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ORIGINAL RESEARCH PAPER

Isolation of adipose-derived stem cells: a comparison among different methods

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Abstract Adipose-derived stromal cells (ASCs) are usually isolated by digestion with collagenase. We have compared alternative methods to isolate ASCs in a more economically viable protocol. Nine protocols using red blood cells lysis buffer solution, trypsin, collagenase and centrifugation were compared; the isolation rate, cell viability, expansion rate, immunophenotype and differentiation in adipogenic and osteogenic lineages were analyzed. ASCs were isolated and successfully maintained by digestion with trypsin. Cells presented similar immunophenotypes, adipogenic differentiation and in vitro proliferation but an osteogenic differentiation capacity up to seven times higher than ASCs isolated by collagenase. This alternative protocol is thus efficient and more cost-effective than the commonly-used methods and may represent a promising protocol for obtaining ASCs for bone tissue engineering.

Keywords Adipose-derived stromal cell · Bone tissue engineering · Isolation methods · Mesenchymal stem cells · Osteogenic differentiation · Trypsin digestion

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Introduction

Mesenchymal stem cells (MSCs) are defined by their capacity to self-renewal, to differentiate into one or more specialized cell types, to adhere to plastic and by a specific panel of surface markers, which includes surface-positive antigens for CD44, CD90 and surface-negative antigens for CD11b and CD45, among other markers (Dominici et al. 2006). MSCs reside in virtually all post-natal organs and tissues and present a great potential for therapy due to their ease of isolation, ability to proliferate in vitro, differentiation in several cell types and potential to repair vital tissues (da Silva Meirelles et al. 2006). There are three main sources of MSCs for human studies and therapies: bone marrow, umbilical cord and adipose tissue. The stem cells derived from human adipose tissue (ASCs) are similar to bone marrow-derived MSCs in morphology and phenotype but show important differences in regard to harvest and cell yield. Adipose

tissue collection is less traumatic than bone marrow harvest, and the frequency of stem cells is higher in that tissue, indicating that ASCs represent an attractive and abundant cell type for regenerative medicine (Lee et al. 2004; Mizuno et al. 2012). Initial clinical studies with ASCs revealed new perspectives for the treatment of several conditions, such as complex fistulas and loss of soft tissue (Mizuno et al. 2012).

Zuk et al. (2001) described the most widely used ASCs isolation protocol. The procedure consists basically in four steps: washing of the adipose tissue, enzymatic digestion, centrifugation and red blood cell lysis. This method results in a fraction of stromal cells including adipocytes, pre-adipocytes, endothelial cells, macrophages, fibroblasts and a subpopulation of MSCs. Although efficient, this procedure could be expensive when considered for clinical therapy purposes. Therefore studies have attemped to develop alternative methods for the isolation of ASCs (Baptista et al. 2009; Francis et al. 2010; Fadel et al. 2011; Al Battah et al. 2011).

The isolation of ASCs with alternative enzymes, such as trypsin, is rarely described and tends to be compared only against the collagenase method (Fadel et al. 2011). A broader comparison among different protocols could contribute to determine their effectiveness, as well as to define the in vitro characteristics of ASCs isolated with each method. This work aims to compare alternative methods of isolation of ASCs in order to develop a more cost-effective protocol to obtain ASCs for future therapeutic purposes.

Materials and methods

Reagents, culture media and solutions

Complete culture medium (CCM) was composed of Dulbecco's modified Eagle's medium (DMEM) with 10 mM HEPES (2.5 g l^{-1} and 10 % (v/v) fetal bovine serum. Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution containing 10 mM sodium HEPES (HB/CMF/HBSS) was used for washing tissues and cells.

Isolation and culture of ASC

Subcutaneous adipose tissue samples were obtained from 10 patients undergoing elective liposuction surgery. The mean age was 36 years (24–51 years), mean weight was 61.4 kg (55–70 kg) and the mean body mass index was 24.9 (21.5–28.3). All patients signed an informed consent form, and the study was approved by the Research Ethics Committee of Irmandade Santa Casa de Misericordia de Porto Alegre, under protocol number 3515/11. The liposuction was performed under general anesthesia and local infiltration of the abdomen consisted in a solution containing normal saline with epinephrine and bupivacaine. The adipose tissue was aspirated from the lower abdomen with a 3.5 mm diam. blunt cannula. Each isolation method tested used aliquots of 10 ml washed adipose tissue. Cells from each patient were obtained by at least three different methods. Total cells were plated in one well of a 6-well culture plate and cultured in 5 % (v/v) CO_2 at 37 °C. The medium was changed every 2-3 days. Cells were passaged after washing with HB/CMF/HBSS, by trypsin release (0.25 % trypsin/EDTA), counting and seeding at appropriate rates.

