Vol 19 No 2, p 115-121

IN VITRO DIFFERENTIATION OF ENDOTHELIAL PROGENITOR STEM CELLS DERIVED FROM PERIPHERAL BLOOD OF CAMEL

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ABSTRACT

The peripheral blood-derived mononuclear cells (PBMNCS) obtained from camel blood when set in culture gave rise to adherent cells. The count of colony forming unit (CFU) after culturing PBMNCS for 7 days were significantly lower in the non pregnant animals than in the pregnant ones (32.4 ± 26.7 versus 65.2 ± 18.3, respectively). Flow cytometry analysis shows the expression of CD34 + (33.8%), CD14 + (33%) and CD29 + (22.8%), the latter is a marker for mesenchymal stem cells although direct evidence for the presence of mesenchymal progenitor cells (MPCS) in the peripheral blood does not exist. The stem/ progenitor cell characteristics were evidenced by their proliferative capacities and their ability to differentiate into osteoblasts, chondrocytes and neural cells after culture for 2 months in a modified conditioned medium. The stemness capacity of the PB-MNCS of camel was proven in this study and further study was needed to establish the clinical application of these stem cells.

Key words: Camel, differentiation, endothelial progenitor cells, peripheral blood, stem cells

A number of stem cell treatments exist, although most are still experimental and / or costly, with the notable exception of bone marrow transplantation (Laughlin *et al,* 2004). Several very promising treatments of serious diseases have already been attempted, using adult stem cells (Kuehnle and Goodell, 2002)

Mesenchymal stem cells (MSCs) derived from bone marrow (BM-MSCs) and adipose tissue (AD-MSCs) are the most highly characterised and are considered comparable (Parker and Katz, 2006). Both have demonstrated broad multi potency with differentiation into a number of cell lineages, including adipo-, osteo-, and chondrocytic lineages. Veterinarians have used autologous AD-MSCs to treat tendon and ligament injuries and joint disease in horses on a commercial basis since 2003 (Harman *et al,* 2006 and Dahlgren, 2006).

The circulatory capacity of the hematopoietic stem cells has been established in transplantation studies as these cells were found in umbilical cord blood (Rubinstein *et al,* 1995) and in peripheral blood (Siena *et al,* 1989).

The aim of our work was to examine the potency of (PBMNCS) harvested from camel blood as a source of stem\ progenitor cells rather than AD-MSCS harvested from the fat. On the other hand, for the future potential to treat human conditions with camel stem cells has not been overlooked.

Materials and Methods

Isolation and culture of peripheral blood-derived mononuclear cells:

PB samples were collected from the 14 animals (7 pregnant and 7 non-pregnant). Total camel PB-MNCs were isolated from 50mL of blood from each of the 14 enrolled animals by density gradient centrifugation using Histopaque- 1077 (Sigma, St Louis, MO, USA), as described previously (Jung *et al,* 2008) and in Supplementary Methods. PB cells were cultured using a protocol as described by (Gehling *et al* 2000) with slight modifications. Briefly, PB cells were pooled, cultured on fibronectin (10 µg/ cm2,Cellsystems, St. Katharinen, Germany) and maintained in endothelial basal medium (EBM) (Clonetics) supplemented with 10% fetal calf serum (PAN-Biotech, Aidenbach, Germany) supplemented with penicillin/streptomycin (Gibco), 50 ng/ml stem cell factor (SCF, R&D Systems, Abingdon, UK), 50 ng/ml vascular endothelial growth factor (VEGF, R&D Systems), 20 ng/ml basic fibroblast growth

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factor (FGF-2, R&D Systems) and 20 ng/ml stem cell growth factor (SCGF, Peprotech, London, UK). This medium was replaced 3 times a week. After four days, cells were stained with 1, 1′-dioctadecyl-3, 3, 3′, 3′- tetramethylindocarbocyanine-labeled acetylated LDL (DiLDL,) and FITC-labeled Ulexeuropaeus agglutinin I (lectin, Sigma), and double stained cells were counted.

