Effect of Mouse Liver Extract on in Vitro Differentiation of Amniotic Membrane Stem Cells into Hepatocyte-Like Cells

Vahideh assadollahi (MSc)
Department of Biochemistry and Genetic, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

Masoumeh Jalalvand (MSc)
Department of Biotechnology, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

Shahrokh Bagheri (MSc)
Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran

Hamed Esmail Lashkarian (PhD)
Department of Biochemistry and Genetic, Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran

Corresponding author: Masoumeh Jalalvand

Email: masumehjalalvand@gmail.com

Tel: +989305708980

Address: Lorestan University of Medical Sciences, Khorramabad, Iran

ABSTRACT

   Background and Objective: Multipotent placental amniotic membrane mesenchymal stem cells (MSCs) are capable of differentiating into specialized tissues under different conditions. The aim of this study was to induce differentiation of placental amniotic membrane MSCs from NMRI mouse into hepatocytes using liver extract.

   Methods: Placental amniotic membrane MSCs from a 14-day pregnant female mouse was used in this study. The cells were incubated with trypsin solution, followed by pipetting. The resulting suspension was cultured in 12-well plates. After confirming their mesenchymal nature, differentiation of the aforementioned cells was induced via exposure to 6, 18, 30 and 60 μg/ml of liver extract. On the 16th day of treatment, immunocytochemical reaction for albumin and periodic acid-Schiff (PAS) test were performed for detection of hepatocyte-like cells.

   Results: Change was observed in the shape of differentiating cells from spindle-like shape to polygonal shape. The immunocytochemical reaction of the differentiated cells was positive. PAS staining also confirmed the accumulation of glycogen particles in the aforementioned cells. Concentration of 6 μg/ml liver extract was found as the effective dose for induction of differentiation.

   Conclusion: The findings of this study show that the placental amniotic membrane-derived MSCs of mouse can differentiate in vitro from spindle-like cells to polygonal hepatocyte-like cells with large nuclei and under the influence of the liver.

Keywords: Placental Amniotic Membrane Mesenchymal Stem Cells, Hepatocyte, In Vitro.

INTRODUCTION

liver is an organ that plays an important role in metabolism, synthesis and storage of nutrients, detoxification, and defense against invading macromolecules. Liver failure and chronic liver disease are among the main causes of mortality worldwide (1, 2). Although liver tissue is able to repair itself, hepatocytes become impaired in some cases and need to be replaced (3). Currently, liver transplantation is the accepted therapy that have some limitations including high cost, use of immunosuppressant drugs, organ transplant rejection and shortage of donors (4). Therefore, researchers are trying to find new sources of hepatocytes. Hence, stem cells have been suggested as new source for the production of hepatocytes in the laboratory due to their high proliferation and differentiation capability. On the other hand, the need for a hepatocyte source for testing drugs and various therapeutic compounds before administration to patients, have prompted scientists to conduct studies on the in vitro production of hepatocytes from stem cells (5). Since mesenchymal stem cells (MSCs) have the ability to self-renew and differentiate into other cells, they have an important role in healing and cell therapy processes (6). Flexibility and plasticity of these cells in differentiation into nerve cells, epithelial cells, pneumocytes, hepatocytes, and intestinal, renal and spleen epithelial cells has been demonstrated (6,7). The researchers have managed to isolate MSCs from different tissues including muscle, skin, trabecular bone, adipose tissue, periosteum, umbilical cord blood and synovial membranes as well as fetal tissues including amnion, Wharton's jelly, umbilical cord and placenta (8-10). Access to a large number of cells, ease of extraction with minimal pain and tissue damage, the high proliferation and differentiation capability, possibility of autogenic or allogeneic transplantation without fear of rejection for treatment are the characteristics of an ideal stem cell (11). Relatively easy isolation and expansion of mesenchymal cells from placental amniotic membrane, low immunogenicity, and lack of tumorigenesis and inflammatory properties have prompted some scientists to focus on the efficacy of these cells for the treatment of diseases (12). In 2004, Zhang et al. were the first to show that placental amniotic membrane has many

MSCs with the ability to differentiate into osteogenic and adipogenic cells (13). Later in 2007, Solomon et al. succeeded to isolate multipotent MSCs from the placental amniotic membrane with ability to differentiate into adipogenic, chondrogenic, osteogenic and skeletal myogenic cells (14). It has been revealed that the stem cells derived from the amniotic membrane are similar to embryonic stem cells with less ethical issue for use in studies (15). The aim of this study was to evaluate the impact of mouse liver extract on differentiation of placental amniotic membrane MSCs into hepatocyte-like cells in vitro. The nature of the resulting cells was verified by immunocytochemistry and periodic acid– Schiff (PAS) reaction.

