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Cryopreservation of Spermatogonial Stem Cells of Native Goat of Iran

Research Article

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ABSTRACT

This study deals with cryopreservation of spermatogonial stem cells (SSCs) of native goat using different media along with cryoprotectant. Morphological differentiation and immunocytochemistry tests were used to identify the cells. Furthermore anti-vimentin and anti-Oct-4 immuno staining methods were used for identification of sertoli cells and SSCs, respectively. Cryopreservation of SSCs was done with two sets of media. One with 40% dulbecco's modified eagle's medium (DMEM), 50% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO). The second medium included 90% FBS and 10% DMSO. Post thaw cryopreserved cells were subjected to viability and colony area formation on 4th, 8th and 12th days of culturing after and before cryopreservation. Results clearly indicated the viability, area and number of colonies in the first medium registered to 61.37%, 0.87 mm^{2}and 246.88 averagely in every 25 cm^2 culture flask, respectively. Similarly with second medium, post thaw cryopreserved sperm registered viability, area and number of colonies to 73.87%, 2.74 mm^2 and 364.36 in every 25 cm^2 culture flask, respectively. After the thawing of spermatogonial cells, the best viability percentage was obtained in the freezing medium containing 90% FBS. Thus the study demonstrated that serum concentration had a distinct positive effect on the maintenance and proliferation of SSCs in culture after cryopreservation.

KEY WORDS co-culture, cryopreservation, fetal bovine serum, freezing, cell isolation, goat, sertoli cells, spermatogonial cells, thawing.

INTRODUCTION

Stem cells can change into many different cell types in the body. In addition, they mend in many tissues, dividing essentially without limit to replenish other cells in person or animal's life. When a stem cell divides, each new cell capable to remain a stem cell or become another type of cell with a more specialized role. Stem cells have characteristics to distinguish from other cell types. First, they are undifferentiated cells that can renew themselves through cell division. Second, they can be induced to become tissue- or organ-specific cells with special functions under ensure physiological or experimental conditions.

It means that stem cells have the capacity of both self-renewal and differentiation (Tokas *et al*. 2011). Rare testis stem cells support spermatogenesis. These cells are called spermatogonial stem cells (SSCs). Amounting to 35000 SSCs are in per mouse testis (Bahadorani *et al*. 2012). Therefore, the scientists in medicine and agriculture course try to find efficient methods for *in-vitro* culture and expansion of SSCs.

The goat (*Capra hircus*) is one of the best choices for the transgenic production of recombinant proteins (Freitas *et al*. 2014; Yoisungnern and Paul, 2014). Therefore, the scientists have interest to the application of SSCs as another way to produce transgenic animals.

However, there are no reports on goat SSC culture and just direct transplantation of crude testicular cells to the recipient goat testis has been studied in previous experiments (Honaramooz *et al*. 2005).

Not only is the goat a very important animal economically, but its reproduction system possesses some interesting characteristics. The seminiferous epithelium cycle duration in goat is 10.6 days (Franca *et al*. 1999). Total period of spermatogenesis in goat is 4.5 epithelium cycles. So, the length of spermatogenesis is 47.7 days (Franca *et al*. 1999). Seminiferous epithelium period in goats was divided into 14 stages. There are three generations of A type (A_1, A_2 and A_3), one generation of intermediate type (In) and two generations of B type (B_1 and B_2) spermatogonia (Bilaspuri and Guraya, 1984).

Sertoli cells and co-culture: sperm has been produced in the process that is called spermatogenesis, and in the adult the stem cells in this system are the SSCs. These cells are in the base of the seminiferous epithelium and coexist with sertoli cells (Russell *et al*. 1990). Sertoli cells provide niche for SSC (Minaee Zanganeh *et al*. 2013).

Immediately after seeding, both the spermatogonial cells and somatic sertoli cells have spherical form, but 1 day after seeding, sertoli cells become attached to the bottom area of the dish and start to form a feeder layer, but some of the spermatogonial cells start to form clusters on the feeder (Miryounesi *et al*. 2013).

Spermatogonial culture need medium plus serum or a feeder layer to successful long term- culture (Dirami *et al*. 1999).

