Short Communication

Cloning Efficiency of Canine Mesenchymal Stem cells Isolated from Bone Marrow of Femoral Head and Subcutaneous Adipose Tissue

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Abstract

Most recently, adipose tissue (AD) has become an alternative source for mesenchymal stem cells (MSCs) instead of the invasive method of bone marrow (BM) aspiration both in human and canine. In this study, we compared MSCs derived from adipose tissue (AD-MSCs) and bone marrow (BM-MSCs) regarding morphology and cell yield for instant usage by using the standard protocol of counting colony forming unit-fibroblast (CFU-F). MSCs from both sources showed fibroblast-like morphology and formed colonies termed as CFU-F after culturing in plastic surface for 10 days. The colony number per mononuclear cells (MNCs) and the colony number per adherent cells derived from AD were significantly higher than those derived from BM. Our study suggested that AD not only was a suitable source to harvest and but also had a higher performance of clonal efficiency of MSCs than BM. Thus, subcutaneous AD might be an appropriated source for stem cells therapy in canine.

Keywords: canine, colony forming unit-fibroblast, mesenchymal stem cells

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บทคัดย่อ

การศึกษาความสามารถในการสร้างโคโลนีของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ ที่เก็บจากไขกระดูกของหัวกระดูกต้นขาหลังและเนื้อเยื่อไขมันใต้ผิวหนังของสุนัข

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ปัจจุบันเนื้อเยื่อไขมันเป็นแหล่งของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่น่าสนใจแหล่งหนึ่ง นอกเหนือไปจากแหล่งไขกระดูก ซึ่งการเจาะดูดไขกระดูกเป็นวิธีที่ทำให้เกิดความเจ็บปวดและเสี่ยงต่อการติดเชื้อแทรกซ้อนได้ งานวิจัยนี้มุ่งเน้นการหาแหล่งของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่เหมาะสมในร่างกายโดยใช้เซลล์ของสุนัขเป็นแม่แบบ ศึกษาเปรียบเทียบคุณลักษณะของเซลล์และความหนาแน่นของเซลล์จากความสามารถในการสร้างโคโลนีของเซลล์ต้นกำเนิดชนิดมีเซนไคม์ จาก 2 แหล่งคือ ไขกระดูกและเนื้อเยื่อไขมันใต้ผิวหนัง พบว่าเซลล์ต้นกำเนิดชนิดมีเซนไคม์จากทั้ง 2 แหล่งสามารถเกาะติดกับพื้นผิวพลาสติก มีรูปร่างคล้ายกระสวยและมีคุณสมบัติในการสร้างโคโลนีได้ในระยะเวลาประมาณ 10 วันของการเพาะเลี้ยง สัดส่วนจำนวนโคโลนีต่อจำนวนเซลล์นิวเคลียสเดี่ยวและสัดส่วนจำนวนโคโลนีต่อจำนวนเซลล์ที่เกาะติดพื้นผิวพลาสติกของเซลล์ที่เก็บจากเนื้อเยื่อไขมันมีสัดส่วนที่มากกว่าเซลล์ที่เก็บจากไขกระดูกอย่างมีนัยสำคัญ จากงานวิจัยนี้จึงสรุปว่าเนื้อเยื่อไขมันเป็นแหล่งที่เหมาะสมสำหรับการเก็บเซลล์ต้นกำเนิดชนิดมีเซนไคม์เพื่อนำไปใช้ประโยชน์ทางการแพทย์ต่อไปในอนาคตและเซลล์ต้นกำเนิดที่ได้จากแหล่งนี้ยังสามารถเพิ่มจำนวนได้เร็วกว่าเซลล์ต้นกำเนิดที่เก็บได้จากแหล่งไขกระดูกอีกด้วย

คำสำคัญ: สุนัข การสร้างโคโลนี เซลล์ต้นกำเนิดชนิดมีเซนไคม์

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Introduction

MSCs are defined as multipotential non-haematopoietic stem cells which have an ability to differentiate into mesenchymal lineages such as osteoblasts, chondrocytes (Kadiyala et al., 1997), tendinocytes, myocytes, adipocytes, and also cells of non-mesenchymal origin such as neuronal progenitors (Kamishina et al., 2008; Lim et al., 2010) in vitro and in vivo under appropriate conditions (Awad et al., 1999; Ouyang et al., 2003; Ge et al., 2005; Grogan et al., 2009). With that great capacity, MSCs have become the most interesting object for regenerative therapy of both human and veterinary medicine since their first description in 1976. MSCs appear *in vi*tro as spindle shaped cells or fibroblast-like morphology termed as CFU-F (Friedenstein et al., 1976). Based on studies in human and laboratory animals MSCs are identified in tissues by an expression of a group of specific markers including Stro-1, CD105/endoglin (transforming growth factor receptor III) and CD90/Thy-1 and lacking of hematopoietic (CD34 and CD45) and endothelial surface antigen (CD144) (Kern et al., 2006; Dvorakova et al., 2008).