MATERIAL AND METHODS

In this fundamental study, the white NMRI mice with a weight range of 26-30 g were used. The mice were maintained under suitable conditions (12-hours light/darkness cycle) at 23 °C. They were used after a week, adapting to laboratory conditions. To obtain embryos required, 40 female NMRI mice were caged separately with mice of the same race in a way that two female mice and a male one were placed in each cage. Intercourse was usually done at midnight, leading to development of vaginal plug. For isolation of placental amniotic membrane mesenchymal cells, 14-day pregnant mice were used.

Placental amniotic membrane mesenchymal cells were isolated using a technique introduced by Marongiu et al. in 2012 (12, 16). The DMEM culture medium containing sodium bicarbonate and L-glutamin were supplemented with 100 mg/L penicillin, 100 mg/L streptomycin and 15% FBS. The medium was sterilized by syringe filter and pH of the medium was adjusted to 7.4.

It is demonstrated that MSCs can differentiate into osteocytes and adipocytes (17). In addition to evaluation of morphology for verification of the mesenchymal nature of the resulting cells from the last phase, some samples were exposed to adipogenic differential medium containing DMEM with50 µg/ml ascorbic2-phosphate, 100 nmol ascorbic2-phosphate and 50 µg/ml indomethacin for two weeks, and osteogenic differential medium containing DMEM with

50 μg/ml ascorbic2-phosphate, 10 nmol ascorbic2-phosphate and 10 mmol ß-Glycerol for three weeks (18).

In this study, liver from 7-day old newborn mice was used. The liver extract was prepared according to method described by Solati et al. (19). After removing the liver of newborn mice, they were immediately taken under laminar hood on dry ice, and then transferred into concentrated liver extract by a homogenizer. Then, necessary amounts of the extract were added to the culture medium.

Second and third passages of stem cells were used for the differentiation of placental amniotic membrane MSCs into hepatocyte-like cells. In this way, when cell density in the flask reached 70-80%, the normal culture medium was removed from the dish and replaced with the prepared differential media.

The experimental groups of this study were as follows:

1. Control group: amniotic membrane MSCs with medium containing 10% FBS

2. First experimental group: in addition to DMEM containing 10% FBS, 100 μl of extract (6 g of liver in 10ml DMEM solution) per 1 ml of medium equal to 60 μg/ml liver extract was added to the MSCs in culture flasks.

3. Second experimental group: in addition to DMEM containing 5% FBS, 50 λ of extract (6 g of liver in 10ml DMEM solution) per 1 ml of medium equal to 30 μg/ml liver extract was added to the MSCs in culture flasks.

4. Third experimental group: in addition to DMEM containing 5% FBS, 30 λ of extract (6 g of liver in 10ml of DMEM solution) per 1 ml of medium equal to 18 μg/ml liver extract was added to the MSCs in culture flasks.

5. Fifth experimental group: in addition to DMEM containing 2% FBS, 10 λ of extract (6 g of mice liver in 10ml DMEM solution) per 1 ml of medium equal to 6 μg/ml of liver extract was added the MSCs in culture flasks.

Cell culture medium was replaced every four days. Dead cells were removed from the medium and the mentioned concentrations of the extract were added to the culture flasks. The flasks from the third and fourth experimental groups had less number of dead cells and more differentiated cells than the other groups. In the fourth experimental group that was treated with the lowest amount of serum and extract, the cells with polygonal morphology and large nuclei in the center of cytoplasm (signs of hepatocytes) were observed on 16th day (Figure 1). The fourth group was used for further evaluations.

