

Osteogenic differentiation of Wharton's Jelly - derived mesenchymal stem cells

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Abstract

Induced osteogenesis of mesenchymal stem cells (MSCs) may provide an important tool for bone injuries treatment. Human umbilical cord is routinely discarded as clinical waste and may be used as noncontroversial MSCs sources. It still remains to be verified which source of MSCs is the most suitable for bone regeneration. The aim of this research was to investigate the osteogenic potential of MSCs derived from Wharton's jelly of the human umbilical cord. Osteogenic differentiation of MSCs was detected and quantified by Alizarin Red S (ARS) staining for calcium deposition.

Keywords: Mesenchymal stem cells, Umbilical cord stem cells, Wharton's jelly derived mesenchymal stem cells, osteogenic differentiation

1 INTRODUCTION

Bone defects caused by trauma, inflammation or cancer result in various functional problems and repair of these injuries is a significant challenge in reconstructive surgery. In this research, induced osteogenesis of Wharton's jelly mesenchymal stem cells (WJ-MSCs) may provide an important tool for bone defect treatment. Multipotent MSCs which possess self-renewal and can differentiate into different cell types, such as osteoblasts, chondrocytes or adipocytes, can be isolated from: bone marrow, adipose tissue, and birth-associated tissues, such as umbilical cord, cord blood, placenta and amnion [8],[12].

Among these sources, human umbilical cord is routinely discarded as clinical waste [35]. In contrast to MSCs obtained from birth-associated tissues, adult tissue derived MSCs are more susceptible for cellular damage, which leads to loss of regenerative capacity and differentiation potential. It is also suggested that MSCs derived from birth-associated tissues, in comparison to MSCs obtained from adult tissues, showed increased proliferative capacity and shorter doubling times in vitro, especially under hypoxic conditions [8],[22].

So far, bone marrow derived MSCs (BM-MSCs) have been mainly used as a cell source for bone engineering. However, their utility in bone tissue-engineering is restricted for the reasons of complicated invasive procedures

and limited ability to provide sufficient cell numbers for clinical applications [35]. Also, the number and differentiation potential of the BM-MSCs decreases with increasing age of the MSCs donor [19].

Therefore, the interest of obtaining MSCs from other tissues that provide the same beneficial functions as BM-MSCs while overcoming their disadvantage has increased [13],[19]. Several studies have reported superior cell biological properties, such as improved proliferative capacity, life span and differentiation potential of MSCs from birth-associated tissues over BM-MSCs [13], [22], [12].

This led us to investigate the osteogenic potential of Wharton's jelly derived mesenchymal stem cells (WJ-MSCs). We assessed the osteogenic differentiation by staining with Alizarin Red stain to assess the mineralization capability.

2 MATERIALS AND METHODS

2.1 Ethical considerations

All protocols were approved by the ethical committee of Mansoura University, Faculty of Medicine (code # MS/528).

2.2 Sampling procedure

According to the policy approved by the local Ethical Committee, all tissue samples were collected after written informed consent from mothers at Obstetrics and Gynecology department of Mansoura university hospital. The collection was with inclusion and exclusion criteria according to College of American Pathologists (CAP) recommendations:

Inclusion criteria are:

- Cesarean section.
- Age of the mother ranging from 18-36 years old.
- Full term infants.
- Females faced no complications throughout pregnancy.

Exclusion criteria are:

Maternal criteria:

- Normal delivery
- Mothers younger than 18 or older than 36 years old.
- Preterm deliveries or deliveries at ≥ 42 weeks.
- Multiple gestations.
- Maternal disorders:
 - Diabetes during any portion of pregnancy
 - Hypertension
 - Autoimmune disorders
 - Hematologic disorders
 - Mothers had a positive result for blood-transmitted diseases
 - Abnormal hemoglobin electrophoresis
 - Ruptured membranes.

Fetal criteria:

- Fetal or perinatal death.
- Fetal or neonatal congenital anomalies.
- Intrauterine growth retardation or macrosomia (>4500 g for term infants).
- Hydrops fetalis.

- Persistent hypotonia.
- Apgar scores <6
- 5 minutes Ventilatory assistance >10 minutes
- Hct $<35\%$

3 ISOLATION OF MESENCHYMAL STEM CELLS FROM WHARTON'S JELLY:

About 15-20 cm of the term umbilical cord after caesarian sections were rapidly collected and rinsed in normal saline and placed in a container with (DMEM) low glucose, 100 U/ml penicillin and 100mg/ml streptomycin and transported to Mansoura Research Center For Cord Stem Cells (MARC_CSC) at Mansoura University.

