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TITLE: Comparative Study of Cloning Efficiency of Canine Mononuclear Cells Isolated from Bone Marrow of Femoral Head and Subcutaneous Adipose Tissue.

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THESIS

COMPARATIVE STUDY OF CLONAL EFFICIENCY OF CANINE MONONUCLEAR CELLS ISOLATED FROM BONE MARROW OF FEMORAL HEAD AND SUBCUTANEOUS ADIPOSE TISSUE

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A Thesis Submitted in Partial Fulfillment of
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Stem cell therapy is expected to be used for orthopedic purposes. Mesenchymal stem cells (MSCs) are increasingly being interested for therapeutic purposes and have been identified in various tissues from many species. Like those in other species, canine MSCs show a great capacity to generate into various cell types under appropriate *in vitro* conditions. Because of ease to access and being a common by-product of surgical procedure, subcutaneous adipose tissue became an alternative source for MSCs apart from bone marrow. In this study, we compared clonal formation ability of mononuclear cells (MNCs) isolated from adipose tissue and bone marrow sources using the standard protocol of counting colony forming unit-fibroblast (CFU-F). MNCs from both sources showed fibroblast-like morphology and formed colonies termed as CFU-F after culturing in plastic surface for 10 days. The colony numbers per MNCs and the colony numbers per adherent cells derived from adipose tissue were significantly higher than those derived from bone marrow. Our study suggested that adipose tissue not only is a suitable source to harvest and also has a higher performance of cloning efficiency of MNCs than bone marrow. Thus subcutaneous adipose tissue might be an appropriated source for stem cells therapy in canine.

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Student's signature Thesis Advisor's signature

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Nada Tanamai
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LIST OF ABBREVIATIONS

ACT	=	Autologous chondrocyte transplantation
ALP	=	Alkaline phosphatase
BDNF	=	Brain-derived neurotrophic factor
bFGF	=	Basic fibroblast growth factor
BHA	=	Butylated hydroxyanisole
BME	=	Beta-mercaptoethanol
BMP	=	Bone morphogenetic protein
ºC	=	Celcius
CD	=	Cluster of differentiation
CFU-F	=	Colony forming unit-fibroblast
CNTF	=	Ciliary neurotrophic factor
CO_2	=	Carbon dioxide
cm^2	=	Cubic centimeter
DMEM	=	Dulbecco's Modified Eagle Medium
DMEM-KO	=	Dulbecco's Modified Eagle Medium-Knock Out
DMEM-LG	=	Dulbecco's Modified Eagle Medium-Low Glucose
DMEM-HG	=	Dulbecco's Modified Eagle Medium-High Glucose
DMEM/F12	=	Dulbecco's Modified Eagle Medium/ Ham F-12
DMSO	=	Dimethylsulfoxide
EGF	=	Epidermal growth factor
ES cell	=	Embryonic stem cell
FBS	=	Fetal bovine serum
FCS	=	Fetal calf serum
FIT-C	=	Fluorescein isothiocyanate
g	=	Gram
GFAP	=	Glial fibrillary acidic protein
HCl	=	Hydrochloric acid
HLA	=	Human leukocyte antigen

LIST OF ABBREVIATIONS (Continued)

HSCs	=	Haematopoietic stem cells
GH	=	Growth hormone
IFA	=	Interferon
IGF	=	Insulin-like growth factor
ISCT	=	The International Society for Cellular Therapy
ITS	=	Insulin, human transferring, and selenous acid
kg	=	Kilogram
L	=	Liter
MEM	=	Minimum Essential Medium
MHC	=	Major histocompatibility complex
ml	=	Milliliter
MNCs	=	Mononuclear cell
MSCs	=	Mesenchymal stem cell
NaH2PO4	=	Sodium hydrogen phosphate
Na2HPO4	=	Disodium hydrogen phosphate
NaCl	=	Sodium chloride
NaOH	=	Sodium hydroxide
NBT/BCIP	=	Neutron-blue tetrazolium/indolylphosphate
NF	=	Neurofilament
NSAIDs	=	Non-steroidal anti-inflammatory drugs
PBS	=	Phosphate buffer saline
PCR	=	Polymerase chain reaction
PDGF-RB	=	Platelet-derived growth factor receptor beta
PE	=	Phycoerythrin
PL	=	Platelet lysate
SIRP	=	Signal regulatory protein
SD	=	Standard deviation
SSEA	=	Stage-specific embryonic antigen

LIST OF ABBREVIATIONS (Continued)

TGF	=	Transforming growth factor
VEGF	=	Vascular endothelial growth factor
v/v	=	Volume by volume
α	=	Alpha
β	=	Beta
γ	=	Gamma

COMPARATIVE STUDY OF CLONING EFFICIENCY OF CANINE MONONUCLEAR CELLS ISOLATED FROM BONE MARROW OF FEMORAL HEAD AND SUBCUTANEOUS ADIPOSE TISSUE

INRODUCTION

Stem cells are known as the basic building block cells that are able to differentiate into different types of tissue (Centeno *et al.*, 2008;De Bari *et al.*, 2003;Jang *et al.*, 2004;Keating, 2012;Kroon *et al.*, 2008). Mesenchymal stem cells (MSCs) defined as multipotential non-haematopoietic stem cells which have an ability to differentiate into mesenchymal lineages such as osteoblasts (Dvorakova *et al.*, 2008), chondrocytes (Kadiyala *et al.*, 1997), tendinocytes (Young *et al.*, 1998), myocytes, adipocytes (Kassis *et al.*, 2006), and also cells of non-mesenchymal origin such as neuronal progenitors (Kamishina *et al.*, 2008a;Lim *et al.*, 2010) *in vitro* and *in vivo* under appropriate conditions (Awad *et al.*, 1999;Ge *et al.*, 2005;Grogan *et al.*, 2009). Increasing knowledge of clinically use of stem cells have been gained by many clinical trials and therefore enables stem cell-based therapies to become a new choice of treatment for degenerative disorders and tissue injuries (Castro-Malaspina *et al.*, 1980;Pountos *et al.*, 2007). Since their first description in 1976, MSCs became the most interesting object for regenerative therapy of both human and veterinary medicine because of their great capacity. MSCs appear *in vitro* as spindle shaped cells or fibroblast-like morphology termed as colony forming unit-fibroblast (CFU-F) (Friedenstein *et al.*, 1976). Based on studies in human and laboratory animals, MSCs are identified in tissues by an expressing 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 (CD44) (Dvorakova *et al.*, 2008;Kern *et al.*, 2006).

Many MSCs sources were identified in laboratory animals and also human. including spleen (Krampera et al., 2007), heart (Hoogduijn et al., 2007), placenta

(Filioli Uranio et al., 2011), umbilical cord blood (Kern *et al.*, 2006;Koch *et al.*, 2007;Lee *et al.*, 2004) , peripheral blood (Kassis *et al.*, 2006;Koerner *et al.*, 2006), thymus (Krampera et al., 2007), periosteum (Park *et al.*, 2007;Yoshimura *et al.*, 2007), cartilage(REF), joint tissues (De Bari *et al.*, 2003;Fan *et al.*, 2010;Ju *et al.*, 2008), muscle (Jackson et al., 2010), and adipose tissue (Black *et al.*, 2008;Black *et al.*, 2007;Lim *et al.*, 2010). However, the most suitable source in canine has not been mentioned. To establish MSCs *in vitro* production and MSCs cell banking, the accessibility and availability of MSCs in such tissue sources are major factors that have to be considered. From this aspect both bone marrow and adipose tissue are very attractive although differences in collecting techniques of MSCs from both tissue sources in patients are obvious. Bone marrow is collected with bone marrow aspiration which is invasive and might cause increasing risk of infection, adipose tissue may be easier to access.

MSCs *in vitro* production is required for therapeutic proposes and stem cell banking. Since femoral head and subcutaneous adipose tissue are known to be common by-products of surgical procedures in canine patients, the use of these tissues as candidate sources need to be evaluated especially in term of the quality of the tissue sources such as cell density and the quality of the isolated cells. Adherent mononuclear cells (MNCs) are MNCs isolated from known stem cell sources and are often used for evaluation of MSCs characterization. In this study we will compare colony formation ability of MNCs which are collected from bone marrow of femoral head with those are collected from subcutaneous adipose tissue of adult healthy dogs.

OBJECTIVE

To compare the ability of colony formation of canine MNCs from two sources, bone marrow and subcutaneous adipose tissue for *in vitro* cell production.

LITERATURE REVIEWS

STEM CELLS

Stem cells are known as undifferentiated cells which are derived from zygote, pre-implantation embryo, and post-natal tissues as well. They have unique properties for regenerative therapy. Their self-renewal enables the stem cells to replicate repeatedly without differentiation. The differentiation potential of stem cells make them giving rise to various cell types under appropriate influences such as biochemical, hormonal, and mechanical stimuli. Their different differentiation potential depends on whether they are derived from either embryo or adult. Totipotency, the capacity of stem cells derived from the fertilized egg or zygote, can develop into all cell types of embryonic and extra-embryonic tissue. Pluripotency, the capacity of stem cells derived from inner cell mass of an early pre-implantation embryo stage called blastocyst, can give rise to more than 220 cell types of the derivatives of the three primary germ layers including ectoderm, mesoderm, and endoderm of adult tissues except extra-embryonic membranes or the placenta. Multipotency, the capacity of stem cells derived from post-natal tissue, can differentiate into certain cell lineages that are restricted by their tissue origin (Blanpain *et al.*, 2004;Patel *et al.*, 2008).

TYPES OF STEM CELLS

Most of all, stem cells are divided broadly into two types: embryonic stem cells and adult stem cells.

Embryonic stem cells

Embryonic stem cells (ES cells) are derived from inner cell mass of early stage of embryo called blastocyst. ES cells have a capability to propagate themselves indefinitely to retain undifferentiated state and are pluripotent which is a capability to differentiate into all cell types under defined conditions. Inoculation with human ES

cells in severe combined immunodeficient beige mice causes teratocarcinoma consisting of gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm) (Thomson *et al.*, 1998).