The following methods for isolation of ASCs were tested:

Collagenase (COL)

The adipose tissue was washed extensively with phosphate buffered saline (PBS) and digested with 1 mg collagenase type I ml^{-1} in DMEM with 10 mM HEPES for 30 min at 37 °C, under gentle agitation. The enzyme was inactivated with an equal volume of CCM and centrifuged at $600\times g$ for 10 min. After centrifugation the supernatant was discarded and the pellet was incubated with 10 ml of red blood cell lysis buffer for 5 min at room temperature and centrifuged at $600\times g$ for 10 min.

Red blood cell lysis buffer solution (LBS)

This method, described by Baptista et al. (2009), is based on simultaneous mechanical shaking incubation with red blood cell lysis solution (150 mM NH_4Cl, 10 mM $NaHCO_3$ and 1 mM EDTA diluted in distilled water) 1:1 (v:v) for 15 min. After incubation, the suspension was centrifuged at $600\times g$ for 10 min.

Trypsin (T025-30, T025-60, T050-30, T050-60, T075-30)

ASC were isolated by digestion at 37 °C with shaking every 10 min with different trypsin concentrations

(025, 050 or 075, corresponding, respectively, to 12.5, 25 or 37.5 μg trypsin/ml of PBS with 0.2 % EDTA) and incubation for either 30 or 60 min. The enzyme was inactivated by adding an equal volume of CCM and the suspension was centrifuged at 600×*g* for 10 min. The supernatant was discarded and the pellet incubated with 10 ml red blood cell lysis buffer for 5 min at room temperature and re-centrifuged at 600×*g* for 10 min.

Centrifugation (CENT1 and CENT2)

This method did not use any enzyme, only the varying centrifugation speed (800 or 1,280×*g*, respectively) for 15 min. After centrifugation, the supernatant was discarded and the pellet incubated with 10 ml red blood cell lysis buffer for 5 min at room temperature and centrifuged at 600×*g* for 10 min. After centrifugation the pellets of all groups were resuspended in CCM.

Determination of population doubling time

Cells were grown to 80–85 % confluence and counted at every passage from passage 3 to 12. Cell number was assessed using a Neubauer chamber after Trypan Blue staining. The mean population doubling time of cultures derived from at least three independent donors was expressed in days. The population doubling time of the different cultures was calculated by the formula: population doubling time: log (final cell number) − log (initial cell number) = K × T, where K is the generation constant (0,008963) and T is time in days (Roth 2006).

Immunophenotyping

Surface markers of the isolated ASCs were analyzed by flow cytometry. The cells were dissociated with trypsin, centrifuged, and incubated for 30 min at 4 °C with phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-conjugated antibodies specific for human CD90.2, CD45, CD34, CD44, CD73, CD117 and CD11b (Pharmingen BD, San Diego, CA, USA). The cells were analyzed using a FACSCalibur flow cytometer equipped with 488 nm argon laser (Becton–Dickinson, San Diego, CA, USA) with the CellQuest software. At least 10,000 events were collected.

ASC differentiation

Differentiation of isolated ASCs was induced by cells between passages 4 to 7 at 2.2×10^5 cells/cm^2 in specific media and cultivating for 4 weeks, with medium being changed every 3 days. For osteogenic differentiation, CCM was supplemented with 10^{-8} M dexamethasone, 5 mg ascorbic acid 2-phosphate ml^{-1}, and 10 mM β-glycerophosphate. Calcium deposition in differentiated cultures was revealed by washing once with PBS, fixing with 4 % (v/v) paraformaldehyde in PBS (v:v) for 15–30 min at room temperature and staining with Alizarin Red S. For adipogenic differentiation, ASCs were cultured in CCM supplemented with 10^{-8} M dexamethasone, 2.5 mg insulin ml^{-1}, 100 mM indomethacin (Merck), and 3.5 mM rosiglitazone (GlaxoSmithKline, Middlesex, UK). Adipocytes were visualized by fixing with 4 % (v/v) paraformaldehyde in PBS for 1 h at room temperature and staining with Oil Red O solution (3.75 % Oil Red O in 2-propanol water; 3:2 v/v) for 5 min at room temperature.

