Original Article

Comparison of isolation techniques for bone marrow derived canine mesenchymal stem cells (MSCs) and the compatibility of MSCs loaded onto polycarpolactone hydroxyapatite

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Abstract

Bone marrow was collected from 7 dogs and submitted to 3 different MSC isolation techniques (direct plating, red blood cell lysis and gradient density). The number of cells, expression of MSC markers and *in vitro* the osteogenic differentiation obtained from each technique were examined. In order to study *in vivo* the osteogenic capability of derived MSCs, non-union ulna lesions (n=3) were firstly induced by transplantation of a composite of polycarprolactone/ hydroxyapatite (PCL/HAp) scaffold experimentally on to a critical-size ulna bone defect. The MSCs were injected into the lesions. The non-union sites were examined by radiography, angiography and histology at 2, 4, 6, 8 and 12 weeks after MSC injection. Gradient density and RBC lysis techniques yielded higher numbers of putative MSCs on day 7 of culture compared with the direct plating technique. A large proportion of isolated MSCs, irrespective of the isolation techniques, expressed all MSC markers (CD 44 and CD 90). For all MSC transplanted dogs, neither radiological changes at scaffold-ulna interface nor callus formation was observed, although all donors used demonstrated *in vitro* osteogenesis. At 16 weeks after MSC injection, the angiogram indicated increased neovascularization. This was confirmed by the histological finding that there was an improvement of vascularization within the thick-fibrous tissue surrounding the scaffold. Gradient density and RBC lysis treatment are suitable MSC isolation techniques, in terms of the numbers of cells obtained and also their MSC properties. However, potential use of these MSCs following injection to a non-union bone site was compromised possibly because of a lack of osteogenic stimulation.

Keywords: bone marrow, dogs, mesenchymal stem cells, non-union bone, polycarprolactone/ hydroxyapatite (PCL/HAp) scaffold

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Introduction

In dogs, incidence of non-union is 3.4% and the most common site (40-60%) is the radius and/or ulna (Bartels, 1987). The etiology of non-union involves multiple factors including poor blood supply to the fracture site, poor apposition of the fracture end, fracture motion, large fracture gap, pathologic fracture, a foreign body, necrotic bone, infection and non-justified corticosteroid therapy (Vertenten et al., 2010; Calori et al., 2011). Autologous bone graft is the gold standard for non-union treatment leading to bone union through osteogenesis, osteoconduction and osteoinduction. However, it causes a high risk of fracture and infection, pain at the donor site and limited cancellous bone (Lee et al., 2009).

Stem cell therapy serves as an interesting tool for regenerative medicine in human and in veterinary medicine (Barry and Murphy, 2004; Ribitsch et al., 2010). Mesenchymal stem cells (MSCs) are able to self-renew and differentiate into bone, cartilage or adipose tissue (Zuk et al., 2002). MSCs have been used for the cell-based therapy of bone and soft tissue regeneration (Jang et al., 2008; Kraus and Kirker-Head, 2006; Zucconi et al., 2010). Although bone marrow are the major accessible and enriched source of MSCs, the numbers of MSCs obtained from bone marrow is generally insufficient for transplantation since the population of MSCs has been reported to range about 0.001 to 0.01% of bone marrow in rodents and felines (Kadiyala et al., 1997; Martin et al., 2002). This therefore highlights the importance of the isolation technique for MSCs. The characterization of MSCs usually relies upon plastic adherence, expression of some surface markers such as CD 90, CD 105 and Stro-1 while lacking the expression of hemopoietic stem cell marker (CD 34) (Wagner et al., 2005; Dominici et al., 2006). In dogs, direct plating of bone marrow aspirate is frequently used for bone marrow derived MSCs. However, a lack of information about the comparison of techniques for MSC isolation has been reported in dogs. It is therefore important to standardize the MSC isolation technique in order to increase the cell homogeneity of the MSCs population, thereby improving efficiency for bone regeneration (Roberts et al., 2008).

A scaffold is a three-dimensional structure with interconnection between pores allowing cell attachment and replacing bone defects. Polycaprolactone (PCL) is a biodegradable synthetic polymer (Williams et al., 2005; Amato et al., 2007) with advantages over other polymeric materials for the bone formation and remodeling phase including good biocompatibility, providing mechanical strength, giving more stability in ambient conditions and slow biodegradation rate (Gunatillake and Adhikari, 2003; Shor et al., 2007). Hydroxyapatite (HAp) ($Ca_{10}[PO_4]_6[OH]_2$) is known as the ceramic of choice for bone tissue engineering because its chemical and crystal properties resemble the mineral component of bone. In addition, it has an excellent biocompatibility, osteoconductive capacity and ability to bind directly to host bone (Di Silvio et al., 2002; Wang, 2006; Hutmacher et al., 2007; Neuendorf et al., 2008). The combination of PCL and HAp improves mechanical properties and provides osteoconductive and osteointegrative potential (Di Silvio et al., 2002; Wang, 2006; Neuendorf et al., 2008). The objectives of this study were to compare the effects of isolation techniques on the derivation of bone marrow MSCs and also to examine the effect of MSC transplantation on bone healing of an experimentally 'induced' non-union ulna bone defect in dogs.

Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, USA, unless otherwise specified.

