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Original paper

# Comparison of survival time of *Echinococcus granulosus* protoscolices in different culture media and temperatures and evaluation of their ability to generate cysts in mice

**Mahnaz MANSOUR[1,2], Ali JELOWDAR[1,2], Abdollah RAFIEI[1,2], Bahman CHERAGHIAN[3]**

[1]Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Parasitology, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[3]Department of Statistics, School of Health, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Corresponding Author: Ali Jelowdar; e-mail: jelowdar@gmail.com

**ABSTRACT.** Hydatidosis is a disease caused by the larval stage of *Echinococcus granulosus*, which has great importance in medicine and veterinary medicine. Protoscolices (PSCs) of fertile hydatid cysts play a critical role in secondary echinococcosis after surgery. Fertile cysts were acquired from infected sheep at the local municipal abattoir in Ahvaz, southwest of Iran. PSCs were obtained aseptically and transferred to 10 different culture media and kept at 4°C and 37°C to determine the duration of PSCs' survival. Then, 2000 live PSCs from each of the culture media were injected into the peritoneal cavity of BALB/c mice. After five months, the mice were evaluated in terms of cyst number, size, and weight. The highest PSCs survival time at 4°C was related to RPMI-1640 medium and cyst fluid for 50 and 45 days, respectively. Also, at 37°C, the longest survival time of PSCs was related to cyst fluid and RPMI-1640 media for 30 and 29 days, respectively. The highest level of infection and median cyst number was observed in mice received PSCs from the RPMI-1640 medium at 4°C, and the highest level of infection in mice at 37°C was related to the DMEM low glucose (L) medium. The current study indicated that 4°C was a more suitable temperature for *in vitro* storage of live PSCs. The maximum amount of infection was observed in mice received PSCs from the RPMI-1640 medium at 4°C. The present study is the first attempt to compare the ability of PSCs to generate hydatid cysts in mice after being cultured in different media and at various temperatures.

**Keywords**: hydatidosis, protoscolices, *Echinococcus granulosus*, culture medium, temperature

## Introduction

Human echinococcosis (HE) is an important parasitic disease and one of 17 tropical diseases that have been prioritized by the World Health Organization. It is caused by the larval stage of the genus *Echinococcus* found in the small intestine of canines and infects a wide range of domestic and wild mammals as well as humans, as intermediate hosts [1,2]. The disease is endemic to Australia and has a high prevalence in the Middle East, India, and South America [3].

Human echinococcosis is a significant health problem that imposes a heavy burden to the health systems in different regions of the world. Planning for permanent control of the disease in humans and livestock is one of the WHO active programs [4]. Fertile cysts with live PSCs in the intermediate host are an essential indicator of disease transmission [5]. Researchers need to find a suitable and cost-effective medium to keep PSCs alive for longer times. The present study was designed to determine *E. granulosus* PSCs' survival duration in different culture media such as DMEM (H & L), RPMI-1640, PBS, and cyst fluid at different temperatures and the ability to generate cysts in mice after being cultured in the mentioned media. In this study, we compared different culture media and temperatures and showed their effects on the pathogenesis and strength of live PSCs. The results of this research

can help gain a better understanding of culture media and select the most suitable medium and temperature to keep live PSCs. Finally, finding a suitable medium can help to save cost and time.

## Materials and Methods

*Preparation of PSCs and culture media*

The organs infected with hydatid cyst such as liver and lungs were collected from a municipal abattoir in Ahvaz, southwest of Iran, and rapidly transferred to the parasitology laboratory of Ahvaz Jundishapur University of Medical Sciences. After washing the outer surface of the cysts with sodium chloride 0.9%, cyst fluid was extracted by a 19 gauge needle. PSCs were washed three times in sodium chloride 0.9% injectable solution. Then, they were evaluated to determine the PSCs' survival rate by using eosin 0.1% and methylene blue.

Besides using a microscope, the presence of evaginated shapes, motility characteristics, and flame cells activity were investigated. The condition of entering PSCs to this study was determined alive by more than 95% of them.

Initially, 30 ml of DMEM (H & L) (Sigma-Aldrich, USA), RPMI-1640 (Sigma-Aldrich, USA), phosphate-buffered saline (PBS, Sigma-Aldrich, USA), and cyst fluid was moved into a culture flask. To prevent the growth of bacterial and fungal agents, penicillin 100 i.u./ml (Gibco™) and streptomycin 100 µg/ml (Gibco™) were added. Afterwards, PSCs were counted by neobar lam, and eventually, 30,000 live PSCs were added to each medium [6–8].

*Determining the survival duration of PSCs in different media and temperatures*

The culture media were incubated at 4°C and 37°C, and PSCs were counted on the appointed days. To determine the survival of the PSCs, 0.5 ml of the culture medium containing PSCs was transferred into a 2-ml microtube and 0.5 ml of eosin 0.1% was added. Also, for more accurate results, we used methylene blue staining. After 15 min under the microscope, the numbers of alive and dead PSCs were counted and tabulated.

