Journal of Biological Research-Thessaloniki **16**: 194 – 201, 2011
J. Biol. Res.-Thessalon. is available online at http://www.jbr.gr
Indexed in: WoS (Web of Science, ISI Thomson), SCOPUS, CAS (Chemical Abstracts Service) and DOAJ (Directory of Open Access Journals)

Mesenchymal cells isolation from Wharton's jelly, in perspective to clinical applications

Iro KOLIAKOS[1*], Nikos TSAGIAS[2] and Vassilis KARAGIANNIS[2]

[1] *Stem Cells Bank*, *National Research Foundation*, *Athens*, *Greece*

[2] *C Gynecology Clinic*, *Hyppokrateion Hospital*, *Medical School*, *Aristotle University of Thessaloniki*, *Greece*

Received: 16 September 2010 Accepted after revision: 22 November 2010

Human umbilical cord tissue, termed as Wharton's jelly, is known to be an important source of mesenchymal cells (MSCs) with considerable therapeutic potential. A rapid enzymatic isolation method in order to cryopreserve large numbers of mesenchymal cells is described and evaluated by culture expansion, flow cytometry and post-thawing viability. The purpose of this study is to provide a rapid and safe method for collection and cryopreservation of mesenchymal cells, for current and future clinical applications. Sixty-seven umbilical cords were stripped of their blood vessels, minced, enzymatically digested and cryopreserved in appropriate media containing no animal serum. MSCs were also culture expanded before cryopreservation and after thawing and characterised for MSC surface marker expression. Post-thaw viability was measured by flow cytometry. An average of 260×10^3 viable cells per cm cord were successfully isolated from all samples. Isolated cells grew adherently with a homogeneous mesenchymal morphology and expressed high levels of CD105, CD90, CD29 and low levels of CD45. They showed a high replicative potential and retained MSC marker expression over six passages. Their viability and post-thaw proliferation were recorded in high values ($96.9 \pm 1.5\%$) and the expression of MSC markers was similar to pre-cryopreserved cells. Taking together, these findings indicate a fast and efficient cryopreservation of large numbers of MSCs that retain their mesenchymal character after thawing. In accordance to their high expansion capabilities and fast growth, they can be seen as a prominent source for cell therapy protocols.

Key words: mesenchymal cells, surface marker expression, cryopreservation, post-thaw viability, Wharton's jelly.

Abbreviations: MSCs: mesenchymal cells; CCM: complete culture medium; CPD-A: citrate-phospate-dextrose-adenine; DMSO: dimethyl sulfoxide; FITC: fluorescein isothiocyanate; HES 200/0.5: hydroxyethyl starch 200/0.5; PBS: phosphate buffered saline; PC5: phycoerythrin-cyanin 5; PE: phycoerythrin; 7-AAD: 7-amino-actinomycin D.

INTRODUCTION

Recent years have seen progress in isolation and expansion of mesenchymal cells (MSCs) from human umbilical cord tissue, namely the Wharton's jelly. Situated between the subamnion and the perivascular zone that surrounds the blood vessels (Troyer & Weiss, 2008), Wharton's jelly is a matrix of mucous connective tissue, composed of fibroblast-like stromal cells, collagen fibers and proteoglycans, mainly hyaluronic acid (Can & Karahuseyinoglou, 2007). Several studies have demonstrated the proliferative capacity and multipotentiality of these stromal cells; they were shown to differentiate into a neural phenotype *in vitro* (Mitchell *et al*., 2003; Ma *et al*., 2005; Yang *et al*., 2005; Lu *et al*., 2006; Ma *et al*., 2006; Karahuseyinoglou *et al*., 2007) and into chondrocytes (Wang *et al.,* 2004; Karahuseyinoglou *et al*., 2007), osteocytes (Wang *et al*., 2004; Sarugaser *et al*., 2005; Conconi *et al*., 2006; Karahuseyinoglou *et al*., 2007), cardiomyocytes (Wang *et al*., 2004), skeletal myocytes (Conconi *et al*., 2006), adipocytes (Conconi *et al*., 2006; Lu *et al*., 2006;

*Corresponding author: tel.: +30 6944 677746, fax: +30 2310 474285, e-mail: iro.koliakou@gmail.com

Karahuseyinoglou *et al*., 2007) and hepatocytes (Anzalone *et al*., 2010). *In vivo* studies indicated that these cells can differentiate to dopamine neurons (Fu *et al*., 2006; Weiss *et al*., 2006), to mature islet-like clusters with insulin-producing ability (Chao *et al*., 2008), and are able to rescue photoreceptors (Lund *et al*., 2007). Increasing interest is now based on their hematopoiesis-supportive function by means of long-term culture with cord blood hematopoietic stem cells or co-transplantation (Lu *et al*., 2006; Bakhshi *et al*., 2008). The mechanisms by which mesenchymal stem cells escape allogeneic rejection have already been discussed (Ryan *et al*., 2005), while the immunological features of Wharton's jelly stromal cells are now being stressed (Cho *et al*., 2008; La Rocca *et al*., 2009a, b).