Cutting the cord into 2-3 pieces by using a sterile scalpel and forceps, Washing each piece 2-3 times with phosphate buffered saline (PBS) in a sterile petri dish to remove any blood, Make a longitudinal section of the cord to remove the vein and the arteries by a sterile scalpel and forceps then , Scratching the cord to obtain the Wharton's Jelly .The cells were isolated with enzymatic mixture consisting of collagenase, hualuronidase and trypsin .

Cells isolated by using 10 ml of collagenase type one(sigma Aldrich-c1639) preheated at 37°C and 5 ml of Hyaluronidase type 5(sigma Aldrich-CH3757) for 1 hour at 37°C in a shaker water bath .Then 5 ml of trypsin EDTA (Sigma Aldrich T4049) was added and shaking was done again for 30 min . After incubation ,dilute the digested suspension with PBS(Gibco-c10010,USA) then centrifuge the suspension at 1100 g for 10 minutes . After centrifugation, remove the supernatant and resuspend the pellet in a PBS solution for another centrifugation at 1100 g for 10 minutes . After the 2nd centrifugation , remove the supernatant then resuspend the pellet in 5 ml complete media consists of:

- DMED low glucose 90% (Sigma Aldrich D5523)
- Fetal bovine Serum 10% (Gibco-C.16000044,USA)
- Antibiotic Antimycotic solution 1 ml (penicillin 100 unit /ml and streptomycin 100mg/ml) (Sigma Aldrich-c A5955,USA).

Then , Transfer it into a sterile 25 cm² tissue culture flask, incubate in a co₂ incubator at 37°C , Change the complete media every 2-3 days until reaching 80% confluency (21 day). [15]

7 ASSESSING THE OSTEOGENIC DIFFERENTIATION WITH ALIZARIN RED S (ARS) STAIN

4 SUBCULTURE AND EXPANSION OF THE CELLS:

We used in these processes complete medium (DMEM low glucose (Sigma Aldrich D5523), fetal bovine serum (Gibco-C.16000044,USA), streptomycin and penicillin. The medium was changed every 3 days for 21 days. Cells were allowed to adhere to culture flask and non-adherent cells were removed by changing medium. When the adherent cells reached to covering 80% of flask, the cells washed by 5 ml PBS then add 5ml of 0.25% trypsin-EDTA (sigma Aldrich-T4049), and examined under microscope. When the adherent cells began to separate from each other, the trypsin EDTA was removed and the flask incubated for 3 minutes at 37°C. About 10 ml of culture media was added to the flask, pipetted and transferred to new 75 cm tissue culture flask and increase the culture media to 25 ml. For future passage the above procedure done again.[3]

5 CHARACTERIZATION OF WJ-MSCs BY FLOW CYTOMETRY

After the third passage, about 5×10^5 were resuspended in 1 ml culture media. The cells were stained by a combination of antibodies: PE-conjugated CD90 and FITC conjugated CD45 (BD Bioscience) and incubated in the dark for 30 min. at room temperature. A 2 ml stain buffer fetal bovine serum (BD Bioscience) was added to each tube and centrifuged at 2000 r.p.m for 10 minutes. The supernatant was discarded and 500µl stain buffer was added to each tube. The cells ready for analyzed on a flow cytometer (FACS calibur, Becton Dickinson) by collecting 10,000 events and the data analyzed using the cellquest Software (Becton Dickinson).[26]

6 OSTEOGENIC DIFFERENTIATION OF WJ- DERIVED MESENCHYMAL STEM CELLS

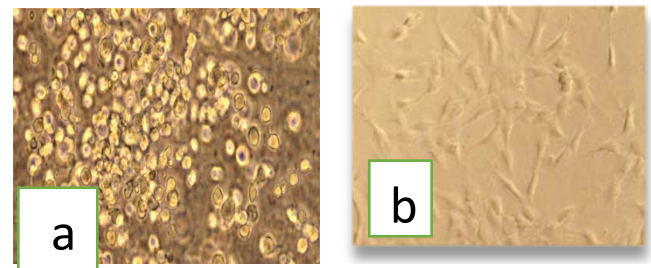
MSCs from the third passage were seeded at (24-well plates; 2×10^4 cells/well). The growth media were replaced with the osteogenic medium(Gibco c- A1007201,USA) containing inducers, supplements and growth factors such as dexamethasone, ascorbate, β -glycerophosphate, mesenchymal cell growth supplement, L-glutamine, and penicillin/streptomycin. Control cells were cultured in an appropriate growth medium(complete media 90%). Both media were changed every 3 days. All of the cultures were grown for 21 days. [36]