Collection and isolation of canine bone marrow derived mesenchymal stem cells: This study was approved by the Committee for the Ethical Care of Animals of the Chulalongkorn University (No. 0931055). Seven mixed-breed dogs, weighing between 10 to 15 kg were enrolled in this study. All dogs were physically examined and remained healthy throughout the experiment. Routine blood analysis was performed preoperatively. Acepromazine 0.02 mg/kg and morphine 0.5 mg/kg were administered intramuscularly and anesthesia was induced with propofol (Fresenius Kabi, Austria GmbH, Graz, Austria) and maintained with isoflurane in 100% oxygen. Cefazolin (25 mg/kg) was administered intravenously as a prophylactic antibiotic. Epidural anesthesia using 0.5% bupivacaine (1 mg/kg) combined with morphine (0.1 mg/kg) was additionally performed to relieve the pain sensation caused by the bone marrow aspiration procedure. Fifteen milliliters of bone marrow were harvested from the iliac crest of each dog with a 1 ml heparinized syringe (100IU/ml).

The bone marrow aspirate (15 ml) was equally divided into 3 aliquots, and each aliquot of 5 ml bone marrow was then submitted to one of the following isolation techniques: 1) direct plating, 2) red blood cell lysis treatment and 3) gradient density.

Direct plating: Direct plating was performed by adding bone marrow aspirate directly into a 10 cm Petri-dish (BD-Falcon™, Franklin Lake, NJ, USA) containing 7 ml of MSC culture medium with approximately 30-40% confluence. After 24 h of culture, the attached cells were washed with Dulbecco's phosphate buffered saline without calcium and magnesium (DPBS, Invitrogen, Carlsbad, CA, USA) 2-3 times and the fresh MSC medium was then added.

Red blood cell lysis treatment: Red blood cell lysis buffer (8.3 g/L ammonium chloride in 0.01 M Tris-HCl buffer, pH 7.5 $\pm$ 0.2) was used to eliminate the contaminated red blood cells (RBC) in the bone marrow aspirate. The RBC lysis buffer was mixed at a ratio of 1: 1 with bone marrow aspirate and then incubated at room temperature (approximately 25-26°C) for 5 min. The mixture was then centrifuged at 1000 rpm for 5 min. After the supernatant was discarded, the pellet was resuspended with MSC medium, and the isolated cells were cultured in MSC

culture medium with approximately 30-40% confluence. The non-attached cells were washed out the following day.

Gradient density: Bone marrow aspirate (5 ml) was gently layered onto a histopaque® 1077 (density 1.077 ±0.001) in a 15 ml conical tube (BD-Falcon™, Franklin Lake, NJ, USA). The centrifugation was set at 26°C and 400g for 30 min, and the interface containing mononuclear cells was then collected. The presumptive MSCs were washed with MSC culture medium two times prior to culture.

Culture of bone marrow derived mesenchymal stem cells: Following MSC isolation (day 0) as previously described, the isolated cells were allowed to attach to the culture plate for 24 h prior to washing with DPBS. These cultured cells were assigned as primary cells at passage 0 (P_0). MSC culture medium was composed of a low-glucose Dulbecco's modified Eagle's medium (low glucose DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen, Carlsbad, USA), 100 unit/ml penicillin G, 100 µg/ml streptomycin, 40µg/ml gentamicin and 5 µg/ml amphotericin B.

A subculture of MSCs was performed at day 7 after MSC isolation by treating the cells with 0.05% (w/v) trypsin-EDTA (Invitrogen). The disaggregated cells were then centrifuged and split into a new Petri-dish at a ratio of 1:3. Putative MSCs at passages 3 (P_3) were used for flow cytometric analysis in order to examine the expression of cell-surface antigens and also for *in vitro* differentiation. MSCs at P_3 were subcultured to passage (P4) for in vivo transplantation. In all cases, the culture condition was performed at 37°C in a humidified condition of 5 % CO_2 in air.

Assessing the characteristics of canine mesenchymal stem cells

Flow cytometry: The examination of MSCs was performed as essentially described by Tharasanit et al. (2011). Canine MSCs at the 3rd passages from a total of 5 dogs (Dog no. 1, 4, 6, 7 and 8) were immunologically examined for surface markers. These markers included the positive (CD 44, CD 90) and negative (CD 34) markers. A total of 200,000 to 300,000 cells were used for immunolabeling. Rat monoclonal anti-canine CD 90 (AbD serotec, Kidlington, UK) with rabbit anti-rat FITC secondary antibodies and monoclonal anti-canine CD44 antibody conjugated with allophycocyanin (APC) (R&D system, Minneapolis, USA) were used as MSC positive markers. A rat monoclonal anti-canine CD 34 antibody conjugated with fluorescein isothiocyanate (FITC) was used as the negative MSC marker. Fluorescently-labeled MSCs were washed and fixed with 1% (w/v) paraformaldehyde in PBS and stored at 4°C in the dark until analysis. Non-staining MSCs and MSCs labeled with only the secondary antibody were used as controls. At least 20,000 MSCs were analyzed by flow cytometry.

