Comparative analysis of isolation, characterization and differentiation of human mesenchymal stem cells derived from adipose tissue and umbilical cord blood: An in-vitro study.

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Abstract

Background: Human mesenchymal stem cells have therapeutic value. They can be derived from different sources like umbilical cord blood, adipose tissue, placenta, whartons jelly etc. **Aim**: Aims and objectives of the study was for isolation, characterization and differentiation of Adipose derived Stem Cells (ASCs) and Umbilical Cord Blood derived Stem Cells (UCB-SCs) in human and comparison of ASCs and UCB-SCs for proliferation capacity.

Material and Methods: Samples of adipose tissue and umbilical cord blood were collected from Belagavi Institute of Medical Sciences with due consent from the subjects after obtaining local ethical committee clearance. Adipose tissue (AT) was collected from apparently healthy individuals undergoing surgeries. Umbilical Cord Blood was collected from newborns delivered by full term normal deliveries and Caesarean Sections.

Results: Mesenchymal stem cells were isolated from adipose tissue and umbilical cord blood. Cultures were done with DMEM and RPMI1640 media. The cultures were observed till 80 to 90 percent confluence. The cells were studied for stem cell markers and differentiation.

Conclusion: Mesenchymal stem cells could be derived from both adipose tissue as well as umbilical cord blood. The cells showed differentiation into other tissues. The growth characteristics differed as per culture media.

Key words: Adipose tissue, umbilical cord blood, stem cell culture, stem cell isolation

Introduction

Bone marrow, adipose tissue, umbilical cord blood, umbilical cord itself, dental pulp and fetal tissues are the multiple sources of stem cells. Mesenchymal stem cells (MSCs) are suitable for tissue engineering and cell therapy because of their multipotentiality, rapid proliferation and self renewal capability. Bone marrow, umbilical cord blood and adipose tissue were initial material of choice. In the 1960s Alexander Friedenstein, Soviet researcher discovered multipotent progenitors of conjunctive tissue in bone marrow. Arnold Caplan in 1990 showed the differentiation capacity of stem cells into not only bone and medullar stroma but also cartilage, tendons and muscle. In 2006 three minimal criteria were proposed for MSCs. 1) MSCs must be plastic adherent when maintained in standard cultured conditions. 2) MSCs must express CD105, CD73 and CD90 and lack expression of CD45, CD35, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. 3) MSCs must differentiate to osteoblasts, adipocytes and chondroblasts in vitro[1,2].

Bone marrow is the most studied and also only approved therapy for stem cell transplant but age is the significant factor affecting usage. Although bone marrow is the initial material of choice, tissues such as fat, fetal tissue and liquids (amniotic fluid, placenta, umbilical cord blood, Wharton's jelly) become alternative sources of Mesenchymal progenitor cells and have greater proliferation and differentiation potential than bone marrow. MSCs are also found in dental pulp or periodontal ligaments and synovia but the quality and quantity of tissue has limitation.

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Fat tissue contains at least hundred times more ASCs than bone marrow contains MSCs. These cells also have greater potential for proliferation. The differentiation and immunomodulatery potential of these cells is equivalent to that of MSCs. Hence suitable for tissue engineering and regenerative medical applications. In 1994 report was published regarding obtaining adherent stroma from Umbilical Cord Blood(UCB). Time interval between harvest and set up of culture affects the success of repairing MSCs. Cryopreservation also reduces chances of success. UCB MSCs have been found to differentiate not only in the mesodermal but also in the endodermal and ectodermal pathway. UCB needs to be cryopreserved while the AT is available at any age. Hence this study was undertaken for comparison between adipose tissue and umbilical cord blood derived Mesenchymal stem cells[3].

Materials and methods:

Sample collection- Samples of adipose tissue and umbilical cord blood were collected from Belagavi Institute of Medical Sciences with due consent from the subjects after obtaining local ethical committee clearance.

Inclusion criteria- Adipose tissue were collected from apparently healthy individuals undergoing surgeries. Umbilical Cord Blood was collected from newborns delivered by full term normal deliveries and Caesarean Sections.

Exclusion criteria- AT was not collected from patients undergoing cancer related surgical procedures. Subjects having infections like TORCH, IDV or subjects delivering babies having external birth anomalies were excluded from collection of Umbilical Cord Blood samples. All procedures were done under sterile conditions with aseptic precautions.

