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·论著·

体外不同培养条件对多房棘球蚴活力的影响研究*

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【摘要】 **目的** 为了使多房棘球蚴在非寄生离体的情况下保持活力,利于移植,需要多房棘球蚴(*Echinococcus multilocularis*,Em)在体外不同影响因素下的最佳培养条件。 **方法** 选择不同的培养条件:生理盐水培养组、无钙镁PBS培养组、血清培养组、无血清培养组,在两种温度(37 ℃,4 ℃)下培养多房棘球蚴,每组 1×10^4 个虫体,连续培养5 d,以经典的染色方法:台盼蓝染色法、亚甲基蓝染色法、伊红染色法、Dil染色法对虫株进行活力染色并计数。第一组小鼠注射新鲜离体虫株、第二组小鼠注射4 ℃生理盐水处理24 h的虫株,一月后观察两处理组小鼠肝脏变化。 **结果** 随培养时间延长,台盼蓝染色结果显示,第1~5 d离体虫株生存率平均值分别为86.575%、94.600%、63.387%、51.225%、32.775%。差异有统计学意义($P<0.05$)。生理盐水组、无钙镁PBS组、血清组、无血清组5 d体外培养平均生存率为50.95%、64.78%、72.50%、74.62%。台盼蓝染色结果显示,无血清组在4 ℃、37 ℃时平均生存率分别为56.475%、87.995%,两组间有统计学差异($P<0.05$)。无血清组四种染色方法间无统计学差异($P>0.05$)。离体虫株4 ℃静置24 h,生理盐水混悬注射组较新鲜离体虫株生理盐水混悬注射组小鼠动物造模感染程度低。 **结论** 37 ℃无血清培养的多房棘球蚴绦虫,较其余三组培养条件的体外生存率更高。进行多房棘球蚴体外移植时效果更佳,为实验室提高泡球蚴动物造模成功率,延长虫体体外生存周期奠定了基础。

【关键词】 多房棘球蚴;体外培养;培养条件;染色方法
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Study on the effect of different culture conditions *in vitro* on the activity of *Echinococcusmultilocularis*

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【Abstract】 **Objective** In order to maintain the vitality of *Echinococcus multilocularis* (Em) in non parasitic ex vivo conditions and facilitate transplantation, the optimal culture conditions for Em under different influencing factors *in vitro* are required. **Methods** Select different culture conditions: physiological saline culture group, calcium magnesium free PBS culture group, serum culture group, and serum free culture group. Cultivate multiple hydatid cells at two different temperatures (37 ℃, 4 ℃), with 1×10^4 cells in each group for 5 consecutive days. Use classic staining **Methods** such as trypan blue staining, methylene blue staining, eosin staining, and Dil staining to stain and count the viability of the cells. The first group of mice were injected with fresh isolated worm strains, while the second group of mice were injected with worm strains treated with 4 ℃ physiological saline for 24 hours. One month later, the liver changes of the two treatment groups of mice were observed. **Results** With the extension of cultivation time, the results of trypan blue staining showed that the average survival rates of isolated insect strains from the first day to the fifth day were 86.575%, 94.600%, 63.387%, 51.225%, and 32.775%, respectively. The difference was statistically significant ($P<0.05$). The average 5-day in vitro survival rates of the physiological saline group, calcium magnesium free PBS group, serum group, and serum free group were 50.95%, 64.78%, 72.50%, and 74.62%, respectively. The results of trypan blue staining showed that the average survival rates of the serum-free group at 4 ℃ and 37 ℃ were 56.475% and 87.995%,

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respectively, with statistical differences between the two groups ($P<0.05$). There was no statistically significant difference ($P>0.05$) among the four staining methods in the serum-free group. The isolated strain was left to stand at 4 ℃ for 24 hours, and the infection level in the physiological saline suspension injection group was lower than that in the fresh isolated strain physiological saline suspension injection group. **Conclusion** The in vitro survival rate of Em cultured at 37 ℃ without serum was higher than that of the other three groups of culture conditions. The effect of *in vitro* transplantation of Em is better, which lays the foundation for improving the success rate of animal modeling of AE in the laboratory and prolonging the in vitro survival cycle of the parasite.