Many MSCs sources were identified in human and laboratory animals. The investigations into MSCs tissue sources in companion animals also showed that BM (Martin et al., 2002; Csaki et al., 2007), blood (Koerner et al., 2006), amniotic fluid (Filioli Uranio et al., 2011), umbilical cord blood (Seo et al., 2009), and subcutaneous AD (Neupane et al., 2008) were readily available for MSCs. The accessibility and availability of MSCs tissue sources are major factors that have to be considered. For MSCs harvesting, both BM and AD are very attractive although differences in collecting techniques of MSCs from both tissue sources in patients are obvious. BM is collected by BM aspiration. With invasive and increased risk of infection, this procedure has to be concerned. AD may be a more attractive source from its ease of access. Since femoral head and adipose tissue have been known to be common by-products of surgical procedures in canine patients, the evaluation of these tissues as candidate sources was required in order to establish MSCs *in vitro* production and stem cells banking. We hypothesized the difference in capability of canine MSCs derived from two sources including BM and subcutaneous AD to expand *in vitro*. The objective of this study was to compare clonal expansion of canine MSCs from these sources.

Materials and Methods

Animals: Ten dogs, aged between 2-5 years and weighing between 3-10 kg, were used. All dogs were selected from patients that required the femoral head and neck excision from orthopedic problems including hip luxation and hip dysplasia in the Veterinary Teaching Hospital, Kasetsart University. Prior to the operation, all dogs got generalized anesthesia. Femoral heads and subcutaneous AD samples from each dog were collected aseptic technique.

MNC isolation and CFU-F assay: Each femoral head and subcutaneous AD sample was collected from the same dog. AD was excised at hip region. The tissue was minced and digested for 1 hour at 37^0C with collagenase type I (1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) and shook every 15 min. AD was removed by filtering through the Steriflip Unit (Millipore Corporation, Billerica, MA, USA). The cell suspension was then added to Minimum Essential Medium Alpha (α-MEM, Gibco, Invitrogen, Carlsbad, Calif., USA) supplemented with 20% heat-inactivated fetal bovine serum (FBS) and 1% v/v penicillin/streptomycin at an equal volume to stop the reaction of collagenase type I and then centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was discarded. MNCs pellet was added with 1 ml of α-MEM supplement with 20% heat-inactivated FBS and 1% v/v penicillin/streptomycin and mixed well.

BM was flushed from femoral head with 100mM phosphate buffer saline (PBS) under sterile condition. Subsequently, MNCs were isolated from the BM cell suspension by density gradient centrifugation using Ficoll-Paque plus® (d = 1.077 g/cm^3, GE Bioscience, Westborough, MA, USA) at an equal volume. The suspension was centrifuged at 1500 rpm at 4^0C for 30 min and middle layer of MNCs was collected into 10 ml PBS with 1% v/v penicillin/streptomycin and mixed well. The cells were washed by centrifugation at 3500 rpm for 10 min twice and then the supernatant was discarded. The α-MEM supplemented with 20% heat-inactivated FBS and 1% v/v penicillin/streptomycin was added. The MNC yield from both AD and BM was determined using a hemocytometer under a light microscope.

MNCs from BM and subcutaneous AD were plated at 10^6 and 10^4 cells/100-cm^2 dish relatively in triplicate. The cell cultures were incubated at 37^0C with 5% CO_2 and maintained for 10 days with medium exchange every 3 days. After 24 hours of incubation every dish was washed twice with PBS to remove non-adherent cells and a number of adherent cells were counted at a magnification of x100 under a microscope (Kern et al., 2006; Yoshimura et al., 2007). At 10 days after initial plating, the medium was discarded and cells were washed gently twice with PBS. The cells were fixed with fresh 4% paraformaldehyde in PBS for 20 min at room temperature. Subsequently, the cells were stained with 0.5% crystal violet in methanol for 5 min and washed twice with distilled water. The visibly intensely stained colonies and colonies which diameter was more than 2 mm were counted. Then, the colony number per MNCs and the colony number per adherent cells were evaluated (modified from Kern et al. (2006) and Yoshimura et al. (2007)).