Studies demonstrating the multipotential capacity of Wharton's jelly stromal cells have employed various isolation, expansion and characterisation techniques. Cells can be isolated either by enzymatic digestion of umbilical cord tissue or by culture expansion of small pieces of tissue, known as the explant method. Various culture conditions have also been used for their *in vitro* expansion; their growth conditions are poorly characterised (Troyer & Weiss, 2008). However, attempts on the large-scale production of bone marrow MSCs (Sotiropoulou *et al*., 2007), expansion of marrow MSCs in animal serum-free medium (Lange *et al*., 2007), the oxygen effects on MSCs cultures (Moussavi-Harami *et al*., 2004), as well as, the effect of various culture media on the expansion of Wharton's jelly explants (Friedman *et al*., 2007), have already been documented. Characterisation of these cells is usually performed by immunophenotype analysis in flow cytometry, while fluorescence-activated cell sorting can be used to sort the desired cell population (Zohar *et al*., 1997). Freezing and thawing of Wharton's jelly stromal cells have already been described (Sarugaser *et al*., 2005; Weiss *et al*., 2006), although characterisation of primary isolates following expansion before and after thawing is not documented.

In view of the potential therapeutic applications of Wharton's jelly stromal cells, we report a fast and efficient isolation and cryopreservation method that ensures its practical use by both research laboratories and clinical practice. The objective of this study is to isolate and cryopreserve large numbers of MSCs that remain viable and retain all their mesenchymal characters post-thaw, as can be determined by flow cytometry and appropriate markers.

MATERIALS AND METHODS

Collection of human umbilical cords

Sixty-seven fresh human umbilical cords were obtained from consenting parents. Immediately after delivery, samples were transferred to sterile containers in Hank's Balanced Salt solution (Biochrom AG) supplemented with 100 U penicillin/streptomycin (100 μg ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, Biochrom AG) at 4°C and stored for 1-24 hrs before tissue processing.

Isolation, culture and cryopreservation

Under sterile conditions, the cord was disinfected in Betadine solution (Lavipharm Hellas) followed by 100% ethanol wash and cleared off in normal saline. Using sterile scalpel and forceps the cord was dissected and unfolded and the exposed arteries and vein were removed and discarded. The tissue was washed free of contaminating blood with normal saline throughout the process and cut into 2-5 mm^3 pieces with a scalpel. The pieces were immersed in an enzymatic cocktail comprising 4 mg ml^{-1} Collagenase Type I (Gibco) and 1 mg ml^{-1} Hyaluronidase (Applichem) and incubated for 1 hr at 37°C with gentle agitation, followed by the addition of 2.5% Trypsin (Biochrom AG) and a further 30 minutes incubation under the same conditions. The digested suspension was diluted 1:2 with PBS (Biochrom AG) to reduce the viscosity of the suspension and passed through a 70 μm nylon mesh (cell strainer BD Falcon^{TM}; BD Biosciences, Bedford, MA, USA) to obtain a single-cell suspension. Cells were centrifuged at 500 g for 15 minutes at 37°C and the pellet was resuspended in a medium containing 25% human albumin (12.5 g per 50 ml; Bayer), HES 200/0.5 (HEAS-steril 10%, Fresenius Kabi, Deutschland GmBH, Germany) and citrate-phosphate-dextrose-adenine anticoagulant (CPD-A). Viable cells were counted using a trypan blue dye exclusion assay on a Neubauer Hemocytometer.

A small portion of the suspension was used for culture in uncoated 6-well plates (CELLSTAR) at an initial plating density that ranged from 1.7 to 9.4×10^3 cells cm^{-2}. Cells were grown in complete culture medium (CCM) composed of Dulbecco's modified Eagle's medium, low glucose with 2 mM L-glutamine (Biochrom AG), supplemented with 20% fetal bovine serum (Biochrom AG), 100 U penicillin/streptomycin and 50 mg ml^{-1} ascorbic acid (Applichem). Cultures were maintained at 37°C in a humidified atmosphere

containing 5% CO_2. Non-adherent cells were removed after 24 hrs by a first change of medium, followed by subsequent changes every 2-3 days. To assess MSC marker expression and morphology over six passages, three samples were passaged in T25 and T75 flasks (CELLSTAR) at a constant plating density of 1×10^3 cells cm^{-2}.

