## Letter to the Editor

# *Echinococcus Granulosus:* Suitable *in vitro* Protoscolices Culture Density*

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The present study is to determine the suitable protoscolices (PSCs) density for long-time culturing *in vitro*. The PSCs were divided into eight groups with different densities and the viability tests were carried out with 0.1% methylene blue staining. Then the infection ability of cultured PSCs was assessed by the mean cyst weight of mice inoculated intraperitoneally with PSCs after 8 months post-infection. During the 22 days of culture, the numbers of live PSCs in each experimental group reduced as extension of the culturing time. It was found that the PSCs sustained best at the density of 1 800 PSCs/mL with slight decline in percentage survival. At the end of this experimentation, no live PSCs had been seen in all groups apart from 1 600, 1 800, and 2 000 PSCs/mL groups. Moreover, the infection ability of PSCs at 1 800 PSCs/mL was later assessed and proved to persist until culturing for 3 weeks. The results of this study suggested that the density of PSCs *in vitro* is an important factor for successfully sustaining PSCs with extended culture time *in vitro* and the density about 1 800 PSCs/mL was favorable in the culture system used in this experiment.

Hydatidosis, also called cystic echinococcosis (CE), is a severe zoonotic disease caused by tapeworm *Echinococcus granulosus* at larval stage and is world-widely distributed. Occurring in all continents, it affects humans and domestic livestock including sheep, cattle, camels, pigs, horses, and others[1]. In China, *E. granulosus* is endemic in at least 23 provinces, autonomous regions and municipalities. It is estimated that at least 50 million individuals are threatened by this disease, with approximately 380 000 cases[2]. *E. granulosus* metacestodes proliferate mainly in liver and lungs, leading to space-occupying lesions in humans. However, their initial proliferation phase is always asymptomatic for many years or permanently if cysts exert pressure on adjacent tissues and induce other pathological events. The development of cysts will eventually result in organ malfunction and death if not effectively treated.

Surgical resection and chemotherapy are two therapies for CE. Chemotherapy is the only option for inoperable and recurrence cases. Until now, mebendazole (MBZ) and albendazole (ABZ) are the only chemotherapy agents for hydatid diseases as WHO recommended. However, their effect is lower than 30% due to their poor absorption[3]. Novel chemotherapeutical agents are thus needed. Previously, rodent animals infected with protoscolices (PSCs) were used as an *in vivo*-model for the study on treatment, especially for evaluating the efficacy of drugs. However, this *in vivo*-model does not work well for drug screening because it is time consuming and expensive, and the culture of PSCs and metacestodes *in vitro* is therefore an alternative[4]. It is known that the culture condition is important for the survival of PSCs *in vitro* and the effect of culture medium and supplements has been well studied. Given the few available data about the effect of PSCs density on their *in vitro*, the present study is focused on the suitable PSCs culture density in order to improve the culture condition and increase the survival time of those infected with PSCs.

Sheep liver hydatid cysts were obtained from abattoirs in Qinghai, China. PSCs were removed from liver cysts and immersed in cyst fluid at 4 °C for not more than 72 h. The collected PSCs were rinsed 5-8 times with physiological saline solution containing penicillin (500 U/mL) and streptomycin (500 U/mL) before use. Viability of PSCs was assayed by inverted microscopy with 0.1% methylene blue staining. The PSCs were counted and placed into a 25 mL culture flask with RPMI 1 640 culture medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10% calf serum at 37 °C in an atmosphere containing 5% $CO_2$, and then divided into 400 (385±9) mL group, 600 (611±15) mL group, 800 (802±25) mL group, 1 200 (1 240±43) mL group, 1 600 (1 588±54) mL group, 1 800 (1 819±61) mL group, 2 000 (2 031±52) mL group, and 2 200 (2 220±31) mL group.

In order to assay the viability of PSCs, 100 μL pooled PSCs was transferred over a slide and mixed with 100 μL 0.1% methylene blue. After 30 secs, the dead PSCs were stained blue and the surviving PSCs remained colorless under inverted microscope. The viability test was carried out on days 3, 4, 5, 6, 7, 8, 9, 14, and 22, respectively. The medium was changed every 3-4 days.

