Original Article

Combination of *In Vivo* Cryptorchid Testis and *In Vitro* Co-Culture System to Obtain High Purification and Proliferation of Mouse Spermatogonial Stem Cells

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Abstract

Background: The present study was designed to evaluate the survival and proliferation of spermatogonial stem cells from cryptorchid mouse testis in co-culture system over a 3 weeks period.
Materials and Methods: Sertoli and spermatogonial cells were isolated from bilateral cryptorchid mouse model testes. Isolated spermatogonial cells were co-cultured with Sertoli cells in minimal essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS) for three weeks. The identity of the cells was confirmed through immunocytochemistry against Oct-4 and Vimentin.
Results: Best results were achieved from the co-culture system spermatogonia which continued to proliferate, and eventually, type A spermatogonia colonies were found. Most of the cells in these colonies were Oct-4 positive.
Conclusion: Bilateral cryptorchid surgery model is a good model for enrichment of spermatogonial stem cells (SSCs). These cells can be used for molecular characterization, genetic manipulation and restoration of male fertility.

Keywords: Co-Culture, Colonization, Spermatogonial, Cryptorchidism

Introduction

During spermatogenesis the spermatogonial stem cells (SSCs), which undergo self-renewal throughout the animal's adult life, go through complicated differentiation steps to produce progeny cells. As in other rare stem cell populations enrichment strategies are required to study SSC morphological and biochemical characteristics (1). Several approaches to enrich stem cells have been attempted. Bellve, et al (2) obtained a 90% pure fraction of type A spermatogonia from immature mice. Similarly, highly purified spermatogonia have been obtained from immature rat and pig testes (3, 4). In addition, vitamin A-deficient animals can be used as a source of spermatogonia as, in these animals, spermatogenesis stops at a spermatogonial differentiation step (5). Either testis hyperthermia or cryptorchidism may be used to increase the percentage of SSCs *in vivo*. Although these experimental conditions cause depletion of more differentiated germ cells, they do not appear to affect SSCs survival and biological activity, thus resulting in an increase in the ratio of SSCs versus the total number of germ cells (6). In this investigation, we use an enrichment strategy combining the *in vivo* cryptorchid testis with an *in vitro* co-culture system. To date, various culture systems for viability, survival and proliferation of spermatogonia of various species have been established. These systems include: co-culture with feeder cell monolayer Sertoli cells or STO (7), adding serum to culture medium (8), serum-free culture system that is cultured in a potassium-rich medium called KSOM (9) and supplementation with various growth factors (10, 11). In the present study, we have created a method in which this enriched population of SSCs is used to develop a co-culture system that consists of Sertoli cell feeders for an extended period of time, during which spermatogonia proliferate and form colonies.

A major impediment to our understanding of the biology of these stem cells is the inability to distinguish them from spermatogonia that are committed to differentiation. During the isolation and purification steps, the viability of cells have been determined using live/dead staining. The differentiation of the germ cells in the colonies is determined by specific biochemical markers for undifferentiated spermatogonia, including

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Oct-4. The Oct-4 POU transcription factor is expressed in mouse totipotent embryonic stem and germ cells. Differentiation of totipotent cells to somatic lineages occurs at the blastocyst stage and during gastrulation. Stem cell lines derived from the inner cell mass and epiblast of the mouse embryo express Oct-4 only if undifferentiated (12). In the present study we attempt to establish an *in vitro* culture system that recreates some *in vivo* characteristics of seminiferous tubules. This co-culture system could be useful for studying spermatogonial proliferation and differentiation at the molecular level and elucidating the role of several newly discovered surface receptors in germ cell-Sertoli cell interaction and recognition. In general, spermatogonial colonization *in vitro* is useful for the *in vitro* enrichment and purification of spermatogonial cells for further studies on cryopreservation, restoration of male fertility by transplantation of spermatogonial cells, genetic modification, gene transfection, and *in vitro* differentiation.