For cryopreservation purposes, the suspension was diluted 1:2 in a freezing medium containing 20% dimethyl sulfoxide (DMSO) (Applichem), human albumin (25%), HES 200/0.5 and CPD-A. The freezing medium was added slowly at a rate of 1 ml min^{-1} with continuous gentle stirring, to aid a better uptake of DMSO. The cell suspension was transferred in cryotubes (Nalge Nunc International, Rochester, NY, USA) covered with cryoflex (Nalge Nunc International), which were gradually frozen to –80˚C overnight in a Mr Frosty freezing container (Nalgene Labware) filled with fresh isopropanol and then transferred permanently to –196˚C liquid nitrogen.

Flow cytometry

On reaching 80-100% confluence, cells were rinsed three times in PBS (preheated at 37˚C) and detached from the plastic with 0.05% Trypsin at 37˚C for 3-7 minutes or until 90-100% of cells were floated. Trypsin was inactivated with fresh medium and the cell suspension was centrifuged at 500 g for 6 minutes. The pellet was resuspended in culture media and the viable cells counted by Trypan blue staining on a hemocytometer. Cells were stained with phycoerythrin (PE)-conjugated antibody against CD105, fluorescein isothiocyanate (FITC)-conjugated antibody against CD29 and phycoerythrin-cyanin 5 (PC5)-conjugated antibody against CD90. Non-viable cells were stained with 7-amino-actinomycin D (7-AAD). Phycoerythrin-cyanin 5 (PC5)-conjugated antibody against CD45 was also used as a negative hematopoietic marker in 30 of the 67 samples. Matched isotype antibodies for FITC, PE and PC5 served as controls. Cells were incubated for 20 minutes at room temperature, in the dark, and analysed in a Beckman-Coulter FC500 flow cytometer (Beckman-Coulter, Miami, FL, USA). Flow cytometry was also utilised immediately after cell isolation (n = 5) to assess viability by 7-AAD staining.

Post-thaw

Cells removed from liquid nitrogen and rapidly thawed by shaking in a 45˚C water bath, until a small particle of ice was still visible and transferred in CCM preheated at 37˚C. Post-thaw viability was evaluated, in nine samples, by 7-AAD staining in the flow cytometer. Five of these samples were also culture expanded and further characterised by flow cytometry at the first passage. Plating density ranged from 6.3 to 21×10^3 cells cm^{-2}.

Explants

Five of the cord samples were also tested with the explant method, in terms of culturing, freezing and thawing small pieces of Wharton's jelly. Briefly, the cord was disinfected, blood vessels removed and tissue cut into pieces as described above. A small part (10-15 pieces) was directly cultured in a well of a 6-well plate, under the same conditions used for the enzymatic method. The plate was left undisturbed for 3-5 days until the first change of medium and changed every 2-3 days thereafter. The remaining pieces were diluted in the same freezing medium as for the enzymatic method (reaching a final concentration of 10% DMSO) and transferred in cryotubes for the freezing process. Explants were rapidly thawed as described above and culture expanded as before cryopreservation.

Bacteriology

Aerobic and anaerobic bacteria contamination was tested on the supernatant following centrifugation of the cells and before cryopreservation, using the Bactec (Beckton Dickinson, Mountain View, CA, USA) or Bact/ALERT (Biomerieux) systems. Cell cultures were also checked for bacterial and fungi infection under the microscope.

RESULTS

Isolation and culture

Mesenchymal cells were isolated from 67 umbilical cords and counted on a Neubauer Hemocytometer. Cell yield was calculated at 260×10^3 viable cells per cm of cord used (range 45-594×10^3 cm^{-1} cord). Viability, checked by trypan blue, was 97.6 ± 3.1% (data not shown).

Cells started showing homogeneous fibroblast-like morphology 24 hrs after seeding (Fig. 1). Most cultures were initiated at a plating density of 5.2×10^3 cells cm^{-2}, by which cells reached confluence in 14.3 ± 7.9 days (Table 1). At this starting density, cells were expanded 300-fold over six passages (n = 3).

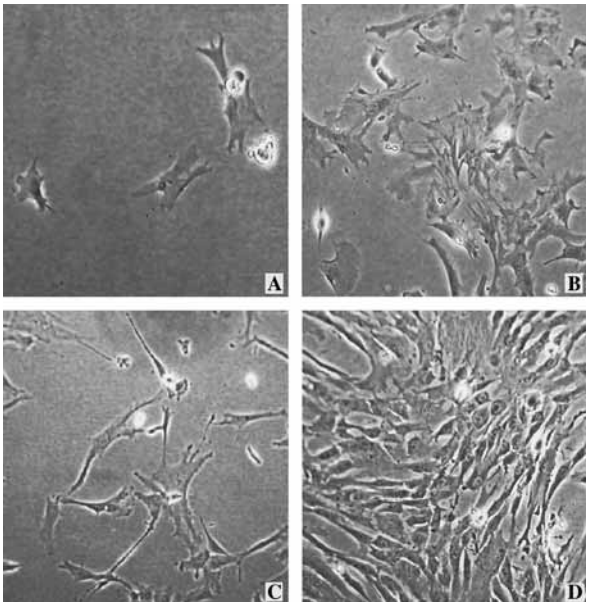

FIG. 1. Morphological features of primary cultured cells at day 1 (A) and 3 (B) following seeding. Cells after the first passage at day 3 (C) and 7 (D). (Original magnification ×10).