【Keywords】 *Echinococcus multilocularis*; *in vitro* culture; cultivation conditions; staining method

多房棘球蚴病(Alveolar echinococcoisis, AE)是一种人畜共患性疾病,在我国西北多发[1]。多房棘球蚴病是误食了棘球蚴卵而引发的疾病[2]。因本病病灶类似缓慢生长的"肝癌"而被称为"虫癌"[3]。目前多房棘球蚴的体外培养成为了实验室研究其药理学机制及体外造模的制约因素[4]。本文从多变量讨论虫体体外培养生存率,以得到最佳虫体活力的培养条件,对多房棘球蚴体外实验模型的建立及体外干预虫体药理学周期的延长奠定基础。

材料与方法

1 材料

1.1 *动物* 沙鼠购自新疆医科大学医学动物实验中心。C57BL/6小鼠购自北京维通利华实验动物技术有限公司。

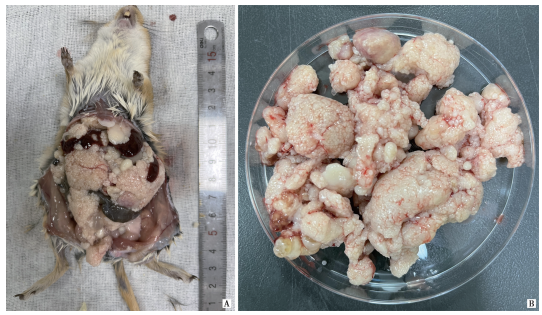
1.2 *主要试剂与仪器* 生理盐水注射液购自国药集团有限公司、无钙镁PBS缓冲液、1640基础培养基、胎牛血清、谷氨酰胺、HEPES、双抗(青霉素和链霉素)购自美国Gibco公司。70目细胞过滤筛、台盼蓝、亚甲基蓝、Dil染色液购自北京索莱宝公司。伊红染色液购自北京中杉金桥公司。培养板购自美国Corning公司。

2 方法

2.1 *多房棘球蚴虫体的剥离* 将感染Em六个月的沙鼠采用脱颈法处死。取出病灶(图1),剪切成约0.3 cm^3的病灶团,置于24目筛网研磨杵研磨至过筛。二次70目过筛,产物置于盛有PBS的皿,利用虫株与钙颗粒流动性不同吸取中层虫体收集。PBS重悬并沉淀,至上清清澈,弃上清。重复3～5次,对虫株计数。

2.2 *虫体的体外培养* 将培养板分37 ℃和4 ℃培养组。设置不同培养条件:生理盐水、无钙镁PBS、血清、及无血清培养组。血清培养组由10%胎牛血清、1% HEPES、1%谷氨酰胺、1%双抗、87%1640基础培养基配置。无血清培养组由1% HEPES、1%谷氨酰胺、1%双抗、97%1640基础培养基配置。每孔约1×10^4只虫体,连续培养5 d。

2.3 *肝门静脉注射离体虫株* 虫体一份用生理盐水混悬,另一份生理盐水混悬后4 ℃静置24 h。经肝门静脉进行动物造模,每鼠3 000个。1月后小鼠脱颈处死取肝脏。



A 沙鼠脱颈处死后腹腔中充斥菜花样病灶 B 病灶及虫株离体后的大体观察

图1 沙鼠体内多房棘球蚴病灶

A Cauliflower like lesions in the abdominal cavity of gerbils after decapitation B Gross observation of lesions and insect strains *in vitro*

