Isolation, characterization and transduction of canine bone marrow-derived mesenchymal stem cells (cBM-MSCs)

Rezaei, M.[1], Jamshidi, Sh.[1], Saffarpour, A.[2], Ashouri, M.[3], Sharifi, D.[4], Zamankhan Malayeri, H.[1], Tamimi, N.[1], Rokn, A.R.[5*]

[1]*Department of Internal Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[2]*Department of Periodontology, International Campus School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran*

[3]*Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Shahed University of Medical Sciences, Tehran, Iran*

[4]*Department of Surgery and Radiology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran*

[5]*Dental Implant Research Center and Department of Periodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran*

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Correspondence
Rokn, A.R.
Dental Implant Research Center and Department of Periodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98(21) 22895324
Fax: +98(21) 66438327
Email: arrokn@yahoo.com

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Abstract:

BACKGROUND: Stem cell therapy in small animal medicine is still in its infancy and few in vitro and in vivo research projects regarding animal Mesenchymal Stem Cells (MSCs) have been carried out. On the other hand, Cell tracking is the first step of the cell-based therapies and is essential to recognize cell fate post transplantation. **OBJECTIVES:** The aim of this study was to isolate, characterize, and transduce cBM-MSCs. **METHODS:** Canine Bone Marrow-derived Mesenchymal Stem Cells (cBM-MSCs) were isolated from bone marrow of dogs and characterized based on morphology, differentiation capacities, and surface marker expressions. For the first time, we labeled cBM-MSCs by GFP-encoding lentiviral vector to track them. **RESULTS:** cBM-MSCs were successfully isolated and proliferated. Morphologically, these cells were similar to other MSCs from other sources and species and were able to differentiate into osteocytes and adipocytes. cBM-MSCs expressed surface marker CD44 but were not able to express CD34. Approximately, 70% of cells efficaciously expressed GFP after labeling; **CONCLUSIONS:** We found that GFP labeling is an easy and effective technique to track transplanted cBM-MSCs. Our results also provide fundamental information about canine BM-MSCs in order to use in veterinary medicine.

Introduction

Stem cell transplantation is a recent attractive therapeutic approach for tissue regeneration in both human and veterinary medicine (Aruna and Hemalata, 2010; Yen and Yelick, 2011; Marklein and Burdick, 2012). Various types of stem cells have been recognized to restore and regenerate lost tissues (Horst et al., 2012). In 1966, Friedenstein et al. isolated mesenchymal stem cells (MSCs) for the first time from bone marrow (Friedenstein et al., 1966). These stem cells were defined by three characteristics: plastic-adherence, expression of surface markers, and differentiation capacity into multiple mesenchymal lineages (Guo et al., 2011; Hodgkiss-Geere et al., 2012). MSCs are applied to enhance tissue regeneration with self-renewal, proliferative and immunomodulatory properties (Mitrano et al., 2010; Kisiel et al., 2012). Among multiple MSCs, bone

marrow-derived mesenchymal stem cells (BM-MSCs) have been introduced as easily accessible and suitable sources of adult MSCs for cell transplantation therapy (Caton et al., 2011; Fortier and Travis, 2011).

Stem cell therapy in veterinary medicine, especially small animal practice, is still in its infancy and few ex vivo and in vivo researches about animal MSCs have been carried out (Kisiel et al., 2012; Takemitsu et al., 2012). So, further studies are required to investigate the isolation, characterization (differentiation and identification of cell surface markers) and labeling of animal MSCs, especially canine MSCs. Moreover, many problems exist regarding stem cell therapies, such as poor understanding of basic information, distribution and cell fate after transplantation, and maintaining the desired number of cells (Horst et al., 2012). These points are key to the future of cell therapy. One approach to solve some of these issues is cell tracking. It is the first step of cell therapy (Yan et al., 2007) which is essential to recognize cell's fate, post transplantation. Various labeling techniques have been announced for cell tracking, among which labeling with green florescence protein (GFP) has frequently been used by researchers (Guo et al., 2011).