TABLE 1. Time needed by primary cultured cells to reach confluence in respect to the initial plating density

Plating density ($\times 10^3$ cm^{-2})	Days to confluence	Number of samples
1.7	39	1
2.1	22.7 ± 11.4	4
3.1	26.2 ± 8.3	5
5.2	14.3 ± 7.9	44
6.3	10 ± 3.8	4
7.3	8.8 ± 2.3	6
8.4	11.5 ± 4.9	2
9.4	11	1

Flow cytometry

The viability of five primary isolates as determined by 7-AAD staining was 98.9 ± 1.1%. At first passage cells stained positive for CD105 (81.9 ± 17.8%), CD90 (99.4 ± 1.4%) and CD29 (95.5 ± 5.3%) (n = 67) and negative for CD45 (11.1 ± 5.5%) (n = 30). Culture viability by 7-AAD staining was 96 ± 3.6% (Fig. 2) (n = 67). It was also shown that from first to sixth passage the mean percentage of CD105 dropped in three samples and was 4.5% (n = 3), while there was no drop for CD90 and CD29 expression (data not shown). After the second passage all cultured cells were negative for CD45. The course of MSC marker expression over six passages is shown in Figure 3.

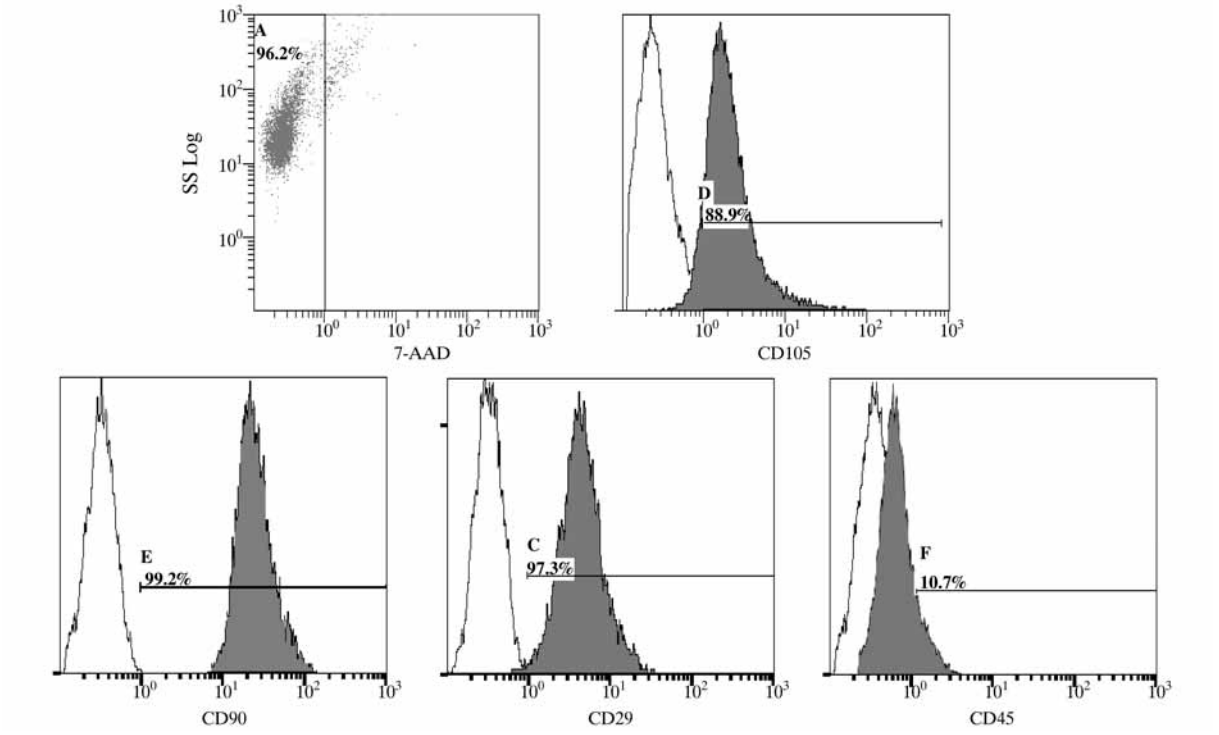

FIG. 2. Expression of MSC surface markers by cultured cells at P0 in a representative sample. Matched isotype IgG controls are shown as non-shaded areas. A minimum of 10000 events was recorded in all cases.