Umbilical cord blood stem cell culture:

In two 6cc heparin vacutainers total 10ml of Umbilical Cord Blood was collected from newborns delivered by full term normal deliveries and Caesarean Sections in Dept of OBG. Cord blood collection was done immediately after delivery from the cut end of the umbilical cord remaining with the placenta as per standard protocol after clamping the cord. Collected blood was diluted with PBS (with 2% antibiotic) in 1:1 proportion slowly with the help of pipette. Diluted blood was overlayed over Ficoll in 2:1 proportion. Then the solution was centrifuged at 2000 rpm for 30 minutes. Then supernatant plasma was pipetted out and discarded. Leukocyte ring was separated out with the help of pipette and was added to sterile centrifuge tube. Further it was washed with PBS twice at 1800 rpm for 10 minutes each. After centrifugation supernatant was discarded and culture media was added to pellet. The culture media used were high glucose DMEM and RPMI 1640. The cultures were setup in 24 well culture plates. Plates were kept in Co2 incubator at 37 deg. C and media was changed after 48 hrs initially and then daily till 80-90% of cell confluence was observed. Subculture were done after separating the adherent stem cells by using enzyme trypsin and planting the cell suspension in new culture wells upto three passages. All procedures were done in sterile condition in a laminar air flow.

Adipose tissue stem cell culture:

Unwanted extra fat about 15-20 ml was collected in sterile Phosphate buffer saline from apparently well-built patients aged between 20 to 50 years undergoing liposuction and reduction plastic surgeries. The adipose tissue collected was cut into small pieces (1mm) with the help of sterile instruments. The pieces were washed with PBS (+2% antibiotic), then it was centrifuged at 1800 rpm for 10 minutes. The supernatant fat was taken in clear sterile test tube. Collagenase solution (30% enzyme+70% DMEM) equal in volume to fat was added. The test tube was kept in shaker incubator for enzyme digestion at 37° C for 30 minutes at 100rpm. After adding equal amount of DMEM culture media to neutralize enzyme activity, centrifugation was done at 3000 rpm for 10 minutes. Supernatant was discarded and pellet was dissolved in 2mL of culture media. It was filtered through 70 mm cell strainer and was centrifuged at 2000 rpm for 20 minutes. The supernatant was discarded and 1.5mL of fresh media was added to it The culture media used were low glucose DMEM and RPMI 1640. The culture was setup in 24 well culture plates. Plates were kept in Co2 incubator at 37 deg. C and media was changed after 48 hrs initially and then daily till 80- 90% of cell confluence was observed. Subculture were done after separating the adherent stem cells by using enzyme trypsin and planting the cell suspension in new culture wells up to three passages. All procedures were done in sterile condition in a laminar air flow[3,4].

The derived mesenchymal stem cells from adipose tissue as well as umbilical cord blood were verified for mesenchymal stem cell markers like CD45 and CD105. Differentiation into different lineages was done using specific RD biosystems differentiating kits into adipocytes, chondrocytes and osteocytes which was confirmed by standard culture and staining procedures mention in the kit instructions.

Results:

Table No. 1 showing comparative growth of adipose tissue derived mesenchymal stem cells in low glucose DMEM and RPMI 1640 culture media

Sr No.	Parameter	Low glucose DMEM	RPMI 1640
1.	Rate of growth	Slow	Fast
2.	Days to reach confluence	8 -10 days	6-8 days
3.	Morphology of cells	Varied	Spindle shaped
4.	Yield	Medium	Less
5	Long term growth	Well sustained	Not well sustained
6.	Short term growth	Good	Good

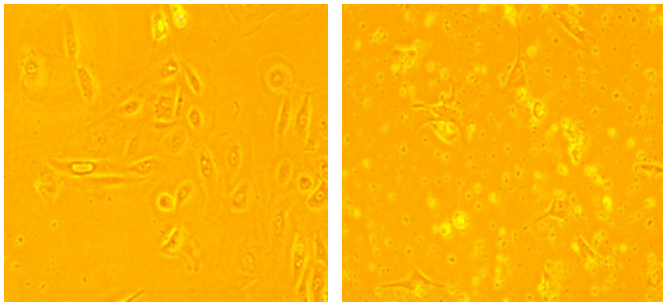
From Table no. 1 it is clear that adipose tissue derived stem cells grow in good sustained quality and quantity in low glucose DMEM media.