Fig. 1 *E. multilocularis* lesions in gerbils

2.4 *体外染色及显微计数* 通过四种染色方法:台盼蓝、亚甲基蓝、伊红、Dil对上述培养组染色;四种培养组进行四种染色,得到37 ℃培养板16组,4 ℃培养板16组。生理盐水终止染色,自然沉降弃上清,留沉淀,重复一次。混悬于载玻片上观察虫体着色情况。确保多房棘球蚴在非寄生离体的情况下保持活力,利于移植。显微镜下观察虫体染色情况,并计算各种培养条件变化下及不同染色条件影响下虫体的生存率。

结 果

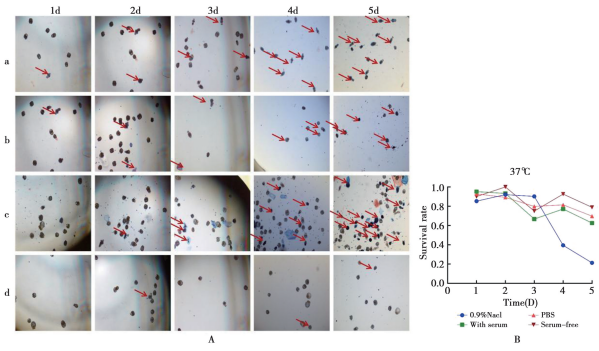
1 培养时间、培养条件对体外虫体生存率的影响

台盼蓝染色结果显示,着色数目与培养天数成正比。第1～5 d离体虫株生存率平均值分别为86.58%、94.60%、63.39%、51.23%、32.78%。随时间增加,虫体生存率呈显著性下降($P<0.05$)(图2)。生理盐水组、无钙镁PBS组、血清组、无血清组5 d体外培养平均生存率为50.95%、64.78%、72.50%、74.62%。不同培养组间虫株生存率差异显著,生理盐水组平均生存率最低,无血清组平均生存率最高(图3)。37 ℃和4 ℃不同条件台盼蓝染色的生存率及显著性差异见表1、表2。

2 培养温度对无血清组虫株生存率的影响

对无血清组不同培养温度分析,其中37 ℃培养中

虫体平均生存率为88.00%，4 ℃培养中虫体平均生存率为56.48%。37 ℃时离体的虫株生存率与4 ℃相比，差异有统计学意义（$P<0.05$）（图4）。



A 37 ℃条件下观察五天四组台盼蓝染色法的镜下生存率（40×） B 37 ℃条件下四组台盼蓝染色生存率折线图。

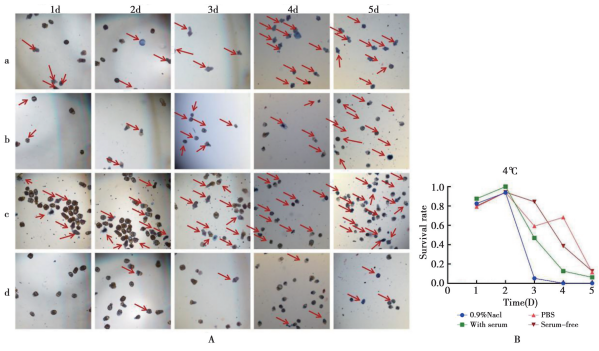
注：a、b、c、d，分别为生理盐水、无钙镁PBS、血清培养组、无血清培养组，红色箭头所指为着色死细胞

图2 37 ℃条件下四组台盼蓝染色后虫体生存率情况

A Microscopic survival rate of four groups of trypan blue staining method observed at 37 ℃ for five days (40×) B Four groups of trypan blue staining survival rate line plots under 37 ℃ conditions.

Note: a, b, c and d are physiological saline, calcium magnesium free PBS, serum medium, and serum free medium groups, respectively. The red arrow indicates stained dead cells

Fig. 2 Survival rate of four groups of trypan blue stained worms at 37 ℃



A 4 ℃条件下观察五天四组台盼蓝染色的镜下生存率（40×） B 4 ℃条件下四组台盼蓝染色生存率折线图。

注：a、b、c、d，分别为生理盐水、无钙镁PBS、血清培养组、无血清培养组，红色箭头所指为着色死细胞

图3 4 ℃条件下四组台盼蓝染色后虫体生存率情况

A Microscopic survival rate of four groups stained with trypan blue for five days at 4 ℃ (40×) B Four groups of trypan blue staining survival rate line plots under 4 ℃ conditions.

