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Morbidity due to *Schistosoma mansoni - Entamoeba histolytica* coinfection in hamsters (*Mesocricetus auratus*)

Morbidade em hamsters (*Mesocricetus auratus*) devido à co-infecção *Schistosoma mansoni - Entamoeba histolytica*

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ABSTRACT

Data on Schistosoma mansoni-Entamoeba histolytica *coinfection are scarce in the literature. In the present study, hamsters that had been infected for 70 days with* Schistosoma mansoni *(LE strain) were inoculated via the portal vein with two strains of trophozoites of* Entamoeba histolytica*: ICB-EGG (highly virulent) and ICB-RPS (non-virulent). The most evident result of coinfection was increased morbidity and mortality, in comparison with either of the infections alone. Histologically, there were no evident signs of interaction between these two infections. The morphological findings of schistosomal granuloma and amoebic abscesses in the liver were similar to those seen in the respective single-infection controls. However, there was severe wasting of the animals with both infections and greater numbers of amoebic lesions in their livers. The results obtained indicated that schistosomiasis aggravates the course of amoebiasis in hamsters.*

Key-words: Entamoeba histolytica. Schistosoma mansoni. *Coinfection. Morbidity. **Mortality.***

RESUMO

Dados sobre a co-infecção Schistosoma mansoni-Entamoeba histolytica *são escassos na literatura. No presente estudo, hamsters com 70 dias de infecção por* Schistosoma mansoni *(cepa LE) foram inoculados com trofozoítos de* Entamoeba histolytica*, cepa ICB-EGG (virulenta) e cepa ICB-RPS (não virulenta), via veia porta. O mais evidente resultado da co-infecção foi o aumento da morbidade e mortalidade, quando comparado com os animais com somente uma das infecções. Histologicamente, não houve sinais evidentes da interação entre as duas infecções. O aspecto morfológico do granuloma esquistossomótico e do abcesso hepático amebiano são similares aos observados nos controles, com somente uma infecção. Entretanto, foi observado que os animais co-infectados apresentavam-se mais debilitados e com maior número de lesões amebianas no fígado. Os resultados obtidos indicam que a esquistossomose agrava o curso da infecção amebiana em hamsters.*

Palavras-chaves: Entamoeba histolytica. Schistosoma mansoni. *Co-infecção. Morbidade. Mortalidade.*

Schistosomiasis caused by the presence of *Schistosoma mansoni* in the visceral system of its hosts affects about 130 million people worldwide. The egg-laying in the tissues, mainly in the liver, leads to a granulomatous inflammatory process that changes the hepatic microenvironment (hypertension and periportal fibrosis). This may, in some patients, result in severe or hepatosplenic forms of the disease, which is potentially lethal because of the bleeding caused by disruption of the esophageal varices.

Amoebiasis is a disease caused by the protozoan *Entamoeba histolytica*, with estimated worldwide prevalence of 500 million infected people[22]. The natural habitat of the parasite is the large intestine, and many cases are asymptomatic. Nevertheless, several factors (parasite strain, bacterial flora or host immunological condition) allow the protozoan to invade the intestinal mucosa, and possibly to reach a site distant from its usual habitat, thus resulting in extra-intestinal amoebiasis. The liver is commonly the extra-intestinal organ most affected[6,9,20].

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The prevalence of *E. histolytica* infection in the human population is high in most of the different regions of the world where schistosomiasis is also an endemic disease, with consequently increased morbidity[1 8 12]. By reviewing the literature, it can be seen that few studies have dealt with *S. mansoni/E. histolytica* interaction. To our knowledge, there are no experimental studies using intrahepatic inoculation for coinfection of hamsters, although the liver is recognized as the main site for the lesions resulting from granulomas caused by *S. mansoni* eggs, as well as being the most common site for extra-intestinal amoebiasis[1 10 12 15].

Multiparasitism is a frequent event in human populations living in areas that are endemic for schistosomiasis, and it mainly affects disadvantaged populations. Thus, studying schistosomiasis in association with other pathogens may be pivotal in updating with new approaches on this subject. Several studies have indicated that there are endemic areas for schistosomiasis and other areas for amoebiasis, and also many regions where both diseases coexist[2 11 14].

