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Fat harvesting site is an important determinant of proliferation and pluripotency of adipose-derived stem cells

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ABSTRACT

To define the optimal fat harvest site and detect any potential differences in adipose-derived stem cells (ASCs) proliferation properties in camels, aspirates from the abdomen and hump sites were compared. Obtained results revealed that ASCs from both abdomen and hump exhibited spindle-shaped and fibroblast-like morphology with hump-derived ASCs being smaller in size and narrower in overall appearance than abdominal ASCs. Abdominal ASCs required a greater time for proliferation than the hump-derived cells. These results were further confirmed with a tetrazolium-based colorimetric assay (MTT) which showed a greater cell proliferation rate for hump ASCs than for the abdomen. Under inductive conditions, ASCs from both abdominal and hump fat deposits maintained their lineage differentiation potential into adipogenic, chondrogenic, and osteogenic lineages during subsequent passages without any qualitative difference. However, expression of alkaline phosphatase was higher in osteogenic differentiated cells from the hump compared with those of the abdomen. Moreover, the increase in calcium content in hump-derived stem cells was higher than that in abdominal-derived stem cells. In conclusion, our findings revealed that ASCs can be obtained from different anatomical locations, although ASCs from the hump fat region may be the ideal stem cell sources for use in cell-based therapies.

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1. Introduction

Stem cells are identified by their unique capability to self-renew, as well as to generate progenitor cells, which then give rise to an inexhaustible supply of differentiated cell types [1]. In this respect, adult mesenchymal stem cells (MSCs) have received great interest and are currently the most appealing cell subsets, due mainly to their extensive self-renewal and differentiation potential [2]. Several adult tissues have been shown to contain rare populations of stem cells with mesenchymal characteristics. In our previous studies, as well as in studies from other laboratories, MSCs have been isolated and cultured from bone marrow [3], umbilical cord blood [4], muscle tissue [5], neuronal tissue [6], periosteum [7], periodontal ligament [8], dental pulp [9], deciduous teeth [10], dental follicle [11], and pancreatic [12] and hepatic tissues [13].

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Adipose tissue is another alternative source for MSCs [14] that can be isolated by a less invasive method and in large quantities [15] than from other sources. These MSCs, which are appropriately named adipose-derived stromal cells (ASCs), are isolated with higher efficiency, have a great expansion potential, and also appear to differentiate efficiently into the same cell lineages as other sources do [16–18]. This source of multipotent cells has been obtained and expanded efficiently from humans [19,20], but also from species such as mouse [21], rat [22], rabbit [23], sheep [24], horse [25], dog [26], as well as non-human primate [27].

Differences in differentiation potential have been observed among ASCs obtained from various species [28]. Our recent experimental findings [29] have provided some support for the hypothesis that camel ASC populations from abdominal tissue like human lipoaspirate [3,19,20] were able to maintain their unique properties during multi passages and that those cells had potential to differentiate into multilineage cells.

Another important aspect to be determined is whether the diversity existing in depots of adipose tissue within the same organism could lead to different outcomes of differentiation. A recent study in dogs demonstrated that the anatomical origin of the adipose tissue has an evident effect in the differentiation potential of the ASCs [30]. In this regard, it has been shown that ASCs from the neck region may be the ideal stem cell sources for tissue engineering approaches for the regeneration of nervous tissue in rat [31]. Therefore, it seems that settling preferred donor origins and sites for lipoaspiration and isolation of ASCs will certainly contribute to a more successful use of fat transplantation enriched with mesenchymal cells. To the best of our knowledge, no previous study has evaluated the effect of anatomical origin of the adipose tissue on proliferation of ASCs and the extent of their pluripotency in camel. A camel model was selected for this study largely due to its different fat depot physiologies.

Therefore, in this study we isolated camel ASCs from abdominal and hump tissues to compare their ease of isolation and to characterize their proliferation ability along the cultured time. The multipotency of these cells was assessed by inducing their differentiation into different cellular lineages, including adipogenic, chondrogenic, and osteogenic and analyzing the production of specific extra cellular matrix and cytoskeletal elements.

