crónicas de la infección, mientras que la testosterona disminuye, lo que nos indica que probablemente el parásito provoca este desequilibrio hormonal en etapas crónicas para poder permanecer por largos periodos en su huésped.

Palabras clave: Hidatidosis murina, testosterona, estradiol, dimorfismo sexual.

INTRODUCTION

Porcine hydatidosis was not known as an animal health problem in Zacatecas, México neither in other states of our country. Currently this is known as an animal health problem, also, some particular characteristics have been observed with regards to the parasite's cycle related to the environmental conditions. The porcine strain is known to be the cause of the hydatidosis in the state of Zacatecas as opposed to the ovine strain as it has been traditionally reported in other countries (Tavizón, 1981, Mondragón and Tavizón, 1991; Mondragón *et al*., 1993; 2002).

In some countries the hydatid disease increased drastically and its prevalence is higher in women than in men. The atmosphere and the style of life favours the dispersion of the disease (Matosian *et al*., 1997; Thompson and Lymbery, 1988; Carmona *et al*., 1999; Abu Hassan *et al*., 2002).

Hydatidosis is an attractive model for immunologic studies due to the significant influence of the parasite over the host's immune response (Brehm *et al.,* 1999); some models have been used to study the host-parasite relation.

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These have shown the ability of *E. granulosus* to evade the humoral and cellular immune response of the host. With respect to the immune response, the parasite is capable of inciting a granulomatous inflammation response in different organs, such as the liver, lungs and others (Nicod *et al*., 1994; Bresson-Hadni *et al*., 1999). Cytokines play a very important role in the host immunological response against *E. granulosus,* but cyst location conditions this release in patients with liver hydatidosis (Torcal *et al*., 1995).

There is a sexual dimorphism in the normal immune response, thus suggesting a link between the immune system and the reproductive endocrine system, demonstrated by the fact that cellular immune response in females is less effective than in males while the humoral immune response is most effective in females (Morales *et al*., 2001). The gender difference of the host can influence on the parasites survival, as well as its level of infectiveness, as has been seen in other models of murine infections (Watanabe *et al*., 1999; Travi *et al*., 2002, Kausic *et al*., 2000). For example, in animal models, the development of *E. granulosus* has shown a correlation between the immune state of the host and particularly with cellular immunity (Annen *et al*., 1981; Mondragón, 1995, Ecker and Diplazes, 2004).

OBJECTIVE

To determine the immunological effect of the metacestode of *E. granulosus* dependant on gender in murine experimental hydatidosis.

MATERIAL AND METHODS

Murine Model

Six groups (n = 10) of 5 to 12 week old, male and female, BALB/c mice were used. They were treated for parasites and kept in biotery conditions. Two groups of mice, male and female were used for control purposes (FC, MC).

Gonadectomization

10 male and 10 female four week old mice were gonadectomiced in compliance with the following technique: each of them were weighed from both lots and the weights were recorded for each mouse, the females weighed and average of 18.8 g c/u and the males 21.5 g c/u. After the anesthesia was prepared (sodic penthobarbital) diluted 1:2 with injected water in a dose of 1 ml/2.5 kg of weight. The mice were anesthetized. The surgery in females was done through lateral incisions in order to facilitate locating the ovaries. In males it was done by making an incision in the scrotum and the testicles were extracted. They were sutured using chromic 3-0 catgut and they were disinfected with **piotanic blue**. The mice recovered in two days, and then males and females were put in two different lots (Morales *et al*., 2002 b,c).

Hydatid cysts

Pigs' livers, which were infected with parasites, were obtained from the municipal slaughter houses of Guadalupe, Zacatecas and Fresnillo, Zacatecas, México. The livers were kept in aseptic conditions and from these the hydatid cysts were used in order to extract the *E. granulosus* protoscoleces.

Inoculation of the E. granulosus *protoscoleces*

The male and female gonadectomized (GIF, GIM) mice, and those that had not been gonadectomized (IM, IF), were inoculated at 12 weeks of age with 2000 PSC/doses (Ganguly *et al*., 1986) in 400 µl of injected water.

Bleeding and tissue sampling

The mice were bled and sacrificed at 16 weeks post-inoculation, including the control groups. The number of cysts in the abdominal cavity was counted and samples of liver were recollected in order to be processed using histological techniques. (Mondragón *et al*., 2002).