Expression of cell surface and molecular markers has used to define the stem cell identity. It is now well established that mouse ES cells express stage-specific embryonic antigen (SSEA)-1 as a surface marker, in contrast with ES cells from human and non-human primate which are characterized by expressing SSEA-3 and SSEA-4. Furthermore, human ES cells express keratan sulfate-associated antigens, TRA-1-60 and TRA-1-81 (Henderson *et al.*, 2002). ES cells also possess enzyme activities for alkaline phosphatase and telomerase (Armstrong *et al.*, 2005;O'Connor *et al.*, 2008). The key regulators of pluripotentiality including Nanog and Oct-3/4 maintain pluripotency of ES cells (Chambers *et al.*, 2003;Mitsui *et al.*, 2003;Nichols *et al.*, 1998).

In regenerative medicine, ES cells from various species including rodents (Kim *et al.*, 2002), porcine, equine (Paris and Stout, 2010), canine (Schneider *et al.*, 2007), and non-human primates such as rhesus monkeys (Thomson *et al.*, 1995), common marmosets (Thomson *et al.*, 1996), cynomolgus monkeys (Suemori *et al.*, 2001), and baboons (Chang *et al.*, 2010) are commonly used in preclinical stem cell study. Attempts to produce mature functional cells from ES of laboratory animals have been reported. For vascular structure formation, *in vitro* study showed that after exposing to culture medium containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF), Rhesus ES cells can differentiate into endothelial cells with relatively uniform morphology. For neuronal repair, mouse ES cells differentiated into dopaminergic neurons and promoted partial recovery in a rat model of Parkinson's disease (Kim *et al.*, 2002). Oligodendrocyte progenitor cells which were differentiated from human ES cells *in vitro* could enhance remyelination and substantially improve locomotor ability after implantation in mice with early period of

spinal cord injury (Keirstead *et al.*, 2005). For diabetes, human ES cells could generate pancreatic endoderm *in vitro* which efficiently produced glucose-responsive insulin secreting cells after implantation into mice (Kroon *et al.*, 2008).

ES cell therapy is thought to have an advantage over other cell or organ transplantation due to the fact that ES cells are derived from non-antigenic tissues and their expression of the class I major histocompatability complex (MHC-1) protein is very low. These allow allogeneic transplantation without or with minimal immunosuppressive treatments (Nelson *et al.*, 2009)**.** However, this advantage is hampered by some studies which demonstrated expression of specific human leukocyte antigen (HLA) subclasses in ES cells when induced with interferon (IFN)-γ (Draper *et al.*, 2002). This caused some concerns about graft rejection after allogeneic transplantation. Because ES cells contain the potential to differentiate spontaneously into multiple cell types, there were some reports that revealed teratoma formation in mouse models after transplantation of undifferentiated ES or ES-derived insulin producing (Fujikawa *et al.*, 2005). Furthermore, there are controversies for the ES cells therapy because of destruction of life from isolating the inner cell mass of pre-implantation embryos so it has raised significant ethical and political concerns in many countries (Jurgens *et al.*, 2008;Kiatpongsan and Pruksananonda, 2006). These evidences might prevent the usage of ES cells in clinical approaches.

Adult or somatic stem cells

Adult or somatic stem cells are also called lineage-restricted stem cells. They are more preferable candidates for research and clinical application than ES cells because they are found in several organs and tissues from fetal or adult animals. Unlike pluripotent ES cells, multipotent adult stem cells have more limited differentiation capacity for producing certain cell lineages. Therefore, they can be categorized in to organ/tissue-specific subsets such as haematopoietic stem cells (HSCs) (Schuster *et al.*, 2012), mammary stem cells (Guo *et al.*, 2012), MSCs (Alt *et al.*, 2011), neural stem cells (Bottai *et al.*, 2003;Galli *et al.*, 2003), olfactory adult

stem cells (Nivet *et al.*, 2011;Roisen *et al.*, 2001), neural crest stem cells (Teng and Labosky 2006), corneal stem cells (Daniels *et al.*, 2001), and testicular cells (Goossens and Tournaye, 2006) or adult germline stem cells (Dansereau and Lasko, 2008;Xie, 2008).

Researches on adult stem cells revealed that each organ or tissue has its own compartment of stem cells which are responsible for normal turnover and repair by replacement of proliferated and differentiated cells from cell death by apoptosis or tissue injury. However, recent research found that lineage-restrict adult stem cells may have a greater plasticity to develop into other differentiated cell types which differ from their tissue origin both *in vitro* and *in vivo.* This capacity is called trans-differentiation capacity (Minguell *et al.*, 2001). For example, adult rat hepatic stem cells which normally differentiate into hepatocytes and bile duct epithelium, however, can be induced into pancreatic endocrine hormone-inducing cells when cultured in a high-glucose environment (Yang *et al.*, 2002). Liver stem cells transplanted in neonatal mouse brain can differentiate to cells with neuronal markers (Deng *et al.*, 2003). Transplantation of HSCs into liver-injured mice was found to give an improvement of liver functions (Jang *et al.*, 2004).

MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) are one type of adult multipotential non-haematopoietic stem cells that recently become an asset to regenerative medicine. With stem cell properties, MSCs have self-renew capacity and can give rise to cells of mesodermal lineages including bone, cartilage, adipose tissue (Grogan *et al.*, 2009;Kamishina *et al.*, 2008b;Sakaguchi *et al.*, 2005), skeletal muscle (De Bari *et al.*, 2003), tendon and ligament (Young *et al.*, 1998), and the stroma of connective tissues (Muschler *et al.*, 2003). Despite ubiquitous presence in many tissues, MSCs is considered as a rare population with very low frequencies between 0.001-0.01% of isolated mononuclear cells (MNCs) (Pittenger *et al.*, 1999).

For many years bone marrow was indicated to be a major source of MSCs for tissue engineering, recently, the study in mice indicated that MSCs characteristics can be isolated and propagated *in vitro* from many organs and tissues (Da Silva Meirelles *et al.*, 2006). Several sources of human MSCs have been established such as trabecular bone (Sottile *et al.*, 2002), placenta (In 't Anker *et al.*, 2004), umbilical cord blood (Lee *et al.*, 2004), thymus (Krampera *et al.*, 2007), periosteum (Park *et al.*, 2007), adipose tissue (Bunnell *et al.*, 2008;Nakagami *et al.*, 2006), spleen capsule, heart and peri-renal adipose tissue (Hoogduijn *et al.*, 2007). A small number of MSCs was found in peripheral blood (Kassis *et al.*, 2006), muscle (Jackson *et al.*, 2010), several joint tissues including synovial membrane and synovial fluid (De Bari *et al.*, 2003;Fan *et al.*, 2010), amnion (Manochantr *et al.*, 2010) and cord blood (Kern *et al.*, 2006;Lee *et al.*, 2004). Recently, sources of canine MSCs are identified including bone marrow (Csaki *et al.*, 2007), cartilage, adipose tissue (Black *et al.*, 2008;Black *et al.*, 2007;Nakagami *et al.*, 2006). In addition, there was a success in isolation and expansion of MSCs from canine umbilical cord blood and fetal blood *in vitro* (Seo *et al.*, 2009).

Human and large animal bone marrow was commonly aspirated from the superior iliac crest of the pelvis (Hoogduijn *et al.*, 2007;Suzuki *et al.*, 2001), the tibia (Suzuki *et al.*, 2001), the femur (Hoogduijn *et al.*, 2007;Kern *et al.*, 2006), the thoracic and lumbar spine (Emery, 1957), and the sternum in horses (Baghaban-Eslaminejad, 2009;Colleoni *et al.*, 2009). In rodents, they are generally harvested from the mid-diaphysis of the tibia and femur by flushing the marrow out of the bones with culture medium (Tropel *et al.*, 2004). In canine, the proximal humerus, the proximal femur and the wing of the ilium are generally used to obtain the marrow (Laura *et al.*, 2008).

The Characteristics of Mesenchymal Stem Cells

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed some minimal standard criteria to

identify human MSCs for both laboratory-based scientific investigations and pre-clinical studies. The three defining criteria include the adherence to plastic surface, the specific surface antigen expression and the multipotent differentiation potential (Dominici *et al.*, 2006). MSCs can be isolated from mononuclear cells of adult mice bone marrow with plastic adherence capacity (Friedenstein *et al.*, 1976). Adherent MSCs can expand *in vitro* in standard culture conditions and form spindle-shaped cell layer which is also considered a characteristics of MSCs (Castro-Malaspina *et al.*, 1980;Yoshimura *et al.*, 2007). MSCs have self renewal potential which is proved by colony formation as fibroblastoid colony-forming unit (CFU-F). The capability of the cells to form colonies originated from a single cell is called clonogenicity (Dimitrov *et al.*, 2008). The comparative study of the human MSCs from three sources: bone marrow, adipose tissue and umbilical cord blood indicated that clonal efficiency of MSCs was different between those of three sources. Bone marrow and adipose tissue-derived cells formed CFU-F within 4-5 days whereas those isolated from umbilical cord blood took 2-4 weeks after initial plating of cells at $1x10^6$ MNCs per cm^2 (Kern *et al.*, 2006).