Figure 1. Image of dividing nucleus and granular cytoplasm of a polygonal hepatocyte-like cell Immunofluorescent assessment: On the 16th day, cells were fixed in 10 μl of 4% paraformaldehyde solution at room temperature for 20 minutes. Then, blocking phase was done using incubation for 45 minutes at room temperature using a solution containing 0.1% Triton X-100 and 10% goat serum and the primary antibody against albumin. Next, the cells were incubated with FITC-conjugated secondary antibody. Cells were washed with PBS between different stages of staining. Finally, the cells were examined by inverted fluorescence microscope (20). PAS staining was perfumed according to the method previously described by Raoufi et al. (20). First, differentiated cells were fixed by paraformaldehyde and then incubated with 1% PAS for 5 minutes. The cells were then washed with deionized water. Cells were incubated with Schiff solution for 15 minutes and then washed with deionized water for 10 minutes. Finally, the cells were examined under a microscope. In the primary culture of placental amniotic membrane cells, mesenchymal cell colonies were observed after a few days that included spindle cells that were drawn radially from the center of the colony to the sides. Mesenchymal cells from the third passage that were placed in the adipogenic medium for 12 days and in the osteogenic medium for 21 days were differentiated to adipocytes and osteocytes, respectively. The differentiation was confirmed by Oil-Red and Alizarin Red staining (Figure 2). Immunofluorescent staining for albumin: Cell differentiation was confirmed with immunohistochemistry staining method. After examination by a fluorescent microscope, green fluorescent signals were observed, indicating the positive test result (Figure 3).

Figure 3. Albumin expression in differentiated cells was confirmed by immunohistochemistry. Images of the differentiated cells from: A) contrast and B) fluorescent phases. The results of the PAS staining showed that differentiated cells are capable of storing glycogen. After 10 minutes of adding the Schiff solution (colorless) to differentiated cells, the solution turned purple,

a response not observed in undifferentiated cells (Figure 4).

Figure 4. Image of a polygonal cell with a central nucleus in red color after PAS staining (400X magnification).

Results

Change was observed in the shape of differentiating cells from spindle-like shape to polygonal shape. The immunocytochemical reaction of the differentiated cells was positive. PAS staining also confirmed the accumulation of glycogen particles in the aforementioned cells. Concentration of 6 μg/ml liver extract was found as the effective dose for induction of differentiation.

