Assessment of the effect of different concentration of foetal bovine serum (FBS) in freezing media on the viability of spermatogonial stem cells

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Abstract

An in vitro system that supports spermatogonial stem cells survival and proliferation is useful for enhancement of stem cell number and efficient transplantation. The purpose of this study was to assess the effect of different concentrations of foetal bovine serum on survival of bovine spermatogonial stem cells and Sertoli cells after cryopreservation. Samples were surgically obtained from testes of 3-5 months old calves and digested twice in enzymatic media to get the isolated cells. These cells were co-cultured for two weeks to obtain the adequate amount of cells. In the freezing step, the purified spermatogonial stem cells were frozen in four different freezing media including bio-freeze medium® as Control and 3 other groups containing: Dulbecco’s modified Eagle’s medium (DMEM) and 50%, 70% and 90% fetal bovine serum (FBS) plus 10% dimethyl-sulfoxide (DMSO) as treatments. After 2 weeks of preserving in liquid nitrogen, to assess the viability, the cells were rapidly thawed by swirling in 38°C water bath for two minutes. The viability of the cells was assessed by using flow cytometry technique. The results showed that the frozen cells in 50 and 70% FBS groups had significantly (P<0.05) higher viability rates than control and 90% FBS groups. It is concluded that spermatogonial stem cells can be cryopreserved in DMEM plus 10% DMSO and 50 to 70% FBS.

Keywords: Spermatogonial stem cell; Sertoli cell; cryo-preservation; viability; freezing medium

Introduction

Bovine spermatogonial stem cells (SSCs) have unique potential to both self-renewal and reproduction of differentiated daughter cells, which will ultimately form spermatozoa (Oatley and Brinster, 2006). SSCs are the unique stem cells which are capable of transmitting genetical information to subsequent generations (Dobrinski, 2006). A short-term co-culture with Sertoli cells showed a significant increase in number and diameter of colonies in compared with other techniques (Koruji et al., 2009). It was shown that the number of SSCs is very low presumably ≈1 in 3000-4000 cells in adult testes compared with young ones (Tegelenbosch and de Rooij, 1993).
Scientists have reported that post freezing and thawing SSCs had still their fertility and could reinitiate spermatogenesis with full functional ability (Izadyar et al., 2002; Wyns et al., 2008). Cryo-preservation of spermatogonia has many advantages over cryopreservation of spermatozoa (Koruji et al., 2007). Cryo-preservation of SSCs may be the single way to preserve fertility in young patients who get chemotherapy and anticancer drugs (Ogawa, 2001). The closest consensus for freezing of SSCs is using 5-10% dimethyl-sulfoxide (DMSO), which was added quickly, and then cooled at 1-2.5°C/min, was followed by rapid thawing (Woods et al., 2007). Additionally, frozen testis cells in comparison with fresh testis cells contain significantly higher stem cell concentration (Koruji et al., 2007; Wyns et al., 2007), as well as SSCs survival in the freeze-thawing process better than other cell types (Kanatsu-Shinohara et al., 2003).

It has been reported that foetal bovine serum (FBS) added to cryo-preservation media had significant effect on cell survival after thawing (Freshney, 2006; Alipoor et al., 2009). Furthermore, it has been shown that DMSO could significantly support the cell viability comparable to other cryoprotectants (Jahnukainen et al., 2007; Goossens et al., 2008). On the other hand it is postulated that the DMSO, Dulbecco’s modified Eagle’s medium (DMEM) and FBS have synergistic effect on cell viability (Izadyar et al., 2002; Freshney, 2006).

In the present study, the viability rate of bovine spermatogonia stem cells has been investigated after cryo-preservation in DMEM supplemented with DMSO and different concentration of FBS. Also, the effects of different concentration of FBS on cell viability were investigated to find out the best foetal bovine serum concentration.

Materials and Methods

Samples

Five healthy pre-pubertal Holstein bulls about 3-5 months of age were selected from the Aminabad Research Institute, University of Tehran. Above mentioned age range was selected to have more chance to get type A spermatogonia germ cells (Aponte and de Rooij, 2008). This study was conducted in accordance with Animal Ethics Committee of the University of Tehran. Samples (approximately 1 cm³ each) from decapsulated testis were placed in 15 ml falcon tube (TPP, Trasadingen, Switzerland) containing DMEM (Sigma Chemical Co, St. Louis, MO, USA) supplemented with 10% FBS (Biochrom AG, Berlin, Germany), 100 IU/ml penicillin and 100 mg/ml streptomycin. Sample tubes were transferred to the laboratory, near ice bag within 2 hours.

