Influence Factors of *in vitro* Culture of Goat Spermatogonial Stem Cells

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Abstract: *This study aims to determine the condition factors influencing the in vitro proliferation culture of spermatogonial stem cells (SSCs) through isolation and culture of SSCs. With mouse embryonic fibroblast (MEF) cells, SIM-6-thiogunanie-oualiain (STO) cells and Hela cells used as the feeder layers, we compared the numbers of cell clones treated with mitomycin C as well as the proliferation conditions with and without the treatment with trypsin. In the culture solution, we respectively added serum and vitamin C (of different concentrations, VC) to culture goat SSCs; furthermore, we tested their pro-proliferation effect on goat SSCs and its mechanism. We determined that one-month-old goats were the most suitable for the in vitro culture; 1.5 h, 2.5 h and 2.5 h were respectively the most suitable treatment times in the feeder layers of MEF cells, STO cells and Hela cells; 2.5% was the optimal serum concentration; VC (30 μg/mL) had a promoting effect on the in vitro culture of goat SSCs. We conclude that the age of goats, different feeding layers and their preparation conditions, serum concentrations and VC concentrations are the condition factors influencing the in vitro culture and proliferation of SSCs.*

Keywords: *Goats, SSCs, in vitro, Influence factors.*

Introduction

In recent years, the relevant researches have made great contributions to the treatment of diseases such as leukemia, cancer and eye diseases [17]. Currently, reproduction-related stem cell is also one of the hottest research issues. Spermatogonial stem cells (SSCs) are situated near the basement membrane of seminiferous tubule in the testis of mammals [12]. It is the only stem cell in the body that can pass on genetic information to its offspring. Under the regulation of reproductive hormones, a large number of sperms are generated through proliferation and differentiation in the manner of a continuous wave, so that the lifetime fertility of male animals is maintained [6]. Spermatogonia can proliferate their daughter cells through continuous mitosis which makes the cell division incomplete, namely, their daughter cells are still connected by a cytoplasmic bridge, thus forming coenocytes clones obtained from the division of a stem cell [4]. Non-primate testicular spermatogonia can be divided into type A spermatogonia, middle type spermatogonia and type B spermatogonia [3]. Type A spermatogonia include three sub-types: single type (A-S), matching type (A-P) and balanced type (A-A). Single type spermatogonia are SSCs in the actual sense.

In 1968, De Rooij et al. [2] suggested that SSCs were the daughter cells of type A spermatogonia of the last generation. In 1971, Oakberg et al. [10] thought A-S type spermatogonia were stem cells. Huckins et al. [5] put forward the form of murid spermatogonia as well as the characteristics of their proliferation and maturity. In 2003, Izadyar et al. [7] performed a study on the autograft and allograft transplantation of cattle spermatagonia. So far, researchers have discovered the integrated technology of separation, purification, allotransplantation, cultivation, identification, induced differentiation, cryopreservation and genetically modified technology of SSCs [13]. This paper studied the factors influencing the proliferation of goat SSCs in the *in vitro* culture system.

Materials and methods

Experimental materials

The animals and cells used in this study included mouse embryonic fibroblast (MEF) cells, SIM-6-thiogunanie-oualiain (STO) cells, Hela cells, STO and milk goat testicular tissue (from the testicles of 3 to 6 month-old male Saanen dairy goats) which were all purchased from Cobioer Biotechnology Co., Ltd. (Nanjing City, Jiangsu Province, China).

The regents used in the study included double resistance DPBS liquid alcohol (5%), IV collagenase (2 mg/mL) DMEM solution, pancreatic enzyme/EDTA (0.25%) solution, 0.04% trypan blue, 0.2% gelatin solution, 75% alcohol, 0.25% trypsin, 0.04% EDTA digestive juice, mitomycin C broth (10 μg/mL) and vitamin C solution of different concentrations (0-40 μg/mL).

The following instruments were used in the study: an experimental scalpel, ophthalmic forceps and scissors, a CO_2 incubator, 200-mesh and 300-mesh gauze, culture plates, a microplate reader, a CCK 8 kit, centrifugal tubes, a centrifuge and a microscope.

