UNIVERSITI PUTRA MALAYSIA

IN VITRO STUDY OF VARIOUS FACTORS IN ISOLATION, EXPANSION AND DIFFERENTIATION OF MESENCHYMAL STEM CELLS FROM HUMAN UMBILICAL CORD

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By

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Chairman: Noorjahan Banu Binti Mohamed Alitheen, PhD

Faculty: Biotechnology and Biomolecular Sciences

The Wharton's Jelly (WJ) of the umbilical cord is a rich source of mesenchymal stem cells (MSCs) with a various potential therapeutic applications. Human umbilical cord mesenchymal stem cells (hUCMCs) can be isolated with different methods, expanded and differentiated into neuron, astrocyte and oligodendrocyte *in vitro*.

In the first study, four methods to isolate hUCMCs were compared. Three of them were based on using an enzyme cocktail containing collagenase, hyaluronidase and trypsin (CHT), collagenase /trypsin (CT) or trypsin alone (TRP) and the other was based on the explant culture (Exp) method. In the second experiment, the competence of DMEM/ F12 and alpha-MEM/Glutamax (α-MEM/GL), also the epidermal growth factor (EGF) and fibroblast growth factor (FGF) role to expand hUCMCs were evaluated. Finally, hUCMCs were examined for their differentiation rate into neural lineage cells by six various cocktails made of a base media

(DMEM/LG), 10 % FBS, retinoic acid (RA), dimethyl sulfoxide (DMSO), EGF and FGF.

Comparison of the four isolation methods of the hUCMCs showed that more cells were isolated in the CT, CHT and TRP groups, respectively. Cells were successfully isolated in TRP group but they did not propagate normally. Flow cytometry analysis revealed that CD44, CD73, CD90 and CD105 were expressed on the cell surface in all groups, but there was no expression of hematopoietic lineage markers, CD34 and CD45, in any of the groups. In addition, the expression of C-kit and OCT-4 in enzymatic (CT and CHT) groups was greater than Exp group. One-way ANOVA showed that cell activity rate in the Exp group was significantly higher ($P<0.001$) than the other groups. A positive alkaline phosphatase activity and differentiation to adipogenic and osteogenic lineage was detected in the hUCMCs of all groups.

The ratio of CD44 and CD105 expression cells were similar in DMEM/F12 and α-MEM/GL groups, while in both of the groups expression of the hematopoietic cells surface marker CD34 were negative. Differentiation potential into adipogenic and osteogenic cells were similar in both groups. Student`s t-test for evaluation of cell metabolism with WST-1 showed that the quality of the expanded cells in α-MEM/GL group was significantly higher ($P<0.001$) than DMEM/F12 group. Population doubling time (PDT) was calculated at 60 hours for DMEM/F12 group, while it was 47 hours in α-MEM/GL group that this difference was statistically significant ($P<0.001$). Data sets were compared using two- way ANOVA.

Semi-quantitative RT-PCR revealed that the TERT in hUCMs/EGF was 0.26 ± 0.06. Cell cycle analysis showed that EGF has caused 37.34± 9.8% and 7.71± 0.53% of the hUCMs to move in the S and G2/M phases, respectively. While, after exposure to FGF 29.92± 2.55% and 9.92± 0.24% of the cells progressed to S and G2/M phases, respectively.

Induction of neuronal differentiation with various cocktails showed that there was no statistically significant difference (one-way ANOVA with a post hoc Tukey's test) among the groups. Despite, the cocktail consist of DMEM/LG, FBS, RA, FGF, and EGF (DF/R/Fg/E group) resulted in the expression of the highest percentage of nestin, ß-tubulin III, neurofilament, and CNPase 69.7 ± 6%, 76 ±1.7%, 89 ±9.5%, and 16.3 ±5.2%, respectively. Also, in this group and the DF/Ds/Fg/E group, the highest cell proliferation was observed. On the other hand, DF/Ds/Fg/E group had the highest percentage of GFAP expression (36 ±12.5%). While, the expression level of NF, GFAP, and CNPase was at least in the DF group. The least percentage of nestin and ß-tubulin III expression was observed in the DF/Ds group.

