Isolation and characterization of bone marrowmesenchymal stem cells from rat and rabbit; a modified method

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Abstract

Bone marrow MSCs were obtained from New Zealand White rabbits (~3 kg, male) and also from white rat *(Ratusratus)* by the Guideline for Animal Care and use Committee for Teaching and Research using a modified procedure [1,2] Briefly, after the animals was sacrified with carbon monoxide cabinet, tibias and femurs were excised and the bone marrow was extracted under sterile conditions.The collected marrow was mixed and dispersed with PBS. After that the (MSCs) were separated by density-gradient centrifugation over ficoll. Viability of the separated MSCs was determined via trypan blue staining technique. Then, cells were seeded into ten tissue culture flasks containing complete culture medium suplimented with 10% fetal bovine serum (FBS) and antibiotics, incubated at 37^0C in a humidified atmosphere with 5% CO2. Once the colonies reached 80–90% confluence, they were ready to be detached with trypsin/EDTA and suspended in medium for continuous culture.

عزل وتوصيف خلايا نخاع العظم ألجذعيه الميزنكيميه من الجرذانوالأرانب .طريقة محورة

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مفتاح البحث: خلايا نخاع العظم الجذعية الميزنكيمية

الخلاصة

تضمنت الدراسة عزل و تنمية الخلايا الجذعية الميزنكيمية من نقي العظم للارنب الابيض الذكر والذي يزن حوالي 3 كغم والجرذ الابيض المختبريين طبقا لقوانين هيئة العناية بالحيوان واستعماله للأغراض البحثية و التعليمية وذلك باتباع طريقة محورة لهذا الغرض. بأيجاز, بعد قتل الحيوان داخل كابينة غاز أحادي أوكسيد الكاربون تم استئصال عظمي الفخذ وعظمي القصبة وبعدها تم استخراج نقي العظم منها والذي خلط مع محلول الفوسفات الملحي (PBS) . ثم تم فصل الخلايا الجذعية الميزنكيمية من الخليط بوساطة السنترفيوج مع فارق الكثافة وذلك بأضافة مادة الفيكول. بعد ذلك تم تحديد حيوية الخلايا المفصولة عن طريق تقنية التصبيغ بصبغة التريبان الزرقاء. بعدها تم استنبات الخلايا في عشرة اطباق زرعية نسيجية يحوي كل منها على الوسط الزرعي النسيجي المتكامل مدعما بالمصل البقري والمضادات الحيوية حيث تم الحضن بدرجة 37 مئوية في محيط رطب يحوي 5% غاز ثاني أوكسيد الكاربون. عندما أصبحت درجة اتصال المستعمرات الخلوية 80-90% كانت جاهزة لفصلها بواسطة محلول أنزيم التربسين مع EDTA واستنباتها في الوسط الزرعي الدائمي

INTRODUCTION:

Literature data concerning the biology and differentiation potential of mesenchymal stem cells (MSCs) have become huge in less than 10 years, although some of these data still remain contradictory. MSCs seem to be a very promising tool for cell therapy because of their peculiar characteristics, which mimic partially those of embryonic stem cells, but with some advantages in terms of availability, expandability, transplantability, and ethical implications[3]

In bone marrow, MSCs make up approximately 0.001%-0.01% of all cells in each aspirate, depending on the technique[4].Specific differentiation media can easily reveal MSC's nature of expanded, adherent stromal-like cells, which do not express specific markers, but a complex pattern of molecules , including CD105 (SH2), CD73 (SH3 and SH4) , CD106 (VCA M-1), CD54 (ICA M-1), CD44, CD90, CD29, STRO -1[5-11], as well as immune molecules such as HLA class I and II (the latter only upon the effect of interferon-gamma, IFN- γ) and CD119 (IFN - γ receptor)[11]. Hemopoietic markers, such as CD45 and CD34, are normally not expressed[5-11].

Source of MSCs is not only bone marrow, but also other adult tissues such as fat[12] , hair follicles and scalp subcutaneous tissue[13] , periodontal ligament [14], thymus and spleen (personal data) , as well as pre-natal tissues, such as placenta [15] , umbilical cord blood [16], fetal bone marrow, blood, lung, liver and spleen[17] . Circulating MSC s canbe detected in peripheral blood[18] , similarly to what happens for hemopoietic stem cells.

On the other hand, because the therapeutic application of MSCs often requires a large number of cells, these require *ex vivo* expansion post-harvest. Thus, in order to obtain sufficient quantities for therapeutic uses of isolated MSCs, it is important to expand these cells *ex vivo*. However, MSCs show limited proliferation and differentiation capacity in an *ex-vivo* setting despite their capability of continuous regeneration and expansion throughout an individual's life. It was shown that their ability to expand is highly variable, even amongst two samples from the same patient[19].Herein, we try to isolate bone marrow MSCs from rat and rabbit via a modified technique, to test their viability by tripan blue **.**

Study design and objectives: This in vitro experiment was done in college of medicine in Malaya University (Malaysia) during March through May, 2011. It aims to isolate bone marrow Mesenchymal stem cells (MSCs) from rat and rabbit by a modified technique. It aims also to expand these cells in vitro making them ready for subsequent differentiation and further experimental use.