Those surface antigens are groups of functional membrane proteins involved in cell communication, adhesion, or metabolism. To date, no specific surface antigen of MSCs has been established, several positive and negative selection cell surface antigen have been purposed to be a major criteria for MSCs identification. The positive surface marker should be showed more than 95 percentages expression. Several cell surface molecules especially CD90 (Thy-1) and CD105 (SH2/endogrin) are commonly used (Csaki *et al.*, 2007;Dvorakova *et al.*, 2008). CD90 referred to be a marker of T-cell, hematopoietic stem cells and MSCs. CD105 is a receptor for transforming growth factors (TGF) family membrane such as TGF-β1, TGF-β3, bone morphogenetic protein (BMP)-2 and BMP-7. Surface proteins which are associated with stem cells migration include CD44 and CD73. A hyaluronic receptor, CD44, is believed to be associated with migratory capacity of MSCs to the area damage of the bone (Sackstein *et al.*, 2008). Some study reported that antibody neutralization of CD44 resulted to decreasing the MSCs migration (Raheja *et al.*, 2008). CD73 as ecto-5'-nucleotidase involved in migration of MSCs (Dominici *et al.*, 2006). However,

another study in equine found unexpected negative expression to CD73 of adipose derived MSCs (Pascucci *et al.*, 2011). MSCs also express several cell adhesion molecules including CD106 (VCAM-1), CD166, CD164, CD54 (ICAM-1), CD102 (ICAM-2), CD29 (Intergrin-β1), CD61 (Intergrin-β3) and CD49b (Intergrin-α2) (Conget and Minguell, 1999;Doyonnas *et al.*, 2000;Tare *et al.*, 2008). CD9, a transmembrane tetraspanin which is expressed on human adipose-derived MSCs modulate cell adhesion, migration and also differentiation (Kim *et al.*, 2007). CD55, CD59 and CD140b (PDGF-RB) are reported in playing a role in signal transduction pathway (Pountos *et al.*, 2007;Solomon *et al.*, 1995;Terstappen *et al.*, 1992). CD172a or signal regulatory protein (SIRP) inhibit cell signaling by receptor tyrosine kinases and cytokine receptor has also been reported on MSCs expression (Adams *et al.*, 1998;Vogel *et al.*, 2003). The expression of a membrane-bound aminopeptidase CD13, on MSCs are still inconclusive as positive expression of this molecule was found on human bone marrow derived MSCs (Vogel *et al.*, 2003) but not on bone marrow derived MSCs from rhesus macaques (Izadpanah *et al.*, 2005).

Lack of both of hematopoietic and endothelial specific surface antigens is used to define the MSCs identity. Hematopoietic surface antigens include CD34, a primitive hematopoietic progenitors and endothelial cells marker, CD144, a endothelial cell marker (Kern *et al.*, 2006), and CD133, a hematopoietic and angioblast marker (Grogan *et al.*, 2009). CD45 is a pan-leukocyte marker. CD11b, CD114 and CD14 mark as monocytes and macrophages (Dvorakova *et al.*, 2008), CD19 and CD79α are markers of B cells and CD31 marks platelet endothelial cell adhesion molecule-1 (Izadpanah *et al.*, 2005). Other markers include CD235a (Glycophorin A) which is a glycoprotein expressed on mature erythrocytes and erythroid precursor cells (Tirelli *et al.*, 2011) and HLA-DR (Dvorakova *et al.*, 2008;Tapp *et al.*, 2008).

Nevertheless, there were significant differences concerning surface marker expression of MSCs isolated from adipose tissue, bone marrow and umbilical cord blood. The expression of CD90 of MSCs derived from umbilical cord blood was significant lower intensity than the other tissues. The percentages of CD105

expression were lower in umbilical-derived MSC than adipose-derived MSC and bone marrow-derived MSCs significantly. The expression of CD106 was shown lower in adipose-derived MSCs than umbilical-derived MSCs and bone marrow-derived MSCs significantly (Kern *et al.*, 2006).

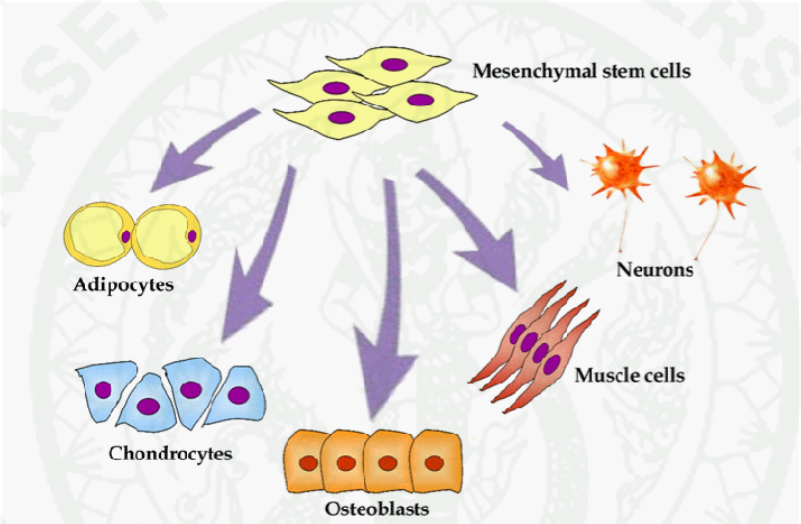

Figure 1 Differentiation potential of mesenchymal stem cells (MSCs). MSCs possess ability to self-renewal and differentiate into several cell types include osteogenic, chodrogenic and adipogenic lineage. Recently, it was demonstrated the differentiation into neutonal lineage. Some previous studies indicated myogenic transcriptional factors were expressed after inducing MSCs with myogenic medium.

Source: (Meregalli *et al.*, 2011).

Based on its unique capacity to differentiate into three mesodermal cell lineages including osteogenic, adipogenic and chondrogenic lineages MSCs can be proved for this multipotency by *in vitro* tissue culture with specific differentiating conditions (Pittenger *et al.*, 1999). Osteogenesis was induced with dexamethasone,

ascorbic acid and β-glycerol-phosphate supplemented culture medium (Dvorakova *et al.*, 2008;Kassis *et al.*, 2006). Although bone nodule formation still occurred even without the presence of dexamethasone, the formation was maximal under influence of dexamethasone (Aubin, 1999). MSCs undergo *in vitro* chondrogenesis in the culture medium of–high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with ITS-premix (insulin, human transferring, and selenous acid, dexamethasone, praline, ascorbic acid, and transforming growth factor-β3 (TGF- β3) (Dvorakova *et al.*, 2008;Pittenger *et al.*, 1999). In addition supplementation of these growth factors including fibroblast growth factor-2 (FGF-2), insulin-like growth factor-I (IGF-I), TGF β-I, growth hormone (GH), BMP-7 (or osteogenic protein-I), and BMP-2 enhance chondrocyte proliferation and chondrogenic differentiation. Adipogenesis was induced in the presence of dexamethasone, indomethacin, and isobuthymethylxanthine (Kassis *et al.*, 2006). Furthermore, MSCs have been shown myogenic capacity. Mouse MSCs can be induced to form multinucleated fibers resembling myotubes after the exposure to amphotericin B (Phinney *et al.*, 1999). Induction with myogenic medium for human adipose tissue-derived MSCs for 6 week resulted in the expression of myogenic transcriptional factor myod1, myf5, myf6, and myogenin and the formation of multinucleated cells containing the myosin heavy chain (Zuk *et al.*, 2002).

Comparative studies in human MSCs of three tissue sources– bone marrow, adipose tissue, and umbilical cord blood in term of mesodermal differentiation capacity revealed that tissue specific MSCs contain different levels of this capacity. Umbilical-derived MSCs could not be induced into adipogenic lineage but still could differentiate into osteogenic and chondrogenic lineages (Kern *et al*., 2006). Another piece of evidences showed that umbilical cord bloods-derived MSCs could differentiate into adipogenic lineage but with lower capacity when compared to the ability of differentiation into osteogenic lineages (Chang *et al.*, 2006;Rebelatto *et al.*, 2008). No differences in the adipogenic, chondrogenic and osteogenic potential between MSCs derived from bone marrow and adipose tissue was reported in many studies (Havlas *et al.*, 2011;Rebelatto *et al.*, 2008;Takemitsu *et al.*, 2012). However, the expression of osteoblastic markers, ALP and osteocalcin, was higher in human

bone marrow derived MSCs than adipose tissue derived MSCs (Al-Nbaheen *et al.*, 2012;Peng *et al.*, 2008).

Because of increasingly requirement of MSCs for treatments of chronic diseases or incurable injuries, attempts on *in vitro* manipulation of MSCs to differentiate into another lineage such as ectoderm have been made. The neuronal induction in human and rat bone marrow MSCs was successful using the culture medium supplemented with dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) (Woodbury *et al.*, 2000). The neuronal induction in feline bone marrow MSCs was accomplished by modification from Woodbury et al. (2000) method by substitution of α-MEM for DMEM (Martin *et al.*, 2002). Supplement of basic FGF (bFGF) can also induce neuronal differentiation of MSCs into neural cell types including neural stem cells, neurons, astrocytes, and oligodendrocytes with higher potency than the use of other growth factors including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Mobarakeh *et al.*, 2012).

The clinical application of mesenchymal stem cells

Early regenerative applications of adult stem cells have been focused on the use of haematopoietic stem cells for the treatment of blood diseases such as leukemia, anemia, auto-immune diseases, and immunodeficiency cases in human (Copelan, 2006). *In vitro* produced stem cells such as neural stem cells and corneal stem cells have been considered for treatment of Parkinson's disease and severe corneal injury, respectively (Rama *et al.*, 2010).

Recently, MSCs are thought to be a major candidate for various therapeutic procedures due to their potential to differentiate into various cell types. However, the limitations of usage of adult stem cells are concerned by several reasons including difficulty and risk that might occur during harvesting stem cells from the internal

organs or tissues, decreasing quality in aging donor (Stolzing *et al.*, 2008), and immunological reactions between transplanted cells and recipient. The autologous stem cell transplantation has been more preferable to avoid the immunological reactions (Hildebrand *et al.*, 2002). Nevertheless, allogeneic transplantation of mouse bone marrow MSCs–to rabbit showed neither immunological rejection nor graft versus host diseases despite of the detection of grafted MSCs in several organs including lung, liver, bone marrow and femoral head (Li *et al.*, 2011). The application of allogeneic transplantation of MSCs has been implemented in treatment of stem cells disorders-involved autoimmune diseases. In cases of patients with refractory systemic lupus erythematosus allogenic MSCs modulated host immune system, resulting in clinical improvement (Liang *et al.*, 2010).

