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Original Article

Impact of multiple isolation procedures on the differentiation potential of adipose derived canine mesenchymal stem cells

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Abstract: Objective: In regenerative biology, the most commonly used cells are adipose tissue-derived mesenchymal stem cells (AD-MSCs). This is due to the abundance and easy accessibility of AD-MSCs. Methods: In this study, canine AD-MSCs were harvested from different anatomical locations, i.e., subcutaneous (SC), omental (OM), and perirenal (PR). Various isolation techniques namely explants (TRT-I), collagenase-digestion (TRT-II), collagenase-digested explants (TRT-III), and trypsin-digested explants (TRT-IV) were used to segregate the MSCs to evaluate cell doubling time, viability, and adipogenic/osteogenic lineage differentiation potential. Results: The study showed that the SC stem cells had superior growth kinetics compared to other tissues, while the cells isolated through TRT-II performed better than the other cell isolation procedures. The metabolic status of cells isolated from dog adipose tissue indicated that all cells had adequate metabolic rates. However, SC-MSCs derived from TRT-III and TRT-IV outperformed those derived from TRT-I and TRT-II. The differentiation analysis revealed that cells differentiate into adipogenic and osteogenic lineage regardless of treatment, as demonstrated by positive oil red O (ORO) and Alizarin Red S (ALZ) stain. It is worth mentioning that cells derived from TRT-III had larger and more intracellular droplets compared to the other treatments. The TRT-I, -II, and -III showed greater osteogenic differentiation in cells isolated from PR and OM regions compared to SC-derived cells. However, the TRT-IV resulted in better osteogenic differentiation in cells from SC, followed by the OM and PR-derived cells. Conclusion: It is concluded that all methods of MSCs isolation from adipose tissues are successful; however, the TRT-II had the highest rate of cell re-assortment from the SC, while, TRT-II and -IV are most suitable for isolating cells from PR and OM adipose tissue.

Keywords: Mesenchymal stem cell, dog, adipogenesis, osteogenesis, cell isolation

Introduction

Stem cells are the “blank” cells of the mammalian body with an unlimited capacity to divide and proliferate into distinct lineages. Stem cells are widely regarded as an important component of regenerative medicine. In recent years, regenerative medicine has become a major focus of human and veterinary research. As research progresses, and the idea of one health evolves, companion animals like dogs, cats and horses, as well as farm animals like cows, sheep and goats and pigs, have been identified as the best translational models of human diseases [1]. Based upon sources, stem cells (SCs) are classified into three categories: embryonic stem cells (ESCs), adult/mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs) [2]. Adult stem cells are preferable to embryonic stem cells for ethical and immunological reasons and are better suited for disease biology research [3, 4]. Mesoderm-derived MSCs help in body growth, wounds healing, and are capable of self-repair [5]. In

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canines, MSCs can be isolated from adipose tissue [6], bone marrow, umbilical cord, Wharton's jelly [7], and synovial fluid [8]. The ISCT (International Society of Cellular Therapy) recommends three key characteristics of MSCs: they express CD73, CD90, and CD105; they stick to plastic surfaces; and they can differentiate into adipogenic, chondrogenic, and osteogenic lineages [9].

A large number of adipose-derived mesenchymal stem cells (AD-MSCs) are needed for transplantation that can be obtained using an efficient source and isolation technique. Previous studies compared the isolation, characterization, and differentiation of AD-MSCs from different sources while using the same method to isolate MSCs [8-10]. Due to the diversity of tissue structures, different protocols can be used to achieve optimal stem cell isolation. Different cell types also have diverse cell-cell/matrix interactions, resulting in different MSC yields. As a result, there is a pressing need to develop optimal separation techniques for different tissues. One of the oldest techniques for mesenchymal stem cell isolation and *in vitro* propagation is tissue explants, which involves cutting tissue into small pieces to allow diffusion of gases and nutrients [11, 12]. One of the disadvantages of tissue explant cultures is that each tissue requires a different size of cut to absorb sufficient gases and nutrients and release cells. An alternative approach to isolating MSCs is to use enzymes to ensure good tissue digestion, optimal cell yield, optimal cell survival, and minimal cytotoxicity. The enzyme that is the most commonly used for this purpose is collagenase, which is obtained from the bacterium *Clostridium histolyticum* [13]. However, trypsin can also be used for this purpose [14]. During enzymatic digestion, the exposure time of the enzyme with the tissue is critical for cell survival and yield, as it damages the cell membrane and makes it difficult for the cells to adhere to the culture flask. Therefore, the purpose of this study was to perform a comparative analysis of various isolation assays for yield generation, in vitro propagation, and differentiation of canine AD-MSCs.