Despite the rapid progression in stem cell field and the considerable importance of stem cell therapy in small animal practice, few studies have been performed regarding cBM-MSCs basic information and tracking. The aim of the present study is to isolate cBM-MSCs and characterize them based on morphology, differentiation and surface marker expressions. Finally, this study aims at labeling the cultured cells by GFP for tracking BM-MSCs after transplantation.

Materials and Methods

Animal Model: Three intact, 1 year old, male, mongrel dogs weighing (20±5 kg) with apparently good general condition and appetite were used in this study. To select healthy animals, general clinical examination and complete blood count (CBC) measurement were done. Dogs were maintained two weeks before the beginning of the study for adapting to the keeping environment and taking Polyvalent and Rabies vaccines (Biocan®, Biovita, Czech Republic) plus antiparasitic drug (univerm, Larmrose, UK). The dogs were kept in a kennel and fed with maintenance traditional ration two times a day. The protocol of this study was approved by the Animal Care Committee of Veterinary college of University of Tehran.

Isolation and expansion of cBM-MSCs: Under general anesthesia using acepromazine (Alfasan, Holland, 0.05 mg/kg, IM) and ketamine (Alfasan, Holland, 20 mg/kg, IM), after scrubbing and creating small skin incision over the iliac crest of each animal, bone marrow (10 mL) was aspirated by sterilized Jamshidi needle (16G) into syringe containing 200 units of heparin (SalariSedigh et al., 2010). Bone marrow samples were immediately transported on ice to the reference cell culture laboratory. Culture of cBM-MSCs was performed as previously described. Briefly, dilution of aspirate was achieved with PBS (1:3). Then, separation of mononuclear cell fraction was carried out with density centrifugation over a Ficoll-Hypaque gradient (d=1.077g/mL, Pharmacia-Amersham, UK). The cells were cultured in high-glucose DMEM (Dulbecco's modified Eagle's medium; Gibco-BRL) supplemented with 10% FBS (Gibco-BRL, Grand Island, NY), and 2 mM L-glutamine (Gibco-BRL) and incubated in a humidified atmosphere consisting of 95% air with 5% CO_2 at 37^{o}C. After 1 week, the adherent cells (with 70%-80% confluence) were trypsinized, detached by 0.25% Trypsin-EDTA solution (Gibco-BRL) and sub-cultured in T75 tissue culture flask. Finally, trypan blue dye exclusion method was used for cell counting and viability measurement (SalariSedigh et al., 2010; Rahbarghazi et al., 2013).

For each animal, Ceftriaxone (Dana pharma co, Iran, 30 mg/kg q12h), metronidazole (Alborzdarou, Iran, 20 mg/kg q12h) and Tramadol (Alborzdarou, Iran, 2 mg/kg) were administered for three consecutive days, post-surgery.

Differentiation: Osteogenic differentiation was performed by osteo-inductive medium with supplementation of the cells with 10 mM beta-glycerol-phosphate (Merck), 50 µg/mL ascorbic acid bi-phosphate (Sigma), and 100 nM dexamethasone (Sigma) for three weeks. The medium was changed twice a week. Alizarin Red staining was used for evaluation of osteogenic differentiation capacity to find mineralized depositions. To investigate adipocyte

differentiation capacity, the authors treated cells with 250 nM dexamethasone and 0.5 mM 3-Isobutyl-1-methylxanthine (Sigma) for three weeks. Accumulation of oil droplets was assessed by Oil Red O staining (SalariSedigh et al., 2010).

