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Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation

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ABSTRACT

Adult stem cells are nowadays used for treating several pathologies. A putative stem cell population was found in the adipose tissue of mammals and canine adipose tissue-derived-mesenchymal stem cells (cA-MSC) have been shown to possess the capacity to differentiate into several lineages. The main goal of our research was to fully characterize cA-MSC and examine the effects of cryopreservation on their *stemness* features. Each sample of cA-MSC was analyzed immediately and then again after being frozen in liquid nitrogen for one year. After the cryopreservation period cells conserved their fibroblast-like morphology, alkaline phosphatase positivity and CD expression but showed a lower proliferation ratio and a lower telomerase activity in comparison with fresh cells. Finally, the cryopreservation protocol did not change the cA-MSC adipogenic, osteogenic and myogenic differentiative potential. Our data demonstrate that stored cA-MSC might represent a promising type of progenitor cell for autologous cellular-based therapies in veterinary medicine.

1. Introduction

Mesenchymal stem cells (MSC) are found in several adult tissues and represent an attractive stem cell category for regenerative medicine and cell therapy purposes (Mimeault and Batra, 2008; Strauer et al., 2009). MSC possess a robust self-renewal potential, show a constant growth and a known proliferation kinetic; furthermore, MSC are able, *in vitro*, to differentiate into numerous specialized cell types (Zuk et al., 2002; Yamamoto et al., 2007). An adult mesenchymal progenitor cell population was identified in human adipose tissue and lipoaspirate (Zuk et al., 2002; Lee et al., 2004; Kern et al., 2006; Rebelatto et al., 2008). Numerous studies (Lee et al., 2004; Kern et al., 2006; Rebelatto et al., 2008) have demonstrated that human mesenchymal stem cells derived from adipose tissue (A-MSC) express classic MSC markers as CD90, CD44, CD73, CD105, CD117, CD106 and possess the same capacity of bone marrow-derived stem cells (BMSC) to differentiate into osteogenic, adipogenic and chondrogenic lineages. Moreover, less than 1% of genes are differentially expressed between A-MSC and BMSC; this data should support the notion that A-MSC and BMSC are originated from a common precursor (Bianco et al., 2008). The adipose tissue presents some advantage with respect to other investigated stem cells sources because the resulting cell numbers obtained from the isolation is very high and, indeed, it is sufficient a small chunk of fat for their isolation (Kern et al., 2006). In mammals the adipose tissue is found in different anatomical compartments and in dogs the visceral fat is particularly easy to collect because this species is subjected to a large number of ovariohysterectomies.

The plasticity of mesenchymal stem cells has made it possible to develop cell-based therapies and for this purpose it is very useful to cryopreserve these cells in order to gain a ready source of abundant autologous stem cells. Mesenchymal stem cells may be frozen to preserve their vitality and when thawed MSC have to maintain their pluripotent phenotype (Attarian et al., 1996; Gonda et al., 2008); obviously, the possible clinical application should be based on the abundance, frequency and expansion potential of the cells.

In the veterinary field the experimental stem cell therapy has been applied mainly in horses while in pets the research is still in progress (Richardson et al., 2007; Martinello et al., 2009). The dog is an important patient in veterinary medicine and it is often used as an experimental model for human diseases. Indeed, the golden retriever dog has been used as a muscular dystrophy model and was successfully treated with mesoangioblast stem cells (Sampaolesi et al., 2006).

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In this study we have analyzed specific features and plasticity of mesenchymal stem cells isolated from adipose tissue of *Canis familiaris* (cA-MSC). Furthermore, we have examined the effect of cryopreservation on cA-MSC "stemness" by means of a complete cellular characterization investigating phenotype morphology, vitality assay, telomerase activity and flow cytometry. The capacity of these multipotent cells to differentiate into adipocytes, osteoblasts and myoblasts has been compared in both fresh and cryopreserved cells. Our results demonstrate that cryopreservation alters some characteristics of *stemness* of cA-MSC although it does not cause any changes in their differentiation potential. Therefore, the opportunity to cryopreserve cA-MSC may offer the prospective for clinicians to use autologous stem cells in canine cell-based therapies.

2. Materials and methods

2.1. Cell culture

Samples were collected from visceral adipose tissue of female dogs during ovariohysterectomy surgery. The average age of the dogs spanned from 1 to 5 years; a total of 20 different samples were collected and processed. The weight of sampled adipose tissue was on average 7 g. Each sample was cleaned of large blood vessels, chopped adding 5–10 ml of phosphate-buffer saline (PBS) and incubated with collagenase I (Sigma–Aldrich S.r.l. Milan, Italy) at 40 μg/ml for 2 h at 37 °C. Subsequently, each sample was filtered with 100 μm filter and centrifuged at 1400*g* for 20 min. The supernatant was removed and the pellet washed with PBS. After a second centrifugation at 1600*g* for 10 min the supernatant was removed and the pellet was set in T25 flask (Falcon, BD Bioscience, Basel, Switzerland) in non-inductive medium consisting of Dulbecco modified Eagle's medium (DMEM D5671, Sigma–Aldrich) with 10% fetal serum (FBS) (ECS0180-L, Euroclone, Milan, Italy), containing penicillin, streptomycin (Sigma–Aldrich) and glutamine (Sigma–Aldrich). The isolated cells were put in an incubator, (Jouan IG150, labogerateborse, Burladingen, Deutschland) supplied with humidified air and 5% CO_2. After the overnight incubation, non-adherent cells and lipid droplets were removed and fresh medium was added to the flask. After three days of incubation canine adipose-derived-mesenchymal stem cells (cA-MSC) were developed, recovered, counted and set for amplification. In order to evaluate stemness characteristics of MSC both human dermal fibroblasts and HeLa cells were used as controls, respectively for differentiation studies and for telomerase assay.