Material and methods

Materials acquired for the study

Cell culture grade chemicals were used in this study. Low glucose Dulbecco's Modified Eagle's Medium (LG-DMEM) and FBS were purchased from BioWest (Nuaillé, France). Penicillin-streptomycin, amphotericin-B, and trypsin-EDTA (0.5% and 5.3 mM w/v, respectively) were acquired from Caisson (Smithfield, UT, USA). Minimum essential medium-alpha (α-MEM) was purchased from Gibco (USA), and Ex-Cyte from Millipore (Billerica, MA, USA). Insulin (3.5 mg [100 IU]/ml) was obtained from Novo Nordisk (Søborg, Denmark). Collagenase Type-I, MTT dye, and TRIZOL reagent, were procured from Solarbio (Fengtai, China). Dimethyl sulfoxide (DMSO), formalin, Triton X-100, isobutylmethylxanthine (IBMX), Dulbecco phosphate buffer saline ($DPBS^{-/-}$; without Ca^{2+} and Mg^{2+}), and Alizarin Red S stain (ARS) were acquired from Sigma-Aldrich (Taufkirchen, Germany). The antifade mounting media were obtained from Vecta Shield (St. Neots, UK). The Oil Red O (ORO) was acquired from Thermo Scientific (Chino, California, USA). T-25 and T-75 cell culture flasks, serological pipettes, 6-well, 24-well, and 48-well cell culture plates, and cell strainers were received from Corning (NY, USA).

Tissue collection

Severely injured dogs (n = 3) aged 6 to 12 months were admitted to Veterinary Medical Teaching Hospital, Faculty of Veterinary and Animal Sciences, PMAS-Arid Agriculture University, Rawalpindi. Dogs were thoroughly examined and after the hospital surgeon's approval, euthanized following AVMA guidelines [15]. Briefly, animals were placed under deep anesthesia by intramuscular injection of xylazine and ketamine (1:5 mg/kg), followed by intravenous administration of $MgSO_4$ (1 g/kg) to induce cardiac arrest. Subcutaneous (SC), omental (OM), and perirenal (PR) adipose tissue was then isolated (**Figure 1**) and stored in $DPBS^{-/-}$ supplemented with 5% penicillin-streptomycin (100 U/mL-100 µg/mL) and amphotericin-B (250 mg/L) and transported to the Stem Cell Laboratory. The experiments were approved by the Institutional Ethics Committee (Protocol Code PMAS-AAUR/IEC/665).

AD-MSCs isolation and culture

The following four treatment (TRT) methods were used to isolate and culture the AD-MSC.

Plain explants (TRT I): The fat samples collected from the SC, OM, and PR regions were cut into the same size fractions, placed in a six-well

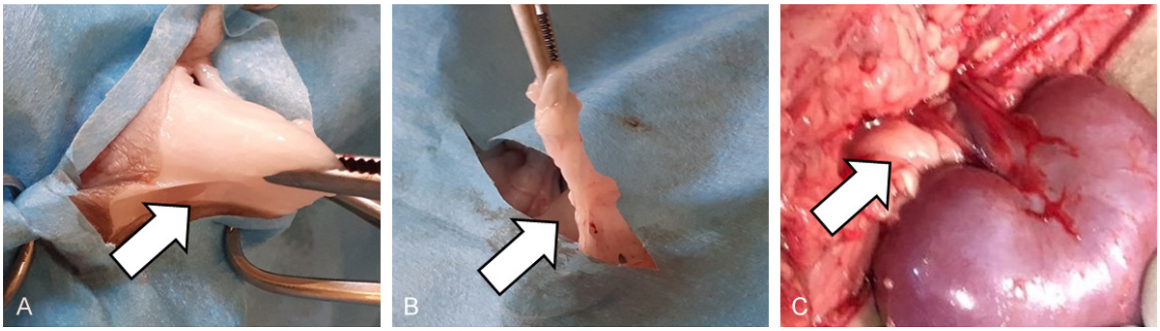

Figure 1. Adipose tissue collection form dogs. A: Subcutaneous. B: Omental. C: Perirenal.

cell culture plate, and supplemented with general media (LG-DMEM, augmented with 10% FBS, 1% penicillin-streptomycin, and amphotericin-B). The culture plate was placed in a well-maintained and humidified incubator at 37°C with 5% CO_2.

