The effects of Glucose and Ascorbic acid on *in vitro* development of *Echinococcus granulosus* Metacestodes

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Abstract

Echinococcus granulosus-developed metacestodes in the cultured medium are used for the assessment of its susceptibility to different compounds; however, this procedure is time-consuming and risky. In the present study, aspirated protoscoleces from the infected sheep were used to evaluate the effects of glucose, as an energy source, as well as ascorbic acid, as an antioxidant vitamin, on larval development. Protoscoleces were maintained in $RPMI_{1640}$ culture media containing 10% fetal calf serum, as well as different concentrations of glucose (6 and 8 mg/ml) and ascorbic acid (25, 50, and 100 µg/ml). A culture medium containing 4 mg/ml of glucose was served as the control. Larger cysts were achieved in a shorter time from the medium enriched with 6 mg/ml of glucose (740 ± 20 µm) compared to the control group (420 ± 40 µm). However, in the groups treated with ascorbic acid, the number of cysts was higher in 100 µg/ml (32.5 ± 0.7) compared to the control group (12.5 ± 0.7). Additionally, the mature cysts were achieved on the 7th day of cultivation with 100 µg/ml of ascorbic acid compared to 18 days in the control group.

Introduction

The protoscoleces of *Echinococcus granulosus* could differentiate in two directions depending on its environmental conditions. The ingested protoscoleces in a dog exposed to the gut environment was differentiated in strobilation to form an adult cestode. On the other hand, in the intermediate hosts, protoscoleces obtained from ruptured cysts escaped into the body cavity, in order to form more hydatid cysts (secondary hydatidosis). In the cultured medium, both the adult worms and the cysts were obtained from protoscoleces (Smyth and Davies 1974). The *in vitro* cultivation of *E.granulosus* metacestodes (protoscoleces or cysts) have been used to study parasite's physiology and biochemistry. In addition, they have been used for the primary assessment of their susceptibility to different compounds (Hemphill et al. 2010; Macpherson and Smyth 1985; Shan et al. 2013; Sharafi et al. 2017).

As the cystic stage of *E.granulosus* develops slowly, the risk of contamination is high and because of the complication of nutritional conditions, only 1 to 11 percent of protoscoleces develop into small cysts (Elissondo 2017; Elissondo et al. 2004; Rodriguez-Caabeiro and Casado 1988). Thus, *in vitro* culture conditions were needed to be improved.

It has been shown that tapeworm's metabolism is dependent on glucose. In this regard, protoscoleces of *E.granulosus* transport glucose via both passive diffusion and the mediated transportation (Jeffs and Arme 1988). It has been shown that the mean concentration of excretory/secretory (E/S) proteins is higher in phosphate- buffered saline (PBS) medium enriched with glucose compared to those of DMEM and $RPMI_{1640}$ (Haniloo et al. 2011).

Many similarities were found between cancer cells and parasites, including multiplying in host organs, resistance to the programmed cell death, escaping from host immunity, and using proteolytic enzymes to

reach the suitable tissues. Of note, there are many common drugs between the parasites and cancer cells (Klinkert and Heussler 2006).

Ascorbic acid (vitamin C), which is an antioxidant vitamin, has been shown to play a biphasic role in cancer cells according to dose, time, and expression level of sodium-dependent vitamin C transporter 2 (SVCT-2) (Cho et al. 2018; Park et al. 2004). In low SVCT-2 expressing cell lines, deficient delivery of ascorbic acid was indicated to increase the cancer's proliferation activity. In the *E.granulosus* genome, three sodium- dependent transporter genes (named as EG_05623, EG_05624, and EG_05625) playing uncharacterized roles exist, which may also play a role in the delivery of acid ascorbic inside the parasite (Zheng et al. 2013) (Sanger Institute).

According to the above-mentioned information, in the current study, the effects of glucose and ascorbic acid on *in vitro* development of *E.granulosus* metacestodes were investigated.