Experiment method

Separation and culture of goat testicular tissue

Under aseptic conditions, testicular tissues of male Saanen dairy goats (of different ages) were flushed with 5% double resistance DPBS liquid alcohol; the albuginea of the tissue was removed. A testicle of each goat was cut into three equal pieces (each in the size of about 3 mm^3). The seminiferous tubule was separated, and the parenchyma was sheared into pieces with ophthalmic forceps and scissors. After 10-minute still standing, IV collagenase (2 mg/mL) DMEM solution was added. Then they were incubated at 37 °C for 30 min.

After the free settling of the seminiferous tubule, the supernatant was removed. PBS was added. It was blown for several times.

After 5-min centrifugation at 1500 r/min, the supernatant was separated. Isovolumetric pancreatic enzyme/EDTA (0.25%) solution was added. Then they were transferred to a CO_2 incubator for 10 min incubation at 37 °C. Isovolumetric basal cell broth was added to terminate the digestion.

Primary cell broth was added for suspension. Through successive filtration with 200-mesh and 300-mesh gauzes, testis cell suspension of the goats was obtained and mixed with 0.04% trypan blue (1:1). At indoor temperature, the survival rate was detected through staining and counting.

The cells (5×10^4 cells/ml) were inoculated to a petri dish (processed with 0.2% gelatin). Then it was transferred to an incubator (37 °C, 5% saturated humidity of CO_2) for culture and purification; meanwhile, the adherence status of the cells was observed timely.

After 2~3 hours of culture, the inadherent cells could be separated from the adherent ones; the inadherent spermatogonia were collected and centrifuged at 1200 r/min at room temperature, then the supernatant was removed.

SSCs broth was added for suspension. The cells (1.5×10^4 cells/ml) were cultured for 10 days in a CO_2 incubator at 37 °C. The broth was replaced with fresh broth every three days. On the 10^{th} day, after clones appeared, the total cell clone count and area of each experimental group were collected.

Preparation of feeder layer cells

MEF cells, STO cells and Hela cells were cultured separately under aseptic conditions. After the confluence degree of the cells grew to 80-90%, they were preserved in a freezer at -80 °C overnight. Then, the three kinds of feeder layer cells were taken out and thawed in the water at 37 °C. As per the following steps, the feeder layers were cultured.

(1) The culture plate was soaked and sterilized in alcohol (75%) for one day. Then it was dried in the air and radiated by ultraviolet for 0.5 hour. Gelatin solution (0.2%) was added and the culture plate was placed in the constant temperature stage for 2 h before the gelatin was sucked out.

(2) The cells were digested with 4 ml of 0.25% trypsin + 0.04% EDTA digestive juice for about 5 min. Then an equal amount of MEF broth was added to terminate the digestion. The cells were blown gently until single cell suspension formed, then they were added to a centrifugal tube (10 ml).

(3) They were centrifuged (1500 r/min, 5 min) and the supernatant was removed. Cell broth was added so that the cell precipitate was made into suspension. After adjusting the concentration of the cells to 5×10^5 cells/mL, we added them to a culture plate without coating of gelatin and cultured them under the conditions of 37 °C and 5% CO_2.

(4) The broth was sucked out from the well-grown cells and the broth containing mitomycin C (10 μg/mL) was added to process the cells for different periods of time (0-3.5 h); then the cells were cultured in an incubator (37 °C, 5% CO_2).

(5) The broth containing mitomycin C was sucked out and the cells were rinsed with DPBS for 4 to 5 times so that mitomycin C was completely removed.

(6) Step (2) was repeated.

(7) The cells were centrifuged (1500 r/min, 5 min). The supernatant was removed. Cell culture solution was added to generate suspension from the sediment. With their concentration adjusted to 5×10^5 cells/mL, the cells were added to the culture plate coated with gelatin and cultured at 37 °C and 5% CO_2.

Analysis on cell proliferation

The culture plate was placed in an incubator (37 °C, 5% CO_2). After 24 hours, the three kinds of feeder layer cells were grouped as per the processing time with mitomycin C. The cells were inoculated and cholecystokinin (CCK 8) solution was added. The culture continued according to different processing methods and time (processing without trypsin for 1 h / 2 h /

3 h; processing with trypsin for 0.5 h / 1 h / 2 h / 4 h). Optical density (OD) value at 450 nm of each sample pore was measured with a microplate reader.

