Goat fetal fibroblasts frozen and in vitro culture *

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Abstract: Goat fetal fibroblasts were frozen at different temperature with various protecting solution in order to find a proper procedure to preserve its rare resources for further study both in transgenic and cloning practice. The result showed that fetal fibroblasts froze at - 196 ℃ with 900 mL/L fetal bovine serum (FBS) grew faster than that at - 80℃ after thawing ($P < 0.05$). - 20 ℃ was not an effective temperature in frozen cells. 100 mL/L dimethyl sulfoxide (DMSO) contained in frozen solution was better than 50 mL/L in improving protection effectiveness ($P < 0.05$). *In vitro* culture of fetal fibroblasts experiment showed that both DMEM and M 199 can support cell growth and proliferation and that a supplementation of 2 mmol/L 2-mercaptoethanol (2-Me) was better than not in improving tissue vitality and growth speed. In cell culture procedure, 2-Me could improve the effect of 100 mL/L FBS in stimulating cell proliferation. And individual 2-Me supplementation in the culture medium could stimulate cell growth. As a result, 2-Me was more likely a cell proliferation stimulator than an oxidation retarding factor. High serum concentration 100 mL/L was vital for cell proliferation, serum starvation (5 mL/L FBS) could not only inhibit cell division but also cause cell loss during long term culture.

Key words: fetal fibroblasts frozen; *in vitro* culture; 2-mercaptoethanol; dimethyl sulfoxide; goat

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Fetal fibroblasts had been used as transgenic and cloning donor cells in cattle, sheep, pigs and goats because of their rapid growth and potential for multiple cell divisions before senescence in culture[1~3]. However, unlike alive animals, the primary tissue could be obtained only once and then discarded, which made it difficult for further comparison of genotypes between cloned animals and their original donors. In this point, fetal fibroblasts preservation became apparently important. Our study focused on the freezing effectiveness of factors, such as freezing temperature, the percentage of freezing protectants and serum. They were considered to have the remarkable influence in freezing procedure.

In cloning practice, 2-mercaptoethanol (2-Me) was added into donor cell culture medium[1, 4, 5]. It was believed that 2-Me acted as serum in cell culture[6]. We wondered if it could replace the role that fetal bovine serum (FBS) played in culture procedure. Therefore we designed an experiment to examine the hypothesis by comparing it with serum within the culture medium.

1 Materials and methods

1.1 Preparation of goat fetal tissue

A total of 5 goat fetuses were collected from abattoir within 15 min after slaughter and were transported in 9 g/L sodium chloride, at 37 ℃ to the lab within 4 hours. The fetuses were washed in PBS; then the heads and inner organs were excised. The remnants of each fetus were pooled,

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minced into small pieces and divided into 2 groups. (1) 20 pieces of tissue were digested and fetal fibroblasts were collected and cultured in four different culture media; (2) 40 pieces of tissue were planted on the plastic Carrel's cultural bottle and cultured with different culture media.

1.2 Tissue culture method

1~3 mmol/L 3 of tissue pieces were cultured by using Carrel's bottle. Every 10 tissue pieces were placed in one bottle. The culture medium could be one of the following: (1) DMEM with 100 mL/L FBS; (2) DMEM with 100 mL/L FBS supplemented with 2 mmol/L 2-Me; (3) M199 with 100 mL/L FBS; (4) M199 with 100 mL/L FBS supplemented with 2mmol/L 2-Me. Tissue culture was performed at 37 ℃ and 50 mL/L CO_2 until fibroblasts reached subconfluency. The observation was taken frequently and growth data were taken down.

1.3 Tissue digestion and cell collection method

Tissue pieces were digested with TE (0.5 g/L trypsin+ 0.2 g/L EDTA) at 37 ℃ for 0.5 h. The supernatant containing single cells was decanted and added to DMEM supplemented with 10% FBS. The cells were separated by centrifugation at 500 g for 10 min, and the cells pellets were resuspended in DMEM containing 100 mL/L FBS, penicillin (100 IU/mL) and streptomycin (100 μg/mL). One square centimeters of culture area was seeded per 2.5×10^5 cells. Culture was performed at 37 ℃ and 50 mL/L CO_2 until fibroblasts reached subconfluency.