In the present experimental study, the interaction between *S. mansoni* and *E. histolytica* was investigated. Animals previously infected with *S. mansoni* (LE strain) were then inoculated with trophozoites of *E. histolytica* (ICB-EGG and ICB-RPS strains) via the portal vein and intracecal inoculation. The ICB-EGG strain presented a high degree of virulence, whereas the ICB-RPS strain was used as an infection control, since it was not virulent for the animals used in this experiment. Animals bearing coinfection (*S. mansoni* - LE strain and *E. histolytica* - ICB-EGG strain) exhibited higher morbidity and mortality than did single-infected controls, although no special features were evident in the histological patterns of the hepatic lesions due either to amoebiasis or to schistosomiasis.

MATERIAL AND METHODS

***Entamoeba histolytica* strains. ICB-EGG strain.** This was isolated in May 1988, by Dr. Silva, from mucosanguinolent feces of a symptomatic male patient, coming from Manaus (Amazonas, Brazil), containing cysts and trophozoites. The patient presented dysenteric colitis and hepatic abscess, with positive serology for HAI, ELISA and IFI. Inoculation of this strain into experimental animals resulted in 100% infection in hamsters (grades III and IV), and 63% intracecal infection in rats (grades II and III), when an inoculum of 2.5 x 10^5 trophozoites was used. Lesions with inocula ranging from 1.0 x 10^3 to 2.0 x 10^6 trophozoites could be observed. Inocula with higher burdens led to more significant lesions. This strain presents pathogen zymodeme XIX and has been maintained cryopreserved in liquid nitrogen for 18 years[13 19]. **ICB-RPS strain.** This strain was isolated in May 1989, by Dr. Silva, from cysts in feces of a 7-year-old child, coming from Manaus (Amazonas, Brazil). The patient was asymptomatic with positive stool examination for cysts of *E. histolytica* and *E. coli,* but with negative serology for HAI, ELISA and IFI. Lesions in the liver of hamsters or in the cecum of rats could not be observed, when inocula of 1 x 10^5 to 1 x 10^6 trophozoites were used. Procedures such as addition of cholesterol, passage in the liver of hamster or association with the flora of pathogenic strains were not able to render this strain virulent. Nevertheless, this non-virulent strain was able to adhere to and phagocytize human erythrocytes, but in smaller quantities than seen for pathogenic strains. This strain presents zymodeme I and has been maintained cryopreserved in liquid nitrogen for 16 years[13 19].

Experimental animals. Four to six-week-old hamsters (*Mesocricetus auratus*) of both sexes, weighing approximately 100g, were provided by the animal house of the Institute of Biological Sciences/UFMG and kept in separated cages (8-14 animals). They were given a standard balanced allowance of food every day, and water *ad libitum*. For intracecal inoculations, Wistar rats of both sexes and weighing approximately 200g were used.

Parasites and experimental infection. In order to produce *S. mansoni* infection, the animals were subcutaneously inoculated with approximately 25 cercariae/animal (LE strain). After 70 days of cercarial infection, the animals were inoculated intraportally with trophozoites, in accordance with the procedure described by Rocha and Coelho[17]. Shortly afterwards, under asepsis and anesthesia, the abdominal wall of the animals was opened by means of a horizontal incision of 1.5cm length. Using a sterilized hypodermic syringe, the trophozoites were inoculated into the portal vein and soon afterwards, the vein was compressed with a gauze embedded in 0.9% saline, to control the hemorrhage. Then, the peritoneum and the skin were individually sutured with nonabsorbent threads. The trophozoites were obtained from monoxenic cultures with Crithidia fasciculata at the log phase of growth (48-72 hours). The inoculum was calculated so as to obtain 2.5 x 10^4 trophozoites in a total volume of 200µl.

In order to perform intracecal inoculations, the rats were kept without solid food for a 24h-period. After anesthesia and asepsis of the abdomen, the cecum was observed via an incision in the lower left quadrant. The exposed cecum was inoculated with 2.5 x 10^5 trophozoites of *E. histolytica* (EGG strain), in 200µl volume. Inoculations were carried out into the normal or traumatized cecum, and the peritoneum and skin were sutured using nonabsorbent thread[18].

The animals subjected to surgery received analgesic (Banamine®/fluxinin - 2mg/kg) subcutaneously every 24h, for the first 48h following the surgery.