Materials And Methods

The Protoscoleces were aseptically aspirated from hydatid cysts of livers of sheep that were slaughtered in a local abattoir in Zanjan, Iran. Thereafter, the protoscoleces were passed through two layers of sterile gauze, which were then washed several times with PBS solution (pH 7.2) containing penicillin-streptomycin. Parasite's viability was also assessed by muscular movements using the 0.1% eosin exclusion test. Those samples with more than 90% of living protoscoleces were included to be used in this study.

The effect of glucose on development of E.granulosus metacestodes

The protoscoleces with the density of approximately 1800 ml were incubated in a $RPMI_{1640}$ medium (R1383, Sigma-Aldrich, USA) containing penicillin (100 IU/ml) and streptomycin (100 µg/ml), which was also supplemented with 10% (v/v) fetal bovine serum (FBS) and 4 mg/ml of D-glucose in 5% CO_2 at 37°C (Liu et al. 2013). Changes in development, including vesiculation, the appearance of laminated layer, mature cyst formation, and number and size of the cysts were all followed using an inverted microscope (Motic®, AE31, Spain) in each day for 50 days (Elissondo 2017). The medium was also changed every 3–4 days. To evaluate the effect of the glucose concentration on the development of *E.granulosus* metacestode, two other groups supplemented with 6 and 8 mg/ml of glucose were selected, as well.

The effect of ascorbic acid on the development of E.granulosus metacestodes

As stated in the previous section, $RPMI_{1640}$ medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 4 mg/ml of D-glucose was served as the control group. Thereafter, L-ascorbic acid (Sigma-Aldrich, A4544) with different concentrations (25, 50, and 100 µg/ml) were added to the above-prepared medium, in order to evaluate the effect of ascorbic acid on the development of *E.granulosus* metacestode. The developmental changes were then evaluated as described earlier. It is noteworthy that

the average length of two diameters of all the formed cysts $(\frac{D+d}{2})$, was used to calculate the cysts' size. The measurements were done by the microscope using an eyepiece reticle (Fig. 1).

Statistical analysis

All the experiments were repeated three times and the obtained data were presented as the mean value ± standard deviation (mean ± SD). The differences between the groups were determined by ANOVA using SPSS (version 22.0) and a *p*-value less than 0.05 was considered as statistically significant.

Results

Posterior bladder formation or vesiculation of evaginated protoscoleces, as the first signs of evolution, have started by passing 2 to 6 days from the incubation in different culture flasks. After 14 days of incubating in the control group, a fine membrane has started to form the laminated layer and by the day 17, some completely developed cysts (mature cysts) were detected (Fig. 2).

The effect of glucose on development of E.granulosus **metacestodes**

As shown in Table 1, the appearances of laminated layer as well as the formation of mature cysts in the cultured medium supplemented with 6 mg/ml of glucose were observed to be faster than the use of 4 and 8 mg/ml of this sugar ($p < 0.05$). Moreover, larger cysts were achieved from the cultured medium with 6 mg/ml of glucose (740 ± 20 μm) compared with the control group (420 ± 40 μm) ($p < 0.05$).

Table 1
The effect of glucose on *E. granulosus* metacestodes development. (Mean ± SD)

Glucose (mg/ml)	Beginning of vesiculation (days)	Appearance of laminated layer (days)	Appearance of mature cysts (days)	Cysts number in 50 days	Cysts size in 50 days (μm)*
4 (control)	5.3 ± 1	14 ± 0	17 ± 0	18 ± 2	420 ± 40
6	4.7 ± 1.1†	10.3 ± 0.6†	14 ± 0†	6.7 ± 0.6	740 ± 20†
8	5.7 ± 0.6	15 ± 1.7	18 ± 1.7	4.3 ± 1.1	340 ± 13

* All formed cysts were measured, Cysts size measured as diameters mean $(\frac{D+d}{2})$, †$p < 0.05$

The effect of ascorbic acid on the development of E.granulosus **metacestodes**

The appearance of laminated layer and the formation of mature cyst occurred after 5 and 7 days in the cultured media supplemented with 100 μg/ml of ascorbic acid; whereas it took place after about 14 and

18 days in the control group, respectively (Table 2). Although the size of the cysts was not very different between the study groups, the number of the obtained cysts was significantly higher in the groups supplemented with 100 µg/ml of ascorbic acid (32.5 ± 0.7) compared to the control group (12.5 ± 0.7) ($p < 0.05$).