Passage 3 cells were seeded at a concentration of 1.87×10^4 cells/cm^2 in two 24-well dishes for each of the 4 groups. Cells were cultured in DMEM containing (1) 100 mL/L FBS and 2 mmol/L 2-Me; (2) 2mmol/L 2-Me; (3) 100mL/L FBS; (4) 5 mL/L FBS. After every 24 h, 3 wells of cell of each group were trypsinized and counted, average cell number/mL was calculated and after 8 days, growth curves of fetal fibroblasts were made.

1.4 Fibroblasts frozen

For the preservation experiments, passage 4 of goat fetal fibroblasts were stored at a concentration of 5×10^5/mL in a mixture of DMEM containing (1) 100 mL/L FBS and 100 mL/L DMSO; (2) 100 mL/L FBS and 50 mL/L DMSO; (3) 900 mL/L FBS and 100 mL/L DMSO; (4) 950 mL/L FBS and 50 mL/L DMSO. These tissues were stored at 4 ℃ for 40 min in a plastic box then stored at - 20 ℃, - 80 ℃ and - 196 ℃ respectively for 3 weeks. They were thawed at 37 ℃, washed by DMEM for 3 times and cultured in DMEM supplemented with 100 mL/L FBS and 2 mmol/L 2-Me. 24 h later, the percentage of adhered cells were calculated. The experiments were repeated three times and average data were made.

1.5 Statistical analysis

All data are analyzed by χ^2 or student t-test.

2 Results

2.1 Viability of goat fetal fibroblasts after thawing

After thawing, the goat fetal fibroblast's growth potency increased steadily as the freezing temperature became lower. In brief, at every temperature condition, a higher percentage of serum (900 mL/L) contained in the culture medium resulted in higher protecting effectiveness than that of 100 mL/L serum ($P<0.05$). Furthermore, the protecting efficiency was also in relation with the percentage of DMSO contained in the solution. 100 mL/L DMSO usually had a better protection effect, compared with 50 mL/L DMSO ($P<0.05$). - 20 ℃ had proved to be of little use in preserving fibroblasts; - 80 ℃ had a significant protection effect than - 20 ℃ ($P<0.01$); - 196 ℃ was even better than - 80 ℃, the difference was also significant (Table 1).

Table 1　Fetal fibroblast's viability after thawing

DMSO/(mL·L⁻¹) concentration	Temperature/℃					
	-20		-80		-196	
	100 mL/L serum	900 mL/L serum	100 mL/L serum	900 mL/L serum	100 mL/L serum	900 mL/L serum
100	0	30.2	70.1	82.6	88.9	95.7
50	0	11.7	65.8	77.1	86.4	93.6

2.2 The influence of culture medium, serum and 2-Me on cell proliferation

In tissue culture group, tissues grew faster in both DMEM and M199 medium containing 2-Me than that of without 2-Me. If supplemented with 2-Me, newly grew cells at the edge of tissues could be observed 22 h post plantation, otherwise the same results could only be observed 36 h post plantation. Both DMEM and M199 medium could support fetal tissue culture to term (Table 2).

Table 2　Tissue growth status

Time/h	DMEM		M199	
	100 mL/L FBS+ 2 mmol/L 2-Me	100 mL/L FBS	100 mL/L FBS+ 2 mmol/L 2-Me	100 mL/L FBS
22	2	1	1	1
36	2	1	3	2
48	2	2	2	2
72	1	2	1	1
96	1	1	1	1
120	1	1	0	0
144	0	0	0	0
168	0	0	0	0
Total alive tissue	9	8	8	7

In digestion group, fibroblasts grew at different speed according to various culture media. If 100 mL/L FBS was added into medium, fibroblasts divided continuously and reached confluncy within 3 - 4 days. 2-Me could promote such role but not significantly ($P > 0.05$). Individual 2-Me could not match the role with FBS in stimulating cell growth ($P < 0.01$). The difference between 2-Me and 0.05% FBS was also significant ($P < 0.05$). In serum starvation group, cells grew only a little at the beginning, then became steady for 3- 4 days. From sixth day a considerable cells lost and cells became smaller morphologically (Fig. 1).