Evaluate the differentiation with Alizarin Red S stain(Sigma-Aldrich-TMS-008-C) analysis to detect calcium deposits (mineralization) and bone nodules. After the cells have differentiated, aspirate the osteogenic differentiation media from the wells and rinse with PBS, Fix cells with 2 ml of 4% formaldehyde solution for 30 minutes, Rinse cells twice with PBS, Stain the cells with 1 ml alizarin red S working solution for every well for 3-5 minutes, Rinse cells 2-3 times with PBS. Cells can visualized under inverted microscope (Nikon Eclipse TS100). The differentiated cells (mineralized osteoblast-like cell with extracellular calcium deposits) will appear in orange red color [36]. For staining quantification, calcium deposits stained with ARS were eluted with leaching solution of 20% methanol and 10% acetic acid in distilled water (all chemicals from Sigma Aldrich) for 15 min[8]. The absorbance of the extracted stain was measured at 450 nm.

8 RESULTS

8.1 Expansion of WJ-MSCs

Attachment of the cells to tissue culture plastic flask was observed after 3 days of culture which was rounded and flat cells. After 7 days, spindle shaped cells with short processes appear. After 14 days, spindle-shaped cells reached 80% confluency. After third passage, cultures were composed of a homogenously fibroblastic cell monolayer .fig. 1 (a-b-c-d).



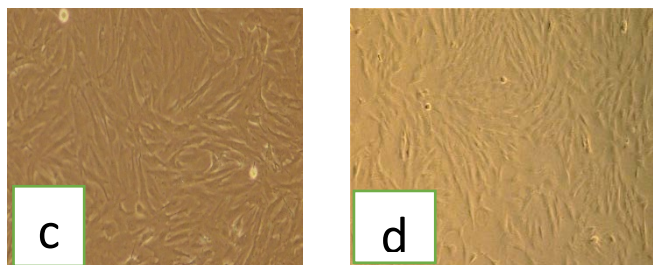
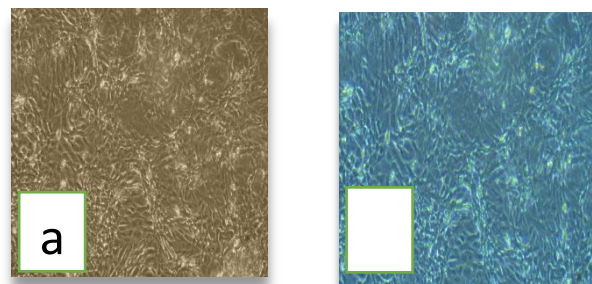


Fig. (1): Showing images of WJ-MSCs
(a) just after culture
(b) 7 days after culture
(c) 14 days of culture
(d) 3rd passage.

the cells became thinner, more elongated with longer extension Fig3 (d)



8.2 Characterization of WJ-MSCs by flow cytometry analysis

Cultures of WJ-MSCs were analyzed for expression of cell-surface markers. WJ-MSCs were negative for the hematopoietic markers CD45 with percentage 9.2% and positive for CD90 with percentage 88.7% (fig .2)

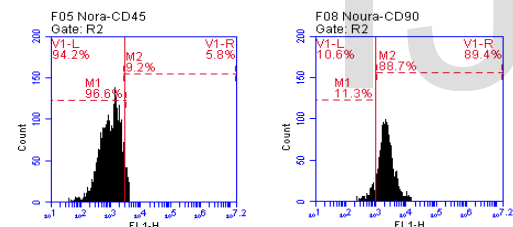


Fig. (2): Immunophenotypic analysis of WJ-MSCs representing the flow cytometry performed on the WJ-MSCs for CD45, CD90.