Table 2
The effect of ascorbic acid on *E. granulosus* metacestodes development. (Mean ± SD)

Ascorbic acid (µg/ml)	Beginning of vesiculation (days)	Appearance of laminated layer (days)	Appearance of mature cysts (days)	Cysts number in 50 days	Cysts size in 50 days (µm)*
Without ascorbic acid (control)	6 ± 0	13.5 ± 0.7	18 ± 0	12.5 ± 0.7	530 ± 35
25	5 ± 0	9.5 ± 0.7†	13.5 ± 0.7†	17 ± 0	560 ± 14
50	2 ± 0	6.5 ± 0.7†	8.5 ± 0.7†	20.5 ± 0.7	560 ± 18
100	2 ± 0	5 ± 0†	7 ± 0†	32.5 ± 0.7†	570 ± 60

* All formed cysts were measured, Cysts size measured as diameters mean $(\frac{D+d}{2})$, †$p < 0.05$

Discussion

In this study, the effects of glucose and ascorbic acid on *E.granulosus* metacestodes development were examined under *in vitro*. Larger cysts were achieved from the cultured medium with 6 mg/ml of glucose in a shorter time compared to other groups; however, the highest number of cyst was found in the groups treated with 100 µg/ml of ascorbic acid. As well, mature cysts were achieved in the 7th day of cultivation with ascorbic acid in100 µg/ml compared to the 18th days in the control group. To the best of our knowledge, this is the first report in which the effect of ascorbic acid on *E. granulosus* metacestodes development was studied under *in vitro*.

Cestodes lack an alimentary tract, which interact with their environment only via the tegument. Accordingly, the tegument contains many structural proteins and enzymes (Thompson and Geary 2003). Glucose absorption in *E.granulosus* protoscoleces is through passive diffusion, which is mediated of both Na^+-dependent and Na^+-independent mechanisms (Jeffs and Arme 1988).

In *E. multilocularis*, EmGLUT1 was introduced as a simply facilitated glucose transporter that may play an essential role in glucose uptake by these parasites (Kashiide et al. 2018). As well, for *E. granulosus*, some sugar transporters were identified based on the gene database (Zheng, 2013) (Sanger institute).

In the present study, the cysts obtained from the 6 mg/ml glucose culture medium were larger (×1.5), but these were fewer in number compared with the control group. Accordingly, this could be explained by an adaptation phenomenon through which parasite biomass adjusts to the host's (environment) capacity (SCHMIDT G.D 2013).

Gordo and Bandera in 1997 cultivated *E.granulosus* protoscoleces in a CMRL-1066 culture medium that was supplemented with fetal calf serum (FCS), 0.014 ml glucose (30% in distilled water) per ml, and yeast extract. Additionally, in this study, the mature cyst formation occurred between days of 19 to 37(Gordo and Bandera 1997), while in our study, the mature cyst formation with 6mg/ml of glucose and 100 µg/ml ascorbic acids was achieved on days 14 and 7 post- incubation, respectively.

In 2004, Elissondo *et al*. in their study for the first time reported the cysts formation from protoscoleces of cattle origin using a M199 culture medium containing 4 mg/ml of glucose (Elissondo et al. 2004). As reported, after 14 days of incubation, some laminated layers appeared and on day 20, some cysts with a complete laminated layer were observed, which is almost similar to our results obtained from the control groups (4 mg/ml of glucose in $RPMI_{1640}$).

Moreover, these authors have cultivated protoscoleces from sheep origin in 2005, and reported that there are no differences between the ovine and bovine during the development process and at the time of cysts' formation (Elissondo et al. 2005).

To improve the *E.granulosus* cultivation process, Elissondo *et al.* designed another study in 2017 by the use of insulin in a M199 cultured medium. As a result, they reported that a laminated layer appeared after 11 days of culturing under *in vitro*, and on day 14, some cysts with a complete laminated layer were detected. Accordingly, this was in line with our results with 6 mg/ml of glucose (Elissondo 2017). Of note, insulin is a peptide hormone that can regulate the metabolism of carbohydrate and promote the absorption of glucose; therefore, the observed similarity was expected.

