Improvement of the *In Vitro* Culture of *Echinococcus Granulosus* Metacestodes

Abstract

This work introduces a novel modification to the *in vitro* experimental vesicular development of the parasite. Our findings showed that protoscoleces exposed to insulin developed into microcysts in shorter times. Moreover, a tenfold increment in the yield of microcysts per tube was observed. An improvement of the *in vitro* culture of *E. granulosus* metacestodes was achieved. On the basis of this axenic *in vitro* system, biochemical, molecular and chemotherapeutical studies of *E. granulosus* will be greatly facilitated.

Keywords: *Echinococcus granulosu*; Protoscoleces; Metacestodes; *In vitro* vesicular development; Insulin

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Introduction

A mayor development in parasitology has been the increased interest in attempts to culture parasites outside their hosts. It is well recognized that the value of *in vitro* techniques is that not only do they allow experiments to be carried out without the use of laboratory animals but also they allow the nutrition, physiology and biochemistry of a parasite to be studied in isolation from the interacting physiology of its host [1].

Historically, the primary assessment of antiechinococcal drug candidates has often been performed in rodents, which has led to the extensive use of animal experimentation. Subsequently, the *in vitro* culture of *Echinococcus* metacestodes has proven to be a suitable tool for the primary assessment of parasite susceptibility to certain compounds, with a focus on broad-spectrum anti-infective agents, and also represents an ideal model system for studies on drug uptake and associated metabolic changes imposed upon the parasite [2].

As the cystic stage of *E. multilocularis* grows more rapidly than that of *E. granulosus*, it has proved to be more amenable to culture [1]. *E. multilocularis* metacestodes, besides increasing in size, also proliferate asexually and, provided with the corresponding nutrients and growth factors, will form new vesicles either endogenously or exogenously [3].

E. granulosus protoscoleces behaviour in *in vitro* cultures has been quite well described previously [4-7]. A low percentage of protoscoleces develops into microcysts in *in vitro* systems supplemented with fetal calf serum, independently of their host origin (Table 1). Thus, the amount of metacestodes that could be generated was not sufficient for large-scale drug screening activities.

Table 1: Comparison of present and previous works on *in vitro* vesicular development of protoscoleces of *Echinococcus granulosus* of sheep and cattle origin.

Author	Geographical Origin of Samples	Host of Procedence	Composition of Culture Medium	Beginning of Vesicularization (In Days)	Appearance of Laminar Layer (In Days)	Appearance of Miniature Cysts (In Days)	Obtained Cyst (%)	Maximum Survival (In Days)
Present work	Southeast Buenos Aires Province (Argentina)	Cattle	Medium 199 + serum + insulin	2	11	14	11	≥120
Elissondo et al. [10]	Southeast Buenos Aires Province (Argentina)	Cattle	Medium 199 + serum	5	14	20-38	1.6	95
Elissondo et al. [10]	Tierra del Fuego (Patagonia Argentina)	Sheep	Medium 199 + serum	7	14	19-27	3	119
Rodríguez-Caabeiro [6]	Spain	Sheep	Medium 199 + serum	6	9	15	1	90

On the other hand, the concept of hormonal cross-communication between evolutionary conserved signaling systems plays an important role in host-parasite interaction in systemic flatworm infections [8]. Konrad et al. [9] demonstrated that the insulin-receptor-like RTK EmIR can interact with host-derived insulin. Moreover, using the axenic cultivation system, Brehm et al. [8] established host cytokines which stimulate RTKs, such as insulin, EGF or FGF, all have positive effects on *E. multilocularis* growth whereas cytokines of the TGF-β/BMP-family appear to inhibit metacestode growth and rather stimulate protoscolex development.

In order to improve the *in vitro* culture of *E. granulosus* metacestodes, the achievement of shorter development times and an increase in the yield of microcysts per tube are needed. The aim of the present work was to study the effect of insulin on the *in vitro* development of *E. granulosus* metacestodes.

Materials and Methods

Protoscoleces of *E. granulosus* were isolated under aseptic conditions from liver and lung hydatid cysts of infected cattle slaughtered in an abattoir located in the southeast of the Buenos Aires province, Argentina. Viability was assessed by muscular movements (evaluated under light microscope) and by the methylene blue exclusion test [10]. The culture protocol was carried out as described previously by Elissondo et al. [4] with the addition of different concentrations of insulin to the medium. Briefly, viable and free protoscoleces (1500 per Leightont tube) were cultured in medium 199 (Gibco), containing 60 µg/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamicin, 4mg/ml glucose and 20% (v/v) fetal calf serum. Insulin (Eli Lilly and Co., USA) was added to the medium resulting in final concentrations of 1.2 and 0.6U ml^{-1}. Cultures without insulin were used as controls. Cultures were performed in 10ml of incubation medium at 37ºC and the medium was changed every 3-4 days. Development was followed microscopically every day. The data recorded for each sample correspond to the moment when a stage was reached by the most advanced individuals of the culture.

Results

Evolution times and yield per tube of control cultures coincides with the previously reported results by our group [5] (Table 1). The percentage of obtained cysts was around 1.6%. On the other hand, cultures with the addition of insulin showed some remarkable differences (Table 1). At the beginning of the culture protoscoleces were invaginated (Figure 1a). Vesiculated protoscoleces appeared after 2 days incubation (Figure 1b).