In the field of veterinary medicine, MSC therapy has become a valuable and alternative strategy for the treatment of several diseases and injuries to which current therapeutic strategies have minimal effectiveness. The MSC therapy has been commercial available in the restoration of tissue functions in musculoskeletal disorders and injuries in dogs such as delayed or malunion bone fracture repair (Haghighat *et al.*, 2011;Rush *et al.*, 2009), cartilage repair (Koga *et al.*, 2008), repair of tendon injury (Daher *et al.*, 2011;Fan *et al.*, 2009;Ju *et al.*, 2008;Young *et al.*, 1998). There were a significant improvement in orthopedic examination scores for lameness and range of motion in dogs with chronic OA who treated with intra-articular of autologous adipose-derived MSCs (Black *et al.*, 2008;Black *et al.*, 2007). In addition, treatment of cardiac diseases have been conducted with local or systemic administration of MSCs (Ohnishi *et al.*, 2007).

The successful results were reported in other species. Horses with experimentally induced osteoarthritis by excision of the medial meniscus and resection of the anterior cruciate ligament were treated by intra-articular injection of autologous bone marrow-derived MSCs. The results showed marked regeneration of the medial meniscus and detected implanted cells in the newly formed tissue. Although reduction of the degeneration of articular cartilage, osteophytic remodeling, and subchondral sclerosis also were detected, the repair of the ligament was still not

found (Murphy *et al.*, 2003). The significant improvement in tendon fiber architecture combined with reduction in inflammatory cell infiltration and vascularity was found in horses with collagenase-induced tendinitis after treating with adipose-derived nucleated cell fractions containing mesenchymal stem cells (Nixon *et al.*, 2008). Another study showed similar result that the transplantation of bone marrow derived MSCs which seeded in fibrin glue showed the improvement of tendon extracellular matrix and fibrin orientation in horses with collagenase-induced tendinitis (Crovace *et al.*, 2010).

Clinical improvement can be a good indicator for success of MSC therapy. MSCs seeded collagen gel that was transplanted into knee joint caused clinical improvement in patients with osteoarthritis, however, whether hyaline cartilage was formed could not determine. (Wakitani *et al.*, 2007). The orthopedic examination scores and clinical criteria including range of motion, pain, and crepitus improved in human patients with degenerative joint disease and OA after treatment with bone marrow MSCs (Centeno *et al.*, 2008;Davatchi *et al.*, 2011). However efficacy of MSC treatment might require more substantial evidences including cell and tissue regeneration which is based on direct examination and histological finding. For instance, the clinical improvement in human with knee osteoarthritis was not significantly different between bone marrow MSCs transplantation and cell-free control group but arthroscopic and histological finding displayed cart (Wakitani *et al.*, 2002).

MATERIALS AND METHODS

Animals

Ten dogs of several breeds including Yorkshire Terrier, Shih Tzu, Poodle, Pomeranian and Crossbred were selected from the patients that required the femoral head and neck excision from orthopedic disorders including hip luxation and hip dysplasia in the Veterinary Teaching Hospital, Kasetsart University, Bangkaen Campus. All dogs must have no history of any systemic diseases, infectious diseases or bone neoplasia. All dogs had age range between 2-5 years (3.1±1.7, mean±S.D.) and weight range between 3-10 kilograms (6.15±2.18, mean±S.D.). Prior to the operation, all dogs received generalized anesthesia. Bone marrow and subcutaneous adipose tissue samples from each dog were collected with aseptic technique.

Isolation of mononuclear cells from bone marrow

Bone marrow was flushed from the femoral head with 100 mM phosphate buffer saline (PBS) with 1% v/v penicillin/streptomycin under sterile condition. Subsequently, the diluted marrow was gently placed on Ficoll-Paque plus® (d = 1.077 g/cm^3, GE bioscience, Westborough, MA, USA) at the equal volume to isolate MNCs by density gradient centrifugation. The suspension was centrifuged at 1500 rpm, 4 ℃ for 30 min (Buhring *et al.,* 2007). The 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 repeat twice and then the supernatant was discarded. The cell pellet was resuspended in The 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. The MNCs yield was determined using a hemocytometer under a light microscope (Black *et al.,* 2007).

Isolation of mononuclear cells from adipose tissues (Yoshimura *et al.*, 2007)

Subcutaneous adipose tissue was excised at the hip region and weighed. The tissue was washed in PBS with 1% v/v penicillin/streptomycin twice. The tissue was minced, and digested with collagenase type I (1 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) in incubator for one h at 37 ℃ and periodically shaken every 15 min. The cell suspension was filtered through the Steriflip Unit (Millipore Corporation, Billerica, MA, USA) and then added with α-MEM supplemented with 20% heat inactivated FBS and 1% v/v penicillin/streptomycin at the equal volume to stop the reaction of collagenase type I. The cell pellet was obtained after centrifuged at 3000 rpm for 10 min at room temperature (Krampera *et al.*, 2007). The supernatant was discarded. The 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. The MNCs yield was determined using a hemocytometer under a light microscope.

Counting cells with a hemocytometer

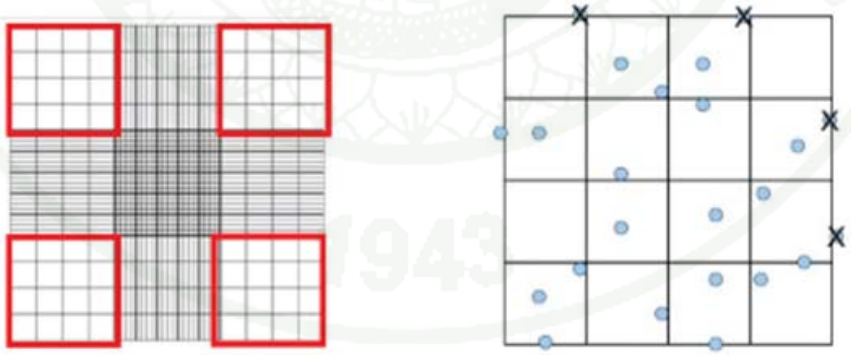

Figure 2 The hemocytometer is a simple method to enumerate MNCs by counting MNCs in the four squares (red line).

After transferred 100 µl of cells suspension into a chamber on the hemocytometer, the MNCs were counted in the four corner squares under a light

microscope. The cells which touched the right line and upper line were eliminated, the cells which located in the small sixteen squares and touched the left and bottom line were counted (Figure 2). The average cell number from four large squares was calculated and the MNCs concentration was then calculated by using the following formula.

Total number of nucleated cells/ml

$$= \text{average cell count per square} \times \text{dilution factor} \times 10^4$$

$$\text{Dilution factor} = \frac{\text{ratio of final volume}}{\text{aliquot volume}}$$

Culture of canine MSCs

MNCs from the bone marrow and adipose tissue were plated at densities of 10^6 and 10^4 cells/100-cm^2 dish relatively in triplicate. The cell cultures were incubated at 37 ºC with 5% CO_2. After 24 h of incubation, every dish was washed twice with PBS to remove non-adherent cells and adherent cells were maintained for 10 days in α-MEM supplement with 20% heat-inactivated FBS and 1% v/v penicillin/streptomycin with medium exchange every 3 days. Number of adherent cells was counted at a 100X magnification of under an inverted microscope (Yoshimura *et al.*, 2007).

Colony-Forming Assays

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 (Kassis *et al.*, 2006). Subsequently, the cells were stained with 0.5% crystal violet in methanol for 5 min at room temperature and washed twice with distilled water. Then the visible and intensely stained colony was counted while the colony whose the diameter was less than 2 millimeters and stained

faintly was ignored. The colony formation unit was calculated (Yoshimura *et al.*, 2007). Colony-forming efficiency (CFE) was calculated by using the formula modified from Wang, M. et al. (Wang *et al.*, 2010).

$$\text{Colony forming efficiency} = \frac{\text{The number of colonies} \times 10^6}{\text{Initial cell number}}$$

Statistical Analysis

Data were presented as mean ± standard deviation (S.D.). A paired t test was used to compare the colony number per MNCs and colony number per adherent cells from adipose tissue and bone marrow. Differences were considered significant at $p < 0.05$. Correlation between age and colony number per adherent cells, gender and colony number per adherent cells were made using Spearman's rank-order correlation test. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Results

Densities of mononuclear cells isolated bone marrow and adipose tissue

The nucleated cell yield from bone marrow of femoral heads ranged from $1.2x10^6$ - $10x10^6$ cells. Average cell numbers were $4.6x10^6$. Densities of MNCs per gram of subcutaneous adipose tissue ranged from $6.3x10^4$ up to $33x10^4$. After plating overnight, adherent cells were enumerated in each culture plate. Average numbers of adherent cells per 10^6 nucleated cells from bone marrow were 100.9±45.9 (mean ± S.D.) whereas those from adipose tissue were 26550±14079.79 (mean±S.D.). It showed significant differences between ratios adherent cells per mononuclear cells between these two sources (Figure 3).