Colony-forming unit counts

A CFU was defined as a central core of rounded cells surrounded by elongated spindled-shaped cells, and CFUs were counted after culturing MNCs for 7 days. Cell clusters without emerging spindle cells were not counted. Colonies were counted manually in a minimum of 3 wells in 12-well plates by two independent observers; results are expressed as average number of CFUs per well (Jonathan *et al,* 2003).

Colony formation and *in vitro* multidifferentiation:

Generation of colonial cell lines and *in vitro* differentiation into osteoblasts, chondrocytes or neural cells from sorted circulating CD34+, CD14+ cells was preformed as described elsewhere (Pittenger *et al,* 1999 and Boquest *et al,* 2005).

For *in vitro* differentiation into osteoblasts, chondrocytes or neural cells, cells were cultured under conditions known to induce differentiation into various cell types. For osteogenic induction adherent cells were cultured in a-MEM, 10% foetal calf serum, containing 100 nM dexamethasone, 50 µM ascorbic acid and 2 mM ß-glycero-posphate (all from Sigma). The medium was changed twice a week for 3 weeks. At the end of the treatment, cells were fixed with 10% formalin and stained with 2% Alizarin red S (Sigma) for 3 min, followed by extensive washing and staining with hematoxylin to detect intracellular calcium deposits.

For chondrogenic differentiation, cells were incubated in DMEM high glucose containing 10% FBS (Hyclone), supplemented with insulin, ascorbate 2-phosphate (37.5 µg/ml), dexamethasone (10-8M), and recombinant human TGFß3 (10 ng/ml). Aggregate cultures were incubated at 37°C, 5% CO2, and medium was changed every 2–3 days. Aggregates were harvested after 2–4 weeks for pathological Alcian blue staining (Pelttari *et al,* 2011).

For neurogenic differentiation cells were plated in DMEM, 10% FBS. After 24 h medium was replaced with DMEM hg, 10% FBS containing B27 (Invitrogen), 10 ng/ml EGF (Peprotech) and 20 ng/ml bFGF (Peprotech). After 5 days, cells were washed and incubated with DMEM containing all-trans retinoic acid (0.5 M), and two neuronal growth factors at a concentration of 10ng/ml which were brain-derived growth factor (BDNF), and nerve growth factor (NGF). After 7-14 days, a small proportion of BMSC changed morphology and RT-PCR was done to detect mRNA gene expression of nestin (a marker of cells destined to become neural cells), and tyrosine hydroxylase (TH) (Romagnani *et al,* 2005 and El-Menoufy *et al,* 2010). The expression of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA integrity. Then, cDNA was synthesized using ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The PCR mixture was composed of 1X PCR reaction buffer [15 mM Tris–HCl (pH 8.0) and 50 mM KCl], 0.5 lM of each primer pair, 250 lM of each dNTP, and 1.25U AmpliTaq Gold DNA polymerase (Applied Biosystems). The MgCl2 concentration in the reaction mixtures was 2.5 mM. The cDNA encoding GAPDH was amplified for 30 cycles (15 s at 94°C, 30 s at 55°C, and1min at 72°C) using the following primers sense 5′- CATTTGGAGTAAAAGGTGTTTCTT-3′and antisense 5′-TTTCACCAATGTGTCACAGTCA -3′ (UniSTS:28969). The products of the PCR amplifications (127 bp) were analysed on a 1.5% agarose gel stained with ethidiumbromide and visualised under ultraviolet light. A similar protocol was used for detection of genes expression of tyrosine hydroxylase and nestin in MSCs cells homogenate using these pairs of primers (UniSTS: 155073) and (UniSTS: 30080) respectively. Sequence of tyrosine hydroxylase forward primer: 5′-CAGCTGCCCTAGTCAGCAC-3′; reverse primer: 5′-GCTTCCGAGTGCAGGTCACA-3′and nestin forward primer: 5′-CAGAGACTTCAGGGTTTC -3′; reverse primer: 5′-AGAGTGTTCAGCATTATGCC -3′, to give a PCR products of 230 bp and 187 bp for tyrosine hydroxylase and nestin genes, respectively (Abdulaziz *et al,* 2010).