Adipose tissue-collagenase digested (TRT II): Each adipose source yielded ~3 grams of fat samples, washed, and cut into slurry. The slurry was then kept in 5% penicillin-streptomycin/amphetamine-B solution, and digested with Type I collagenase (0.1 mg/mL) in LG-DMEM without FBS and incubated for 135 min under 37°C. The enzyme activity was terminated by adding the general medium and free cells were filtered by a cell strainer (100 µm). The obtained cells were centrifuged for 10 min at 548 g. The recovered cell pellet was re-suspended in the general medium which was then transferred to a T-25 cell culture flask and incubated in a humidified environment with general media changes every 48 h until confluence levels reached 80-90%.

Collagenase digested explants (TRT III): In this group, no adipose tissue was removed from the upper strainer. Instead, the remaining chunks of it were used as explants in 6-well cell culture plates. The general media was added to the sticky explants and the plate was transferred to the humidified incubator.

Trypsin digested explants (TRT IV): Each tissue source contains ~3 g of fat samples. The fat samples were washed and then cut into a slurry composed of 5% penicillin/streptomycin and amphotericin-B solution. Then, the slurry was digested with 0.06% Trypsin EDTA at 37°C for 30 min, and then a general medium was added to inhibit the enzyme activity. The digested fat was sieved through a 100 µm cell strainer and the remaining chunks on the strainers were used for explants. The explants were carefully placed on a 6-well culture plate and general media was poured over them. The plates were incubated at in a humidified incubator at 37°C.

AD-MSCs passaging: After reaching 80-90% confluence, the cells of each treatment were transferred to the subsequent passages. Briefly, the confluent state of the cells was washed with $DPBS^{-/-}$ and treated with the working solution of trypsin EDTA (0.05% + 0.53% w/v) and placed in a humidified environment at 37°C with 5% CO_2 for 10 min. The enzymatic activity was blocked with general media, and the recovered cell pellet was centrifuged at 548 g, and then sub-cultured until passage number two (P-2).

AD-MSCs growth analysis

Added 5,000 cells to each well of a 48-well plate to obtain a growth curve. The media was removed on the 3rd and 6th days after seeding. The cells were washed with $DPBS^{-/-}$, trypsinized as described in the preceding section. The collected cell pellet was resuspended with 1 mL of general medium and quantified in a modified Neubauer chamber. The cell viability was evaluated by trypan blue exclusion (> 92%) and the growth curve was determined as follows: $DT = CT \times \log^2/\log (N_1/N_0)$.

Where DT = Doubling time, CT = culture time, N_1 = cells harvested, N_0 = cells seeded.

AD-MSCs proliferation test

The AD-MSCs proliferation at P3 on days 3 and 6 was quantified by the application of 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT dye). A total of 5,000 cells

were seeded in each well of a 48-well culture plate. Viable cells were evaluated by adding MTT dye at 0.25 µg/ml. Cells were then cultured in a humidified incubator at 37°C, 5% CO_2 for 180 min. MTT was reduced in viable cells. The supernatant was carefully removed, blue formazan cell crystals were mixed with 100 µL of DMSO. Thereafter, the absorbance was recorded at 630 nm using a microplate reader (BioTek 800TS, USA).

AD-MSCs cellular differentiation assay

AD-MSCs isolated from TRT I to IV at P-3 were induced to differentiate into adipogenic and osteogenic lineages according to our previously reported study [16]. For adipogenic differentiation, cells were cultured in 24-well culture plates with general media. After 80-90% confluence, general medium was replaced by adipogenic induction medium (LG-DMEM, supplemented with 10% FBS, 10 mM isobutylmethylxanthine (IBMX), 10 µM Rosiglitazone, 1 mM dexamethasone, 5 µg/mL insulin, and 1% penicillin-streptomycin and amphotericin-B) for 48 h. Subsequently, adipogenesis maintenance medium containing LG-DMEM supplemented with 1% Ex-cyte, 5 µg/mL insulin, and 1% penicillin-streptomycin and amphotericin-B was administered to the cells for 7 d. To confirm adipogenesis, Oil red O (ORO) staining was used. Briefly, the culture media from each well was removed, cells were washed with $DPBS^{-/-}$ and fixed with 4% formalin for 30 min. Finally, cells were incubated with 6:4 working ORO solution for 30 min in the dark. The stain was removed and cells were re-washed with $DPBS^{-/-}$ and inspected under an inverted light microscope. The ORO stain from developed adipocytes was eluted with anhydrous Isopropanol, and the absorbance was measured at 490 nm with a microplate reader.

