UNIVERSITI PUTRA MALAYSIA

ISOLATION, PURIFICATION AND CRYOPRESERVATION OF SPERMATOGONIAL STEM CELLS FROM TESTIS OF AZOOSPERMIA PATIENTS

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By

TOOBA MIRZAPOUR

Thesis Submitted to the School of Graduate of Studies, Universiti Putra Malaysia, in Fulfilment of Requirement for the Degree of Doctor of Philosophy

April 2011

Dedicated

To

This thesis dedicated to my dear and lovely daughter Mahtab, to my beloved husband, Tooraj Sohrabi Langaroudi, to my dear mother Iran Pourkhosro and my lovely father Rahmat Mirzapour that I owe them all of success in my life

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

ISOLATION, PURIFICATION AND CRYOPRESERVATION OF SPERMATOGONIAL STEM CELLS FROM TESTIS OF AZOOSPERMIA PATIENTS

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Chairman: Professor Tengku Azmi bin Tengku Ibrahim, PhD

Faculty: Veterinary Medicine

Isolation and proliferation of human spermatogonia stem cells (hSSCs) *in vitro* will allow powerful new approaches in treatment of selected causes of male infertility. Leukaemia inhibitory factor (LIF) has been considered an essential component for long-term culture of primordial germ cell and its combination with basic fibroblast growth factor (bFGF) has been expected to elicit a much higher effect. The objective of the present study was to develop an *in vitro* culture system for cellular proliferation of spermatogonia stem cells (SSCs) from the human testes by adding different concentrations of LIF and bFGF and co-culture germ cells on Sertoli cells.

Human testis biopsies were routinely obtained from patients diagnosed with maturation arrest of spermatogenesis through the clinical practice of Imam Khomeini Hospital

(Tehran, Iran) patients (n=25, average 35.6±0.5 years). Then hSSCs and Sertoli cells were isolated by two- step enzymatic digestion method, plated and grown on DSA-Lectin coated dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% CO_2 for 5 weeks.

The culture groups include (1) SSCs cultured without Sertoli cells, (2) SSCs co-cultured with Sertoli cells (as control group), and (3) SSCs co-cultured with Sertoli cells and adding different concentration of bFGF (0.1, 1, 10 ng/mL) and LIF (1000, 1200, 1500 unit/mL) as experimental groups. Spermatogonial-cell-derived colonies were evaluated based on the number and the diameter of colonies after 10 days of culture and continued every 10 days during 5 weeks culture using an inverted microscope (Zeiss, Germany).

Cultured cells were evaluated by RT-PCR using mRNA of *Dazl, Oct-4, Nanog, α6-Integrin, β1-Integrin, Piwil2, Stra8, Vasa, Bax* and *Dmc1*genes and examined by immunocytochemistry using anti-Vimentin, anti-CDH1 and anti-Oct-4 to confirm Sertoli cells and spermatogonia stem cells. Effects of cryopreservation on cell viability and proliferation of SSCs *in vitro* was also evaluated in different stages of culture. The presence of functional spermatogonia stem cells in the culture system was evaluated by xenotransplantation of different concentration of cultured cells in immunodeficient mice testis. The number of colonized seminiferous tubules after transplantation was detected by BrdU fluorescence and Hoechst staining during eight weeks after transplantation.

Results showed that spermatogonial-cell-derived colonies were formed in the culture system after one week. In the first group, the average number and diameter of the colonies were significantly lower than in the two other groups ($P<0.05$). The largest

number of colonies was observed in the control group (32.29± 9.15) in day 30. The largest diameter of colonies was formed in combinated doses of 1ng/mL bFGF +1500unit/mL LIF (302.93±37.68). Isolated SSCs were positive for spermatogonial cell markers such as *Oct4, Dazl, Stra8, Piwil2, Vasa* and *α6-Integrin*, but negative about *Nanog and β1-Integrin* Genes. Expression of an apoptotic gene (*Bax*) was observed approximately 35 days after culture but *Dmc1* that is a meiotic gene was not observed in the culture system. Assessment of proliferation ability of SSCs after cryopreservation showed that the number and the diameter of colonies in the co-culture of frozen-thawed SSCs on fresh Sertoli cells was more than co-culture of fresh SSCs on fresh Sertoli cells (19.60± 2.80 and 17.33±2.20 for the number and 269.70±52.10, 204.34± 24.10 for the diameter, respectively). It indicated that cryopreservation of SSCs before culture will increase purified SSCs and they formed many big size colonies in comparison of the control group.