For fully understanding the infection ability of PSCs *in vitro*, every 2 000 PSCs cultured at the

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density about 1 800 PSCs/mL were inoculated intraperitoneally in female mice weighing 18-22 g at weeks 0, 1, 2, 3, respectively. The animals were housed in animal facility of our institute with free access to rodent food and tap water. The mice were sacrificed 10 months post-infection with their cysts isolated and removed out from peritoneal cavity for weighing. The infection ability of PSCs was assessed by the mean cyst weight.

Statistical analysis was performed by Microsoft excel software version 2007 and SPSS17.0 software. $P<0.05$ was considered statistically significant.

The PSCs in liver were even, smooth, and thin, with distinct internal structures, including rostellum hook, sucker, calcareous corpuscles *etc*. (Figure 1). Once the PSCs were placed onto the culture medium, their mobility increased and evagination appeared in some of them with protrudenting suckers. However, the dead PSCs were shrunken or turgescent in shape with a vague internal structure, the hooks and calcareous corpuscles came off from PSCs. The staining and count methods for assessing the activity and viability are most widely used[5], the activity of PCSs can also be roughly observed from their morphological changes (Figure 1). Although the methods work well, they are still considered inaccurate when the morphology of PSCs became so irregular that their viability is hard to describe. In short, though the changes in morphology of PSCs may give some useful information, the staining and count are still the standard methods.

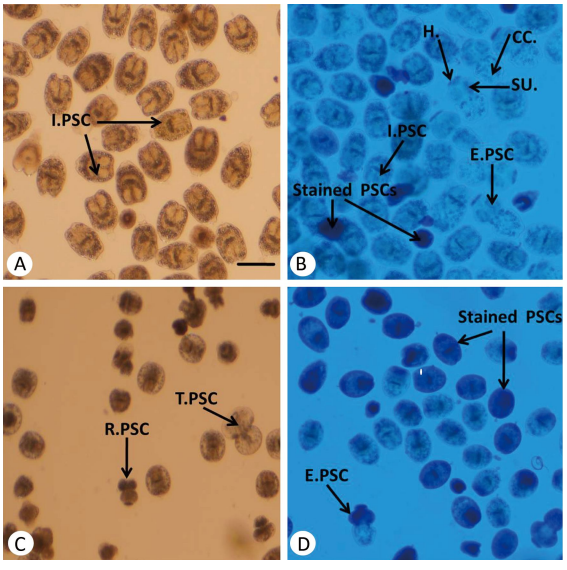


**Figure 1.** Inverted microscopy showing morphology of cultured *Echinococcus granulosus* PSCs. (A) PSCs (viability>95%) without staining; (B) 0.1% methylene blue-stained PSCs (viability>95%); (C) PSCs (viability<50%) without staining; (D) 0.1% methylene blue-stained PSCs (viability<50%). Bar=100 μm; H: hooks; SU: suckers; CC: calcareous corpuscles; IPSC: invaginated PSCs; EPSC: evaginated PSCs; TPSC: turgescent PSCs; RPSC: retracted PSCs.

In the present study, the number of PSCs in live reduced with the culture time, but the viability of PSCs was different (Figure 2). The PSCs sustained best at 1 800 mL with a slight decline in survival rate which was still higher than 80% on day 22. The survival rate of PSCs was still over 80% after cultured for another 2 months. The PSCs died in other groups except in 1 600 (1 588±54) mL group, 1 800 (1 819±61) mL group, 2 000 (2 031±52) mL group. The survival rate of PSCs in 1 600 mL group and 2 000 mL group was higher than 70% during the first 2 weeks of culture and then decreased to 32.8%±7.4% and 41.5%±12.0% after another one week of culture (Figure 2 and Figure 3). The survival rate of PSCs seemed to be recovered in every group when the culture medium was changed due to the effect of discarded dead PSCs. The viability of PSCs decreased with the culture time, and was lower than 50% in 2 000 mL group and 2 200 group. Once the PSCs with different densities were put onto the culture medium, the influence is irreversible. In a word, the density of PSCs is important in the culture of PSCs and 1 800 mL is most suitable for assessing the viability of PSCs.

The infection ability of PSCs still persisted after 3 weeks of culture *in vitro* and decreased with the culture time (Table 1). The weight of cysts from non-cultured PSCs-infected mice was 5.77±3.98 mg ($F=2.821$, $P=0.078$), confirming that the infection ability of PSCs still persists after culture.