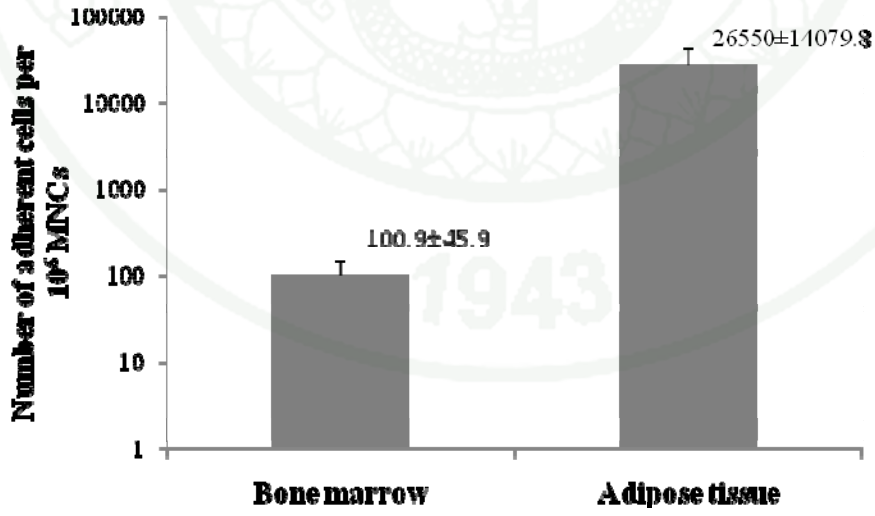

Figure 3 Adherent cell numbers per 10^6 nucleated cells; Adherent cells were counted at day 1 after plating. Data was showed as mean±S.D.; $p<0.05$ (n=10).

Morphology of mononuclear cells and adherent cells

MNCs isolated from bone marrow of femoral head and adipose tissue appeared as round cells with heterogenic by cell size attached to plastic plate surface within 24 hours after initial plating (Figure 4). Heterogeneity of the observed as some adherent cells became fibroblast-like cells. This occurred after 3-5 days in the culture of adipose tissue-derived cells and 5-7 days in that of the bone marrow-derived cells (Figure 5). At these time-points adherent cells from both cell sources exhibited identical morphology appearing elongated and spindle-shaped with long processes. As the culture proceeded, the cell of both sources increased in densities and formed the monolayer after 4-7 days and 5-7 days of incubation for adipose tissue and bone marrow relatively (Figure 6).

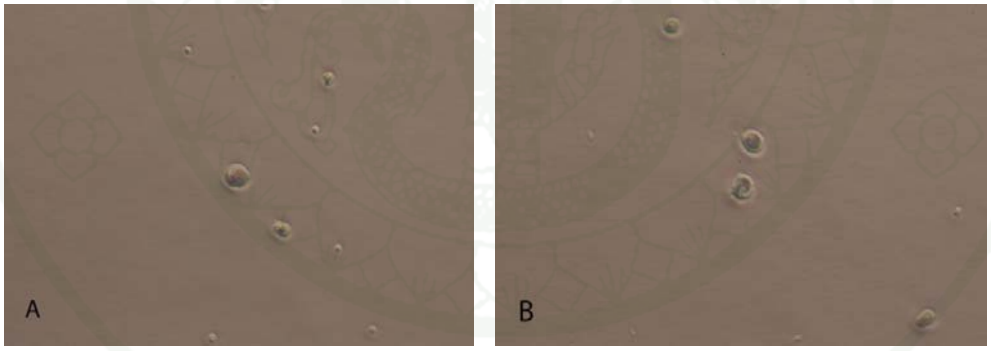

Figure 4 Adherent mononuclear cell at day 1 post-plating. The cells derived from both bone marrow (A) and adipose tissue (B) were spherical and contained heterogeneous cell sizes; magnification x400.

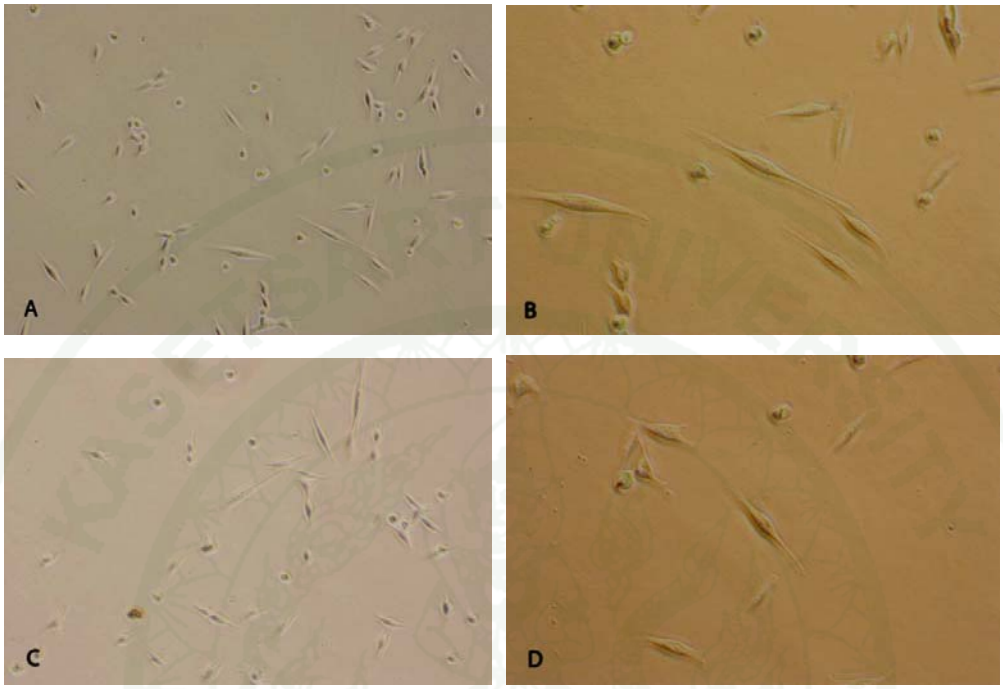

Figure 5 MSCs obtained from bone marrow (A, B) and adipose tissue (C, D). The spindle-shaped morphology was observed in cells from both sources at day 5 post-plating; magnification x200 (A, C) and magnification x400 (B, D) relatively.

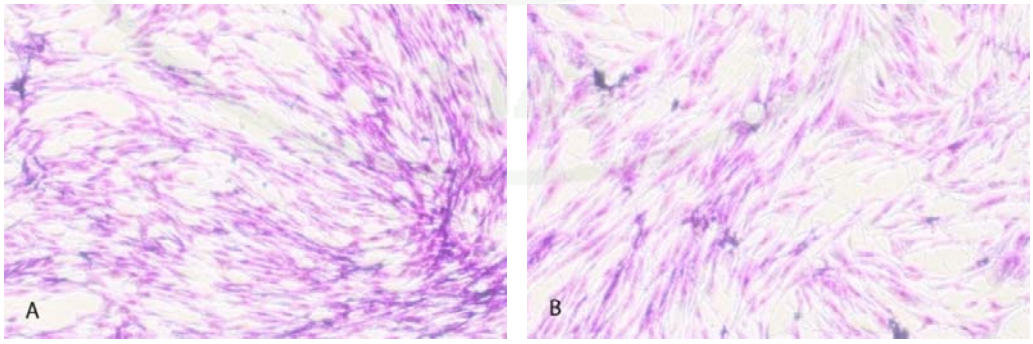

Figure 6 MSCs monolayer from bone marrow-derived (A) and adipose tissue-derived (B) MNCs at day 7 of incubation.

Colony Formation

The colony formation was detected within 5-7 days after initial plating for adipose cells and 10-14 days for bone marrow (Figure 7). However, some replicates from bone marrow-derived MNCs did not form colonies. Under microscope, colonies were composed of–spindle-shaped cells and some polygonal cells. Colonies were found evenly distributed. No difference in morphology was observed between those two sources. The colonies' size showed the range between 2-7 mm in diameter. An increase in colonies' size was observed throughout incubation period.

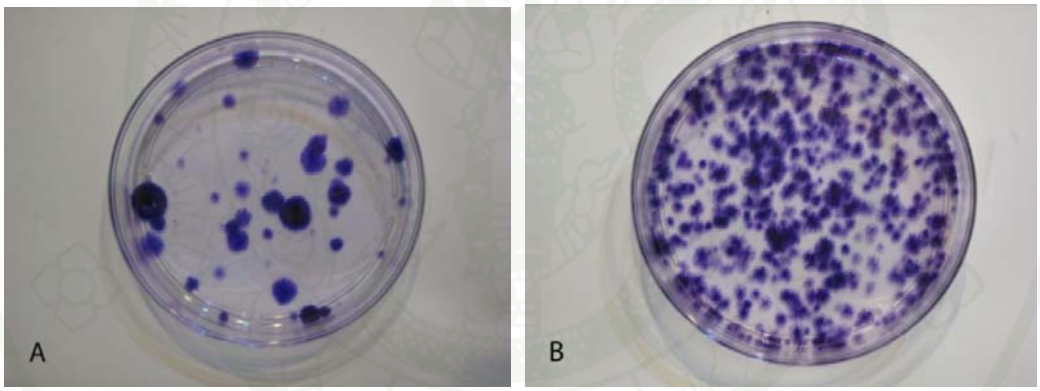

Figure 7 Colony formation of MSCs: (A) MNCs derived from bone marrow were plated at 10^6 cells/100-cm^2 dish and (B) those derived from adipose tissue which plating at 10^4 cells/100-cm^2 dish. Both were cultured for 10 days and then were observed the colony number by stained with 0.5% crystal violet in methanol. All samples formed CFU-F but some replication did not.

Clonal efficiency

Average colony numbers per 10^6 MNCs from adipose tissue was 8200±5731.98 (ranged between 1000-20200) whereas that from bone marrow was 14.8±13.68 (ranged between 0-31). Colony number per adherent cells derived from bone marrow and adipose tissue were not influenced by ages and gender ($p>0.05$) (Appendix B).There was a significant difference in colony numbers per 10^6 MNCs between these two sources ($p<0.05$) (Figure 8). Average colony number per 100 adherent cells from adipose tissue was 37.05±25.78 (ranged between 5.1-74) while that from bone marrow was 16.31±15.79 (ranged between 0-38.85). The similar results was found as the colony number per 100 adherent cells of adipose tissue were also higher than which of bone marrow significantly ($p<0.05$) (Figure 9).