To enable osteogenic differentiation, the cells were cultured in the 24-well culture plates supplemented with general media until 80-90% confluence was reached. The cells were then kept with osteogenic differentiation media [Alpha-Minimum Essential Media (α-MEM) added with 10% FBS, 100 mM ß-glycerophosphate, 50 mM ascorbate-2-phosphate, 1 mM dexamethasone, 1% penicillin-streptomycin and amphotericin-B]. Cells were maintained in the osteogenic medium for 21 d, with medium changes every 48 h. To confirm mineralization, cells were stained with Alizarin red-S staining (ALZ). Briefly, after removing the medium, cells were washed with $DPBS^{-/-}$ and fixed (4% formalin) for 30 min at room temperature. Cells with ALZ (40 mM) were incubated for 45 min in the dark, and then the stain was carefully removed, washed with $DPBS^{-/-}$, and observed under a light microscope.

Statistical analysis

The statistical analysis was performed using Sigma Plot 12.0 software by Systat Software Inc. (San Jose, CA, USA). All ORO, growth and proliferation experimental data were analyzed using ANOVA and expressed as Mean ± SD. To determine whether there was a difference between the groups, the Holm-Sidak test was used. All experiments were performed in duplicate. Statistical significance was presented using a *P*-value < 0.05.

Results

AD-MSCs isolation and culturing

The AD-MSCs were isolated from the SC, OM, and PR adipose tissues and cultured using four different isolation methods: TRT-I, TRT-II, TRT-III, and TRT-IV. Isolated adipose tissue-derived AD-MSCs exhibit a typical fibroblastic phenotype and adheres to the plastic culture flasks (**Figure 2**).

AD-MSCs growth time

The cumulative doubling time at P3 was determined on day 3 after culture of MSCs isolated from all three adipose sources (SC, PR, and OM). A non-significant increase in cell count was observed in cultured cells harvested by TRT-IV. Overall, there were no significant differences in cell doubling times between all treatment regimens and tissue sources (**Figure 3A**). On day 6 post-culture, there was also a non-significant increase in the cell number in TRT-II as shown in **Figure 3B.** In general, there is no difference in cell doubling time between treatments and tissue sources. Cumulative doubling time results showed that neither tissue source nor cell isolation treatment affected cell proliferation.

AD-MSCs viability assay

At P3, the cell metabolism of AD-MSCs was evaluated at days 3 and 6 post-culturing, isolated from the SC, PR, and OM fat sources,

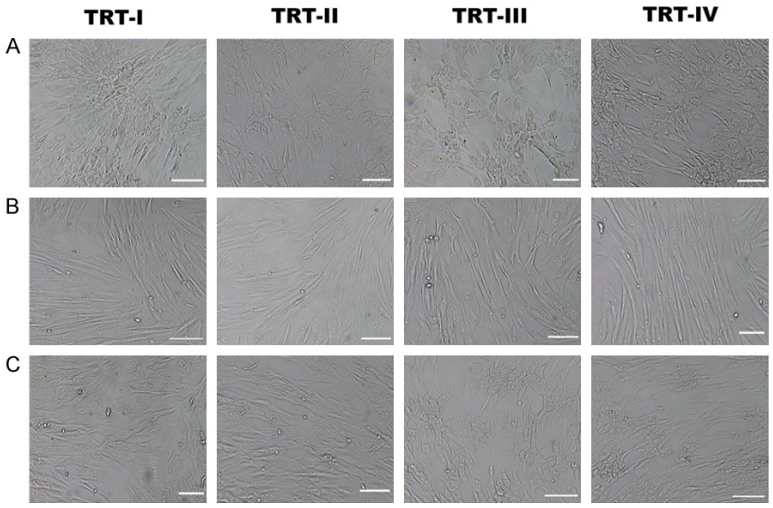

Figure 2. Culturing of canine AD-MSCs at P3 (Objective 25×). A: Subcutaneous fat-derived AD-MSCs. B: Omental fat-derived AD-MSCs. C: Perirenal fat derived AD-MSCs. All isolated AD-MSCs have a typical fibroblastic appearance. Abbreviation: TRT: Treatment. Scale Bar (25 μm).

