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Evaluation of the age and anatomical areas impact on the properties of canine adipose-derived multipotent mesenchymal stromal cells

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Abstract. Multipotent mesenchymal stem cells (MMSCs) are potentially promising cells for non-pharmacological therapy. We have carried out a comparative analysis of MMSCs populations isolated from different anatomical areas of canine adipose-derived tissue (subcutaneous and visceral adipose tissue) with different age (one year and ten years) characterized by immunophenotype CD44 + / CD90 + / CD105 + / CD45-. Histochemical analysis revealed that the number of cells in per unit volume of canine adipose tissue decreases with age. The number of cells derived of stromal-vascular fraction by filtration method (filters 80 microns) also revealed the dependence on the age and anatomical location of the collected adipose tissue. In dogs of one year age, the number of cells in this population was $2.4 \geq$ times more than in the experimental groups of 10 years. The necessity of collection adipose tissue from dogs of 10 years for further isolation of MMSCs has been justified, compared with dogs aged one year.

1. Introduction

In cell biotechnology, medicine and veterinary there are observed a rapidly growing interest to the multipotent mesenchymal stem cells (MMSCs) due to their regenerating and immunomodulating properties which are used with high efficiency [1, 2]. Numerous experimental data show the prospects and universality of the clinical use of MMSCs, demonstrating their significance in cell therapy [3]. They are successfully used not only for allogeneic, but also for xenogenic transplantation without immunosuppressive drugs necessity [4, 5, 6]. MMSCs are found and characterized in almost all organs and tissues of the trabecular bone and periosteum, umbilical cord blood [7] of the synovium, skin, teeth, periodontal ligament and etc. At the same time, MMSCs isolated from bone marrow (BM) [8, 9] and adipose tissue (AT) [10, 11, 12, 13] are still remain the most studied. However, for therapy MMSCs are usually isolated from AT as it has got more superior properties compared with BM. According to the International Federation for Adipose Therapeutics and Science (IFATS) and International Society of Cell Therapy (ISCT) adipose tissue as biological material, is obtained by less traumatic and safe methods for the donor. MMSCs derived from AT have a high adipogenic, osteogenic and chondrogenic differentiation potential. The concentration of MMSCs in a unit volume of material is higher in comparison with the BM while isolating. In addition, this type of cells, isolated from AT has a high proliferative capacity with a long life time in vitro. Besides, they have a shorter doubling time and more in vitro aging compared with MMSCs isolated from BM [14]. Despite the all above mentioned unique



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properties of AT-MMSCs [15, 16], their introduction into Veterinary Medicine requires more extensive laboratory research. In this regard, the **goal** of this study was to analyze the populations of the multipotent mesenchymal stem cells isolated from different anatomical areas of canine adipose tissue of different ages.

2. Materials and methods

Adipose tissue was harvested from 12 healthy mongrel dogs of different sexes subjected to elective ovariohysterectomy at the Veterinary Clinic with previous informed consent of the owners. The body mass index of all dogs in the experiment was 3 (optimal body weight) in a five-step assessment of somatic status. All animals were divided into four groups (n = 3 × 4) table 1. The first two groups consisted of animals up to one year old. Samples were collected from the subcutaneous adipose tissue and visceral region of the ventral abdomen (inguinal fold). The third and fourth group consisted of animals aged over 10 years. The same material was obtained from similar areas. All samples were obtained by a veterinarian in the operating room, by resection method following all aseptic and antiseptic rules. The sample sizes were 4 grams.

Table 1. Experimental groups of dogs.

Experimental groups of dogs			
Group 1	Group 2	Group 3	Group 4
Matured dogs of different sex, age up to 1 year		Dogs of different sex, over 10 years old	
AT Samples were collected from the subcutaneous adipose tissue (inguinal fold)	AT samples were collected from the visceral region	AT Samples were collected from the subcutaneous adipose tissue (inguinal fold)	AT samples were collected from the visceral region