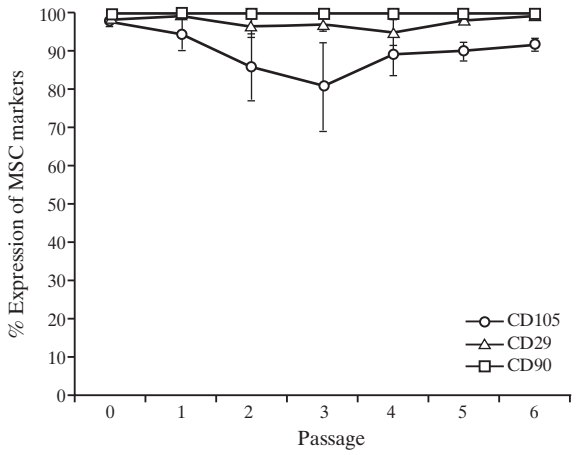

FIG. 3. Mean values of percentage expression of MSC markers of three samples from P0 to P6. Error bars denote standard deviation.

Post-thaw

Post-thaw viability of primary isolates, by 7-AAD staining, was 96.9 ± 1.5% (n = 9). Expression of MSC surface markers was evaluated after culture expansion and compared to values obtained before cryopreservation (n = 5). Post-thaw cultures showed higher levels of MSC surface marker expression (Table 2). These cultures reached confluence in 14 ± 4.4 days, while the same cultures before cryopreservation needed 10 ± 0.5 days to reach confluence.

Explant

Five explant cultures were compared morphologically to identical cultures initiated by the enzymatic method. Explant cultures were shown to form clumps and colonies with dense centres, some of them were detached from the plastic (Fig. 4). Confluence was reached much slower compared to the same enzymatic cultures (25 ± 8.7 days compared to 15 ± 9.9 days, respectively). Due to clump formation, explant cultures could not be characterised in the flow cytometer. Post-thaw culture expansion of cryopreserved explants saw no spreading of cells (0 out 5 cultures).

TABLE 2. Mean ± SD of the percentage MSC marker expression of first passage cultures for 5 samples evaluated before cryopreservation and post-thaw

Marker	Before cryopreservation (%)	Post-thaw (%)
CD105	91.7 ± 9.2	98.7 ± 0.9
CD29	97.2 ± 2.3	99 ± 1.4
CD90	99.7 ± 0.2	99.9 ± 0.1

DISCUSSION

We have reported a detailed enzymatic method where large number of cells can be efficiently isolated from the umbilical cord matrix, and cryopreserved on the same day of the arrival in the laboratory. Cells were successfully isolated from 100% of samples, in a rapid procedure relative to the particularity of isolating tissue embedded cells, as finely commented by Karahuseyinoglu *et al*. (2007); the entire isolation and cryopreservation procedure for a 15 cm cord sample lasts approximately 3-4 hrs.

Complete enzymatic degradation of vessel-free tissue by collagenase and hyaluronidase followed by trypsin released much higher cell numbers than that reported previously by a similar enzymatic method (Weiss *et al*., 2006). The combination of three enzymes is appropriate to digest the different types of ex-

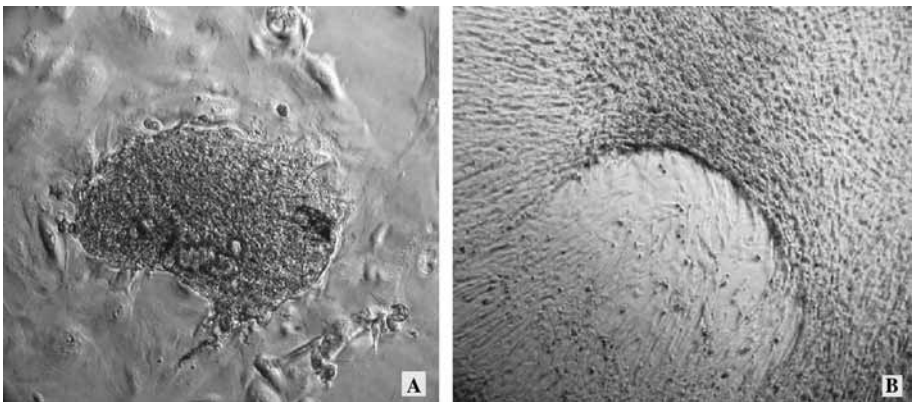

FIG. 4. Culture expansion of explant pieces after 12 days (A) and 26 days (B). (Original magnification ×20 for A and ×10 for B).