MATERIAL AND METHOD:

Lab animals:

Bone marrow MSCs were obtained from White rabbit (2–3 kg, male) and also from white rat *(Ratusratus)* by the Guideline for Animal Care and Use Committee for Teaching and Research using a modified procedure [1,2].

Culture medium:

Complete Dulbecco's modified Eagle medium (DMEM), high-glucose formulation, containing: 10% fetal bovine serum (FBS), heat-inactivated 1 hr at 56°C; 1% nonessential amino acids, 2 mM L-glutamine, 50 µM 2-ME 100 U/ml penicillin 100 µg/ml streptomycin sulfate[20].

Isolation of bone marrow MSCs:

This was perfomed by a modified procedure [1,2] .First of all, the samples (bones) were extracted from the experimental animal in special operation theater (experimental animal operation theater) as follows:The animal was sacrificed by carbon monoxide inhalation inside a CO-cabinet for 2 min. Then, the animal was dissected to obtain the long bones; femurs and tibiae.The bones werewashed by saline and directly put in the falcon tube with PBS 10 % and kept in the ice box.

Secondly bones were processed in the tissue engineering lab to extract their marrow content as follows: Bones' ends were cut by bone cutter and diaphysis were put it in petri dish and then by 1-ml syringe pushing and sucking the PBS in and out of the bone cavity to obtain from each bone 1 ml bone marrow that wasdispensed in a centrifuge tube . Finally, 4 ml of bone marrow suspended with PBS was obtained. The collected bone marrow was then carefully layered over ficoll-paque solution (3 ml) in new tube. Then, the sample (7ml) was centrifuged with 2200 rpm for 25 min. at 25^0C.After that, cloudy layer in the middle between the ficolllayer and the bone marrow, represented the stem cell layer, was drawn carefully by a 500-micro liter pipette by circular movement and put it in a new tube. The aspirate was centrifuged with 1600 rpm for 10 min. at 25^0C. The supernatant wasdiscarded and 10 mlDMEM media without supplement was added and mixed with the cells' pellet. Then, the mixture was transferred to tenflasks with 10 mlcompleteDMEM(supplemented with FBS, glutamate and antibiotics) and was incubated in 5%CO_2, at 37 ^{0}C. Non-adherent cells were removed after 24 h, and MSCs adhered to the flask bottom were tested for viability indicated by negative trypan blue staining and were gradually proliferated to form fully confluent colonies in eight of the ten flaskswithin 4or 5 dayswhen examinedby inverted microscope.

RESULTS:

Of the ten culture flasks, eight (80%) showed full-confluence MSCs layer by the 4^{th}-5^{th} day of incubation, while the remaining two flasks (20%) failed to expand full-confluence cell layer at that time (Table:1).

Table 1: Rates of confluence of Mesenchymal stem cells' layers in 10 culture flasks containing DMEM:

Culture Flasks	Incubation days/confluence rates(~%)				
	Day 1*	Day 2	Day 3	Day 4	Day 5
1	-	20	50	75	90
2	-	30	55	80	95
3	-	25	70	80	90
4	-	35	60	85	90
5	-	45	65	70	95
6	-	40	55	75	90
7	-	30	70	75	90
8	-	30	60	80	85
9	-	40	65	85	95
10	-	35	55	80	95

*Growth on day 1 was mixed with other cell types (before washing) and thus confluence is immeasurable.

DISCUSSION:

Researches dealing with MSCswererevolutionalized at the last decade, initially MSCs were isolated from humans, thereafter, they had been successfully harvested from many other species including: mouse, rat, dog and horse[21-29]. They have also been isolated from almost every type of tissue, including: periosteum, brain, liver, bone marrow, adipose, skeletal muscle, amniotic fluid and hair follicle[30-39]. Expansion of the isolated MSCs is mandatory to make them ready for subsequent therapeutic application. Many different protocols have been applied in order to isolate and further expand MSCs including variation in materials and techniques used[1,2,40]. Many of such works have estimated a low *ex vivo* expansion potential of the separated MSCs[19].

Some studies have also suggested that MSCs have the capacity to undergo malignant transformation *in vitro,* but this is still a controversial phenomenon [41]. This highly variable expansion ability made it difficult to compare data from different researchers. Many factors may be implicated in affecting MSCs' *in vitro* expansion potential; including culture conditions such as nutritional level, cell confluence, oxygen level, number of passages and plastic surface quality[42].