Haniloo *et al.* in their study in 2011 reported that protoscoleces maintained in PBS enriched with glucose produced a higher concentration of E/S protein compared to protoscoleces maintained in DMEM and $RPMI_{1640}$ media during 24 h of culturing. Correspondingly, this indicates the effect of glucose on the protoscoleces' metabolism (Haniloo et al. 2011).

Vitamin C, also known as L-ascorbic acid, is one of the water- soluble vitamins. In this regard, most of animals make their own vitamin C, but some others cannot do that. Although it is challenging to investigate the vitamin requirement of parasites, some works are available that measured ascorbic acid levels as well as the maturity and growth of cestodes (Ramalingam et al. 2006).

Hayanjeh in 2014 studied naturally infected sheep with hydatid cysts in comparison with healthy sheep. As a result, he reported that the plasma level of ascorbic acid in sheep infected with hydatid cyst was below the normal range and lesser than that of the control group (Hayajneh 2014).

It was observed that the release of reactive oxygen species (ROS) from the activated phagocytes against infectious agents could be harmful to host cells (Hemila 2017). Vitamin C may protect host cells and also reduce the rate of infection during infection diseases such as echinococcosis. However, Cinar *et al.* in a study in 2018 reported that vitamin C plasma level in sheep naturally infected with hydatid cysts was not affected by infection (Cinar et al. 2018). However, further studies are needed to determine the exact effect of ascorbic acid on *E.granulosus* larval stages.

Conclusion

The cysts obtained from the cultured medium enriched with glucose in the concentration of 6 mg/ml were bigger in size and achieved in a shorter time compared to the cultured medium supplemented with glucose with the concentration of 4 mg/ml. The achievement time of the cysts was even faster when the cultured medium supplemented with ascorbic acid with the concentration of 100 µg/ml was used, and more cysts were then gained.

Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

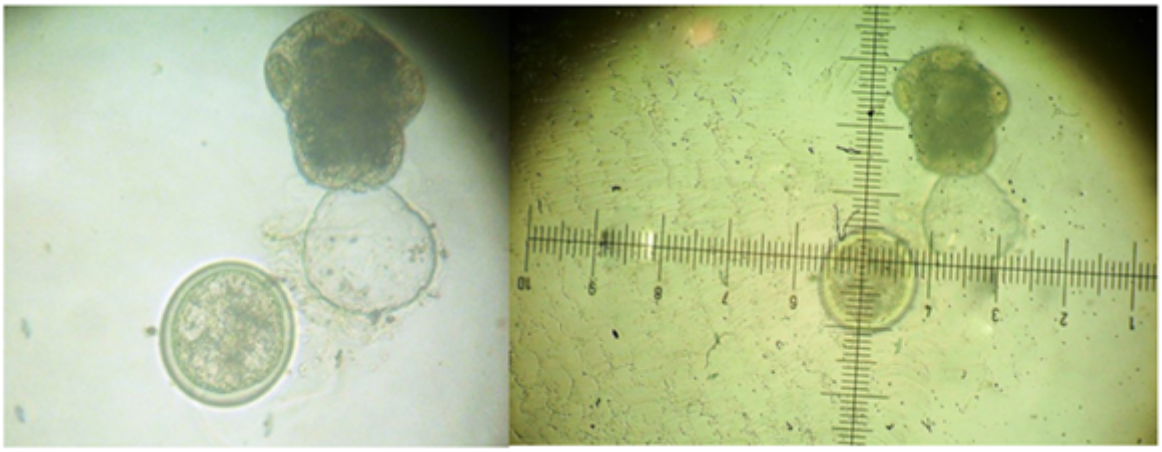

Figure 1

The cysts measurement under the microscope using an eyepiece reticle.