Untreated cells

Treated cells after 4 days

Treated cells after 8 days

Treated cells after 12 days
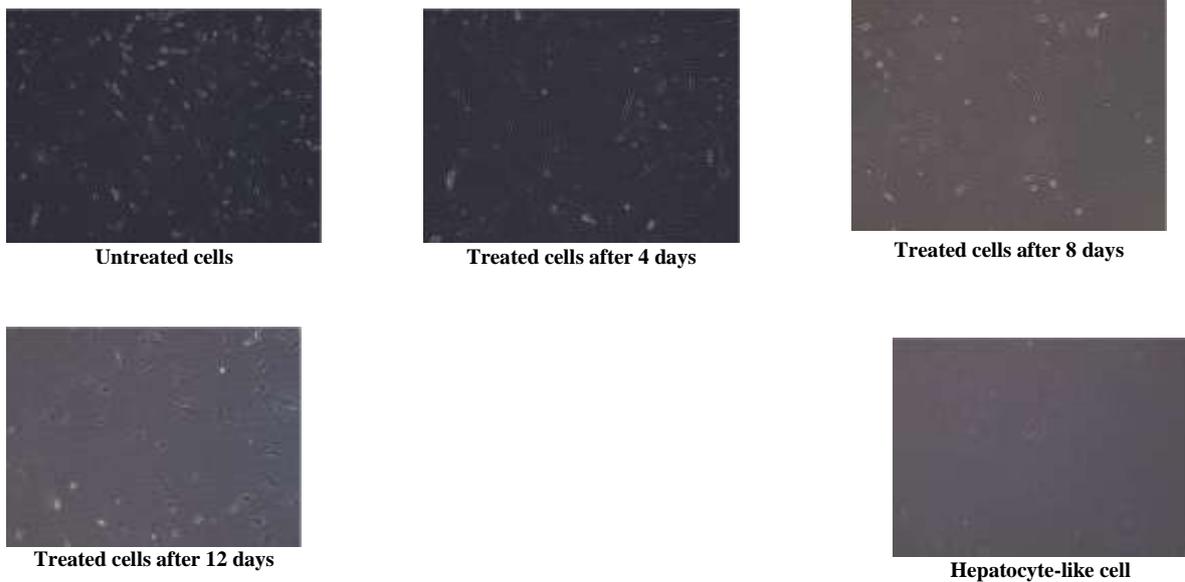

Hepatocyte-like cell

Figure 1- Image of dividing nucleus and granular cytoplasm of a polygonal hepatocyte-like cell (400X).

Figure 2- a) Image of Alizarin Red staining of mouse amniotic membrane mesenchymal cells differentiated into osteocytes. b) Image of Oil-Red staining of mouse amniotic membrane mesenchymal cells differentiated into adipocytes (100X)
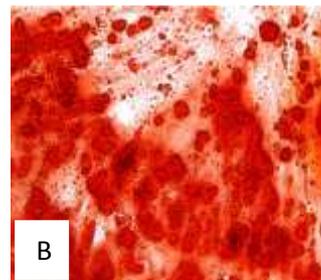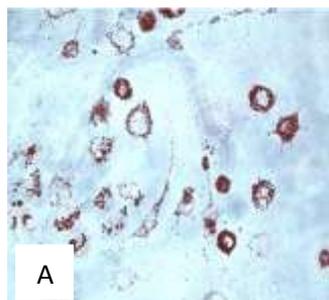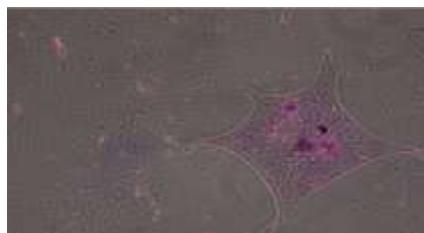

A

B

Figure 3- Albumin expression in differentiated cells was confirmed by immunohistochemistry. Images of the differentiated cells from: A) contrast and B) fluorescent phases (200X).

Figure 4- Image of a polygonal cell with a central nucleus in red color after PAS staining (400X magnification).

DISCUSSION

MSCs are used as a valuable source for cell therapy in animal models due to the high proliferation and self-renewal capacity, the ability to differentiate into other mesenchymal lineages, inhibition of immune responses and lack of teratoma formation after transplantation (13). These cells have been used in the treatment of genetic disorders such as osteogenesis imperfecta, hematopoietic recovery, bone regeneration, repairing the necrosis site in myocardial infarction patients and treatment of joint diseases (2, 21-25). Because of the importance of MSCs in cell therapy, preclinical studies using animal models are required to utilize these cells for the treatment of human disease.

The liver of 7-day NMARI mouse was removed completely and the liver extract was used as an induction agent. The results of this study showed that the liver extract induces the differentiation of amniotic membrane MSCs into hepatocyte-like cells. Since the hepatocyte-like cells were able to synthesize glycogen, PAS test was positive and the purple color was observed in some areas. The differentiated cells were capable of expressing albumin, which is the index for detection of hepatocytes.

In this study, the cells in the first and second groups (30 μg/ml and 60 μg/ml) died after three days of treatment with the extract. The cells in the third group were treated with 18 μg/ml of the extract and had a hepatocyte-fibroblastic morphology that experienced aging and apoptosis with time. After eight days of treating the MSCs in the fourth group with 6 μg/ml of the extract, the cells lost their ability to reproduce under the influence of the extract. On the 14th day, a small number of cells with nucleus divided and became balanced. The cell division was in fact a sign of differentiation on the surface of hepatocyte-like cells. The process of cell differentiation was stopped on the 16th day when the cells had large cytoplasm, changes that were quite similar to morphology of hepatocytes. such as inhibition of differentiation pathways. In contrast to previous studies, the present study was performed in the absence of induction factors such as hepatocyte growth factor, epidermal growth factor, transforming growth factor beta, acidic fibroblast growth

