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ANIMAL EXPERIMENTATION

Achieving high survival rate following cryopreservation after isolation of prepubertal mouse spermatogonial cells

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Abstract

Purpose Isolating spermatogonia cells with high purity and viability and achieving better survival rate following cryopreservation

Methods Isolating the cells by Magnetic Activating Cell Sorting (MACS) method using anti CD49f (*α6 integrin*) antibody and Dynabeads and freezing in DMSO-based freezing mediums containing three different FBS concentrations of 50%, 60% and 70%.

Results The mean (±SD) purity of the isolated cells was 92.52±3.57 (range 92.43–98.25). The cells frozen in group I, II and III had mean 39.60±1.48 (range 37.98–41.62), 89.05±3.83 (range 80.83–90.33) and 90.52±1.71 (range 89.07–92.52) viability, respectively.

Conclusion Higher viable cell counts and purity can be attained by the use of α6 integrin and magnetic beads. After the thawing of spermatogonial cells, optimum viability was achieved in freezing media containing 60% FBS.

Keywords Spermatogonia cells · Magnetic activated cell sorting · Cryopreservation · Infertility

Capsule In this study spermatogonia cells from mouse testis were isolated by MACS method and the effect of FBS concentration on cell survival was assessed during cryopreservation.

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Introduction

Spermatogenesis is the paradigm of development that continues throughout the adult life in most mammals. This process occurs in seminiferous tubules containing an epithelium populated by a mixture of germ cells and Sertoli cells [1]. Spermatogenesis is a complex process that originates in a small population of Spermatogonial Stem Cells. Male fertility depends on SSCs which are the only stem cells in adults that divide to contribute genes to subsequent generations [2].

Approximately 1 in 475 children will develop cancer before reaching the age of 15 [3]. Therapeutic advance in the last two decades has improved the long-term survival rate of patients with cancer. More than 60% of patients treated for their malignancies are now expected to live at least 5 years [4]. Chemo- and radiotherapy cure rates of as high as 80% have also been reported [5].

These treatments destroy malignant cells, but also, unfortunately, have many cytotoxic effects on the rapidly dividing spermatogonia. As a result, spermatogenic failure and infertility may occur during adulthood [6–9].

Adolescents and adult men have the option of cryobanking their semen before cancer treatment, but prepubertal boys can not benefit from this approach since they don't have complete spermatogenesis [10].

Recently, however, alternative strategies for preserving fertility have been introduced, including transplantation of SSCs and grafting of testicular tissue [10].

One possibility of fertility preservation is autologous germ cell transplantation. This technique was introduced by Zimmerman and Brinster [11], and may become a method with potential clinical use for fertility protection. Transplantation has already proved to be successful for SSCs in mice, rats and recently even in primates [12]. In the future

SSCs may be reintroduced into the testis of oncological patients to re-induce spermatogenesis [9, 13, 14]. Isolating SSCs with high purity and preserving these cells until the patient has been treated for cancer are essential for SSCs transplantation. The important clinical concern for spermatogonia transplantation is the low number of SSCs [8].

Initial attempts at isolating SSCs started with the isolation of "type A" spermatogonia (containing SSCs) by enzymatic digestion of testicular tissue [15–18]. This initial step has been combined with different techniques, such as the use of prepubertal [17, 19], cryptorchid [20–23], and vitamin A deficient animal models [18, 24]; discontinuous density gradient [18]; differential plating [17, 25]; fluorescent activated cell sorting (FACS) [26, 27]; and MACS [22, 23, 28, 29].

Another problem in transplantation is the preservation of SSCs. One option is the culture of SSCs. Culture of SSCs by both short- and long-term culture have been reported (for review see [30]). Another option is the cryopreservation of these cells. Izadyar et al have reported a protocol for cryopreservation of SSCs [31]. Kanatsu-Shinohara et al showed that both fresh and cryopreserved SSCs can be colonized in received testis [32].

In this study a new method is suggested for the isolation of spermatogonia from prepubertal mouse testis with high viability and purity, and for the first time, the effect of FBS concentration on SSCs cryopreservation with a common medium for cell freezing has been assessed.