All the procedures relating to the EGG strain were conducted using a non-pathogenic RPS strain.

Animal sacrifice and evaluation of infection. One week after infection with *E. histolytica*, the animals were sacrificed by cervical fracture and subjected to necropsy. After opening the abdomen, the gross changes in the liver were evaluated. The proportion of animals with lesions containing amoebae per number of inoculated animals and the severity of the lesions were determined. The hepatic lesions were classified as reported by Diamond et al[5]: grade 0 – liver without visible lesion or just whitish points at the inoculation site; grade I – single abscess at

the inoculation site, less than 5mm in diameter; grade II – single abscess of more than 5mm in diameter or several small abscesses up to 2mm in diameter; grade III – abscess in the inoculated lobe, of more than 5mm in diameter, and some metastases at other sites in the liver; and grade IV – large abscess in the inoculated lobe, with extensive metastases and damage in at least 50% of the organ.

The viscera were carefully examined, and the cecum and part of the large intestine were removed and transferred to Petri dishes containing buffer saline solution. They were then opened longitudinally and their contents were removed for dilution in saline buffer. The cecum was then washed and its contents, plus the mucosa scrapings, were microscopically examined for *E. histolytica* trophozoites.

Histopathology. Representative fragments of the liver were fixed in buffered formalin 10%. They were then dehydrated, clarified, embedded in paraffin, sliced into thin sections of 3-5µm and stained with hematoxylin-eosin for conventional optical microscopy.

RESULTS

Inoculation by portal vein route. In the group of animals infected with *E. histolytica* only, of ICB-EGG strain (Table 1 and Figure 1), 16 animals presented lesions of degrees III and IV one week after sacrifice. It was also observed that one animal showed only one small focus of amoebic infection, and four animals presented no lesions.

Among the animals with both infections, one of them was sacrificed before the planned time, because it presented cachexia with frequent diarrhea. At necropsy, this animal's liver presented multiple foci of amoebic appearance. The microscopy on direct smears and cultures for trophozoites was positive. Among the 23 remaining animals, 11 died between 4 and 5 days after amoebic infection, presenting diffuse lesions (grades II and III degrees) in the liver. The rest of animals showed dissemination of small, well marked amoebic lesions (grades II and III), diffusely throughout the liver.

In the group of animals infected with *S. mansoni* only, all hamsters exhibited numerous granulomas within the hepatic parenchyma and there was no mortality.

Hamsters inoculated with trophozoites from the RPS strain (non-pathogenic) did not show amoebic hepatic lesions when inoculated with amoebae only or in association with *S. mansoni*.

Intra-cecal inoculation. When the intracecal route was used for performing inoculation of trophozoites, no amoebic lesions could be seen in the coinfected group. The cecal mucosa was thick, due to the presence of *S. mansoni* eggs. However, the content was altered and more diarrheic in the group coinfected with the ICB-EGG strain, and six animals presented trophozoites of *E. histolytica* when observed under the microscope.

Histopathology. The lesions produced by schistosomal eggs were characterized by granulomas, formed by a small number of macrophages around viable eggs and surrounded by a cuff of lymphocytes and mild fibrosis (Figure 2A). These lesions were totally independent from necrotic areas associated with amoebic infection. The lesions tended to be more focal in the hamsters with *S. mansoni* coinfection, and were characterized by areas of coagulative necrosis, involving lobular parenchyma, probably reflecting earlier lesions. Trophozoites were easily identified at the periphery of necrotic areas and sometimes within sinusoids in the vicinity. Around these necrotic areas, mild non-specific chronic inflammatory infiltrate composed mainly of mononuclear cells was observed. In animals without *S. mansoni* infection, the necrotic areas were more extensive and lytic. At the periphery of these necrotic areas, where numerous trophozoites were detected, the necrotic material stained more basophilic due to nuclear condensation and cellular debris. Surrounding these necrotic areas, non-specific moderately chronic inflammatory infiltrate composed mainly of mononuclear cells including lymphocytes and plasma cells was also observed, accompanied by a mild degree of fibrosis (Figure 2B).

Table 1 - Results from experimental inoculations of trophozoites (ICB-EGG and ICB-RPS strains), into the portal vein of hamsters (with and without* Schistosoma mansoni *infection). The animals were sacrificed seven days after receiving the amoebic inoculum.