Culture in vitro of goat SSCs with serum and vitamin C of different concentrations

A contrast experiment was performed to observe the effect of serum and vitamin C (with different concentrations) on the culture of goat SSCs. The purified SSCs (1×10^5 cells/mL) were cultured in the environment of 37 °C and 5% CO_2. Serum (0%, 1%, 2.5%, 5%) and vitamin C (0-40 μg/mL) were used as the influence factors for the proliferation *in vitro* of SSCs. The results were evaluated based on the number of SSCs on the third day, 6th day and 10th day as well as the OD values of the clones on the 7th, 8th, 9th and 10th days.

Statistical analysis

SPSS 17.0 was applied to the statistical analysis on all the data. When $p < 0.05$, there was statistical significance.

Experimental results

Separation and purification effects of goat SSCs

The goat testicles were collected and separated. Table 1 shows the number and survival rate of the cells before and after purification.

Table 1. Number and survival rate of goat testis cells before and after purification

Group	One-month-old	Three-month-old	Five-month-old	Over two-year-old
Number of the digested and separated cells, (10^8/g testis)	1.30 ± 0.07	1.51 ± 0.17	1.73 ± 0.13	1.62 ± 1.02
Cell survival rate, (%)	93.24 ± 2.48	93.04 ± 1.33	92.97 ± 2.34	94.02 ± 2.17
Total cell number after purification, (10^6/g testis)	4.51 ± 3.08	4.88 ± 2.71	4.93 ± 1.63	4.73 ± 3.19
Cell survival rate, (%)	79.11 ± 2.01	75.29 ± 1.64	76.04 ± 2.73	75.68 ± 3.19

Table 2. Cell clone area of goat testis after 10-day culture

Group	One-month-old	Three-month-old	Five-month-old	Over two-year-old
Clone area, (μm^2)	824.39 ± 145.23	796.68 ± 241.61	816.12 ± 187.31	790.75 ± 163.35

According to Table 1, five-month-old goats had the most separated cell count after digestion, while the cell count of one-month-old goats was the least; there was a significant difference between them ($p < 0.05$). After purification with 0.2% gelatin, five-month-old goats had the highest cell count, while one-month-old goats had the lowest cell count; there was a significant difference between them ($p < 0.05$). In Table 2, after 10-day culture, there was no significant difference in the average clone area of testis of the four groups of goats ($p > 0.05$).

Proliferation ability of the goat SSCs cultured in different feeder layers

Culture of MEF cells and their feeder layer

MEF cells are the most common feeder layer cells [11]. In the primary stage of culture in vitro, the shape of an MEF cell was like a shuttle, while the cluster of MEF cells was in the shape of a chrysanthemum with clear cell boundaries [14]. On account that the first and second generations of cells contained impurities, the third and fourth generations of cells were preferable for their higher purity. As shown in Fig. 1, the fourth generation of cells spread more completely and uniformly [19]. In the feeder layer shown in Fig. 2, proliferation ability of cells declined. The cells were distributed uniformly and their density was moderate, which provided favorable conditions for the proliferation of SSCs.

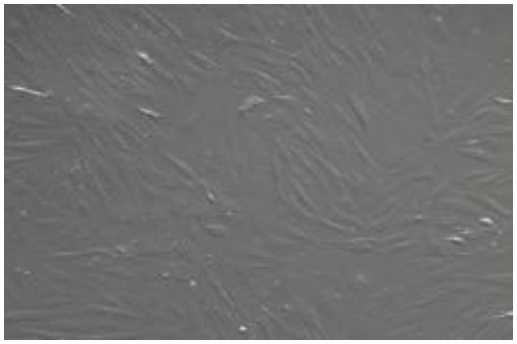

Fig. 1 The fourth generation of MEF cells

Fig. 2 The fourth generation of the feeder layer

As shown in Table 3, when the culture time was within two hours, the proliferation rate of the cells in the 0 h group was higher than in the other groups ($p < 0.05$). When the culture time was 2 h, there was a significant difference between the 1.5 h group and the other groups ($p < 0.05$). In the case of trypsin processing (Table 4), when the culture time was between 0.5 h and 4 h, the culture ability of the 0 h group was obviously higher than in the other groups; the difference was of statistical significance ($p < 0.05$).