*Mice infection*

Fifty female BALB/c mice within the age range of 6–8 weeks with a mean weight of 25±5 g were prepared. The mice were divided into ten groups of five, and five mice were assigned to each culture medium. Then, 0.5 ml of each culture medium that contained 2000 viable PSCs was injected into the peritoneal cavity of each mouse [9] as follows: group 1: received PSCs from RPMI-1640 at 37°C, group 2: received PSCs from DMEM (L) at 37°C, group 3: received PSCs from DMEM (H) at 37°C, group 4: received PSCs from PBS at 37°C, group 5: received PSCs from hydatid cyst at 37°C, group 6: received PSCs from RPMI-1640 at 4°C, group 7: received PSCs from DMEM (L) at 4°C, group 8: received PSCs from DMEM (H) at 4°C, group 9: received PSCs from PBS at 4°C, and group 10: received PSCs from hydatid cyst at 4°C.

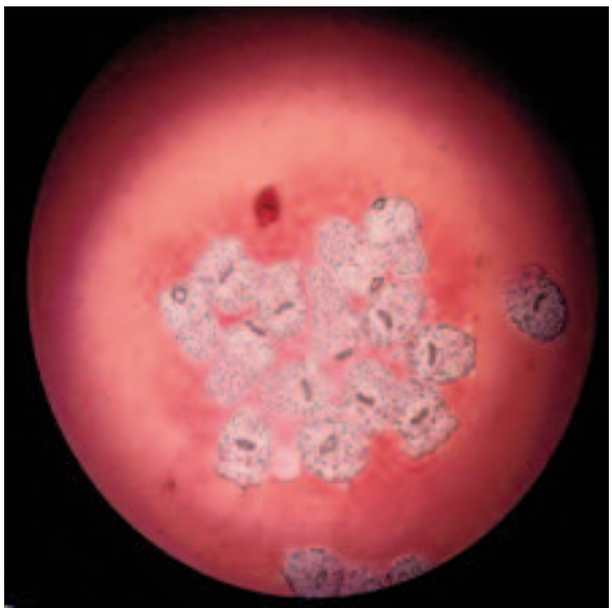

Figure 1. Colored PSCs with eosin 1%. Live PSCs remained colorless and dead PSCs turned red.

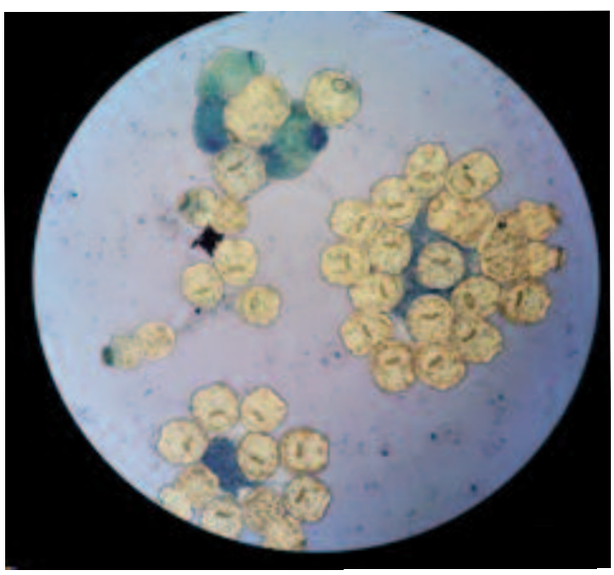

Figure 2. Colored PSCs with methylene blue. Live PSCs remained colorless and dead PSCs turned blue.

Table 1. The percentage of PSCs survival in hydatid cyst in different culture media at 37°C

| Day | Cyst fluid | RPMI 1640 | DMEM(H) | DMEM(L) | PBS |
|---|---|---|---|---|---|
| 1 | 93.1 | 90.5 | 90 | 89.9 | 83 |
| 10 | 77.6 | 68.9 | 58 | 57 | 43.5 |
| 12 | 71.1 | 60 | 55 | 30 | 26.2 |
| 17 | 55.6 | 45 | 24.2 | 10 | 0 |
| 20 | 50 | 35.1 | 10 | 0 | |
| 29 | 22.2 | 5.2 | 0 | | |
| 30 | 18.3 | 0 | | | |
| 32 | 0 | | | | |

For five months, the mice were kept at a controlled temperature (22–28°C), light-cycle (12 h light and 12 h dark), with free access to food and water. Then, they were sacrificed and the abdominal cavity was opened for removing, counting, and measuring cysts. The obtained data was recorded in relevant tables, and finally, Kruskal-Wallis and Mann-Whitney (nonparametric tests) tests were run in SPSS to compare the differences in weight, size, and number of cysts between different groups.

*Ethical standards*

The study protocol No: IR.AJUMS.ABHC.REC.1398.047 was approved by the Ethics Committee on Research in School of Medicine, Ahvaz Jundishapur University of Medical Sciences.