8.3 Osteogenic differentiation and calcium deposition

The induced osteoblasts (mineralized osteoblast -like cell with extracellular calcium deposits) appeared in orange red color using an inverted microscope (Nikon Eclipse TS100; magnification of 100×) Fig 3 (e). Control group: the cells cultured in primary culture media shown fibroblast spindle shape like cells Fig 3(a) and after staining not induced into osteoblast differentiation Fig 3 (b) .Test group at day 10 of staining :the cells were elongated and more spindle shaped Fig 3 (c). Test group at day 21 of staining:

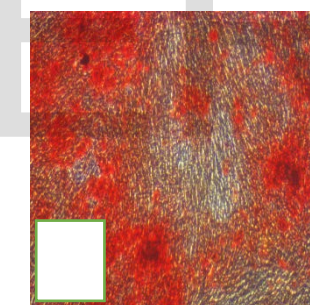
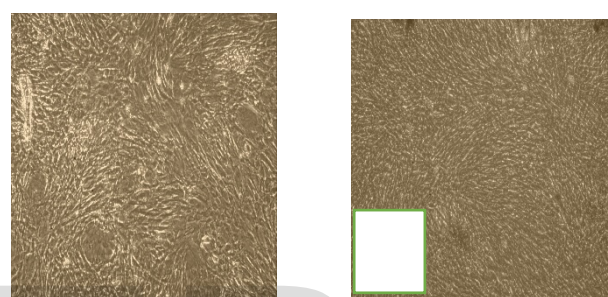


Fig. 3: (a) Control after 21 days of the primary culture media
(b) Control after staining
(c) Test after 10 days of the differential media
(d) Test after 21 days of the differential media
(e) Test after staining

The ARS amount [nmol/well] extracted from extracellular matrix of WJ-MSCs after 21 days of culture in the osteogenic medium was 1082.93±190.5 nmol/well. Fig.4

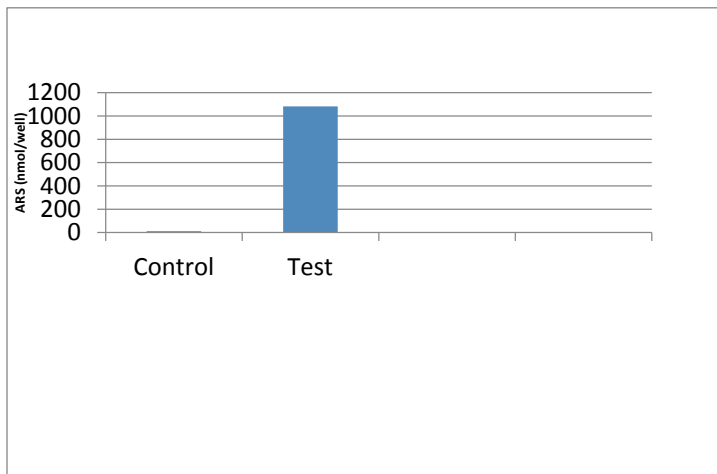


Fig.(4).The amounts of ARS [nmol/well] extracted from extracellular matrix of WJ-MSCs. Data calculated as the mean \pm S.D. from three experiments.

9 DISCUSSION

MSCs are suggested as a suitable source for tissue engineering [22], [12]. MSCs are found in adult tissues such as bone marrow, dental pulp, adipose tissues, and in neonatal tissues such as amnion, placenta, umbilical cord [7]. BM was the main traditional source of MSCs [27], [37],[23]. However, collection of MSCs from bone marrow is an invasive and painful procedure [20]. Also, the number of bone marrow MSCs significantly decreases with age and infirmity [26], [4]. Also, there is a potential risk of viral contamination during the isolation of MSCs from bone marrow [27],[14]. All these factors limit the use of bone marrow as a suitable source of MSCs for cell therapy.

Therefore, searches increased on tissues containing cells with a higher proliferative and differentiation potency and a lower risk of viral contamination. In this regard, Wharton's Jelly [25] has been shown to be a suitable source of MSCs with many advantages. The UCMSCs have surface markers [34], immune properties [32] and a differentiation potential similar to MSCs derived from bone marrow [21]. WJ-MSCs have the ability to specialize into ectoderm (neural cells), mesoderm (osteocyte, adipocyte, chondrocyte, smooth muscles) and endoderm (Pancreatic progenitor).[27]

There are many different isolation techniques of WJ-MSCs as explant (Exp), collagenase/trypsin (CT), collagenase/hyaluronidase/trypsin (CHT) and trypsin/EDTA (Trp). The utilization of collagenase/trypsin enzymes gives better results than the other isolation methods (i.e. collagenase/hyaluronidase/trypsin, trypsin

and explant) (29). Cell isolation using trypsin followed by digestion of the remaining tissue with collagenase proved to be very effective for obtaining MSCs [30] as Trypsin converts pro-collagenase into the active form of the enzyme [33], so most of the connective tissue cells will be released.