Surface marker analysis: The third passage of cBM-MSCs was subjected to flow cytometry analysis with a panel of antibodies, including mouse anti-dog FITC: CD34 (10ul for 10^6 cells, catalog No.MCA2411F; Serotec, UK), mouse anti-dog CD44 (20µL for 10^6 cells, catalog No. ab95138; Abcam, UK) and mouse IgG1 (FITC) isotype control (10µL for 10^6 cells, Catalog No. ab106163; abcam, UK). In sum, passage 3 cells were trypsinized, harvested, and centrifuged at 3,500 rpm for 10 min at 4 °C. After twice washing by PBS, 1×10^6/mL, single cell suspension was collected in 15 mL conical tube. The cell suspension was blocked by 1% Bovine Serum Albumin (BSA) for 20 min at room temperature, then centrifuged 1500 rpm for 5 min at 4°C and supernants was discarded. After that, the primary labeled antibody was added and cell suspensions were incubated for 30 min at 4°C in the dark. Finally, the cells were washed 3 times by centrifugation at 1500 rpm for 5 minutes and re-suspended in 200 µL of ice cold paraformaldehyde (1%). Flow cytometry was performed by FACSCalibur (Becton Dikinson), and the output data was processed with WinMDI 2.8 software (Rahbarghazi et al., 2013).

Transduction of cBM-MSCs with GFP-encoding Lentiviral Vector: To label cBM-MSCs, cultured cells at 70-80% confluence were exposed to GFP-encoding lentiviral backbone vector, namely pLV-IRES-GFP. The cells were transduced with pLV-IRES-GFP at the multiplicity of infection in the presence of 5 mg/mL polybrene followed by a second transduction after 48h. Four days later, the cells were evaluated for expression of GFP using inverted fluorescent microscope (IX70, Olympus, Japan) (Gheisari et al., 2012).

Results

Isolation and expansion of cBM-MSCs: In the present study, isolation and expansion of cMSCs was performed on the bone marrow of three dogs. After 3 days of initial seeding, several colonies of adherent fibroblast-like cells were observed at the bottom of tissue culture flasks and became 80-100% confluent after 1 week. These cells were then sub-cultured and

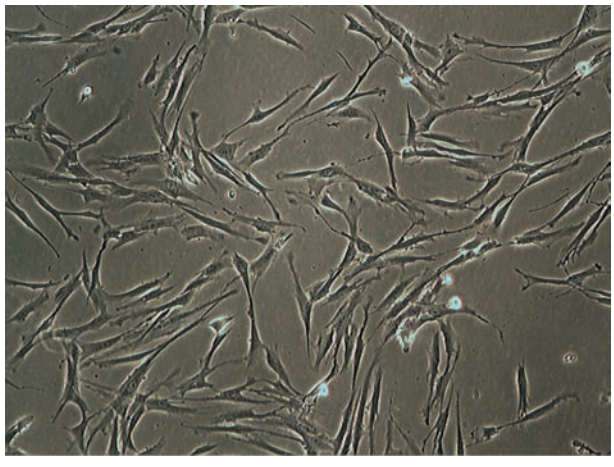

Figure 1. In vitro morphology of cBM-MSCs (passage 2) by Phase-contrast microscope.

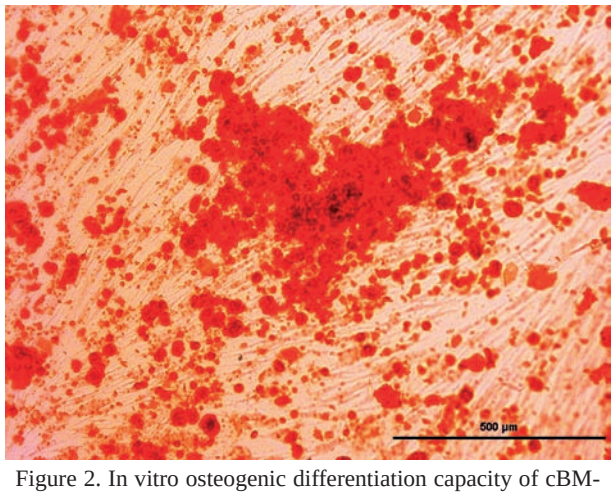

Figure 2. In vitro osteogenic differentiation capacity of cBM-MSCs by Alizarin Red staining, scale bar: 500 µm.