Materials and Methods

Donor mice and cell collection

Cryptorchid testes were produced in NMRI mice (age: 4-6 weeks, n=55 derived from original stocks obtained from Razi Laboratory, Tehran, Iran), by making two horizontal flank incisions 2 mm caudal to the last rib in the abdominal wall skin and peritoneum followed by suturing the fat pad of the testis to the peritoneum. Cryptorchid testes were used to provide donor cells 2 months after surgery. They were placed on ice and transferred to the laboratory within 10 minutes. After decapsulation, the testes were minced into small pieces and suspended in minimal essential medium (α-MEM; Gibco, Paisley, UK), supplemented with 14mM $NaHCO_3$ (Sigma Chemical Co., St. Louis, MO, USA), 0.2% Bovine Serum Albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA), 100 $IUmL^{-1}$ penicillin, 100μgmL^{-1} streptomycin (both from Gibco, Paisley, UK) 0.1% insulin-transferrine selenium (Sigma Chemical Co., St. Louis, MO, USA) and 2mM glutamine (Sigma Chemical Co., St. Louis, MO, USA). The minced pieces of testis were suspended in α-MEM, which contained 0.5mg mL^{-1} collagenase/dispase, 0.5 mg mL^{-1} trypsin and 0.08 mg mL^{-1} DNase, for 30 minutes (with shaking and slight pipetting) at 37°C. All enzymes were purchased from Sigma. After three washes in α-MEM medium with removal of most of the interstitial cells, spermatozoa and some spermatid cells (fig 1A), a second digestion step (45 minutes at 32°C) was performed in α-MEM by adding fresh enzymes to the seminiferous cord fragments. Most of the cell aggregates that remained after this treatment were sheared gently by repeated pipetting with a Pasteur pipette for 5 minutes. The cells were separated from the remaining tubule fragments by centrifugation at 30g for 2 minutes at 37°C (fig1 B). The cells were washed twice and fresh medium α-MEM was added.

Spermatocyte cell isolation by PNA binding

The spermatocyte cells were isolated using a procedure described by van Pelt, et al (11) with some modifications (6). Petri dishes with a diameter of 60 mm were coated with 5 ml, 100µg/ml PNA in PBS+ for at least 1 hour at 37°C. Next, dishes were washed three times with α-MEM containing 0.5% BSA. The dishes were stored with α-MEM containing 5µg/ml DNase for at least 1 hour at 37°C. 5-25 million cells/dish were incubated for 1.5 hours at 32°C in an atmosphere of 5% CO_2. After cell binding to PNA, the remaining non-bound cells were collected by repeated washing of the dishes with a pipette. The cell suspension collected after this PNA binding contained 30-45% type A spermatogonia. The collected cells were used for further cell separation on coated plastic dishes by DSA-lectin.

Sertoli cell isolation by DSA-lectin binding

Sertoli cells were isolated using a procedure described by Scarpino, et al. with some modifications (13). Petri dishes with a diameter of 60 mm were coated with a solution of 5μgmL^{-1} of datura stramonium agglutinin (DSA; Sigma, Germany) in phosphate-buffered saline (PBS) at 37°C for 1 hour. The coated plastic dishes were washed three times with α-MEM containing 0.5% BSA (Sigma, Germany). The mixed population of cells obtained by enzymatic digestion was placed on lectin-coated dishes at a concentration of 1.5×10^5 cells/cm^2 and incubated for 1 hour at 32°C in a humidified atmosphere of 5% CO_2. After incubation the non-adhering cells were collected by washing twice with medium. Alternatively, 48 hours after being plated on the lectin-coated dishes, the Sertoli cells were detached by ethylenediamine tetraacetic acid (EDTA) – trypsin treatment (0.02% EDTA, 0.1% trypsin in Ca - and Mg-free PBS) for 5 minutes at 37°C, counted and adjusted to desired densities into each well of a 24-well multi dish (1.5×10^5 cells cm^2) for secondary culture in α-MEM at 32°C in the presence of 10% fetal bovine serum (FBS; Gibco, Paisley, UK). This method helped isolate Sertoli cells with more than 95% purity. The collected cells were then used for culture cells.

