Equine marrow-derived mesenchymal stem cells: isolation, differentiation and culture optimization

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Summary

Most studies regarding the marrow-derived equine mesenchymal stem cells (MSCs) have mainly focused on the cell transplantation without considering the capacity of differentiation and *in vitro* requirements of the cells. These concerns were investigated in the present study. Equine MSCs were isolated from the sternal marrow aspirates and expanded through two successive subcultures. Passage-2 equine MSC cultures were then treated with appropriate supplements in order to examine the cell osteogenic, chondrogenic and adipogenic differentiation potential. Furthermore, the culture of the cells was investigated in terms of the optimal concentration of fetal bovine serum (FBS) and the initial cell-seeding density. Additionally, a growth curve was plotted for the cells to study their growth characteristics. According to our findings, equine MSCs were easily generated specialized bone, cartilage and adipose cell lineages as confirmed by specific staining and RT-PCR analysis. Moreover, the cells exhibited rapid expansion when being cultivated in the medium with 15% FBS at 100 cells/cm^2. Growth curves indicated that these cells rapidly entered the log phase after a brief lag (adaptation) period. In summary, marrow-derived equine MSCs possess tripotent differentiation capacity and rapid growth rate in the appropriate culture conditions.

Key words: Equine MSCs isolation, Cartilage, Bone, Adipose differentiation, FBS concentration

Introduction

Mesenchymal stem cells (MSCs) were first described by Friedenstein *et al.* (1970) who provided definitive evidences that bone marrow contained a population of colonogenic cells in addition to well-recognized hematopoietic precursors. At that time, the most striking feature of the cells was reported to be their colonogenic property; the colon that they produced showed resemblance to bone and cartilage deposits. Subsequent investigations indicated that MSCs were also able to differentiate into adipocytic cell lineage (Piersma *et al.*, 1985; Owen, 1988). Nowadays, this tripotent differentiation capacity is considered to be a golden standard for MSC identification (Pittenger *et al.*, 1999; Dominici *et al.*, 2006). Although,

MSCs have been first isolated from marrow tissue, but some reports have indicated the existence of these cells in other tissues such as fat, liver, amniotic fluid and umbilical cord blood (Erices *et al.*, 2000; Campagnoli *et al.*, 2001; Zuk *et al.*, 2001; In't Anker *et al.*, 2003).

MSCs have been reported to enjoy more differentiation potential than that they were originally thought in that they can give rise to various cells such as neurons, cardiomyocytes, keratinocytes, lung and intestinal epithelial cells (Petersen *et al.*, 1999; Mezey *et al.*, 2000; Orlic *et al.*, 2001; Chapel *et al.*, 2003; Pousom *et al.*, 2003). Furthermore, an investigation by Herrera *et al.* (2004) has indicated that MSCs engrafted in the damaged kidney, differentiated into tubular epithelial cells and promoted the recovery of morphological and functional

alterations. Such property is referred to as MSCs' plasticity or trans-differentiation.

Because MSCs can easily be isolated from the bone marrow aspirates and expanded in culture conditions, they are considered to be an appropriate cellular candidate for gene therapy, cell therapy and tissue engineering purpose (Baksh *et al*., 2004; Eslaminejad *et al*., 2007). Up to now, MSCs from human and several other species have successfully been isolated (Jessop *et al*., 1994; Martin *et al*., 2002; Ringe *et al*., 2002; Eslaminejad *et al*., 2006; Shao *et al*., 2006; Abdallah and Kassem, 2007). MSCs from animal models could be valuable tool in term of preclinical investigations.

MSCs could also be used to cure the tissue damages occurring in expensive animals such as the race horses. The most frequently occurring tissue damages in horse include tendon and cartilage lesions which may diminish the useful lifespan of the animal for competitions (Jeffcott *et al*., 1982; Litzke *et al*., 2004). Regeneration of these tissues is indeed considered as challenging task in the field of orthopedic surgery owing mainly to their inherent insufficient repair capacity (Vachon *et al*., 1992; Awad *et al*., 2003).