There is an important problem in transplantation of stem cells. That is the preservation of SSCs. The culture of SSCs is a one way. *In vitro* culture of SSCs is suitable way to study of SSCs self-renewal, differentiation, and manipulation. There is no organization for SSC cultures more than 2 months in domestic animals, but short term SSC cultures have been reported in goat, pig, and cattle (Zheng *et al*. 2014). Another way for preservation of SSCs is the cryopreservation of these cells. Izadyar (2002) has reported a protocol for freezing of SSCs. In that method DMSO and FBS were used for cryopreservation and reached good results. Kanatsu-Shinohara (2003) reported that both fresh and cryopreserved SSCs can be colonized in received testis. FBS was used in cryopreservation of stem cells and had a positive effect on the viability of frozen cells (Yuan *et al*. 2009). In other study Jannat Alipoor (2010) reached a better result when DMSO was used instead of glycerol.

In this study a new method for the isolation of spermatogonia from prepubertal goat testes with high viability is obtained, and for the first time, the effect of FBS concentration on the cryopreservation of SSCs with a common medium for cell cryopreservation has been studied.

MATERIALS AND METHODS

Sampling

Two to three months old goat in Khalat-Poshan research center of the University of Tabriz were used. Ten replicates were performed. The animals had been locally anesthetized and testicular sperm extraction (TESE) technique was performed for getting samples from goats.

After incision of the testicular membrane, including the scrotal skin, Tunica dartos, parietal Tunica vaginalis, visceral Tunica vaginalis, and Tunica albogines, the seminiferous tubules were observed. Samples were transferred to the laboratory in Falcon tubes containing DMEM (Bio Idea, cat no. BI 1024-500) and AB medium at low temperature.

Cell-culture, cryopreservation and thawing

At first, the seminiferous tubules were minced into small pieces, and then stem cell mechanical digestion and enzymatic digestion were performed. This step was according to method of Izadyar (2002) with little modification. This method included two steps: Briefly, after mechanical digestion, the samples were left in DMEM containing 1 mg/mL collagenase (Gibco, 17100-017), 1 mg/mL trypsin, 1 mg/mL hyaluronidase type II and 5 µg/mL DNase I and were incubated at 37 ˚C for 60 min. At this first step interstitial cells were removed and seminiferous tubules were the product of this step (Figure 1A). Second digestion step was 45 min. In this step seminiferous tubules were incubated in DMEM that containing collagenase, hyaluronidase and DNase. For achieving the favored cell population, the obtained cellular suspension was centrifuged at 30 *g* for 2 min.

After isolation of SSC_s and co-culture with sertoli cells for 12 days, the samples were divided in two parts and were frozen using two solutions for cryopreservation. The first freezing medium contained 40% DMEM, 50% FBS (Gibco, cat no. 10270) and 10% DMSO (Gibco, cat no. 25200). The second freezing medium included 90% FBS and 10% DMSO. Viability rate of cells and number and area of colonies were measured in 4, 8 and 12 days of culturing after and before cryopreservation (Figure 1B).

The cryoprotected medium was added slowly to the cryotube that containe the cell suspension over a period of 10-15 minutes. Slow method for cryopreservation of cells was used. Eppendorf vial were put in a refrigerator at -3 ˚C for 1 hour and then transferred to a -20 ˚C freezer for 6 hours. Finally after 2-3 min of exposure to evaporate of nitrogen, cells were put in a liquid nitrogen tank at -196 ˚C for 1 month.

Cells were thawed after 1 month rapidly by swirling in a 37 ˚C water bath for 1 min. The contents of the vials were transferred to a Falcon tube.