Note: a, b, c and d are physiological saline, calcium magnesium free PBS, serum medium, and serum free medium groups, respectively. The red arrow indicates stained dead cells

Fig. 3 Survival rate of four groups of trypan blue stained worms under 4 ℃ conditions

3 四种染色方法对分析虫体生存率的影响

无血清组在台盼蓝、亚甲基蓝、伊红、DiI染色的平均生存率分别为74.62%、64.72%、76.14%、73.46%。四种不同染色方法的总计平均生存率为72.24%，染色方法之间差异无统计学意义（$P>0.05$）（图5），染色方法对无血清组虫株体外生存率无影响。

表1 37 ℃条件下四组虫体观察5 d生存率分析

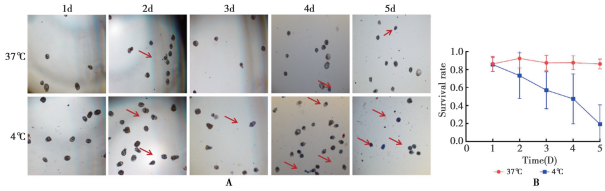
Table 1 Observation of four groups of insects under 37 ℃ and analysis of natural survival rate

条件 Condition	第1 d Day 1	第2 d Day 2	第3 d Day 3	第4 d Day 4	第5 d Day 5	P
生理盐水组	0.778	0.933	0.810	0.563	0.501	0.019
无钙镁PBS组	0.772	0.917	0.783	0.820	0.633	0.011
血清培养组	0.882	0.650	0.823	0.783	0.761	0.157
无血清培养组	0.863	0.924	0.875	0.877	0.862	0.817
P	0.457	0.029	0.418	0.004	0.062	

表2 4 ℃不同条件虫体生存情况

Table 2 Survival of insects under different conditions at 4 ℃

条件 Condition	第1 d Day 1	第2 d Day 2	第3 d Day 3	第4 d Day 4	第5 d Day 5	P
生理盐水组	0.822	0.281	0.124	0.034	0.000	0.000
无钙镁PBS组	0.826	0.733	0.428	0.118	0.180	0.001
血清培养组	0.888	0.684	0.550	0.433	0.164	0.003
无血清培养组	0.855	0.732	0.571	0.474	0.192	0.006
P	0.113	0.260	0.025	0.007	0.301	



A 不同温度五天无血清组台盼蓝染色法的镜下观察（40×） B 不同温度无血清组台盼蓝染色生存率折线图。

注：红色箭头所指为着色死细胞。

图4 不同温度台盼蓝染色检查无血清组虫体生存率情况

A Microscopic observation of trypan blue staining method in serum free groups at different temperatures for five days (40×) B Line chart of trypan blue staining survival rate in serum-free groups at different temperatures.

Note: The red arrow indicates stained dead cells.

Fig. 4 Trypan blue staining at different temperatures to examine the survival rate of serum-free groups of parasites

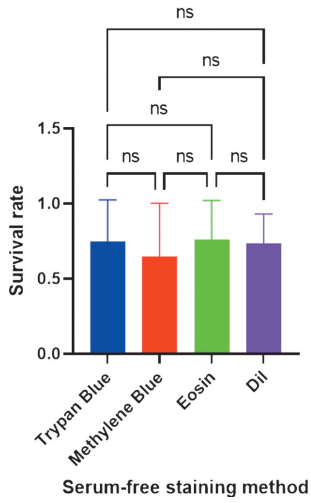
4 虫体活力对动物模型感染严重程度的影响

4 ℃生理盐水24 h处理组，肝脏隔面处无大病灶，呈毛玻璃状。新鲜分离生理盐水重悬的多房棘球蚴组肝脏表面见弥散性白色囊泡，边缘囊泡连接成团，0.3 cm * 0.3 cm病灶5处（图6）。4 ℃处理24 h组一月感染情况明显较无处理组程度轻。