The density of PSCs mainly influences their proliferation, which is crucial for cell culture *in vitro*. It was reported that the effect of PSCs density on their viability is influenced by various factors, such as temperature, voltage, and medicine[5]. It has been shown that the density of PSCs is different and incomparable because of their different culture conditions[6-10]. Since the information from these studies

**Table 1.** Weight of Cysts from PSCs-infected Mice after Culture *in vitro*

| Culture Period | No. of Mice | Mean Cyst Weight mg (SD) |
|---|---|---|
| 0 w | 5 | 5.77 (3.98) |
| 1 w | 5 | 7.83 (6.99) |
| 2 w | 5 | 0.65 (0.91) |
| 3 w | 4 | 1.75 (3.50) |

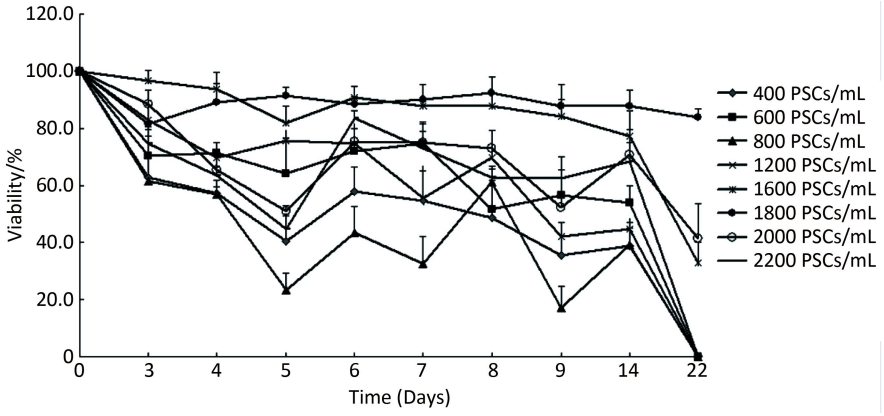


**Figure 2.** Viability of *E. granulosus* PSCs at different densities *in vitro*.

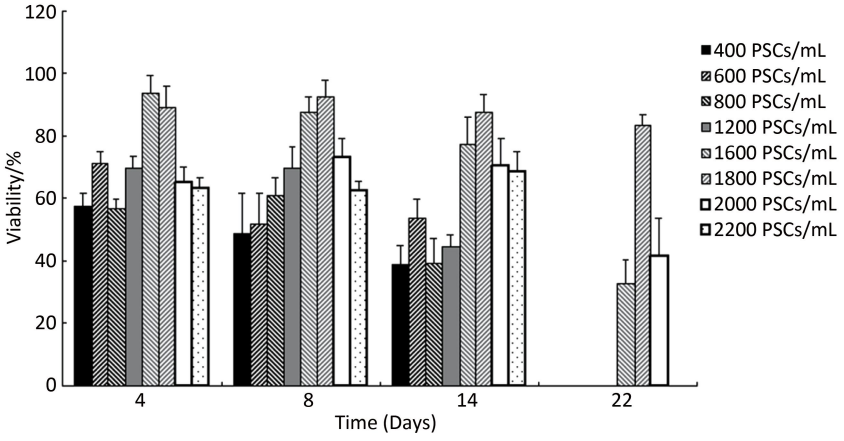


**Figure 3.** Viability of *E. granulosus* PSCs at different densities on days 4, 8, 14, and 22.

is quite enough to determine the optimal density of PSCs cultured *in vitro,* the effect of different densities of PSCs on their viability was observed in this study, showing that 1 800 mL is the optimal density. Furthermore, large-scale cultivation of PSCs *in vitro* is possible, which can last more than 3 months. Continuous culture of PSCs *in vitro* can overcome the difficulties in collecting PSCs from endemic areas and promote further studies on hydatid diseases.

In conclusion, the viability of PSCs is affected by their density, and the best survival rate of PSCs can be harvested at 1 800 PSCs/mL in a 25 mL culture flask with RPMI 1 640 culture medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 10% calf serum in an atmosphere containing 5% $CO_2$ at 37 °C. The optimal density of PSCs can be used in drug screening, studies on immunology and molecular biology or in other relevant fields.

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