factor, insulin-like growth factor, oncostatin M and non-protein factors and agents including dexamethasone, retinoic acid, sodium butyrate, nicotinamide, norepinephrine and dimethyl sulfoxide (3). Due to the use of liver extract from newborn mouse, differentiation of placental MSCs of NMRI mouse into hepatocyte-like cells occurred within the 16-day period. The concentrations of 6, 18, 30 and 60 μg/ml were used to induce differentiation. The MSCs acquired the morphology of hepatocytes on the 16th day of treatment with 6 μg/ml of the extract. PAS test and immunohistochemistry staining were positive for albumin in these cells, which indicated the successful differentiation of these cells into hepatocyte-like cells. Study of Solati et al. used rat liver extract to differentiate stem cells into hepatocytes. In the mentioned study, the extract induced differentiation of rat derived omentum tissue MSCs (rOT-MSCs) into hepatocyte-like cells. Positive PAS test for differentiated cells indicated the differentiation of rOT-MSCs into hepatocyte-like cells. The morphology change of MSCs toward hepatocyte-like cells during the induction period was also investigated. RT-PCR was performed for *WT-1, ALB, AFP* and

CK-18 genes , and urea test was used to measure the function of these cells. Finally, immunocytochemistry test and western blot for albumin production demonstrated the differentiation of MSCs into hepatocyte-like cells (19). The final maturity of hepatocytes in rodents such as mouse occurs in a few days after birth (28). The extract from liver of newborn mouse seems to contain factors and substances that can differentiate MSCs into hepatocyte-like cells in vitro.

CONCLUSION

The findings of this study show that the amniotic membrane-derived MSCs of NMRI mouse can differentiate into hepatocyte-like cells under the influence of the liver extract.

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Xu YQ, Liu ZC. *Therapeutic potential of adult bone marrow stem cells in liver disease and delivery approaches*. Stem cell reviews. 2008; 4(2): 101-12. doi: 10.1007/s12015-008-9019-z.

2. Baksh D, Song L, Tuan R. *Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy*. Journal of cellular and molecular medicine. 2004; 8(3): 301-16. PMID:15491506.

3. Pournasr B , Farzaneh Z, Shahsanvani M, Baharvand H. *Liver development and in vitro differentiation of embryonic stem cells to hepatocytes*. Yakhteh Medical Journal. 2010; 11(4): 348-73. [persian]

4. Banas A, Yamamoto Y, Teratani T, Ochiya T. *Stem cell plasticity: learning from hepatogenic differentiation strategies*. Developmental Dynamics. 2007; 236(12): 3228-41.

5. Teramoto K, Asahina K, Kumashiro Y, Kakinuma S, Chinzei. *Hepatocyte differentiation from embryonic stem cells and umbilical cord blood cells*. Journal of hepato-biliary-pancreatic surgery. 2005; 12(3): 196-202. PMID:15995807.

6. Sugaya K. *Potential use of stem cells in neuroreplacement therapies for neurodegenerative diseases*. International review of cytology. 2003; 228: 1-30. PMID:14667041.

7. Chapel A BJ, Bensidhoum M, Fouillard L, Young RG, Frick J, Demarquay C, et al. *Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrom*e. The journal of gene medicine. 2003; 5(12):1028-38.

8. Lee OK, Kuo TK, Chen W-M, Lee K-D, Hsieh S-L, Chen T-H. *Isolation of multipotent mesenchymal stem cells from umbilical cord blood*. Blood. 2004; 103(5): 1669-75. PMID:14576065.

9. Vater C, Kasten P, Stiehler M. *Culture media for the differentiation of mesenchymal stromal cells*. Acta biomaterialia. 2011; 7(2): 463-77. doi: 10.1016/j.actbio.2010.07.037.

10. Mafi R, Hindocha S, Mafi P, Griffin M, Khan W. *Suppl 2: Sources of Adult Mesenchymal Stem Cells Applicable for Musculoskeletal Applications-A Systematic Review of the Literature*. The open orthopaedics journal. 2011; 5: 242. doi: 10.2174/1874325001105010242.

11. Bunnell BA, Flaat M, Gagliardi C, Patel B, Ripoll C. *Adipose-derived stem cells: isolation, expansion and differentiation*. Methods. 2008; 45(2): 115-20. doi: 10.1016/j.ymeth.2008.03.006.