In spite of the significant importance of MSCs in horse, relatively a few investigation has been devoted to this subject. By far, the isolation of MSCs from equine bone marrow (Douglas and Herther, 2001; Smith *et al*., 2003; Carstanjen *et al*., 2006; Vidal *et al*., 2006), equine umbilical cord blood (Koch *et al*., 2007) as well as equine peripheral blood has been described (Koerner *et al*., 2006). In most of these studies, isolated MSCs have directly been transplanted into tissue defects. For instance, Douglas and Herther (2001) used autologous MSC transplantation to repair equine pelvic ligaments. Two- to four-year follow-ups indicated that the tissue damage had successfully been cured. Autologous MSC transplantation to cure equine flexor digitorum tendonitis also could improve the animal crippling and relieve their pain (Smith *et al*., 2003). Transplanted autologous culture-expanded MSCs into damaged equine soft plate regenerated the tissue (Carstanjen *et al*., 2006). While MSCs Committee of the International Society for

Cell Therapy has proposed the certain criteria for MSCs, including their *in vitro* differentiation ability into osteoblast, chondrocyte and adipocyte cell lineages (Dominici *et al*., 2006); the most relevant investigations have ignored to explore this in their isolated cell population. The present study was designed to investigate the *in vitro* differentiation potential of the equine marrow-derived MSC into bone, cartilage and adipose cell lineages in culture.

Furthermore, since MSCs occur in a very low number in marrow samples, they must be expanded prior to any experimental work. Little is known about the culture requirements of the equine MSCs. This subject has been investigated in the present study. In this regard, the culture of the cells was investigated in terms of the optimal concentration of fetal bovine serum (FBS) and the initial cell-seeding density. In addition, a growth curve was plotted for the cells to study their growth characteristics. Our hypothesis is that the marrow-derived MSCs from horse differentiate into skeletal cell lineages in culture and the rate of their *in vitro* expansion is dependent on the certain concentration of serum as well as the certain density of seeded cell at culture initiation.

Materials and Methods

Marrow aspiration: Five adult, normal, mixed breed, male horses, with a mean age of 4.3 years (range 2-6 years) and a mean weight of 368.5 kg (range 350-400 kg) were included in this study. The design and procedures were performed in accordance with the regulations and approval of Institutional Animal Care and Use Committee of the University of Medical Sciences of Tehran. The horses were dewormed and hoof care was performed two weeks before the beginning of the project. The animals were confined to individual stalls, receiving water and feeding a balanced diet consisting of concentrate and alpha hay. The horses were sedated, and the harvest site (sternum) was prepared aseptically. Using ultrasonography, the boundaries between the sternum segments were determined and marked. The area was scrubbed and five ml of 2% lidocain with

adrenalin (Nasr Company, Iran) was injected on ventral surface of the sternum. Under aseptic conditions, skin incision was made in the area and a biopsy needle (Jamshidi, VMR Scientific, Bridgeport, NJ) was inserted through the skin incision. Ten ml marrow was then aspirated into a 20 ml syringe and shipped to the Royan Institute (Tehran, Iran) cell culture lab.

Cell culture: Bone marrow aspirates were diluted with two parts of Dulbecco modified Eagle medium (DMEM, Sigma, USA), loaded on lymphodex (Sigma, USA) and centrifuged at 300 g for 30 min. Mononuclear fraction was then isolated, suspended in 5 ml DMED and washed twice with centrifugation at 250 g for 5 min. The cells were plated at 10^5 cells/cm^2 in 150-cm^2 plastic culture flask in 20 ml DMEM medium containing 10% fetal bovine serum (FBS, Gibco, UK), 100 IU/ml penicillin (Sigma, USA) and 100 µg/ml streptomycin (Sigma, USA). The cultures were incubated at 37°C and 5% CO_2 with medium replacement twice weekly utill confluency after which the cultures were expanded through two successive subcultures.

Osteogenesis: After passage-2, confluent cells cultivated in 6-well plates were used to induce bone differentiation. The proliferation medium of the cultures was replaced by osteogenic medium that consisted of DMEM supplemented with 50 mg/ml ascorbic2-phosphate (Sigma, USA), 10 nM dexamethazone (Sigma, USA) and 10 mM ß-glycerol phosphate (Sigma, USA) (Eslaminejad *et al*., 2006). The cultures were incubated at 37°C and 5% CO_2 for 21 days with medium replacement of three times a week. Occurrence of differentiation was examined by alizarin red staining and RT-PCR analysis.

Alizarin red staining was used to detect whether the mineralized matrix has been deposited in the cultures or not. For staining, the cultures were fixed by methanol for 10 min and then subjected to alizarin red (Sigma, USA) solution for 2 min.