Alkaline phosphatase (ALP) assay

After 4 weeks of osteogenic differentiation induction, the cultures were rinsed with PBS and incubated for 2 h at 37 °C with 200 μl of the BCIP/NBT substrate kit (Invitrogen). After addition of 200 μl SDS/10 % HCl, cells were incubated overnight at 37 °C in 5 % (v/v) CO_2. The A_{595} of the supernatant was determined. Results are expressed as the A_{595} of differentiated cultures minus control results.

Statistical analysis

Data are expressed as mean ± standard deviation. All tests were performed as at least three independent experiments. Data were analyzed and graphs were generated using the Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). Results were evaluated by a one-way analysis of variance followed by a Tukey's test or by Student's *t* test. $p < 0.05$ was considered statistically significant.

Results and discussion

Alternative methods for ASCs isolation have been proposed (Al Battah et al. 2011; Baptista et al. 2009;

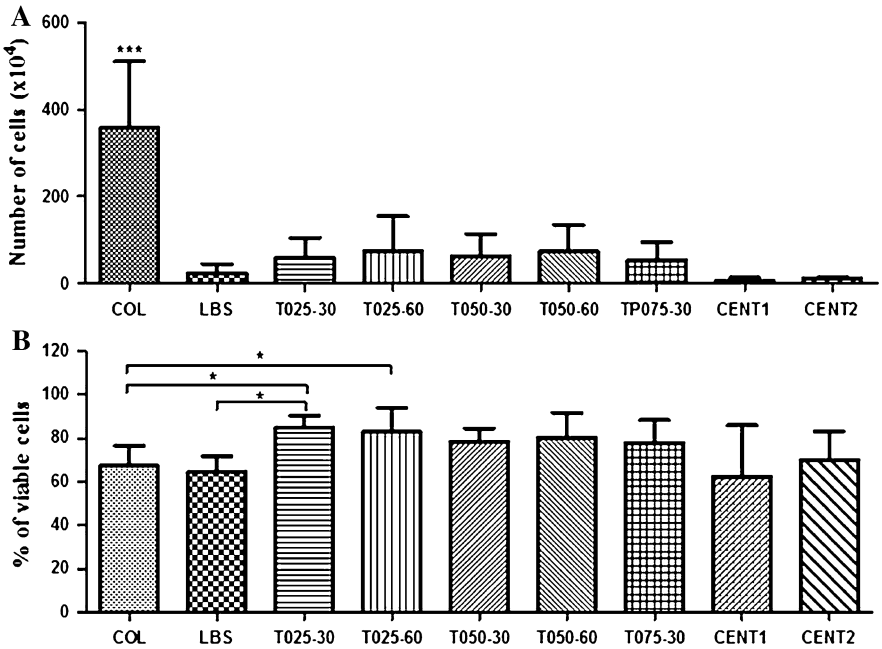

Fig. 1 Yield and viability of cells resulting from the different isolation protocols. Cell yield and viability were assessed immediately after isolation by Trypan Blue staining using Neubauer counting chamber. CENT1 and CENT2, $n = 3$. LBS and T075-30, $n = 5$. COL, T025-30, T025-60, T050-30 and T050-60, $n = 10$. **a** Cell yield, expressed in number of viable cells ($\times 10^4$). **b** Cell viability, expressed in percentage of viable cells. $*p < 0.05$; $***p < 0.001$

Fadel et al. 2011). The present work compared cell yield, cell viability, expansion rate, immunophenotype and differentiation capacity of cultures established using nine different protocols for ASCs isolation from human adipose tissue.