2. Materials and methods

All chemical reagents were obtained from Sigma (Sigma, USA) unless otherwise noted.

2.1. Harvesting of camel adipose tissue

Adipose tissues were collected from abdominal and hump depots from 5 adult dromedary camels, between 2 and 5 years of age. For sampling, using sterile scalpel blades, forceps, and scissors, adipose tissues were harvested from abdominal and hump tissues after slaughter of each of the five animals.

2.2. Isolation and expansion of camel ASCs

Camel ASCs were isolated by enzymatic digestion as previously performed in our laboratory [29]. Briefly, adipose tissues were washed extensively by phosphate buffer saline (PBS) and dissected into 1 mm pieces, followed by treating with 0.2% collagenase type I in Dulbecco's modified Eagle's medium (DMEM). The cell suspension was centrifuged at 350 *g* for 5 min, and the supernatant was discarded. Cell pellet was suspended in DMEM (low glucose) with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin 100 U/ml, streptomycin 0.1 mg/ml) at 37 °C atmosphere of 5% CO_2. Cells were seeded in tissue culture polystyrene plates and grown in monolayer culture under above conditions. When the cells reached 80% of confluence, they were detached from the culture dish using trypsin/EDTA. Cells were then divided into two aliquots; the first aliquot was passaged into fresh culture flask upon reaching confluence and the second was frozen in 90% FBS and 10% DMSO and stored in liquid nitrogen for later use. The survival rate, evaluated by trypan blue staining exclusion test, of all thawed MSC lots was >85% before use in this study. To study the variability of cryopreserved progenitor cells, all ASCs thawed cells were subjected to the trypan blue test and examined for proliferative capacity. Cells from passage 2 to passage 3 were used for all experiments.

Population doubling time (PDT) was calculated according to the equation PDT = culture time (CT)/population doubling number (PDN). To determine PDN, the formula PDN = log N/N0 × 3.31 was used. In this equation, N stands for the cell number at culture end and N0 the number of the cells at culture initiation. To determine the culture time and N and N0 in each passage, cells were counted and plated at 10^4 cells/cm^2 in 25-cm^2 culture flasks for a period when one of the cultures reached confluence. At this time, the cells were trypsinized and counted. Using the data, PDT was calculated for ASCs from abdominal and hump fat depot in each passage until passage 8 (P8).

ASCs at passage 2 (P2) were seeded with an initial cell density of 5000 cells/well in 24-well tissue culture polystyrene plates and were cultured for 5 days. The proliferation of these cells was evaluated via tetrazolium-based colorimetric assay (MTT test). Thus on each day, 50 μl of MTT solution (5 mg/ml in DMEM) was added to each well, to evaluate the conversion of MTT to formazan crystals by the mitochondrial dehydrogenases of the living cells. The plate was incubated at 37 °C, 5% CO_2. After 3.5 h incubation, the supernatant was removed to examine the dissolution of the dark-blue intracellular formazan, and 250 μl dimethyl sulfoxide (DMSO) as an appropriate solvent was added. The optical density was read at a wavelength of 570 nm in a micro-plate reader (ELx-800, BIOTEK instruments, Winooski, VT, USA).

2.3. Multilineage differentiation potential of ASCs

Different batches of cryopreserved cells from passage 0 were thawed and expanded to the second passage (P2). When 80% confluent, the cells were used for induction of differentiation into three lineages.

2.3.1. Adipogenic

Induction of adipogenic differentiation was accomplished as previously described [29]. Briefly, the cells were seeded at a density of 2 × 10^4 cells/cm^2 into four-chamber slides and cultured in basal media (DMEM with 10% FBS) supplemented with 0.5 mM hydrocortisone, 60 mM indomethacine, and 0.5 mM isobutylmethylxanthine. To assess adipogenic capacity, lipid accumulation was identified in differentiated cells with *in situ* Oil Red O (ORO) staining 21 days after induction. An undifferentiated batch of cells was allocated to the control group without differentiating supplements.