## Results

*The results of PSCs survival in culture media*

In Figures 1 and 2, colored PSCs are shown with the color of eosin 0.1% and methylene blue. Live PSCs retain their natural color. However, dead PSCs were stained by absorbing colors that appeared as red in eosin and blue in methylene blue.

Table 1 shows the results obtained from the PSC cultures at 37°C. As observed in the table, the maximum duration of PSCs survival at 37°C was associated with cyst fluid for 30 days, and the minimum survival duration was associated with PBS for 12 days.

Table 2 shows the results obtained from the PSC cultures at 4°C. As shown in the table, the maximum duration of PSCs survival at 4°C was related to the RPMI-1640 medium for 50 days, and the minimum survival duration was associated with the DMEM (L) for 22 days.

Figure 3 indicates the duration of PSCs survival in different media and temperatures. The maximum PSCs survival was associated with the temperature of 4°C in RPMI-1640 medium followed by cyst fluid for 50 and 45 days, respectively, and the minimum PSCs survival was related to PBS at 37°C for 12 days.

Table 2. The percentage of PSCs survival in different culture media at 4°C

| Day | RPMI 1640 | Cyst fluid | DMEM(H) | PBS | DMEM(L) |
|---|---|---|---|---|---|
| 1 | 93.5 | 94.2 | 91 | 85 | 92.4 |
| 10 | 80 | 80 | 79.2 | 70.5 | 70.1 |
| 20 | 65 | 70 | 76.1 | 42.8 | 32 |
| 22 | 62.1 | 68 | 60 | 40 | 17.2 |
| 30 | 49 | 43.2 | 39.5 | 20.7 | 0 |
| 40 | 28.2 | 20 | 7.8 | 0 | |
| 45 | 18.5 | 5.5 | 0 | | |
| 50 | 14.2 | 0 | | | |
| 52 | 0 | | | | |

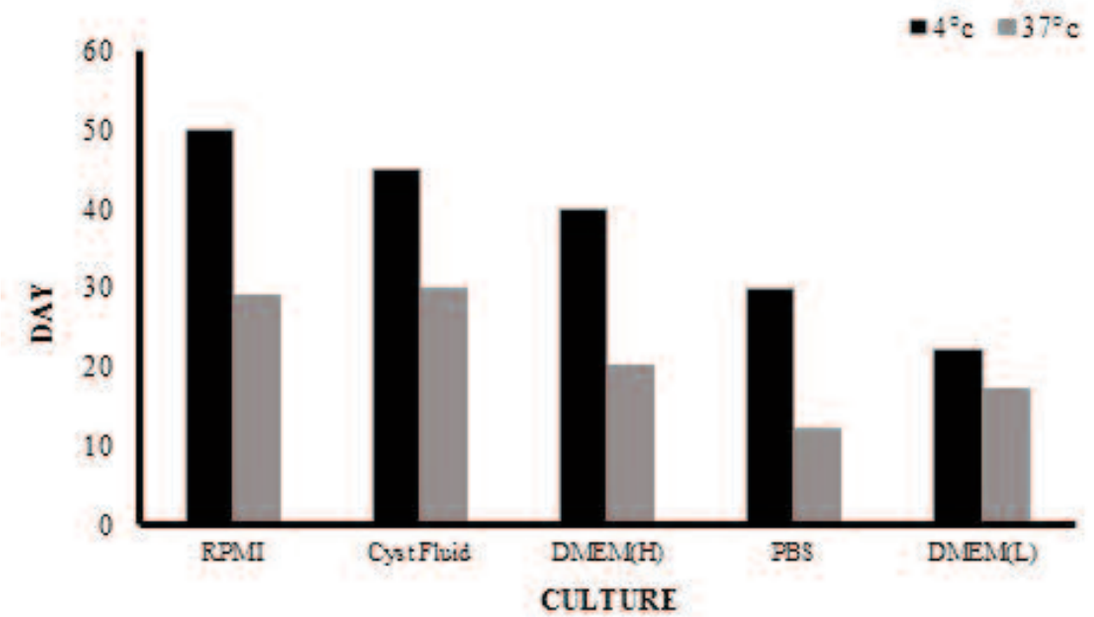


Figure 3. The duration of PSCs survival in various media and temperatures

*The results of mice infection with cultured PSCs in different culture media*

In the course of this study, three mice from the RPMI-1640, PBS, and cyst fluid groups were lost at 37°C. Also, cysts were not generated in one case of PSCs injection from DMEM (L) at 37°C.

Figure 4 exhibits that the highest median number of cysts was related to the RPMI-1640 group at 4°C (median 4), and the lowest median number of cysts at this temperature was in the DMEM (H) and cyst fluid (median 1) groups. The highest median number of cysts was in the DMEM (L) mice group at 37°C (median 2), and the lowest median number of cysts at this temperature was in RPMI-1640, DMEM (H) PBS, and cyst fluid (median 1).