Adipogenesis: After passage-2, confluent cultures were used to evaluate the adipogenic ability of the isolated cells. The proliferation medium of the cells was replaced by adipogenic DMEM medium containing 100 mM dexamethasone (Sigma, USA) and 50 mg/ml indomethacin (Sigma, USA) (Eslaminejad *et al*., 2006). The cultures were then incubated for 21 days at 37°C and 5% CO_2. The medium was changed 3 times a week. At the end of the differentiation period, cultures were evaluated by oil red staining as well as RT-PCR analysis to detect adipose differentiation.

For staining, cultures were fixed with 4% formalin at room temperature, washed by 70% ethanol and stained by oil red (Sigma, USA) solution in 99% isopropanol for 15 min. At the end, the stain solution was removed and cultures were washed with 70% ethanol before they were observed by light microscopy.

Chondrogenesis: To induce cartilage differentiation, a micro mass culture system was used. For this purpose, 2.5×10^5 passage-2 cells were pelleted under 250 g for 5 min and cultured in a chondrogenic medium containing DMEM supplemented by 10 ng/ml transforming growth factor-ß3 (Sigma, USA), 10 ng/ml bone morphogenetic protein-6 (Sigma, USA), 50 mg/ml insulin transferin selenium + premix (Sigma, USA), 1.25 mg bovine serum albumin (Sigma, USA) and 1% fetal bovine serum (Gibco, UK) (Johnston *et al*., 1998). The chondrogenic culture was maintained at 37°C and 5% CO_2 for 21 days with a medium replacement of three times a week. At the end of this period the cultures were evaluated for cartilage differentiation by histochemical staining and RT-PCR analysis.

To examine cartilage differentiation, two specific stainings for proteoglycan detection were utilized. For this purpose, the pellets were subjected to the following process: fixing in 10% formalin; dehydrating in an ascending ethanol; clearing in xylene; embedding in paraffin wax and sectioning in 5 µm by microtome. Some sections were stained in toluidine blue for 30 seconds at room temperature and the others with alcian blue for 20 min at 37°C.

RNA extraction and RT-PCR analysis of gene expression: Total RNA was collected from cells that had been induced to differentiate into osteoblastic, chondrocytic or adipocytic lineages as detailed above, using RNX-Plus^{TM} solution (CinnaGen Inc., Tehran, Iran). Before reverse transcription,

the RNA samples were digested with DNase I (Fermentas, Germany) to remove contaminating genomic DNA. Standard reverse-transcription reaction was performed with 5 μg total RNA using Oligo (dT) 18 as a primer and RevertAid[TM] H Minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. For each sample in RT-PCR, two tubes including RT[+] and RT[-] were used. RT[+] was included reverse transcriptase enzyme while RT[-] was not included reverse transcriptase enzyme. The latter was as a negative control and could show any DNA contamination. In this sample no cDNA was synthesized due to the absence of enzyme and any PCR product in this sample would be an indication of the contamination with DNA. The control group was the MSCs cultivated in the DMEM medium without differentiation-inducing supplements. Subsequent PCR was as follows: 2.5 μl cDNA (50 ng), 1X PCR buffer (AMS, Germany), 200 μM dNTPs, 0.5 μM of each primer pair and 1 unit/25 μl reaction Taq DNA polymerase (Fermentas). Program for PCR reaction was as follows: step 1, first denaturation at 94ºC for 4 min. Step 2, denaturation at 94ºC for 45 sec. Step 3, annealing at 61ºC [for Coll I (collagen I) and bone sia (bone sialoprotein)] at 60ºC [for PPAR-γ, -α (peroxisome proliferators activated-receptor gamma, alpha) Coll II (collagen II), Coll X (collagen X) and agc (aggrecan)], at 58ºC for LPL (lipoprotein lipase), at 59ºC for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for 45 sec. Step 4, extension at 72ºC for 45 sec. Go to step 2 repeat 32 cycles for all except GAPDH which needs 30 cycles. Step 5, final extension at 72ºC for 10 min. The primers indicated in Table 1 were utilized to detect the differentiations.

Colony forming unit-fibroblast (CFU-F) assay: The cells were plated at 1 cell/cm^2 in 100 cm^2 Petri dish for 5 days and then the cultures were examined under phase contrast inverted light microscope. The colonies developed at the culture were then counted.