2.3.2. Chondrogenic

Chondrogenic differentiation of ASCs was induced by a 21-day culture in micropellet, as described elsewhere [29,32]. Briefly, MSCs (2.5 × 10^5 cells) were pelleted by centrifugation in 15 ml conic tubes and cultured in DMEM supplemented with 0.1 μM dexamethasone (Sigma), 50 μg/ml ascorbic acid 2-phosphate (Sigma), 1% insulin–transferrin–sodium selenite supplement (Gibco), and 10 ng/ml TGF-β (Peprotech).

2.3.3. Osteogenic

For osteogenic differentiation, the adherent cells were cultured in osteogenesis induction medium containing basal medium (DMEM + 10% FBS) supplemented with 10 mM β-glycerophosphate (Sigma), 0.1 μM dexamethasone, and 50 μg/ml ascorbic acid 2-phosphate. This was followed by detection of early mineralization using Alizarin Red S 21 days after culture. An undifferentiated batch of cells was allocated to the control group without differentiating supplements [20,29].

2.4. Alkaline phosphatase (ALP) activity

For ALP activity assay, total protein of cells was extracted using 200 μl radioimmunoprecipitation (RIPA) lysis buffer. ALP activity was detected with an ALP assay kit (Parsazmun, Tehran, Iran) using p-nitrophenyl phosphate (p-NPP) as substrate. Amount of ALP in the cells was normalized against total protein content [29].

2.5. Calcium content

The amount of deposited calcium on stem cells following osteogenic induction was measured using Cresolphthalein Complex one method with a calcium content assay kit (Parsazmun, Tehran, Iran). The extraction of calcium was performed using 0.6 M HCl as previously described [29].

2.6. Karyotype analysis

For karyotype analysis, cells were collected from primary culture and every 2 passages up to passage 8. The collected cells were treated with 0.15 μg/ml Colcemid solution (Gibco) for 2 h, followed by exposure to 0.075 M KCl at 37 °C for 16 min. Thereafter, the cells were fixed in ice-cold 3:1 methanol:glacial acetic acid and were then dropped onto pre-cleaned chilled slides. Chromosomes were visualized using standard G-band staining technique. At least 20 metaphase spreads were screened and five banded karyotypes were evaluated for chromosomal rearrangements [29].

2.7. Statistical analysis

At least three replications were performed for each experiment. Values were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to evaluate all data sets. A P-value of less than 0.05 was considered statistically significant.

3. Results

Camel ASCs were isolated successfully from abdominal and hump fat depot. The yield of ASCs from camel abdominal source was around ($1 \pm 0.3 \times 10^6$) ASCs per gram of adipose tissue which was not statistically different ($P > 0.05$) from that yielded from hump fat depot ($1.1 \pm 0.12 \times 10^6$) ASCs per gram adipose tissue. Almost three to five days after culture, the colonies were identified in abdominal (3.1 ± 1.3) and hump (5.0 ± 1.2) derived ASCs by their morphology which was not statistically different between the two groups ($P > 0.05$). Thereafter, the cells were trypsinized and subsequently re-plated on a new 75 cm^2 flask. In this regard, 10 to 15 days after culture of abdominal (14.5 ± 1.2) and hump (10.1 ± 1.4) derived ASCs, when the cells became confluent they were considered as passage 0 and propagated under culture conditions with careful monitoring and medium change. The hump derived cells, as above result is shown, revealed a greater ability ($P < 0.05$) to reach confluence by the end of culture at passage 0. The cells from both fat depots were then either passaged or frozen for later use.