In vitro bone differentiation of MSCs: Canine bone marrow derived MSCs at passage 3 from four dogs were simultaneously induced to osteogenic lineage (each dog represented one replicate). Osteogenic differentiation was performed as essentially described by Bosch et al. (2006) with minor modifications. The MSCs were loaded into a petridish at a density of 35,000 cells per cm^2. The bone induction was performed when the cells were approximately 80% confluent, the osteogenic medium containing MSC medium supplemented with 0.1 µM dexamethasone, 50 µM ascorbic acid, 10 mM β-glycerophosphate (Merck, Darmstadt, Germany) was then added into the culture dish. The *in vitro* bone differentiation was performed for 21 days. Von Kossa staining was used to detect the deposition of calcium phosphate indicating *de novo* bone formation.

Scaffold preparation and MSC transplantation: PCL/HAp composite scaffolds were prepared as previously described (Wutticharoenmongkol et al., 2007). The scaffolds were cut into 10x5x25 millimeters and sterilized with 100% (v/v) absolute ethanol for 1 h. The scaffolds were washed thoroughly with sterilized distilled water and then DPBS to remove the ethanol.

Anesthesia and surgical procedures were performed similar to previously described. Brachial plexus block using 0.5% bupivacaine (1.3 mg/kg) was additionally performed prior to bilateral ulnar osteotomy to reduce pain. A total of 3 dogs were induced to bilateral ulnar non-union (n=6) by cutting the mid-shaft of the ulnar bone (2.5 cm) using an oscillating saw. The ostectomy sites were implanted with the PCL/HAp composite scaffold to improve bone stability. After the operation, all forelimbs were applied with modified Robert Jones bandages reinforced with thermoplastic splints (Vet-lite, Bangkok, Thailand). All dogs also received 5 mg/kg enrofloxacin (Bayer, Bangkok, Thailand) and 4 mg/kg carprofen (Pfizer, Bangkok, Thailand) orally for 7 days after implantation. All dogs demonstrated a non-union of ostectomy site at 12 weeks after scaffold implantation. For transplantation, autologous MSCs were subcultured to passage 4 as previously described. After trypsinization, approximately $1x10^7$ cells/ml were loaded into a syringe containing a minimum volume (0.5 ml) of DMEM supplemented with 1% (v/v) FBS. Three MSC transplanted sites (proximal, middle and distal parts of the PCL/HAp scaffold implant) were injected via a 23 G indwelling intravenous catheter with MSCs under a fluoroscope (Philips healthcare, Eindhoven, the Netherlands).

Examination of bone formation

Radiography: Lateral radiographs were taken immediately and at 2, 4, 6, 8, 10 and 12 weeks post-operatively in all dogs. Bone healing was evaluated using the radiographic scoring system previously described (Johnson et al., 1996b) as shown in table 1. Bone union was justified grading from 0 to 3 at the proximal and distal of implanted material. In addition, the new bone formation was scored 0 to 4.

Angiography: Fluoroscopic angiography was performed in two experimental dogs at 12 weeks before MSC injection (dog no. 1, 2) and 16 weeks after MSC injection (dog no.1) in order to observe the blood

vessels at the implantation site. Anesthesia and surgical procedures were performed as previously described. A 23 G intravenous catheter connected with an extension tube was inserted into the axillary vein and the radiographic contrast medium (iohexol, Omnipaque®, G E healthcare, Buckinghamshire, UK) was slowly administrated in order to observe the presence of the blood vessel at the MSC transplanted site using fluoroscopy.

Table 1 Radiographic scoring system for evaluation of bone healing (Johnson et al., 1996b).

	Score	Description
Bone formation	0	No new bone; graft approximates density of soft tissue
	1	Minimal new bone composed mostly of noncontiguous Areas of minimal density
	2	New bone present as mostly contiguous areas of normal Density and fills approximately 50% of the defect
	3	New bone present as mostly contiguous areas of normal Density and fills approximately 51-95% of the defect
	4	New bone a solid contiguous mass that fills > 95% of the defect
Bone union	0	No contact between new bone and noninvolved adjacent normal bone
	1	Partial bridge (< 50%) from new bone to adjacent normal bone
	2	Partial bridge (> 50%) from new bone to adjacent normal bone
	3	Complete bridge from new bone to adjacent normal bone

Histological examination of MSCs loaded scaffold: The biopsy was performed both before (n=2) and after MSC transplantation (n=2) at 12 and 20 weeks post-transplantation. The biopsy samples were fixed with 10% (v/v) buffered formalin, and the fixed samples were embedded in paraffin and processed following the guidelines for a routine histological procedure. The sections were stained with hematoxylin & eosin (H&E) and examined for neovascularization and bone formation under a light microscope. Masson's trichrome staining was additionally performed to detect the presence of collagen fibers.

Statistical analysis: Values are present as means ± standard deviation (SD). The efficacy for different MSC isolation techniques on the number of isolated MSCs, viability and expression of MSC markers were compared by one-way analysis of variance (ANOVA) and post-hoc analysis with the least significant difference (LSD). Angiographs and histological findings were descriptively analyzed. The differences in radiographic scores at 2, 4, 6 and 8 weeks post MSC transplantation were evaluated using Kruskal-Wallis and Mann-Whitney U statistical tests. In all cases, statistical analysis was performed using the SPSS statistical program (version 17.0). P values <0.05 were considered statistically significant.