Histological techniques

The tissues were fixed with a Bouin solution (picric acid 750 ml, formaldehido 40% 250 ml, acetic acid, glacial 50 ml) for 10 days. The tissue was washed using running water for 1 h in order to remove the excess stain. Later, the tissue was processed in order to place it in paraffin. The tissue was placed in boxes for its automatic processing through the HISTOKINETT using ethylic alcohol 80% 2 h, ethylic alcohol at 95% two phases of 2 h each, absolute ethylic alcohol 3 phases of 2 h each, xylene two changes of 2 h each one and paraffin up to its inclusion in molds to be cut afterwards. Histological cuts were made in rotary microtome, with a thickness of 4µ. The cuts were placed in laboratory bath of 45ºC and they were put on slide cover glasses that had a sheet of albumin of Mayer (egg white 50 ml, glycerin 50 ml.) that were adhered using heath. The slides were the cuts were placed underwent the Hematoxylin-Eosin stain technique: xylene two treatments during 10 min. each. Absolute ethylic alcohol 1 min., ethylic alcohol at 80% 1 min, ethylic alcohol at 60% 1 min., running water 1 min., Harris hematoxylin 10 min., washed down with running water, washed down with acidic alcohol, washed down with running water, baths using lithium carbon

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in a watery saturated solution, running water baths, eosin 3 min., running water baths, alcohol at 96%, absolute alcohol, xylene-absolute alcohol, and xylene, it was covered with a synthetic resin and it was observed under the light microscope. (Schopf *et al*., 2002).

Radioimmunoassay technique

Kits were used (Coat-a-count, Diagnostic products corporation) in its solid phase designed for determining the amount of testosterone and estradiol in serum in a competitive form with testosterone marked with ^{125}l and estradiol marked with ^{125}l for this purpose 50 ml of serum from each mouse were used competing with 1ml of testosterone marked with ^{125}l in polypropylene tubes, which had rabbit anti-testosterone antibodies, they were incubated at room temperature for three hours. Later, they were decanted and read in a gamma counter as well as the tubes of the calibration curve which contained known concentrations of testosterone (0, 20, 100, 400, 800 1600 ng/dl) and later the results were processed in the data base (RIA WHO given by the OMS) and the concentration of testosterone was obtained from each serum ng/dl. Also, 100 µl of serum from each mouse was used and it was put in competition with 1ml of estradiol marked with ^{125}l following the same procedure. (Grasso *et al*., 1997).

Statistics:

ANOVA and Tukey´s studentized range (HSD) test.

In order to evaluate the results, the data were captured and they analyzed with program SAS System.

RESULTS

By this investigation we were able to induce experimental hydatidosis in Balb/c mice, after intrabdominal injection of a standardized dose of protoscoleces. Under this experimental condition we further investigated whether sexual dimorphism affect the rate of infection. Interestingly female mice were more susceptible to infection than males, because the number of hepatic cysts was almost the double; therefore differences in both groups was statistically significant $p < 0.0001$ (Figs. 1 and 2). To answer the question whether estrogens influence in the susceptibility of infection, a group of female mice was gonadectomised. As expected the estradiol level decreased (Figs. 3 and 4) and surprisingly we were able to show a decrease in the rate of infection, when the rate of infection of gonadectomised females was compared with males, it was a little bit higher (Table 1).

To reply the question whether estrogenic factor affected the infection rate in males, an additional experiment

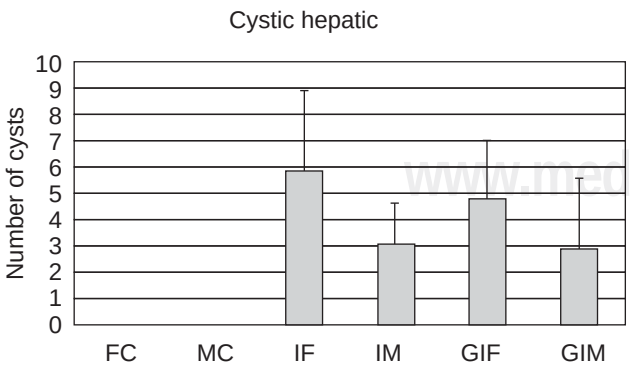

Figure 1. *Average of number cysts by group. The result of the ANOVA and the test of Tukey indicate that there were significant differences between groups and that IF and GIF had the highest values (P < 0.0001).*

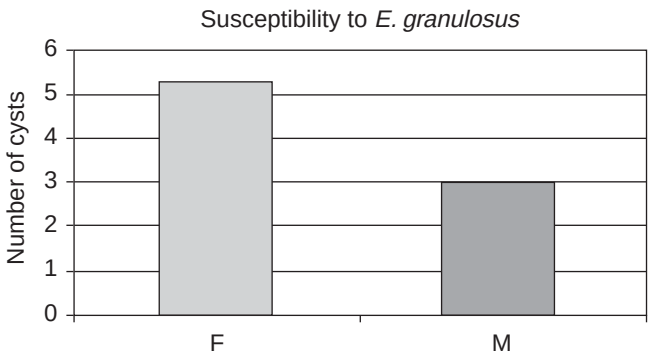

Figure 2. *Females (F) are more susceptible to the infection by* E. granulosus *that the males (M), analysis of difference of averages by Tukey (P < 0.0001).*