3.1. Effect of fat harvesting site on morphology and stemness properties of camel ASCs

In vitro morphological characterization was performed on ASCs from camel abdominal and hump fat of the one-hump camel. The ASCs from both abdominal (Fig. 1A) and hump (Fig. 1B) exhibited spindle-shaped and fibroblast-like morphology, similar to those of MSCs derived from other species. The morphological comparison of these two sources of ASCs demonstrated that ASCs derived from hump fat depot were smaller in size and narrower in overall appearance than ASCs derived from abdominal fat depot. Our observation revealed that the ASCs from both sites of harvest, i.e. abdomen (Fig. 1C) and hump (Fig. 1D), also had high proliferative ability *in vitro*.

3.2. Effect of fat harvesting site on cell proliferation and senescence of camel ASCs

The cell growth kinetic data demonstrated that the biological characteristics and proliferation capacity of camel ASCs from abdominal and hump fat depot did not alter during expansion *in vitro*. However, further comparative analyses revealed a variation in proliferation rate between ASCs from abdomen and hump. In this regard, PDT calculated after successive sub-culturing of camel ASCs showed that ASCs from the abdomen had a greater ($P < 0.05$) time for proliferation than the hump-derived cells (Fig. 2). These results are in agreement with MTT assay results which showed a greater cell proliferation rate for hump ASCs during primary culture (Fig. 3).

Irrespective to their anatomical origins, the camel ASCs, after a number of passages stopped proliferating and entered senescence as determined by well-structured granulation, increased vacuolization, and typical flattened appearance. However, ASCs from abdominal fat depot entered senescence earlier ($P < 0.05$) than their hump counterparts (6.0 ± 1.3 days vs. 8.0 ± 1.1 days, respectively).

3.3. Karyotype analysis

Karyotyping was performed with the interval of every two passages until passage 8 to evaluate the chromosomal stability and unwanted transformation of ASCs following sequential *in vitro* subculture. The stem cells, irrespective of their harvesting site, retained a normal karyotype ($n = 74$), and no chromosomal instability was observed during multi passages.

3.4. Effect of fat harvesting site on multi-lineage differentiation potential of camel ASCs

3.4.1. Adipogenic differentiation evaluation

Eighteen days differentiation of ASCs from abdominal and hump fat depot was accompanied by an increased accumulation of lipid droplets, confirmed by a positive reaction of ASCs with ORO staining. No lipid deposition was observed in cytoplasm of undifferentiated control cells (data not shown). A qualitative comparison of lipid staining suggested that no differences existed in lipid accumulation in the cytoplasm of ASCs from abdominal fat depot (Fig. 4A) versus ASCs from hump fat depot (Fig. 4B).

3.4.2. Chondrogenic differentiation evaluation

The positive staining of cartilage matrix components demonstrated the ability of camel ASCs from abdominal and hump adipose tissue to differentiate into the chondrogenic lineage (Fig. 4C,D). No visible differences were observed when comparing samples from different anatomical origins; both types of samples revealed round