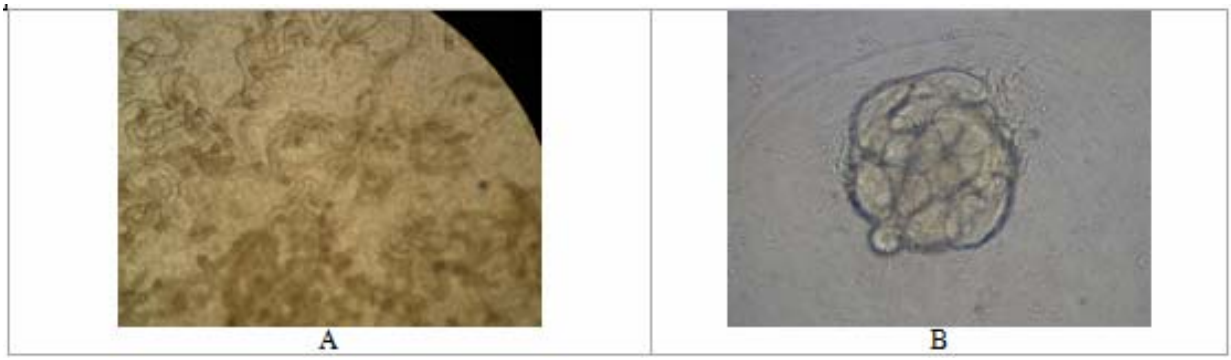

Figure 1 (A) Semineferous tubule; light microscope 40x
(B) Colony of spermatogonial cells and sertoli cell; inverted microscope 1000x

Then, DMEM containing 10% FBS was poured on the suspension of the cells. The Falcon was centrifuged and the cells were cultured ongoing. The viability of cells was measured immediately after thawing and the number and area of colonies were assayed 4, 8 and 12 days after culturing.

Viability of cells and number and area of colonies assessment

The viability of the cells was assayed before and after cryopreservation using trypan blue. Two kinds of colonies with different concentrations of FBS were counted one by one using an inverted microscope at days 4, 8 and 12 after cryopreservation. Areas of colonies that were cultured with different concentrations of FBS were also measured using the inverted microscope equipment at days 4, 8 and 12 after cryopreservation.

Identification of spermatogonial and Sertoli cells

Sertoli cells were identified with anti-vimentin as procedure of Tajik *et al.* (2010). Briefly, anti-vimentin diluted in tris-buffer saline/bovine serum albumin (TBS/BSA) (5 µg/mL and 2 µg/mL, respectively) was applied over slides for 60min at 25 ˚C. After washing as above, fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse Ig was diluted in TBS/BSA at a ratio of 1:50 and then incubated further continued for 45min at 25 ˚C. After washing with TBS/BSA, the slide was exposed to DAPI for 5 min. It was then washed again and mounted in PBS-glycerol 90% and examined under a fluorescence microscope.

Oct-4 is a marker for undifferentiated cells. Colonies of spermatogonial cells were immunocytochemically stained with anti-Oct4 conjugated with FITC. Briefly, anti-Oct4 (Abcam) was diluted in TBS/BSA and applied over slides for 60 min at 25 ˚C. After washing, donkey polyclonal secondary antibody to goat Ig G conjugated with FITC was added.

It was incubated for 45 min at 25 ˚C. After washing with TBS/BSA, it was mounted in PBS-glycerol 90%. Finally it was seen under a fluorescence microscope (Qasemi-Panahi *et al*. 2011; Dominguez *et al*. 2014).

Statistical analysis

The results were analyzed with SAS software (SAS, 2004). Statistical analysis was done by two-sample t-test. Differences were compared by Tukey-test and the difference was study when $P < 0.05$.

RESULTS AND DISCUSSION

Isolation and characterization of spermatogonia and Sertoli cells: After co-culturing, the sertoli cell population created a monolayer of cells. The sertoli cells were identified by their morphology.

Identification of cells: As is obvious form Figure 2, the spermatogonial cells are round cells and the sertoli cells are triangle cells. Immunocytochemistry showed that vimentin was detected in sertoli cells (Figure 2A).

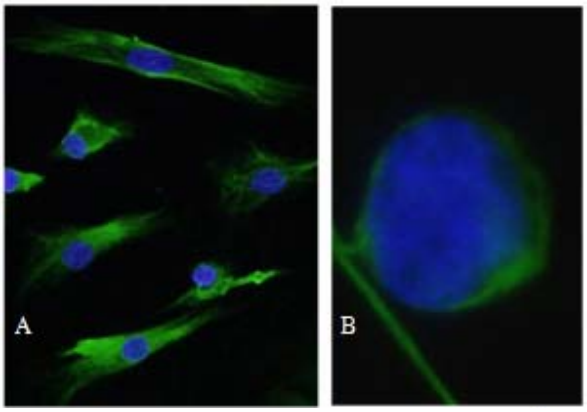

Figure 2 Immunocytochemistry tests
(left: A) sertoli cells (200x) and (right: B) spermatogonial cells (400x)
The volume of nucleus is greater in spermatogonial cells than in sertoli cells

Vimentin localized in the sertoli cells indicates expression of this intermediate filament protein in these cells. Spermatogonial cells shwed expression of Oct-4 by immunocytochemistry staining (Figure 2B).