It may be concluded that isolation technique affects both the amount and the quality of the isolated hUCMCs. When collagenase/trypsin enzymes are used for isolation of hUCMCs, the waiting time for primary culture is shorter, the number of isolated cells is higher and pluripotency properties of them were greater. The type of culture media also affects the quality of the harvested cells. α-MEM/GL supports hUCMCs growth more strongly than DMEM/F12. Furthermore, growth factors also affect the TERT expression and cell cycle phases. In this case, EGF increases TERT expression and the number of cells that progress towards S/G2/M phases. In addition, hUCMCs

differentiate into various neuronal cell types successfully; the amount of different neuronal cell types is influenced by inducing agents. FGF and EGF are important inducers for differentiation of hUCMCs into neuron, astrocyte and oligodendrocyte. RA can induce hUCMCs to differentiate into neuron and oligodendrocyte; while in contribution to astrocyte differentiation, DMSO had a pivotal role.

From this study, it can be concluded that different isolation methods can effect on characteristics of the mesenchymal cells from human Wharton's Jelly. These cells are able to expand in culture with various media and growth factors and differentiate into neural cells.

Abstrak Tesis Untuk Dikemukakan Kepada Senat Universiti Putra Malaysia Sebagai Memenuhi Keperluan Untuk Ijazah Doktor Falsafah

KAJIAN IN VITRO MENGENAI PERBAGAI FAKTOR DALAM PEMENCILAN, PERKEMBANGAN, DAN PEMBEZAAN SEL INDUK MESENKIMAL DARI TALI PUSAT MANUSIA

Oleh

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Whaton Jeli (WJ) dari tali pusat adalah sumber yang kaya dengan sel induk mesenkimal (MSCs) dan mempunyai pelbagai potensi terapeutik. Sel induk mesenkimal dari tali pusat manusia (hUCMCs) boleh diasingkan dengan pelbagai kaedah, dan boleh berkembang dan dibezakan menjadi neuron, astrocyte dan oligodendrocyte secara in vitro.

Dalam kajian pertama, empat kaedah untuk mengasingkan hUCMCs telah dibandingkan. Tiga daripada kaedah itu berdasarkan penggunaan koktel enzim yang mengandungi kolagenase, hyaluronidase dan trypsin (CHT), kolagenase / trypsin (CT) atau trypsin sahaja (TRP) dan selebihnya adalah berdasarkan kaedah kultur eksplan (Exp). Dalam kajian kedua, kemampuan media DMEM / F12, α-MEM/Glutamax (α -MEM/GL dan juga peranan faktor pertumbuhan dalam perkembangan hUCMCs dikaji. Akhir sekali, pemeriksaan dijalankan ke atas sel hUCMCs untuk mengenal pasti kadar perkembangan mereka menjadi sel keturunan

neural menggunakan kombinasi enam koktail yang dibuat daripada media (DMEM / LG), 10% FBS, Retinoic asid (RA),sulfoxide dimetil (DMSO), EGF dan FGF.

Perbandingan antara empat kaedah pengasingan sel hUCMCs menunjukkan bahawa lebih banyak sel dapat diasingkan dalam kumpulan CT, CHT dan TRP. Sel-sel telah berjaya diasingkan di kumpulan TRP tetapi tidak dapat berkembang secara normal. Analisis aliran sitometri menunjukkan bahawa CD44, CD73, CD90 dan CD105 telah diekspreskan di atas permukaan sel dalam semua kumpulan, tetapi CD34 dan CD45 tidak diekspreskan pada keturunan hematopoietik, dalam mana-mana kumpulan. Di samping itu, ekspresi C-kit dan OCT- 4 pada kumpulan enzim (CT dan CHT) adalah lebih baik daripada kumpulan Exp. Kadar perkembangan sel dalam kumpulan Exp adalah jauh lebih tinggi ($P < 0.001$) berbanding kumpulan lain. Aktiviti fosfatase alkali yang positif dan pembezaan kepada keturunan adipogenik dan osteogenik dapat dikesan pada sel hUCMCs dari mana-mana kumpulan.