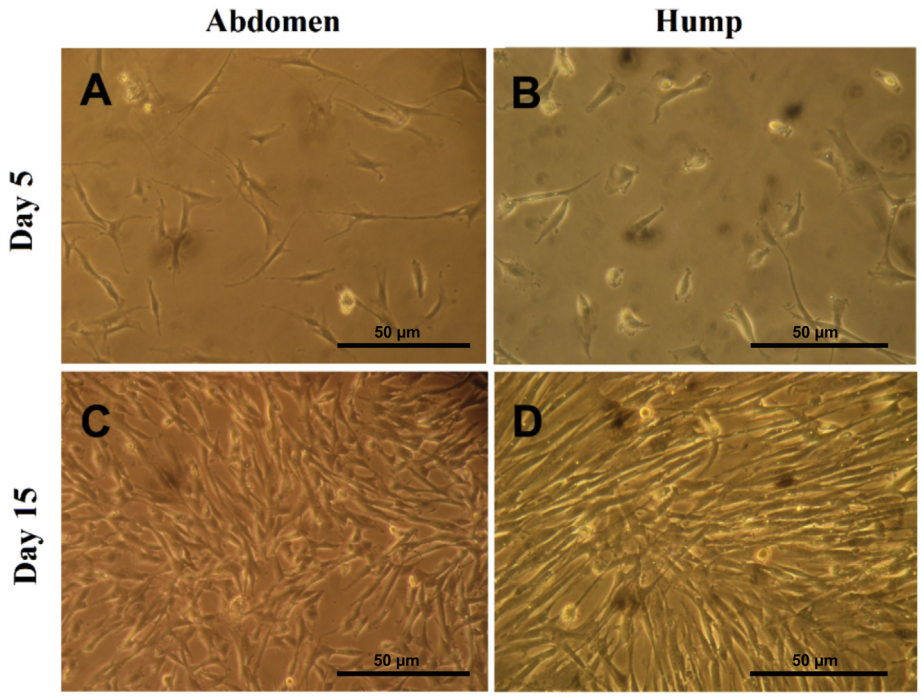


Fig. 1. Morphology of camel ASCs derived from abdomen (A and C) and hump (B and D) fat deposits at Day 5 and Day 15 phases of population growth, respectively.

cell morphology, low cell density and extensive cartilaginous matrix deposition.

3.4.3. Osteogenic differentiation evaluation

The osteogenic differentiation was first demonstrated as observed by Alizarin Red staining of the cells after 21 days of culture, which showed an increase of matrix mineralization along culturing time. Analysis of cells stained with H&E revealed no evident differences in terms of cellular morphology and matrix mineralization between camel ASCs obtained from abdominal (Fig. 4E) or hump (Fig. 4F) adipose tissue.

3.5. Alkaline phosphatase activity

The expression of ALP, as an early marker of osteogenesis, was measured at three time points during the period of study of induction of osteogenesis. Although the differentiated cells of hump-derived stem cells and abdominal-derived stem cells represented the same ALP activity at Day 7 of culture, a significant increase in ALP activity was observed in differentiated cells of hump-derived stem cells in comparison with those of abdominal-derived stem cells at Days 14 and 21 of culture (Fig. 5).

3.6. Calcium content

In contrast to the activity of ALP, which is assessed as an early marker of osteogenesis, mineralization can be considered as a late marker of osteogenesis. After seven days of culture of ASCs to induce them to differentiate into osteogenesis lineage, a significant increase of calcium deposition in both abdominal derived stem cells and hump derived stem cells was observed. The same pattern was observed after Days of 7, 14 and 21 days of culture. However, the increase in calcium content in the hump-derived stem cells was higher than that in the abdominal-derived stem cells (Fig. 6).

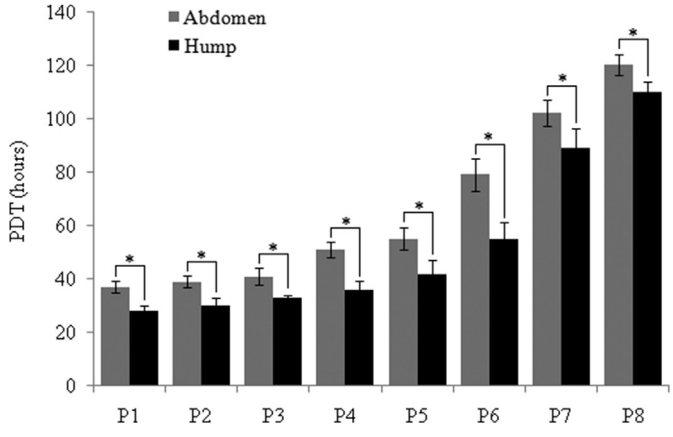


Fig. 2. Population doubling time (PDT) shows time required by camel ASCs derived from abdomen and hump fat deposits for cell proliferation.