Viable cells were obtained from all protocols investigated in this work. The number of isolated cells is shown in Fig. 1a. The conventional isolation method of ASCs using collagenase yielded significantly more cells than the other protocols ($p < 0.001$). Comparison among all trypsin groups and LBS showed no statistically different cell yield, probably due to the wide standard deviation resulting from differences among the patients. The assessment of viability in cells obtained with the different isolation protocols showed that the percentage of viable cells was higher in two groups using trypsin (T025-30 and T25-60) when compared to collagenase ($p < 0.05$) (Fig. 1b). The group T025-30 also presented a higher percentage of viable cells when compared with LBS ($p < 0.05$). There was no statistical difference among all trypsin protocols. The same was observed in groups COL and LBS (Fig. 2). The age of the patients did not significantly influence cellular yield or viability (data not shown). Both collagenase and LBS protocols were more toxic than the group using trypsin at the lowest concentration and time (0.25 %, 30 min). LBS is toxic for the cells since after only 15 min of incubation the mean cell viability was 64.4 %. The least toxic protocol used was TP025-30, which resulted a mean cell viability of 84.8 %.

Although viable cells were obtained with the CENT1 and CENT2 protocols, they did not proliferate after 14 days of culture. Therefore these groups were discontinued after three isolation procedures. Cells isolated with LBS did not achieve confluence in 14 days, unlike described by Baptista et al. (2009) and Al Battah et al. (2011). The cultures from the other groups reached confluence within 7 days (data not shown) and displayed a typical fibroblast-like morphology, which was not modified until passage 12 (Fig. 2).

Several factors may contribute for the conflicting results observed in this and previous studies attempting to establish in vitro culture of ASCs isolated with the LBS protocol. These factors might include: differences in LBS preparation and composition, inclusion or not of the washing step of the adipose tissue with the preservation of the saline portion, and uneven composition of cells from the stromal fraction isolated due to possible selection of specific cell types. Francis et al. (2010) described the presence of ASCs in the saline portion of adipose tissue that is usually discarded in the washing step of the conventional