Results

Following MSC isolation, putative MSCs were attached on to a Petri-dish. These MSCs irrespective of the MSC isolation techniques demonstrated a typical MSC morphology (Fig. 1 A-C). There were several cell types including thin spindle shaped, typical fibroblast-like and mantle cells. On day 7 after MSC isolation, the total number of MSCs obtained from a 5-ml of bone marrow aspirate was ranged from 0.35×10^6 to 2.8×10^6, 0.04×10^6 to $1.43\times$, 0.03×10^6 to 0.75×10^6 for gradient density, RBC lysis treatment and direct plating, respectively (Table 2). The gradient density techniques significantly increased the numbers of isolated MSCs when compared with the direct plating technique ($P<0.05$), while cell yields obtained from this gradient density were efficiently comparable to RBC lysis treatment.

Table 2 Mean ± SD (range) of the percentage of MSCS on day 7 and the proportion of MSCs (passage 3) positive to cell surface markers. The MSCs were isolated from bone marrow using three different techniques.

	Total cell number ($x10^6$)	Cell surface markers		
		CD 44	CD 90	CD 34
Whole aspiration	0.26 ± 0.28^a	98.74+1.73	95.84+3.05	0.018+0.03
	(0.03-0.75)	(95.66-99.59)	(91.93-99.15)	(0-0.01)
RBC lysis treatment	$0.94 \pm 0.55^{a,b}$	99.28+0.82	93.48+10.76	0.014+0.03
	(0.04-1.43)	(99.18-99.89)	(74.3-99.21)	(0-0.06)
Gradient	1.08 ± 0.92^b	99.2+0.96	93.62+6.65	0.012+0.027
	(0.35-2.8)	(99.02-99.89)	(82.53-98.88)	(0-0.06)

Cell-surface antigen profiles of canine MSCs were ascertained after immunolabeling with canine-specific/cross-reacted monoclonal antibodies and examined with a flow cytometer. Ranges and averages of percentage of cells positive for CD 44, CD 90 and CD34 are shown in table 2. Canine MSCs at passage 3 highly expressed CD 44 (95.66-99.89) and rarely expressed CD 34 in all cases (Table 2). Furthermore, the

cultured MSCs were demonstrated to have the capability to differentiate into osteogenic lineage as they positively stained with Von Kossa following osteogenic induction (Fig. 2).

Following MSC transplantation into non-union sites, all three dogs could walk properly within 24 hours of surgery. Neither radiological changes at the scaffold-ulna interface nor callus formation were presented at the implant sites of PCL/HAp alone (Fig. 3A) and PCL/HAp combined with MSCs (Fig. 3B) at 2, 4, 6, 8 and 12 weeks post-operatively. The density of the implant grafts was comparable to soft tissue density. The radiographic scores were 0 in all cases.

Fluoroscopic angiography indicated the presence of blood vessels within the PCL/HAp scaffolds at 12 weeks after implantation (Fig. 4A). These blood vessels descriptively appeared to increase when examined at 16 weeks after MSC injection (Fig. 4B). This result was in an accordance with the histology of the host-PCL/HAp transplanted sites that neovascularization was observed in PCL/HAp both with and without MSC injection. However, no such osteoid formation was observed in the histological sections. The transplanted PCL/HAp scaffolds were filled with loose and unorganized connective tissue (Fig. 5A) but the thickness of the fibrous tissues and the number of small blood vessels were likely to increase at 20 weeks after MSCs injection (Fig. 5B). The fibrous tissue mainly comprised of the spindle cells with extensive collagen deposition which was confirmed by Masson's trichrome staining (Fig. 5B). Interestingly, we also found a number of multinucleated gaint cells and lymphocytes within the scaffolds indicating an inflammatory response.

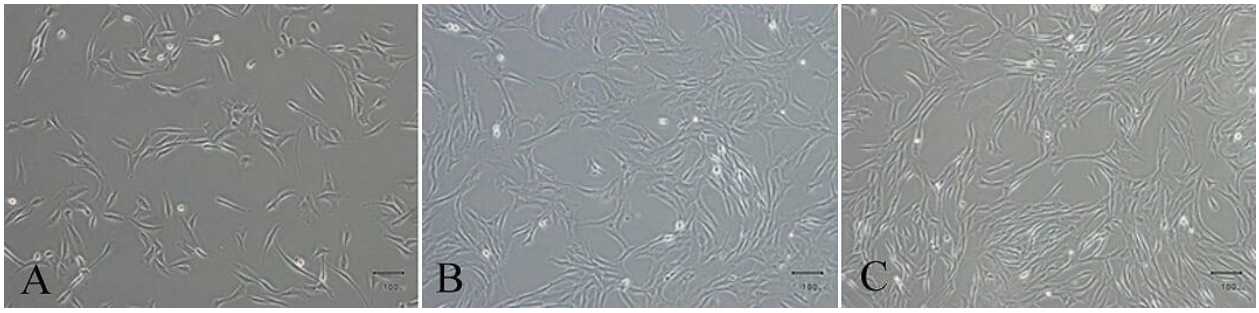

Figure 1 Morphology of canine MSCs derived from bone marrow using direct plating (A), RBC lysis treatment (B) and gradient density (C).