Two-step enzymatic digestion was used to isolate cells as described by Van Pelt et al. (1996) with some modification. First, the testis samples were transferred to sterile petri dishes and were cut mechanically into small pieces. After washing with PBS, first enzymatic digestion step was done in which small pieces of testis suspended in DMEM contained 1 mg/ml collagenase type 1 (Biochrom, AG Berlin, Germany), 1 mg/ml trypsin and 1 mg/ml hyaluronidase (Sigma Chemical Co, St. Louis, MO, USA) and were incubated at 37°C for an hour in a humidified atmosphere containing 5% CO₂. To remove interstitial cells, the suspensions were centrifuged three times at 1400 g for 2 min at room temperature. The supernatant was replaced by fresh media and mixed with the precipitant each time. For second digestion step, the suspensions which contained the released seminiferous cord fragment were incubated with fresh enzymes as described above for 15 min at 37°C in a humidified atmosphere containing 5% CO₂. For hastening of dispersion and separation, the seminiferous tubules were gently agitated by a Pasteur Pipette. The enzymatic reaction was halted by adding 10% FBS and centrifuged at 800 g for 5 min. The supernatant were carefully removed and centrifuged again at 2000 g for 1 min at room temperature. The obtained cells which separated from the tubule fragments were used for cell culture.

Cell culture

The cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂ as previously described by Tajik et al. (2012). Co-culture of Sertoli cells and SSCs lasted for two weeks.

Spermatogonia stem cells isolation

The cultured cells were detached from 25 cm² cell culture flasks (Greiner, Frickenhausen, Germany) by using trypsin and then enzymatic reaction neutralized by DMEM medium containing 20% FBS. Spermatogonia stem cells were extracted from Sertoli cells as previously described by Scarpino et al. (1998) with little modification. Briefly, culture dishes were coated with 5 µg/ml of Datura Stramonium agglutinin-Lectin (DSA; Sigma Chemical Co, St Louis, MO, USA) in phosphate-buffered saline (PBS) at 37°C for an hour. The cell suspension was placed on lectin-coated dishes and incubated for an hour at 37°C in a humidified atmosphere of 5% CO₂. The non-adhering cells were collected and were incubated with DMEM plus 10% FBS.

Recognition of bovine SSCs from Sertoli cells

The presence of Vimentin protein was confirmed on firmly attached Sertoli cells to lectin-coated dishes by using anti-vimentin mouse antibody (Abcam, Cambridge, UK) optimally diluted 1:500 in TBS/BSA (5µg/ml and 2µg/ml, respectively) at room temperature.
for 60 min. After washing, sheep anti-mouse secondary antibody conjugated with Fluorescein isothiocyanate (Sigma Chemical Co, St Louis, MO, USA) was diluted in TBS/BSA in a ratio of 1:50 and added in media and incubated for 45 min at room temperature. After three times washing with TBS/BSA, the cells were exposed to 0.1 µg/ml DAPI (4′-6-diamidino-2-phenylindole dihydrochloride; Calbiochem, Nottingham, UK) for 5 min. After washing with TBS/BSA and mounting by PBS-glycerol 90%, the slide was observed under a fluorescence microscope (Olympus, Tokyo, Japan). Vimentin positive cells indicated the Sertoli cells properly isolated (Fig 1).

In order to identify bovine spermatogonia stem cells among the non-adhering cells to lectin-coated dishes, immunocytochemical staining with anti-Oct-4 conjugated with FITC was used according to Koruji et al. (2009) with some modification. Briefly, anti-Oct-4 (Abcam, Cambridge, UK) diluted in TBS/BSA, was applied onto cells for 60 min at room temperature. After washing, FITC conjugated polyclonal donkey anti Goat IgG (Sigma Chemical Co, St Louis, MO, USA) was added. Incubation continued at room temperature for 45 min. Following washing with TBS/BSA, the cells were counterstained by DAPI (Calbiochem, Nottingham, UK) at 0.1 µg/ml for 5 min. After washing with TBS/BSA and mounting by PBS-glycerol 90%, the slide was observed under a fluorescence microscope (Fig. 2).