tracellular matrix components that Wharton's jelly contains. The lack of mechanical chopping of the cord that increases the interacting surface area of digestion reagents (Can *et al*., 2007) and the removal of large pieces of undigested tissue before centrifugation, used in the Weiss method, could account for the difference in cell yield. In another study where umbilical cords were enzymatically digested, but without vessel removal, the isolation efficiency was higher, probably reflecting the inclusion of endothelial, subendothelial and perivascular cells in the counting (Lu *et al*., 2006). Removal of vessels could represent an important reason for the uniform cell morphology observed in our primary cultures. In previous studies, enzymatic treatment of the umbilical cord vein endothelium and subendothelium yielded cultures with numerous small flattened cells with typical endothelial morphology and few fibroblast-like cells, which nicely contrasts to our observations and gives an idea of how cultures would appear if vessels were included in the isolation (Covas *et al*., 2003; Romanov *et al*., 2003). Since it is known that Wharton's jelly stromal cells had greater expansion efficacy and lower doubling times than perivascular cells (Karahyseyinoglou *et al.*, 2007), and thus they represent a different cell population, blood vessels along with the perivascular region of the cord were not included in this isolation procedure. Perivascular mesenchymal cells stain for pan-cytokeratin stronger than Wharton's jelly and do not differentiate to neuronal cells (Karahuseyinoglou *et al*., 2007). These findings indicate that this type of cells is more differentiated than Wharton's jelly and perhaps are originated from the adjacent vascular cells. Wharton's jelly is derived from extra-embryonic or embryonic mesoderm and it is possible that some stem cell populations continue to exist along with Wharton's jelly mesenchymal cells and this is may explain the multipotentiality of Wharton's jelly mesenchymal cell populations.

The cord is chopped in small pieces and completely digested, just to secure a high cell yield. In addition, the homogeneous cell morphology in our cultures is believed to be shaped by immature and highly proliferative cells, in contrast to the highly differentiated fibroblasts in the perivascular regions, which for this reason were discarded along with the vessels (Nanaev *et al*., 1997). In addition to the morphological inspection of our primary cultures, cell surface marker expression determined by flow cytometry, was an important part of this study for characterising the cells. Upon culture, cells consistently expressed CD105, CD90 and CD29 at high percentages, but not CD45. The expression of these markers is one criterion for defining multipotent mesenchymal stromal cells (Dominici *et al*., 2006). The phenotypic characteristics of the cells in this study are in agreement with other studies employing the same mesenchymal stem cell markers (Wang *et al*., 2004; Conconi *et al*., 2006; Friedman *et al*., 2007; Bakhshi *et al*., 2008). In particular, expression of CD90 and CD29 showed low variations between samples, while CD105 expression exhibited the highest variations between samples, as demonstrated for the first time herein. After six passages, CD90 and CD29 expression remained intact, while CD105 after a temporal drop, followed a rising course from third passage onwards, reflecting an increase in cellular attachment, growth and spreading (Guo *et al*., 2004a) through the passages. The rising course of CD105 expression could also indicate that cells were still expanded in an undifferentiated state, since CD105 expression levels tend to decrease during differentiation of MSCs, isolated from various fetal tissues into adipocytes (Guo *et al*., 2004b).

Post-thaw mean viability of primary isolates, according to our data, are reported to be higher than 96% ($96.9 \pm 1.5\%$) as determined by 7-AAD staining in flow cytometry. Accordingly, post-thaw cell harvesting efficiency was 100% (9 out of 9 cultures). Decrease in post-thaw viability ($52 \pm 2.3\%$) has been reported by Karahuseyinoglou *et al.* (2007). The serum-free freezing medium, that had been used to cryopreserve our samples, proved to sustain high viability and cell expansion, in contrast to serum dependent concentration, according to Karahuseyinoglou *et al.* (2007). In addition, the cells not only retain the mesenchymal marker and high proliferative capacity post-thaw, but they showed a slight increase in marker expression, compared to pre-cryopreservation primary cultures. Post-thaw cells showed a fast proliferative status, which was slower to their pre-cryopreservation counterparts, but similar to the general mean of 14.3 ± 7.9 days reached by primary isolates cultured at 5.2×10^3 cells cm^{-2}.

A rapid same-day procedure for obtaining and cryopreserving Wharton's jelly mesenchymal cells has already been proposed using the explant method, by direct culture of small pieces of umbilical cord, as an alternative to freezing cells after culture expanded or primarily isolated native cells (Friedman *et al*., 2007). Our attempt is to compare the proliferative capacity and phenotypic characteristics of primary cultured or native cells, as well as post-thaw, using both the enzy-

matic and explant methods. Our preliminary results indicate that, albeit a faster preparation, the explant method does not produce an efficient and safe for clinical use number of MSCs, rendering them impossible to characterise by flow cytometry. Total tissue cryopreservation ensures the cryopreservation of the superficial layer of cells. Cryoprotectants, like DMSO, can not penetrate the total thickness of the tissue and insert to each cell separately. So the superficial layer of the cells is cryopreserved safely, but the one underneath will die during post-thaw, because of cytoplasm crystal formation. Cutting the umbilical cord in small pieces will expose more cells to DMSO and finally the number of viable cells after post-thaw will be more. Since a number of superficial cells die during cutting and the recovering of cells after thawing is low, a significant, initial cell number for culturing can not be reached. On the other hand, the explant method has been used successfully in various differentiation studies (Mitchel *et al*., 2003; Conconi *et al*., 2006), there is not any risk of cellular degradation after enzymatic treatment, but we can not exclude spontaneous differentiation during culture expansion.