Table No. 2 showing comparative growth of umbilical cord blood derived mesenchymal stem cells in low glucose DMEM and RPMI 1640 culture media

Sr No.	Parameter	High glucose DMEM	RPMI 1640
1.	Rate of growth	Slow	Fast
2.	Days to reach confluence	8 -10 days	6-8 days
3.	Morphology of cells	Varied	Spindle shaped
4.	Yield	Medium	More
5	Long term growth	Well sustained	Not well sustained
6.	Short term growth	Good	Good

From Table no. 2 it is clear that umbilical cord blood derived stem cells grow in good sustained quality in high glucose DMEM media but excellent in quantity in RPMI 1640 media. RPMI 1640 media in both cases gives only spindle shaped cells but DMEM media gives varied morphology.

Photograph No. 1 Photograph No.2

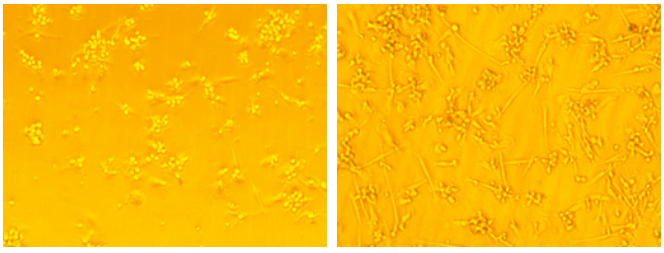


Photograph no.1 and 2 showing growth of adipose tissue stem cell culture in low glucose DMEM media and umbilical cord blood derived stem cells in high glucose DMEM media respectively.

Photograph no. 1 shows growth of adipose tissue derived mesenchymal stem cell in low glucose DMEM liquid culture media. The growth of cells is slow and sustained, taking 8 to 10 days for confluence to be seen. The morphology of cells varies from spindle shape to fibroblast shape. The yield was good.

Photograph no. 2 shows growth of umbilical cord blood derived mesenchymal stem cell in high glucose DMEM liquid culture media. The growth of cells is slow and sustained taking 8 to 10 days for confluence to be seen. The morphology of cells varies from spindle shape to fibroblast shape. The yield was less.

Photograph No. 3 Photograph No. 4

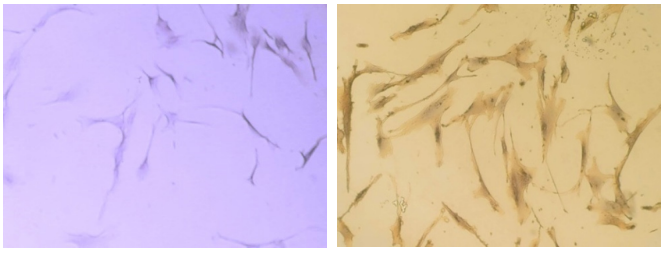


Photograph no.3 and 4 show growth of adipose tissue stem cell culture and umbilical cord blood derived stem cells in RPMI 1640 media respectively.

Photograph no. 3 shows growth of adipose tissue derived mesenchymal stem cell in RPMI 1640 liquid culture media. The growth of cells is rapid and sustained taking 6 to 8 days for confluence to be seen. The morphology of cells was spindle shape. The yield was medium.

Photograph no. 4 shows growth of umbilical cord blood derived mesenchymal stem cell in RPMI 1640 liquid culture media. The growth of cells is slow and sustained taking 6 to 7 days for confluence to be seen. The morphology of cells was spindle shape. The yield was excellent.

Photograph No. 5 Photograph No. 6

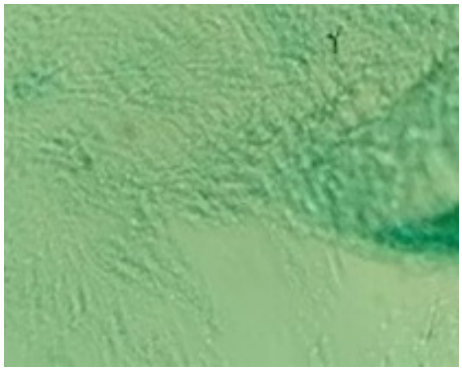


Photograph no.5 and 6 shows Mesenchymal stem cells derived from AT and UCB show negative results to CD45 and positive results to CD105 markers respectively.

Photograph no. 5 & 6 shows mesenchymal stem cells

negative for CD45 and positive for CD90 markers respectively.