Table 3. The proliferation conditions of MEF cells processed with mitomycin C (10 μg/mL, without trypsin)

T_2 \ T_1	1 h	2 h	3 h
0	0.82	0.83	0.92
1.5	0.70	0.75	0.76
2	0.70	0.66	0.73
2.5	0.69	0.60	0.70
3	0.71	0.61	0.69
3.5	0.69	0.59	0.68

Note: T_1 refers to the culture time;
T_2 refers to the time of processing MEF cells.

Table 4. The proliferation conditions of MEF cells processed with mitomycin C (10 μg/mL) and trypsin

Culture time / Time of processing MEF cells	0.5 h	1 h	2 h	4 h
0	0.56	0.59	0.65	0.85
1.5	0.42	0.54	0.41	0.48
2	0.43	0.53	0.42	0.47
2.5	0.42	0.52	0.41	0.46
3	0.45	0.52	0.42	0.49
3.5	0.42	0.52	0.39	0.45

Culture of STO cells and their feeder layer

As shown in Fig. 3, STO cell lines were transparent and irregular-shaped under the microscope. They were of high refraction while the outline was not clear. There were vacuoles, lipid droplets or other particulate matter [8]. STO cells had plenty of sheeny vacuoles. As shown in Fig. 4, cells in the culture layer were quite smooth and had few vacuoles and most were adherent cells.

Fig. 3 STO cells

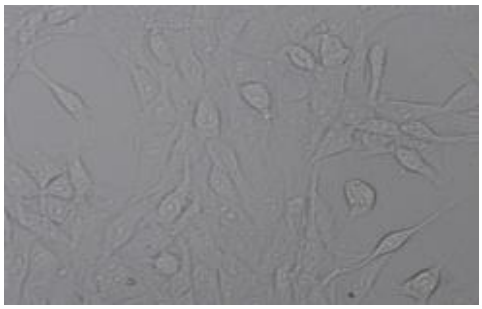

Fig. 4 The feeder layer of STO cells

As shown in Table 5, when the culture time was 1 h, there was a significant difference between the untreated group (0 h) and the other groups ($p < 0.05$); the proliferation rate in the 3.5 h group was significantly lower than that in the other groups, and the difference was significant ($p < 0.05$), which indicated that the 3.5 h group had a greater inhibition effect. When the culture time was 2 h / 3h, the differences between the untreated group and the treated groups were insignificant ($p > 0.05$).

Table 6 shows that with trypsin treatment, when the culture time was 0.5 h, the proliferation rate of the untreated group was significantly higher than in the other groups with a significant difference ($p < 0.05$); the difference between the other groups was insignificant. When the culture time was 1 h and 2 h, the proliferation rate of the untreated group was still the highest with a significant difference ($p < 0.05$). Among the treated groups, the proliferation rate of the 2.5 h group was higher than the other four groups with a significant difference ($p < 0.05$). When the culture time was 4 h, the proliferation rate of the 1.5 h group was the lowest.

Table 5. The proliferation conditions of STO cells processed with mitomycin C (10 μg/mL, without trypsin)

Culture time / Time of processing STO cells	1 h	2 h	3 h
0	0.60	0.78	0.91
1.5	0.51	0.76	0.82
2	0.50	0.63	0.69
2.5	0.49	0.62	0.68
3	0.50	0.61	0.66
3.5	0.40	0.48	0.51

Table 6. The proliferation conditions of STO cells processed with mitomycin C (10 μg/mL) and trypsin

Culture time / Time of processing STO cells	0.5 h	1 h	2 h	4 h
0	0.65	0.75	0.89	1.09
1.5	0.52	0.57	0.42	0.48
2	0.51	0.57	0.43	0.58
2.5	0.53	0.60	0.52	0.62
3	0.51	0.57	0.42	0.49
3.5	0.51	0.57	0.42	0.49

Culture of Hela cells and their feeder layer

The cell nucleus of cancer cells was bigger than that of normal cells. Due to the disordered cytoskeleton, cancer cells were in irregular shapes and varied sizes. As shown in Fig. 5, most cancer cells were malformed. In Fig. 6, Hela cells were capable of proliferation with a fast adherent speed. Under the microscope, they were in a multilateral form, which made it easier for them to cluster and grow more compactly [1, 9].