Flow cytometry cell analysis and sorting

Endothelial phenotypes was confirmed by flow cytometry analysis of cell surface molecules as detailed elsewhere (Annunziato *et al,* 2002) for $CD34^+$ and $CD14^+$. Cells were sorted by using FITC-labeled anti-CD34 (1:20; DAKO, Carpinteria, CA, USA), anti-CD14 (1:20; DAKO). Mesenchymal stem cell surface marker was identified by using FITC-labeled anti-$CD29^+$ (1:20; DAKO, Carpinteria, CA, USA).Briefly, after staining with appropriately

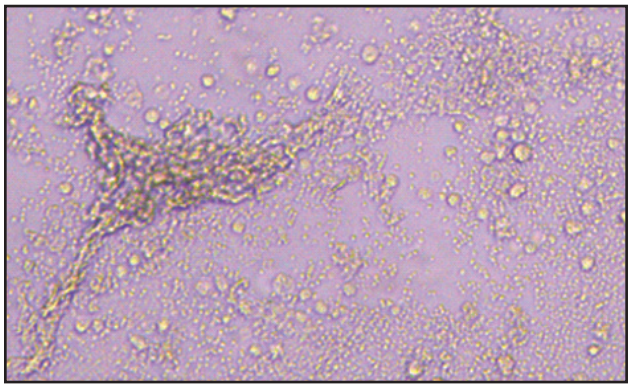

Fig 1. EPC at 0 day culture on fibronectin plate. EPCs were rounded in their shape.

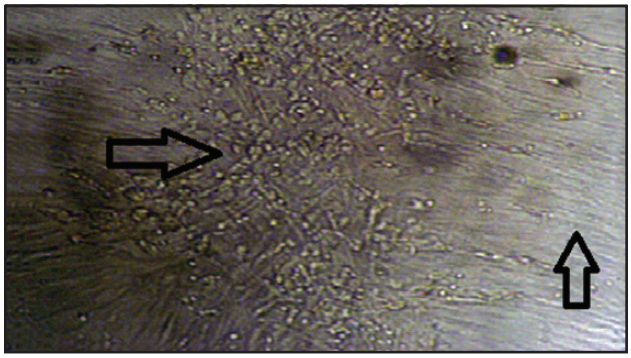

Fig 2. EPC-CFU at 72 hours culture on fibronectin plate. Arrows show EPC-CFU as a central core of rounded cells (horizontal arrow) surrounded by elongated spindled-shaped cells (vertical arrow).

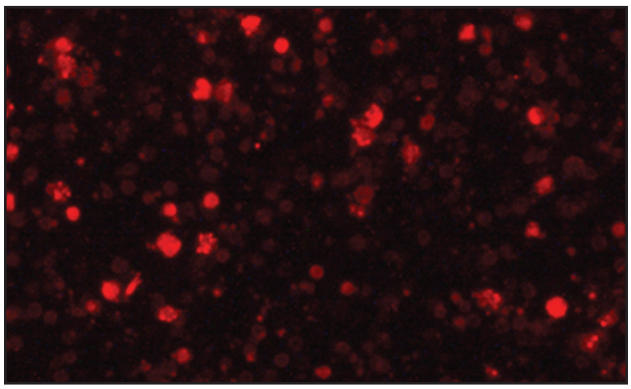

Fig 3. Characterization of circulating EPCs by expression of endothelial markers, Di-LDL and lectin dual staining.

conjugated Abs and washings, cells were analysed on a BDL cytofluorimeter, (BD Biosciences). The area of positivity was determined using an isotype matched control Ab. 10^4 events for each sample were acquired.