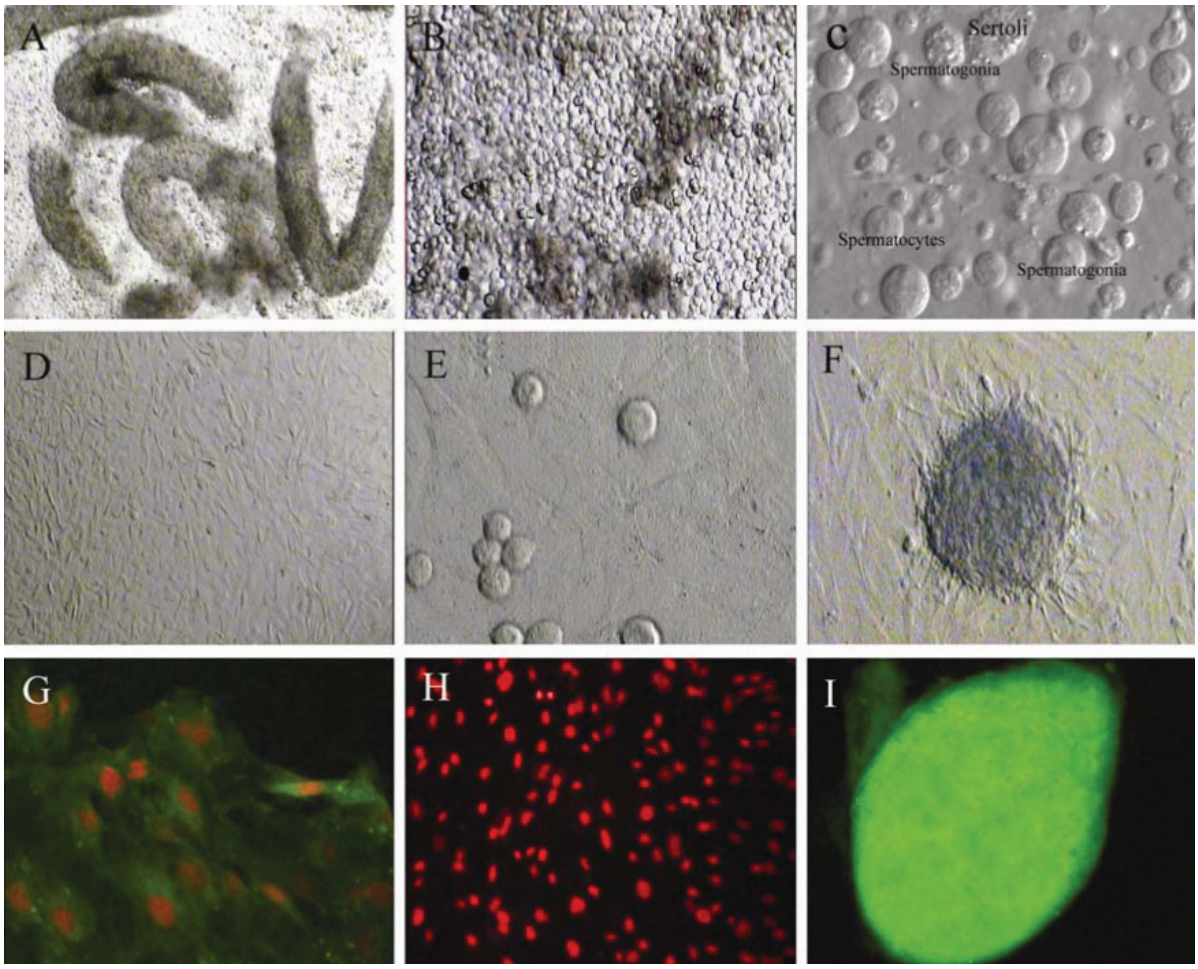

Fig 1: A. Seminiferous tubular separation after first step of enzymatic digestion, B. Cell suspention after second step of enzymatic digestion, C. Spermatogonia, spermatocytes and Sertoli cells after isolation in medium, D. Monolayer of Sertoli cells formation after 36-48 houres, E. co culture of Sertoli and Spermatogonial stem cells, F. Colony formation in culture medium, G. Vimentin localization for Sertoli cells, H. Control group of Sertoli cells without primary antibody, I. Oct-4 localization for Spermatogonial stem cells. bar for A×100, I, G, E, C×400, B, D, F, H×200

Cell number and viability

The number of Sertoli and spermatogonoal cells was determined with a hemocytometer. Cell viability was evaluated by means of the dye exclusion test (0.04% trypan blue solution).

