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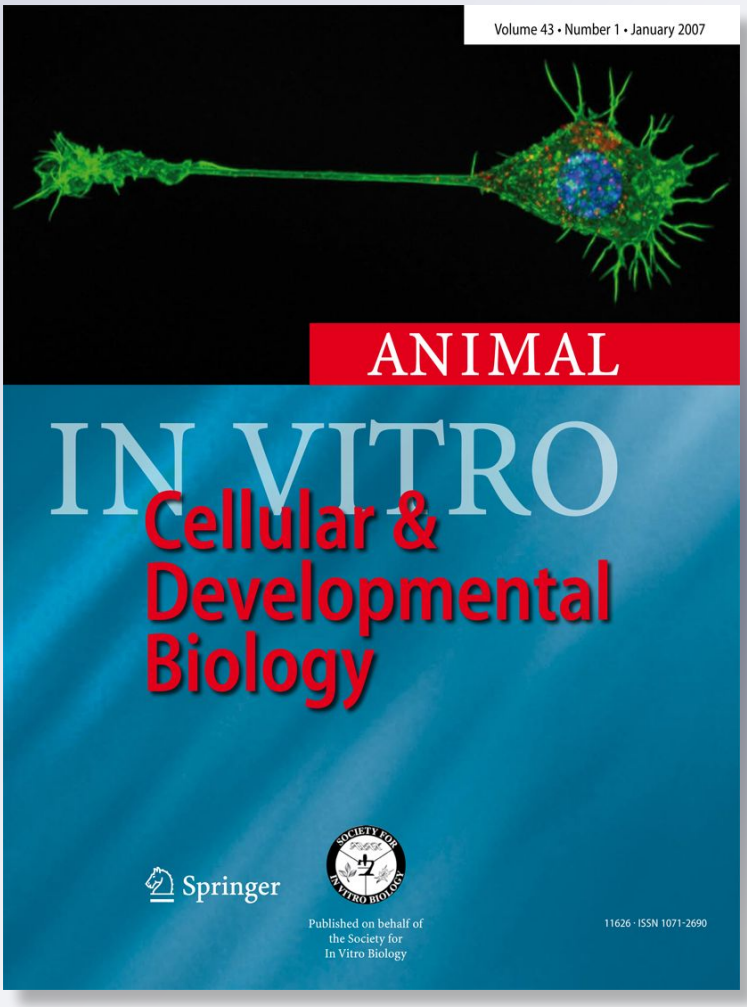

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Abstract Several techniques have been devised for the dissociation of tissues for primary culture. These techniques can affect the quantity and quality of the isolated cells. The aim of our study was to develop the most appropriate method for the isolation of human umbilical cord-derived mesenchymal (hUCM) cells. In the present study, we compared four methods for the isolation of hUCM cells: three enzymatic methods; collagenase/hyaluronidase/trypsin (CHT), collagenase/trypsin (CT) and trypsin (Trp), and an explant culture (Exp) method. The trypan blue dye exclusion test, the water-soluble tetrazolium salt-1 (WST-1) assay, flow cytometry, alkaline phosphatase activity and histochemical staining were used to evaluate the results of the different methods. The hUCM cells were successfully isolated by all methods but the isolation method used profoundly altered the cell number and proliferation capacity of the isolated cells. The cells were successfully differentiated into adipogenic and osteogenic lineages and alkaline phosphatase activity was detected in the hUCM cell colonies of all groups. Flow cytometry analysis revealed that CD44, CD73, CD90 and CD105 were expressed in all groups, while CD34 and CD45 were not expressed. The expression of C-kit in the enzymatic groups was higher than in the explant group, while the expression of Oct-4 was higher in the CT group compared to the other groups. We concluded that the collagenase/trypsin method of cell isolation yields a higher cell density than the others. These cells expressed a higher rate of pluripotent cell markers such as C-kit and Oct-4, while the explant method of cell isolation resulted in a higher cell proliferation rate and activity compared to the other methods.

Keywords Enzymatic isolation · Explant · Umbilical cord matrix-derived cells

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Introduction

Mesenchymal stem cells (MSC) have been considered as an appropriate source of cells in the treatment of a number of congenital and degenerative diseases (Baksh et al. 2004). At present, bone marrow is the main, traditional source of