Results

EPC-CFU culture count:

The count of EPC-CFU was significantly lower in the non –pregnant animals than in the pregnant

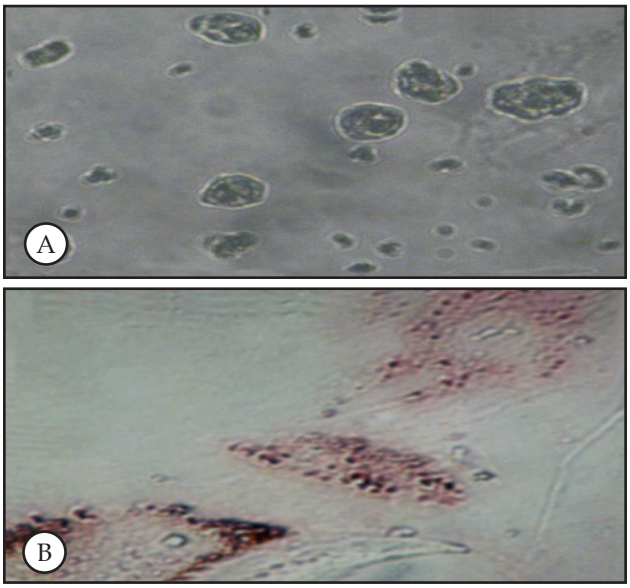

Fig 4. (A) Morphological changes of EPCs, arrows indicate differentiated EPCs into osteoblasts. (B) Pathological Alzarin red staining of differentiated EPCs into osteoblasts.

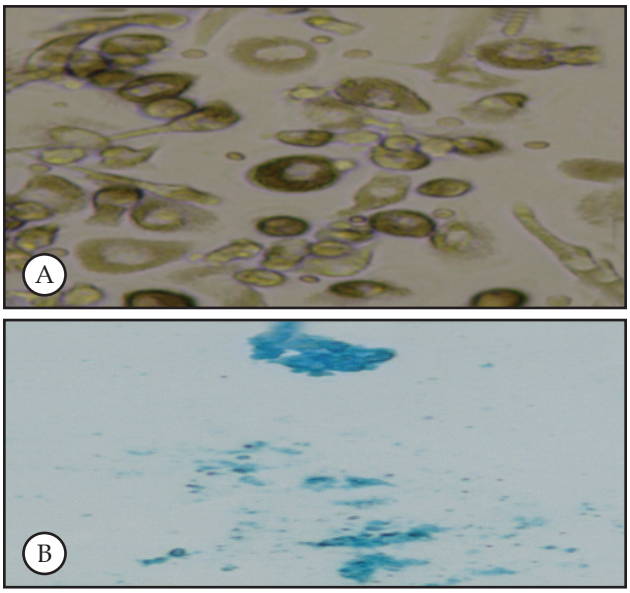

Fig 5. (A) Morphological changes of differentiated EPCs, arrows indicate differentiated EPCs into chondrocytes. (B) Pathological Alcian blue staining of differentiated EPCs into chondrocytes.

animals (32.4±26.7 versus 65.2±18.3, respectively) table (1). The MNCs ($3x10^6$cells/well) obtained from pregnant and non-pregnant animals were cultured for at least 2 weeks in modified medium to form outgrowth cells (Figs 1&2). To ensure that CFUs observed reflected endothelial differentiation, colonies were stained with acetylated LDL (DiLDL,) and FITC-labeled lectin. All colonies which were examined displayed staining with both markers (Fig 3).