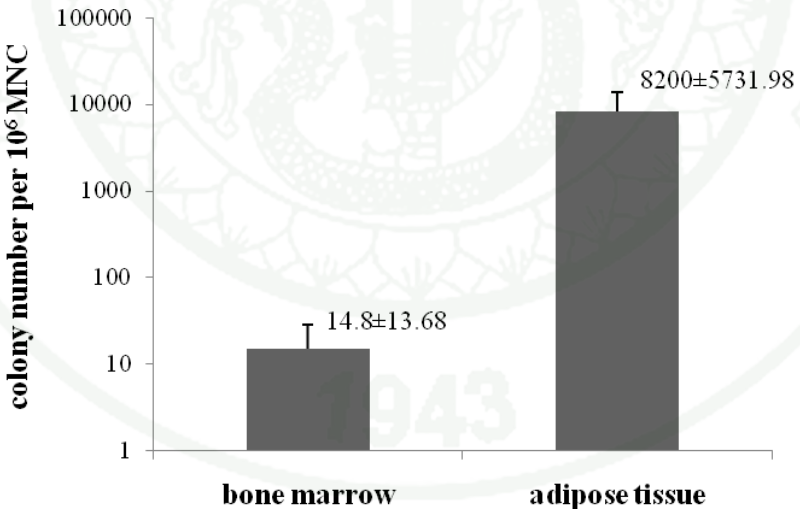

Figure 8 The colony number per 10^6 MNCs; the initial 10^6 bone marrow MNCs and 10^4 adipose MNCs were cultured in 100-cm^2 dishes for 10 day and stained with crystal violet to count the colonies. The colony number per 10^6 MNCs was evaluated from both sources. Data was showed as means±S.D.; $p<0.05$ (n=10).

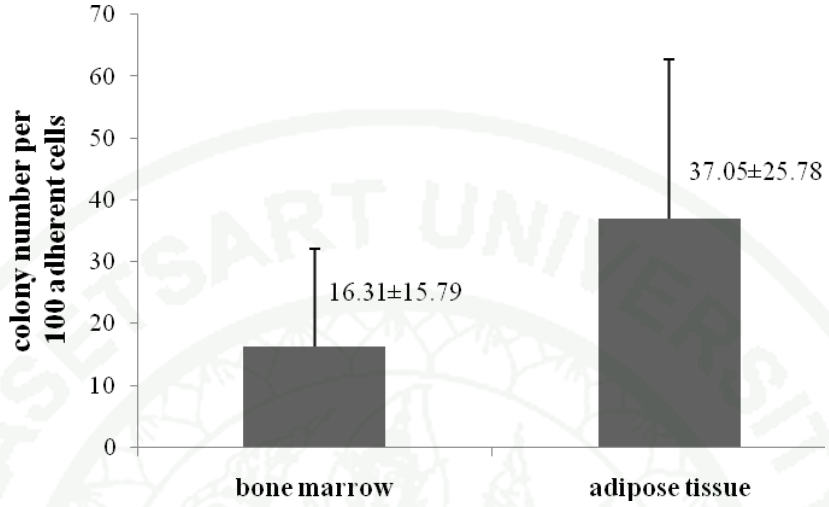

Figure 9 The colony numbers per 100 adherent cells; numbers of adherent cells were counted after one day plating. The cell colonies were counted at day 10 post-plating. Data was showed as means±S.D.; $p<0.05(n=10)$.

Discussion

Cell yields from bone marrow of femoral head and subcutaneous adipose tissue

To identify MSCs population within isolated MNCs, we used the colony formation as an indicator (Dominici *et al.*, 2006), although other characteristic i.e. expression of specific surface markers were not identified. Thus, the ratios of plastic adherent cells per nucleated cell number in our study may be related the frequencies of MSCs in tissues. The frequency of adherent cells per nucleated cells isolated from bone marrow of femoral heads in canine was small amount (100.9±45.9; mean±S.D.), whereas, adherent cells per nucleated cells isolated from adipose tissue was much significant higher (26,550±14,079.79; mean±S.D.). These corresponded to the previous report in rat which about 300 and 30,000 adherent cells per 10^6 MNCs from bone marrow and adipose tissue, respectively (Yoshimura *et al.*, 2007).

Clonal efficiency

Colony forming capacity indicates an ability to expand *in vitro* of MSCs using CFU-F assay (Alt *et al.*, 2011;Dominici *et al.*, 2006;Rojewski *et al.*, 2008;Stolzing *et al.*, 2008). Clonal efficiency is defined by numbers of colony generated per nucleated cells and per adherent cells. The CFU-F assay is a simple method that characterized the MSCs by the establishment of discrete colonies of fibroblast-like cells initiated by single cells. Colony-forming efficiency was calculated by the ratio of the number of visible colonies to the number of initial cells plated. Several factors may influence the colony-forming efficiency include medium formation, cell seeding density, culture surface substrate, physiochemical environment i.e. oxygen concentrations, carbon dioxide concentrations, temperature and pH along with culture period, age, gender and strains (Xu and Han, 1990). In this study, we compared the colony forming ability of canine MNCs which derived from two sources, bone marrow and adipose tissue, of the same donor to minimize some biological factors that might affect densities and qualities of the cells such as ages (Friedenstein *et al.*, 1976). According to our result both colony number per adherent cells and colony number per MNCs obtained from both sources had high ranges, suggesting there must be some biological factors that affect colony formation ability of MNCs. However, it was proved that no correlation between colony numbers per adherent cells and per MNCs with ages and gender was found. Therefore it might suggest that breed or individual donor's health status also affect cloning efficiency (Peister *et al.*, 2004). Cell signaling pathway such as the Wnt and their downstream signaling pathway was reported in playing an important role in the regulation of MSCs proliferation (Etheridge *et al.*, 2004;Ling *et al.*, 2009).

Several reports have been revealed that human and mouse MSCs of different sources have different abilities on *in vitro* expansion and also differentiation potency (Musina *et al.*, 2005;Yoshimura *et al.*, 2007). In dogs, the adherent cells and MNCs isolated from subcutaneous adipose tissue produced much higher colony numbers per MNCs and colony numbers per adherent cells of MSCs than those derived from bone marrow (Figure 8 and 9). This finding added one more piece of evidence that tissue-

specific MSCs exist. It is possible that tissue-specific MSCs are likely defined due to the distinction of growth ability of MSCs from different tissues (Baksh *et al.*, 2007;Hwang *et al.*, 2009;Zhu *et al.*, 2008). There were a study indicated that the presence of CD271, a neurotrophin receptor, was related to the CFU-F activity, however, the function of CD271 on MSCs still remained incompletely understood (Battula *et al.*, 2009;Kuci *et al.*, 2010;Rogers *et al.*, 2008). Moreover, the different expression of other surface markers between tissue sources of MSCs was observed. Adipose-derived MSCs expressed CD49d (integrin-α4) but bone marrow-derived MSCs did not, while CD106 (VCAM-1) expressed on bone marrow-derived MSCs but negative on adipose-derived MSCs (Zuk *et al.*, 2002). The higher expression of CD54 (ICAM-I) was found on adipose-derived MSCs compared to MSCs from bone marrow (De Ugarte *et al.*, 2003). These might be related with the endogenous signal of MSCs via the expression of cytokines including interleukin, macrophage and granulocyte colony stimulating factor (G-CSF and M-CSF), and stem cell factor (SCF) which enhance the proliferation, migration, and differentiation of MSCs (Majumdar *et al.*, 2000). The report of down regulation of CD106 expression in the presence of G-CSF and SCF was performed (Levesque *et al.*, 2001). It is interesting to determine that the presence of MSCs cytokines may affect up- or down-regulation of surface marker expression of MSCs themselves.

Periods of colony formation of MSCs are related to growth rates of the cells and colony formation ability as well. It has been showed in many species that MSCs derived from different tissue sources had different growth rates and formed colony at different time period (Kern *et al.*, 2006;Shetty *et al.*, 2010). The previous study in canine reported that colony formation from bone marrow derived MSCs was observed in 3-5 days after plating (Csaki *et al.*, 2007), whereas cells from adipose tissue was not indicated. However, other species including rat and human showed that adipose derived MSCs formed colonies within 5-7 days (Sakaguchi *et al.*, 2005;Xu *et al.*, 2010). Our study showed that canine subcutaneous adipose tissue derived MSCs formed visible colony in shorter periods of time than bone marrow derived-MSCs as the colony formation was found within 5-7 days after initial plating for adipose tissue and 10-14 days for bone marrow. These assumed that the colony formation rate of

canine MSCs derived from bone marrow was lower than that of MSCs derived from adipose tissue which similar to the study in rat (Yoshimura *et al.*, 2007). This may related to cell doubling time of MNCs that were different between tissue-specific MNC populations. The previous study in canine indicated that doubling time of adipose tissue derived MSCs is shorter than bone marrow derived MSCs which is 2.4±0.3 days and 3.3±0.6 days respectively (Spencer *et al.*, 2012).

For CFU-F assay, the initial cell seeding density determines the clonal efficiency and rate of expansion of MNCs (Colter *et al.*, 2000;Fossett and Khan, 2012;Fossett *et al.*, 2012). The optimal initial cell densities were varied among tissue sources however low densities of seeding were preferable. To date optimal initial cell densities of various species except canine were reported by many researchers. Therefore, Yoshimura et al. (2007) method that used for mouse MNCs cultures was adopted. This protocol suggested the initial nucleated cell number plating of rat bone marrow was at 10^6 cells/60-cm^2 dish, while those from synovium, periosteum, and adipose tissue was at 10^4 cells/60-cm^2 dish. These numbers were similar to the study of Sakaguchi et al. (2005) which indicated that the optimal initial nucleated cell density of human bone marrow should plate at 10^3-10^4 cells/cm^2 and other mesenchymal tissues such as adipose tissue, synovial tissue, periosteum, and muscle should plate at 10^3-10^4 cells/60-cm^2 dish. Likewise another study used initial plating the MNCs at 10^3-10^4, 10^6, and 10^2-10^3 cells/cm^2 for human bone marrow, umbilical cord blood, and adipose tissue, relatively (Kern *et al.*, 2006). These indicated that our initial cell seeding densities for both adipose tissue and bone marrow are suitable for cloning expansion.