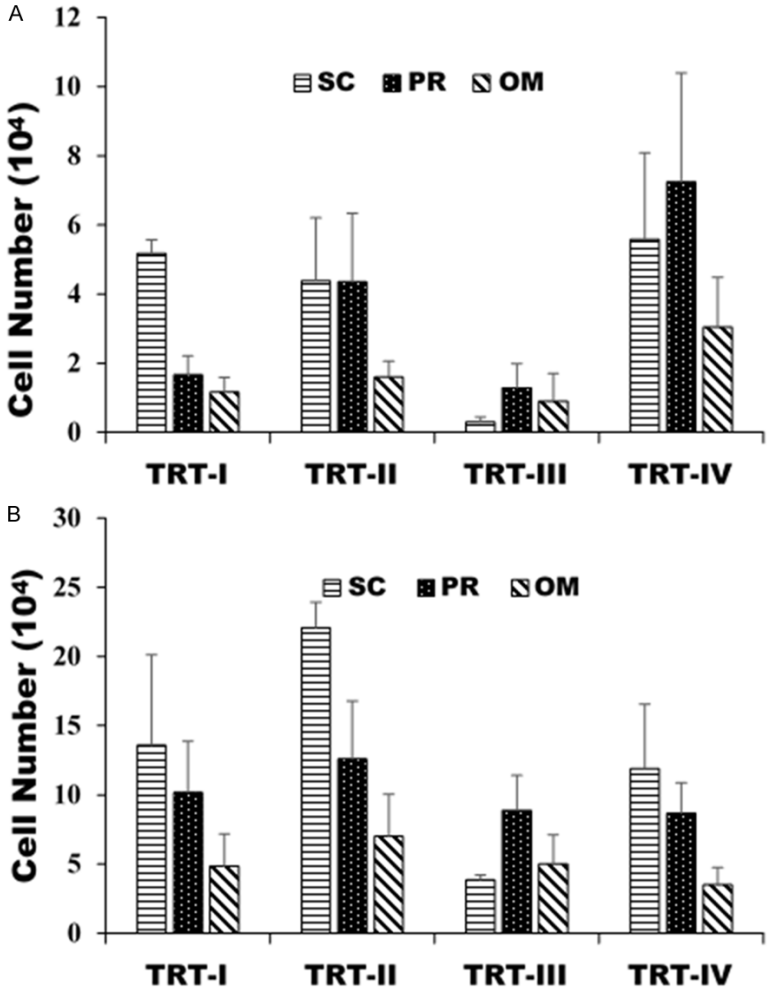

Figure 3. AD-MSCs growth curve analysis at P3. A: Doubling time of AD-MSCs on the 3rd day post-culturing. B: Doubling time of AD-MSCs on the 6th day of post-culturing. Abbreviation: TRT: Treatment.

and subjected to four different treatment (I-IV) protocols (**Figure 4A**, **4B**). On day 3, cellular metabolism of AD-MSCs isolated by TRT-IV was significantly elevated ($P > 0.001$), whereas cellular metabolism from TRT-I to III remained non-significant. Correspondingly, at day 6 of post-culturing the AD-MSCs isolated via TRT-IV showed significantly higher cellular metabolism compared to the other treatment methods.

AD-MSCs adipogenic differentiation

The MSCs of the adipogenic lineage (SC, OM, PR) were differentiated into adipocytes for 7 days. The cells were stained with ORO for the detection of intracellular lipids (**Figure 5A**). TRT-IV-harvested AD-MSCs (OM and PR-derived) showed dispersed small lipid droplets while MSCs derived from SC from TRT-IV exhibited large fat droplets compared to other fat sources, and both large and small fat droplets were present in PR. Plain explants and collagenase-digested cells demonstrated the uptake of diffused lipids. For quantitative analysis, ORO stain was eluted in an anhydrous isopropanol alcohol and absorbance was measured by spectrophotometer at 490 nm. Non-significant differences were observed for the tissue sources. However, there was a statistically significant difference between the TRT-III and TRT-IV ($P < 0.03$; **Figure 5B**).