Photograph No. 7

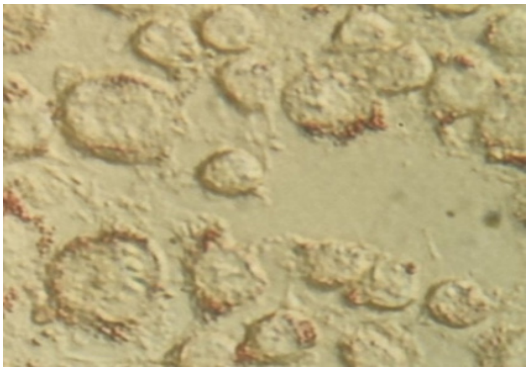


Photograph no. 7 shows differentiation of adipose tissue stem cell and umbilical cord blood stem cell into chondrocyte stained by alcian blue.

Photograph No. 8

Photograph no. 8 shows differentiation of adipose tissue stem cell and umbilical cord blood stem cell into osteocyte stained by alizarin red.

Photograph No. 9



Photograph no. 9 shows differentiation of adipose tissue stem cell and umbilical cord blood stem cell into adipocyte stained by oil R.

Photograph no. 7 to 9 shows differentiation of adipose tissue and umbilical cord blood derived stem cells into chondrocyte, osteocyte and adipocyte.

Discussion: Previous studies show that ASCs isolated from different locations, cell types, and species have different density and different procedures may affect ASC quality, functionality, and plasticity. The protocol reported by Zuk et al is still the most widely used method for ASC isolation. The same protocol has been followed in our study. The modifications suggested to this protocol were experimented by Palumbo et al. He found that the differentiation ability of ASCs is not influenced by any of the handling methods. After either spontaneous stratification at 20 min or centrifugation at 400 × g, the middle layer obtained from lipoaspirate samples can provide sufficient ASCs and maintain adipocyte integrity, demonstrating that the two approaches are effective[4-6].

In our study the use of low glucose DMEM media showed slow and sustained growth as observed by others. We could not find studies where RPMI 1640 media was used. The growth rate difference seen in different media may be due to variety of cells stimulated in stromal vascular fraction. Further studies with pre and post growth analysis of stem cells with automated FACS counter are needed. Many studies attempted to determine the localization of ASCs within adipose tissue, but the precise localization remains unclear. Some believe that ASCs are located in the vasculature of adipose tissue, other findings suggest that ASCs reside within adipose tissue in a perivascular location[5,6].

Although ASCs are of mesodermal origin, it has been shown that they can differentiate into cells of ectodermal, endodermal, and mesodermal origin. In our study, differentiation into chondrocyte, osteocyte and adipocyte was seen. Studies prove that Adipogenic differentiation can be induced in medium that typically contains DMEM (with 10% FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 50 mM indomethacin, 0.5 mM dexamethasone, and 10 mg/ml insulin. For differentiation of ASCs into osteoblasts, the culture medium should contain DMEM (with 10% FBS), 10 mM β-glycerol phosphate, 40 µg/ml gentamicin, 8 M dexamethasone, 2 mM L-glutamine, 8 M dihydroxyvitamin D3, and ascorbic acid. Chondrogenic differentiation medium should be supplemented with basic fibroblast growth factor (bFGF), dexamethasone, transforming growth factor-β1, insulin, ascorbate-2-phosphate, and BMP-6. The kit used in this study was prepared on the same basic principles mentioned above[7,8].

For chondrogenic differentiation, a micro mass pellet culture system should be preferred because cells require a three-dimensional environment to increase cell-to-cell interaction and produce a cartilage-like

matrix. Some studies have shown that the cultured ASCs can also differentiate into other cells types including vascular smooth muscle cells, keratinocytes, hepatocytes, and beta islet cells. ASCs also have the capacity to differentiate toward cells of ectodermal origin, such as neuronal and glial lineages[9-11].

The proliferating rate of UCB-MSCs was reported to widely range from about 30 h to hundred hours. Even Some studies denied the presence of MSC in UCB, whereas others have claimed that under suitable conditions an isolation rate up to 60% can be achieved. Generally, an enrichment rate of around 30% is the widely reported result in most studies. In our study we saw slow but sustained growth in DMEM media as observed by above authors. But in RPMI 1640 the growth seen was best and fast. We couldn't find references for comparison. Rebelatto et al. observed that sample volume did not have any influence on the success rate of MSC isolation from umbilical cord blood. Others have proposed that sample volume is a critical parameter for isolation of MSC. Attiteh et al faced the crucial problem of the low number of MSC in UCB in comparison to bone marrow. Many publications have pointed out the function of coating components on MSC growth and survival. Without surface coating the culture took longest of doubling time. Research conducted by T. Nazari-Shafti et al and Alexander Popov et al, shows that the presence of human serum in culture improved the proliferation capacity of UCB-MSCs[12-17].