Isolation method and MNC yield

The method of MSCs isolation is considered a main factor for retrieving appropriate MSCs yield, density gradient-based cell separation and collagenase digestion were used to collect MNC from bone marrow and subcutaneous adipose tissue, relatively. Yoshimura *et al.* (2007) showed that the expansion and

differentiation properties of MSCs were not affected by these two separation method. So this may support our comparison between bone marrow-derived MSCs and adipose-derived MSCs that difference in clonal expansion between BM-derived and adipose-derived adherent cells in our study was not caused by differnt isolation methods.

Density gradient-based cell separation of bone marrow derived MNCs is a conventional method due to its high efficiency of isolation. Nevertheless, using a Ficoll gradient to separate bone marrow MNCs may cause a relevant loss of MNCs compared to the initial MNC concentrations in native bone marrow or by using Percoll gradient protocol, however, the difference was not significant (Bourzac *et al.*, 2010;Kasten *et al.*, 2008). On the contrary, another study found that isolation with the Ficoll protocol was higher MNCs yield than the native bone marrow but with no significant difference. One study indicated the correlation between the volume of bone marrow and the initial number of MNCs was obviously performed (Isaikina *et al.*, 2008). In our study average nucleated numbers from flushing bone marrow of one femoral head can be as high to $4.48x10^6 \pm 3.53x10^6$ MNCs while total cell numbers isolated from bone marrow aspiration in dogs can be as high as $3.4\times10^7 \pm 1.6\times10^7$ MNCs (Crovace *et al.*, 2008). This difference may be caused by volumes of bone marrow that were obtained. Bone marrow aspiration in dogs, at least 10 ml bone marrow was retrieved but the volumes of bone marrow in each femoral head were less than 1 ml (personal observation). Despite of this difference, the ratio of bone marrow MNC yield between our study and the previous report was only 1:7.56. It suggested bone marrow femoral head can give appropriate numbers of MNCs that can be further investigated and bone marrow MNCs are well preserved after this method.

Culture technique

Difference in culture condition and techniques, and differences in FBS preparation and lot contribute to proliferation rate variability (Caplan, 2005;Kamishina *et al.*, 2008b;Neuhuber *et al.*, 2008). Several different basal media

were studied for optimal culture condition of MSCs. Dulbecco's modified Eagle medium low glucose (DMEM-LG), DMEM high glucose (DMEM-HG), DMEM-knock out (DMEM-KO), DMEM/Ham's F-12 (DMEM/F12) and α-MEM were indicated able to support MSCs expansion and long-term growth (Ji *et al.*, 2009;Pal *et al.*, 2009). The comparison between two culture media, α-MEM and DMEM, was indicated that α-MEM medium have higher efficiency rate than DMEM upon cell plastic adherence capacity, viability and proliferation capacities, and distinct colonies with fibroblast-like morphology (Laura *et al.*, 2008). Later, DMEM-KO and DMEM/F12 supplemented with 10% FBS were demonstrated to be an optimal condition for MSCs growth till 30 passages, while DMEM-LG and DMEM-HG failed to support human MSCs growth since 10 and 5 passages relatively (Totey *et al.*, 2009). However, the most optimal exact medium for optimal expansion of MSCs is not indicated. Our study which cultured with α-MEM supplemented with 20% FBS also showed appropriate cell growth.

Animal serum such as FBS which provides hormonal and growth factors, transport proteins, fatty acids, minerals and trace elements is usually added into a basal culture media to support cell growth (Gstraunthaler, 2003). In this study, we used 20% FBS because 10% FBS which had been used in our preliminary study did not support sufficient MSCs growth. Castro-Malaspina *et al.,* was also reported the relationship between growth and concentration of fetal calf serum (FCS) in human bone marrow MSCs. Their study found that at 5% concentration FCS, the growth of MSCs was stimulated and then reached the highest growth stimulation at 20% concentration (Castro-Malaspina *et al.*, 1980). However, the risk of prion diseases transmission and zoonoses from animal's serum were certainly concerned for complete clinical application. Xenogenic protein could not completely eliminated by washing MSCs which were cultured in media supplemented FBS, so several serum-free media were attempted with the addition of cytokines and growth factors (Totey *et al.*, 2009).

CONCLUSION AND RECOMMENDATION

Conclusion

CFU-F assay is a standard method to characterize MSCs which indicate an ability of the cells to expand in culture. Frequencies of adherent cells per nucleated cells were evaluated to show some characteristic of MSCs. However, the ideal techniques to identify MSCs in tissue sources are Magnetic Activated Cell Sorting and Fluorescent Activating Cell Sorting even though no available of specific marker of MSCs (Raynaud *et al.*, 2012). Related to our study, MNCs derived from subcutaneous adipose tissue showed colony number per nucleated cells and colony number per adherent cells higher than MNCs derived from bone marrow. So we conclude that subcutaneous adipose tissue MNCs has higher potential of expansion and proliferation which suit for *in vitro* production and stem cell banking.

Recommendation

Our study is a preliminary study to demonstrate the suitable source of MSCs for *in vitro* culture and stem cell banking. However, there are other sources which were interested. Synovium was indicated a source of high proliferation and differentiation potential of MSCs in human and rat (De Bari *et al.*, 2003;Ju *et al.*, 2008;Sakaguchi *et al.*, 2005). In addition, umbilical cord blood derived MSCs 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). It's interesting to study about factors that play a role in MSC adhesion, expansion, migration, proliferation and maintenance of plasticity of MSC. For the substitute from using of animal's serum media, molecules and their sufficient concentration which promote optimal MSCs proliferation and differentiation should be investigated.

Furthermore, the investigation of concentration and route of transplantation of MSCs should be performed prior to the clinical application. Moreover, while the autologous MSCs transplantation has been widely studied and successful reported in improvement the function of an injured musculoskeletal, the allogeneic MSCs transplantation still has to be evaluated the safety and effectiveness.

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APPENDICES

Appendix A

Solution Preparation

1. Cell culture media (alpha-MEM + 20% FBS)

   1000 ml contain

Minimum essential medium eagle alpha powder	10.2 g
NaHCO3	2.2 g
Distilled water	1 L

   Added powdered medium to 1 L volumetric flask filled with 990 ml sterile distilled water and gentle stirred. Then, added 2.2 g of $NaHCO_3$ and dissolved well. Sterile distilled water was then added to final volume and adjusted medium pH to 7.0 with HCl and NaOH. Membrane filtration was used to sterilize the medium, after that the pH units rose to 7.2.

2. 1 mM Phosphate buffer saline (PBS)

   1000 ml contain

NaH2PO4	15.6 g
Na2HPO4	26.8 g
NaCl	9 g
Distilled water	1 L

   Weighted out each substance and transferred into volumetric flask. 1 L of distilled water was then added and mixed well. The pH value was adjusted to 7.2 with HCl and NaOH. Finally, the PBS was sterilized by autoclave at 121ºC for 1-2 h.

3. 4% Paraformaldehyde fixative

   100 ml contain

Paraformaldehyde	4 g
PBS	100 ml

   Weighted out 4 g of paraformaldehyde and transferred into 100 ml conical flask. 100 ml PBS was measured into a measuring cylinder and then poured 90 ml PBS into the conical flask containing 4 g of paraformaldehyde. Dissolved it well by using the stir bar under the heat about 58-60ºC under a hood. Added 5N NaOH to raise the pH until the solution was clear. After the

formaldehyde was dissolved, the solution was removed from heat and added PBS until the final volume reached 100 ml.

4. 0.5% Crystal Violet

   10 ml contain

Crytal violet	0.05 g
Methanol	2.5 ml
Distilled water	7.5 ml

Weighted out 0.05 g of crystal violet (Sigma-Aldrich, St.Louis, MO) and dissolved it with 2.5 ml methanol in 15 ml conical tube. After that, 7.5 ml distilled water was added to 10 ml final volume of 0.5% crystal violet.

Appendix B

Correlation Analysis

Spearman Correlation Coefficients, N = 10
Prob > |r| under H0: Rho=0

	FATMNC	BMMNC	FATCFU	BMCFU	FATCO	BMCO
AGE	-0.27131	-0.32078	-0.36657	-0.00631	-0.20503	-0.03145
	0.4483	0.3662	0.2975	0.9862	0.5699	0.9313
SEX	0.00000	0.21583	0.42640	-0.07217	0.14213	0.14389
	1.0000	0.5493	0.2191	0.8430	0.6953	0.6917

Pearson Correlation Coefficients, N = 10
Prob > |r| under H0: Rho=0

	FATMNC	BMMNC	FATCFU	BMCFU	FATCO	BMCO
WT	0.60171	0.47127	-0.45474	-0.61776	-0.76926	-0.79120
	0.0657	0.1692	0.1867	0.0570	0.0093	0.0064
FATWT	0.54604	0.61406	-0.17194	-0.62833	-0.55066	-0.63109
	0.1025	0.0589	0.6348	0.0517	0.0990	0.0504
FATMNC	1.00000	0.72354	0.29195	-0.23149	-0.15881	-0.34043
		0.0180	0.4131	0.5199	0.6612	0.3358
BMMNC	0.72354	1.00000	0.33342	-0.27929	-0.04973	-0.31327
	0.0180		0.3465	0.4345	0.8915	0.3781
FATCFU	0.29195	0.33342	1.00000	0.18014	0.62438	0.29681
	0.4131	0.3465		0.6185	0.0536	0.4050
BMCFU	-0.23149	-0.27929	0.18014	1.00000	0.82360	0.89578
	0.5199	0.4345	0.6185		0.0034	0.0005
FATCO	-0.15881	-0.04973	0.62438	0.82360	1.00000	0.89275
	0.6612	0.8915	0.0536	0.0034		0.0005
BMCO	-0.34043	-0.31327	0.29681	0.89578	0.89275	1.00000
	0.3358	0.3781	0.4050	0.0005	0.0005	

WT = weight

FATWT = fat weight

FATMNC = adipose derived MNCs

BMMNC = bone marrow derived MNCs

FATCFU = colony number per nucleated cells from adipose tissue

BMCFU = colony number per nucleated cells from bone marrow

FATCO = colony number per 100 adherent cells from adipose tissue

BMCO = colony number per 100 adherent cells from bone marrow

Appendix C

Flow Cytometry

MATERIALS AND METHODS

Animals

Four dogs including Shih Tzu, Pomeranian and Crossbred with hip luxation which required the femoral head and neck excision at the Veterinary Teaching Hospital, Kasetsart University were selected. All dogs, aged range between 2-4 years and weighed range between 2-4 kg but only one dog was 30 kg, were examined without other complications. Aseptic technique was used to collect subcutaneous adipose tissue and femoral head from each dog.