Figures 5 and 6 indicate that the highest median cyst size was in the PBS group at 37°C with 5.3 mm, and the lowest median cyst size was in the DMEM(L) group at 4°C with 2.3 mm.

Figures 7 and 8 show that the highest median cyst weight was in the RPMI-1640 group at 4°C with 0.35 mg, and the lowest median cyst weight belonged to the DMEM (L) group at 4°C with 0.09 mg.

According to table 3, the highest mean rank number of the cyst was related to the RPMI-1640 culture medium with 30.9, and the lowest mean rank number belonged to the DMEM(H) culture medium with 17.5 (P=0.14), which indicates no significant correlation between them. Also, the highest mean rank rating of size was related to cyst fluid with 27.7 mm, while the mean rank grade was in the RPMI-1640 medium with 19.4 mm (P=068), which indicates the lack of a significant correlation between them. The highest mean rank rating for weight was associated with PBS with 27.6 mg, and the mean weighted mean rank grade of who was the lowest in the DMEM(L) medium with 18.4 mg (P=0.5), indicating no significant correlation between them.

Table 3. Comparison of cyst number, size, and weight between different groups of mice

| Group | Number of mice | Mean rank number | P-value | Mean rank size (mm) | P-value | Mean rank weight (mg) | P-value |
|---|---|---|---|---|---|---|---|
| RPMI | 9 | 30.9 | | 19.4 | | 27.4 | |
| DMEM(L) | 9 | 26.2 | | 20.7 | | 18.4 | |
| DMEM(H) | 10 | 17.5 | 0.14 | 25.3 | 0.68 | 20.6 | 0.5 |
| PBS | 9 | 23.7 | | 24.1 | | 27.6 | |
| Cyst fluid | 9 | 19.9 | | 27.7 | | 23.7 | |

Table 4. Comparison of cyst number, size, and weight at different temperatures

| Temperature | Number of mice | Mean rank number | P-value | Mean rank size (mm) | P-value | Mean rank weight (mg) | P-value |
|---|---|---|---|---|---|---|---|
| 37°C | 21 | 19.17 | 0.028 | 29.71 | 0.004 | 25.6 | 0.33 |
| 4°C | 25 | 27.14 | | 18.28 | | 21.74 | |

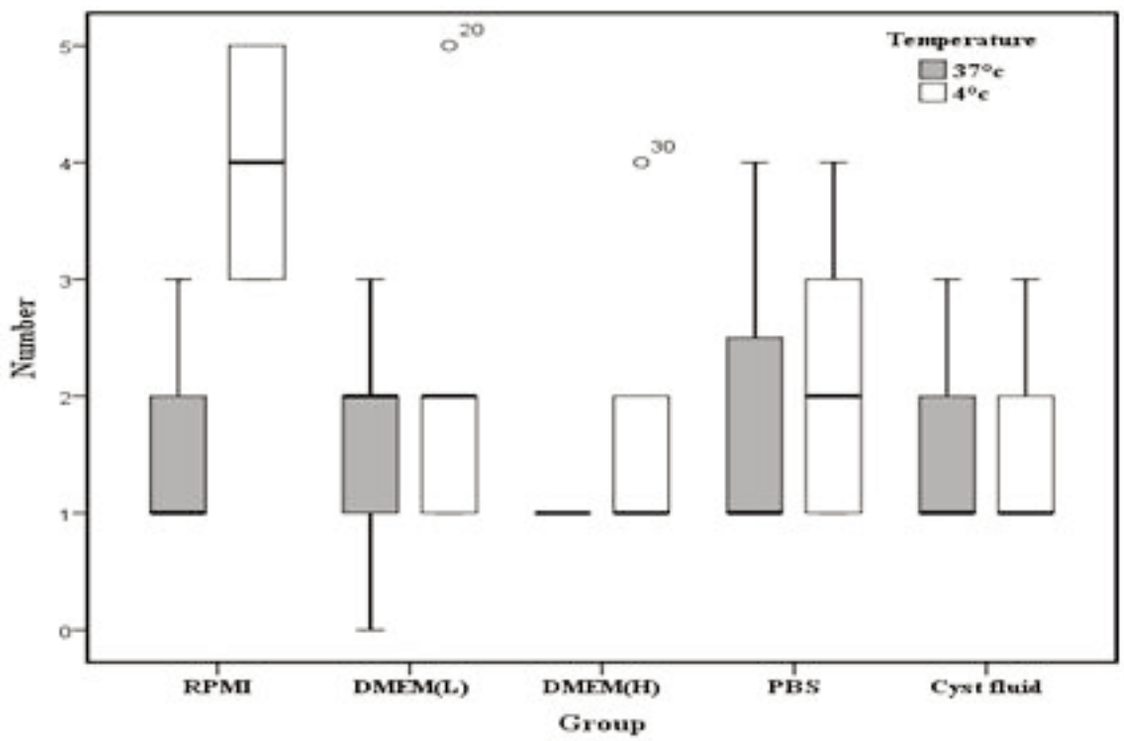


Figure 4. The box plot diagram of hydatid cyst numbers in various groups of mice at different temperatures