After 11 days of culture, a considerable increment in the size of vesiculated protoscoleces could be observed and a laminated layer appeared like a fine membrane in one of the extremes (Figure 1c). On day 14 some microcysts with a complete laminated layer were detected (Figure 1d). By day 20, microcysts completely developed could be observed (Figure 1e). Some cultures could be maintained for more than 120 days when the cultivation was interrupted for an *in vitro* drug screening experiment (Figures 1f-h). The percentage of obtained cysts per tube was approximately 11% (Table 1). A tenfold increase per tube was registered after the addition of insulin. The used concentrations of insulin on the cultures did not differ in relation to evolution times and yield per tube.

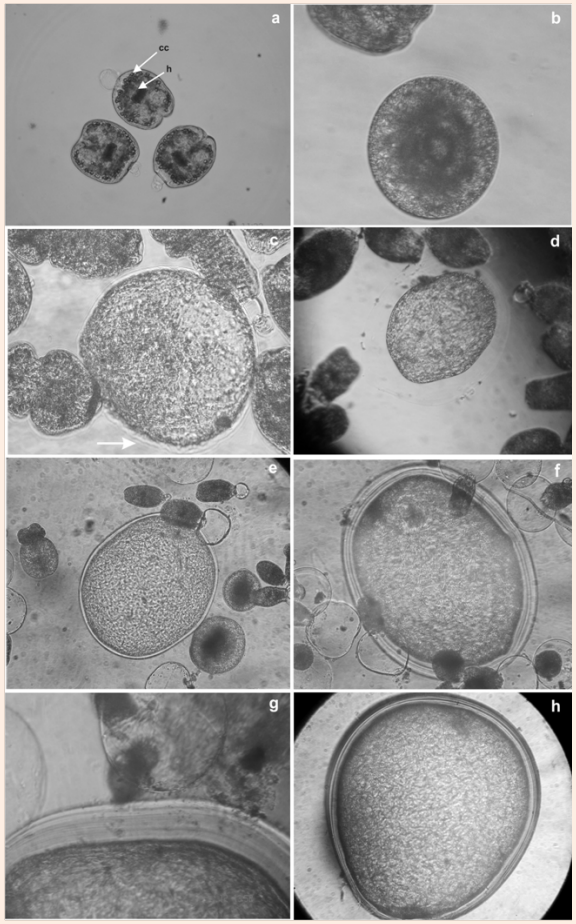

Figure 1: In vitro development of E. granulosus protoscoleces of cattle origin.

a. Invaginated protoscoleces. The typical morphology of protoscoleces is observed (h hooks, cc calcareus corpuscles, 300x).

b. Vesiculated protoscolex (2 days of culture, 600x).

c. Vesiculated protoscolex (11 days of culture) showing the appearance of laminar layer (white arrow, 480x).

d. Complete laminar layer (14 days of culture, 190x).

e. Completely developed microcyst (20 days of culture, 150x).

f. Microcyst (45 days of culture; 150x). Note the increment on size.

g. Detail of the laminated layer (100 days of culture, 340x).

h. Microcyst after 115 days of culture (20x).

Discussion

The relationship between parasites and their hosts implies close biochemical co-evolution and communication between their

complex physiological and metabolic systems [11]. This work describes the effect of insulin on *E. granulosus* protoscoleces *in vitro* development to microcysts. On the other hand, this study introduces a novel modification to the *in vitro* experimental vesicular development of *E. granulosus*. Our findings showed that protoscoleces exposed to insulin evolved in shorter times. Moreover, a tenfold increment in the yield of microcysts per tube was observed.

Our results are consistent with those reported by Brehm [12], where significant effects of insulin on the formation of *E. multilocularis* metacestode vesicles from primary cells *in vitro* were observed. Furthermore, in recent years the effects of insulin on the *in vitro* development of other cestodes has been reported. Insulin showed significant effects on *Taenia crassiceps* reproduction, growth, viability and on parasite infectivity [11]. In addition, the in vivo response of *Mesocestoides vogae* to human insulin was demonstrated [13]. In the mentioned work, parasite larvae were challenged with different levels of insulin for variable periods. The parameters tested were influenced by human insulin, and suggested a host-parasite molecular dialogue.

We consider that our findings lead to an improvement of the *in vitro* culture of *E. granulosus* metacestodes. The achievement of better axenic culture system for *E. granulosus* metacestodes would introduce several advantages to the study of this parasite. As it was mentioned, the *E. multilocularis* behavior *in vitro* cultures is substantially different from *E. granulosus*. An improvement on the differentiation of *E. granulosus* metacestode vesicles under host cell free conditions would allow the performance of biochemical and physiological studies without the influence of the intermediate host. For example, different signaling pathways could be investigated. Moreover, the study of the influence of selected host factors on development for prolonged periods of time without the presence of interfering host cells will be possible. Performing of molecular and proteomic studies also would be feasible.

CE is one of the several parasitic diseases of difficult chemical control, where chemotherapy with currently used drugs is highly variable. This disease is included by the WHO in the list of the neglected tropical diseases, in which the use of integrated approaches to cure, prevent and control the disease at the human-animal interface is needed in order to be successful in its prevention and control [14]. This *in vitro* model does not require prior development of secondary hydatid cysts, and thus prevents the use of laboratory animals (which is desirable in itself). The development of cyst using the murine model of cystic echinococcosis last at least 6-8 month after the intraperitoneal inoculation of protoscoleces. This improved *in vitro* system reduces the time needed for the *in vitro* drug screening of putative therapeutic agents and could allow the discovery of novel potential drug targets associated with the parasite signalling network.

More exhaustive evaluation of the effect of insulin on *E. granulosus* should be undertaken. In a next step, we will investigate the effects of the hormone when the parasite is cultured under low oxygen conditions and in the presence of reducing agents.

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