Transplantation of hSSCs to infertile mice model testis indicated that these cells have good efficiency for colonization of seminiferous tubules after proliferation in the culture system, however meiotic divisions were not observed eight weeks after transplantation.

An ultrastructure study of cultured germ cells showed that many various types of spermatogonia were identified in culture system but were not observed remarkably difference between spermatogonial cells in culture system and testis tissue.

Taken together, SSCs and Sertoli cells derived from adult human testes were maintained and proliferated in the *in vitro* culture condition, but did not differentiate to meiotic cells. Also proliferation did not change their structure morphologically.

Abstrak tesis yang dkemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi Keperluan untuk ijazah Doktor Falsafah

PENGASINGAN, PENULENAN DAN PENGAWETAN KRIO SEL STEM SEL TESTIS DARI PENDERITA AZOOSPERMIA

Oleh

TOOBA MIRZAPOUR

April 2011

Pengerusi: Profesor Tengku Azmi bin Tengku Ibrahim, PhD

Fakulti: Perubatan Veterinar

Pengasingan dan pengembangbiakan sel stem spermatogonia manusia (human spermatogonia stem cells, (hSSC) *in vitro* membolehkan pendekatan-pendekatan baru yang berkeupayaan tinggi di bidang rawatan beberapa penyebab kemandulan lelaki. Faktor penyekat leukaemia (LIF) telah dianggap sebagai suatu komponen terpenting untuk kultur jangka-panjang sel germa primordium dan gabungannya dengan faktor pertumbuhan fibroblast asas (basic fibroblast growth factor, bFGF) telah dijangka akan memberi kesan yang lebih tinggi. Objektif kajian ini ialah untuk membangunkan suatu sistem kultur *in vitro* untuk pembiakan sel-sel dasar spermatogonia dari testes manusia secara menambahkan pelbagai kepekatan LIF dan bFGF dan kultur-bersama sel-sel germa ke atas sel-sel Sertoli.

Biopsi testis manusia telah didapatkan secara rutin dari pesakit yang telah didiagnosiskan dengan penyekatan kematangan spermatogenesis melalui amalan klinikal

Imam Khomeini Hospital (Tehran, Iran) ke atas pesakit (n=25, purata berumur 35.6±0.5 tahun). Kemudian sel hSSC dan Sertoli diasingkan melalui kaedah penghadaman enzim dua-peringkat, diplatkan dan dibiakkan di piring yang disaluti dengan DSA-Lecitin di dalam bahantara Dulbecco's modified Eagle's (DMEM) dan ditambahkan dengan 10% FCS di suhu 37^{o}C di 5% CO_2 selama 5 minggu.

Kumpulan-kumpulan kultur termasuklah (1) SSC yang dikultur tanpa sel Sertoli, (2) SSC yang dikultur bersama sel Sertoli (sebagai kumpulan kawalan), dan (3) SSC yang dikultur bersama dengan sel Sertoli dengan tambahan pelbagai kekuatan bFGF (0.1, 1, 10 ng/mL) dan LIF (1000, 1200, 1500 unit/mL) sebagai kumpulan uji kaji. Koloni terbitan sel spermatogonia dinilai berasaskan jumlah dan diameter koloni selepas 10 hari dikultur dan diteruskan setiap 10 hari selama 5 minggu kultur tersebut, menggunakan mikroskop songsang (Zeiss, Germany).