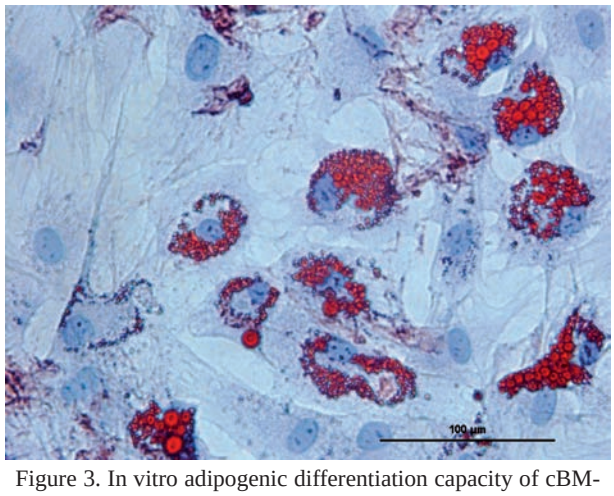

Figure 3. In vitro adipogenic differentiation capacity of cBM-MSCs by Oil Red O staining, scale bar: 100 µm.

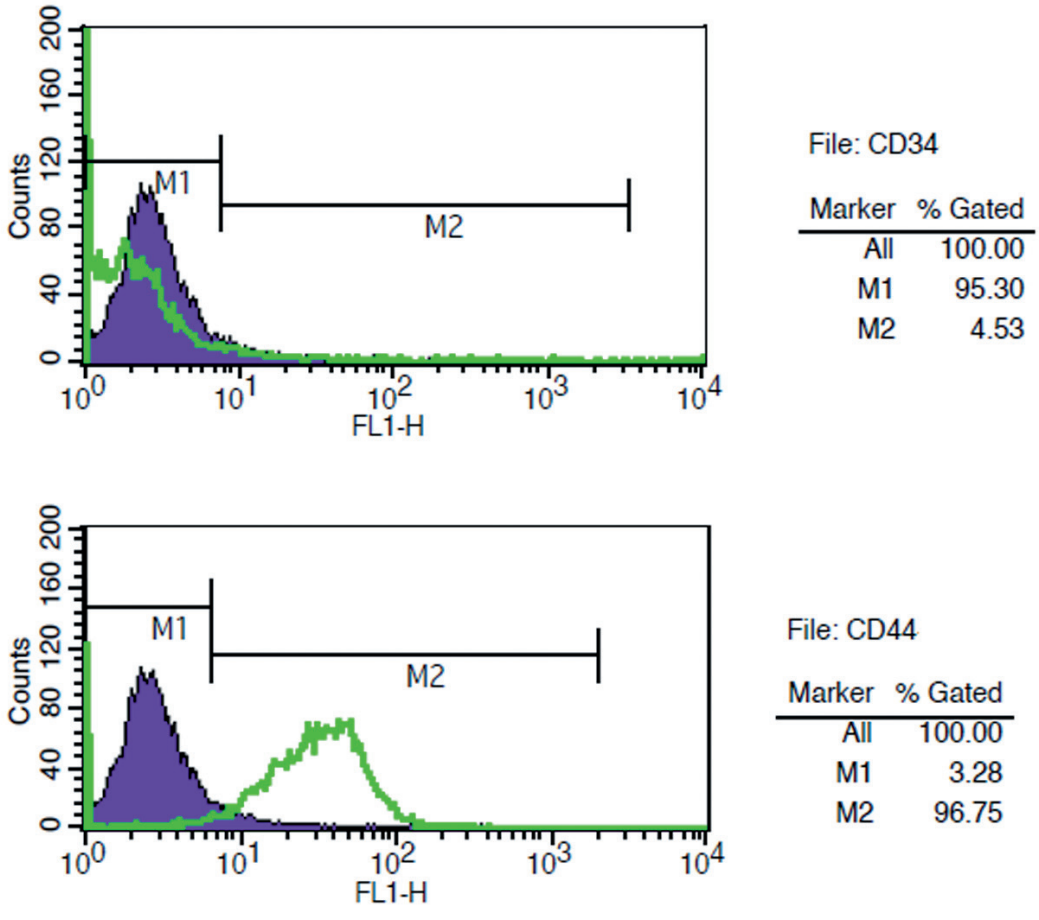

Figure 4. Histogram of flow cytometric analysis (The data demonstrated that cBM-MSCs were positive for CD44 but negative for CD34).