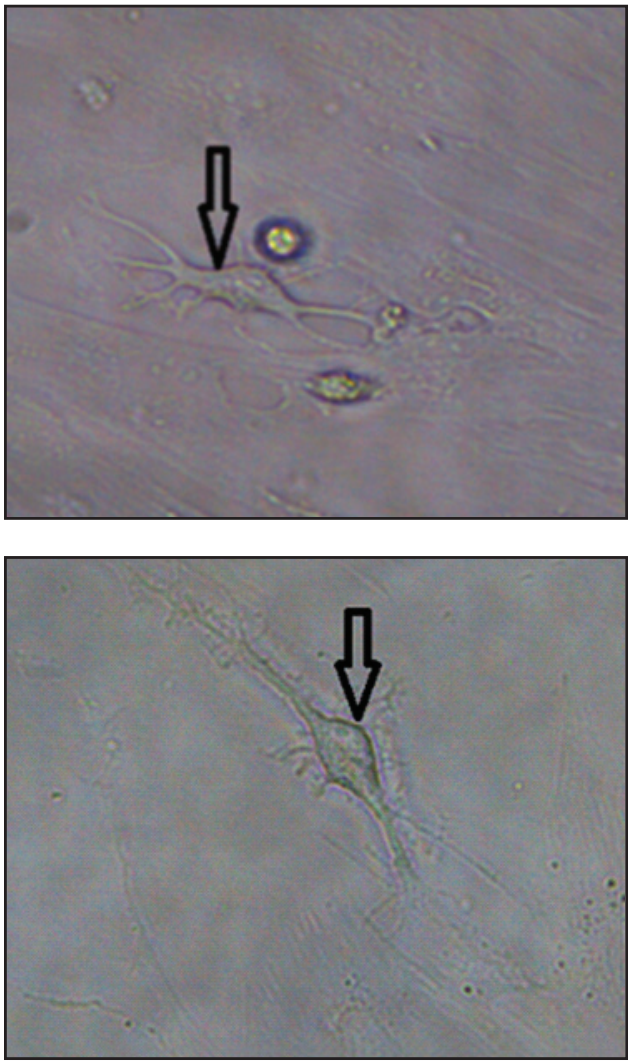

Fig 6. (A&B) Morphological changes of differentiated EPCs, arrows indicate differentiated EPCs into neural cells.

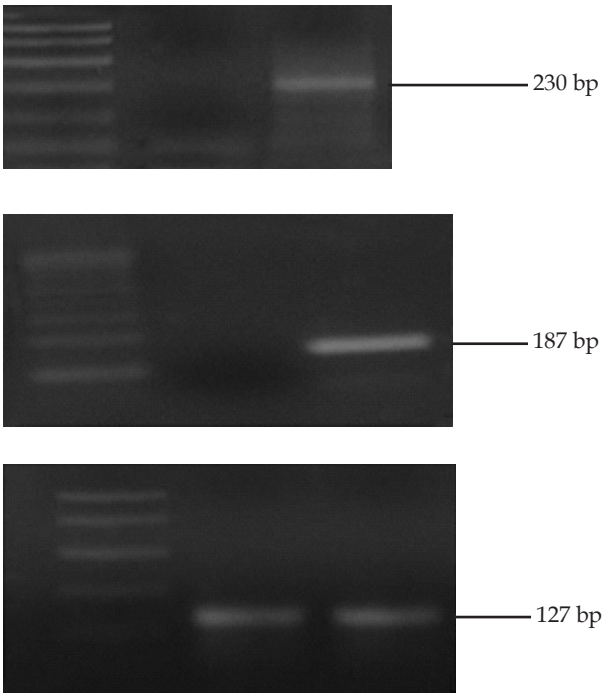

Fig 7. Molecular characterisation of the Neuronal cells (PCR gene expression of tyrosine hydroxylase and nestin). Agarose gel electrophoresis shows (A) gene expression of tyrosine hydroxylase (B) gene expression of nestin and (C) gene expression of GAPDH in undifferentiated and differentiated EPC.

Lane 1: DNA marker (100, 200, 300 bp…)

Lane 2: No PCR product neither for tyrosine hydroxylase gene (A), nor nestin gene in undifferentiated EPCs (B). But there is PCR product for GAPDH gene (C) in undifferentiated EPCs.

Lane 3: PCR products for tyrosine hydroxylase gene (A), nestin gene (B) and GAPDH gene (C) in differentiated EPCs.