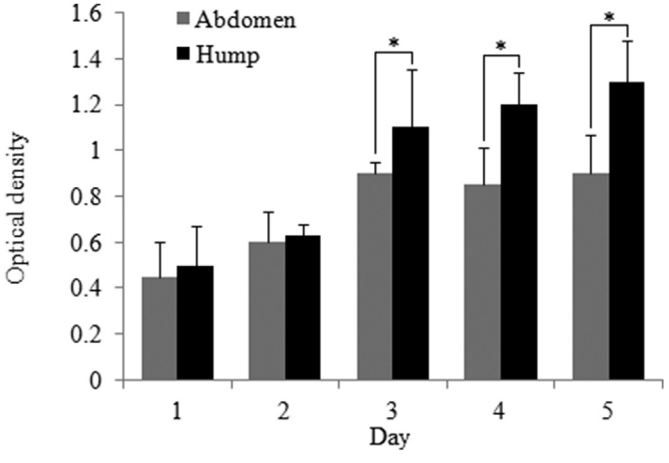


Fig. 3. The tetrazolium-based colorimetric assay (MTT test) shows cell viability of camel ASCs derived from abdomen and hump fat deposits.

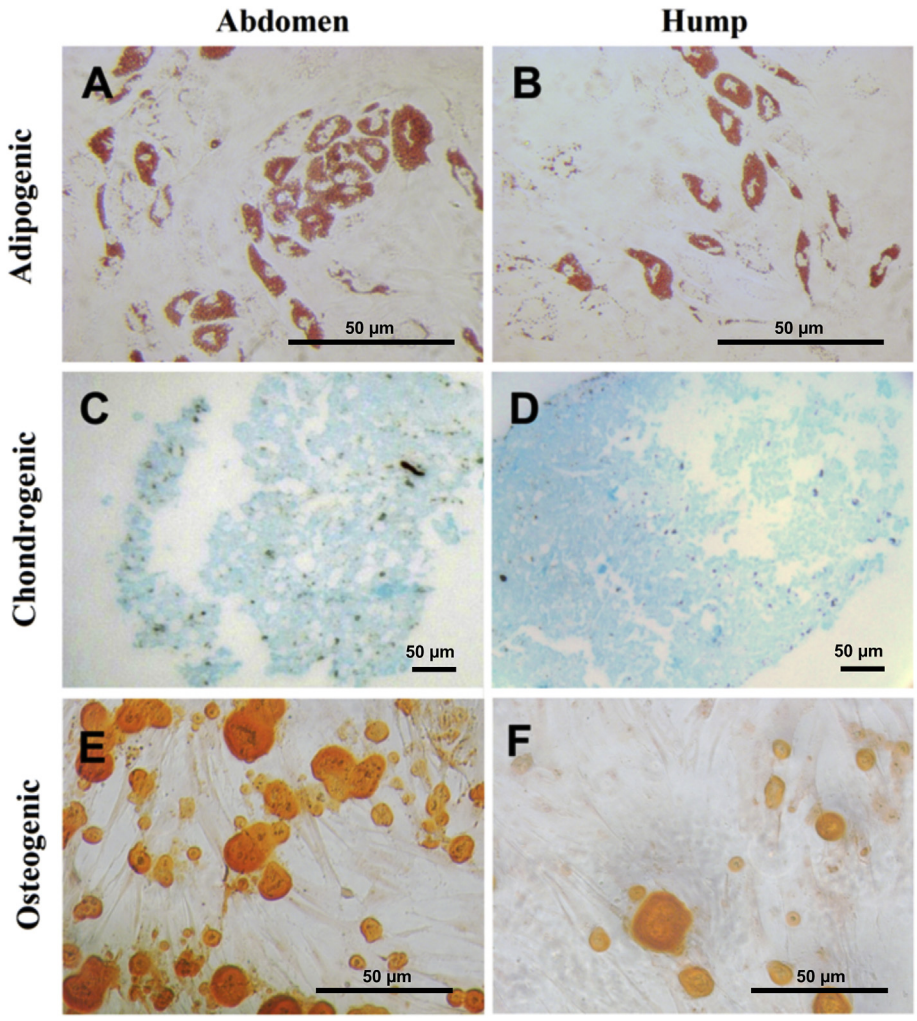


Fig. 4. Plasticity of camel ASCs in normal phase examined by differentiation of cells into particular lineages in the presence of appropriate induction media. Cells derived from both abdomen (A) and hump (B) had the potential to differentiate into adipocytes as assessed by ORO staining. Alizarin red S staining confirmed the differentiation of abdomen- (C) and hump- (D) derived stromal cells into the osteoblast. Alcian blue staining confirmed differentiation into chondrocytes derived from both abdomen (E) and hump (F) fat deposits.

4. Discussion

Most adult stem cells, such as MSCs, are minor populations found in adult organs and can differentiate into specific cell lineages. Much recent interest conducted on adult stem cells has focused on adipose or fat tissue because this tissue contains the highest concentration of adult stem cells [14,15]. Adipose tissue can be harvested in large amounts with minimal morbidity, and thus

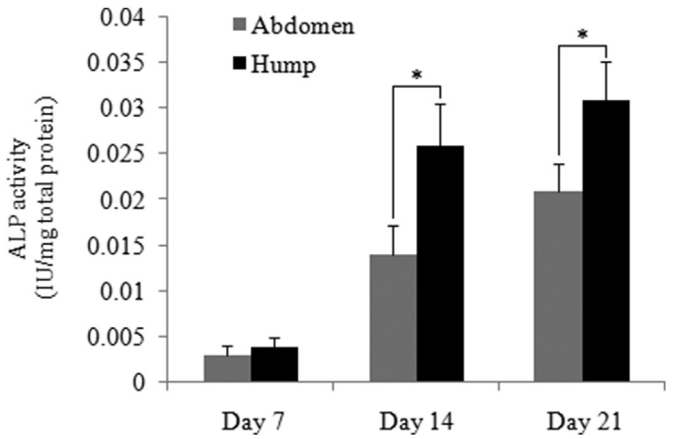