MSCs (Romanov et al. 2003; Zhang et al. 2004; Qiao et al. 2008). However, collection of MSCs from bone marrow is an invasive and painful procedure (Logeart-Avramoglou et al. 2005). Also, the number of bone marrow MSCs significantly decreases with age and infirmity (Rao and Mattson 2001; Bobis et al. 2006). Furthermore, there is a potential risk of viral contamination during the isolation of MSCs from bone marrow (Romanov et al. 2003; Kadivar et al. 2005). All these factors limit the use of bone marrow as a suitable source of MSCs for cell therapy. Therefore, attention should be focused on tissues containing cells with a higher proliferative and differentiation potency and a lower risk of viral contamination. In this regard, Wharton's Jelly (Petsa et al. 2009) has been shown to be a suitable source of MSCs with practical advantages. It is demonstrated that cells derived from Wharton's jelly (WJ) of the umbilical cord, the so-called umbilical cord matrix cells (UCMCs), have similar characteristics to MSCs (Conconi et al. 2006; Lu et al. 2006). The UCMCs have surface markers (Weiss et al. 2006), immune properties (Sotiropoulou et al. 2006) and a differentiation potential similar to MSCs derived from bone marrow (Lu et al. 2006). These cells are also more similar to foetal MSCs in terms of their in vitro expansion capability (Sotiropoulou et al. 2006). Studies have shown that hUCMs have the potential of in vitro and in vivo differentiation into many cell types, including neurons, cardiomyocytes, striated muscle, insulin secreting cells, cartilage and osteocytes (Aghaee-Afshar et al. 2009; Ishige et al. 2009; Leeb et al. 2009; Latifpour et al. 2011). However, up until now, the question of which was the optimal method for efficiently isolating MSCs from umbilical cords (UC) remained unanswered.

The diverse nature of tissue architecture requires different techniques for cell dispersal; for example, bone, the brain, skeletal muscle, the liver and the spleen all have different cell–cell and cell–extracellular matrix associations. A consequence of this is that no single protocol will serve for all tissue types. One of the earliest techniques in cell isolation and in vitro cell propagation is the explant of tissue fragments. This technique is characterized by reducing the tissue size small enough for gases and nutrients to diffuse freely (Atala and Lanza 2002). Success in the primary explanted culture is dependent on the migratory ability of the type of cells (Saward and Zahradka 1997).

Another common technique is the use of defined enzyme systems to dissociate of different tissues. The type of enzyme employed has an effect on digestion outcome in terms of efficiency, yield, viability and toxicity (Costa et al. 1987; Konig et al. 1993). Commercial collagenases from *Clostridium histolyticum* are often used in tissue digestion (Williams et al. 1995; Bowman et al. 1999). In addition, trypsin, with or without ethylene tetra acetic acid (EDTA), is increasingly used as a common dissociative enzyme (Freshney 2005). The duration of the enzymatic treatment is critically important, as it may degrade the extracellular matrix and cell membrane, and prevents the cells from adhering to the culture substrate after isolation (Can and Karahuseyinoglu 2007), so the enzymatic treatment time should be kept to a minimum.

Taking into consideration the lack of a comparative study showing the influence of different isolation methods of hUCMs on cell quantity and quality, in this study we attempted to compare various isolation conditions in order to identify the optimal protocol for hUCM isolation while still maintaining their growth capacities.

Materials and Methods

Isolation of human umbilical cord matrix-derived mesenchymal cells. All materials were purchased from Sigma Company (Sigma-Aldrich, MO) unless stated otherwise. Ethical approval was obtained from the Institutional ethical review board (approval number 69-1780) at Kerman University of Medical Sciences, Kerman, Iran. UC were obtained from patients who delivered full-term infants by Caesarean section after written consent was obtained (n=15). These patients faced no complications throughout pregnancy. Fifteen-centimetre-long cords were immersed in sterile Hanks' balanced salt solution, at 4°C, supplemented with penicillin (300 units/ml), streptomycin (300 μg/ml) and amphotericin B (7.5 μg/ml), and immediately transferred to the laboratory. The surface of each cord was rinsed with phosphate buffered saline (PBS) to remove as much blood as possible. The cord was cut into 3–5-cm-long pieces using a sharp, sterile blade. Blood vessels were removed from each piece after incising the cord lengthwise and the WJ was carefully separated from the amniotic membrane. The WJ was cut into small fragments with sharp scissors and scalpels. The fragments were weighed using a digital weighing machine and divided into four equal parts among four experimental groups as follows: explant (Exp), collagenase/trypsin (CT), collagenase/hyaluronidase/trypsin (CHT) and trypsin/EDTA (Trp) groups.

In the Exp group, pieces with an approximate diameter of 2 mm were seeded onto the surface of a culture dish with DMEM-F12 supplemented with 10% (*v*/*v*) foetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml) and amphotericin B (2.5 μg/ml) for 2 wk. Within this period, half of the culture medium was refreshed every 5 d until the cells migrated from the fragment borders and reached approximately 80% confluence.