AD-MSCs osteogenic differentiation

AD-MSCs from SC, OM, and PR were induced using an

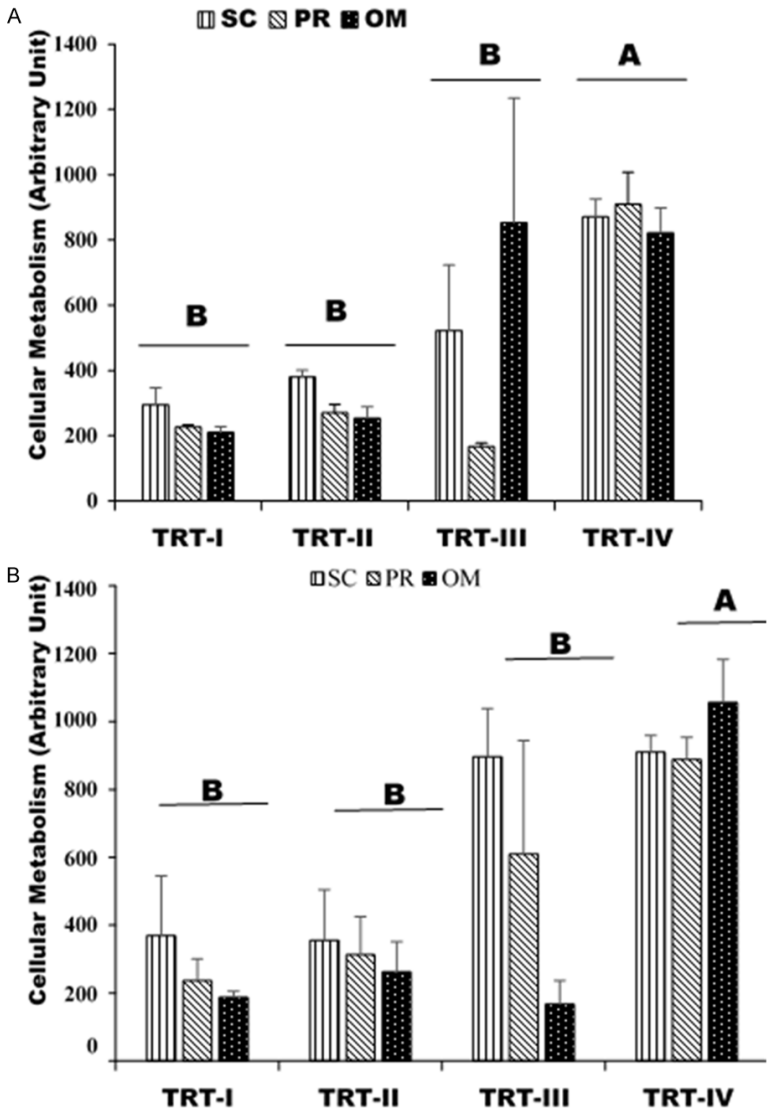

Figure 4. Cellular viability AD-MSCs at P3. A: Cellular viability at 3rd day of post-culturing of AD-MSCs. B: Cellular Viability at 6th day of post-culturing of AD-MSCs. Superscript A, B indicates the $P < 0.005$. The data is represented in Mean ± SEM. Abbreviation: TRT: Treatment.

osteogenic medium for 21 days and compared with the control group treated with a general growth medium. ALZ staining was observed in all induced groups compared with the control group (**Figure 6**). High ALZ staining levels were observed in the PR and OM of all explant groups (**Figure 6**), i.e., trypsin, collagenase-digested, and normal explants.

Discussion

A perfect and excellent MSC isolation source must have a high volume of stem cells and be readily available for clinical use. Adipose tissue is the most suitable source for MSC culture because it fulfils all the requirements for regenerative medicine [17, 18]. This paper compared four different isolation techniques for MSCs extracted from SC, OM, and PR fat. The study compared the traditional enzyme digestion technique with the new digested adipose explant implantation technique. Comparison of isolation methods will help the clinician to obtain a reasonable number of high-performing MSCs. Armed with this information, clinicians can not only select an appropriate source but also choose a specific approach to target fat in specific areas.