Sel yang dikultur dinilai dengan RT-PCR menggunakan mRNA gen-gen *Dazl, Oct-4, Nanog, α6-Integrin, β1-Integrin, Piwil2, Stra8, Vasa, Bax* dan *Dmc1* dan diperiksa secara immunositokimia menggunakan anti-Vimentin, anti-CDH1 dan anti-Oct-4 untuk mengesahkan sel Sertoli dan juga sel dasar spermatogonia. Kesan pengawetan krio ke atas daya hidup dan pengembangbiakan SSC di dalam kujltur *in vitro* juga dinilai di pelbagai peringkat kultur. Kewujudan sel dasar spermatogonia berfungsi di dalam sistem kultur tersebut dinilai secara pemindahan asing pelbagai kepekatan sel kultur ke dalam testis tikus yang berkurangan keimunan. Jumlah tubul semen yang berkoloni selepas pemindahan dikesan menggunakan pendarfluor BrdU dan pewarnaan Hoechst selama lapan minggu selepas pemindahan.

Hasil menunjukkan bahawa koloni terbitan-sel-spermatogonia dibentuk di dalam sistem kultur tersebut selepas satu minggu. Di dalam kumpulan pertama, purata jumlah dan diameter koloni adalah nyata lebih rendah berbanding dengan dua kumpulan yang lain itu ($P<0.05$). Jumlah koloni yang terbesar telah diperhatikan di dalam kumpulan kawalan (32.29± 9.15). Diameter koloni yang terbesar didapati di dalam dos berkombinasi 1ng/mL bFGF +1500unit/mL LIF (302.93±37.68). SSC yang diasingkan adalah positif untuk penanda sel spermatogonia seperti *Oct4*, *Dazl*, *Stra8*, *Piwil2*, *Vasa* dan *α6-Integrin* tetapi negatif berkenaan dengan gen-gen *Nanog* dan *β1-Integrin.* Penjelmaan suatu gen apoptosis (Bax) diperhatikan lebihkurang 35 hari selepas kultur tetapi Dmc1 yang merupakan suatu gen meiosis tidak diperhatikan di dalam sel sistem kultur tersebut.

Penilaian kebolehan pengembangbiakan SSC selepas pengawetan krio menunjukkan bahawa jumlah dan diameter koloni di dalam kultur-bersama SSC yang dicairkan dari beku ke atas sel Sertoli adalah lebih dari kultur-bersama SSC ke atas sel Sertoli yang segar di penghujung kultur (masing-masing19.60± 2.80 dan 17.33±22 untuk jumlah dan 269.70±52.10, 204.34± 24.10 untuk diameter). Ini menunjukkan bahawa pengawetan krio SSC sebelum kultur akan meningkatkan SSC yang ditulenkan dan mereka membentuk banyak koloni yang besar berbanding dengan kumpulan kawalan.

Pemindahan sel dasar spermatogonia kepada model testis tikus mandul menunjukkan bahawa sel ini adalah tinggi efisiennya untuk penkolonian tubul semen selepas pengembangbiakan di dalam sistem kultur tersebut, tetapi pembahagian meiosis tidak dilihat lapan minggu selepas pemindahan.

Suatu kajian ultrastruktur sel germa dikultur menunjukkan bahawa sel walaupun banyak pelbagai jenis spermatogonia dikenalpasti dalam sistem budaya, tapi kira-kira tidak diamati sangat perbezaan antara sel spermatogonium dalam sistem kultur dan rangkaian testis.

Jika dipertimbangkan semuanya sekali, SSC dan sel Sertoli terbitan testes manusia dewasa diperkekalkan dan mengembangbiak di dalam keadaan kultur *in vitro* ini tetapi tidak membeza menjadi sel meiosis. Juga, pengembangbiakan tidak mengubah struktur mereka dari segi morphologi.

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I certify that a Thesis Examination Committee has met on 27 April 2011 to conduct the final examination of Tooba Mirzapour on her thesis entitled "Isolation, Purification and Cryopreservation of Spermatogonial Stem Cells from Testis of Azoospermia Patients" in accordance with the Universities and University College Act 1971and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follow:

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

TOOBA MIRZAPOUR

Date: 27April 2011

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