In enzymatic groups, different dosages and time were examined to obtain the most appropriate dosages and time (Fig. 1). We kept the time of exposure to the enzymes at minimum. Thus, in the CT and CHT groups, we added either 1 mg/ml collagenase type B for 3 h or a cocktail consisting of

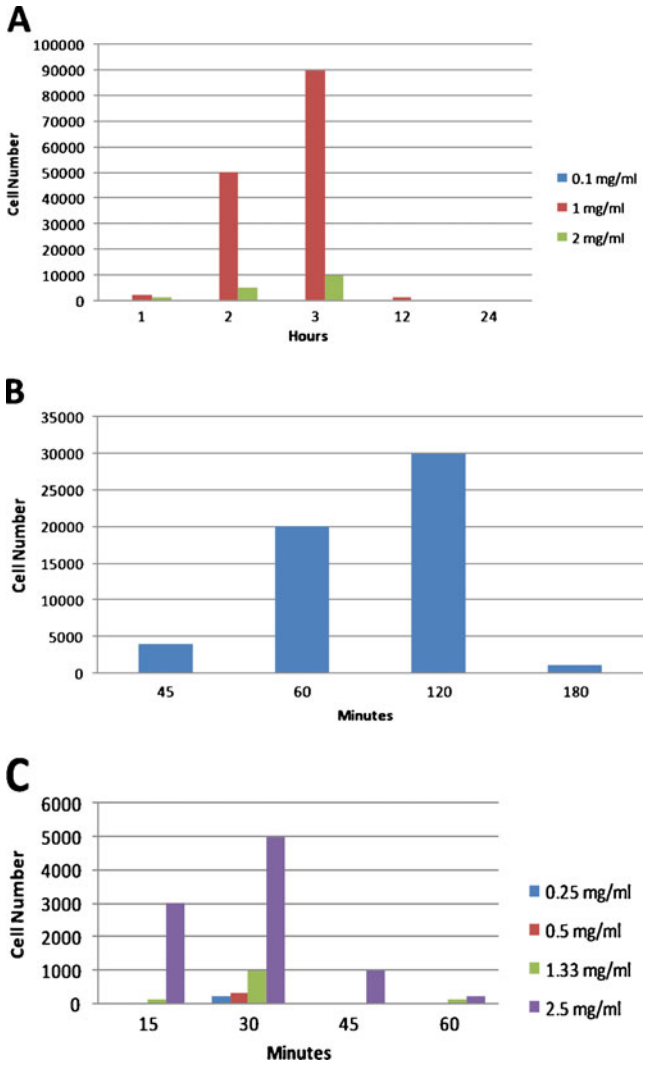

Figure 1. The appropriate duration of the enzymatic treatment on human umbilical cord was assessed in a preliminary study. (*A*) 1 mg/ml collagenase for 3 h; (*B*) 300 μg/ml hyaluronidase for 120 min and (*C*) 2.5 mg/ml trypsin for 30 min resulted in a higher number of hUCMs.

1 mg/ml collagenase and 300 μg/ml hyaluronidase to the tissues for 2 h at 37°C. These tissues were then washed with PBS and 0.25 mg/ml was added for 15 min at 37°C with agitation. In the Trp group, the tissue fragments were treated with 2.5 and 0.2 mg/ml EDTA for 30 min at 37°C with agitation. The isolated cells were washed with PBS and the cell pellet was resuspended in the culture medium. The cells were cultured in 25 cm^2 tissue culture flasks and incubated in a 37°C humid atmosphere with 5% CO_2 in the air.

Detection of cell viability. The trypan blue assay was performed on enzymatically isolated cells before the cells were cultured in the culture medium. A total cell count was accomplished using a haemocytometer chamber after staining the cells with 0.4% trypan blue. The cells that were not stained with trypan blue were considered viable cells.

Detection of cell proliferation. In order to detect cell growth rate in the various groups, we calculated the period of time from seeding the fragments (Exp group) or the onset of cell culture of the cells in the enzymatic groups until cell confluence was reached ≤80%.

In order to detect the propagation potential of the isolated cells, WST-1 assay was used for living cells after the fourth passage of hUCMCs. It is an ELISA-based one-step method that determines mitochondrial respiration (Nematollahi-Mahani et al. 2007). One-hundred-microliter cell suspensions (1×10^4 cells/ml) were introduced to each well of a 96-well plate and incubated at 37°C for 48 h. At the end of the incubation period, 10 μl of WST-1 solution was added to each well. After 1 h incubation at 37°C the absorbance was measured at a 450 nm wavelength and reference wavelengths of 630 nm with an ELISA reader (Bioteck, IA).

Flow cytometry. Cells were prepared at a concentration of 1×10^5 cells/ml in DMEM/LG with 10% FBS. The cells were washed, fixed and permeabilized with 0.2% Triton X-100 (Oct-4 only) and incubated for 15 min at 4°C with a 1:9 dilution of normal goat serum in PBS to block non-specific binding of the primary antibody. The cells were then labelled with the following antibodies: FITC-conjugated anti-CD44, FITC-conjugated anti-CD34 (Chemicon, IL), FITC-conjugated anti-CD45 (eBioscience, USA), PE-conjugated-anti-CD73 (Becton Dickinson [BD], NJ), PE-conjugated anti-CD90 (Dako, Denmark) and PE-conjugated anti-CD105 (R&D Systems, MN) for 1 h. Mouse primary antibodies and FITC-conjugated anti-CD117 and FITC-conjugated anti-Oct-4 were labelled for 45 min followed by goat anti-mouse IgG for CD117 and goat anti-rat IgG for Oct-4 (R&D Systems, MN). The cells were washed with 2% FBS in PBS and analysed using a FACSCalibur (BD, NJ) machine. The control population was stained with matched isotype antibodies (FITC- and PE-conjugated mouse IgG monoclonal isotype standards), which were confirmed by positive fluorescence of the limbal samples. At least 10,000 events were recorded for each sample and data were analysed using WinMDI software (BD Biosciences).