The effect of passage number on MSCs' characteristics was studied by comparing early (< 5 passages) to late MSCs (> 15 passages), and results demonstrated that late MSCs had characteristics associated with cell aging as depicted by actin accumulation and reduced substrate adherence and that early MSCs remain pluripotent, while late MSCs had limited differentiation capacity[43].

In accordance with these data, investigators have attempted to study the stem cell niche in hopes of mimicking this environment in an *ex-vivo* setting to allow for more predictable cellular behavior.

The amount of oxygen to which the stem cells are exposed is a key difference between *in vivo* and the *in vitro* settings. IT is well known that tissue oxygen concentration is always significantly less than atmospheric oxygen levels[44]. Bone marrow has a characteristically hypoxic environment with oxygen concentrations very similar to ischemic tissue, but still higher than culture environment[45]. It was shown that MSCs that are cultured in hypoxic environments demonstrate greater expansion and differentiation potential. Some studies have suggested that these characteristics may in part relate to the up-regulation of telomerase activity in cells cultured in hypoxic conditions[46]. Accordingly, it was suggested that MSCs proliferate and renew *in vivo* under low oxygen conditions, while differentiate only when they approach higher oxygen tension represented by the blood vessels' environment.

In addition, with minor differences in expression patterns from one tissue source to another, all MSCs express embryonic cell markers such as Oct4, Nanog, and stage-specific embryonic antigen-4 (SSEA-4) [47]. Further variation in MSC characteristics has been associated with the age of the donor, with a direct correlation exiting between advanced age and decreased osteogenic potential.

This fact may in part contribute to disorders, such as osteoporosis, seen primarily in the aging population[48]. A similar decrease in cell number as a function of age has also been documented in satellite cells[49]. In addition, MSCs isolated from older donors demonstrate lower proliferation potential which may provide an explanation for the reduced healing capacity observed in older patients[50].

Conclusion and Recommendation:

Our modified method of isolation of bone marrow MSCs yielded very good separation rate with considerable *in vitro* expansion capability of the isolated MSCs in relatively short incubation time. It is recommended to perform more advanced experiments involving *in vivo* expansion and differentiation of the separated MSCs inoculated into lab animal models with varying diseases applicable for MSCs'cytotherapy.

REFERENCES:

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Science. 1999;284:143-147.[PubMed] [DOI]
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Science 1999;284:143 –7.
3. Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ. Stem Cells 2004;22:823–31.
4. Horwitz EM, Prockop DJ, Fitzpatrick LA, Koo WW, Gordon PL, Neel M, et al. Nat Med 1999;5:262– 4.
5. Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al. PNAS 1995;92:4857 –61.

6. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, et al. Nature 2002;418:41–9.
7. Krampera M, Pasini A, Rigo A, Scupoli MT, Tecchio C, Malpeli G, et al. Blood 2005;106:59– 66.
8. Krampera M, Cosmi L, Angeli R, Pasini A, Liotta F, Andreini A, et al. Stem Cells 2006;24:386 –98.
9. Lee RH, Kim B, Choi I, Kim H, Choi HS, Suh K, et al. Cell PhysiolBiochem 2004;14:311–24.
10. Shih DT, Lee DC, Chen SC, Tsai RY, Huang CT, Tsai CC, et al. Stem Cells 2005;23:1012–20.
11. Trubiani O, Di Prim io R, Traini T, Pizzicannella J, S carano A, Piattelli A, et al. Int J ImmunopatholPharmacol 2005; 18 :2 13 –21.
12. In 't Anker P S, Scherjon SA, Kleijburg -v an de r Keur C , de Groot-Swings GM , C la as FH, F ib be WE, e t a l. Stem Cells 2004 ;22:1338–45.
13. Erices A, Conget P, Minguel JJ. Br J Haematol 2000;109:235–42.
14. In't Anker PS, Noort WA , Scherjon SA, Kleijburg-van der Keur C, Kruisselbrink AB, van Bezooijen RL, et al. Haematologica 2003;88:845 –52.
15. Roufosse CA, D irekz e NC, Otto WR, Wright NA. Int J Biochem Cell Biol 2004;36:585 –97.
16. Digirolamo CM, Stokes D, Colter D, Phinney DG, Class R, Prockop DJ. Br J Haematol. 1999;107:275-281.[PubMed] [DOI]
17. Current Protocols in Immunology (1999) sections A.2A.1-A.2A.8 Copyright © 1999 by John Wiley & Sons, Inc.]
18. Ting Chen &Ya n Zhou & We n -Song Ta n (2009): Cell BiolToxicol 25:573 –586.
19. Røsland GV, Svendsen A, Torsvik A, Sobala E, McCormack E, Immervoll H, Mysliwietz J, Tonn JC, Goldbrunner R, Lønning PE. Cancer Res. 2009;69:5331-5339.[PubMed]
20. Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC. Tissue Eng. 2006;12:3007-3019.[PubMed] [DOI]
21. Igura K, Zhang X, Takahashi K, Mitsuru A, Yamaguchi S, Takashi TA. *Cytotherapy*. 2004;6:543-553.
22. Airey JA, Almeida-Porada G, Colletti EJ, Porada CD, Chamberlain J, Movsesian M, Sutko JL, Zanjani ED. *Circulation*. 2004;109:1401-1407.