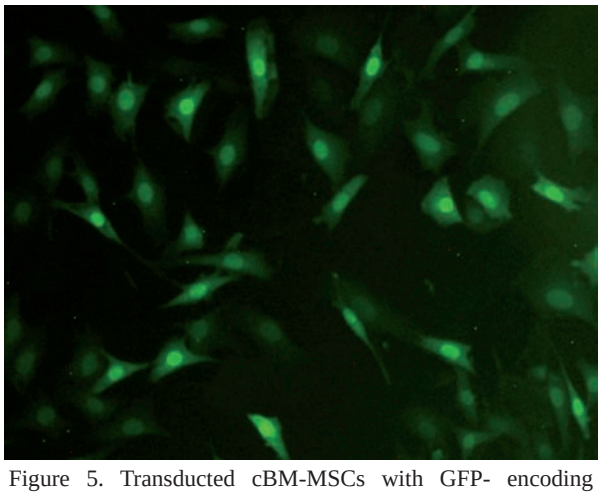

Figure 5. Transducted cBM-MSCs with GFP- encoding lentiviral vector (passage 2).

by high rate proliferation, passage 3 of cBM-MSCs was achieved after 21 days. As shown in Figure 1, morphology of cBM-MSCs was similar to other MSCs with regard to being spindle-shaped, triangular, and elongated. Cultured cells showed 100% viability by trypan blue dye exclusion method.

Differentiation potential: In vitro osteogenic, differentiation capacity of cultured cBM-MSCs was evaluated by Alizarin Red staining, and after the incubation period; existence of mineralized deposits was confirmed (Figure 2). Adipogenic induction of cBM-MSCs was evaluated with Oil Red O staining and accumulation of cytoplasmic oil droplets was confirmed in induced cells (Figure 3).

Surface marker analysis: Furthermore, in vitro immunophenotype, characterization of cBM-MSCs was analyzed by detection of expressed cell surface CD markers with flow cytometry. Based on the flow cytometry results, isolated cells were positive for cell surface antigen CD44 and negative for hematopoietic markers CD34. cBM-MSCs showed high expression of CD44 (Figure 4).

Transduction of cBM-MSCs with GFP- encoding lentiviral vector: In this study, transduction of cBM-MSCs was done by GFP-encoding lentiviral backbone vector, namely pLV-IRES-GFP. Transduction was successfully performed with high

efficiency (70%) and low toxicity. Using an inverted fluorescent microscope, cBM-MSCs were observed as green florescent after 4 days and reserved their characteristic until transplantation (Figure 5). The morphologic and biologic characterizations of cells did not change after transduction,; however, the number of GFP positive cells was reduced by passaging (approximately 5%).

Discussion

Cells are the key factors in tissue regeneration. Therefore, recruitment of precursor cells is considered one of the critical events in successful healing (Zhang et al., 2007; Chen et al., 2010; Hughes et al., 2010). Despite growing interests in stem cell therapies, many issues still remain to be elucidated before clinical applications (Caton et al., 2011; Yoshida et al., 2012). Basic information about stem cells, their best sources, isolation, characterization, and enrichment are some of the major obstacles in the field of stem cell research (Takemitsu et al., 2012), still being investigated to optimize cell-based therapies. For this purpose, the present study was designed, and we successfully isolated, characterized, and labeled cBM-MSCs.