Materials and methods

Animals Male neonatal (6 days of age; n=200) Naval Medical Research Institute (NMRI) mice, derived from original stocks obtained from Pasteur Institute (Tehran, Iran).

Cell isolation

Bilateral testes from 15 neonatal 6-day old NMRI mice were collected for cell suspension. The collected testes were transferred to a laminar flow hood in a laboratory on ice within 15 min.

The testes were decapsulated and washed twice in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12) medium containing 40 µg/mL gentamicin and 100 IU/mL penicillin G and 100 µg/mL streptomycin (all purchased from Sigma).

Step 1: The testes were placed in 1 ml of DMEM/F12 containing 1 mg/ml Collagenase type I (Sigma), 250 mg/mL trypsin (Gibco) and 50 µg/mL DNase I (Roche) for 12 min (shaken gently) at 39–40°C.

After washing in DMEM/F12 containing 10% FBS and only DMEM/F12, most interstitial cells were removed. The isolated seminiferous tubules were observed under light microscope.

Step 2: Seminiferous tubules digestion was performed to obtain single cell suspension in 1 ml of DMEM/F12 containing the same enzymes as step 1 plus 1 mg/mL Hyaluronidase type I (Sigma) and 0.05 M EDTA (Sigma). After 8 min at 39–40°C, 2 mL DMEM/F12 containing 15% FBS was added to neutralize the effect of the enzymes, and the cell suspension was filtered through a 40 µm nylon filter mesh (BD Biosciences).

The medium was removed after centrifuge and the cells were washed twice and transferred to fresh medium. Viability was assessed by adding 10 µg/mL propidium iodide (PI, Sigma) in 1 mL cell suspension in the dark and analyzed by flow cytometer after 10 min.

Spermatogonia cell separation by MACS method

After filtration, spermatogonial cell separation was performed according to Shinohara et al [33] and Dynal Biotech factory instructions with some modifications.

Briefly, for positive selection, 4×10^7 cells were suspended in buffer 1 (100 µL HSA[Vitrolife,USA], 99/9 µL PBS) and 10 µL of primary antibody (Rat Anti human/mouse CD49f (Serotec)) was added. After 20 min in 4°C, two volumes of buffer 1 were added and centrifuged in 300 g for 8 min at 4°C.

The supernatant was removed and 25 µL secondary antibody, M450 sheep Anti Rat IgG (Dynal Biotech), was added. After 20 min the same volume of buffer 1 was added and the tube was placed on magnetic field (MiniMacs, Miltenyi, USA). The extra medium was removed and the cells were washed four times with buffer and suspended in fresh medium, and the viability was assessed by PI. The majority of micro beads were detached from the cells after being placed in a 37°C and 5% CO_2 incubator for 3 h to 4 h.

Cell purity

Flow cytometric analysis was performed on enriched population of testis cells by standard procedures [34]. Briefly, after magnetic isolation, 1×10^5 cells were fixed in 100 mL paraformaldehide 4% for 10 min and then divided into two tubes, one for test and another for control. 500 µL of blocking serum (goat serum, 10%) was added to every tube, followed by 5 µL of primary antibody after 30 min. Rat Anti human/mouse CD49f was added to the test group

and washed after 25 min with washing buffer(1%FBS in PBS) and then 1 μL of secondary antibody (FITC Anti Rat IgG), was added to both tubes. Finally the cells were washed and 500 μL Paraformaldehide 1% containing 1% FBS was added and analyzed by flow cytometer. All flow cytometry analyses were performed by Partec (PAS Model, Germany).

Freezing and thawing of spermatogonia cells

The isolated cells with magnetic beads were cryopreserved in three groups by the procedure described below:

Group 1: freezing medium containing 10% DMSO (dimethyl sulfoxide, Sigma), 50% FBS in DMEM/F12 medium.
Group 2: freezing medium containing 10% DMSO, 60% FBS in DMEM/F12 medium.
Group 3: freezing medium containing 10% DMSO, 70% FBS in DMEM/F12 medium.

