Canine Infra-patellar and Subcutaneous Adipose Tissue Derived Multipotent Stromal Cells have Similar In Vitro Behavior Before and After Cryopreservation

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Introduction: A key factor in tissue engineering is the source of multipotent stromal cells (MSCs) [1, 2]. Adipose tissue is established as a source of MSCs that has a high density of undifferentiated cells with characteristics similar to those isolated from bone marrow [3, 4]. However, adult adipose tissue derived multipotent stromal cells (ASCs) are not identical among adipose deposits. Due to differences in anatomy and physiology between brown and white adipose tissues, cells harvested from them may have different phenotypes, expansion capabilities and plasticities. Additionally, cryopreservation may influence cell behaviors differently depending on the adipose tissue source. Infra-patellar adipose tissue is largely white while subcutaneous adipose over the gluteal muscles is primarily brown adipose tissue. This investigation was designed to test the hypothesis that canine ASCs isolated from infra-patellar (IFP) adipose tissue have greater MSC immunophenotype percentages, in vitro expansion rates, and multipotentiality that is sustained over multiple cell passages compared to ASCs from subcutaneous (SUB) adipose tissue.

Methods: Subcutaneous (SUB) and infrapatellar (IFP) adipose tissues were harvested from 6 adult dogs (3.0 ± 0.5 years) and cells were isolated from them using standard type I collagenase digestion. After reaching 80%-90% confluence, passage 0 (P0) cells were cultured at 5000 cells/cm2 as were all subsequent passages. P0 aliquots of each cell isolate was cryopreserved for 1 month, revitalized and then evaluated as described below. Doubling times (DT) and cell doublings (CD) were calculated according to standard methods for cell passages (P) 0-3 before and 1-3 after cryopreservation. For the same cell passages, limiting-dilution assays were used to determine fibroblastic (CFU-F), adipogenic (CFU-Ad), and osteoblastic (CFU-Ob) colony forming unit frequencies. The CFU-F was determined after cells were cultured in stromal medium for 7 days, and CFU-Ad and - Ob after 21 days of culture in adipogenic (oil red O staining) and osteogenic (alizarin red staining) differentiation medium, respectively. Wells were considered positive with ≥10 colonies. For chondrogenesis, cell pellets were cultured in chondrogenic medium for 21 days, fixed, embedded in paraffin, sectioned and stained with alcian blue and nuclear fast red. Percentages of cells expressing CD29, CD34, CD44, and CD90 were determined for P0, P1, and P3 fresh and P1 and P3 revitalized cells with flow cytometry. Both mRNA and protein were isolated for RT-PCR and western blot analysis of lineage-specific target gene mRNA (PPARγ and leptin - adipogenesis; osteoprotegerin and collagen I - osteogenesis) and protein (osteopontin - osteogenesis, PPARγ-adipogenesis, SOX2 - progenitor, CD29, CD34, CD44, and CD90 - MSC surface makers) expression for the same passages. All outcome measures were assessed with ANOVA models to quantify effects of cell tissue source, passage and cryopreservation. Significance was considered at P<0.05.

Results: The CDs and DTs of fresh and revitalized IFP and SUB ASCs decreased with passage (Fig. 1a). The CDs were significantly lower for P0 SUB ASCs versus P0 IFP ASCs and the CD was significantly lower for
P3 revitalized ASCs versus P3 fresh ASCs from both tissue sources. Fresh and revitalized IFP and SUB ASCs showed robust adipogenesis and osteogenesis for all passages evaluated. SUB and IFP showed similar cell plasticity before and after cryopreservation (Fig. 1b,c). The majority of fresh cells from both tissue depots were CD29+, CD44+, CD90+, and CD34- for all passages evaluated (Fig. 2a). Cryopreserved cells had lower percentages of CD29+ cells than fresh, and CD44+ cells were significantly lower in P3 versus P1 for both tissue sources (Fig. 2a). Cell surface maker expression was confirmed with immunocytochemistry and western blot in P3 fresh and cryopreserved cells (Fig. 2b, c). Tissue specific target gene expression decreased with increasing passage (Fig. 3a) and cryopreservation diminished osteogenic-specific gene expression changes in P1 ASCs (Fig. 3A). Adipogenic (PPARγ) and osteogenic (osteopontin) target protein expression increased in P3 cells cultured in induction medium while SOX2 decreased (Fig. 3b). Additionally, SOX 2 protein expression is decreased after cryopreservation in cells from both tissues.

**Discussion:** In this study, the in vitro behaviors of two sources of canine adipose tissue derived multipotent stromal cells were investigated. Based on study findings, ASCs from fresh and cryopreserved SUB and IFP adipose tissues have similar in vitro growth characteristics and multipotentiality up to P3, but there are potential differences in cryopreservation effects on cell phenotype among tissue harvest sites. Additionally, cryopreservation appears to decrease SOX2 expression in IFP and SUB ASCS. Future studies are necessary to determine the impact of these findings on cell potential.

**Significance:** Fresh and revitalized canine ASCs from infra-patellar and subcutaneous adipose tissue are comparable cell candidates for tissue regeneration studies based on nearly identical in vitro behavior before and after cryopreservation.

![Figure 1](image-url)

**Figure 1.** a. Cell Doubling (CD) and Doubling time (DT) (mean ± SEM) of P0-3 before and after cryopreservation; b. Fresh and cryopreserved SUB and IFP ASC CFU frequencies after 7 days culture in stromal medium and 21 days culture in osteogenic, adipogenic. The frequencies decrease with increasing passage; c. Light micrographs of P 3 canine ASCs from subcutaneous adipose tissue (middle) and infra-patellar (right) adipose tissue following chondrogenic induction. Extracellular matrix formation is evident in both chondrogenic pellets compared to control.
Figure 2. a) Cell percentages (mean ± SEM) of P0, 1, and 3 SUB and IFP before cryopreservation, and P1 and P3 after cryopreservation (t) of SUB and IFP that were CD29+, CD34-, CD44+ or CD90+; b) Western blot of uninduced (stromal medium) ASCs before and after cryopreservation; c) Immunocytochemistry of P3 SUB and IFP before and after cryopreservation for cell surface markers indicated.

Figure 3. a) Fold change (mean ± SEM, n=6, 3 replicates) of adipogenic (PPAR-G, Leptin) and osteogenic (OPG, Col I) target genes in SUB and IFP ASCs after the indicated induction. Values are normalized to GAPDH and relative to cells cultured in stromal medium. Columns with different letters are significantly different within cell tissue source (p<0.05). OPG=osteoprotegerin, Col I=Collagen I; b) Western blot of uninduced (Stromal medium) and adipogenic, and osteogenic induced ASCs before and after cryopreservation. Lineage specific target protein expression (PPAR-G, adipogenesis; osteopontin (OPN), osteogenesis; Sox-2, stemness) increased following induction while SOX2 decreased. Cryopreservation decreased SOX2 expression as well.

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