					Infection grade*					
Group	Infection	Mortality	%	Lesion	%	0	I	II	III	IV
1	Eh-EGG	0/21	0.0	17/21	81.0	1	-	-	14	2
2	Sm + Eh-EGG	12/24	50.0	24/24	100.0	-	-	16	8	-
3	Eh-RPS	0/20	0.0	0/20	0.0	-	-	-	-	-
4	Sm + Eh-RPS	0/20	0.0	0/20	0.0	-	-	-	-	-
5	Sm	0/10	0.0	0/10	0.0	-	-	-	-	-
6	Control	0/10	0.0	0/10	0.0	-	-	-	-	-

*Infection grade, in accordance with Diamond et al[6]; Sm: *Schistosoma mansoni;* Eh-EGG: *Entamoeba histolytica* (EGG strain); Eh-RPS: *Entamoeba histolytica* (RPS strain); - No: sign of lesion

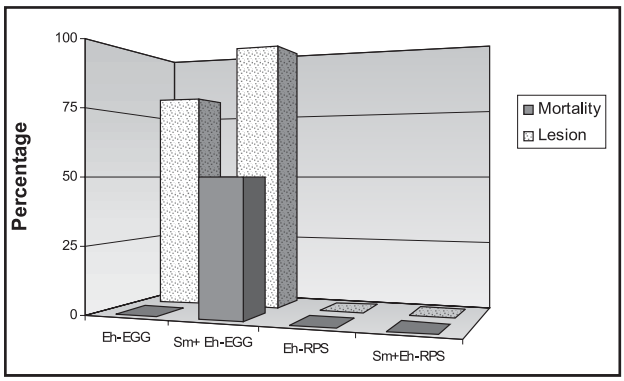

Figure 1 - Histogram - Results from experimental inoculations of trophozoites (EGG and RPS strains), into the portal vein of hamsters (with and without* Schistosoma mansoni *infection).

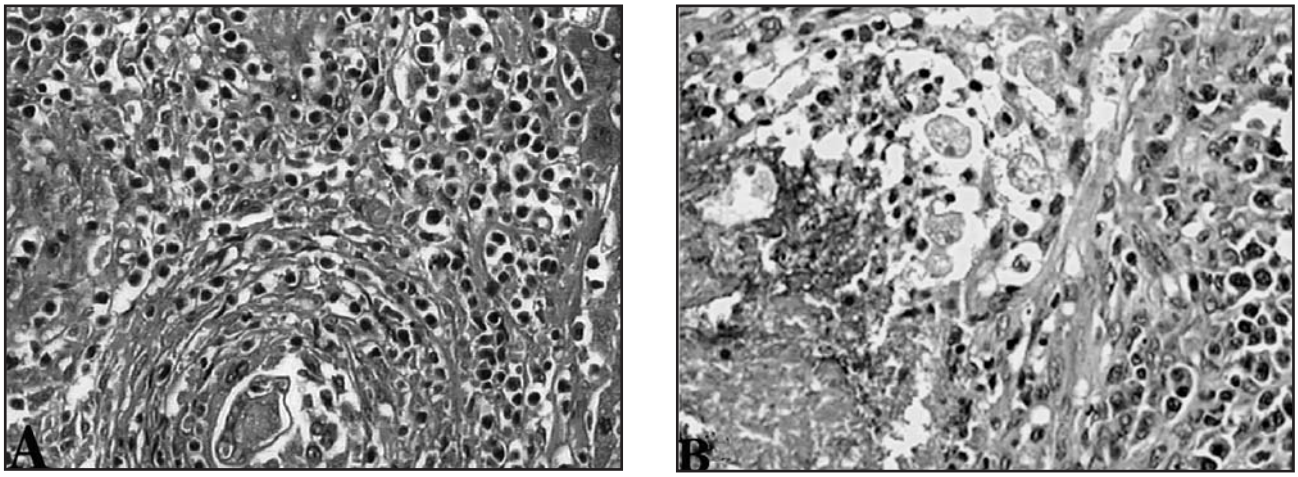

Figure 2 - Histopathological appearance of the liver of hamsters (with and without **Schistosoma mansoni** *infection) with* **Entamoeba histolytica***, ICB-EGG strain. A) Lesion produced by schistosomal eggs, characterized by epithelioid granulomas, containing only a small number of macrophages around the egg and surrounded by a cuff of lymphocytes and mild fibrosis (100x); B) Extensive coagulative and/or lytic necrotic areas can be seen, containing several amoebae inside them. No interaction between amoebic and schistosomal lesions could be observed in the sections examined (400x).*