In each experiment the cryovials and freezing medium were cooled at −20°C. The cooled freezing medium was added to1×10^6 cells/mL and placed in a cryobox and transferred to −70°C. After 24 h the cells were transferred to −196°C liquid nitrogen within a transfer time of maximal 25 s. After 15 days the frozen cryovials were placed in a 37°C water bath. Just before the entire medium thawed 10 ml of DMEM/F12 was gently added to the thawed freeze medium and cells to neutralize the effect of the freezing medium. Viability assessment was then performed by PI.

Statistical analysis

In each step a minimum of five experiments were performed. The data was analyzed by SPSS 13.5 software and results have been expressed as median, SD and also mean and standard error have been presented (Table 1). Normality of values distribution was tested by Kolmogorov-Smirnov test. The statistical difference between groups was determined by the non parametrical Kruskal-Wallis test and for paired comparison non parametrical Mann–Whitney test was used, considering $P<0.05$ as significant.

Results

The first and second steps of enzymatic digestion were assessed under light microscopy.

It was observed that seminiferous tubules had been successfully separated in the first step (Fig. 1a) and the cells were almost completely single after the second step (Fig. 1b). The viability of the cells after enzymatic digestion was 90.84±5.19 (range 81.44–95.33) (Table 1). Fig. 1c and 1d show the cells with attached microbeads separated in a magnetic field and cells that did not attach to microbeads respectively. The purity of spermatogonia cells was 92.52±3.57 (range 92.43–98.25), which was assessed by flow cytometry using Rat Anti human/mouse CD49f antibody, which had been utilized for the separation of these cells (Fig. 2).

The viability of separated cells by MACS method assessed by PI was 96.20±2.27 (range 92.33–98.20) (Table 1).

After freezing and thawing, the viability of the cells was assessed by PI and the results were mean (±SD) 39.60± 1.48 (range 37.98–41.62), 89.05±3.83 (range 80.83–90.33) and 90.52±1.71 (range 89.07–92.52) in Groups 1, 2 and 3 respectively.

These data show that after the addition of 10% more FBS to Group 1, a viability of up to 89% was observed which significantly differs with the 50% FBS concentration (P=0,004), but adding 10% more FBS to group 2 did not result in any significant difference (p=0.056)(Table 1).

Discussion

At present, prepubertal cancerous patients have no option to preserve their fertility after cancer therapy, although sperm cryopreservation has proven to be a valuable pro-

Table 1 Viability of cells after enzymatic digestion, MACS isolation and after thawing of cryopreserved cells

Cells in different steps	Mean	Median	SE	SD	Range
Cells after enzymatic digestion	89.87	90.84	2.32	5.19	81.44–95.33
Cells after MACS isolation	95.89	96.20	1.01	2.27	92.33–98.20
Thawed cells in Group I	39.66[a]	39.60	0.66	1.48	37.98–41.62
Thawed cells in Group II	87.60[a,b]	89.05	1.71	3.83	80.83–90.33
Thawed cells in Group III	90.52[a,b]	90.52	0.61	1.37	89.07–92.52

[a] There were significant differences between Groups I and II (P=0.004) and also between Groups I and III (P=0.008), [b] There was not a significant difference between Groups II and III (P=0.004)