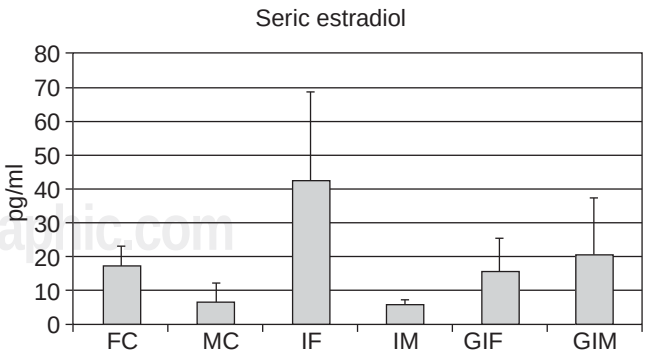

Figure 3. *Chart of the average of the levels of Estradiol per group. It is possible to see the differences among the groups that can be attributed to the treatments and to the infection with* ***hytidosis*** *which increases the differences. The group of IF is the one that represents a higher amount to a level of significance of p < 0.0001.*

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was designed as follows: A group of males were gonadectomised and testosterone and estrogens levels were measured, therefore correlating with the rate of infection. Main results of this experiment did not demonstrate effects in the infection rate, in spite that testosterone drop and estradiol rise in this group of males (Figs. 5, 6 and 7). Thus we suppose that in addition of estrogens there are other constitutional factor in the male that increase resistance to hydatidic infection (Tables 1, 2 and 3).

Intrahepatic inflammatory process was another factor studied, and females exhibited more infiltrates than males, however not differences in degree of infiltrates between gonadectomized and non-gonadectomised were observed (Fig. 8).

DISCUSSION

Present investigation attempts to clarify the role of sexual dimorphism in susceptibility to experimental hydatidosis. Main results of this work demonstrated that: 1. Feminine hormones determine high degree of infection. 2. Parasite infectivity in female animals associates to higher values of estradiol. 3. In male animals no differences in estradiol levels were found, however infection triggers a significant decrease of testosterone levels. By this result we propose that feminine hormones facilitate the infection of *E. granulosus*.

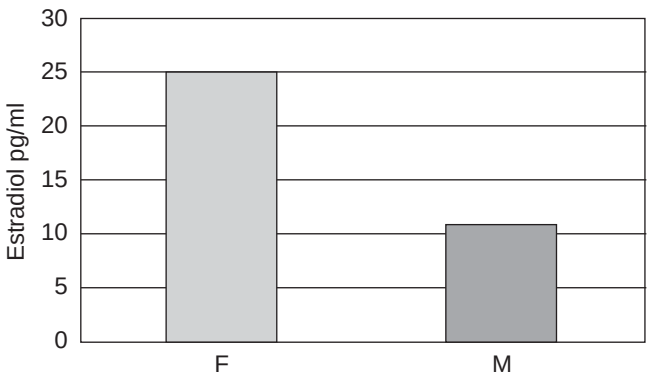

Figure 4. *Chart of the levels of Estradiol in females (F) and males (M). Analysis of the mean differences by Tukey.*

Morales *et al*., (2002a, 2004) found that the castration and the pregnant, doubled the prevalence of the cysticercosis by *T. Solium* near the 25 to 50% due to the hormonal changes that undergo the animals influencing the susceptibility of the pigs castration. The levels found of testosterone and estradiol in castrated animals, can be due to the hormonal production of the adrenal gland (Randall *et al*.,1997).

Maybe *E. granulosus* has a mechanism by which it stimulates the production of estradiol and testosterone of the adrenal gland to maintain a favourable environmental for its survival.

Sexual hormones affect the immune response in infections by cestods, therefore it has been reported that females are more susceptible to infection than males (Arteaga *et al*., 2002). *E. granulosus* belongs to the cestod family, that affects mainly the liver and lung, after infection the parasite develops several evasion mechanisms that determine its long stay in host tissues. We previously studied the cytokine regulation in hydatidosis, which is important in the host-parasite relationship, *E. granulosus* induce a local IL-10 and TGFβ mediated response that cause immune depression and favor the long life span and protoescoleces dissemination in the liver (Mondragón *et al*., 2002).