The cells isolated by Trypsin only did not proliferate as fast as the cells in the other groups [29]. This is due to degradation of the extracellular matrix and disintegration of cell membranes may have caused cellular damage through the use of trypsin alone for a long period [6], because some cells are sensitive to exposure with trypsin but not to collagenase [24].

Other studies searched the isolation of cells from different regions of the umbilical cord e.g. ;WJ, whole cord, cord vein and perivascular area, mixed cord and cord lining [22].

They showed that the cells from all regions of UC contained a high number of adherent MSCs which proliferate rapidly in number [22].

In this research the best result during WJ-MSCs isolation obtained from enzymatic digestion with (collagenase, hyaluronidase and trypsin).

In this research, cells isolated from WJ, are adherent to plastic and negative for the expression of hematopoietic markers (CD 45) however positive for MSCs markers (CD90). According to MSCs criteria described by The International Society for Cellular Therapy (ISCT) [5].

Studies have shown that UC-MSCs have the potential of in vitro and in vivo differentiation into many cell types, including osteocytes, cartilage, neurons, cardiomyocytes, striated muscle, insulin secreting cells [1], [11],[18],[17].

The osteogenic differentiation can be assessed with: mineralization capability, alkaline phosphatase (ALP) activity, osteoprotegerin (OPG) and osteocalcin (OC) secretion in the differentiated cultures of these MSCs [36].

Osteoblasts can be induced to produce extracellular calcium deposits in vitro. This process is called mineralization. It is an indication of successful in vitro bone formation [31].

Mineralization can be assessed by a number of methods including ARS staining, fluorescent calcein binding and von Kossa. The von Kossa method is based on the binding of silver ions to the anions (phosphates, sulfates, or carbonates) of calcium salts in an acidic environment. Photochemical reduction of silver salts leads to dark brown or black metallic silver deposits. Unlike the von Kossa

staining which is not specific for calcium, ARS forms chelates with calcium cations [7].

Osteogenic differentiation of MSCs in vitro can be divided into three stages. During the first stage (days 1–4) the cells proliferate and a peak in cell number is seen [10]. Then (days 5–14) an early cell differentiation characterized by the transcription and protein expression of ALP and OPG occurs [2], [16]. In MSCs OPG is an early indicator of osteogenic differentiation. Its level markedly increases over the days 4–7 and then declines while ALP activity increases. The last stage (days 14–28) is characterized by high expression of osteocalcin followed by calcium and phosphate deposition [10], [9].

In this study Cells undergo osteogenic differentiation for 21 days using osteogenic differentiation media and osteogenesis supplementation and evaluate the differentiation with Alizarin Red S stain analysis to detect calcium deposits which give positive orange-red stained cells.

10 CONCLUSION:

We provide evidence that WJ-MSCs possess the potential to differentiate towards the osteogenic lineage.

11 ACKNOWLEDGEMENT

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12 REFERENCES

1-Aghaee-Afshar M.; Rezazadehkermani M.; Asadi A.; Malekpour-afshar R.; Shahesmaeili A.; Nematollahi-Mahani S. N. Potential of human umbilical cord matrix and rabbit bone marrow-derived mesenchymal stem cells in repair of surgically incised rabbit external anal sphincter. *Dis Colon Rectum*, 2009; 52: 1753–1761.

2-Aubin JE. Regulation of osteoblast formation and function. *Rev Endocr Metab Disord*, 2001; 2: 8194 .

3-Azandeh S, Orazizadeh M, Hashemitabar M, Khodadadi A, Shayesteh AA, Nejad DB, Gharravi AM, Allahbakhshi E. Mixed enzymatic-explant protocol for isolation of mesenchymal stem cells from Wharton's jelly and encapsulation in 3D culture system. *J. Biomedical Science and Engineering*, 2012; 5: 580-586.

4-Bobis S.; Jarocho D.; Majka M. Mesenchymal stem cells: characteristics and clinical applications. *Folia Histochem Cytobiol*, 2006; 44: 215–230.