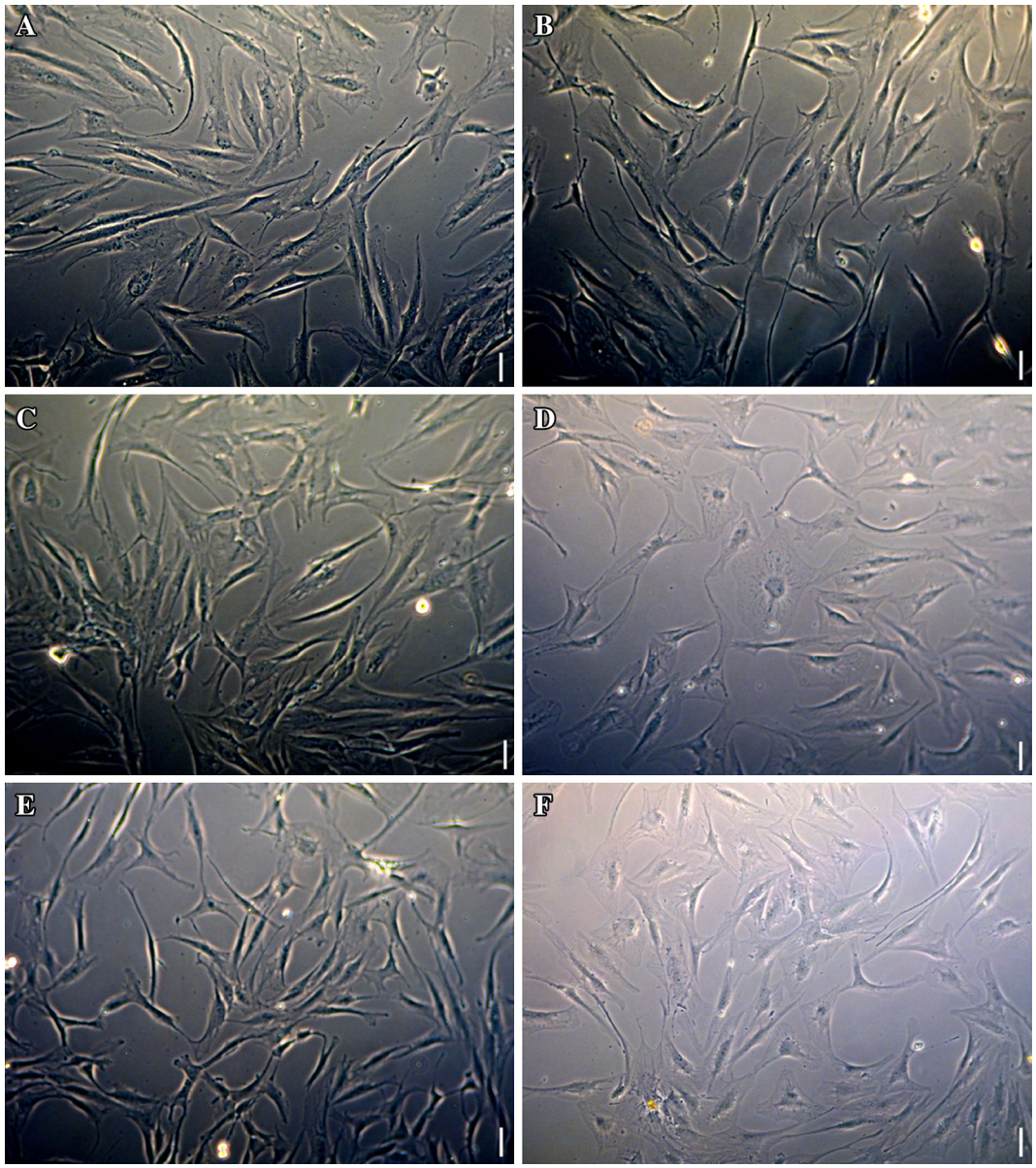

Fig. 2 Cell morphology. Cultures (passage 3–5) resulting from different isolation methods are displayed as following: **a** COL, **b** T025-30, **c** T025-60, **d** T050-30, **e**, T050-60; **f**, T075-30. *Scale bar* 50 μm

protocol. A rapid establishment of ASCs cultures from a LBS protocol is reported (Al Battah et al. 2011; Baptista et al. 2009), however it is not clear whether the saline fraction was discarded before incubation with LBS. In this study we did not analyze the composition of the isolated stromal fraction. It is possible than only a few of the cells isolated with LBS were actually ASCs. The same may have happened with the CENT1 and CENT2 protocols, which did not include enzymatic digestion. A low cell yield may also compromise the rapid establishment of the culture.

The population doubling time was similar in all cultures (Fig. 3) and remained stable until passage 12 (data not shown). Dmitrieva et al. (2012) demonstrated that the population doubling time increased significantly in bone marrow MSC while remaining stable in ASCs from the same patient, suggesting that these cells are more appropriate for long term expansion in vitro.

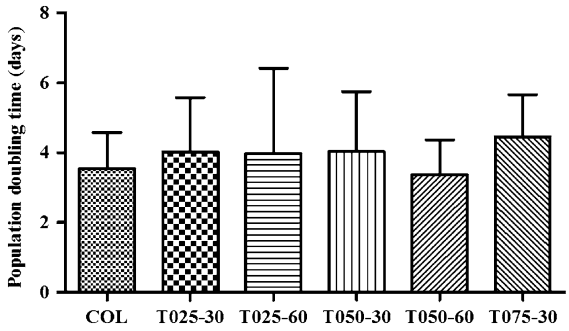

Fig. 3 Cell population doubling times. Cells were grown to 80–85 % confluence and counted at every passage from passage 3 to 12. Cell number was assessed with Trypan Blue staining using Neubauer chamber. The mean population doubling of cultures derived from at least three independent donors is expressed in days

The analysis of surface markers indicated that ASCs isolated with collagenase and all trypsin protocols are similar (Table 1). The cultures were consistently positive for CD90.2, CD44, CD73, and negative for CD45 and CD11b, while the expression of CD34 and CD117 showed some variation. This immunophenotype profile is consistent with recent guidelines for characterization of ASCs (Bourin et al. 2013). According to this statement, the frequency of CD34-positive cells depends on several factors during the collection and digestion of adipose tissue, which might explain the greatest variation observed in our results.

Adipogenic and osteogenic differentiation were obtained in cultures established with the COL, T025-30, T025-60, T050-30, T050-60 and T075-30 protocols (Figs. 4, 5). Adipogenic differentiation could be observed without specific staining in 1 week by the development of intracellular lipid vacuoles (data not shown). The quantification of alkaline phosphatase revealed that cells isolated using the T025-60 and T050-60 protocols presented a greater osteogenic differentiation capacity when compared to COL ($p < 0.05$ and $p < 0.01$ respectively) (Fig. 6).