Alkaline phosphatase activity. The hUCM cells from the fourth passage were grown on clean, sterile glass slides for 2–3 wk. The medium was refreshed every 5 d. Alkaline phosphatase activity was detected using an ALP Kit (R-87 Sigma) according to the manufacturer's instructions. A dark red product after 20 min exposure to the substrate confirmed ALP activity. The cells were then counterstained with haematoxylin and mounted and photographed using an Olympus DP71 digital camera attached to an IX71 inverted microscope (Japan).

Induction of adipogenic and osteogenic differentiation. The hUCM cells (5×10^3 cells/cm^2) were seeded on 3 cm plates in DMEM-F12 supplemented with 10% FBS for 2 d. Osteogenic

and adipogenic differentiation media were then added for 2 and 3 wk for adipogenic and osteogenic differentiation, respectively. The adipogenic differentiation medium was composed of DMEM-F12 supplemented with 10% FBS, 100 nM dexamethasone, 50 μg/ml ascorbic acid and 50 μg/ml indomethacin. The osteogenic differentiation medium contained DMEM-F12 supplemented with 10% FBS, 10 nM dexamethasone, 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. As a negative control, cells were cultured in a proliferative medium deprived of the differentiation factors. Adipogenic differentiation was detected by Oil Red O staining while osteogenic differentiation was detected by Alizarin Red-S staining.

Statistical analysis. Data from the different tests were expressed as mean±SE and were analysed by one-way ANOVA followed by post hoc Tukey's test, after normality assumption; $p<0.05$ was considered statistically significant.

Results

Cell number. The initial cell number was $0.5–1\times10^4$ cells per cm of UC in CT, 0.25×10^4 cells per cm of UC in CHT, and 1×10^3 cells per cm of UC in the Trp groups. The number of isolated cells was 1×10^5, 0.5×10^5 and 0.5×10^4 cells per gram of WJ in the CT, CHT and Trp groups, respectively.

Cell morphology. The primary isolated cells had a heterogeneous shape, including fibroblast-like cells with short and long processes and small round cells with a relatively high nuclear to cytoplasm ratio, as well as flat cells, in the CT and CHT groups Fig. 2*a*, *b*. However, in the Trp Fig. 2*c* and Exp groups Fig. 2*d*, the isolated cell populations were less heterogeneous; with fibroblast-like cells which being predominant.

Cell proliferation. The interval between the primary culture and the first passage was approximately 4 wk in the Exp, 10 d in the CT, and 20 d in the CHT groups. In the Trp group, the cells did not continue to proliferate at a noticeable rate after 30 d in the culture.

The level of cell viability and proliferation was different in the different groups. In the Exp group, cell proliferation was significantly higher ($p<0.001$) than in the other groups. Also, the cells isolated by collagenase/trypsin digestion had a significantly higher ($p<0.05$) proliferation rate compared to the CHT group (Fig. 3).

Flow cytometry. The isolated cells in all groups were negative for the haematopoietic cells surface markers CD34 and CD45. Cells from all groups were positive against mesenchymal stem cell markers CD44 and CD105 with similar expression intensities. However, the mesenchymal stem cell markers CD73 and CD90 were expressed differently in the different groups (Fig. 4). The cells isolated by the CT and Exp methods showed