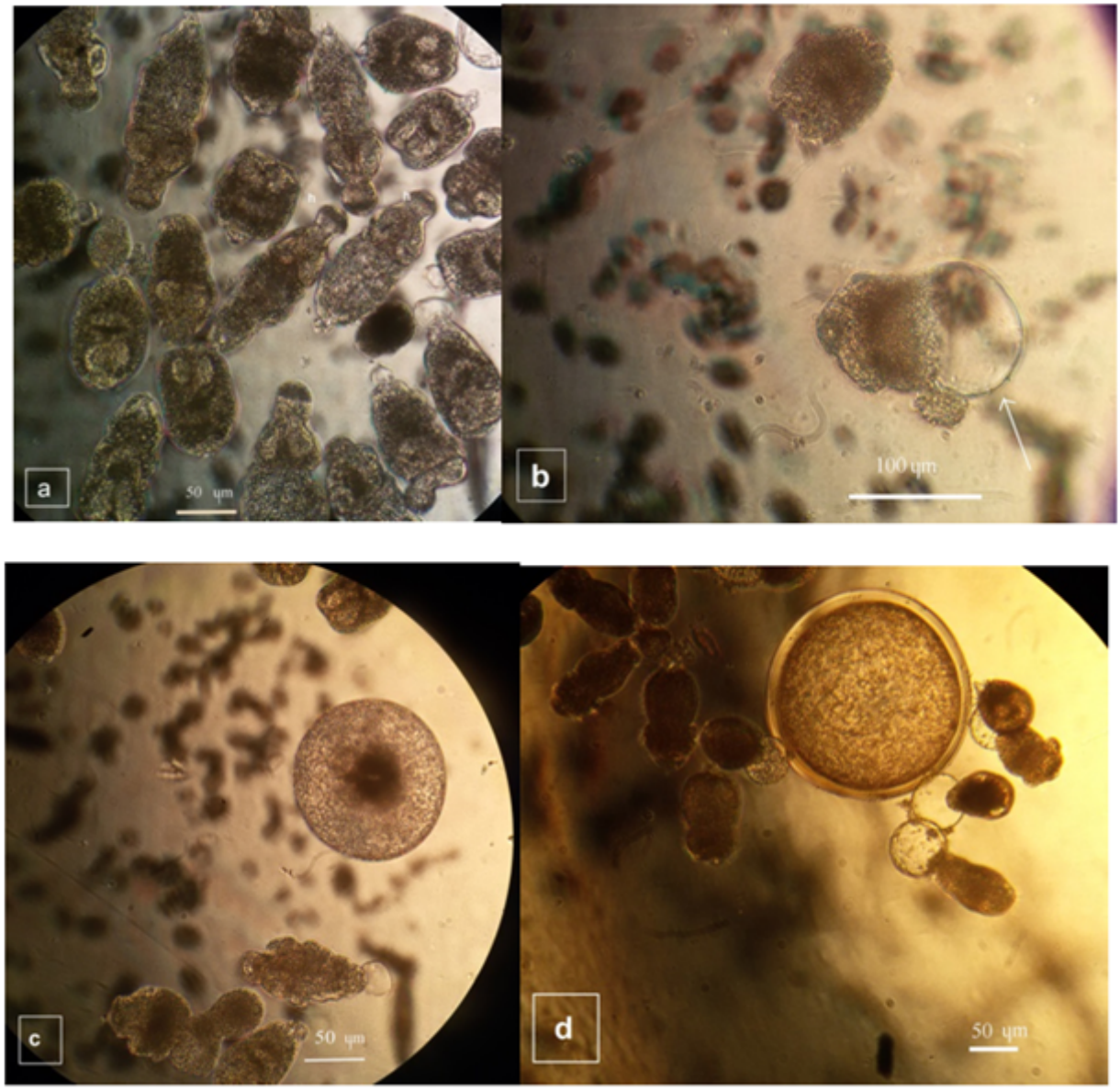

Figure 2

E. granulosus cysts development from protoscoleces in control group. a) Evaginated protoscoleces 1 day post- incubated (p.i.) (h hooks) (×200). b) Protoscolex with posterior bladder, 5 days p.i. (×200) c) Vesiculated protoscolex, 5 days p.i. (×200) d) Mature cyst 20 days p.i. (×100).