According to Tarone et al. (1982), some cell surface molecules that mediate adhesion are resistant to proteases such as trypsin. Among these surface molecules are the integrins. Beta 1 integrin, represented by the CD29 surface marker, is usually analyzed in MSCs. $CD29^+$ and $STRO^+$ ASCs subpopulations may have a greater capacity for osteogenic differentiation (Rada et al. 2010, 2012). Integrins are resistant to trypsin, therefore cultures isolated using this enzyme can represent a cell fraction rich in $CD29^+$ cells. This could explain the significantly greater capacity of osteogenic differentiation observed in this study.

Moreover, ASCs are composed by different subpopulations with similar but not identical characteristics regarding surface markers, proliferation and differentiation capacity (Rada et al. 2010, 2012; Yoshimura et al. 2006). In the present work, we did not evaluate the heterogeneity of ASCs isolated from each group. Nevertheless it is possible that the enzymatic digestion with trypsin results in the isolation of a wider range of subpopulations of ASCs than the collagenase protocol usually provides. This would explain the greater osteogenic potential observed in cultures isolated through incubation with trypsin during the longest periods (60 min).

Although the literature presents methods that are simpler, more economic and quicker for ASCs isolation, their results are usually inferior to conventional protocols using collagenase when considering cell yield (Baptista et al. 2009; Francis et al. 2010). Francis et al. (2010) reported isolation of ASCs from the liquid portion of the adipose tissue, but the yield was ~25

Table 1 Immunophenotyping of human ASC between passages 5–6 during in vitro cultivation

Marker	COL	T025-30	T025-60	T050-30	T050-60	T075-30
CD11b	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0	0 ± 0
CD34	38.3 ± 2.3	12.5 ± 1.7	25.7 ± 1.4	32.2 ± 2.4	38.8 ± 1.6	41.1 ± 2.1
CD44	98.2 ± 0.3	97.8 ± 1.1	92.7 ± 0.6	93.9 ± 1.2	98.5 ± 0.6	97.4 ± 1.5
CD45	0 ± 0	0.0 ± 0	0.0 ± 0	0 ± 0	0.1 ± 0	0 ± 0
CD73	98.2 ± 1.8	91.7 ± 1.3	94.8 ± 0.2	93.3 ± 0.4	98.7 ± 1.7	92.1 ± 0.5
CD90.2	97.6 ± 1.5	94.8 ± 2.1	93.7 ± 1.8	95.2 ± 2.5	91.6 ± 0.9	98.3 ± 1.5
CD117	4.2 ± 0.2	1.4 ± 0.5	12.8 ± 1.3	5.7 ± 1.7	9.2 ± 1.4	6.1 ± 1.1

Data are presented as mean percentage of positive cells ± standard deviation ($n = 3$)