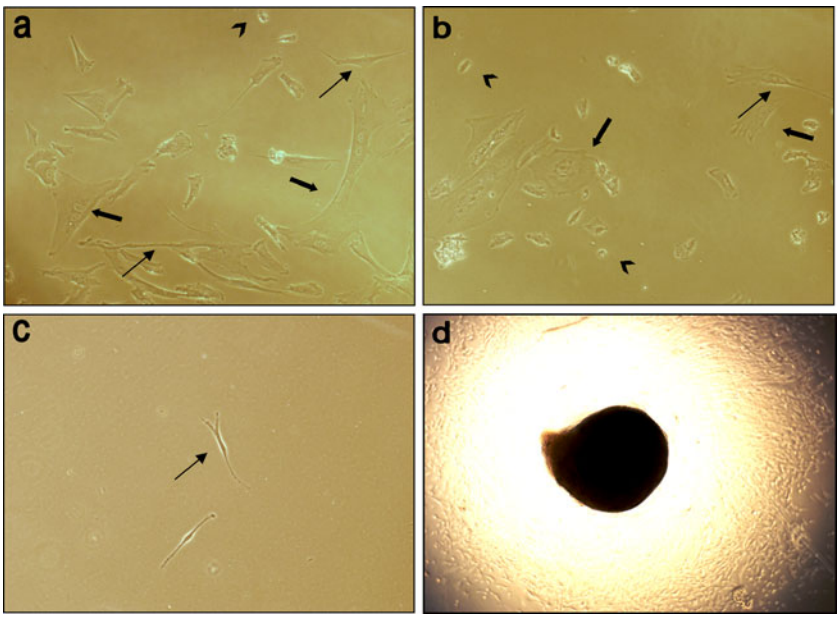

Figure 2. Morphological features of the isolated cells derived from human umbilical cord Wharton's jelly in three enzymatic groups on the second day of isolation and in explant group (*d*) at the third wk of isolation. (*a*) Three types of isolated cells (fibroblast-like cells possessing short and long processes, *arrow*; and flat cells, *short arrow*; as well as small round cells, *arrowhead*) in the collagenase/trypsin group. Note that the cell population was much greater in this group than the other groups. (*b*) Three types of cells isolated in the collagenase/hyaluronidase/trypsin group. (*c*) Spindle-shaped cells (*arrows*) in the trypsin and (*d*) explant groups. Magnification, ×200 in collagenase/trypsin, collagenase/hyaluronidase/trypsin and trypsin groups and ×100 in the explant group.

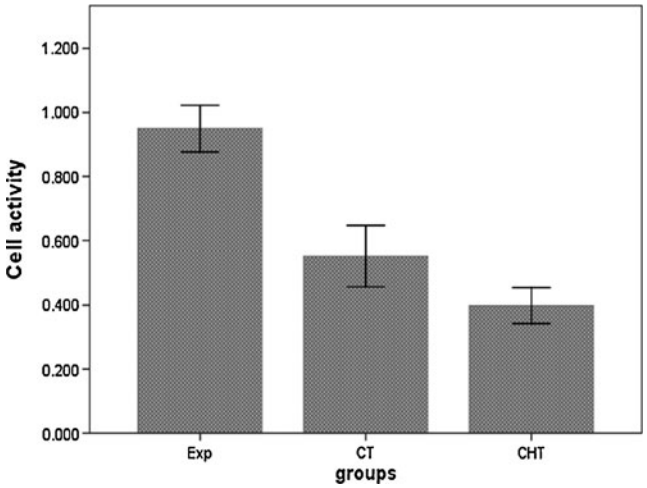

Figure 3. Cell proliferation was assessed by the WST-1 method. The highest proliferation rate was found in the Exp (explant) group followed by the CT (collagenase/trypsin) and CHT (collagenase/hyaluronidase/trypsin) groups.

higher expression rates of CD90 (72.23±3.64 and 55.79±5.95, respectively) than CHT group (28.53±1.81). Furthermore, CD73 was expressed higher rate in the Exp group (74.47±0.67) than the CHT group (13.33±5.02). Both the CT and CHT populations expressed a considerable level of C-kit (33.05±8.41 and 42.14±27.67, respectively) compared to 4.56±0.79 in the Exp population. Cells in the Exp and CHT groups slightly expressed Oct-4 (11.31±1.77 and 6.08±3.64, respectively) compared to 30.66±7.55 in the CT group (Table 1).

Alkaline phosphatase activity. The colonies formed by the hUCM cells exhibited ALP activity, which is consistent with the stem cell identity. The hUCM cells were maintained in culture until colony formation was observed. The reaction product was most intense within and surrounding the colonies. Also, ALP activity was observed in a small number of single cells in all groups Fig. 5a,b,c. There was no significant difference in ALP activity between the groups ($p>0.05$).

Adipogenic and osteogenic differentiation. In this study, an accumulation of lipid-containing vacuoles was detected in all groups as early as 6–7 d after the onset of treatment. Adipocytes began to morphologically appear, and their numbers increased with time. At day 15, approximately 15–20% of the cells appeared to be adipocytes, as determined by Oil Red O staining Fig. 6a,b,c. Osteogenic activity was observed on day 21 of treatment Fig. 6d,e,f. Human bone tissue was stained as the positive control by Alizarin Red-S Fig. 6*h*. No Oil Red O or Alizarin Red-S-positive cells were detected in the control group