Cell seeding density: Since cell seeding density can influence the rate of cell expansion in culture, passage-2 equine MSCs were plated at 100, 500, 1000, 2000 and 5000 cell/cm^2 in 60-mm Petri dishes to determine the best seeding density with the highest rate of cell proliferation outcome. After 10 days, the cells were lifted by trypsin (Gibco, UK) exposure and counted with the cell counting chamber known as hemocytometer. For this purpose, the cells were first suspended in 0.5 ml DMEM medium and mixed with the equal volume of trypan blue (0.8 mM in PBS) to mark the dead cells. Five μl out of 1 ml cell suspension was diluted by 95 μl DMEM medium. Five μl of resultant diluted solution was placed in hemocytometer slide and the cells were counted. The actual number of the cells was calculated by the formula: N/4 × D × 10^4, where N is the sum of the cells counted in four hemocytometer chambers and D is the dilution factor (in this case

Table 1: Primers used in RT-PCR

	Gene name	Direction	Sequence	AT	Product size	PCR cycle
Osteogenic genes	Collagen type I	Forward	F: 5' ccc aga aca tca cct acc ac 3'	61	317	32
		Reverse	R: 5' gga ggg agt tta cag gaa gc 3'			
	Bone sialoprotein	Forward	F: 5'gtg gag aca acg gag aag ag 3'	61	320	32
		Reverse	R: 5'acc atc ata gcc gtc ata gc 3'			
Adipogenic genes	PPAR-alpha	Forward	F:5' aga aca agg aag cgg aag tc3	60	200	32
		Reverse	R: 5' atc ccg tct ttg ttc atc ac 3'			
	PPAR-gamma	Forward	F:5'aag agc aga gca aag agg tg3	60	404	32
		Reverse	R: 5' ggg cttc aca ttc aac aaa c 3'			
	LPL	Forward	F: 5' tct ctt ggg ata cag cct tg 3'	58	228	32
		Reverse	R: 5' atg ccc tac tgg ttt ctg 3'			
Chondrogenic genes	COL2A1	Forward	F: 5' gcg gag act act gga ttg	60	152	32
		Reverse	R: 5' ttt ctt gtc ctt gct ctt gc 3'			
	Collagen type X	Forward	F: 5' act gag cga tac caa aca cc 3'	60	302	32
		Reverse	R: 5'ggt cca ttt agt cct ctc tcc 3'			
	Aggrecan	Forward	F: 5' ttg gac ttt ggc aga ata cc 3'	60	187	32
		Reverse	R: 5'ctt cca cca atg tcg tat cc 3'			
House-keeping	GAPDH	Forward	F: 5'atc atc tct gct cct tct gc 3'	59	280	30
		Reverse	R: 5' cac cag tag aag cag gga tg3'			

equals 20). *Bovine serum concentration:* The expansion of the MSCs is largely dependent on FBS presence in culture medium. To determine the best FBS concentration allowing maximum growth rate, the passage-2 equine MSCs were plated at 100-cells/cm^2 at different serum concentration including 5, 10, 15 and 20%. After 10 days, the cells were lifted, counted and the fold increase for each serum concentration were calculated.

Growth curve plotting: For this purpose, 10^4 passage-2 equine MSCs were plated in 6-well culture plate till day 10 when the culture became confluent. During this period, the cells were counted on daily basis. Using the data, growth curve was plotted.

Statistical analysis: All measurements were performed for each 5 studied horses in triplicate. All values stated as statistical means ± SD. Repeated measure ANOVA with one within subject effect was used to analyze the data. Pairwise comparison of the data of FBS concentrations and initial cell seeding density was performed using LSD post hoc. Furthermore, Mauchly method was used to test the sphericity of the data. The used software was the SPSS version 13. A p-value of <0.05 was considered as statistically significant.

Results

Cell culture: Four days after culture initiation, some adherent elongated cells became visible on the culture surface (Fig. 1A). These cells proliferated and developed round colonies visible on several areas of culture surface one week after culture initiation. There was also a few small clear cell on the cell colonies (Fig. 1B) which gradually disappeared as the culture time progressed. The cultures became confluent by 17-18 days after the initial cell seeding (Fig. 1C). At this time, the culture consisted of a homogenous monolayer of fibroblastic cells with a number of nodule-like cell aggregates (Fig. 1D). Performing two successive passages produced a sufficient cell to be used in the next phase of the experiment. Throughout the cultivation period the cells maintained their fibroblastic morphology.