讨 论

泡型棘球蚴病是一类致死性疾病。针对棘球蚴病的小鼠造模方式主要包括腹腔注射虫体、肝脏穿刺法，肝门静脉穿刺注射等[5]。门静脉穿刺效果最佳，但相对其他方式，操作难度大，耗费时间长[6]。且造模通常不能当天完成，新鲜离体虫株在保证相对活力的基础上其存放成为制约因素[7]。虫株离体后在体外受环境因素影响，发生“成泡”现象，不利于肝门静脉回植，一周内维持离体虫株的相对活力以及减少离体虫株的成泡现象，对维持实验室小鼠造模的重复性和均一性上至关重要[8]。

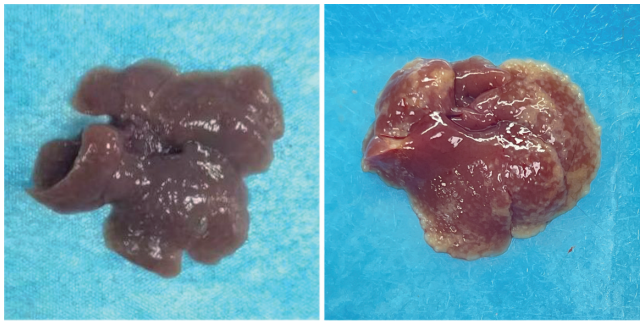
由于其特殊的寄生性质，确定适合其需求的培养基存在困难[9]。多房棘球蚴绦虫对温度和湿度的要求较高[10]。在体外培养中，需为其提供适宜的温度条件，维持活力[11]。37 ℃和4 ℃是细胞培养常用温度，37 ℃模拟体温；4 ℃冷培养抑制虫体酶活性，降低细胞代谢水平，延缓虫体存活率[12-13]，故选择37 ℃和4 ℃进行培养。



注：两组间比较，$P>0.05$。

图5 无血清组不同染色方法对虫体生存率的影响

Fig. 5 The effect of different staining methods on the survival rate of parasites in serum-free groups



A 4 ℃生理盐水24 h处理后多房棘球蚴悬液肝门静脉注射一月小鼠肝脏 B 无处理多房棘球蚴悬液肝门静脉注射一月小鼠肝脏

图6 多房棘球蚴感染一月小鼠肝脏

A After 24 hours of treatment with 4 ℃ physiological saline, the suspension of multilocular echinococcus was injected into the liver portal vein of mice for one month B Untreated multi locular hydatid suspension injected into the liver portal vein of a month old mouse liver

Fig. 6 Infection ofmouse liver with *M. echinococcus* infection for one month

无血清组在37 ℃下培养条件下能获得相对较高的生存率，而生理盐水和无钙镁PBS组则可能与时间的延长且缺乏必要的营养物质，导致的能量耗竭相关[14]。多房棘球蚴培养过程中蚴虫离体后缺乏营养导致能量代谢降低，本研究中血清组生存率相较无血清组低，可能与虫株在体内接受到的营养物质和信号通讯远高于体外培养基相关，血清组中血清的某种成分促进了虫株过成熟[15]导致虫株代谢能力增强，新陈代谢速度加快，维系蚴虫生存的营养物质却缺乏，使得蚴虫死亡。体外4 ℃ NaCl培养组维持活性能力最差，用新鲜离体虫株与体外培养效能最低组进行回植效果比较，验证了实验结果。体外培养多房棘球蚴绦虫仍然是一个相对较新的研究领域。因此，一些培养技术尚处于不断优化和改进的阶段。

通过对多房棘球蚴体外培养时间延长来保证动物造模结果的一致性和重复性，使造模方式更加规范化，提高小鼠造模成功率。借此探究新的诊断方法、发现新药物、新靶点以及深入研究病原体的生物学特性，这些问题的解决有利于对泡型棘球蚴病致病机制研究。

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