Using our protocol we provide a rapid, safe and efficient method to isolate and cryopreserve MSCs for current or future clinical use. Primary isolates herein harbour large number of cells ready to provide homogeneous cultures, exhibiting a high proliferative capacity and the phenotypic characteristics of MSCs. Storage in serum-free freezing medium sustains their proliferative behaviour and mesenchymal phenotype post-thaw and benefits their clinical use. To reduce any possible contamination with viruses or prions, cells are frozen in an animal serum-free medium, while primary isolates are monitored for bacterial infection right before cryopreservation.

Autoimmune (Chao *et al*., 2008) and neurodegenerating diseases (Weiss *et al*., 2006), cardiac failure (Noort *et al*., 2010), bone fractures (Granero-Moltó *et al*., 2009) and osteoarthritis (Djouad *et al*., 2009) are some of the diseases that are proposed to be treated with MSCs. Further studies are needed to demonstrate that these cells are actually multipotent and possess self-renewal property, as mesenchymal cells do. It is also important to study their differentiation capacity post-thaw and the most effective therapeutic dose following *ex vivo* expansion, that will establish MSCs from Wharton's jelly, as an important source for cellular therapies. Their ability to differentiate not only to mesoderm tissue origin, make them valuable for the new era of the Regenerative Medicine.

REFERENCES

Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F, Zummo G, Farina F, La Rocca G, 2010. New emerging potentials for human Wharton's jelly mesenchymal stem cells: immunological features and hepatocyte-like differentiative capacity. *Stem Cells and Development*, 19: 423-438.

Bakhshi T, Zabriskie RC, Bodie S, Kidd S, Ramin S, Paganessi LA, Gregory SA, Fung HC, Christopherson KW 2nd, 2008. Mesenchymal stem cells from the Wharton's jelly of umbilical cord segments provide stromal support for the maintenance of cord blood hematopoietic stem cells during long-term ex vivo culture. *Transfusion*, 48: 2638-2644.

Can A, Karahuseyinoglu S, 2007. Concise review: Human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells*, 25: 2886-2895.

Chao KC, Chao KF, Fu YS, Liu SH, 2008. Islet-like clusters derived from mesenchymal stem cells in Wharton's jelly of the human umbilical cord for transplantation to control Type 1 diabetes. *PLoS ONE*, 1: e1451.

Cho PS, Messina DJ, Hirsh EL, Chi N, Goldman SN, Lo DP, Harris IR, Popma SH, Sachs DH, Huang CA, 2008. Immunogenicity of umbilical cord tissue derived cells. *Blood*, 111: 430-438.

Conconi MT, Burra P, Di Liddo R, Calore C, Turetta M, Bellini S, Bo P, Nussdorfer GG, Parnigotto PP, 2006. CD105(+) cells from Wharton's jelly show *in vitro* and *in vivo* myogenic differentiative potential. *International Journal of Molecular Medicine*, 8: 1089-1096.

Covas DT, Siufi JLC, Silva ARI, Orellana MD, 2003. Isolation and culture of umbilical vein mesenchymal stem cells. *Brazilian Journal of Medical and Biological Research*, 36: 1179-1183.

Djouad F, Bouffi C, Ghannam S, Noël D, Jorgensen C, 2009. Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases. *National Review of Rheumatology*, 5: 392-399.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E, 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*, 8: 315-317.

Friedman R, Betancur M, Boissel L, Tuncer H, Cetrulo C, Klingemann H, 2007. Umbilical cord mesenchymal stem cells: Andjuvants for human cell transplantation. *Biology of Blood and Marrow Transplantation*, 13: 1477-1486.

Fu Y-S, Cheng Y-C, Lin M-Y, Cheng H, Chu P-M, Chou S-C, Shih Y-H, Ko M-H, Sung M-S, 2006. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons *in vitro*: potential therapeutic application for Parkinsonism. *Stem Cells*, 24: 115-124.

Granero-Moltó F, Weis JA, Miga MI, Landis B, Myers TJ,

O' Rear L, Longobardi L, Jansen ED, Mortlock DP, Spagnoli A, 2009. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells*, 27: 1887-1898.

Guo B, Rooney P, Slevin M, Li C, Parameshwar S, Liu D, Kumar P, Bernabeu C, Kumar S, 2004a. Overexpression of CD105 in rat myoblasts: role of CD105 in cell attachment, spreading and survival. *International Journal of Oncology*, 2: 285-291.