12. Parolini O, Alviano F, Bergwerf I, Boraschi D, De Bari C, De Waele P, et al. *Toward cell therapy using placenta-derived cells: disease mechanisms, cell biology, preclinical studies, and regulatory aspects at the round table*. Stem cells and development. 2010;19(2): 143-54. doi: 10.1089/scd.2009.0404.

13. Zhang Y, Li C, Jiang X, Zhang S, Wu Y, Liu B, et al. Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood CD34+ cells. Experimental hematology. 2004; 32(7): 657-64.

14. Solomon A, Wajngarten M, Alviano F, Anteby I, Elchalal U, Pe'er J, et al. *Suppression of inflammatory and fibrotic responses in allergic inflammation by the amniotic membrane stromal matrix*. Clinical & Experimental Allergy. 2005; 35(7): 941-8.

15. Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U. *Stem cells derived from human fetal membranes display multilineage differentiation potential*. Biology of reproduction. 2007; 77(3): 577-88.

16. Marongiu F, Gramignoli R, Sun Q, Tahan V, Miki T, Dorko K, et al. *Isolation of amniotic mesenchymal stem cells*. Current protocols in stem cell biology:1E. 5.1-E. 5.11.

17. NematiSh BH. *Differentiation of human bone marrow mesenchymal stem cells to neural- like cells in vitro*. Tehran University Medical JournalNov 2009;Vol. 67, No. 8 .527-534.

18. Eslaminejad MB, Nikmahzar A, Taghiyar L, Nadri S, Massumi M. *Murine mesenchymal stem cells isolated by low density primary culture system*. Development, growth & differentiation. 2006; 48(6): 361-70.

19. Solati Sarvandi S, Joghataei MT, Parivar K, Khosravi M, Sarveazad A, Sanadgol N. *In vitro differentiation of rat mesenchymal stem cells to hepatocyte lineage.* Iran J Basic Med Sci. 2015; 18: 89-97.

20. Raoufi A, Amini A, Azadbakht M, Abouzari M. *In vitro differentiation of human umbilical vein mesenchymal stem cells into hepatocyte-like cells identified by cellular uptake of indocyanine green*. Khoon. 2010; 7(1): 34-40. [Persian]

21. Ko O, Gerson S, Cooper B, Dyhouse S, Haynesworth S, Caplan A, et al. *Rapid hematopoietic recovery after confusion of autologous-blood stem cells in advanced breast cancer patients receiving high dose chemotherapy*. J Clin Oncol. 2000; 18(2): 307-16.

22. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. *Tissue-engineered bone regeneration*. Nature biotechnology. 2000; 18(9): 959-63.

23. Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov A, et al. *Repair of large bone defects with the use of autologous bone marrow stromal cells*. New England Journal of Medicine2001;344(5):385-6.

24. Grinnemo K, Mansson A, Dellgren G, Klingberg D, Wardell E, Drvota V, et al. *Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium*. The Journal of thoracic and cardiovascular surgery. 2004; 127(5): 1293-300. PMID:15115985.

25. Horwitz EM GP, Koo WK, Marx JC, NeelMD,mcNallRY,MuulL,Hofmann T. *Isolated allogenicbone marrow- drivedmesenchymal cells engraftand stimulate growth in children with osteogenesisimperfecta: Implications for cell therapy of bone*. Proc Natl Acad Sci USA. 2002; 99: 8932-37.

26. Niknejad H, Peirovi H, Ahmadiani A, Ghanavi J, Jorjani M. *Differentiation Factors That Influence Neuronal Markers Expression In Vitro From Human Amniotic Epithelial Cell*s. Eur Cell Mater. 2010; 19: 22-9.

27. Parivar K, Momayez sefat M, Hayati N, Banaj M. *The effect of neonatal NMRI mouse cardiac on differentiation of NMRI mouse umbilical cord stem cells.* Medical Sciences Journal of Islamic Azad University - Tehran Medical Branch. 2014; 24(1): 1-7

28. Barry FP. *Mesenchymal stem cell therapy in joint disease.* Novartis Found Symp. 2003; 249: 86-96.