Fig. 5. ALP activity of camel ASCs derived from abdomen and hump fat deposits during 21 days of osteogenic differentiation.

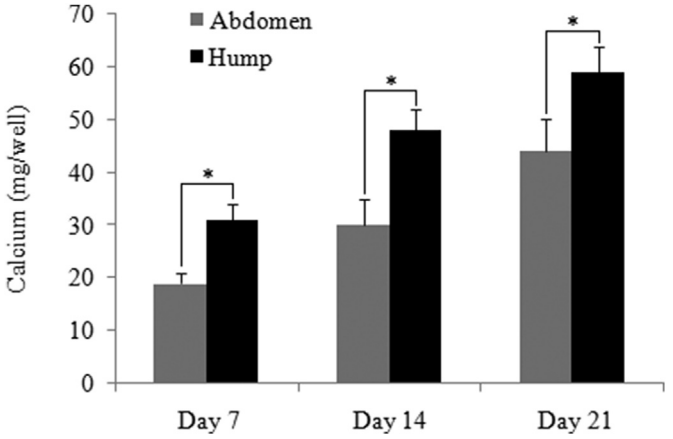


Fig. 6. Calcium content of camel ASCs derived from abdomen and hump fat deposits during 21 days of osteogenic differentiation.

constitutes a very convenient source of stromal stem cells with the capacity of multipotential differentiation [33].

Regions where adipose tissue is stored or concentrated are described as "adipose depots." There are several adipose depots all around the body that can potentially be used for isolation of ASCs. There is evidence that ASCs from different depots of adipose tissue might produce different characteristics [31,34,35]. From the viewpoint of the clinical application, it is of interest to know which adipose depot is the best candidate for isolation, as this would greatly enhance the efficiency of ASC recovery and differentiation in culture. No study except our previous report [29] has examined the expansion and differentiation potential of camel ASC; thus, the factors that affect proliferation and differentiation properties of camel ASCs are still unknown.