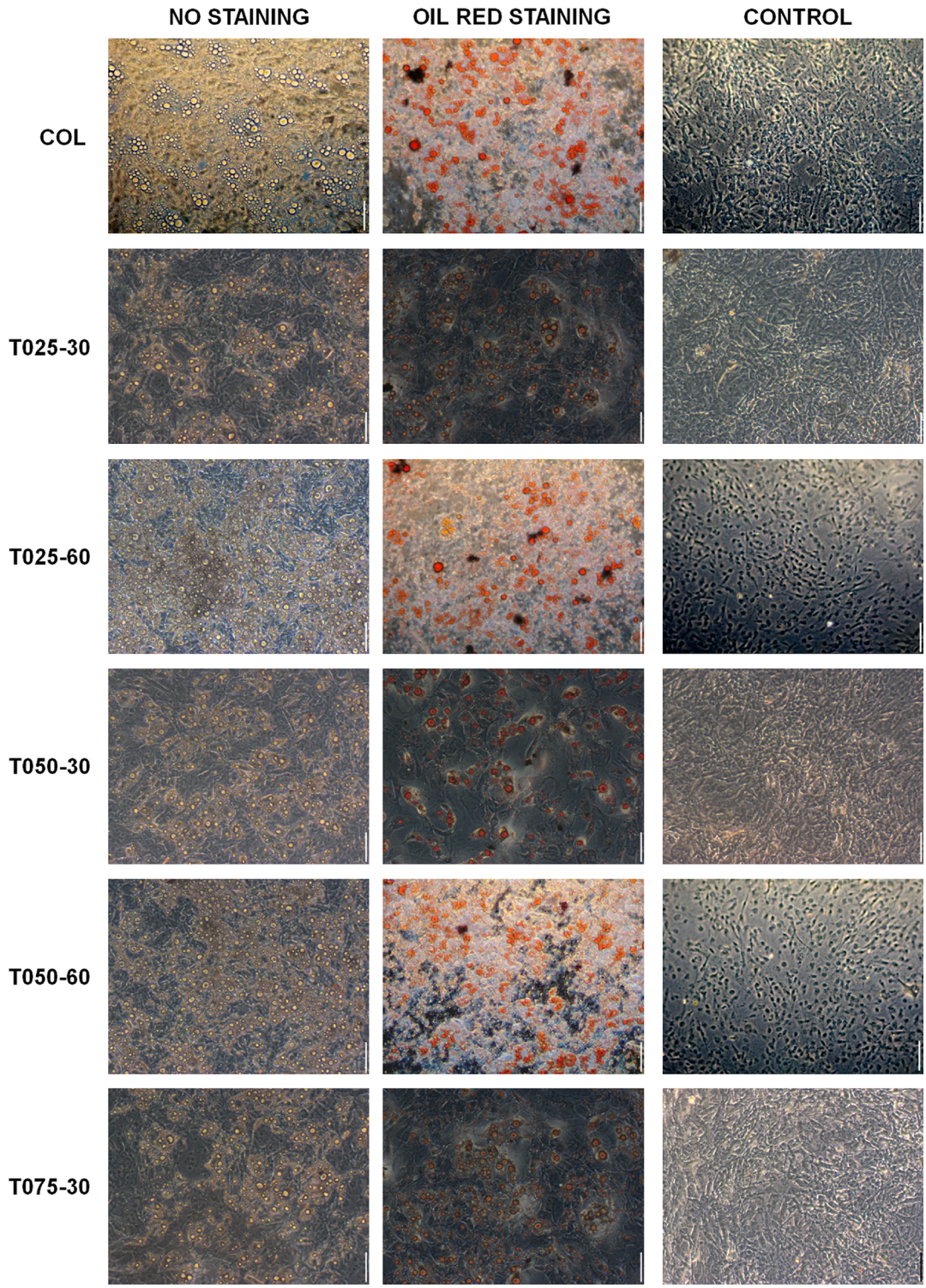

Fig. 4 ASC cultures after adipogenic differentiation. Cultures (passage 4–7) from different isolation protocols are displayed as induced with no staining (*left*), induced and stained with Oil Red O (*center*), controls stained with Oil Red O (*right*). *Scale bar* 100 μm