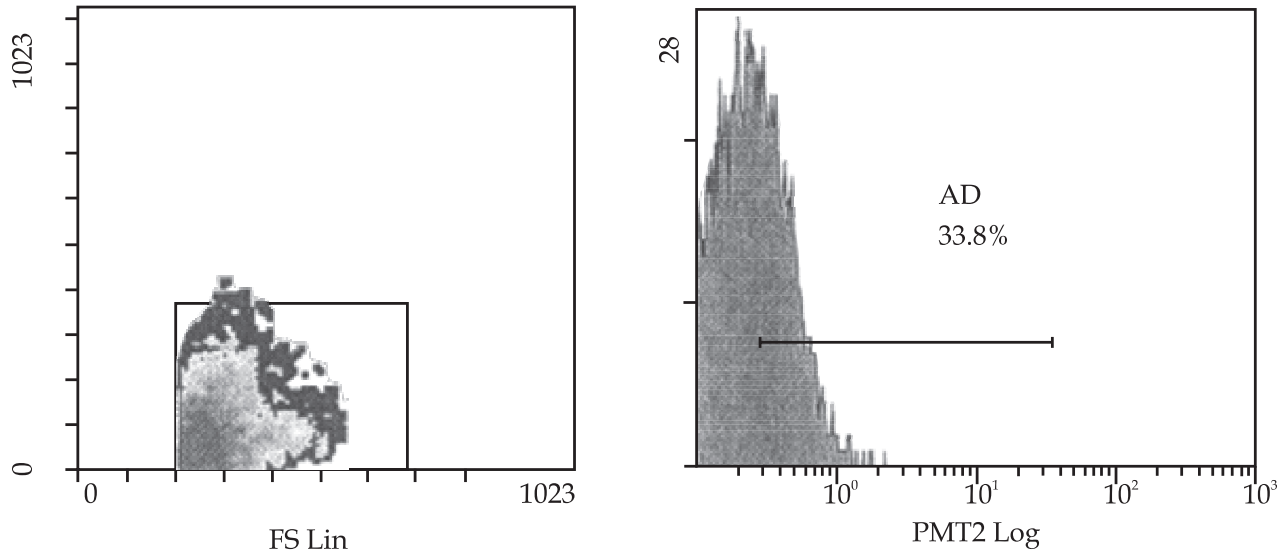

Fig 8. FACS analysis shows expression of CD 34+ (33.8%) for isolated and cultured EPCs for 7 days.

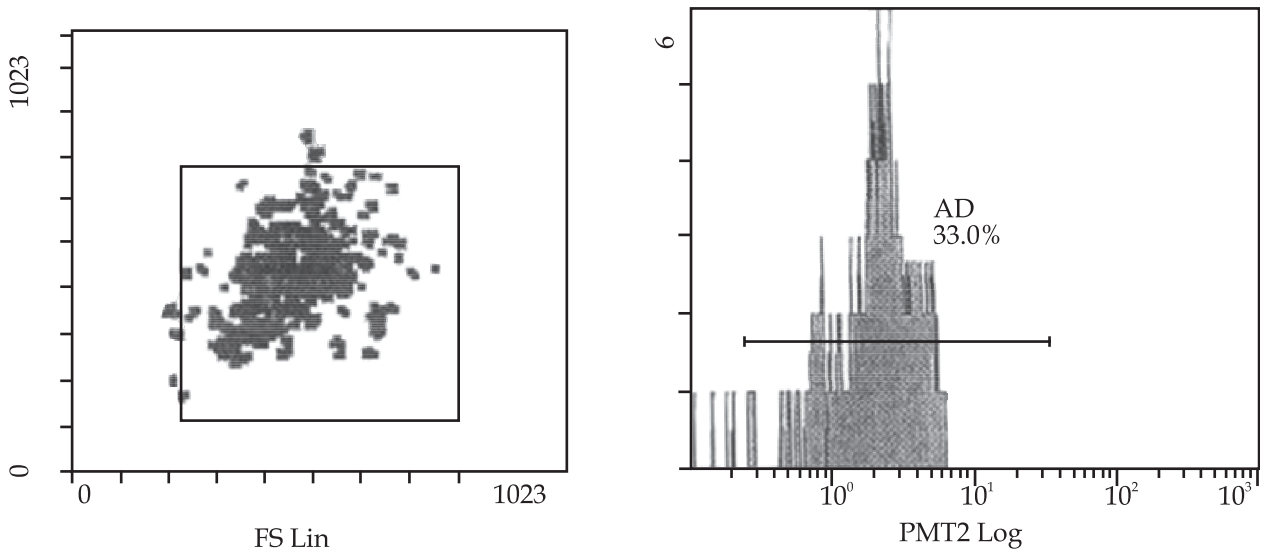

Fig 9. FACS analysis shows expression of CD 14+ (33.0%) for isolated and cultured EPCs for 7 days.