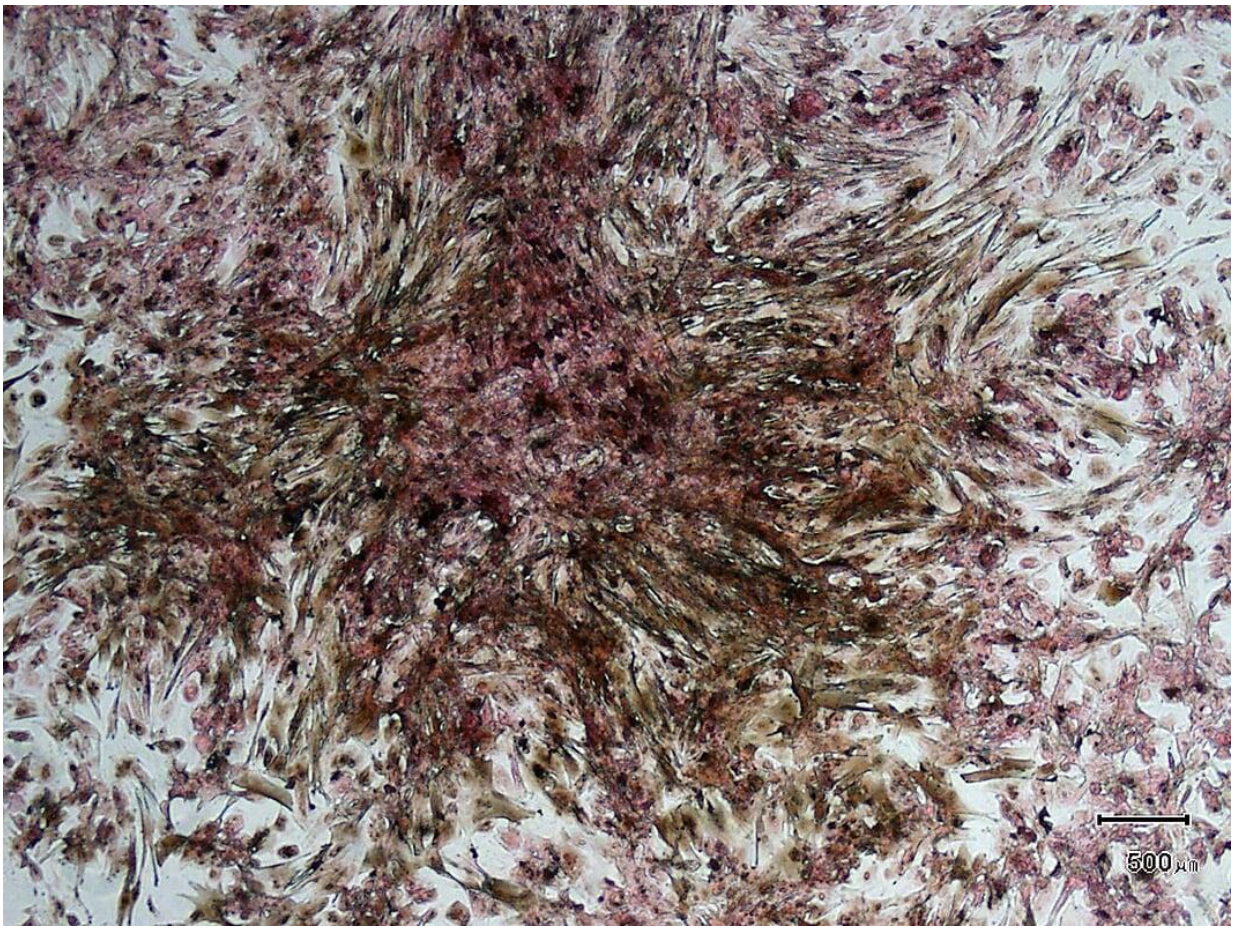

Figure 2 An example of canine bone marrow MSCs that were induced to differentiate into osteogenic lineage as they were positive to Von Kossa staining.