Ekspresi stem sel mesenkimal penanda termasuklah CD44 dan CD105 adalah sama dalam kumpulan media DMEM/F12 dan α-MEM/GL, manakala dalam kedua-dua kumpulan ekspresi penanda CD34 pada permukaan sel-sel hematopoietic adalah negatif. Potensi perkembangan sel kepada sel adipogenik dan osteogenik adalah sama dalam kedua-dua kumpulan. Penilaian kadar metabolisme sel dengan WST-1 menunjukkan bahawa kualiti sel-sel yang berkembang dalam kumpulan media α-MEM/GL adalah lebih tinggi ($P < 0.001$) berbanding kumpulan DMEM/F12. Populasi masa dua kali ganda (PDT) telah dikira pada 60 jam untuk kumpulan DMEM/F12, sementara pada kumpulan α-MEM/GL adalah 47 jam. Perbezaan ini adalah signifikan secara statistik ($P < 0.001$).

Separa-kuantitatif RT-PCR telah menunjukkan bahawa TERT di dalam hUCMs/EGF adalah 0.26 ± 0.06. Analisis kitaran sel juga menunjukkan yang EGF adalah punca kepada pergerakan 37.34± 9.8% dan 7.71± 0.53% hUCMs kepada fasa S dan G2/M. Selepas pendedahan FGF, 29.92± 2.55% dan 9.92± 0.24% sel telah memasuki fasa S dan G2/M.

Induksi pembezaan neuron dengan pelbagai koktel menunjukkan bahawa koktel mengandungi kumpulan DMEM/LG, FBS, RA, FGF dan EGF (DF/R/Fg/E) menghasilkan ekspresi dengan peratusan yang tertinggi untuk nestin, ß-tubulin III, neurofilament, and CNPase 69.7 ± 6%, 76 ±1.7%, 89 ±9.5%, and 16.3 ±5.2%, masing-masing. Juga di dalam kumpulan ini dan kumpulan DF/Ds/Fg/E, perkembangan sel yang paling tinggi diperhatikan. Selain itu, kumpulan DF/Ds/Fg/E mempunyai peratusan ekspresi GFAP yang tertinggi ((36 ±12.5%). Sementara itu, peratusan ekspresi NF, GFAP, dan CNPase adalah yang terendah di dalam kumpulan DF. Peratusan ekspresi terendah nestin dan ß-tubulin III diperhatikan di dalam kumpulan DF/Ds.

MEM/GL menyokong penumbuhan hUCMCs lebih baik daripada DMEM/F12. Di samping itu, faktor penumbuhan juga mempengaruhi ekspresi TERT dan fasa kitaran sel. Di dalam kes ini, EGF meningkatkan ekspresi TERT dan jumlah sel yang memasuki fasa S/G2/M. Selain itu, kejayaan hUCMCs untuk membezak kepada pelbagai sel neuron dan jumlah pelbagai sel neuron adalah dipengaruhi oleh agen-agen induksi. FGF dan EGF adalah ejen pendorong yang penting untuk pembezaan hUCMCs kepada neuron, astrocyte dan oligodendrocyte. RA juga boleh mendorong

hUCMCs untuk membezakan kepada neuron dan oligodendrocyte, DMSO mempunyai peranan yang penting dalam pembezaan astrocyte.

Daripada kajian ini, dapat disimpulkan bahawa teknik pengasingan yang berbeza boleh mempengaruhi ciri-ciri sel mesenchymal daripada Wharton's Jelly manusia. Sel-sel ini boleh berkembang di dalam kultur dengan pelbagai jenis media dan faktor penumbuhan, dan membeza kepada sel neuron.

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I certify that a Thesis Examination Committee has met on 11 June 2012 to conduct the final examination of Parvin Salehinejad on her thesis entitled "*In Vitro* Study of Various Factors in Isolation, Expansion and Differentiation of Mesenchymal Stem Cells from Human Umbilical Cord" in accordance with the Universities and University Colleges Act 1971 and 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 follows:

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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 or concurrently submitted for any degree at Universiti Putra Malaysia or at any other institutions.

PARVIN SALEHINEJAD

Date: 11 June 2012

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