Statistical Analysis: Data are presented as mean±standard deviation. A paired t test was used to compare the colony number per MNCs and colony number per adherent cells between BM and AD. Differences were considered significant at $p < 0.05$.

Results and Discussion

In this study, we compared the colony forming ability of canine MSCs derived from two sources, BM and subcutaneous AD, of the same donor to prevent the variation from different donors by using the CFU-F assay (Friedenstein et al., 1976; Murphy et al., 2002; Rojewski et al., 2008; Stolzing et al., 2008). The CFU-F assay is a simple method to characterize MSCs and perform colony forming capacity (Dominici et al., 2006; Alt et al., 2011). To demonstrate clonal expansion capacity between BM-MSCs and subcutaneous AD-MSCs, the MNCs were plated at low density. Single cells from both sources appeared as round cell attached to plastic plate surface within 24 hours after initial plating (Fig 1). Heterogeneity by cell size of the MSCs population was observed. Some adherent cells became fibroblast-like or spindle-shaped cells after 3-5 days in culture for AD cells and 5-7 days for BM (Fig 2). Adherent

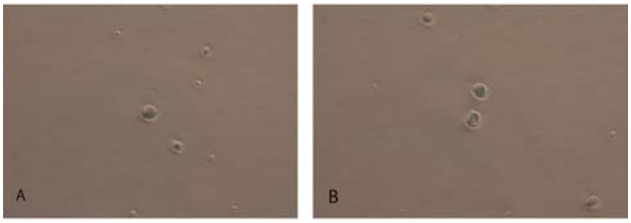

Figure 1 Adherent MNCs at day 1 post-plating. Cells derived from both BM (A) and AD (B) showed similar morphology with heterogeneity in cell size; x400.

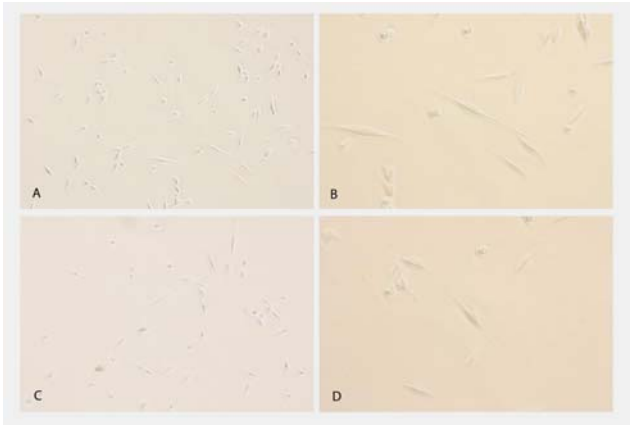

Figure 2 MSCs obtained from BM (A, B) and AD (C, D). The morphology of spindle-shaped cells was observed from both sources at day 5 post-plating; x200 (A, C) and x400 (B, D) relatively.

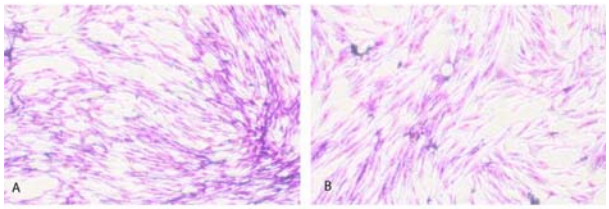

Figure 3 Spindle-shaped morphology of BM-MSCs (A) and AD-MSCs (B) at day 10.

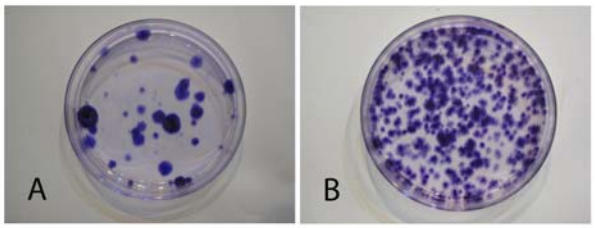

Figure 4 Colony formation of MSCs: (A) MNCs derived from BM were plated at 10^6 cells/100-cm^2 dish and (B) those derived from AD which plating at 10^4 cells/100-cm^2 dish. Both were cultured for 10 days and then were observed the colony number by stained with 0.5% crystal violet in methanol.

cells from both cell sources exhibited the same morphology similar to other adult-derived tissue source of MSCs from many species (Musina et al., 2005; Csaki et al., 2007; Dimitrov et al., 2008; Neupane et al., 2008). About 5-7 days the spindle-shaped cells produced large colonies which indicated clonal expansion, a characteristic of stem cells. Our study found that subcutaneous AD-MSCs formed colony faster than BM-MSCs. The colony formation was found within 5-7 days after the initial plating for adipose tissue and 10-14 days for bone marrow (Fig 3 and Fig 4). However, some replicates from bone marrow derived MNCs did not form colonies. These concluded that the colony formation rate of canine BM-MSCs was lower than subcutaneous AD-MSCs similar to a study in rat (Yoshimura et al., 2007).