Cell culture

After the spermatocyte and Sertoli cells had been isolated, the spermatogonial cells that remained in suspension were collected and kept at 32°C in a humidified atmosphere in the presence of 10% FCS. More than 35-45% of the cells were spermatogonial cells. The number of testicular cells for culture was determined with a hemocytometer. Cell viability was evaluated by means of the dye exclusion test (0.04% trypan blue solution).

Spermatogonial cells co-cultured with Sertoli cells

Four to seven days after the above-mentioned procedure the Sertoli cells formed a confluent layer. Testicular cells collected (5×10^3 cells/cm^2) were co-cultured on top of the Sertoli cell layer for 3 weeks. At the end of the first week the cells were passaged and then cultured for an additional two weeks.

The diameters and number of colonies were determined every 7 days during the culture for 3 weeks. Assay of the spermatogonial-cell-derived colonies was carried out at 7 days. At the end of the first week, the cells were passaged. Colony assay was carried out at 14 and 21 days during culture. An inverted microscope (Zeiss, Germany) was used to determine the number of colonies whose diam-

eters were measured by ocular grid.

Immunohistochemistry for identity confirmation of spermatogonial and Sertoli cells

The cells were fixed for 20 minutes in 4% paraformaldehyde at room temperature before they were rinsed with PBS. Following permeabilization by 0.2% Triton X-100 to facilitate antibody penetration the cells were washed with PBS. Extraneous antibodies were blocked with 10% goat serum (Vector, Burlingame, CA, USA). The slides were then incubated for 1 hour at 37°C with a mouse monoclonal anti-vimentin antibody (diluted 1:200; Sigma Chemical Co., USA), which has been described as a marker for Sertoli cells (14) or mouse anti-Oct-3/4 (R&D system, USA) polyclonal antibody diluted in PBS (1:50) which has been described as a marker for undifferentiated cells (15, 16). After being extensively washed with PBS, the second antibody (goat anti-mouse IgM labelled with fluorescein isothiocyanate (FITC); diluted 1:100; Sigma Chemical Co., USA) was applied for 45 minutes. Nuclei were stained with ethidium bromide (diluted 5µg/ml; Sigma Chemical Co., USA). The control slides were processed under similar conditions except for the removal of the first antibody. All incubations were performed in a moist chamber at 37°C. The slides were then mounted with 90% glycerol in PBS. Examination was carried out using a fluorescence microscope with appropriate filters. Results are expressed as mean±S.D. The statistical significance between mean values was determined by two way analysis variance Tukey-test; $p \leq 0.05$ was considered significant. All animal exprimentation protocels approved by the Institutional Animal Care and Use Committe of Tarbiat Modares University.

Results

Isolation and characterization of spermatogonial and Sertoli cells

The cell population obtained from DSA-lectin isolation proliferated and created a monolayer of cells (Fig 1D). These cells had an irregular outline with a granular appearance (Fig 1C).

Moreover vimentin, which is a molecular marker for Sertoli cells, was detected in the feeder monolayer cells (Fig 1G).

The other cell type, with spherical outline and two or three eccentrically placed nucleoli, created a colony after proliferation during the first week or immediately after passage (Fig 1F). Oct-4 which is a molecular marker for spermatogonial stem cells was detected in the obtained colonies (Fig 1).

Colony assay in co-culture with Sertoli cell groups

At the end of the first week, the cells were passaged and then cultured for two weeks. Colonies of spermatogonia cells appeared after one week of culture. Taken together, as demonstrated in Fig 2 and 3, the average numbers and diameters of colonies significantly increased in a time dependent manner. This increase was especially observed after cell passage.

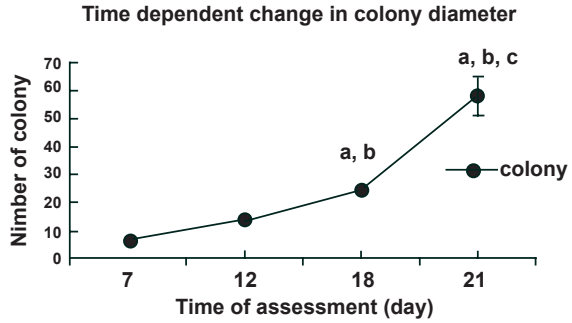

Fig 2: Time dependent changes in Spermatogonial stem cells colony number. a: Significant difference with 6 days. b: Significant difference with 9 days. c: Significant difference with 15 days.