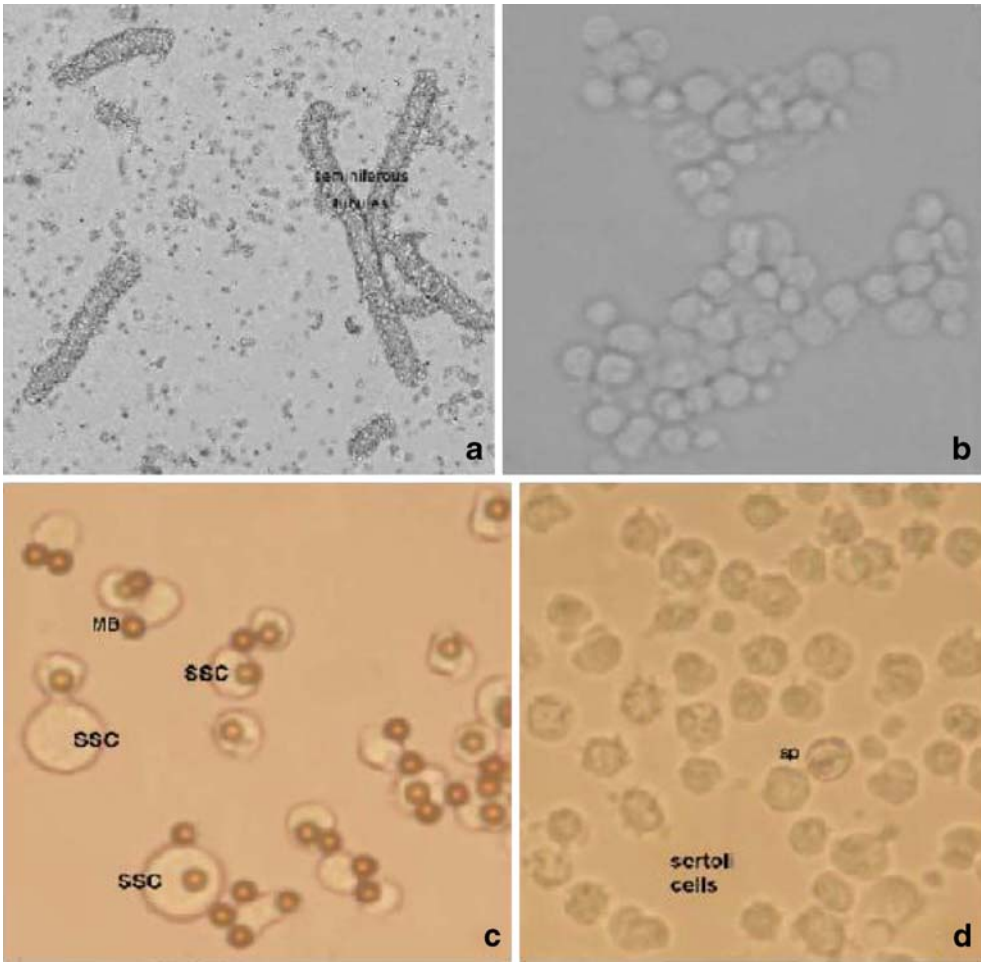

Fig. 1 **a** Seminiferous tubules after first step enzymatic digestion, magnification 100 ×s. **b** isolated cells after second enzymatic digestion. Magnification 400 ×s. **c** the cells after isolation by MACS method; these cells attached to magnetic beads and separated, magnification 1000×. **d** the cells that didn't attach to magnetic beads and removed, magnification 1,000×

cedure for the management of infertility in oncology patients [35–38].

Testicular stem cell transplantation may become a promising technique for prepubertal cancer patients [39]. Spermatogonial stem cells represent an attractive cell population for preservation of male germ lines. These cells, due to their self-renewal activities, have an enormous capacity to regenerate from a small basal population, and to produce limitless numbers of spermatozoa[40, 41]. Rapid and effective preparation of a pure population of spermatogonial stem cells from prepubertal and adult animals is the basis for in vitro experiments and attempts at germ cell transplantation [28]. In the past isolation procedures using elutriation [42] or velocity sedimentation [43–45] have been described. With both these methods, a cell population containing up to 90% spermatogonia can be obtained. With the isolating method using centrifugation in discontinuous gradient described by Morenea [18], up to 85% spermatogonia have been isolated. In 1999 Von Schonfeldt et al [29] reported a fast and effective method for isolating spermatogonia from a variety of species, including mouse. They use magnetic beads coupled to anti C-kit antibody, which recognize the C-kit receptor at the surface of type A spermatogonia. The authors stained the isolated cells with FITC and PI, and analyzed them by flow cytometry [29]. In 2001 Vonder et al [28] used EPCAM and C-kit for isolating spermatogonial stem cells in prepubertal and adult mouse testis. Several different cell types in the adult mouse (e.g. leydig cells and spermatocysts) express C-kit receptor, and spermatogonia were enriched only up to 54% [28]. T.