As shown in Table 7, with the increase of the culture time, the proliferation rate of the untreated group was obviously higher than in the other groups, while the proliferation rate of the 2.5 h group was lower than the other groups ($p < 0.05$). The differences between the other groups were insignificant; their proliferation rates were in the trend of rising first and then decreasing. As shown in Table 8, the proliferation rate of the untreated group was remarkably higher than in the other groups ($p < 0.05$); the differences between the other groups were insignificant ($p > 0.05$).

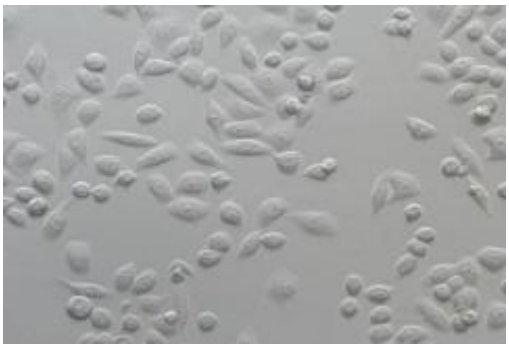

Fig. 5 Hela cells

Fig. 6 Feeder layer of Hela cells

Table 7. The proliferation conditions of Hela cells processed with mitomycin C (10 μg/mL) and without trypsin

T_2 \ T_1	1 h	2 h	3 h
0	0.89	1.18	1.21
1.5	0.78	0.90	0.91
2	0.74	0.91	0.93
2.5	0.50	0.62	0.68
3	0.81	1.02	0.94
3.5	0.76	0.91	0.92

Note: T_1 refers to the culture time;
T_2 refers to the time of processing Hela cells.

Table 8. The proliferation conditions of Hela cells processed with mitomycin C (10 μg/mL) and trypsin

Time of processing Hela cells \ Culture time	0.5 h	1 h	2 h	3 h
0	0.58	0.61	0.68	0.91
1.5	0.49	0.48	0.51	0.59
2	0.48	0.47	0.52	0.60
2.5	0.47	0.47	0.50	0.58
3	0.48	0.47	0.51	0.59
3.5	0.48	0.47	0.51	0.59

The influence of serum and vitamin C (with different concentrations) on the in vitro culture of goat SSCs

The influence of different serum concentration on the in vitro culture of goat SSCs

Serum of different concentrations (0%, 1%, 2.5%, 5%) was added to the basal culture of SSCs. The number of the clones and the proliferation ability were used as the indexes. According to Fig. 7, on the third day (3D), the number of clones and proliferation ability were in direct proportion; significant differences were found between the groups. When the serum concentration was 5%, the number of SSCs clones was the maximum. On the 6th day (6D), the number of clones decreased when the serum concentration was 5%, while the number of clones increased in the cases of other serum concentrations. Thereby, it was concluded that with the increase of time, the high-concentration serum had a greater effect of inhibiting the proliferation of SSCs. As can be seen in the figure, when the serum concentration was 2.5%, the number of the increased clones was the highest; however, the total number of clones was the highest when the serum concentration was 5%. The differences between the groups were significant ($p < 0.05$). On the 10th day, the number of clones in each group reduced; it was the highest when the serum concentration was 2.5% and the lowest when the serum concentration was 0%. The differences between the groups and other groups were significant ($p < 0.05$). The number of clones was higher in the 1% serum group than in the 5% serum group with an insignificant difference.