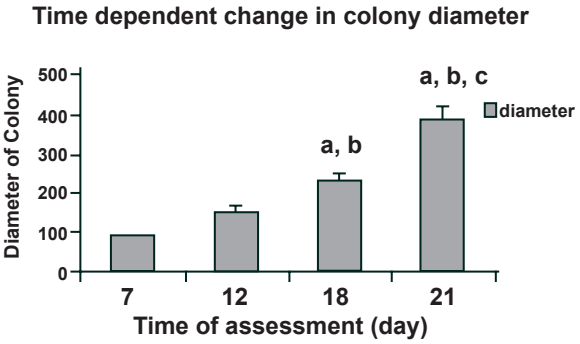

Fig 3: Time dependent changes in Spermatogonial stem cells colony diameters. a: Significant difference with 6 days. b: Significant difference with 9 days. c: Significant difference with 15 days.

Discussion

Rapid and effective preparation of pure populations of type A spermatogonia from prepubertal or adult animals is the basis for *in vitro* experiments and attempts at germ cell transplantation. In the past, isolation procedures using elutriation (17) or velocity sedimentation have been described (4). Morena, et al (3) also described a method for isolating rat spermatogonia using centrifugation in a discontinuous Percoll gradient. Unless the temperature of the body cavity damages stem cells or they are decreased in number, testis cells collected from a cryptorchid mouse should be enriched for stem cells since most differentiating germ cells

have been eliminated. Importantly, some stem cells remaining in the cryptorchid testis are known to differentiate normally after orchidopexy (18). Two important implications arise from our results with cryptorchid testis cells. First, the function of spermatogonial stem cells is not adversely affected by elevated temperature and other changes that occur in the cryptorchid testis during the 2 month period in this study. The second important implication from the cryptorchid experiments is that the procedure produces considerable stem cell enrichment. This finding is in accordance with Shinohara's (19) research. The cryptorchid testis cell suspension contained about 1 stem cell in every 200 cells, which is 5 to 10 times more enriched than following selection with laminin or antibodies to integrines (19). Enrichment procedures that rely on *in vitro* treatment by centrifugation, antibodies, or machine sorting may adversely affect activity of the stem cells-a situation that is difficult to identify or eliminate (20). Thus, the cryptorchid approach is an ideal negative selection method for the enrichment of stem cells and further combination with other cell separation techniques will improve the degree of enrichment. The enrichment of stem cells in cryptorchid testes will facilitate the characterization of these cells and provide an opportunity to develop new methods to achieve greater purity, for example, by fluorescence-activated cell sorting. A combination of *in vivo* and *in vitro* enrichment techniques will be the most effective approach to obtain pure populations of these important cells for molecular characterization and genetic manipulation.

We collected testicular cells from cryptorchid mice (4-6 weeks old) to culture according to Nagano, et al and Jeong, et al (15, 21). In our culture system, irregular outline cells with a granular appearance created a monolayer. In addition specific marker detection was carried out using immunocytochemistry anti-vimentin antibody. Vimentin is a cytoskeletal protein usually found in epithelial cells; this protein is a marker for day 14 postnatal Sertoli cells (13) and is strictly localized at the perinuclear region of the cells (22, 23). This finding is in agreement with that reported by Anway, et al (14). Also spermatogonia, with a spherical appearance and two or three eccentrically placed nucleoli, created round or radial colonies. Our results are similar to Izadyar, et al (5). For confirmation of the presence of spermatogonial cells, in addition to morphology, Oct-4 was traced in the colony cells. Spermatogonial-derived colonies showed Oct-4 expression. This finding is in agreement with that reported by previous investigators (13, 15-16) who demonstrated alkaline phosphatase activity and Oct-4 expression in spermatogonial cells.

Our colony numbers increased during three weeks of culture. One of reason could be due to subculturing the colonies at the end of first week. The remaining colonies that survived were increased in size.

Conclusion

In conclusion, we demonstrate that the cryptorchid mouse model is a suitable tool for enrichment of spermatogonial stem cells. Spermatogonial stem cells co-cultured with Sertoli cells can influence spermatogonial proliferation *in vitro*. These cells can be used for molecular characterization, genetic manipulation and restoration of male fertility.

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