The nuclei of both sertoli and spermatogonial cells were immunostained with DAPI (Figure 2A, B).

Cell growth and colony formation ability were assayed at 4, 8 and 12 days after co- culture (Figure 3).

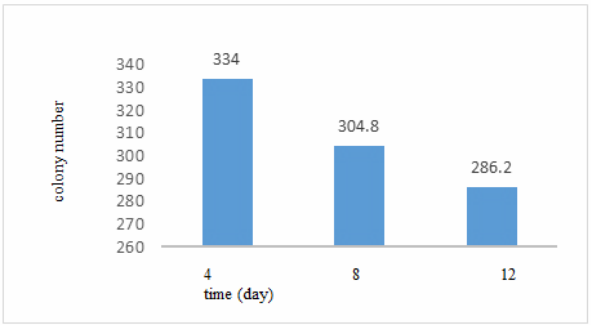

Figure 3 Relationship between colony number and time
There is no significant difference between colony number and different days of culturing

Trypan blue is a dye that penetrates dead cells. The viability of the cells after 12 days co-culture in DMEM with 10% FBS was > 93%.

Immunocytochemistry: As a result of this study, spermatogonia cell clumps were round with a size about 14 µm. The sertoli cells were pyramid-shaped. As mentioned, the first freezing medium contained 40% DMEM, 50% FBS and 10% DMSO; the second freezing medium included 0% DMEM, 90% FBS and 10% DMSO. So, two techniques for cryopreservation were performed. There was an obviously direct effect of serum concentration on the number of colonies developed at day 7 of culture. Accordingly, when the FBS was low, the number of colonies was significantly lower than in the other group (Figure 4).

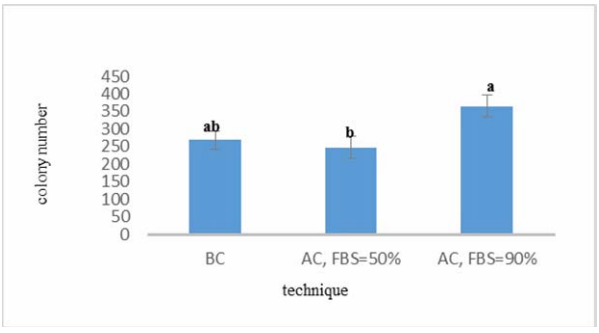

Figure 4 Relationship between colony number and cryopreservation technique
There is significant difference between colony number and cryopreservation technique
BC: before cryopreservation; AC: after 1 month cryopreservation and 12 days culture

Concentration of serum adversely affected *in vitro* culture of SSCs in the goat. They demonstrate for the first time that short term *in vitro* cultures of goat SSCs maintain their activity and allow them to proliferation and to form colony cells.

Assessments of colony numbers

Significant differences were found when data were analyzed according to the effect of cryopreservation techniques and subsequent culture time, and the interaction effects of cryopreservation technique and time. Increase of FBS from 50% to 90% for cryoprotect solution lead to increase of colony number (Figure 5). At both FBS concentration, colony numbers increased gradually over time.

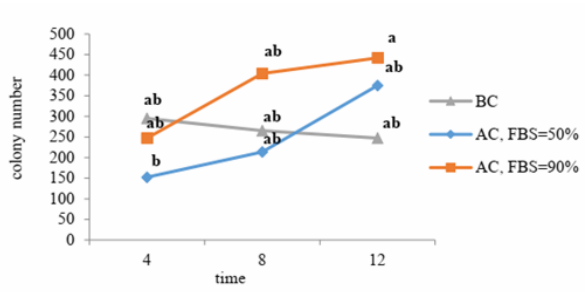

Figure 5 Relationship between interaction effects of cryopreservation technique × time on colony number
There is significant difference

Effect of cryopreservation technique on colony number

The average of colony numbers was 308.16 in every 25 cm^2 culture flask before cryopreservation and 220.72 in every 25 cm^2 culture flask after cryopreservation using 50% FBS and 296.52 in every 25 cm^2 culture flask using 90% FBS. Damage of cell membrane during freeze processing in presence of 50% FBS resulted in a decrease of colony numbers but when the concentration of FBS was 90%, the colony numbers were even higher after 1 month cryopreservation (Figure 4).