There was no significant difference in cell doubling time ($P > 0.05$), but a significant difference in cell viability was observed. Cell metabolic activity was increased ($P < 0.001$) in TRT IV-harvested cells regardless of tissue source. Similar findings were observed in a study comparing SC adipocytes to visceral fat adipocytes. SC fat-derived adipocytes were shown to have a better population doubling time than visceral fat [19]. Another study looked at the proliferative potential of MSCs obtained from SC, OM or mesenteric adipose tissue and showed that the population doubling time of SC adipose tissue was better than that of visceral adipose tissue [20, 21]. The MTT values were greater for SC sources than for fat pads. However, another study in horses showed that retroperitoneal fat was superior to SC fat sources [22]. This finding may be attributed to either the species effect or the age of the animals. A similar study compared nine isolation protocols for AD-MSC using non-enzymatic digestion and enzymatic digestion using collagenase, RBC lysis buffer, and different trypsin concentrations. Studies have found that digestion with trypsin is the

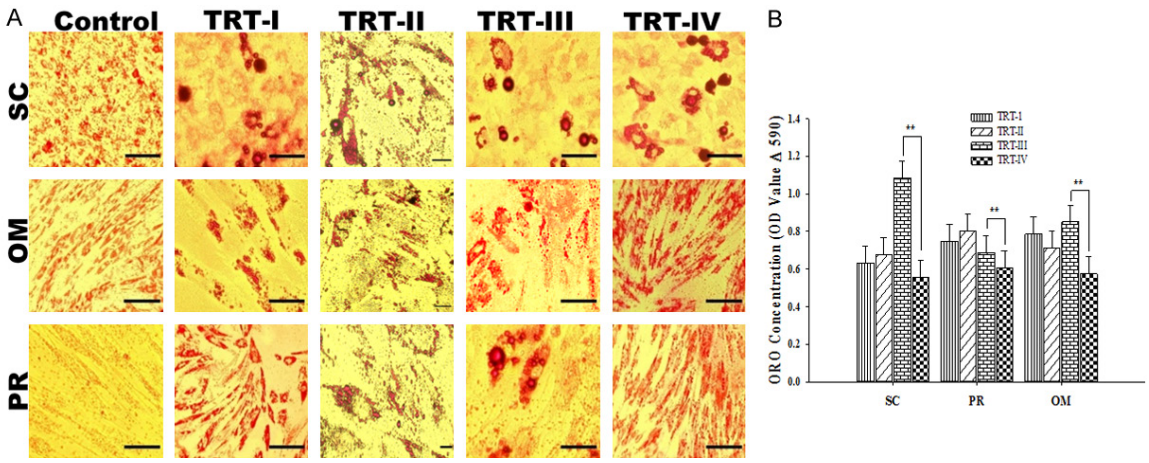

Figure 5. The AD-MSCs after their adipogenic differentiation were stained with oil-red-O stain. A: Control and differentiated AD-MSCs isolated with different (Objective 20×) treatments. Scale bar = 25 μm. B: For quantification, ORO stain eluted from control and differentiated AD-MSCs. The data was represented as the mean ± SEM. Superscripts letter * indicates a level of significance $P < 0.05$. Abbreviations: SC: Subcutaneous fat derived AD-MSCs, OM: Omental fat derived AD-MSCs, PR: Perirenal fat derived AD-MSCs.

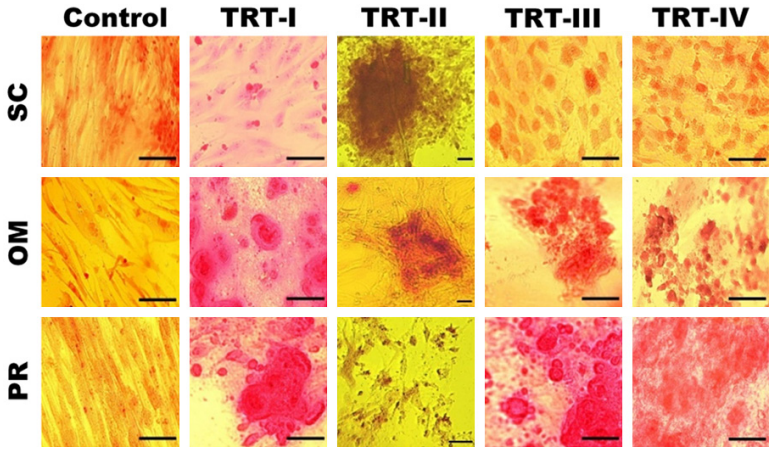

Figure 6. Alizarin red staining of AD-MSCs in osteogenic medium after 21 days. Control and osteogenic cells show deposition of hydroxyapatite mineral stained red with Alizarin red stain (objective 20×). Scale Bar = 25 μm. Abbreviations: SC: Subcutaneous fat derived AD-MSCs, OM: Omental fat derived AD-MSCs, PR: Perirenal fat derived AD-MSCs.