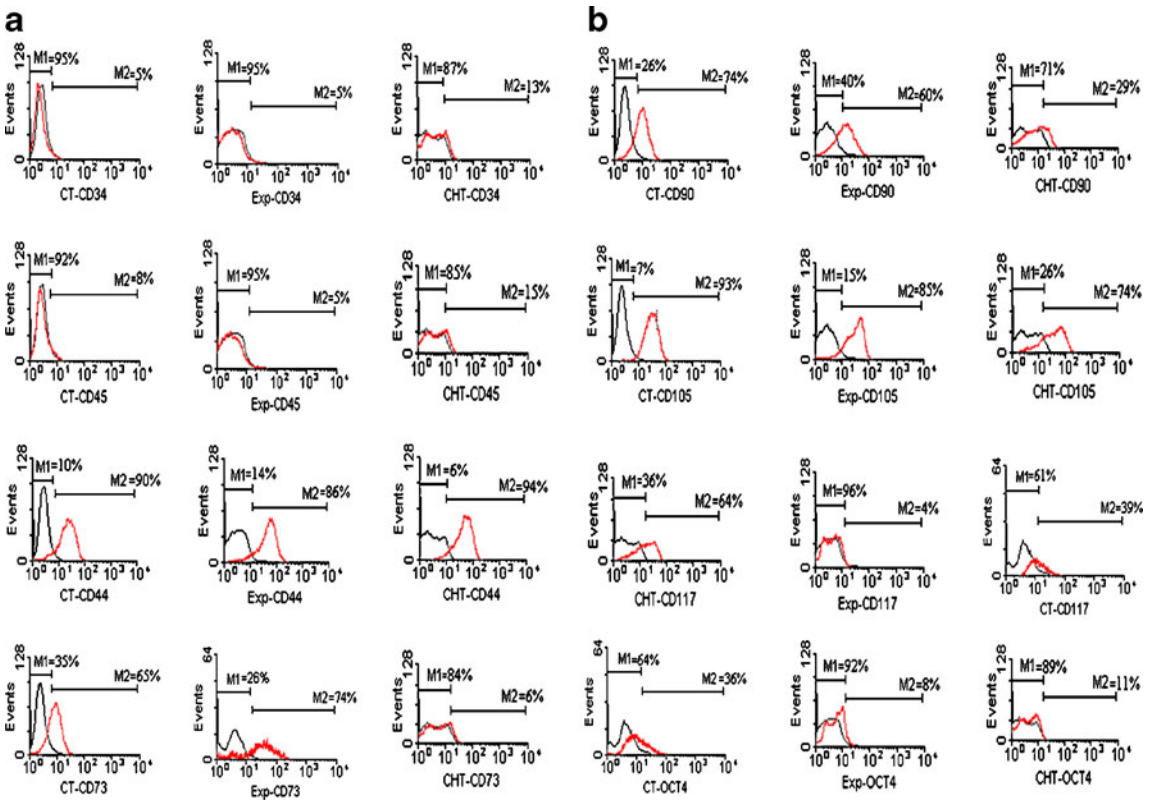

Figure 4. Representative of flow cytometry results. Data were obtained from passage 4. The *black histograms* show the isotype control-stained cells and the red histograms show the antibody-stained cells. The control population was stained with matched isotype antibodies (FITC-conjugated and PE-conjugated mouse IgG1κ monoclonal isotype standards), which were confirmed by positive fluorescence of the limbal samples.

Table 1. Flow cytometry results for the CT, CHT and EXP groups

Intracellular or surface antigens	CT group	EXP group	CHT group
CD 34	5.79±0.21	5.40±2.57	13.61±3.22
CD 45	7.08±1.85	10.38±0.08	15.84±0.58
CD 44	97.69±0.94	93.01±7.92	93.54±0.92
CD 73	67.32±3.28	74.47±0.67	13.33±5.02
CD 90	72.23±3.64	55.79±5.95	28.53±1.81
CD 105	90.30±8.16	83.06±2.73	74.19±0.19
C-Kit	33.05±8.41	4.56±0.79	42.14±27.67
OCT4	30.66±7.55	11.31±1.77	6.08±3.64

The values are mean±SD percentage of cells expressing each antigen and were derived from three independent experiments

Fig. 6*g*. The effects of the isolation methods on adipogenic and osteogenic differentiation were not significant ($p>0.05$).

Discussion

The techniques for the separation of adult stem cells from tissues and foetal mesenchymal stem cells from foetal membranes and tissues need to be refined, since the separation from stromal-contaminating components has not been optimized (Lorenzini et al. 2008). The isolation protocols allow cells to be reproducibly isolated and retain their morphological and phenotypical features throughout passage (La Rocca et al. 2009). However, no single isolation method has still been introduced as a standard protocol. Thus, we investigated whether or not isolation methods can affect cell characteristics. We found that the utilization of collagenase/trypsin enzymes gives better results than the other isolation methods (i.e. collagenase/hyaluronidase/trypsin, trypsin and explant). We also found that the fibroblast-like cell populations obtained in this study have similar properties in regard to morphology, immunophenotype and differentiation potential with MSCs.

The impacts of different enzymes on the morphology of isolated cells have shown some differences. In our study, the trypsin cell isolation protocol yielded cells with a different morphology compared to the cells in the other groups. It was shown that trypsin-dispersed cells consist of comparatively smaller cells, whereas collagenase-dispersed cells consist of larger cells (Miyazaki et al. 1984). Our results showed that the cells obtained via the trypsin method had a thin spindle-shaped morphology whereas the cells obtained by the other enzymatic methods had thick spindle, small round and flat shapes. In addition, the cells in the Trp group did not proliferate as fast as the cells in the other groups. This issue was so severe it prevented further assays being performed on these cells. We suggest that degradation of the extracellular matrix and disintegration of cell membranes may have caused cellular damage through the use of trypsin alone for a long period (Freshney 2005), because some cells are sensitive to exposure with trypsin but not to collagenase (Oyama et al. 1990).