Because each colony is generated from a single cell, according to our study, we found that the colony number per MNCs and colony number per adherent cells of AD-MSCs were higher than BM-MSCs significantly. The colony number per 106 MNCs of AD and BM showed about 8200±5731.98 and 14.8±13.68 (mean±SD; $p < 0.05$), respectively (Fig 5). The colony number per 100 adherent cells of AD and BM showed 37.05±25.78 and 16.31±15.79 (mean±SD; $p < 0.05$) (Fig 6). These indicated that subcutaneous AD source had a higher initial MSC yield than BM source from the same canine donor, similar to another study in human (Musina et al., 2005). Moreover, the study in both human and rat also found that the cells collected from BM had a colony number per nucleated cell numbers lower than which collected from the other mesenchymal tissue. In human, it was indicated that a cell number per colony was much higher in BM than in other mesenchymal tissues (Sakaguchi et al., 2005). In rat, on the contrary, it was found that a cell number per colony from BM source was lower than in the other mesenchymal tissues (Yoshimura et al., 2007). Nevertheless, our

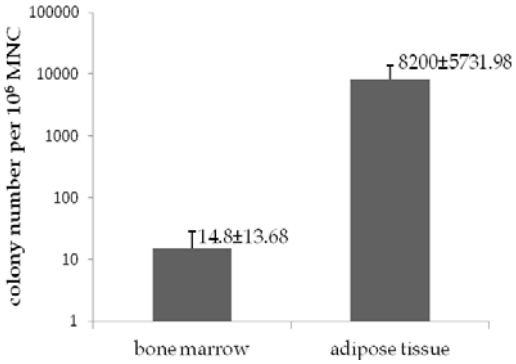

Figure 5 The colony number per 10^6 MNCs; the initial 10^6 BM-MNCs and 10^4 AD-MNCs were cultured in 100-cm^2 dishes for 10 day and stained with crystal violet to count the colonies. The colony number per 10^6 MNCs was evaluated from both sources. Data was showed as means±SD (n = 10).

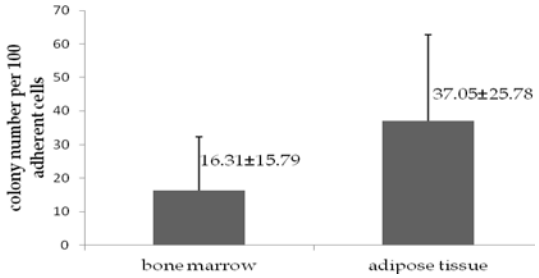

Figure 6 The colony numbers per 100 adherent cells; the adherent cells were counted after 1 day plating. The cell colonies were counted at day 10 post-plating. The colony numbers per adherent cells was evaluated and compared between BM and AD sources. Data was showed as means±SD; $p < 0.05$ (n = 10).

study did not demonstrate this data although the difference in sizes of CFU-F was noticed between BM-MSCs and AD-MSCs (Fig 4).

The ideal techniques to identify MSCs in tissue sources are Magnetic Activated Cell Sorting (MACS®) and Fluorescent Activating Cell Sorting (FACS) (Raynaud et al., 2012). However, limitation of these techniques is that there are no specific markers for MSCs. The clonal expansion ability is the technique that has been used to characterize MSCs in various species. The ability is not only unique to the stem cells but also provides the crucial information for in vitro expansion and clinical application for cell therapy (Alt et al., 2011).

Finally, we can conclude that compared with BM-MSCs, AD-MSCs have great advantages for cell preparation and instant clinical purposes in canine due to their ease of access, higher MSC yields and higher expansion rate. However, there are other sources which are also interesting. For example, synovium was indicated as a source of high proliferation and differentiation potential of MSCs in human and rat (De Bari et al., 2003; Sakaguchi et al., 2005; Yoshimura et al., 2007; Ju et al., 2008). In addition, umbilical cord blood derived MSC which provided a non-invasive procedure was well-known reported in human, equine (Kern et al., 2006; Koch et

al., 2007; Schuh et al., 2009; Toupadakis et al., 2010) and canine (Seo et al., 2009).

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