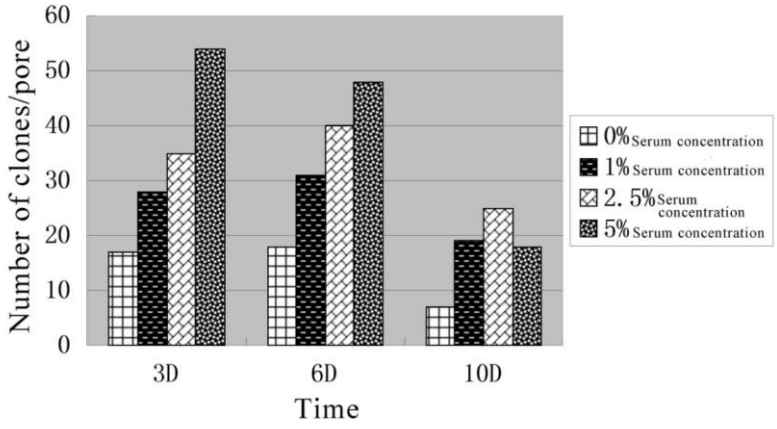

Fig. 7 The average clone amount of goat SSCs of different serum concentrations

Table 9 shows that on the 7th and 8th days, the proliferation speed of the clones was the fastest when the serum concentration was 2.5%, while the number of clones was the lowest in the 0% serum group. Therefore, 2.5% was the optimal serum concentration for the *in vitro* culture of goat SSCs.

Table 9. Optical density of goat SSCs clones with different serum concentrations

Serum / Cultured days	0%	1%	2.5%	5%
7	1.25	1.59	1.41	1.41
8	1.88	2.1	2.22	2.11
9	1.91	2.08	2.18	1.92
10	1.81	1.92	2.01	1.9

The effect of vitamin C (with different concentrations) on the in vitro culture of goat SSCs
In Fig. 8, after 3-day culture, the number of clones was the greatest when the concentration of vitamin C was 30 μg/mL, and the differences between groups were significant. On the 6th day, the number of clones was the greatest when the concentration was 40 μg/mL; there were significant differences between groups, and the clone number increased with the concentration.

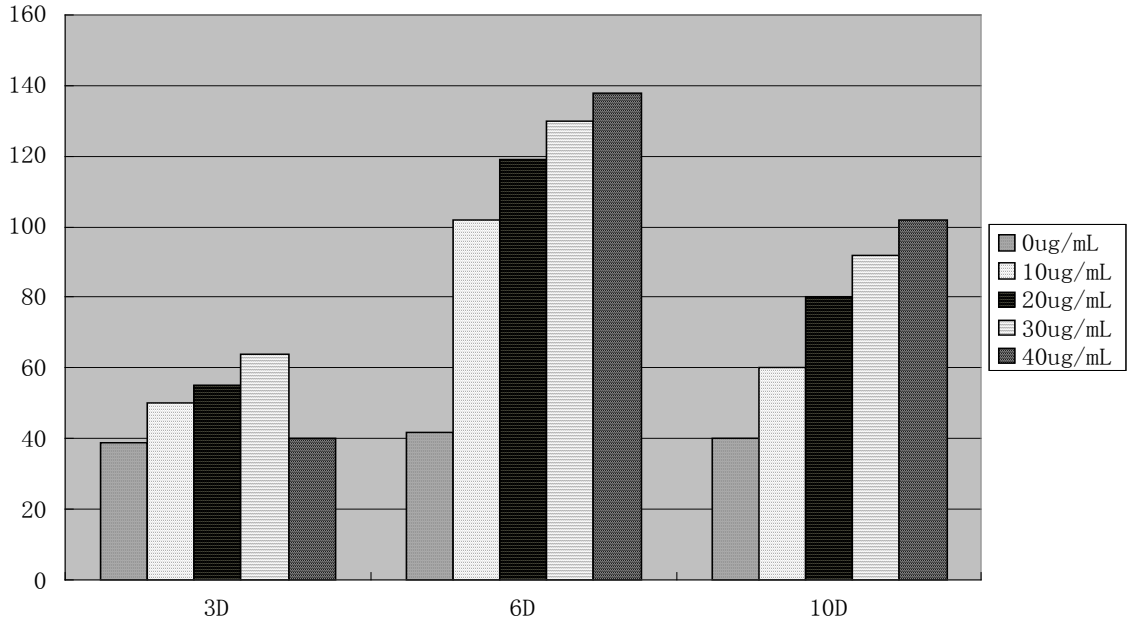

Fig. 8 The average number of goat SSCs clones with vitamin C of different concentrations

On the 10th day, the number of clones in each group declined; the greater the concentration, the more the clones. Table 10 shows that between the 7th day and the 10th day, the OD values were in their maximum when the vitamin C concentration (VC) was 20 μg/mL and in their minimum when the concentration was 40 μg/mL.