Table 4 shows the mean rank number of cysts in the 37°C groups was 19.17, and in the 4°C groups it was 27.14. In other words, the number of cysts generated in mice that had received PSCs from 4°C temperature culture media was higher (P=0.028), indicating a significant relationship between temperature and cyst number. Also, the mean size rate in the 37°C groups was 29.71 mm, and in 4°C groups it was 18.28 mm, which indicates the larger size of cysts at 37°C (P=0.004) and shows the existence of a significant correlation between temperature and cyst size. In terms of cyst weight, the mean weight rate at 37°C was 25.6 mg, and at 4°C it was 21.74 mg (p=0.33), showing that there is no significant correlation between temperature and weight.

## Discussion

So far, several studies have been conducted on PSCs culture media. But this study describes for the first time the best medium to maintain PSCs' pathogenicity and cyst production potency in mice. The study of Moazeni et al. [10] showed that the survival rate of PSCs in cyst fluid at 37°C was 33 days. In the present study, the maximum duration of PSCs' survival was recorded in cyst fluid at 37°C for 30 days, which is compatible with the above research finding and shows that cyst fluid at 37°C is an appropriate option for PSCs maintenance.

Loveless and Andersen [11] indicated that PSCs survived in cyst fluid at the temperatures of 10, 20, 30, 40, and 50°C for 16, 8, 4, 2 days and 2 hours, respectively. Our study showed that PSCs in cyst fluid at the temperatures of 37 and 4°C survived for 30 and 45 days, respectively. Both studies show the direct effect of temperature on the survival of PSCs. However, in our research, the results from the storage of PSCs at 4°C were better than 37°C.

In Casado et al. [6] study, the survival rate of separated pulmonary hydatid cyst PSCs at 37°C was about 15% on the 30th day in cyst fluid. In our study, the survival rate of PSCs in cyst fluid at 37°C on the 30th day was 18.3%, which is almost

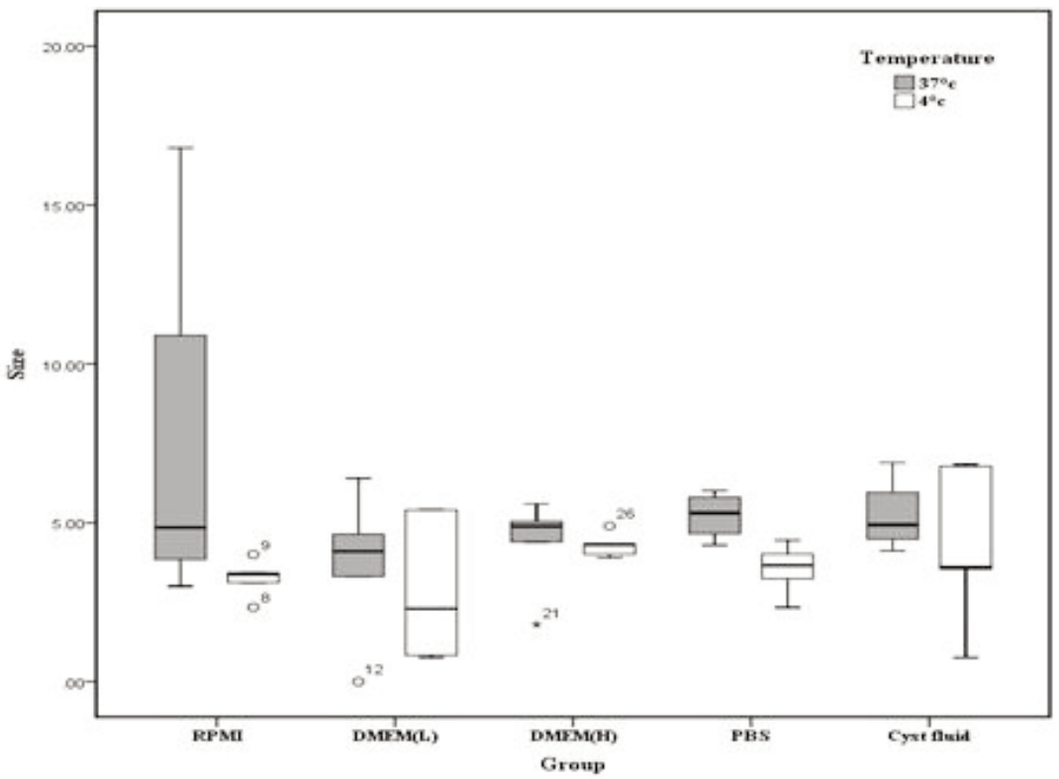


Figure 5. The box plot diagram of hydatid cyst size in different groups of mice at different temperatures