Guo H, Liu J-W, Yang S-G, Cao D-J, Liu J-H, Liao L-M, 2004b. The distribution of $CD105^+$ cells with characteristics of mesenchymal stem cells in diverse fetal tissues and organs. *Chinese Journal of Cell Biology*, 26: 404-408.

Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D, Can A, 2007. Biology of stem cells in human umbilical cord stroma: *in situ* and *in vitro* surveys. *Stem Cells*, 25: 319-331.

La Rocca G, Anzalone R, Farina F, 2009a. The expression of CD68 in human umbilical cord mesenchymal stem cells: new evidences of presence in non-myeloid cell types. *Scandinavian Journal of Immunology*, 70: 161-162.

La Rocca G, Anzalone R, Corrao S, Magno F, Loria T, Lo Iacono M, Di Stefano A, Giannuzzi P, Marasà L, Cappello F, Zummo G, Farina F, 2009b. Isolation and characterization of Oct-4^+/HLA-G^+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. *Histochemistry and Cell Biology*, 131: 267-282.

Lange C, Cakiroglu F, Spiess AN, Cappallo-Obermann H, Dierlamm J, Zander AR, 2007. Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *Journal of Cellular Physiology*, 213: 18-26.

Lu L-L, Liu Y-J, Yang S-G, Zhao Q-J, Wang X, Gong W, Han Z-B, Xu Z-S, Lu Y-X, Liu D, Chen Z-Z, Han Z-C, 2006. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. *Haematologica*, 91: 1017-1026.

Lund RD, Wang S, Lu B, Girman S, Holmes T, Sauvé Y, Messina DJ, Harris IR, Kihm AJ, Harmon AM, Chin F-Y, Gosiewska A, Mistry SK, 2007. Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. *Stem Cells*, 25: 602-611.

Ma L, Feng X-Y, Cui B-L, Law F, Jiang X-W, Yang L-Y, Xie Q-D, Huang T-H, 2005. Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiation into nerve-like cells. *Chinese Medical Journal*, 118: 1987-1993.

Ma L, Cui B-L, Feng X-Y, Law F-D, Jiang X-W, Yang L-Y, 2006. Biological characteristics of human umbilical cord-derived mesenchymal stem cells and their differentiation into neurocyte-like cells. *Zhonghua Er Ke Za Zhi*, 44: 513-517.

Mitchell KE, Weiss ML, Mitchell BM, Martin P, Davis D, Morales L, Helwig B, Beerenstrauch M, Abou-Easa K, Hildreth T, Troyer D, 2003. Matrix cells from Wharton's jelly form neurons and glia. *Stem Cells*, 21: 50-60.

Moussavi-Harami F, Duwayri Y, Martin JA, Moussavi-Harami F, Buckwalter JA, 2004. Oxygen effects on senescence in chondrocytes and mesenchymal stem cells: consequences for tissue engineering. *Iowa Orthopaedic Journal*, 24: 15-20.

Nanaev AK, Kohnen G, Milovanov AP, Domogatsky SP, Kaufmann P, 1997. Stromal differentiation and architecture of the human umbilical cord. *Placenta*, 18: 53-64.

Noort WA, Feye D, Van Den Akker F, Stecher D, Chamuleau SA, Sluijter JP, Doevendans PA, 2010. Mesenchymal stromal cells to treat cardiovascular disease: strategies to improve survival and therapeutic results. *Panminerva Medica*, 52: 27-40.

Romanov YA, Svintsitskaya VA, Smirnov VN, 2003. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells*, 21: 105-110.

Ryan JM, Barry FP, Murphy JM, Mahon BP, 2005. Mesenchymal stem cells avoid allogeneic rejection. *Journal of Inflammation*, 2: 8.

Sarugaser R, Lickorish D, Baksh D, Hosseini MM, Davies JE, 2005. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells*, 23: 220-229.

Sotiropoulou PA, Perez SA, Papamichail M, 2007. Clinical grade expansion of human bone marrow mesenchymal stem cells. *Methods in Molecular Biology*, 407: 245-263.

Troyer DL, Weiss ML, 2008. Concise review: Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells*, 26: 591-599.

Wang H-S, Hung S-C, Peng S-T, Huang C-C, Wei H-M, Guo Y-J, Fu Y-S, Lai M-C, Chen C-C, 2004. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells*, 22: 1330-1337.

Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, Luo Y, Rao MS, Velagaleti G, Troyer D, 2006. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells*, 24: 781-792.

Yang LY, Zheng JK, Wang CY, Xu MD, 2005. Stromal cells from human Wharton's jelly differentiate into neural cells. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 36: 13-16.

Zohar R, Sodek J, McCulloch CAG, 1997. Characterization of stromal progenitor cells enriched by flow cytometry. *Blood*, 90: 3471-3481.