Bone differentiation: During the differentiation period, several osteogenic foci appeared in the culture and their number increased as the culture time proceeded (Fig. 2A). These foci turned into red colour upon the alizarin red staining indicating the deposition of the mineralized matrix (Fig. 2B). RT-PCR results indicated that the mRNA of a number of bone-related genes including bone sialoprotein and collagen I was largely produced in the culture treated with osteogenic supplements (Fig. 3). Collagen type I is the major organic part of the bone matrix and bone sialoprotein is the bone specific glycoprotein that has binding sites for bone matrix component and integrins of osteoblastic and osteocytic cells (Fawcett, 1994; Chen *et al*., 1997).
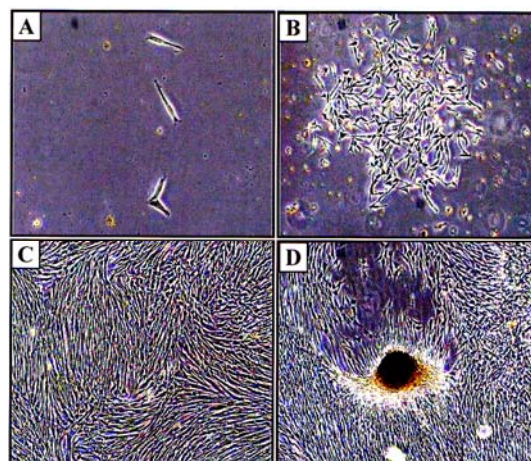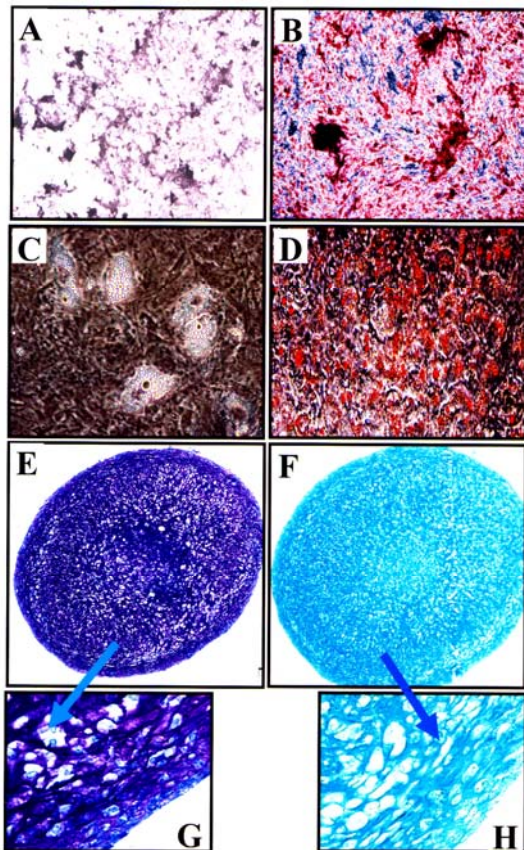

Fig. 1: The culture of the equine marrow cells. A) Four days after culture initiation, single elongated adherent cells were visible, B) These cells proliferated and developed round colonies on day 12, C) The culture became confluent 17-18 days after initiation and D) Some nodule-like cell aggregates became visible in some confluent culture, (magnification of all images: ×100)