In our study we could differentiate the UCB stem cells into chondrocyte, osteocyte and adipocyte as observed by previous researchers like Rebelatto et al. In a surprising study Kern et al contradicts the findings, stating that UCB-derived MSC cannot differentiate to adipose tissue. Previous studies revealed that VEGF-A combined with HGF resulted in a potent angiogenic effect in a mouse model of limb ischemia. The combination of these two molecules can promote neovascularization of endothelial cells [14-18].

Varied morphology of cells was observed by previous researches also. They have mentioned oval/round or elongated cells with visible borders, cells with cytoplasmic extensions and occasional multiple nuclei. These cells were considered as osteocyte like cells (OLC). Interestingly there is mention of OLCs could become confluent within two months which explains multi nucleated cells. We also encountered the presence of OLCs as other researchers. But in our case varied morphology was limited to only DMEM media but surprisingly not seen at all in RPMI 1640 media culture[19].

ASCs as well as UCB stem cells have practical advantages in clinical medicine and research has made their application more feasible. MSCs can secrete numerous growth factors and cytokines that are critical for wound healing. MSCs have been shown to be effective in treating severe refractory acute graft-versus-host disease and hematological and immunological disorders such as idiopathic thrombocytopenic purpura and refractory pure red cell aplasia, indicating that MSCs may have immunomodulatory effects. Fat grafting demonstrates the clinical regenerative potential of ASCs. Further studies are needed to explore among other topics the long-term safety of MSCs, their precise mechanisms of action and the ability to translate experimental findings to the clinical with accurate prediction[20].

Conclusions:

a. Mesenchymal stem cells can be derived from umbilical cord blood as well as adipose tissue
b. Different media give different rate of growth and morphology of cells.
c. Further analysis of pre and post culture of cells in different media using automated cell counter is needed.

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References:

1. *Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991 Sep;9(5):641-50. doi: 10.1002/jor.1100090504. PMID: 1870029..*
2. *Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, Keating A; International Society for Cellular Therapy. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy. 2005;7(5):393-5. doi: 10.1080/14653240500319234. PMID: 16236628.*
3. *Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells. 2006 May;24(5):1294-301. doi: 10.1634/stemcells.2005-0342. Epub 2006 Jan 12. PMID: 16410387.*
4. *Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001 Apr;7(2):211-28. doi: 10.1089/107632701300062859. PMID: 11304456.*
5. *Palumbo P, Lombardi F, Siragusa G, Cifone MG, Cinque B, Giuliani M. Methods of Isolation, Characterization and Expansion of Human Adipose-Derived Stem Cells (ASCs): An Overview. Int J Mol Sci. 2018 Jun 28;19(7):1897. doi: 10.3390/ijms19071897. PMID: 29958391; PMCID: PMC6073397.*
6. *Palumbo P, Miconi G, Cinque B, La Torre C, Lombardi F, Zoccali G, Orsini G, Leocata P, Giuliani M, Cifone MG. In vitro evaluation of different methods of handling human liposuction aspirate and their effect on adipocytes and adipose derived stem cells. J Cell Physiol. 2015 Aug;230(8):1974-81. doi: 10.1002/jcp.24965. PMID: 25736190.*
7. *Bacakova L, Zarubova J, Travnickova M, Musilkova J, Pajorova J, Slepicka P, Kasalkova NS, Svorcik V, Kolska Z, Motarjemi H, Molitor M. Stem cells: their source, potency and use in regenerative therapies with focus on adipose-derived stem cells - a review. Biotechnol Adv. 2018*

Jul-Aug;36(4):1111-1126. doi: 10.1016/j.biotechadv.2018.03.011. Epub 2018 Mar 18. PMID: 29563048.