On the other hand, the immune response is affected by sexual hormones; thus, the focus of present investigation was to determine the influence of sexual hormones in hydatidic infection. As expected, it was found that females are more susceptible to *E. granulosus* infection than males. Furthermore, the gonadectomy in females decreased in one third the infection rate. This suggest that the estradiol abrogation is determinant for infection.

CONCLUSIONS

The sex of the host is an important factor in the susceptibility and the type of response to parasitism, in this case the females are more susceptible to the infection by metacestode of *E. granulosus*, than the males.

The females displayed one better granulomatous response than males; nevertheless this was not sufficient to eliminate the parasite or to inhibit its growth.

Table 1. Average of the number of hepatic cysts by group. Average of the number of cysts found in the liver of the mice and its deviation standard, after 16 weeks of being inoculated with the PSC of *E, granulosus*. 10 BALB/c mice were used by group; the mice castrated themselves to the 4 or 5 weeks of 8 ages and inoculated of a12 weeks of age. The statistics analysis of ANOVA and Tukey´s studentized range indicate that there were significant differences between groups and HI and HGI have the highest values (P < 0.0001).

	CM	CF	IM	IF	GIM	GIF
Cystic	0.00 ± 0.00	0.00 ± 0.00	3.1 ± 1.52	5.8 ± 3.04	3.00 ± 2.52	4.80 ± 2.25

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The sexual hormones importantly influence the immune response of the host, in this case the levels of estradiol and testosterone undergoes a imbalance, seeing that estradiol is increased in chronic stages of the infection whereas the testosterone diminishes, which would indicate that probably the parasite causes a hormonal imbalance in chronic stages to be able to remain by long periods in the host.

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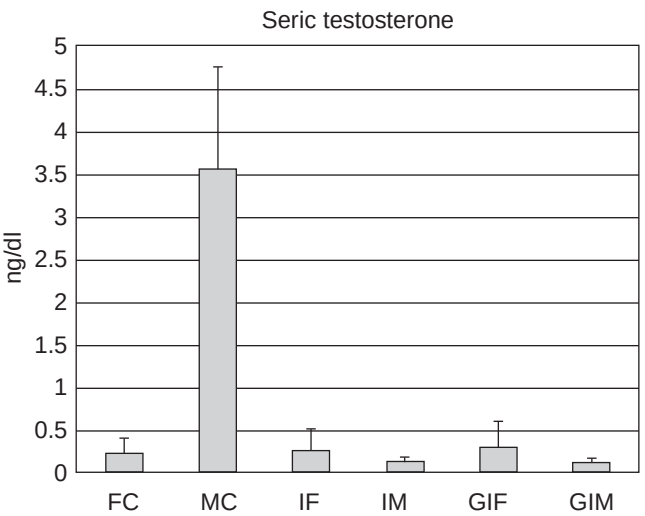

Figure 5. *Average of the levels of testosterone by group. The result of the ANOVA and the test of Tukey indicate that there are significant differences between groups and that the MC is the one that presents/displays the value highest (P<0.0001).*

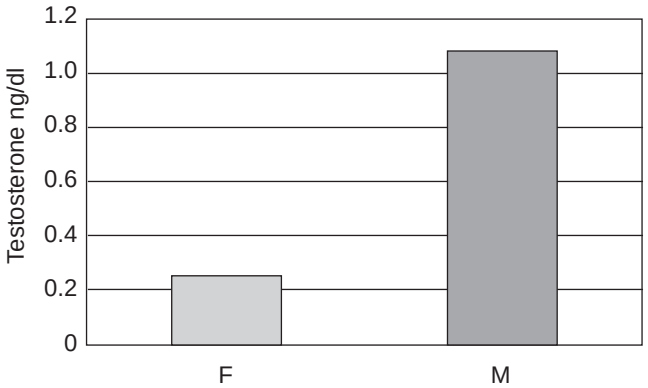

Figure 6. *Chart of the levels of testosterone in females (F) and males (M), where males present higher levels than females.*