Cell isolation using trypsin followed by digestion of the remaining tissue with collagenase proved to be very effective for obtaining MSCs (Semenov et al. 2009). Trypsin converts pro-collagenase into the active form of the enzyme (Stricklin et al. 1977), so most of the connective tissue cells will be released. Also, light trypsinization after using collagenase removes all adherent cells except macrophages (Freshney 2005). We used trypsin after collagenase or collagenase/hyaluronidase to promote the quantity and quality of the isolated cells.

The lack of a standard method for isolating WJ cells originates from the differences between the markers used to characterize such mesenchymal cells. CD73, CD105 and CD44 are just a few of the numerous markers shown to be present on MSCs (Schugar et al. 2009). It has been reported that differences in isolation and subculture protocols may lead to different cell lines in terms of both gene expression and stemness potencies (La Rocca et al. 2009). Many of the markers mentioned vary in their expression due to variations in tissue source, the methods of isolation and culture and species differences (Chamberlain et al. 2007). Interestingly, some antigens can be found on freshly isolated MSCs, but their expression disappears in culture (Bobis et al. 2006). It is important to note that differences in the expression of many cell surface markers can be influenced by factors secreted by accessory cells in the initial passage (Chamberlain et al. 2007). We found some differences between the different groups when the pattern of surface marker expression was considered. Expression of the haematopoietic markers CD34 and CD45, and the mesenchymal stem cell markers CD44 and CD105, was almost similar in all groups. However, the expression of mesenchymal markers CD73 and CD90 were different among the three groups. The results reported by other investigators are inconsistent to some

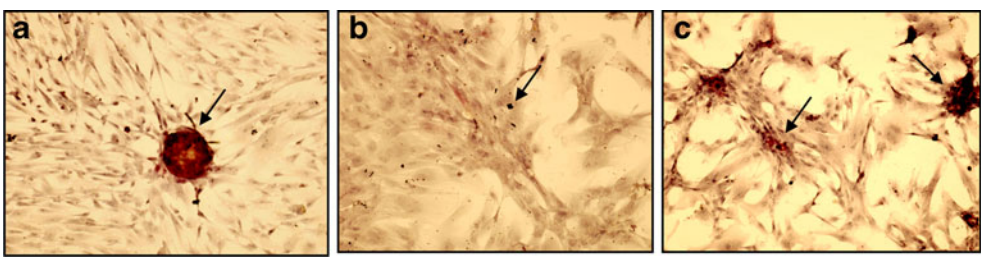

Figure 5. The cell colonies positively stained with alkaline phosphatase (*red colour*) in the (*a*) collagenase/trypsin group, (*b*) collagenase/hyaluronidase/trypsin group and (*c*) explant group. Original magnification ×200.