Table 10. OD values of goat SSCs clones with vitamin C of different concentrations

VC / Cultured days	0 μg/mL	10 μg/mL	20 μg/mL	30 μg/mL	40 μg/mL
7	1.75	1.77	1.81	1.72	1.65
8	1.83	1.85	1.88	1.84	1.81
9	1.84	1.88	1.90	1.84	1.82
10	1.83	1.87	1.89	1.84	1.78

Discussion and conclusion

In this study, age of goats, feeder layers, serum concentrations and vitamin C concentrations were the factors influencing the *in vitro* culture of goat SSCs.

The number of clones and survival rate of goat SSCs vary with the age of goats [15]; therefore, it is important to determine the optimal age of the goats selected for the culture. In this study, the number of cells of one-month-old goats was the least before and after the purification, while their survival rates were high and the cloning area was large. Accordingly, one month was the optimal age for the experimental goats.

In recent years, more and more researchers have achieved great improvement in choosing the suitable feeder layers for the in vitro culture of cows. However, there is no systematic study on the feeder layers designed for the proliferation of goat SSCs.

In this study, after the treatment with mitomycin C, in order to determine whether trypsin digestion was influential to the feeder layers, we performed experiments respectively with and without trypsin treatment. The experimental results showed that the clone proliferation speed of the cells untreated with trypsin was relatively slow and the difference was insignificant. The proliferation quantity of the cells treated with trypsin varied constantly and greatly, which indicated that the treatment with trypsin increased the instability. Therefore, the feeder layers untreated with trypsin were preferable. As the fourth generation of MEF cells showed moderate density and uniform distribution, MEF cells were the most suitable for the feeder layer.

As for the influence of serum concentrations on the *in vitro* culture of SSCs, there are varied opinions. In 2014, Tokas et al. [16] performed a study on the *in vitro* cultivation of buffalo SSCs and proposed that the maintaining effect of 2.5% serum on the *in vitro* cultured cells was the most obvious. In this study, high-concentration serum led to the decrease of the number of SSCs clones, suggesting that high concentration of serum was adverse to the *in vitro* culture; it was determined that 2.5% was the optimal serum concentration for *in vitro* culture.

Concentration of vitamin C, VC, is another influencing factor. In 2014, Wu et al. [18] suggested that VC had a promoting effect on the cloning of mouse embryonic stem cells. According to the experimental data of this study, 40 μg/mL VC could remarkably promote the proliferation of *in vitro* cultured goat SSCs. However, with the increase of the culture time, the number of cell clones gradually reduced at a relatively fast speed. With the addition of 30 μg/mL VC, the number of clones was less than that in the case of 40 μg/mL VC, however, the number of clones decreased more slowly. In summary, 30 μg/mL VC could effectively promote the *in vitro* proliferation.

To sum up, the optimal conditions for the *in vitro* culture of goat SSCs are determined as follows: SSCs cells of one-month-old goats were the most suitable for separation, purification and culture; MEF cells untreated with trypsin were selected as the optimal feeder layer cells; the optimal serum concentration was 2.5%; the VC concentration was 30 μg/mL.