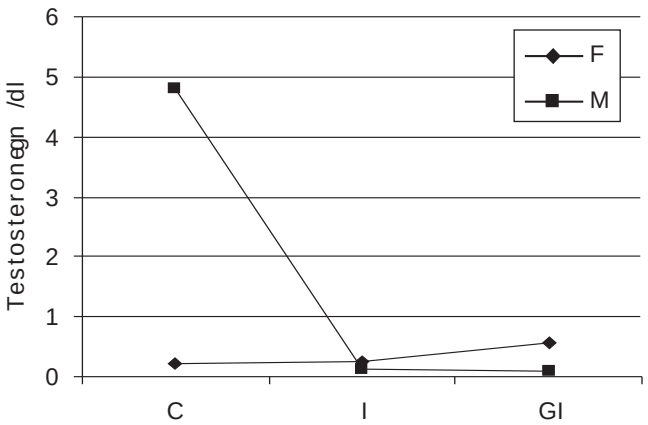

Figure 7. *Sex-variable interaction with the levels of testosterone, observing that the testosterone diminishes considerably because of the effect of the infection with the metacestodo of* E. granulosus *in males (M). Analysis of the mean differences by Tukey.*

Table 2. Effect of the infection in the testosterone levels. One was to moderate the levels of Testosterone in blood of BALB/c mice (n = 10), to the 16 weeks of to be inoculated with *E. granulosus* protoscoleces. The statistics analysis of ANOVA and Tukey´s studentized range indicate that the values of the cm are significantly different from the others (P < 0.0001).

	CM	CF	IM	IF	GIM	GIF
Testosterone ng/dl	3.04 ± 1.2	0.22 ± 0.16	0.12 ± 0.05	0.25 ± 0.2	0.09 ± 0.06	0.28 ± 0.3

Table 3. Effect of the infection in the levels of estradiol. The levels of estradiol in blood of BALB/c mice (n = 10), to the 16 weeks after to be inoculated with *E. granulosus* protoscoleces. The statistic analysis of ANOVA and Tukey´s studentized range indicate that there are significant differences between groups, and that the HI presented/displayed the highest values (P < 0.0001).

	CM	CF	IM	IF	GIM	GIF
Estradiol pg/ml	6.50 ± 5.41	17.08 ± 5.84	5.84 ± 1.01	38.97 ± 27.22	20.36 ± 16.71	15.46 ± 9.70

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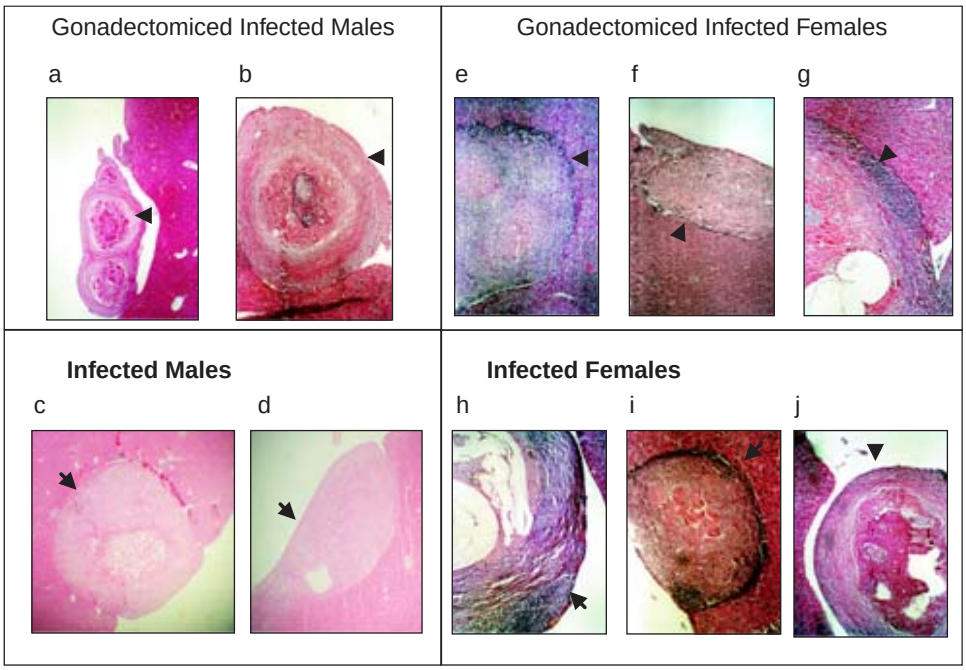

Figure 8. *Implantation of* E. granulosus´ *metacestode in the mice´ livers. a, b) hydatid cyst in GIM' liver where it is possible to see a minimum chronic inflammatory infiltration and a superficial implantation. e, f, g) Hydatid cyst in infected GIF´ liver, where it is possible to see a more abundant chronic inflammatory infiltration and a deeper implantation of the hydatid cyst. c, d) hydatid cyst in infected male livers where it is possible to see a minimal chronic inflammatory infiltration and a superficial implantation. h, i, j) hydatid cyst in infected females' liver, where it is possible to see a more abundant chronic inflammatory infiltration and a deeper implantation of the cyst.(→) hydatid cyst.*

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