Adipocyte differentiation: The first indication of the adipocyte differentiation became evident one week after culture initiation when lipid droplet appeared in the differentiating cells (Fig. 2C). These droplets stained red with oil red, a specific dye for lipid droplets in adipocytic cells (Fig. 2D). RT-PCR analysis indicated that the mRNA of adipocyte-specific genes including LPL, PPAR-gamma and PPAR-alpha were largely produced in adipogenic culture (Fig. 3). PPAR-alpha and gamma
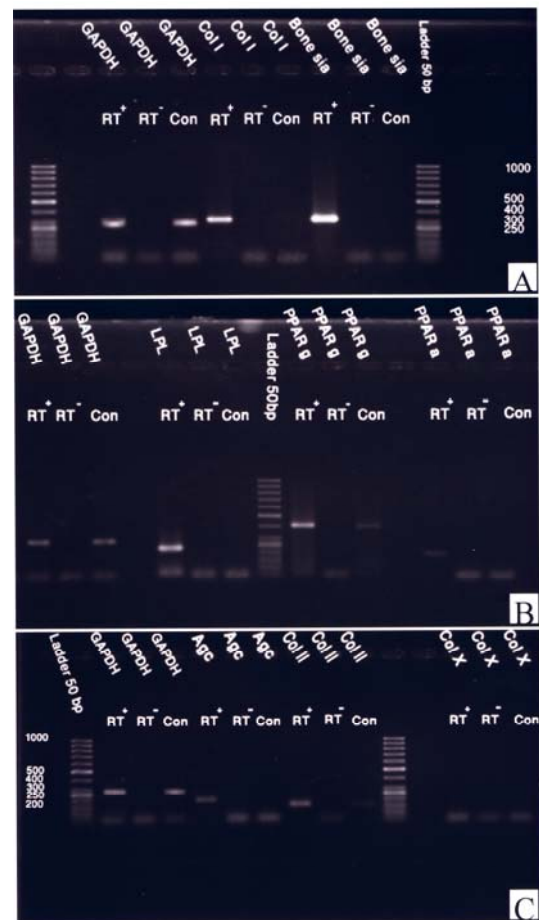
function as important coregulators of lipid homeostasis. PPAR-gamma also serves as a key regulator of adipocyte differentiation and lipid storage. LPL functions in lipid mobilization and storage in adipose tissue. All these molecules have considered as the adipocyte cell markers (Gregoire *et al*., 1998; Yu *et al*., 2003).

Cartilage differentiation: The sections prepared from the cell aggregates treated

with chondrogenic supplements were positively stained purple with toluidine blue and green with alcian blue, confirming that

Fig. 2: *In vitro* differentiation of the equine MSCs. A) during the differentiation period, some osteogenic foci appeared in the culture, B) These foci were positively stained red by alizarin red indicating the deposition of mineralized matrix among the cells, (magnification of A and B: ×40), C) Lipid droplet became visible first on day 7 of adipogenic culture (×200), D) The droplets turned into red upon oil red staining (×40), E) The sections prepared from the chondrogenic cultures were methachromatically stained purple with toluidine blue (×40), F) The sections from chondrogenic culture were stained green with alcian blue (×40), G) The picture of the E with higher magnification (×200) and H) The picture of the F with higher magnification (×200)

Fig. 3: RT-PCR analysis for differentiation detection of the equine MSCs. A) Gel image of the osteogenic culture: in this culture Coll I (collagen I) as well as the bone sialoprotein mRNA were largely produced, B) Gel image of the adipogenic culture: all adipogenic gene markers including PPAR-γ, PPAR-α (peroxisome proliferators activated-receptor gamma, alpha) and LPL (lipoprotein lipase) were expressed in this culture. PPAR-γ was also slightly expressed in the control, C) Gel image of the chondrogenic culture: agc (aggrecan) and Coll II (collagen II) genes were expressed in this culture but the Coll X (collagen X) mRNA was not produced. Col II was slightly expressed in the control. RT⁺ denotes the sample with reverse transcriptase enzyme and RT⁻ the sample without reverse transcriptase enzyme. The control was the MSCs cultivated in the medium without differentiation inducer. In all RT-PCR analysis, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the house-keeping gene

the glycosaminoglycan-rich matrix was produced in chondrogenic cultures (Fig. 2E-H). Based on the RT-PCR analysis, the expression of the cartilage-related genes including collagen II and aggrecan were verified in these cultures (Fig. 3). According to our data, collagen X was not expressed in neither group and the collagen II was slightly expressed in the control groups (MSCs in the medium without osteogenic supplements) in addition to the differentiated groups. Collagen type II is a specific collagen for hyaline cartilage. Aggrecan is the most abundant component of cartilage matrix, having a protein core to which glycosaminoglycan molecules are covalently linked. Collagen X is a minor collagen component of cartilage tissue (Fawcett, 1994; Hall, 2005).

CFU-F: This assay was used to assess the proliferative capacity that MSCs retain after the cells have been expanded in culture. According to our results, the mean number of CFU-F was 60.54 ± 2.23 per dish indicating that the cells are capable of developing fibroblastic cell colonies.

Cell seeding density: The results of this part are shown in Fig. 4. According to the data, significantly more fold increase occurred in the culture initiated at 100 cell/cm^2 ($P<0.05$).

FBS concentration: Based on the results indicated in Fig. 4, 15% FBS creates more fold increase than the other FBS concentrations. The value for 15% FBS was even significantly more than that for 20% FBS.

