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Original Article

Co-culture of mouse spermatogonial stem cells with sertoli cell as a feeder layer, stimulates the proliferation and spermatogonial stemness profile

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ABSTRACT

Objectives: Sertoli cells effect the fate map of spermatogonial stem cells (SSCs) to self-renew via providing the special microenvironments. Maintenance of proliferation and self-renewal activity of SSCs may be usable as a therapeutic strategy, leads to increase the recovery of male fertility. This research was aimed to evaluate the effect of mouse sertoli cells on spermatogonia stem cells proliferation and the expression pattern of stemness markers.

Methods: Spermatogonia stem cells were collected from neonatal mouse testis using a two-step mechanical and enzymatic digestion. SSCs were cultured in three groups: The first group or co-culture group consists of spermatogonia and sertoli cells that were cultured together. The control group, only spermatogonial cells and the group no. 3 included spermatogonial cells in the presence of GDNF. The colony formation of mentioned groups, was monitored during one month in culture. Identification of the colonies, was confirmed using PLZF and Oct4 immunostaining. Spermatogonial stemness genes includes; Stra8, *mvh and piwill2* were analyzed by RT-PCR.

Results: In the co-culture group, cells proliferated rapidly and many colonies were appeared whereas they were rarely formed in the control groups. Colonies were exhibited alkaline phosphatesase activity and were immunopositive to Oct4 and PLZF, strongly. The gene expression of srta8, mvh and piwill2, in SSCs that were cultivated with sertoli cells, were greater significantly than other control groups.

Conclusion: It is concluded that co-culture of SSCs with sertoli cells prepares conditions which leads to efficient proliferation and maintenance of stemness condition of SSCs, that is usable as a therapeutic approach for treatment of male fertility.

1. Introduction

Spermatogonial stem cells (SSCs) are unique cell population that maintains spermatogenesis during via their potential of proliferation and differentiation into male gamete, e.g. spermatozoa [1]. The SSCs are settled in base of seminiferous tubules of testis, surrounded by sertoli cells that support SSCs survival and proliferation [2,3]. In vitro proliferation of spermatogonia stem cells is possible via various growth factors, special; glial cell line-derived neurotrophic factor (GDNF) that is an essential factor promoting the self-renewal and propagation of SSCs in vivo and in vitro [4–6].

GDNF is an essential element for SSCs maintenance of SSCs, via prevent from apoptosis. It can mediate activation of intracellular pathway, in combination with other growth factors that leads to support in vitro proliferation of SSCs [7,8].

Previous reports show that growth rate and colony formation of SSCs in the presence of GDNF to culture medium [9,10]. Extracellular matrix of seminiferous tubule, that consist of sertoli cells can secret GDNF, which influence SSCs maintain and proliferation [11,12]. Sertoli cells are the somatic cells of the testis, responsible to secret of GDNF under regulation of FSH [13]. In fact, specialized interaction between sertoli cells and SSCs and niche paracerine factors, regulate SSCs proliferation [12,13].

In this research, we used sertoli cells as feeder layer to improve proliferation of SSCs and examined the co culturing effect of mouse sertoli cells in spermatogonia enrichment, their colonization activity and gene expression pattern.

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2. Materials and methods

2.1. Testicular cells collection

The present study was done following Animal Ethics Committee at Medical University of Mazandarn. Testicular cells were collected from neonatal mouse (2–6 days old) according previous report [14]. Animal were sacrificed and both testes were collected following small insecure in lower part of the abdomen. After decapsulation of the testis, tissue was mechanically dissociated via a two-step mechanical and enzymatic digestion. First, testis was minced into small pieces and testicular tissues were incubated in the enzymatic solution included: Dulbecco's modified Eagle's medium (DMEM) with 0.5 mg/ml collagenase-dispase, 0.5 mg/ml trypsin, and 0.05 mg/ml DNase for approximately 45 min with shaking and pipetting at 37 C for 30–45 min. The digested testicular tissues were washed and the supernatant was discarded, leading to remove of interstitial cells from seminiferous tubules. The remain seminiferous tubules were digested during second stage of enzymatic digestion until separation of their constituent cells. The obtained dissociated cells consisted of spermatogonia and Sertoli cells that were prepared for further in vitro culture. In total, three groups were designed in our research.

The group no. 1 that consisted spermatogonial cells were co-cultured with sertoli cells, the group no. 2 (control group), only spermatogonial cells (without the sertoli cells) and the group no. 3 included spermatogonial cells in the presence of GDNF. Separation of spermatogonia cells from sertoli cells were performed using differential plating method according the procedure: after 12 h of incubation, sertoli cells attached to culture dish, but SSCs had remained in the suspension.