Flow cytometry

After MNCs were isolated from subcutaneous adipose tissue and bone marrow from femoral head, the MNCs was then washed with PBS twice and resuspended in PBS. The cell suspension from each source was divided into four aliquots. The first one was used as a negative control. Three were stained with fluorescein isothiocyanate (FIT-C) coupled antibodies against CD105 (P17813; Millipore, Bedford, MA, USA) and CD172a (DH59B; VMRD, Pullman, WA, USA) and phycoerythrin (PE) coupled antibody against CD34 (1H6; Abcam, Cambridge, UK), respectively. After 15 min incubation on ice, the cells were washed with PBS 3 times and then resuspended in 1 ml PBS. The cell suspension was kept at 4ºC overnight for analysis at Lerdsin Hospital. Cell fluorescence was evaluated by flow cytometry (Becton Dickinson) and data was analyzed by using CellQuest software (Becton Dickinson).

RESULTS

Results

Appendix Table B1 Number of MNCs per ml (MNCs/ml) and the percentage of positive fluorescent markers including CD105, CD172a and CD34 which isolated from bone marrow of femoral head.

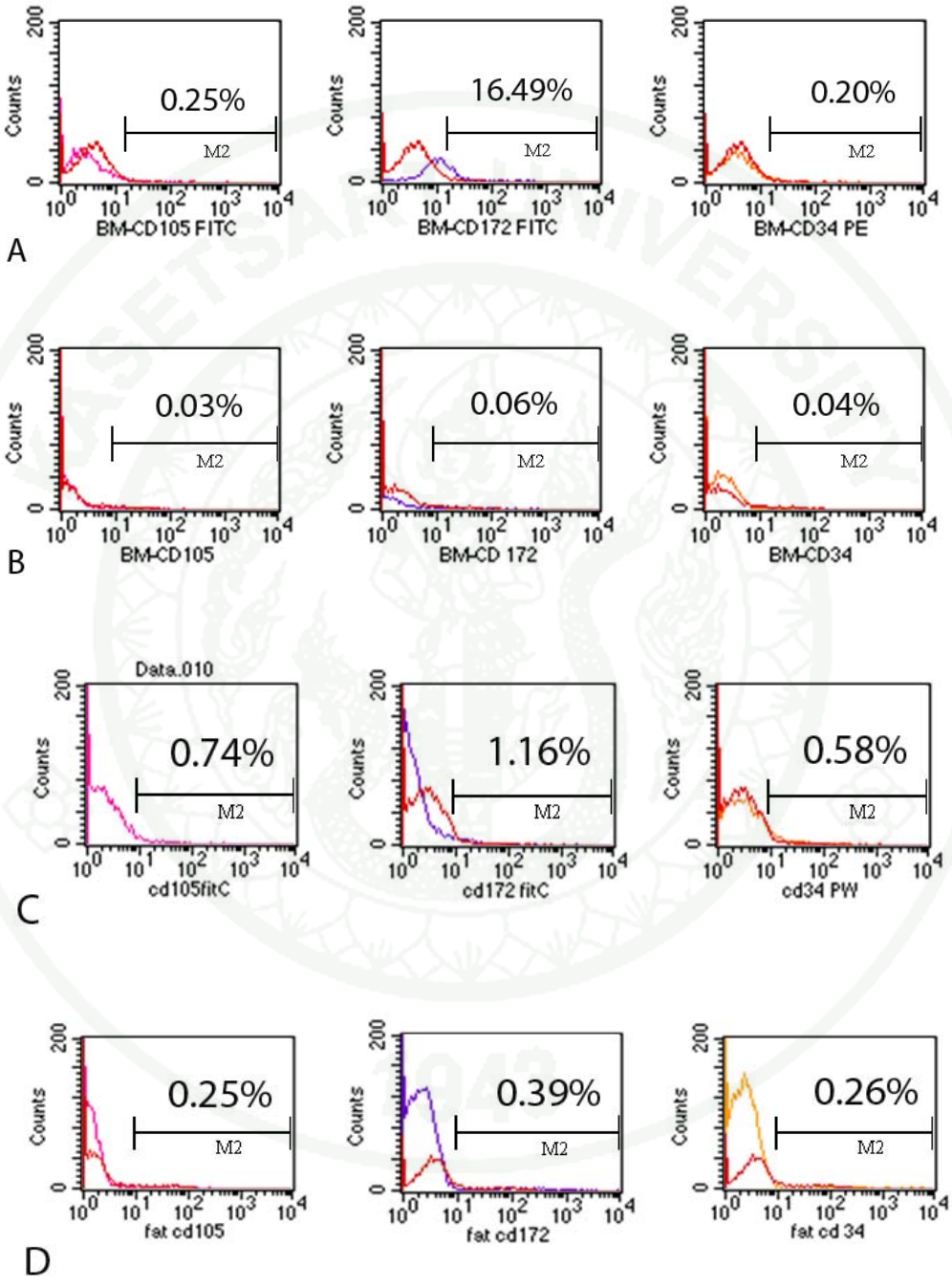
Dog	Age (year)	Weight (kg)	MNCs/ml	CD105	CD172a	CD34
1	4	2	$1.96x10^6$	0.25%	16.49%	0.20%
2	2	2.2	$3.00x10^6$	0.03%	0.06%	0.04%
3	3	30	$8.71x10^6$	0.74%	1.16%	0.58%
4	4	4	$1.88x10^6$	0.02%	0.50%	1.59%

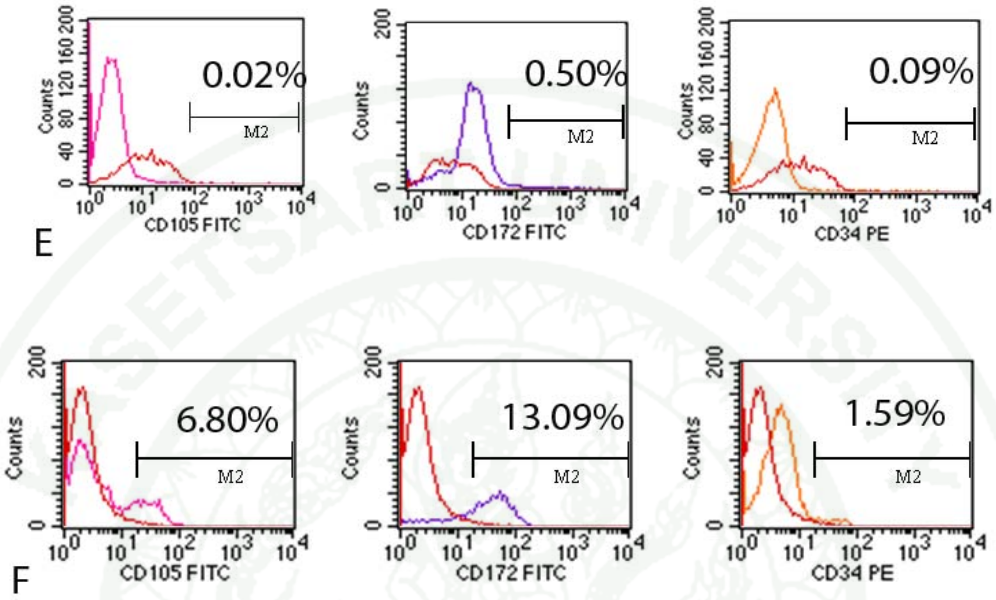
Appendix Table B2 Number of MNCs per ml (MNCs/ml) and the percentage of positive fluorescent markers including CD105, CD172a and CD34 which isolated from subcutaneous adipose tissue.

Dog	Age (year)	Weight (kg)	MNCs/ml	CD105	CD172a	CD34
1	4	2	$6.00x10^4$	-	-	-
2	2	2.2	$4.50x10^4$	-	-	-
3	3	30	$1.38x10^6$	0.25%	0.39%	0.26%
4	4	4	$1.07x10^6$	6.80%	13.09%	1.59%

Immunophenotypic characterization of MSCs derived from bone marrow and subcutaneous adipose tissue using flow cytometry

The first two adipose tissue samples which collected only small amount of MNCs were excluded from analyze by flow cytometry because of low quantity of MNC count. Histogram results from flow cytometry characterization of each sample which were stained with anti-CD105, -CD172a and –CD34 compared with negative control were shown with the percentage of positive markers. The results showed very low percentage of all markers and could not confidentially conclude that the positive-stained against CD105 and CD172a was MSCs. The positive-stained against CD34 expression was also shown in low percentage.





Appendix Figure B1 Flow cytometric analysis of mesenchymal tissue-derived cells, bone marrow (A, B, C and E from four dog) and adipose tissue (D and F from the third and fourth dog), stained with FIT-C coupled antibodies against CD105 and CD172a and PE coupled antibody against CD34. The area under the red line showed as a control (x-axes intensity log values, y-axes cell counts).

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