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References

1. Cairns R. A., I. S. Harris, T. W. Mak (2011). Regulation of Cancer Cell Metabolism, Nature Reviews Cancer, 11(2), 85-95.
2. De Rooij D. G. (1968). Stem Cell Renewal and Duration of Spermatogonial Cycle in the Goldhamster, Cell and Tissue Research, 89(1), 133-136.
3. Garvin A. M. (2005). Method for Processing Samples Containing Sperm and Non-sperm Cells for Subsequent Analysis of the Sperm DNA: US, US 20050032097.
4. Gasnereau I., O. Ganier, F. Bourgain, A. de Gramont, M. C. Gendron, J. Sobczak-Thépot (2007). Flow Cytometry to Sort Mammalian Cells in Cytokinesis, Cytometry Part A the Journal of the International Society for Analytical Cytology, 71A(1), 1-7.
5. Huckins C. (1971). The Spermatogonial Stem Cell Population in Adult Rats. I. Their Morphology, Proliferation and Maturation, The Anatomical Record, 169(3), 533-557.
6. Iwamori N., T. Iwamori, M. M. Matzuk (2011). Characterization of Spermatogonial Stem Cells Lacking Intercellular Bridges and Genetic Replacement of a Mutation in Spermatogonial Stem Cells, Plos One, 7(6), e38914.
7. Izadyar F., K. D. Ouden, T. A. Stout, J. Coret, D. P. K. Lankveld, T. J .P. Spoormakers, B. Colenbrander, J. K. Oldenbroek, K. D. Van der Ploeg, H. Woelders, H. B. Kal (2003). Autologous and Homologous Transplantation of Bovine Spermatogonial Stem Cells, Reproduction, 126(6), 765-774.
8. Lim J. W. E, A. Bodnar (2002). Proteome Analysis of Conditioned Medium from Mouse Embryonic Fibroblast Feeder Layers which Support the Growth of Human Embryonic Stem Cells, Proteomics, 2(9), 1187-203.
9. Mikheev A. M., S. A. Mikheeva, B. Liu, P. Cohen, H. Zarbl (2004). A Functional Genomics Approach for the Identification of Putative Tumor Suppressor Genes: Dickkopf-1 as Suppressor of HeLa Cell Transformation, Carcinogenesis, 25(1), 47-59.
10. Oakberg E. F. (1971). Spermatogonial Stem-cell Renewal in the Mouse, The Anatomical Record, 169(3), 515.
11. Park H. S., S. R. Lee (2012). Method for Co-culture of Human Embryonic Stem Cells and Fibroblast Feeder Cells Using a Polyester Membrane: US, US 8257973.
12. Piacibello W., S. Bruno, F. Sanavio, S. Droetto, M. Gunetti, L. Ailles, F. S. de Sio, A. Viale, L. Gammaitoni, A. Lombardo, L. Naldini, M. Aglietta (2003). Lentiviral Gene Transfer and *ex vivo* Expansion of Human Primitive Stem Cells Capable of Primary, Secondary, and Tertiary Multilineage Repopulation in NOD/SCID Mice, Nonobese Diabetic/severe Combined Immunodeficient, Blood, 100(13), 4391-400.
13. Shang M. (2004). Isolation, Identification, Culture, Cryopreservation, Genetic Transformation and Transplantation of Catfish Germline Stem Cells, Cheminform, 36(18), 1249-1259.
14. Shinohara T., M. Shinohara (2010). Method of Growing Sperm Stem Cells *in vitro*, Sperm Stem Cells Grown by the Method, and Medium Additive Kit to be Used in Growing Sperm Stem Cells *in vitro*: US, US 7666673 B2.

15. Shirazi M. S., B. Heidari, A. Shirazi, A. H. Zarnani, M. Jeddi Tehrani, M. Rahmati-Ahmadabadi, M. M. Naderi, B. Behzadi, M. Farab, A. Sarvari, S. Borjian-Bboroujeni, M. M. Akhondi (2014). Morphologic and Proliferative Characteristics of Goat Type a Spermatogonia in the Presence of Different Sets of Growth Factors, Journal of Assisted Reproduction & Genetics, 31(11), 1519-1531.
16. Tokas J., G. Kaul, V. Kumar (2014). *In vitro* Culture of Buffalo (*Bubalus bubalis*) Spermatogonial Stem Cells: Effect of Serum, Sertoli Cell Coculture and Single Growth Factors, Buffalo Bulletin, 33(4), 407-422.
17. Wong S. C. (2011). Stem Cells: A Personal Perspective, Balkan Journal of Medical Genetics, 14(2), 7-11.
18. Wu C. Y., X. Feng, L. N. Wei (2014). Coordinated Repressive Chromatin-remodeling of Oct4 and Nanog Genes in RA-induced Differentiation of Embryonic Stem Cells Involves RIP140, Nucleic Acids Research, 42(7), 4306-4317.
19. Yang S., S. Shan, Y. Dong (2009). The Simple Method of Reparation for MEF Feeder Layer Cells, Journal of Agricultural Sciences, 35(2), 18-24.

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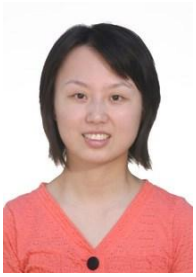

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