8. *Si Z, Wang X, Sun C, Kang Y, Xu J, Wang X, Hui Y. Adipose-derived stem cells: Sources, potency, and implications for regenerative therapies. Biomed Pharmacother. 2019 Jun;114:108765. doi: 10.1016/j.biopha.2019.108765. Epub 2019 Mar 25. PMID: 30921703.*
9. *Varghese J, Griffin M, Mosahebi A, Butler P. Systematic review of patient factors affecting adipose stem cell viability and function: implications for regenerative therapy. Stem Cell Res Ther. 2017 Feb 28;8(1):45. doi: 10.1186/s13287-017-0483-8. PMID: 28241882; PMCID: PMC5329955.*
10. *Zhang M, Zhang F, Sun J, Sun Y, Xu L, Zhang D, Wang Z, He W. The condition medium of mesenchymal stem cells promotes proliferation, adhesion and neuronal differentiation of retinal progenitor cells. Neurosci Lett. 2017 Sep 14;657:62-68. doi: 10.1016/j.neulet.2017.07.053. Epub 2017 Jul 31. PMID: 28774569.*
11. *Fesharaki M, Razavi S, Ghasemi-Mobarakeh L, Behjati M, Yarahmadian R, Kazemi M, Hejazi H. Differentiation of Human Scalp Adipose-Derived Mesenchymal Stem Cells into Mature Neural Cells on Electrospun Nanofibrous Scaffolds for Nerve Tissue Engineering Applications. Cell J. 2018 Jul;20(2):168-176. doi: 10.22074/cellj.2018.4898. Epub 2018 Mar 18. PMID: 29633593; PMCID: PMC5893287.*
12. *Pham PV, Vu NB, Pham VM, Truong NH, Pham TL, Dang LT, Nguyen TT, Bui AN, Phan NK. Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. J Transl Med. 2014 Feb 24;12:56. doi: 10.1186/1479-5876-12-56. PMID: 24565047; PMCID: PMC3939935.*
13. *Van SY, Noh YK, Kim SW, Oh YM, Kim IH, Park K. Human umbilical cord blood mesenchymal stem cells expansion via human fibroblast-derived matrix and their potentials toward regenerative application. Cell Tissue Res. 2019 May;376(2):233-245. doi: 10.1007/s00441-018-2971-2. Epub 2019 Jan 4. PMID: 30610451.*
14. *Rebelatto CK, Aguiar AM, Moretão MP, Senegaglia AC, Hansen P, Barchiki F, Oliveira J, Martins J, Kuligovski C, Mansur F, Christofis A, Amaral VF, Brofman PS, Goldenberg S, Nakao LS, Correa A. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. Exp Biol Med (Maywood). 2008 Jul;233(7):901-13. doi: 10.3181/0712-RM-356. Epub 2008 Apr 29. PMID: 18445775.*
15. *Nazari-Shafti TZ, Xu Z, Bader AM, Henke G, Klose K, Falk V, Stamm C. Mesenchymal Stromal Cells Cultured in Serum from Heart Failure Patients Are More Resistant to Simulated Chronic and Acute Stress. Stem Cells Int. 2018 Apr 1;2018:5832460. doi: 10.1155/2018/5832460. PMID: 29760728; PMCID: PMC5901835..*
16. *Popov A, Scotchford C, Grant D, Sottile V. Impact of Serum Source on Human Mesenchymal Stem Cell Osteogenic Differentiation in Culture. Int J Mol Sci. 2019 Oct 11;20(20):5051. doi: 10.3390/ijms20205051. PMID: 31614651; PMCID: PMC6834181.*
17. *Vasaghi A, Dehghani A, Khademalhosseini Z, KhosraviMaharlooei M, Monabati A, Attar A. Parameters that influence the isolation of multipotent mesenchymal stromal cells from human umbilical cord blood. Hematol Oncol Stem Cell Ther. 2013 Mar;6(1):1-8. doi: 10.1016/j.hemonc.2013.02.002. Epub 2013 Feb 28. PMID: 23664598.*
18. *Makarevich P, Tsokolaeva Z, Shevelev A, Rybalkin I, Shevchenko E, Beloglazova I, Vlasik T, Tkachuk V, Parfyonova Y. Combined transfer of human VEGF165 and HGF genes renders potent angiogenic effect in ischemic skeletal muscle. PLoS One. 2012;7(6):e38776. doi: 10.1371/journal.pone.0038776. Epub 2012 Jun 13. PMID: 22719942; PMCID: PMC3374822.*
19. *Sibov TT, Severino P, Marti LC, Pavon LF, Oliveira DM, Tobo PR, Campos AH, Paes AT, Amaro E Jr, F Gamarra L, Moreira-Filho CA. Mesenchymal stem cells from umbilical cord blood: parameters for isolation, characterization and adipogenic differentiation. Cytotechnology. 2012 Oct;64(5):511-21. doi: 10.1007/s10616-012-9428-3. Epub 2012 Feb 12. PMID: 22328147; PMCID: PMC3432537.*
20. *Eming SA, Krieg T, Davidson JM. Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol. 2007 Mar;127(3):514-25. doi: 10.1038/sj.jid.5700701. PMID: 17299434..*

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