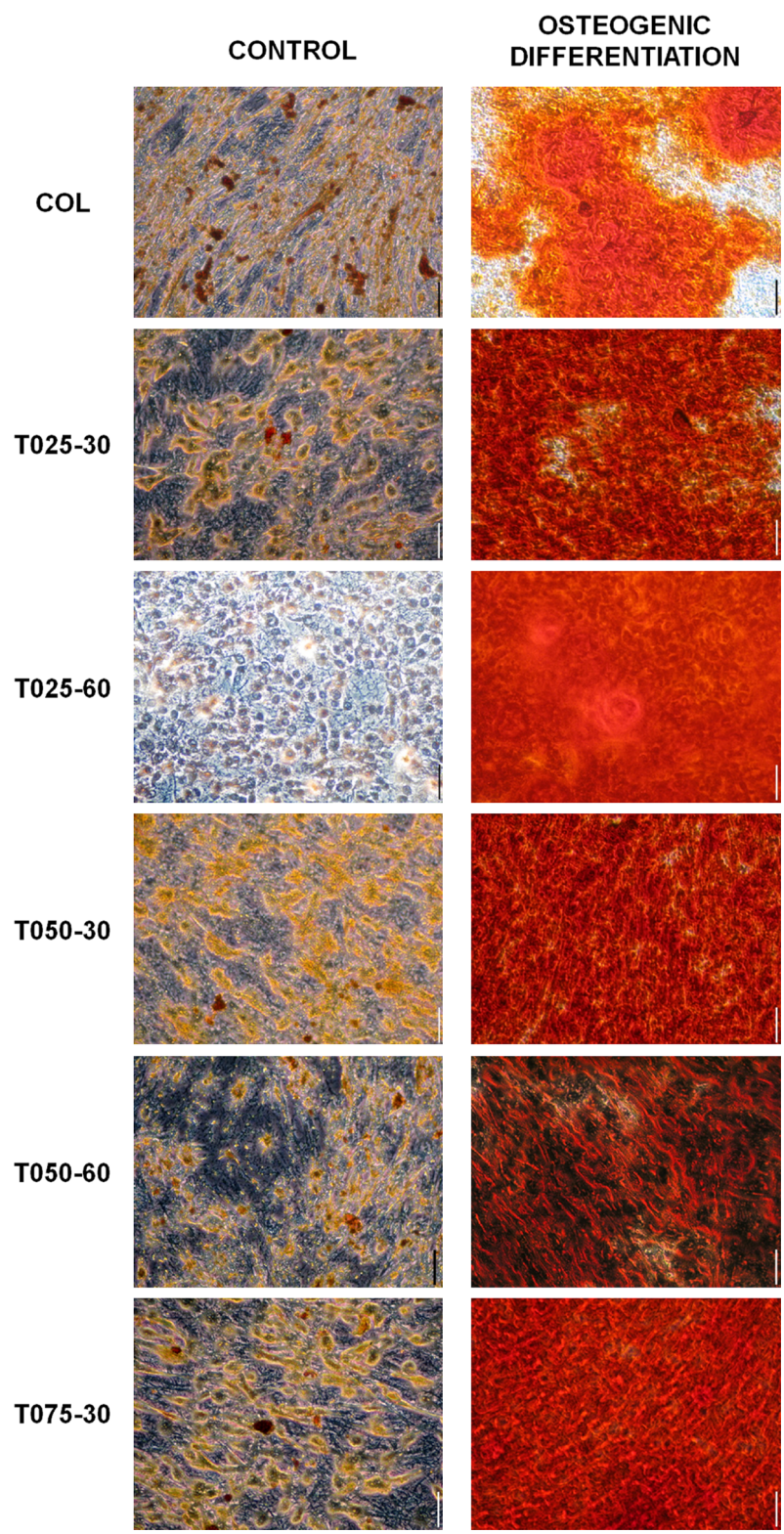

Fig. 5 ASC cultures after osteogenic differentiation. Cultures (passage 4–7) from different isolation protocols are displayed as controls (*left*) and induced (*right*) after staining with Alizarin Red. Control cells were cultured in CCM without addition of inductive factors. *Scale bar* 100 µm

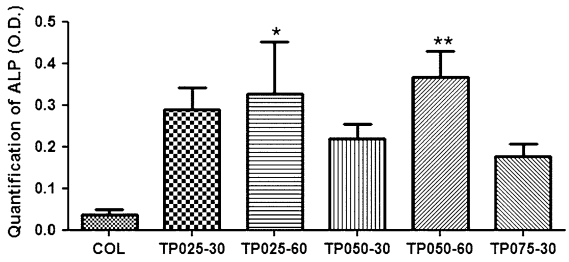

Fig. 6 Quantification of alkaline phosphatase. Analyzes were compared to optical density (O.D.), the values were obtained from the subtraction of each control (undifferentiated cells cultured in CCM with no inductive factors) with its corresponding induced culture (Quantification of ALP: induced O.D.–O.D. uninduced). $*p < 0.05$; $**p < 0.01$, $n = 3$

times less than achieved with trypsin presented in this study, and less than 250 times the conventional protocol with collagenase. Battah et al. (2011) did not compare the cell yield obtained from the protocol with lysis buffer as described by Baptista et al. (2009). In a study describing the use of trypsin and collagenase for the isolation of MSCs from human umbilical cord and placenta, cells isolated with trypsin failed to establish MSCs cultures (Salehinejad et al. 2012).

In conclusion, considering that the trypsin protocol for ASCs isolation is 40 times cheaper, has only a five-fold smaller yield when compared to collagenase, and results in cultures with similar average proliferation capacity, we believe that the use of trypsin represents an important alternative protocol to the conventional method for the isolation of adipose-derived stem cells. Our results suggest that the best protocol to replace collagenase tissue digestion is incubation with trypsin in the smaller concentration (25 %) for 60 min. In addition, based on osteogenic differentiation results, we conclude that this isolation protocol may represent a promising method for studies regarding bone tissue engineering.

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