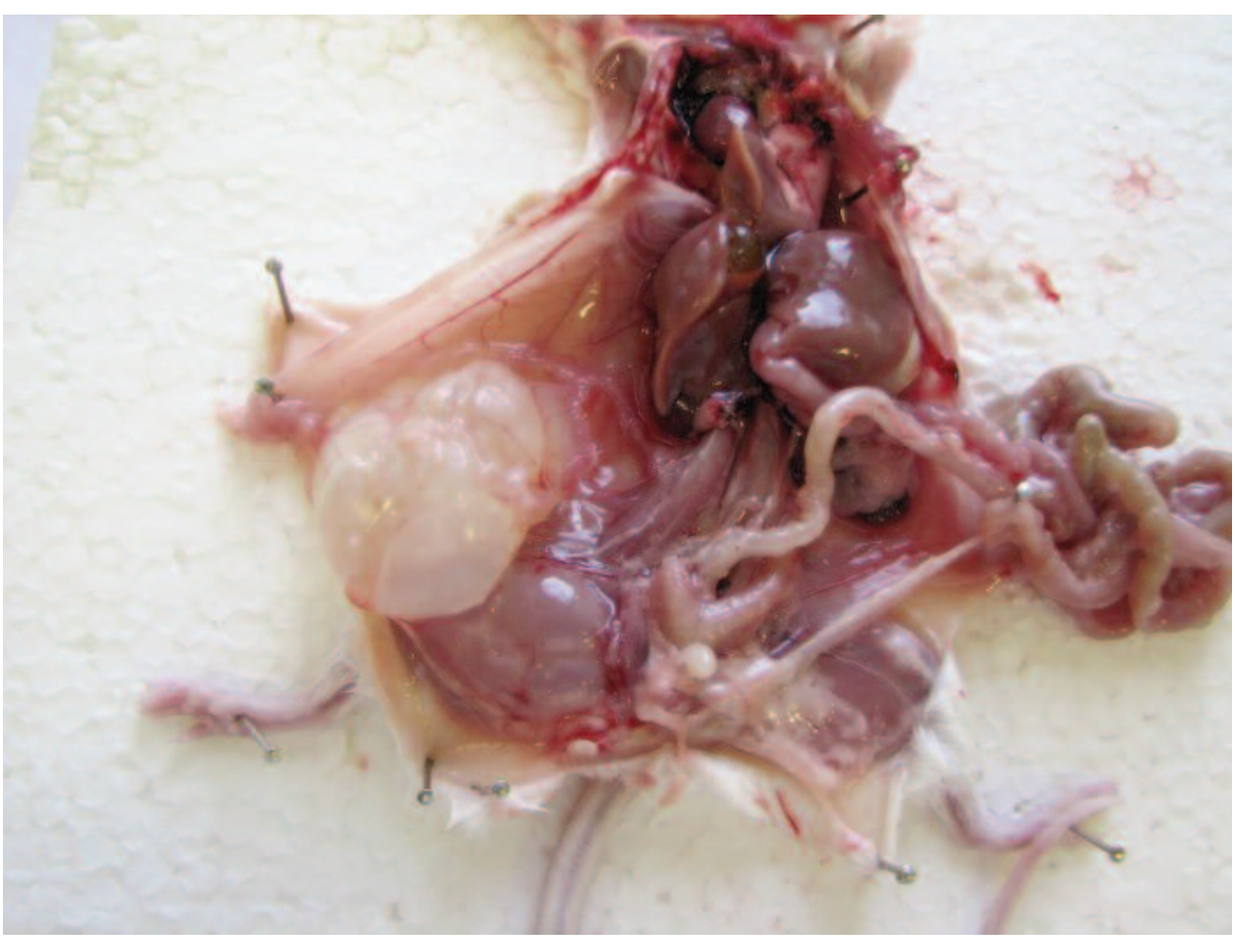

Figure 6. Image from the mouse hydatid cyst produced 5 months after inoculation of PSCs from PBS at 37°C