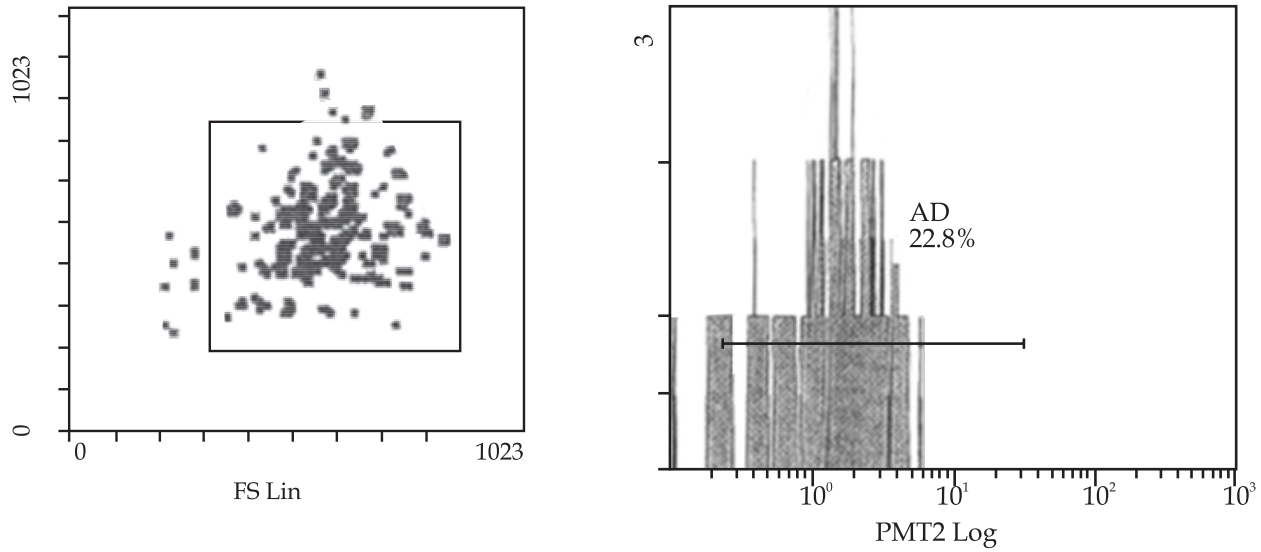

Fig 10. FACS analysis shows expression of CD 29+ (22.8%) for isolated and cultured PB-MNCs for 7 days which is a marker for mesenchymal stem cells (MSCs).

EPCs in vitro multidifferentiation:

The stem/progenitor cell characteristics were evidenced by their proliferative capacities and their ability to differentiate into osteoblasts (Fig 3), chondrocytes (Fig 4) and neural cells (Figs 5&6) after culture for 8 weeks in a modified conditioned medium. Molecular characterisation of the differentiated neuronal cells by PCR is shown in Fig (7).

FACS analysis results:

Flow cytometry analysis revealed high percentage expression for CD 34^+ and $CD14^+$ (33.8% (Fig 8) and 33% (Fig 9) respectively). Both were indicative for EPCs characterization. In comparison those to CD markers for MSCs like $CD29^+$ (Fig 10) which was detected but in lower concentration (22.8%). This is expected as MSCs present mainly in bone marrow and EPCs present in peripheral blood.