5-Dominici M , Le Blane K , Mueller I , Slaper – Cortenbach I , Marini F , Krause D. Minimal criteria for defining multipotent mesenchymal stromal cells . *The International Society for Cellular Therapy position statement Cytotherapy*, 2006; 8 : 315 – 317 .

6-Freshney R. I. *Culture of animal cells; a manual of basic technique*. 4th ed. Wiley, New York; 2005.

7-Gregory CA, Gunn WG, Peister A, Prockop DJ. An alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Anal Biochem*, 2004; 329: 7784

8-Gupta DM, Panetta NJ, Longaker MT .Osteogenic differentiation of human multipotent mesenchymal stromal cells. *Methods Mol Biol*, 2011; 698: 201–214.

9-Hass R, Kasper C, Böhm S, Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal*, 2011; 9: 12.

10-Hoemann CD, El-Gabalawy H, McKee MD. In vitro osteogenesis assays: influence of the primary cell source on alkaline phosphatase activity and mineralization. *Pathol Biol*, 2009; 57: 318–323.

11-Huang Z, Nelson ER, Smith RL, Goodman SB. The sequential expression profiles of growth factors from osteoprogenitors [correction of osteoprogenitors] to osteoblasts in vitro. *Tissue Eng*, 2007; 13: 2311–2320.

12- Ishige I.; Nagamura-Inoue T.; Honda M. J.; Harnprasopwat R.; Kido M.; Sugimoto M.; Nakauchi H.; Tojo A. Comparison of mesenchymal stem cells derived from arterial, venous, and Wharton's jelly explants of human umbilical cord. *Int J Hematol* ,2009;90: 261–269.

13-Jeon YJ, Kim J, Cho JH, Chung HM, Chae JI. Comparative analysis of human mesenchymal stem cells derived from bone marrow, placenta, and adipose tissue as sources of cell therapy. *J Cell Biochem* ,2016;117: 1112–1125.

14-Jin HJ, Bae YK, Kim M, Kwon SJ, Jeon HB, Choi SJ, Kim SW, Yang YS, Oh W, Chang JW. Comparative analysis of human mesenchymal stem cells from bone marrow, adipose tissue, and umbilical cord blood as sources of cell therapy. *Int J Mol Sci* ,2013;14: 17986– 18001.