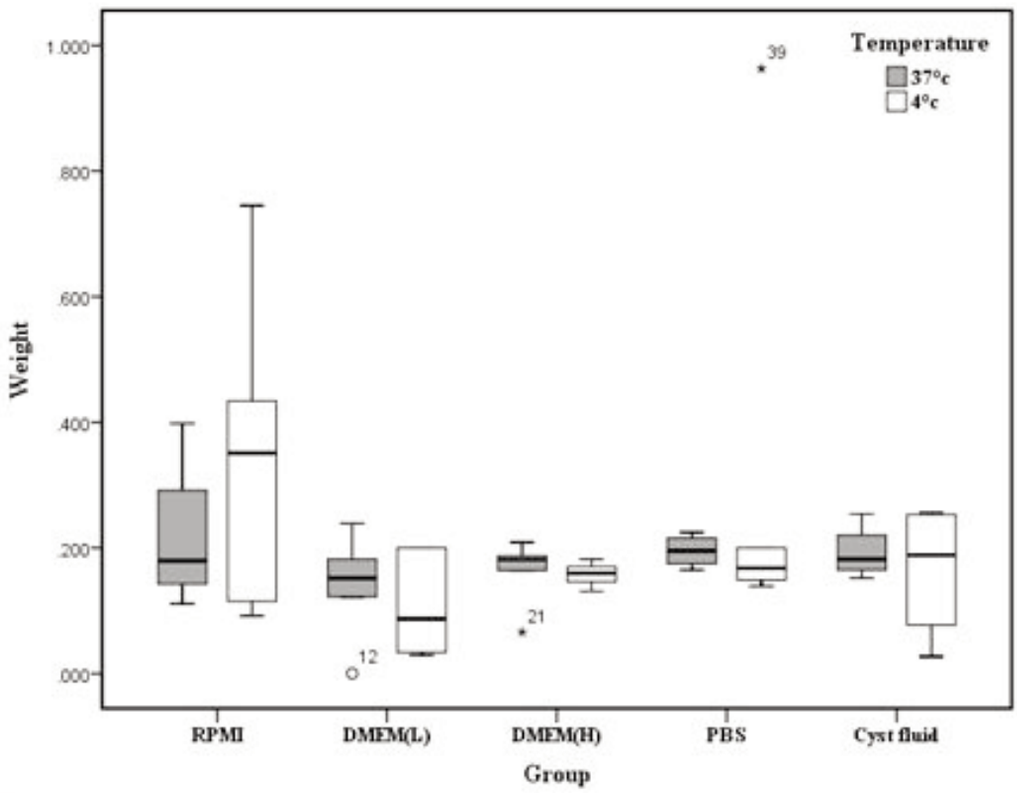


Figure 7. The box plot diagram of hydatid cyst weight in different groups of mice at various temperatures

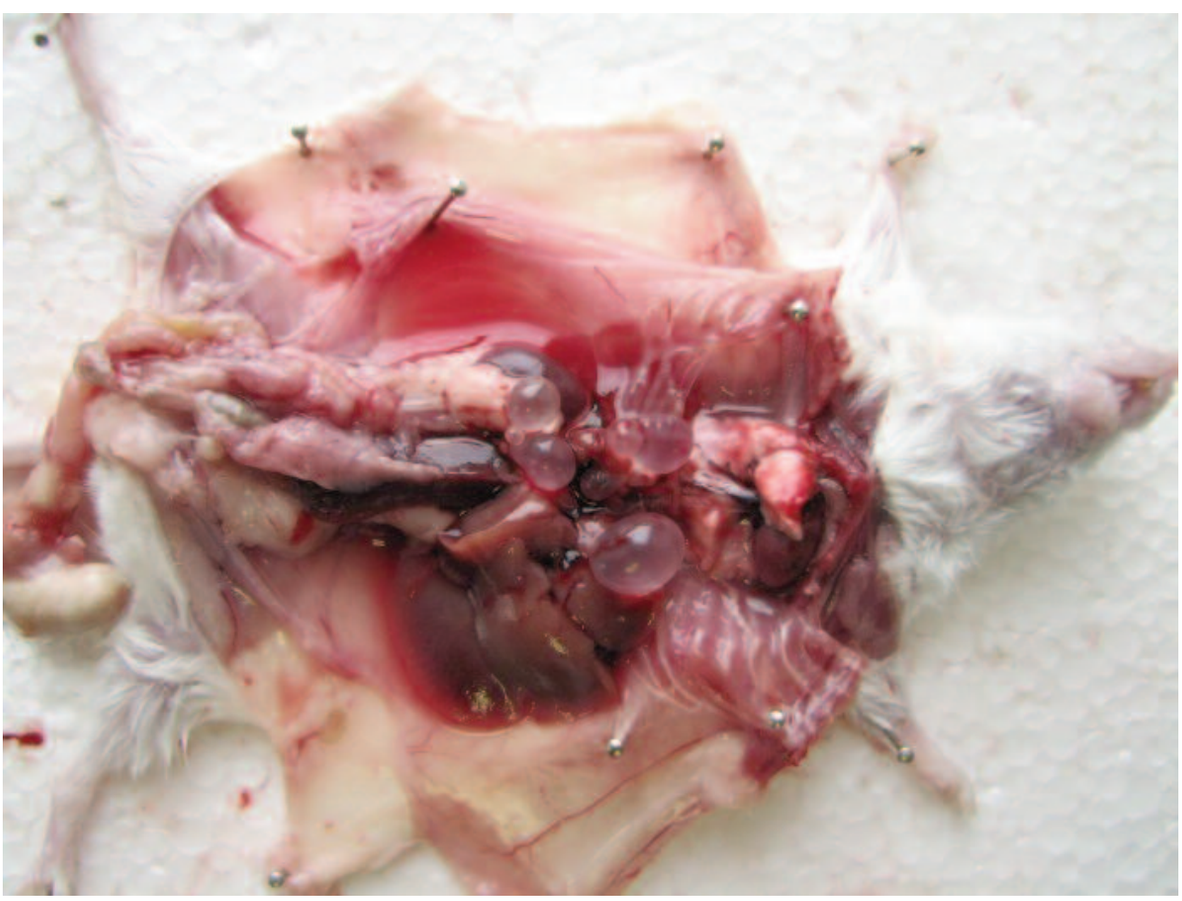

Figure 8. Image from the mouse hydatid cyst produced 5 months after inoculation of PSCs from RPMI-1640 at 4°C