Growth curve: According to this curve, equine marrow derived MSCs possessed high growth capacity in culture (Fig. 5). They rapidly became adaptive to the culture conditions and started to enter the log (logarithmic) phase which led them into plateau phase within 8 days.

Discussion

In the present study, MSCs from equine marrow were isolated and examined in terms of bone, adipose and cartilage differentiations in culture. In most relevant investigations, immediately after isolation, equine MSCs have been directly
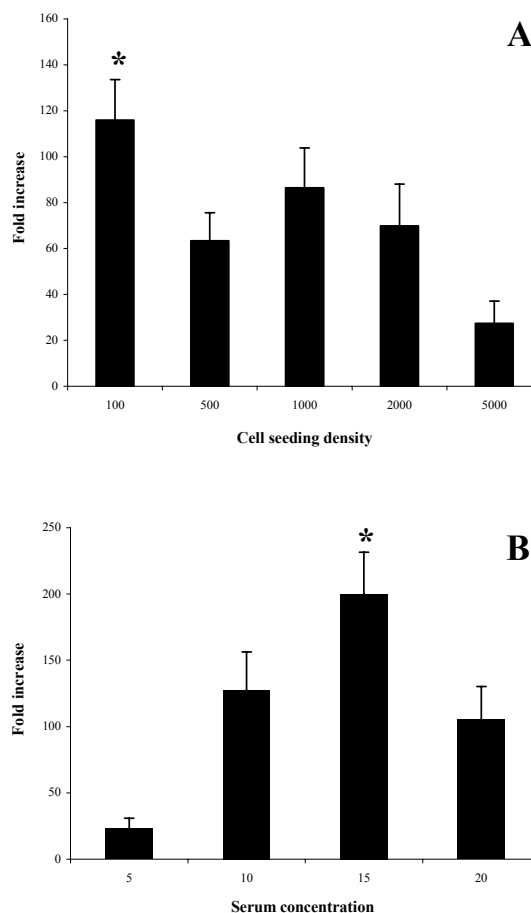
Fig. 4: Culture optimization for maximum fold increase. A) The graph indicated the significantly more fold increase of equine MSCs at 100 cells/cm^2 seeding density (*: P<0.05) and B) The graph indicated that equine MSC possess comparatively more fold increase when being provided with 15% FBS as medium supplement (*: P<0.05)
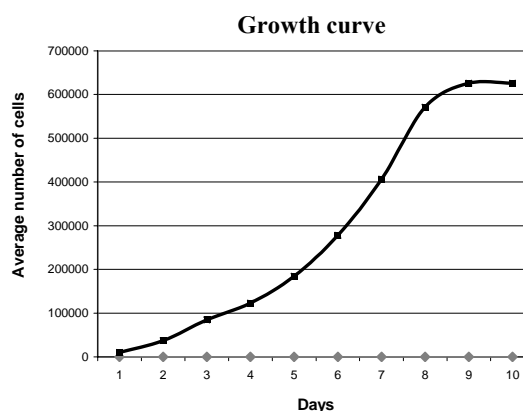
Fig. 5: The growth curve of the equine MSCs. As can be seen from the graph the cells enter rapidly to log phase after a brief lag phase and reach to saturation density on day 9

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transplanted into damaged tissues and the *in vitro* differentiation capacity of the cells has largely been ignored. The ability of MSCs for *in vitro* differentiation could be of great clinical significance. There could be two strategies with respect to the use of MSCs-mediated tissue regeneration. One strategy is the use of cells in undifferentiated state, allowing them to be differentiated within the *in vivo* microenvironment. The disadvantage of this approach is that unwanted cell differentiation, other than the desired cells may occur in the transplantation site. For instance, when the cells are used to regenerate bone tissue, adipocytic cell type may be produced in bone defect site. Therefore, the alternative approach is to fully differentiate the cells down the desired cell lineage prior to their transplantation. Such strategy could indeed guarantee the administration of the intrinsic differentiated cells into the repair site (Weissman, 2000; Pioletti *et al*., 2002).