In general, the exact role of stem cells in regeneration is not fully illustrated (Czernik et al., 2013). The importance of such studies depends on successful isolation, in vitro expansion, and manipulation of stem cells for in vivo transplantation in therapeutic approaches. In the present study, isolation and expansion of cBM-MSCs were successfully carried out from bone marrow of dogs. Cultured cells were comparable to MSCs of other species. Therefore, isolated stem cells were defined by three characteristics: plastic-adherence, expression of surface markers, and differentiation capacity into multiple mesenchymal lineages (Kisiel et al., 2012). Morphology of isolated cells resembled MSCs of other sources or species such as canine periodontal stem cells (SalariSedigh et al., 2010) or human MSCs (Shim et al., 2004), which are spindle-shaped, triangular, or elongated. We observed clonogenicity and morphologic heterogenicity in the primary passage of cells. Isolated cBM-MSCs showed a high rate of proliferation in the present study.

Moreover, we confirmed in vitro differentiation abilities of cBM-MSCs into osteocytes and adipocytes as reported regarding canine BM-MSCs (Hodgkiss-Geere et al., 2012; Kisiel et al., 2012; Takemitsu et al., 2012). In addition, the investigations demonstrated that BM-MSCs of other species could differentiate to adipocyte and osteoblast (Czernik et al., 2013). In the present study, adipogenic induction of cBM-MSCs was done in a 3-week period compared with a 2-week period in human MSCs (Hodgkiss-Geere et al., 2012; Kisiel et al., 2012).

To investigate in vitro immunophenotype characterization of MSCs, a range of surface marker expressions have been evaluated. It is positive for CD29, CD44 and CD90 and negative for CD34 and CD45 in human and dogs (Hodgkiss-Geere et al., 2012; Kisiel et al., 2012; Takemitsu et al., 2012). In the present study which used a standardized technique, immunophenotype characterization of cBM-MSCs was also performed based on surface marker expression. In accordance with other canine MSCs studies (Hodgkiss-Geere et al., 2012; Kisiel et al., 2012; Takemitsu et al., 2012), our results showed that these cells were CD44 positive and CD34 negative. Our findings are also comparable to human MSCs (Gronthos et al., 1994). Furthermore, in line with the results of Takemitsu et al. (2012) high expression of CD44 was considerable in our study (more than 95% of isolated cells).

Although investigating stem cell therapies have contributed to a rapid progress, much more research should be done about the fate and distribution of transplanted cells. It is also important to determine safety and efficacy of therapeutic approaches. Understanding the stem cell's fate after transplantation is achieved by cell tracking. Finding a non-invasive method to track transplanted stem cells is an important step that should be taken before stem cell researches (Yan et al., 2007). The current labeling approaches to track MSCs include 5-bromo-2'-deoxyuridine (BrdU), fluorescent dye, green fluorescent protein (GFP), magnetic, and isotope labeling techniques (Yan et al., 2007). Each method has its strengths and limitations. BrdU and fluorescent dye gradually disappear and cannot be detected after a long period. The limitation of MRI and labeling techniques are false positive results (Guo et al., 2011).

Among these methods, GFP labeling is a safe and simple technique for in vivo cell tracking. This

method enables us to monitor labeled cells and proteins non-invasively (Xu et al., 2010). Because of its biocompatible properties, this method is frequently applied by researchers (Guo et al., 2011). Detection of labeled cells in tissues has been done with high sensitivity and specificity by immunofluorescence and immunohistochemistry methods (Guo et al., 2011). Nevertheless, high levels of GFP result in cell toxicity (Yan et al., 2007). Another disadvantage of this method is gradual loss of GFP expression because of the migration, differentiation, and death of the cells. Consequently, precise time of post transplantation tracking still remains unknown and should be investigated later (Guo et al., 2011).

For the first time, we efficaciously labeled cBM-MSCs by GFP-encoding lentiviral vector to track them. Using an inverted fluorescent microscope, we observed green-florescent labeled cBM-MSCs after 4 days, and their characteristics reserved until transplantation. In comparison with the results of Czernik et al. 2007 who could transduce 46.6% of BM-MSCs, in this study, we were able to transduce approximately 70%. The characterizations of cells did not morphologically and biologically change after transduction, but passaging reduced the number of GFP-positive cells. We found this technique easy and effective.

Finally, it should be noted that the application of stem cells in veterinary medicine, alongside its importance and great potential, is controversial and remains to be investigated further.