**Cells freezing**

After two weeks of co-culture, cells were divided into four different groups:

1. Control: Bio-freeze medium Biochrom AG® (Biochrom AG, Berlin, Germany) without DMSO and FBS
2. Group A: 50% FBS, 40% DMEM and 10% DMSO (Sigma Chemical Co, St Louis, MO, USA)
3. Group B: 70% FBS, 20% DMEM and 10% DMSO
4. Group C: 90% FBS and 10% DMSO

The detached cells were resuspended in 1 ml of each freezing medium within 15 min and were then transferred into 2 ml sterile cryo-tubes (Greiner, Germany). Freezing was initiated immediately. Cryo-tubes were wrapped in sterile bandages that previously pre-cooled for approximately 30 min in the freezer at -24°C and put in the pre-cooled polystyrene box (thickness approximately 10 mm, closable). Immediately the closed polystyrene box was put into the freezer at -24°C, at least for 2 hours. Unwrapped cryo-tubes were then placed in the freezer at -24°C for one hour. Subsequently, unwrapped cryo-tubes were hanging in the nitrogen vapour for 20 min and then cryo-tubes were plunged into the liquid nitrogen tank and were kept for approximately two weeks.

**Thawing procedure**

After two weeks of cryo-preservation, the cells were thawed by immersing the cryo-tubes into 38°C water bath for 2 min, as reported by Izadyar et al. (2002).

**Flow cytometric analysis**

Fluorescein-isothiocyanate (FITC)-labelled Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for negatively charged phosphatidylserine that was exposed to external environment was used to evaluate apoptosis cells (Andree et al., 1990). To obtain more accurate results, flow-cytometric techniques with technical data sheet of FITC Annexin V Apoptosis Detection Kit II (BD Biosciences Pharmingen, San Diego, CA, USA) were
followed. The experiment was repeated five times and in every instance, 10000 cells from content of each cryo-tube were counted by flow cytometry.

**Statistical analysis**

Data were analyzed by ANOVA and those which had statistically insignificant effects were followed by Duncan's multiple range test.

**Results**

When FBS concentration increased from 50% to 70% in Group A and B, survival rate was increased and viability of cells in these concentrations was higher than Control and Group C (P<0.05). The lowest rate of late apoptosis was observed in Group B which had maximum rate of live cells and the highest rate of late apoptosis was observed in group C which had the minimum rate of live cells (Table 1).

**Table 1: Comparison of cell viability and apoptosis between control and experimental groups after freezing (Mean±SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Live</th>
<th>Early apoptosis</th>
<th>Late apoptosis</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65.42±1.86 a</td>
<td>5.92±2.88</td>
<td>9.35±2.69</td>
<td>19.31±5.41</td>
</tr>
<tr>
<td>A</td>
<td>74.41±3.1 b</td>
<td>2.78±1.32</td>
<td>3.27±1.8 ab</td>
<td>19.54±0.34</td>
</tr>
<tr>
<td>B</td>
<td>77.48±2.38 b</td>
<td>1.23±0.52</td>
<td>1.30±0.49 b</td>
<td>19.98±1.94</td>
</tr>
<tr>
<td>C</td>
<td>52.74±2.53 c</td>
<td>8.43±4.52</td>
<td>19.98±1.94</td>
<td>20.21±5.25</td>
</tr>
</tbody>
</table>

* Control: Bio-freeze medium; Group A: 50% FBS + 40% DMEM + 10% DMSO; Group B: 70% FBS + 20% DMEM + 10% DMSO; Group C: 90% FBS + 10% DMSO; a-c: Values in the same column with different superscripts are significantly different (P<0.05).

**Discussion**

Jahnukainen et al. (2007) obtained 73% viability for SSCs in testicular tissue by using cryo-preservation media containing 10% foetal calf serum (FCS) and 1.4 M DMSO. Redden et al. (2009) recorded 46.3±2.2% viability by MEM based freezing medium supplemented with 10% FCS. In a study by Izadyar et al. (2002) increased FBS concentration from 10% to 20% was observed with no significant difference in the survival rate of bovine type A spermatogonia. In our present study, there was no difference in cell surviving in 50% and 70% FBS in freeze medium. In contrast, Alipoor et al. (2009) observed that the SSCs cryopreserved in 70% FBS had more viability rate than 50% FBS in freezing medium. The rates of dead cells are the same in all groups whereas the rates of apoptosis are different, it can indicate that freezing process may just affect the apoptosis.

**Conclusions**

Since in the groups with no DMEM (control and group C), lower survival rate was observed, it can be concluded that cells need some nutrient to stay alive in freezing medium. Our research also showed that higher concentrations up to 70% FBS have better cell viability.

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**References**


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