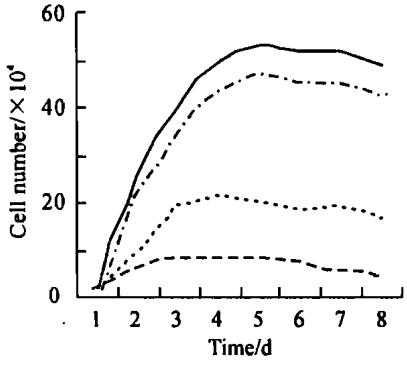

Fig. 1　The influence of serum and 2-Me on cell growth
- --- . 100 mL/L FBS; ----. 2 mmol/L 2-Me;
——. 2 mmol/L 2-Me+ 100 mL/L FBS; - - - . 5 mL/L FBS

3　Discussion

Our data showed that as high as 900 mL/L FBS added into frozen protection medium had a significant influence on cell proliferation provided that the tissue were preserved at -80 ℃ or -196 ℃. 100 mL/L DMSO was more effective in protecting cells from frozen damage than 50 mL/L DMSO did. -20 ℃ failed in freezing process. Fahrudin[7] also demonstrated that tissue stored in 50 mL/L DMSO both at -35 ℃ and -80 ℃ were alive after thawing. These fetal bibroblasts could be used in cloning and have the same efficiency of producing cytoplasts compared with new and fresh digested fetal fibroblasts. All above demonstrated that freezing fetal fibroblasts procedure was practicable. It was not only a good method in preserving rare tissue resources and cells, but also had a potency to be used in cloning transgenic animals.

This study also demonstrated that serum deprivation could inhibit cell division significantly, which is a good method to capture cells at G_0/G_1

stage and has been used widely in nuclear transfer procedure[8,9]. 2-Me could accelerate cell division only a little provided that FBS is added into the medium, and it could stimulate cell proliferation directly, or promote mitogen activated protein kinase and DNA synthesis as well as retarding cell oxidation[6]. 2-Me, though, could not replace serum in culture medium, could stimulate cell division individually during prolonged cell culture which indicates that it is more likely a cell proliferation stimulator than an oxidation retarding factor.

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山羊胎儿成纤维细胞的冷冻保存和体外培养

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[摘 要] 将胎儿成纤维细胞悬浮于不同的冷冻保护液中, 在不同的温度下进行冷冻保存试验。结果显示, 当保护液中含有 900 mL/L 胎牛血清(FBS)时, - 196 ℃下保存的山羊胎儿成纤维细胞解冻后的细胞贴壁率高于 - 80 ℃, 且前者贴壁细胞增殖也较后者快, 二者间差异显著; 而在- 20 ℃条件下只有一小部分细胞能够存活, 与前两者相比差异极显著, 说明- 20 ℃不适合冷冻胎儿细胞。冷冻保护液中含有 100 mL/L 二甲基亚砜(DMSO)的保护效果明显好于 50 mL/L DMSO, 二者间差异显著。用组织块法培养的细胞, 在两种培养基 DMEM 和 M199 中添加 100 mL/L FBS 和 2 mmol/L 2-巯基乙醇(2-Me), 其组织块成活率及细胞生长速度均高于单纯添加 100 mL/L FBS, 同时证明 DMEM 和 M199 都能用于胎儿成纤维细胞的体外培养。以 DMEM 培养液为基础, 分别添加 2 mmol/L 2-Me, 5 mL/L FBS, 100 mL/L FBS 和 2 mmol/L 2-Me+ 100 mL/L FBS 对山羊胎儿成纤维细胞进行体外培养, 结果显示, 细胞在 100 mL/L FBS 和 2 mmol/L 2-Me+ 100 mL/L FBS 培养液中生长迅速, 两者间无明显差异, 2-Me 的存在促进了细胞的增殖; 两者都与添加 2 mmol/L 2-Me 组存在显著差异; 添加 2 mmol/L 2-Me 和 5 mL/L FBS 相比, 前者也明显地起到了促进细胞增殖的作用, 二者间差异显著。说明 2-Me 对细胞的促生长作用大于抗氧化作用, 100 mL/L FBS 能保证细胞的生长和增殖, 5 mL/L FBS 在培养液中长期存在不仅抑制细胞分裂, 而且导致大量丢失。

[关键词] 胎儿成纤维细胞; 体外培养; 2-巯基乙醇; 二甲基亚砜; 山羊

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