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جداسازی، تمایز و نشاندارسازی سلول‌های بنیادی مزانشیمی مغز استخوان سگ

مهدیه رضائی[۱] شهرام جمشیدی[۱] آنا صفارپور[۲] مهدی عاشوری[۳] داوودشریفی[۴] حامد زمان خان ملایری[۱] نقاء تمیمی[۱] امیر رضا رکن[۵*]

۱) گروه بیماریهای داخلی، دانشکدهٔ دامپزشکی دانشگاه تهران، تهران، ایران
۲) گروه پریودنتولوژی، پردیس بین المللی دندانپزشکی دانشگاه علوم پزشکی تهران، تهران، ایران
۳) گروه آسیب شناسی دهان و فک و صورت، دانشکدهٔ دندانپزشکی دانشگاه شاهد، تهران، ایران
۴) گروه رادیولوژی و جراحی، دانشکدهٔ دامپزشکی دانشگاه تهران، تهران، ایران
۵) گروه پریودنتولوژی، دانشکدهٔ دندانپزشکی دانشگاه علوم پزشکی تهران، تهران، ایران

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چکیده

زمینه مطالعه: سلول درمانی با استفاده از سلول های بنیادی در طب دام های کوچک، در مراحل اولیه خود قرار دارد و تعداد اندکی تحقیقات درشرایط آزمایشگاهی و یا در بدن موجود زنده در رابطه با سلول های بنیادی مزانشیمی سگ انجام گرفته است. از طرفی دیگر، ردیابی سلول ها، اولین قدم در درمان های مبتنی بر سلول می باشد. این کار به منظور تشخیص سرنوشت سلول های پیوند داده شده در بدن موجود زنده ضروری است. **هدف:** هدف از این مطالعه، جداسازی، تمایز و نشاندارسازی سلول های بنیادی مزانشیمی مغز استخوان سگ بود. **روش کار:** سلول های بنیادی مزانشیمی از مغز استخوان سگ جداسازی و بر مبنای ریخت شناسی، قابلیت تمایز و بیان فاکتورهای سطحی متمایز گردیدند. برای اولین بار، ما توانستیم سلول های بنیادی مزانشیمی مغز استخوان سگ را به کمک وکتور لنتی ویروسی کدکنندهٔ پروتئین سبز فلورسانت به منظور ردیابی این سلول ها در بدن، نشاندار کنیم. **نتایج:** در مطالعهٔ حاضر سلول های مزانشیمی مغز استخوان سگ به طور موفقیت آمیزی جداسازی گردیده و تکثیر یافتند. بر اساس ریخت شناسی، این سلول ها با دیگر سلول های مزانشیمی جدا شده از سایر بافت ها و دیگر گونه ها مشابهت داشتند و دارای قابلیت تمایز به استئوسیت و آدیپوسیت بودند. سلول های بنیادی مزانشیمی مغز استخوان سگ، مارکر سطحی CD44 را بیان کردند، اما نتوانستند مارکر CD34 را بیان نمایند. به دنبال نشاندارسازی، تقریباً ۷۰٪ سلول ها با پروتئین سبز فلورسانت نشاندار شدند. **نتیجه گیری نهایی:** بنابراین روش نشاندارسازی با پروتئین سبز فلورسانت تکنیکی آسان و مؤثر جهت ردیابی سلول های بنیادی مزانشیمی مغز استخوان سگ به دنبال پیوند می باشد. همچنین نتایج این مطالعه اطلاعات پایه در رابطه با سلول های بنیادی مزانشیمی مغز استخوان سگ را به منظور استفاده از این سلول ها در دامپزشکی ارائه می دهد.

واژه های کلیدی: سلول های بنیادی مزانشیمی مغز استخوان، تمایز، نشاندارکردن، پروتئین سبز فلورسانت

*)نویسنده مسؤول: تلفن: ۲۲۸۹۵۳۲۴ (۲۱) ۹۸+ نمابر: ۶۶۴۳۸۳۲۷ (۲۱) ۹۸+ *Email: arrokn@yahoo.com*