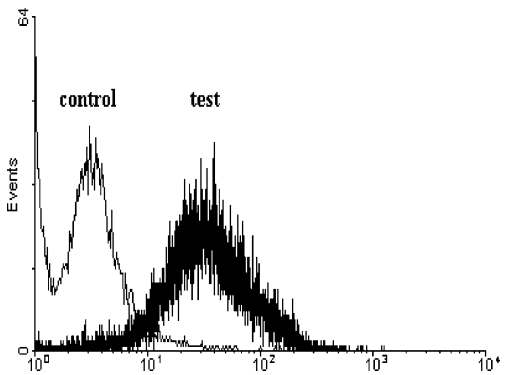

Fig. 2 Cell purity analyzed by flow cytometry

Shinohara et al showed that α6 and β1 integrins are the surface markers of Spermatogonial stem cells. They showed with flow cytometry analysis that β1 and α6 integrins enriched cell population but did not indicate a high number of C-kit positive cells in these integrin-selected cells. Moreover they observed that cells selected by α6 integrin antibody appeared to be twice as effective in colonizing recipient testes as those selected by the β1 integrin antibody [33]. Recently M.Geens et al used CD49F (α6 integrin) antibody by MACS and FACS methods for the enrichment of spermatogonial stem cells from tumor-contaminated testis cells in mouse and humans. [39]. In this study we used Rat Anti human/mouse CD49F antibody which recognizes α6 integrin on the surface of spermatogonia cells. We assessed the purity of the cells that express α6 integrin in their surface, by flow cytometry. FITC conjugated antibody was used as secondary antibody. In comparison to previous studies we had a higher number of viable cells after enzymatic separation (91/66%±0/60) and we achieved better viability in contradiction to other studies by increasing the incubating temperature for enzymatic digestion. In comparison to previous studies we had higher viability (95/25%±0/33 instead of 65%) after MACS isolation, which can be related to the separation of the cells in low temperature (4°C) [39].

After isolation of SSCs from testis, these cells can be preserved in two ways; long term culture and cryopreservation. Short- and long- term culture of SSCs has been described before. An alternative and probably the best method for long-term preservation of SSCs is cryopreservation [31].

Spermatogonial stem cells are easy to handle, as the same protocol and inexpensive equipment may be used to freeze SSCs from a variety of species, including rats, hamsters, cattle, primates and humans [31, 46–51]. Thus SSCs have several unique advantages over frozen sperm. it has been attempted to develop a cryopreservation protocol for type A spermatogonia from bovine [31]. SSCs could reinitiate spermatogenesis after cryopreservation and reintroduction into the seminiferous tubules of infertile recipient males, and this raised the possibility of banking frozen stem cells for male infertility treatment [32]. Moreover it has been shown that germ cells from frozen-thawed stem cells are fertile [32].

It also has been shown that spermatogonial stem cells are relatively resistant to freezing compared with other spermatogenic cells, and suggested that any freezing protocol commonly used for somatic cells would be applicable for stem cells. In an experiment that used fresh and frozen testis cells for transplantation, it was shown that frozen testis cells contain significantly higher stem cell concentration than fresh testis cells [52].

In previous studies a commercially available cryopreservation solution was used for freezing SSCs. This solution was also used for freezing testis pieces [52]. Izadyar et al used a solution containing DMSO, FCS and sucrose for freezing SSCs which showed that freezing with DMSO-based medium results in higher viability after thawing than glycerol-based medium [31]. It has been proved that a rich medium such as DMEM/F12 has a better effect on primary cell culture, and FBS often gives better survival rate than FCS or horse serum [53]. Therefore, in this study FBS was utilized in the cryopreservation solution. After thawing and staining by PI as shown in Table 1, we observed that the cells cryopreserved by 50% FBS did not have reliable viability, but using 60% FBS in the freeze medium had significant effect on viability. Using 70% FBS did not have any significant effect on cell survival after thawing.

Considering previous studies we suggest that FBS with 60% concentration in the cryopreservation solution of spermatogonial cells with DMSO-based medium has a beneficial effect on the viability of these cells after thawing than any other serum concentration. FBS also had a better effect than any other serum types, including FCS.

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