For the isolation and studying the properties of obtained cells we have used a method developed earlier [17]. The calculation of cells number in per unit volume was carried out by a histochemical method, using the standard technique of lipid cells staining by Sudan III (Biochem, France). Frozen sections were prepared from the freshly adipose derived tissue fixed in ice-cold (-20° C) methyl alcohol of 99.8% (Sigma Aldrich, USA) within 15 minutes. Then the sections were placed in the dye for 30 minutes and were washed with methyl alcohol and distilled water. The nuclei were stained with Mayer's hematoxylin (Sigma-Aldrich, USA) for 15 minutes. The calculation of cells was performed by morphometric mesh. The result was analyzed by a phase contrast microscope (Carl Zeiss AG, Germany) and AxioVision Rel. 4.8. Software. The canine adipose derived tissue was exposed to the enzymatic cleavage in a solution of type I and II collagenases - 1:1 (PanEco, Russia), for 30–35 min at T = 37 ° C supplemented with serum-free DMEM-LG medium (Lonza, Belgium). The obtained cell fraction was washed twice in the main growth medium with a low glucose content of 1g / 1 DMEM-LG (Lonza, Belgium) containing L-glutamine (Gibco, USA), 10% fetal serum (HyClone, USA) and antibiotics (100 units / ml penicillin and 100 µg / ml streptomycin) (PanEko, Russia), by centrifuging for 10 minutes at 800 g. After the last deposition, the obtained cell fraction was mixed with the growth medium, counted according to the standard methods in the Goryaev chamber, and filtered through nylon filters with 80 µm size (BD Falcon Cell Strainer, USA) for the selection of stromal vascular fraction (SVF) cells. Thus, in order to analyze the quantitative dependence of the canine adipose-derived multipotent mesenchymal stromal cells, the correlation of the total number of derived cells, the viable and stromal vascular fraction (SVF) cells obtained as a result of separation by filtration was calculated. SVF cells were subcultured with a density of 25 cm³ (SPL, Korea) at the rate of 1 million cells per 1 cm². Cultivation was carried out

under standard conditions in a CO₂ incubator (5% CO₂ concentration and a temperature of 37 ° C). After 24-48 hours of cultivation non-adherent cells were removed by washing the culture surface with phosphate-saline buffer with the absence of Ca²⁺ + and Mg²⁺ + ions (FBS) (Eco-Service, Russia). Further cultivation of adherent cells was continued under the same conditions with replacement of the growth medium every 3–4 days, until the cells reached the 80% of confluent monolayer. The derived populations of cells were used in further experiments from zero to the fourth passage.

The MMSCs morphology of native cells and cells stained with Romanovsky – Giemsa was evaluated by inverted phase contrast microscopy (Carl Zeiss, Germany) and AxioVision Rel. 4.8 software. Average time of cells number doubling was calculated by the standard method, and the proportion of cells in the G₀ phase were not taken into account. The ability of cloning was assessed on the 7-10th day of cultivation. For this, the cells were subcultured with a density of 5×10^3 cells/cm² in a 25 cm² cultural flasks (SPL, Korea) and the correlation of formed clones to the total number of cultivated cells was calculated on the 7-10th day. The evaluation of cells viability was performed using a 0.4% trypan blue solution (Sigma Aldrich, USA) using the standard procedure. The mitotic index for each population was calculated in the logarithmic growth phase as the ratio of mitoses to the total number of counted cells multiplied by 1000 (%).

The differentiation potential in adipo-, osteo- and chondrogenic directions in vitro was assayed using commercial kits for directional differentiation StemPro (Gibco, USA). For adipogenic differentiation, the canine adipose derived MMSCs were incubated in StemPro Adipogenesis Differentiation Kit (Gibco, USA) medium. For osteogenic differentiation the StemPro Osteogenesis Differentiation Kit (Gibco, CIIIA), for chondrogenic differentiation the StemPro Chondrogenesis Differentiation Kit (Gibco, CIIIA) was used. After cultivation in differential mediums of StemPro (Gibco, USA) for 21 days, the cells were washed in phosphate-buffered saline (PBS) and fixed for 15 minutes in ice-cold (-20 ° C) methyl alcohol 99.8% (Sigma Aldrich, USA). The detection of lipids in culture was performed by staining with 1% solution of alizarin red S (Bio-Optica, Italy) for 45 minutes, after which the nuclei were stained over with Meyer's hematoxylin (Sigma-Aldrich, USA) for 10 minutes. For the evaluation of differentiation potential of AT MMSCs staining was carried out using the Cossa silver method, by commercial van Koss set (Bio-Optica, Italy), according to the manufacturer's recommendations at room temperature. The chondrogenic differentiation of canine adipose-derived multipotent mesenchymal stromal cells was confirmed by staining with alcian blue in cartilage (BioVitrum, Russia), which stains the mucopolysaccharides produced by cartilage cells in the extracellular matrix in combination with Harris hematoxylin (Labpoint, Russia). All the results of the directed differentiation were analyzed by phase-contrast microscopy.

For the immunophenotype determination of derived cells populations the immunocytochemical analysis was used. The analysis was performed in accordance with the manufacturer's instructions by kits to the following surface antigens: CD44 (Catalog No. AF6186), CD45 (Catalog No. DF6839), CD90 (Catalog No. DF4804), CD105 (Catalog No. DF7735) (Affinity Biosciences, China). For detection of marker proteins, goat anti-rabbit IgG antibodies labeled with horseradish peroxidase were used (Catalog No. S0001) (Affinity Biosciences, China). The analysis was carried out in accordance with the manufacturer's instructions. The result was evaluated by phase contrast microscopy.