Discussion

Haemopoiesis is sustained by two main cellular components, the haematopoietic cells (HSCs) and mesenchymal progenitor cells (MPCs). MPCs are multipotent and are the precursors for marrow stroma, bone, cartilage, muscle and connective tissues. The circulatory capacity of the haematopoietic stem cells is well documented in

adults by transplantation studies and supported by the observation that these cells circulate in umbilical cord blood (Rubinstein *et al,* 1995 and Wyrsch *et al,* 1999) and under certain circumstances, in peripheral blood (Siena *et al,* 1989). In turn, direct evidence for the presence of MPCs in the human blood does not exist. Recently Kuwana *et al* (2003) described a population of $CD14^+$ monocytes that could differentiate into several distinct mesenchymal cell lineages. These cells, named as monocytes-derived mesenchymal progenitors, were obtained from circulating MNCs cultured on fibronectin for 7 days and had a unique molecular phenotype-$CD14^+$ $CD45^+$ $CD34^+$. MOMPs could be obtained from PB even if deprived of $CD34^+$ cells, but their source, as well as their stem cell nature, was not clearly defined. Our results show that camel peripheral blood-derived mononuclear cells (PB-derived mononuclear cells), when cultured on fibronectin were able to generate adherent layer initially formed by individual cells or colonies of a few cells. These cells demonstrate cell surface molecule for EPCs $CD34^+$ and $CD14^+$ in a percentage of 33.8% and 33% respectively. It also demonstrates mesenchymal stem cell surface marker $CD29^+$ in a percentage of 22.8%. These harvested cells from the peripheral blood of camel have the ability to differentiate in vitro and give rise to osteoblasts, chondrocytes or neural cells. In this respect, Romagnani *et al* (2005) found that the circulating $CD14^+$ CD34 low cells exhibited proliferative response to stem cell growth factors, clonogenicity and multidifferentiation potential, as shown by their ability to give rise not only to ECs, but also to osteoblasts, adipocytes, or neural cells and these data provide the first evidence that a relevant, even if variable percentage of $CD14^+$ cells consist of double-positive $CD14^+$ CD34 low cells showing phenotypic and functional features of multipotent stem cells. In the contrast, Erices, Conget and Minguell (2000) recorded that, approximately 75% of the umbilical cord blood collections gave rise to cultures of adherent cells which displayed the morphology and characteristics of multinucleated osteoclasts and expressed several markers of osteoclasts. 25% of cord blood harvests gave rise to adherent cells that expressed several mesenchymal progenitor (MPCs) related antigens, such as $CD13^+$, $CD29^+$, $CD49^+$e and $CD54^+$. These cells did not express myeloid or endothelial antigens. In addition MPCs upon proper stimulation could be differentiated into osteoblasts and adipocytes. So our results declare for the first time for our knowledge that PB of both pregnant and non-pregnant camels contained both the EPCs and the MPCs. Under certain circumstances and specific growth factors EPCs and/or MPCs have multidifferentiation potentials into osteocytes, chondrocytes and neuronal cells. In addition, for our knowledge, it is the first time to discuss a point of research about detecting a significant increase in counts of colony forming units of stem progenitor cells in peripheral blood of pregnant camel compared to non pregnant ones. The EPCs content of cord blood were discussed by Péault (1996) who hypothesized that both haematopoietic and mesenchymal progenitors traveling, via cord blood, from early fetal haematopoietic sites to the newly formed bone marrow. Ng *et al* (2009) showed that the adipose- derived stem cells from pregnant women show higher proliferation rate unrelated to estrogen. Based on the results of this study, further investigation on the clinical application of EPCs derived from the PB of camel is needed, for examining their abilities in organ transplantaion.

Conclusion

The stemness capacity of the PB-MNCS of camel was proven in this study and its ability for multidifferentiation was also estimated. Further study was needed to establish the clinical application of these stem cells.

Table 1. EPC-CFU count in pregnant and non-pregnant animals.

	1	2	3	4	5	6	7	Mean	±SD
EPC-CFU count in Pregnant	61	67	69	66	63	70	65	65.8	±18.3
EPC-CFU count in Non-pregnant	31	33	30	29	32	34	37	32.2	±26.7

Acknowledgements

Deepest thanks for Prof. Dr. Saber Abuzeid and Prof. Dr. Hesham El Saeid for helping us in this work by providing us the camel blood.

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