2.2. Co-culture of spermatogonial cells with sertoli

The dissociated cells e.g. spermatogonia and sertoli cells were incubated together and were cultured in DMEM (Invitrogen) supplemented with 15% Fetal Bovine Serum (FBS; Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM bmercaptoethanol (Sigma Aldrich), and 1000 units/ml Leukemia Inhibitory Factor (LIF; Sigma Aldrich) at 37 C and 5% CO2, and in a humidified atmosphere.

48 h post plating, most testicular cells were attached to the culture dish, and medium was changed. The cells in all groups were cultured for one month. The number of the cells was determined using a hematocytometer before culture. Cell viability was evaluated by means of dye exclusion test (trypan blue solution).

2.3. Assessment of the colonization of SSCs

The colonization of SSCs in the co-cultured, control and GDNF groups was monitored using an inverted microscope.

2.4. Alkaline phosphates (ALP) activity

ALP reaction that is recognized as a marker of SSCs activity was evaluated in present study. Briefly, the Spermatoginial colonies were washed three times in PBS and then were fixed in a solution containing 2.5 ml citrate, 6.5 ml acetone and 0.6 ml formaldehyde in distilled water at room temperature (RT) for 30 min. The cells were incubated in a solution containing 0.5 mg/ml Fast Red Violet (Sigma) and 40 μl/ml α-naphthol phosphate (0.25% solution) for 30 min. The ALP reaction of cells was observed using light microscopy.

2.5. Quantitative real-time polymerase chain reaction (RT-qPCR)

The expression of a subset of germ cell-specific genes markers incudes; Stra8, Mvh, and Piwill2, was evaluated using RT-qPCR. Alteration in genes expression patterns during in vitro culture of SSCs, these genes were and evaluated again. Total RNA was extracted from cells, using RNXPlus™ (Cinnagen, Iran) according to the manufacturer's recommendations. Concentrations of RNA were determined using UV spectrophotometer (DPI-1, Qiagen). The cDNAs were synthesized from 500 ng of RNA using oligo (dT) primers via Revert-Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). Table 1 shows primer sequences (forward and reverse) which were applied in this study. PCR were carried out with Master Mix and SYBR Green in an Applied Biosystems, StepOne™ thermal cycler (Applied Biosystems, USA). The PCR program started with an initial melting cycle, 4 min at 94 °C, to activate the polymerase, and was succeeded by 40 cycles as follows: a melting step (20 s at 94 °C), annealing (30 s at 57 °C), and an extension (30 s at 72 °C).

Efficiency of the PCR reactions was confirmed by a standard curve (the logarithmic dilution series of testis cDNA). For each sample, the reference gene and the target gene were amplified in the same run. The target genes were normalized to the reference gene. The ratio of gene expression was detected via the comparative CT (cycle threshold). The comparative CT method (2ΔΔCT) was applied to determine the relative quantification of target genes, normalized to a housekeeping gene (β-actin) and relative to a calibrator (untreated SSCs cells). Table 1 shows Quantitative realtime PCR primer sequences.

2.6. Statistical analysis

Data were analyzed and compared using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Data were presented as mean ± SEM and $p < 0.05$ was considered significant.

2.7. Immunocytochemistry staining of SSCs colonies

For immunostaining, the cells were fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) then were washed three times with PBS and incubated in 1% Triton X in PBS for 30 min following, block-

Table 1
Quantitative realtime PCR primer sequences.

Gene Primer (forward/reverse)	Product size	Significance
β actin 5′- CTTCTTGGGTATGGAATCCTG -3 5′- GTGTTGGCATAGAGGTCTTTAC-3′	131	Internal Control
Piwil2 5′-GCACAGTCCACGTGGTGGAAA -3′ 5′-TCCATAGTCAGGACCGGAGGG -3 ’	681	Spermatogonial marker
Stra8 5′- ACGACGCGTCGCTATTCCCTCTCACATCTTC-3′ 5′- AGCGAGCTCGATGCACCTTCGACACTTG -3′	441	Spermatogonial marker
Mvh 5′- CGGAGAGGAACCTGAAGC -3′ 5′- CGCCAATATCTGATGAAGC -3′	145	Spermatogonial marker
Oct4 5′- AGCACGAGTGGAAAGCAAC -3′ 5′-AGATGGTGGTCTGGCTGAAC-3′	73	Stemness marker

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