DISCUSSION

As emphasized earlier, the literature provides only scarce information regarding the *S. mansoni - E. histolytica* association, and most of the data does not relate to studies using experimental models[8 12 15]. Moreover, the conclusions resulted from epidemiological surveys carried out in communities with precarious sanitary conditions, which are a predisposing factor for the seeding of other parasitic infections. Thus, based on this latter observation, it cannot be concluded that the presence of schistosomiasis would be *per se* a facilitating factor for *E. histolytica* infection, since such individuals are routinely exposed to a great number of other parasites.

In this study, we utilized the *E. histolytica* strains ICB-EGG (well known to be virulent) and ICB-RPS (non-virulent). Both of these are kept in the Amoebiasis Laboratory, and the LE strain of *S. mansoni* is kept in the Schistosomiasis Research Unit Laboratory. These laboratories belong to the Parasitology Department, Institute of Biological Sciences, Federal University of Minas Gerais, Brazil. These strains had been standardized with regard to pathogenicity and virulence by using experimental animals. The hamster is the most commonly used animal model in studies on experimental hepatic amoebiasis produced by *E. histolytica*[4 21]. The portal vein has been used as the infection route for *E. histolytica*[16], since this is the natural route used by trophozoites to reach the liver. We observed that the presence of granulomatous schistosomiasis lesions made the amoebic infection a serious risk factor for death among the coparasitized animals. The presence of both infections led to the death of 50% of the animals, and it seemed that the lesions of the two parasitoses were cumulative, although there was no association between the presence of granulomas and trophozoites. Among the factors that could be involved in this increased mortality, it may be suggested that the animal's fragility was due to the effects of schistosomal infection. Regardless of the fact that the amoebic abscesses were smaller in the animals with both infections, these abscesses were diffuse in the liver, thus resulting in numerous foci of infection, with more severe impairment of the organ.

The non-pathogenic RPS strain of *E. histolytica* that was used as control in this study was unable to produce lesions, or even to infect the animals, whether or not of *S. mansoni* was present, thus showing that mixed infection could not convert this strain into a pathogenic type.

Although Knight and Warren[10], using mice as experimental models, demonstrated that previously existing schistosomiasis was a risk factor for the seeding of intestinal amoebiasis, in the present study cecal inoculation of trophozoites in rats did not show lesions in these animals, or even in the presence of *S. mansoni* granulomas (data not shown). The resistance of these animals to intestinal infection with trophozoites of *E. histolytica* was also observed by Ghadirian and Meerovitch[7]. However, the more diarrheic cecal content and the presence of trophozoites in the animals presenting coinfection lead us to wonder about the possibility that lesions might form if a longer period of association were studied. In these animals, thickening of the mucosa could be observed, caused by granulomas that led to formation of an area of constriction of the cecal lumen, with abundant mucus and presence of necrotic tissue. This created an environment to enable the maintenance of *E. histolytica* at this site for a longer period. This could explain the high prevalence of amoebiasis detected in endemic regions for schistosomiasis[1 8 12]. In the animals inoculated with *E. histolytica* only, the presence of trophozoites could not be observed during examination of the cecal contents, probably due to the normal intestinal motility of the organ, as well as the absence of *S. mansoni* granulomas, which could alter the intestinal peristaltism of the animal.

The initial hypothesis was that the presence of *S. mansoni* granulomas would facilitate *E. histolytica* infection, since there would be a change in the hepatic microenvironment and exposure of the extracellular matrix and its components[3]. Moreover, collagen resulting from *S. mansoni* granulomas might serve as adhesion sites for subsequent amoebic colonization. Nevertheless, adhesion of trophozoites on *S. mansoni* granulomas was not observed in the histological sections. However, exacerbation of tissue damage was observed when both diseases were present,

which resulted in a higher percentage of deaths among the animals, which suggests that there is an additive effect between the typical lesions of each disease.

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