2.2. Cryopreservation and thawing

cA-MSC were collected and resuspended in cryopreservation medium (90% FBS and 10% of dimethyl sulfoxide, DMSO, Sigma–Aldrich) at density of 3×10^6 cells/cryovial. Cells were frozen by Mr. Frosty (Nalgene, Roskilde, Denmark) decreasing −1 °C/min until −80 °C for 1 week and then they were transferred to the liquid nitrogen tank for long-term storage. Thawing of cells was performed after 10/12 months; cA-MSC were placed into a 37 °C water-bath for 1–2 min and washed in 10% FBS and 90% DMEM to eliminate DMSO. Finally, the cells were plated into T25 flasks with non-inductive medium.

2.3. Alkaline phosphatase

The AP (alkaline phosphatase) enzyme activity was assayed using a commercial AP detection kit (Kit SIGMA FAST BCIP/NBT; 5-bromo-4chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma–Aldrich, B5655-5TAB). In order to detect the AP enzyme activity, adipose-derived stem cells at 50% of confluence were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature.

2.4. Measurement of population-doubling time (pdt)

We defined the population-doubling time as the time required for a colony area to expand twofold. Population-doubling was determined in continual subculture and growth of cA-MSC harvested at subconfluence, and were calculated using the formula $x = [\log_{10}(N_H) - \log_{10}(N_i)]/\log_{10}(2)$ (Cristofalo et al., 1998), where N_i is the inoculums cell number and N_H the cell harvest number. To yield the pdt, the population doubling for each passage was divided by the time (days). The pdt is showed at passages 2, 4 and 8.

2.5. Telomerase assay

TeloExpress Quantitative Telomerase Detection Kit (Express Biotech International, Thurmount, USA) was used for the detection of telomerase expression. Samples ($n = 6$) were assayed with real-time PCR (ABI 7500 Real-Time PCR System) to measure the enzyme presence.

2.6. Flow Cytometry Assay (FACS)

Adipose-derived stem cells ($n = 6$) were analyzed between passage P3 and P6 using FACS technique. Cells were collected, washed with PBS 1X, and incubated for 10 min at 4 °C in PBS 1X, FCS 2%, EDTA 2 mM. Subsequently the cell suspension was incubated with the primary antibodies directed against the following cell surface markers, each of which was conjugated with phycoerythrin (PE) and conjugated with fluorescein isothiocyanate (FITC). Cells were suspended in 300 μl of PBS in FACS tubes (Falcon, BD Bioscience, Basel, Switzerland). As control mouse isotype IgG1, IgG2a and rat isotype IgG2a (BD Bioscience, Erembodegem, Belgium) were used. For each sample 200,000 cells were passed through a FACScan Canto Flow cytometric, (BD Bioscience, Erembodegem, Belgium) and results were analyzed by FlowJo software (Tree Star, Inc., Oregon Corporation, USA).

2.7. Differentiation assay

cA-MSC at 80–90% of confluence were induced to differentiate towards different lineages by appropriate culture medium.

Osteogenic differentiation: to induce adipose-derived stem cells to differentiate into osteogenic tissue was added to the culture medium 1 nM dexamethasone, (Sigma D1756, Sigma–Aldrich S.r.l. Milan, Italy, http://www.sigmaaldrich.com) 10 mM β-glycerophosphate, (1-800-854, MP Biomedicels Europe, Illkirch, France) and 50 μM ascorbate-2-phosphate (Sigma A4544, Sigma–Aldrich). Cells were maintained in differentiation medium for 21 days and in this period the medium was changed every 2–3 days. After fixation in a 4% solution of formaldehyde (PFA) for 30 min the presence of mineralizated matrix was assessed by von Kossa staining.

Adipogenic differentiation: adipogenesis was induced using culture medium (DMEM-LG D5921 Sigma–Aldrich) supplemented with 0.5 mM isobutylmethylxanthine (Sigma I5879, Sigma–Aldrich), 200 μM indomethacin (Sigma I7378, Sigma–Aldrich), 0.5 μM dexamethasone (Sigma D1756, Sigma–Aldrich), and 10 μM insulin (Sigma I6634, Sigma–Aldrich). The medium was changed every 3 days and cells were maintained in culture differentiation medium for 21 days. The formation of lipid droplets was verified with Oil Red O staining, after fixation of cells in 4% PFA solution for 10 min. *Myogenic differentiation:* adipose-derived stem cells were cultured in differentiation medium consisting of high glucose DMEM, (Sigma–Aldrich) supplemented with 2% HS