3. Results

The results of histochemical analysis showed that the number of cells in the first two groups differed, but not significantly. In the second experimental group, in the unit of material, there has been the maximum number of cells in percentage ratio to the other experimental groups it was 100%. It was 94% in the first group, 65% in the third and 89% in the fourth group. The viability of the cells isolated from the Canine adipose derived cells after fermentation in all experimental groups was similar and amounted to 64%, in the range from 30.1% to 92.6%, without age dependence and area where the samples obtained. The number of stromal-vascular fraction cells, which were isolated by filtration, also had significant differences by groups. The first group - $7 \pm 0.5\%$ of the total number of derived cells, calculated before filtration, the second - $9 \pm 1\%$, the third - $0.08 \pm 0.05\%$, the fourth - $3.8 \pm 0.2\%$. All

the cells population with similar phenotype to MMSCs derived from AT of all experimental groups of dogs analyzed in this research had similar morphological characteristics. There have been presented heterogeneous population in which three types of cells can be distinguished: large fibroblast-like cells with processes (about 60–200 μm), in the cytoplasm of which there are many vacuoles and granules, a population (about 20–60 μm) of dark round cells and a population having narrow spindle-shaped cells with narrow cytoplasm (diameter 10–40 μm).

During subcultivation of the canine adipose-derived multipotent mesenchymal stromal cells there have been observed gradually changed of one type of cell to another. The cells prevailed at passage 1–4 were gradually replaced by cells of the 1st type, which was accompanied by a gradual inhibition of proliferation. The average value of the time of one cytogenesis and mitotic index of MMSCs by groups was: 1st (44 ± 0.01 h), 3%; 2nd (39 ± 0.05 h), 2.7%; 3rd (124 ± 0.05 h), 8.4%; 4th (104 ± 0.05 h), 7.1%; respectively. The populations of all derived experimental cell groups, with a phenotype similar to MMSCs, are highly efficient in forming colonies of fibroblast-like cells. The colony formation was differed in groups and in the first group it was $89 \pm 2\%$, $90 \pm 4\%$ in the second, $63 \pm 5\%$ in the third, $81 \pm 2\%$ in the fourth. All the derived cells had a differentiation potential and chondrogenic cell types. However, the adipogenic, osteogenic, and chondrogenic potential of the third and fourth experimental groups (animals over 10 years old) was lower in comparison with the first and second groups (animals under the age of 1 year). Chondrogenic differentiation in the third group (MMSCs isolated from subcutaneous adipose tissue of dogs aged over 10 years) was significantly lower than the directional differentiation of the fourth group (MMSCs isolated from visceral fat of dogs aged over 10 years).

In groups of young dogs the anatomical areas where the materials were obtained the age differences were not detected. In the expression of surface antibodies, differences between groups of young and aging animals were not found. Phenotypic analysis showed that all canine adipose-derived multipotent mesenchymal stromal cells were positive for CD 44, CD 90 and CD 105, but negative for CD45.

4. Discussion

We carried out a comparative analysis of cell populations with a phenotype similar to MMSCs, isolated from subcutaneous and visceral canine adipose tissue, aged up to a year old and over 10 years old. So it was formed 4 experimental groups of dogs. Absolutely all obtained cell populations possessed all the properties and features of MMSCs. Histochemical analysis showed that the number of AT cells per unit volume decreases with age, which is associated with an increase in the size of adipocytes. The number of cells obtained stromal-vascular fraction by filtration, before culture selection, in animals of experimental groups, aging 1 year, was higher than aging by $2.4 \geq$ times.

There were also observed significant differences between the sources of the derived cells, prior to the culture selection, when obtaining cells from visceral canine-derived AT $1.3 \geq$ times more than from subcutaneous AT. In canine adipose-derived multipotent mesenchymal stromal cells at the level of the first passage of the doubling time and mitotic index in culture in vitro were 2.6–2.8 times higher in the first two experimental groups than in the third and fourth group of dogs. The effectiveness of the directed differentiation of MMSCs, isolated in groups of young animals, was higher than that of the aging ones.

5. Conclusion

Our results show that the decision on the amount of adipose tissue derived from dogs that is necessary for tissue therapy by multipotent mesenchymal stem cells, have to be made considering the areas of the material collection and the age of the animals. In elderly animals, it is necessary to collect AT in a big amount in comparison with young donors. It has been noticed that in elderly dogs it is more preferably to collect the visceral adipose tissue. Thus, it can be concluded that with age, the number of MMSCs isolated from adipose tissue of dogs is depleted, while from subcutaneous AT to a greater extent in comparison with visceral AT. In this regard, when taking biological material from dogs, in order to further isolate stem cells, it is necessary to take into account the age of the animal and the anatomical area of the material collection.

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