identical to the findings of Casado et al. Our study showed that the results obtained from the survival of PSCs in RPMI-1640 at 37°C were very close to those of cyst fluid. In other words, we can use cyst fluid instead of RPMI-1640 to maintain PSCs.

Diker et al. [12] assessed the survival of hydatid cyst PSCs in different conditions and temperatures. The results showed that PSCs at -10, 0, 10, 20, and 30°C respectively survived for 3, 36, 28, 12, and 4 days. In this study was observed the effect of temperature on PSCs survival. Of course, in our study, the temperature of 4°C showed better results than 37°C, which confirms the positive effect of lower temperature on the survival of PSCs. However, it is necessary to provide more decisive results from the 37°C temperature in terms of size and weight of cysts produced in mice at 4°C.

Mansourian et al. [13] kept live hepatic hydatid cyst PSCs in RPMI-1640 medium and 10% fetal calf serum (FCS) for 18 days. In the present study, hydatid cyst PSCs survived in the RPMI-1640 medium without adding FCS for 30 days. Adding FCS does not seem to have a significant impact on the survival of PSCs in RPMI-1640.

Zhang et al. [14] showed that cultivation in PBS and RPMI-1640 before injection to mice can cause an increase in cysts by injecting fewer amounts of PSCs. The direct injection of PSCs to mice without cultivation requires injecting a larger number of PSCs with the chance of creating fewer cysts. The results of the present study also indicated that PSCs in the culture medium have successfully established a more significant number of cysts in mice, which matched well with the investigation. The highest mean rank value in the RPMI-1640 group was 30.9 and the lowest mean number in DMEM(H) group was 17.5 (P=0.14), which indicates the lack of a significant relationship between them. The result of the injection of cultured PSCs in the RPMI 1640 medium, generates more amounts of cysts in mice. It seems, therefore, clinically relevant to the difference.

In terms of cyst number, the results showed that the mean rank rating in the 37°C group was 19.17, and in the 4°C group, it was 27.14. In other words, the number of cysts generated in mice that received PSCs from the 4°C media was higher (P=0.028), suggesting the existence of a significant relationship between them. The number of cysts in 4°C media was higher than in 37°C; thus, culture medium temperature has a significant impact on cyst number.

The highest mean size in the cyst fluid medium was 27.7 mm, and the lowest mean size in the RPMI-1640 medium was 19.4 mm (P=0.68), showing no significant relationship between them. Although the results revealed that cyst size between different groups of mice was not significantly different, in clinical terms, this amount of difference in the two groups of cyst fluid and RPMI-1640 can be of significance.

The bigger size of cysts at the temperature of 37°C shows a significant relationship between temperature and cyst size (P=0.004). Thus, cyst size in the groups that received PSCs from 37°C media was greater than in 4°C media, and in fact, the temperature has a significant association with cyst size. The highest weight mean rank was in the PBS group with 27.6 and RPMI-1640 with 27.4, and the lowest weight mean rank belonged to the DMEM (L) group with 18.4 (P=0.5), which indicates the lack of a significant statistically relationship between them. But this amount of difference between different groups of mice can be of paramount importance.

In terms of cyst weight, the cyst weight mean rank at 37°C was 25.6, and at 4°C it was 21.74 (P=0.33), implying no significant relationship between temperature and cyst weight. It means that the weight means rank was not different at these temperatures. Finally, this study showed that generated cyst number at 4°C was greater than at 37°C. In addition, cyst size at 37°C was larger than at 4°C, but temperature and weight were not correlated, that is, the temperature did not affect cyst weight.

The best medium to keep PSCs is RPMI-1640 followed by cyst fluid. This study showed that the most appropriate temperature for the maintenance of PSCs is 4°C, and PSCs kept in RPMI-1640 at 4°C caused the most infection in mice. It is also because of the highest duration of survival as the most appropriate setting for storing PSCs at 4°C, it is also known for the highest median of the cyst to inject PSCs. Further, the DMEM (L) medium at 37°C with the highest median number of cysts in mice was found the most suitable medium at 37°C for PSCs injection. Considering that the survival rate of PSCs in cyst fluid is very close to the value for the RPMI-1640 medium, cyst fluid can also be used as a suitable medium for preserving PSCs. However, it should be noted that if used PSCs stored in cyst fluid, the PSCs must be several times washed before injecting.

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