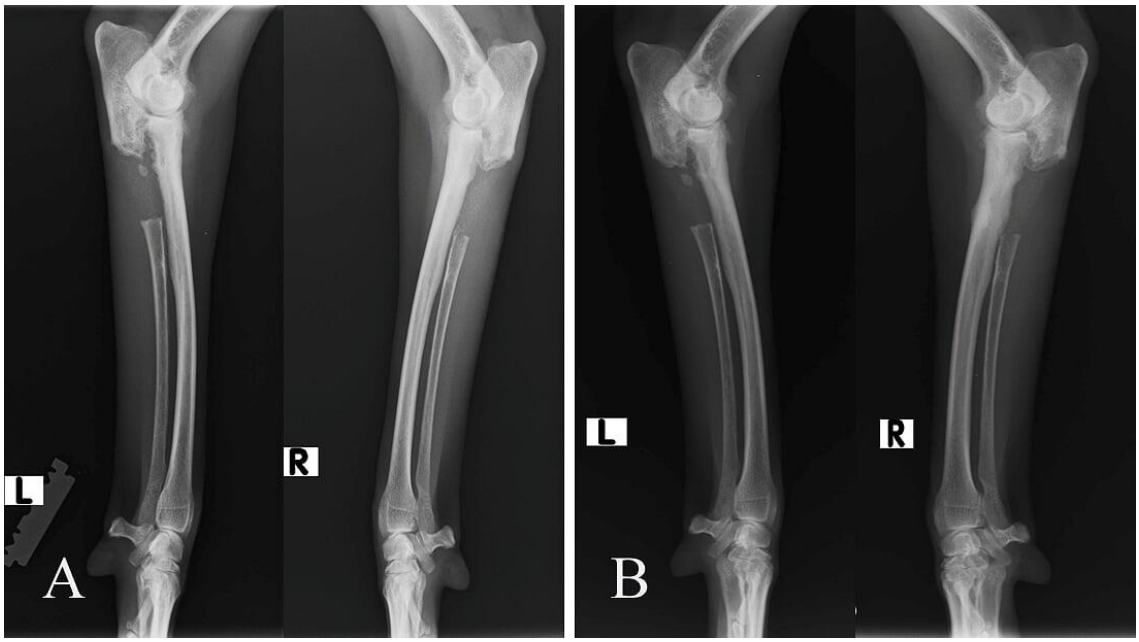

Figure 3 Radiographs demonstrating canine ulnar segment defect with PCL/HAp alone (A) or PCL/HAp combined with MSCs at 12 weeks (B) postoperatively. Note that no *de novo* bone formation was observed.

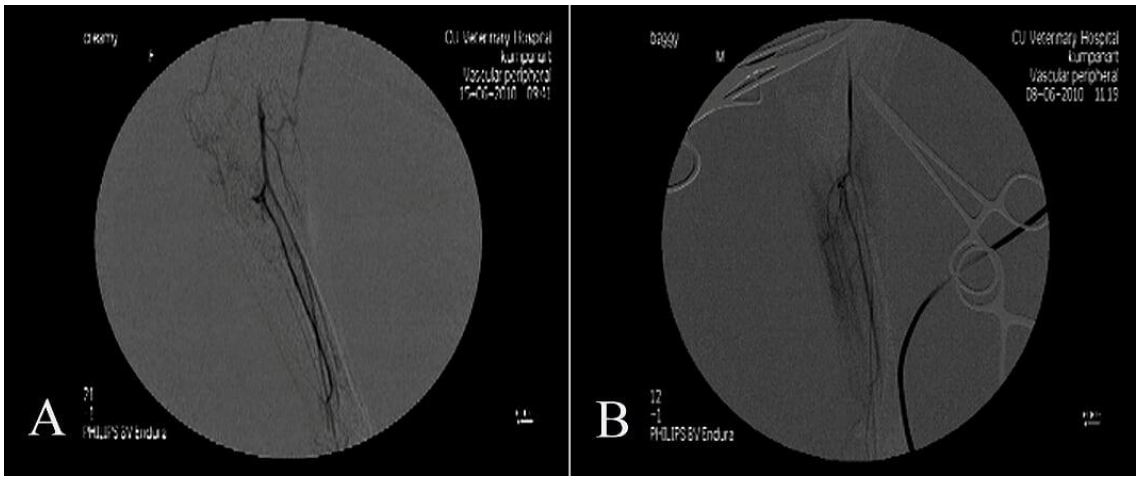

Figure 4 Fluoroscopic angiography at 12 weeks (A) after PCL/HAp scaffold implant and at 16 weeks after MSC injection (B). The increased blood vessels (black and gray lines) were observed at the implantation site after BMSC injection.

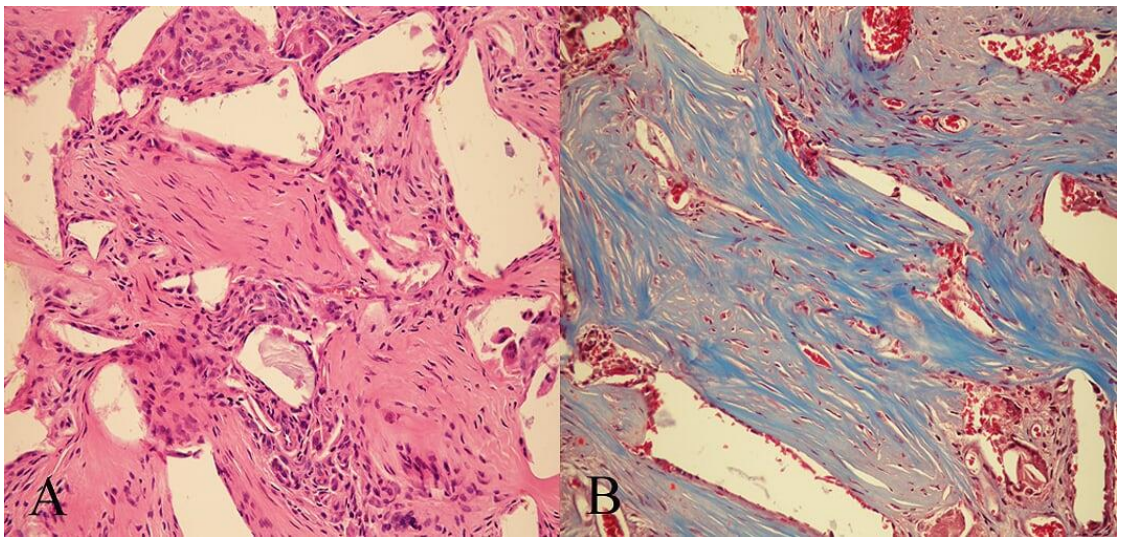

Figure 5 Histology of PCL/HAp implanted site at 12 weeks after surgery (A) and at 20 weeks after MSC injection (B) indicates the newly formation of capillaries and arterioles. Masson's trichrome staining (B) indicates the thickening of fibrous tissue mainly constructed by an extensive collagen fibers.