Time effects on colony numbers

The effect of time was significant on colony number; during 12 days culturing the number of colonies gradually increased for both cryopreservation conditions (Figure 5).

Time and cryopreservation technique interaction effects on colony numbers

There is significant difference in relationship between interaction effects of cryopreservation technique and time on colony number.

Assessments of colony area

According to results of data analysis, the effect of techniq-

ues, time, and interaction effects of technique and time were insignificant when $P < 0.05$ but interaction effects of technique and time were significant when $P < 0.1$ (Figure 6).

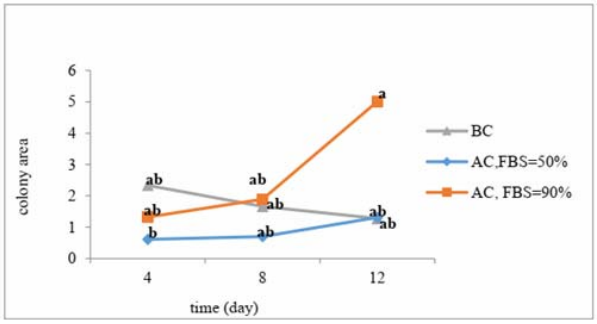

Figure 6 Relationship between interaction effects of cryopreservation technique × time on colony areas
BC: before cryopreservation and AC: after cryopreservation

Assessments of cells viability

The viability of cells was 93% before cryopreservation. The viability rate of cells that had been cryopreserved by 50% and 90% FBS, was 61.17% and 73.67% respectively (Figure 7).

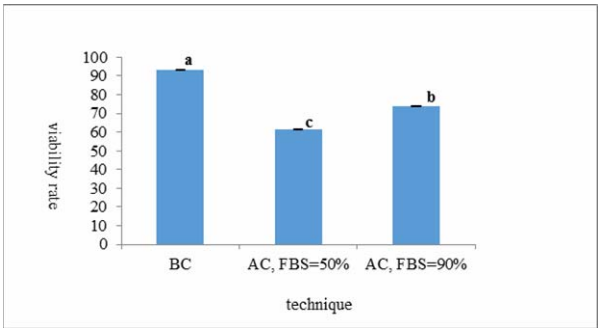

Figure 7 Relationship between cryopreservation technique and viability rate of spermatogoginal cells
There is significant difference

Isolation and culture of human spermatogonial stem cells was done from TESE testicular biopsies (Goossens *et al.* 2013; Goharbakhsh *et al.* 2013) and in this study TESE technique was performed for getting samples from goats.

The first colony formation observed at day 4 of culture in different treatment groups (Bahadorani *et al.* 2012) and in this study the colonies of spermatogonial cells could be observed in day of 4, too.

Damage of cell membrane in freeze processing was cause of decreasing of colony numbers. When the concentration of FBS was high, the colony numbers were high after cryopreservation. This is in agreement with that reported by Bahadorani *et al.* (2012) serum may contain factors that do as anti-apoptotic.

When a cryoprotectants medium was contain of 2.5% PEG (polyethylene glycol) (MW 1000), DMSO and FBS, cryopreservation of murine SSCs was significantly improved (Lee *et al.* 2013).

The effect of time on colonies number was significant on colony number; meaning gradually after culturing, the number of colonies increased, agreeing with the result of Koruji *et al.* (2007). The cause of this result is that colonies need time to increase.

The viability of cells was 93% before cryopreservation, in agreement with previous studies (Anjoamrooz *et al.* 2006; Izadyar, 2002). The viability rate of cells after thawing with high concentrations of FBS was higher because there are high lipopolysacarid and lipoprotein levels in the serum confering resistance in cell membranes. When Izadyar (2002) used sacaroz in the cryopreservation process, the viability rate of cells was 68.3. In this study the viability rate was 73.67%; that is important (Figure7).

If the interaction of time and technique was insignificant, the diagrams would be parallel but the diagrams crossed together. Increasing of spermatogonial cells may be the result of less resistance of sertoli cells to cryopreservation processes. The sertoli cells are sensitive to the cryopreservation process, but spermatogonial cells are resistant to this process, so colony number increased (Figure 5).

CONCLUSION

This study demonstrated that serum concentration had an obviously positive effect on the maintenance and proliferation of SSCs in culture after cryopreservation.

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