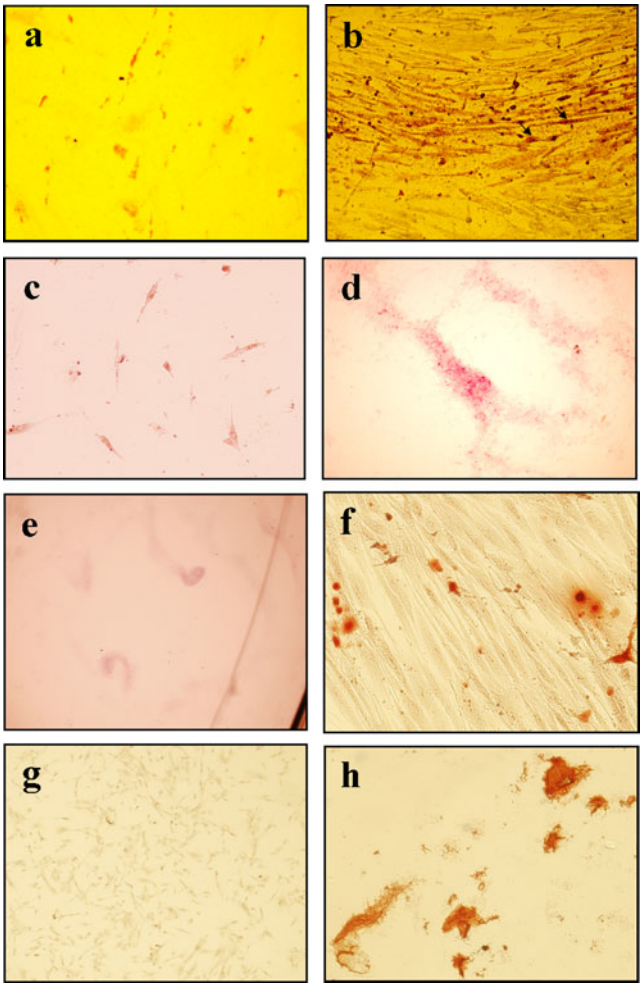

Figure 6. Adipogenic differentiation in the (*a*) collagenase/trypsin, (*b*) collagenase/hyaluronidase/trypsin and (*c*) explant groups. Oil Red-positive adipocytes stained bright orange. Osteogenic differentiation in the (*d*) collagenase/trypsin, (*e*) collagenase/hyaluronidase/trypsin and (*f*) explant groups. Alizarin Red-S-positive calcium deposition appeared as a red precipitate. (*g*) No Oil Red O or Alizarin Red-S-positive cells were detected in the control group. (*h*) Human bone tissue stained as a positive control by Alizarin Red-S. Original magnification ×100.

degree when cell marker expression is considered. For Example expression patterns for CD105, CD73, CD44 and CD90 were reported to be 35%, 42%, 48% and 65%, respectively, on WJ cells following isolation by collagenase digestion (Schugar et al. 2009), whereas, 96% and 91% of the hUCM cells stained positively for CD44 and CD90, respectively. These cells were harvested by an enzyme cocktail (e.g., hyaluronidase, trypsin and collagenase; Weiss et al. 2006). More comparisons are necessary in order to explain the reasons for these differences. It is certain that the collagenase digestion process will consistently isolate a specific cell population expressing MSC surface markers when compared to the dispase digestion and mechanical dissociation/explant culture methods (Schugar et al. 2009). Expression of Oct-4 and C-kit was also dissimilar between the groups. These two markers had a greater level of expression in the CT and CHT groups compared to the Exp group. Stromal cells, as the dominant cells in foetal-derived tissue, possess multipotent properties and are located between embryonic stem cells and adult stem cells (Can and Karahuseyinoglu 2007). Whereas they share common surface markers with bone marrow-derived MSCs, they also express certain embryonic stem cell markers, albeit at low levels, such as Oct-4 (Kadam et al. 2009; Huang et al. 2010a, b), C-kit (Vawda 2008) and Nanog, both at the mRNA and protein levels (Can and Karahuseyinoglu 2007; Nematollahi-Mahani et al. 2008). It is possible that MSCs from WJ are earlier-stage cells, the so-called more primary cells, than the MSCs of adult bone marrow (Wang et al. 2004).

Isolation protocols may also alter epigenetic and genetic changes in the cells, which may dramatically affect their plasticity and therapeutic utility (Phinney and Prockop 2007). Marked morphological and cell-size differences have been observed in the UC-derived primary cultures. The small-sized subpopulation exhibited a higher proliferative capacity compared to the total UC-derived primary cultures and the large-sized cells (Majore et al. 2009). Cell activity determined by the WST-1 assay showed that the cell proliferation rate was highest in the Exp group, followed by CT and then CHT groups. We suggest that the more homogeneous morphology of the cells in the Exp group might have resulted in the highest cell proliferation in this group. Also, damage to the extracellular matrix and cell membranes during enzymatic dissociation could have had an impact on the lower proliferation capacity of the CT and CHT cells.

The proportion of ALP-positive cells was highest in the CT group, followed by the Exp and CHT groups. The isolation procedure (explant versus enzyme treatment) apparently influences ALP levels at the start of osteoblast subcultures, where ALP activity was found to be higher in the enzymatic method compared to the explant method (Declercq et al. 2004). Our results are in accordance with the results of Schugar et al. (2009), who detected an increased presence of ALP in collagenase-isolated cells. Also, like the results of previously reported studies, the results of the present study show that the reaction product of alkaline phosphatase was most intense at the margins of the colonies, and a positive reaction was observed in some of the individual cells (Nematollahi-Mahani et al. 2008) in all groups.

We cultured hUCM cells for 21 d in adipogenic medium and then evaluated them by Oil Red staining. However, Karahuseyinoglu et al. (2007) reported that hUCM cells were shown adipocyte phenotype in 40 d. Our results were also similar to Struys et al. (2011) results that only less than one third of the hMSCs had adipogenic potential. Moreover, in our results the osteogenic differentiation of mesenchymal cells derived from Wharton's jelly was achieved after 28 d of culture that this time has been also noted by Karahuseyinoglu et al. (2007). In any case, whether or not isolation methods including the proper time of incubation can affect the

differentiation potential of these cells into other cell lineages needs to be investigated.

Conclusions

The purpose of this investigation was to develop the most appropriate method for isolating hUCMs. Isolation methods have a significant influence on the purity and growth capacity of isolated mesenchymal cell populations. When collagenase/trypsin enzymes are used, the waiting time for primary culture is shorter and results in (a) a higher number of isolated cells and (b) greater pluripotency properties of the isolated cells. The utilization of collagenase/trypsin enzymes is superior to the other isolation methods when the isolation and harvest of mesenchymal cells from human Wharton's jelly is considered.

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