Therefore, initially, we characterized herein the behavior of ASCs obtained from abdominal and hump fat depots, assessing their morphology and cell proliferation rate, along successive passages, from passage 0 (P0) to passage 8 (P8). Our findings revealed that camel ASCs from both anatomical origins were successfully isolated and expanded with relative ease, simplicity, and speed. The ASCs from both abdominal and hump adipose depots demonstrated a spindle-shaped and fibroblast-like morphology, with the ASCs derived from hump fat depot being smaller in size and narrower in overall appearance than ASCs derived from abdominal fat depot. Although ASCs from both abdomen and hump had high proliferative ability over the time of culture, ASCs from hump represented a higher proliferative rate compared with abdominal ASCs, suggesting that the proliferating capacity of camel ASCs is influenced by the harvesting site. In other words, the stromal cells derived from abdomen fat required more time for proliferation than the cells derived from hump. Additional confirmation provided by MTT assay clearly demonstrated the differences in cell viability between the two adipose depots. Our results revealed that ASCs derived from hump had a greater cell proliferation rate during primary culture than those of abdominal-derived stromal cells. In a study [36] by Neupane et al. (2008), cell yield from subcutaneous fat was higher than cell yield from both omental and inguinal fat, and subcutaneous ASCs showed higher proliferation capacity compared to other adipose depots. Additional evidence is available concerning the factors affecting proliferation capacity of ASCs, which further supports the role of harvesting site as a determining factor influencing cell proliferation rate [36]. For instance [34], indicated that the abdomen seems to be preferable to the hip/thigh region for harvesting adipose tissue, in particular when considering stromal vascular fraction cells for stem-cell-based therapies in one-step surgical procedure for skeletal tissue engineering. These findings suggest that the capacity of hump adipose depots to proliferate might become evident when molecular events in adipocyte development are identified in camel species.

Additionally, we evaluated the potential of ASCs from different anatomical origins to differentiate into adipocytes, chondrocytes and osteoblasts. In this study, no significant differences were observed in multilineage differentiation potential of isolated ASCs from both abdomen and hump, as assessed qualitatively. In accordance with our findings [37], isolated and characterized MSCs from human raw oral fat tissue and subcutaneous fat and showed that buccal fat pad-derived ASCs had a similar phenotype and differentiation potentials in comparison with ASCs derived from abdominal subcutaneous fat tissue.

Cleavage of calcium phosphate groups in ALP causes an increase in mineralization of calcium phosphate cements producing a remarkable final osteogenic differentiation phenomenon of stem cells *in vitro*. Therefore, herein, critical osteogenic markers, i.e. the activity of ALP and the content of calcium, were investigated for further evaluation of osteogenic differentiation potential of these stem cells. Our data revealed a greater increase in ALP activity and calcium content in differentiated cells of hump-derived stem cells in comparison with those derived from abdominal stem cells.

Transformation is referred to as the change that a normal cell undergoes as it becomes malignant and is one of the limitations and challenges in stem cell therapy approaches. Herein, we performed karyotypic analysis after each passage to see the chromosome abnormalities. With respect to the fat harvesting site, cytogenetic analysis showed no abnormal karyotypes before the senescence stage was initiated. Our novel results revealed that camel ASC derived from both abdomen and hump can be successfully expanded and passaged to reach a sufficient amount of transferable cells, which would be applicable for cell therapy and tissue engineering in camel species.

In conclusion, the anatomical origin of the adipose tissue has an evident effect in the differentiation potential of the ASCs. Besides the anatomical origin, other factors such as, age, sex, isolation and conservation methods have been reported to have an effect on the biological behavior of these cells in humans and other species, but the effect of such factors in camel ASC behavior still remains unclear. Furthermore, studies are currently underway in the authors' laboratory to evaluate the osteogenic differentiation potential of the ASCs on different scaffold materials, with respect to cell response to these substrates *in vitro*.

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