- 15-Kadivar M.; Khatami S.; Mortazavi Y.; Soleimani M.; Taghikhani M.; Shokrgozar M. Isolation, culture and characterization of postnatal human umbilical vein-derived mesenchymal stem cells. *Daru*,2005; 13:170-176.
- 16-Koliakos, I., Tsagias, N., & Karagiannis, V. Mesenchymal cells isolation from wharton's jelly in perspective to clinical applications. *Jurnal of Biological Research-Thssaloniki*,2011; 16, 194-201.
- 17-Krause U, Seckinger A, Gregory CA. Assays of osteogenic differentiation by cultured human mesenchymal stem cells. *Methods Mol Biol*,2011; 698: 215-230.
- 18-Latifpour M.; Nematollahi-Mahani S. N.; Deilamy M.; Azimzadeh B.S.; Eftekhar-Vaghefi S. H.; Nabipour F.; Najafipour H.; Nakhaee N.; Yaghoubi M.; Eftekhar-Vaghefi R.; Salehinejad P.; Azizi H.Improvement in cardiac function following transplantation of human umbilical cord matrix-derived mesenchymal cells. *Cardiology*,2011; 120: 9-18.
- 19-Leeb C.; Jurga M.; McGuckin C.; Moriggl R.; Kenner L. Promising new sources for pluripotent stem cells. *Stem Cell Rev*,2009; 6: 15-26.
- 20-Li CY, Wu XY, Tong JB, Yang XX, Zhao JL, Zheng QF, Zhao GB, Ma ZJ. Comparative analysis of human mesenchymal stem cells from bone marrow and adipose tissue under xeno-free conditions for cell therapy. *Stem Cell Res Ther*,2015; 6: 55.
- 21-Logeart-Avramoglou D.; Anagnostou F.; Bizios R.; Petite H. Engineering bone: challenges and obstacles. *J Cell Mol Med*,2005; 9: 72-84.
- 22-Lu L. L.; Liu Y. J.; Yang S. G.; Zhao Q. J.; Wang X.; Gong W.; Han Z.B.; Xu Z. S.; Lu Y. X.; Liu D.; Chen Z. Z.; Han Z. C. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica*,2006; 91: 1017-1026.
- 23-Mennan C, Wright K, Bhattacharjee A, Balain B, Richardson J, Roberts S.Isolation and characterisation of mesenchymal stem cells from different regions of the human umbilical cord. *Biomed Res Int*, 2013: 916136.
- 24-Qiao C.; Xu W.; Zhu W.; Hu J.; Qian H.; Yin Q.; Jiang R.; Yan Y.; Mao F.; Yang H.; Wang X.; Chen Y. Human mesenchymal stem cells isolated from the umbilical cord. *Cell Biol Int*,2008; 32: 8-15.
- 25-Oyama Y.; Hori N.; Allen C. N.; Carpenter D. O. Influences of trypsin and collagenase on acetylcholine responses of physically isolated single neurons of *Aplysia californica*. *Cell Mol Neurobiol* ,1990;10:193-205.
- 26-Petsa A.; Gargani S.; Felesakis A.; Grigoriadis N.; Grigoriadis I.Effectiveness of protocol for the isolation of Wharton's Jelly stem cells in large-scale applications. *In Vitro Cell. Dev. Biol. Anim*.2009.
- 27-Rao M. S.; Mattson M. P. Stem cells and aging: expanding the possibilities. *Mech Ageing Dev*,2001; 122: 713-734.
- 28-Romanov Y. A.; Svintsitskaya V. A.; Smirnov V. N. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells*,2003; 21: 105-110.
- 29-Sabapathy V, Sundaram B, Sreelakshmi V M, Mankuzhy P, and Kumar, S. Human Wharton's Jelly mesenchymal stem cells plasticity augments scar free skin wound healing with hair growth. *PLOS ONE*, 2014;9(4): e93726
- 30-Salehinejad, P., Alitheen, N. B., Ali, A. M., Omar, A. R., Mohit, M., Janzamin, E.,Nematollahi-Mahani, S. N. Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly. *In Vitro Cellular & Developmental Biology - Animal*,2012; 48(2), 75-83.
- 31-Semenov O. V.; Koestenbauer S.; Riegel M.; Zech N.; Zimmermann R.; Zisch A. H.; Malek A. Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. *Am J Obstet Gynecol*,2009; 20: 193 e191-193 e113.
- 32-Sonomoto, K., Yamaoka, K., Oshita, K., Fukuyo, S., Zhang, X., Nakano, K., Tanaka, Y. Interleukin-1 α Induces Differentiation of Human Mesenchymal Stem Cells Into Osteoblasts via the Wnt-5a / Receptor Tyrosine Kinase - like Orphan Receptor 2 Pathway,2012; 64(10), 3355-3363.
- 33-Sotiropoulou P. A.; Perez S. A.; Salagianni M.; Baxevasis C. N.;Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells*,2006; 24: 462-471.
- 34-Stricklin G. P.; Bauer E. A.; Jeffrey J. J.; Eisen A. Z. Human skin collagenase: isolation of precursor and active forms from both fibroblast and organ cultures. *Biochemistry*,1977; 16: 1607-1615.
- 35-Weiss M. L.; Medicetty S.; Bledsoe A. R.; Rachakatla R. S.; Choi M.; Merchav S.; Luo Y.; Rao M. S.; Velagaleti G.; Troyer D. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in

a rodent model of Parkinson's disease. *Stem Cells*, 2006; 24: 781-792.

36-Wen Y, Jiang B, Cui J, Li G, Yu M, Wang F, Zhang G, Nan X, Yue W, Xu X, Pei X. Superior osteogenic capacity of different mesenchymal stem cells for bone tissue engineering. *Oral Surg Oral Med Oral Pathol Oral Radiol*, 2013; 116: e324-e332.

37-Zajdel, A., Kałucka, M., Kokoszka-mikołaj, E., & Wilczok, A. Osteogenic differentiation of human

mesenchymal stem cells from adipose tissue and Wharton's jelly of the umbilical cord. *ACTA BIOCHIMICA POLONICA*, 2017; 64(2016), 365-369.

38-Zhang Y.; Li C. D.; Jiang X. X.; Li H. L.; Tang P. H.; Mao N. Comparison of mesenchymal stem cells from human placenta and bone marrow. *Chin Med J (Engl)*, 2004; 117: 882-887.

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