Discussion

In this study, we demonstrated that bone marrow derived canine MSCs could be enriched by treating the bone marrow aspirate prior to culture, and the harvested cells were highly expressed MSC markers (CD 44 and CD 90) and had the potential to differentiate into osteogenic lineage in vitro. However, the de novo bone formation following transplantation of the MSCs into PCL/HAp scaffold was compromised.

Bone marrow is considered as a primary source of MSCs but only 0.001 to 0.01% of bone marrow mononuclear cells in rodents and felines were identified as MSCs (Kadiyala et al., 1997; Martin et al., 2002). We first examined the different techniques to enrich MSCs from bone marrow aspirate and found that gradient density significantly increased the numbers of isolated MSC on day 7 of culture when compared with the direct plating technique ($P<0.05$, table 2). It had become clear that the presence of RBC in the culture dish negatively affected the number of harvested MSCs probably because the RBC may compromise the MSCs to settle and adhere to the Petri-dish (Horn et al., 2011). In addition, excessive RBC lysis during culture may cause the release of free hemoglobin that can induce cellular stress and apoptosis (Meguro et al., 2001; Kumar and Bandyopadhyay, 2005). Although the efficacy of the gradient density was similar to that of RBC lysis treatment ($P>0.05$), the RBC lysis buffer treatment is preferable for clinical use since it requires less time and is inexpensive. This technique may also be beneficial for the initial growth of the isolated MSCs due to the platelet derived growth factor secreted from the remaining platelets following RBC lysis (Horn et al., 2008). In addition to the effect of isolation techniques on MSC derivation, the numbers of MSCs isolated were considerably variable among donors, irrespective of the isolation techniques used. This result is in an agreement to the facts that the number of bone marrow MSCs significantly decreases with increased age and poor health status (Egrise et al., 1992; Dodson et al., 1996; Rao and Mattson, 2001; Bobis et al., 2006), while the cells obtained from young donors grew more rapidly (Musina et al., 2005). However, there was no effect of isolation techniques on the expression of MSC makers (CD 40 and CD 90) and also in vitro differentiation, suggesting that MSC properties could be maintained using the current culture system. However, study of the effect of long term culture of these MSCs on MSC proliferation activity and also differentiation capability remains elusive. This aspect is important given that the large numbers of cells would be required for cell/tissue engineering (Barry and Murphy, 2004).

To date, several attempts have been made on transplantation of MSC into long-bone defects (Johnson et al., 1996b; Kadiyala et al., 1997; Burastero et al., 2010; Vertenten et al., 2010). However, the de novo bone formation following transplant has been unsuccessful (Kadiyala et al., 1997) especially in dogs that have a slower bone turnover rate compared with mice (Cook et al., 1994). Furthermore, the injured bone site often becomes ischemic due to the excessive vascular destruction (McCartney and MacDonald, 2006). A combination of osteopotential cell and biolodegradable scaffold is one of the most successful strategies in bone tissue engineering. Our previous data suggested that PLC/HAp composite scaffold demonstrated a promising approach for promoting new bone formation when transplanted to calvarial defect in mice (Chuenjitkuntaworn et al., 2010). In addition, previous study suggested that polycaprolactone-tricalcium phosphate for loading MSCs presented a high amount of lamellar bone in dog mandibles (Khojasteh et al., 2013). In order to minimize the risk of ischemic lesion, we therefore first transplanted the PCL/HAp scaffold to the large bone lesion allowing neovascularization within the scaffold before MSC transplantation. After 12 weeks of PCL/HAp scaffold transplant, no bone formation was found but fluoroscopy indicated the neovascularization within the scaffold (Fig. 4A). However, no osteogenic differentiation was observed following MSC transplant into the vascularized scaffolds. From this result, it has become clear that the implanted site of the scaffold and species employed plays an important role for bone regeneration (i.e. cortical versus cancellous bone) (Dickson et al., 2007). Furthermore, a large size and slow degradable PCL-HAp scaffold may also create an unfavorable environment for bone formation (Bostman et al., 1990; Bergsma et al., 1993; Prokop et al., 2004). In this study, several multinucleated giant cells in the PLC/HAp scaffold indicated chronic reactions due to foreign body response. *Similar tissue reaction was previously reported* (Linder and Lundskog, 1975; Volker et al., 1997; Tindel et al., 2001). Although the scaffolds used in this study were contaminated with copper *(probably from hydroxyapatite ceramic)* as we further analyzed the scaffold using *energy dispersive x-ray (SEM/EDX) analysis* (unpublished data), the failure for osteogenesis of transplanted MSCs in the scaffold was probably not caused by the cell cytotoxicity of the contaminated copper. There was good evidence of fibrovascular tissue infiltration with an extent matrix deposition throughout the entire PCL/HAp scaffold, and injection of MSCs into the scaffold seemed to increase the thickening size of the fibroblastic mass and vascularization (Fig. 5B). In fact, though several factors are critical for bone tissue engineering, the main hallmark remains to be the sufficient osteogenic factors to stimulate de novo bone differentiation of the transplanted MSCs (Calori et al., 2011). Several growth factors and pathways have been demonstrated to be necessary for bone development (Lin and Hankenson, 2011). For example, bone morphologic protein-2 (BMP-2) increased osteocalcin release from MSCs promoting and healing response and induced chondrogenic and osteogenic differentiation of human bone marrow MSCs (Schmitt et al., 1999; Cheng et al., 2003; Shen et al., 2010) and also promoted the new bone formation in femoral defects in rats (Burastero et al., 2010) and increased the local population of cells and the connective tissue progenitors in a canine femur defect model with the combination of MSCs (Takigami et al., 2007). Moreover, early markers of osteogenesis were induced in canine MSCs by a combination of BMP-2 and ascorbate (Volk et al., 2005).