23. Baddoo M, Hill K, Wilkinson R, Gaupp D, Hughes C, Kopen GC, Phinney DG.*J Cell Biochem*. 2003;89:1235-1249.
24. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Cell Tissue Res. 2005;319:243-253.
25. Devine SM, Bartholomew AM, Mahmud N, Nelson M, Patil S, Hardy W, Sturgeon C, Hewett T, Chung T, Stock W. *ExpHematol*. 2001;29:244-255.
26. Moscoso I, Centeno A, López E, Rodriguez-Barbosa JI, Santamarina I, Filgueira P, Sánchez MJ, Domínguez-Perles R, Peñuelas-Rivas G, Domenech N. *Transplant Proc*. 2005;37:481-482.
27. Ringe J, Häupl T, Sittinger M.: *Med Klin* (Munich). 2003;98Suppl 2:35-40.
28. Bjerknes M, Cheng H. Methods Enzymol. 2006;419:337-383.
29. Coles BL, Angénieux B, Inoue T, Del Rio-Tsonis K, Spence JR, McInnes RR, *et. al*.ProcNatlAcadSci USA. 2004;101:15772-15777.
30. da Silva Meirelles L, Chagastelles PC, Nardi NB. J Cell Sci. 2006;119:2204-2213.
31. Davis AA, Temple S. Nature. 1994;372:263-266.
32. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, de Groot-Swings GM, Claas FH, Fibbe WE, Kanhai HH. Stem Cells. 2004;22:1338-1345.
33. Liu Z, Martin LJ. J Neurotrauma. 2004;21:1479-1499.
34. Ringe J, Leinhase I, Stich S, Loch A, Neumann K, Haisch A, Häupl T, Manz R, Kaps C, Sittinger M. J Tissue EngRegen Med. 2008;2:136-146.
35. Sinanan AC, Hunt NP, Lewis MP. BiotechnolApplBiochem. 2004;40:25-34.[PubMed]
36. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Tissue Eng. 2001;7:211-228.[PubMed] [DOI]
37. Gregory CA, Singh H, Perry AS, Prockop DJ. J Biol Chem. 2003;278:28067-28078.[PubMed] [DOI]
38. Vacanti V, Kong E, Suzuki G, Sato K, Canty JM, Lee T. J Cell Physiol. 2005;205:194-201.[PubMed] [DOI]
39. M.T. Harris, D.L. Butler, G.P. Boivin, J.B. Florer, E.J. Schantz, R.J. Wenstrup. J. Orthop. Res. 22 (2004) 998–1003.
40. Vacanti V, Kong E, Suzuki G, Sato K, Canty JM, Lee T. J Cell Physiol. 2005;205:194-201.[PubMed] [DOI]
41. Panchision DM. J Cell Physiol. 2009;220:562-568.[PubMed] [DOI]

42. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC. Nat Med. 2004;10:858-864.[PubMed] [DOI]
43. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. J Cell Sci. 2004;117:2971-2981.[PubMed] [DOI]
44. Gang EJ, Bosnakovski D, Figueiredo CA, Visser JW, Perlingeiro RC. Blood. 2007;109:1743-1751.[PubMed] [DOI]
45. D'Ippolito G, Schiller PC, Ricordi C, Roos BA, Howard GA. J Bone Miner Res. 1999;14:1115-1122.[PubMed] [DOI]
46. Gibson MC, Schultz E. Muscle Nerve. 1983;6:574-580.[PubMed] [DOI]
47. Schäfer R, Knauf U, Zweyer M, Högemeier O, de Guarrini F, Liu X, Eichhorn HJ, Koch FW, Mundegar RR, Erzen I. Artif Organs. 2006;30:130-140.[PubMed] [DOI]
48. Kollet O, Shivtiel S, Chen YQ, Suriawinata J, Thung SN, Dabeva MD, Kahn J, Spiegel A, Dar A, Samira S. J Clin Invest. 2003;112:160-169.[PubMed] [DOI]
49. Rochefort GY, Delorme B, Lopez A, Hérault O, Bonnet P, Charbord P, Eder V, Domenech J. Stem Cells. 2006;24:2202-2208.[PubMed] [DOI]
50. Annabi B, Lee YT, Turcotte S, Naud E, Desrosiers RR, Champagne M, Eliopoulos N, Galipeau J, Béliveau R. Stem Cells. 2003;21:337-347.[PubMed] [DOI]