Based on the available data, the *in vitro* differentiation of equine MSCs has gained little attention. As a matter of fact, most studies have focused on the cartilage differentiation of the cells, ignoring the standard tripotent differentiation into bone, cartilage and adipose cells (Fortier *et al*., 1998; Worster *et al*., 2000, 2001). In the present study, this was investigated. In this regard, there is only one report comparing the equine MSCs from the two sources, bone marrow versus peripheral blood (Koerner *et al*., 2006). The results of the current study are indeed in accordance with the findings of Koerner *et al*. (2006). In addition, in the present study, the culture requirement of the equine MSCs was investigated and the growth characteristics of the cells were determined.

According to the previous investigations, MSCs occur at low frequencies in bone marrow samples (Pittenger *et al*., 1999). Therefore, any experimental work with MSCs requires *in vitro* expansion of the cells which is largely dependent on the presence of FBS in the culture medium. On the other hand, MSCs developed in a medium containing FBS would be immunogenic and may transfer bovine pathogens upon transplantation (Spees *et al*., 2004). In spite of the extensive attempts to substitute the bovine serum, little success has been achieved (Kusnetsov *et al*., 2000; Kobayashi *et al*., 2005). In this study, therefore, equine MSCs were cultivated in different concentrations of FBS to determine the least concentration with the highest mitogenic effect, this was achieved by 15% FBS in the medium.

The other factor affecting the rate of MSCs proliferation would be the cell seeding density at culture initiation. Based on our findings, the best seeding density in which the highest fold increase could occur was determined to be 100 cells/cm^2. This data can be used to optimize the culture condition for maximum proliferation of the cells. This is of the utmost importance especially at cell therapy strategies where the rapid expansion of cells is needed.

To well characterize the growth of the cells, in this study, a growth curve was also plotted for MSCs from the equine bone marrow. The most striking feature of this curve was its short lag phase indicating the rapid entering of the isolated cells into mitotic phase (logarithmic phase). This result stands in opposition with the previous findings using the MSCs from human marrow suggesting that these cells remain quiescent in culture for a few days before beginning to multiply.

RT-PCR results indicated that the collagen X was not expressed at chondrogenic culture. To explain this result, it should be mentioned that collagen X constitute a minor collagen component of the hyaline cartilage. The amount of this collagen increases in hypertrophied chondrocyte of cartilage involved in endochondral ossification (Hall, 2005). The lack of collagen X expression in chondrogenic culture would be acceptable considering that *in vitro* microenvironment could never provide an ideal condition for complete cartilage differentiation. In addition, in some lanes of control group (MSCs cultivated in the medium without differentiation inducer), there were some faint band indicating that in these cells the gene have been slightly expressed (for example, Coll II in the control lane of chondrogenic culture). This data indicated that in MSCs as the stem cell for skeletal cell lineages, some differentiated genes

would be slightly expressed and the differentiation medium, indeed, enhances the level of expression.

In spite of considerable attempts to define the antigenic profile of human MSCs, no definitive single marker has so far been introduced. In this regard, several markers including CD133, LNGFR (low affinity nerve growth factor receptor) and STRO-1 have been proposed to be markers of human MSCs (Gronthose and Simmons, 1995; Quirici *et al.*, 2002; Kuci *et al.*, 2003). However, the identification of MSCs, especially those from animal sources, among other cell types and hence their isolation would be a difficult task owing to the lack of distinct specific markers. To overcome this problem, MSCs Committee of the International Society for Cell Therapy has proposed that MSCs from animal sources should possess two characteristic criteria: a) they must be adherent on plastic surfaces of culture dish and b) they must be able to produce bone, cartilage and adipose cell lineages (Dominici *et al.*, 2006). Since the isolated cells of the present study were easily differentiated into bone, cartilage and adipose cells and also they were plastic adherent cell, we convinced that they were the MSCs described elsewhere.

The other interesting result of the current study was the behaviour of equine MSCs at confluent expansion culture. According to our observation, several nodule-like cell aggregates had been developed in these cultures. This could be as a result of the extensive mitotic activity of the cells. Similar nodules have not been reported at MSC culture from human, rabbit or mice (Eslaminejad *et al.*, 2006; Shao *et al.*, 2006; Abdallah and Kassem, 2007).

In summary, we concluded that MSCs from equine bone marrow can be differentiated into bone, cartilage and adipose cell lineages in culture. Furthermore, the optimal culture condition for equine MSC expansion could be achieved by seeding the cells at 100 cells/cm^2 in a medium supplemented with 15% FBS. Equine MSCs possess a great expansion rate in culture rendering them much appropriate as the cellular candidate for regeneration of defects of skeletal tissue.

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