In conclusion, this study revealed that MSCs can be derived from bone marrow. These MSCs could be further enriched by treating bone marrow aspirate with gradient density and red blood cell lysis treatment. Although these MSCs highly expressed the MSC markers and also retained the differentiation potential, the bone differentiation following implantation was compromised. Further novel strategies in particular the combination of tissue engineering and osteogenic substances that can create a proper environment for de novo bone formation remain to be studied.

Conflict of interest statement: None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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บทคัดย่อ

การเปรียบเทียบเทคนิคการคัดแยกเซลล์ต้นกำเนิดมีเซนไคม์จากไขกระดูกสุนัขและความเข้ากันได้ของเซลล์ต่อโครงสร้างชนิดโพลีคาร์โปแลตโตน ไฮดรอกซีอะพาไทต์

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ไขกระดูกจากสุนัขจำนวน 7 ตัว นำมาคัดแยกเซลล์มีเซนไคม์ด้วยการใช้วิธีการคัดแยก 3 วิธีการ (direct plating, red blood cell lysis และ gradient density) ทำการตรวจหาจำนวนเซลล์ที่แยกได้ การแสดงออกของ marker ที่จำเพาะต่อเซลล์มีเซนไคม์และความสามารถในการกลายเป็นเซลล์กระดูกบนจานเพาะเลี้ยงจากเซลล์ที่แยกได้ทั้ง 3 วิธีก่อนการนำมาปลูกถ่ายในกระดูก ulna ที่ถูกกระตุ้นให้เกิดภาวะกระดูกไม่ติดกันจำนวน 3 ตัว เพื่อดูประสิทธิภาพการสร้างกระดูกจากเซลล์มีเซนไคม์ โดยทำการฝังโครงร่างเลี้ยงเซลล์ชนิดโพลีคาร์โปแลตโตน ไฮดรอกซีอะพาไทต์ ไปในตำแหน่งกระดูกที่ตัดไปก่อนทำการฉีดเซลล์ชนิดมีเซนไคม์เข้าไปในโครงร่างเลี้ยงเซลล์ ทำการถ่ายภาพเอกซเรย์ การบันทึกภาพรังสีหลอดเลือดและตรวจดูจุลกายวิภาคของเนื้อเยื่อในสัปดาห์ ที่ 2, 4, 6, 8 และ 12 หลังการฉีด จำนวนเซลล์มีเซนไคม์ที่แยกได้จากวิธี gradient density และ RBC lysis มีมากกว่าจำนวนเซลล์มีเซนไคม์ที่แยกได้จากวิธี direct plating ในวันที่ 7 ของการเลี้ยง เซลล์มีเซนไคม์ที่แยกได้จากทุกๆ วิธีมีการแสดงออกของ marker ที่จำเพาะต่อเซลล์มีเซนไคม์ ได้แก่ CD 44 and CD 90 ไม่พบการเปลี่ยนแปลงจากภาพถ่ายเอกซเรย์หรือการเกิดแคลลัสบนโครงร่างเลี้ยงเซลล์ในสนัขทุกตัวที่ได้รับการ ปลูกถ่ายเซลล์ชนิดมีเซนไคม์ ถึงแม้ว่าเซลล์มีเซนไคม์จากสุนัขทุกตัวจะมีการสร้างกระดูกบนจานเพาะเลี้ยง จากภาพรังสีหลอดเลือดแสดงการสร้างหลอดเลือดใหม่เพิ่มขึ้นในสัปดาห์ที่ 16 หลังฉีดเซลล์มีเซนไคม์ การตรวจทางจุลกายวิภาคของเนื้อเยื่อได้ยืนยันการสร้างหลอดเลือดเพิ่มขึ้นและพบเนื้อเยื่อไฟบรัสอยู่รอบๆ โครงร่างเลี้ยงเซลล์ จากการศึกษาครั้งนี้สรุปได้ว่าการคัดแยกเซลล์ชนิดมีเซนไคม์ที่เหมาะสมได้แก่วิธี gradient density และ RBC lysis โดยจะได้เซลล์ที่มีจำนวนเยอะกว่าและมีคุณสมบัติของเซลล์ชนิดมีเซนไคม์ อย่างไรก็ตามศักยภาพของการใช้เซลล์ชนิดมีเซนไคม์ในการฉีดเข้าไปยังกระดูกที่มีภาวะกระดูกไม่ติดยังต้องอาศัย ปัจจัยอื่นๆ เช่นสารกระตุ้นการสร้างกระดูก

คำสำคัญ: ไขกระดูก สุนัข เซลล์มีเซนไคม์ กระดูกไม่ติดกัน โครงร่างเลี้ยงเซลล์ชนิด โครงสร้างชนิดโพลีคาร์โปแลตโตน ไฮดรอกซีอะพาไทต์

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