most efficient and cost-effective way to obtain MSCs. The cost-effectiveness of trypsin compared to the conventional and frequently used collagenase type 1 is 40 times that of trypsin. The study also found that the reduced concentration (25%) of trypsin resulted in better results [23, 24]. According to Salehinejad *et al.* [13] MSCs were not found in trypsin culture flasks. This may be due to the sensitivity of cells, and long-term exposure to trypsin may cause cell damage [14, 24]. Enzymatic digestion of perivascular dog umbilical cord tissue with collagenase and hyaluronidase generates high-quality MSCs with high multilineage differentiation capabilities [25]. In our study, all treatments actively produced MSCs, possibly because fat was processed with two enzymes on an individual basis.

Enzymatic digestion is the most efficacious isolation method when multiple cells are needed. However, explants are more effective due to lower resource consumption. This claim is backed up by a study, which states that MSC extracted from human cord-derived Wharton's jelly produced by explant culture releases high levels of fibroblast-like cells and high cell counts as compared to enzyme digestion by collagenase type II. Mitotic genes were upregulated in explant cultures, indicating that explants are superior to enzymatically digested tissue [26]. According to one study, explants may take longer to recuperate MSCs compared to traditional enzymatic digestion due to reduced proteolysis [27]. Therefore, we hypothesized that enzymatic digestion of explants might show better MSC debut, thereby promoting MSC migration. Several studies have shown that anatomical sites play an important role in stem cell isolation. For example, in a compara-

tive study, SC fat demonstrated superior MSC potential compared to visceral fat sources [10]. Our study shows that SC fat outperforms other sources when using plain explants, but OM, SC, and PR fat have very similar results in terms of metabolic activity of collagenase-digested cells. The treated explants had a higher physicochemical gumminess compared to standard explants, which led to a decrease in their floatability. In a recent study, enzyme-free isolation of MSCs from the human placenta (decidua basalis) was reported, cut into explants, and cultured directly in a cell culture medium. A high yield of cells was obtained with MSC cell surface marker expression and strong multilineage differentiation capabilities. This study strongly suggests that the enzyme-free explant-based cell culture technique is the preferred method for MSC isolation for humans [28]. Another study showed that stem cell isolates from cryopreserved microfragmented adipose tissue derived from knee osteoarthritis patients showed enhanced cellular metabolic function, enhanced differentiation potential, and higher MSC count by the explant culture method compared to the enzymatic digestion technique [29]. Alizarin staining can also be used to study the osteogenic differentiation characteristics of MSCs [30]. Visceral (Perirenal) adipose tissue and subcutaneous adipose tissue were found to be almost identical in terms of adipogenicity [31, 32]. In another study, MSCs isolated from peritoneal fat were found to secrete intracellular lipids earlier than those isolated from SC adipose tissue [33].

We conclude that all treatments of cells isolated from the tissues and methods described above were successful and resulted in the recovery of MSCs. However, it was discovered that collagenase digestion of explants was the most effectual for increasing cell isolation from SC. Trypsin-treated explants are most effective for isolating cells from visceral adipose tissue. Furthermore, MSCs obtained from all types of treatments showed differentiation into either adipocytes or osteocytes. However, this is not a true indicator of stem cell differentiation into a particular cell type. Therefore, ultrastructural examination of differentiated MSCs is required. Overall, this study provides pilot-scale data on how best to isolate MSC from multiple sources of adipose tissue in dogs. To obtain more efficient connective tissue stem cells, SC- and OM-derived MSCs, it is recommended to use collagenase- or trypsin-digested explants. This study will open new perspectives for future detailed *in vitro* or *in vivo* experiments for each isolation procedure and be applicable to different types of MSC sources from different species. It will also pave the way to identify the most practical, cost-effective, time-saving and high MSC yield options.

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Disclosure of conflict of interest

None.

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