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Cell viability evaluation of transdifferentiated endothelial-like cells by quantitative electron-probe X-ray microanalysis for tissue engineering

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Summary. Development of an efficient vascular substitute by tissue engineering is strongly dependent on endothelial cell viability. The aim of this study was to evaluate cell viability of transdifferentiated endothelial-like cells (Tr-ELC) by using for the first time electron probe X-ray microanalysis (EPXMA), not only to accurately analyze cell viability by quantifying the intracellular ionic concentrations, but also to establish their possible use in vascular tissue engineering protocols.

Human umbilical cord Wharton's jelly stem cells (HWJSC) and endothelial cells from the human umbilical vein (HUVEC) were isolated and cultured. Transdifferentiation from HWJSC to the endothelial phenotype was induced.

EPXMA was carried out to analyze HUVEC, HWJSC and Tr-ELC cells by using a scanning electron microscope equipped with an EDAX DX-4 microanalytical system and a solid-state backscattered electron detector. To determine total ion content, the peak-to-local-background (P/B) ratio method was used with reference to standards composed of dextran containing known amounts of inorganic salts.

Our results revealed a high K/Na ratio in Tr-ELC (9.41), in association with the maintenance of the intracellular levels of chlorine, phosphorous and magnesium and an increase of calcium ($p=0.031$) and sulfur ($p=0.022$) as compared to HWJSC. Calcium levels were similar for HUVEC and Tr-ELC. These results ensure that transdifferentiated cells are highly viable and resemble the phenotypic and microanalytical profile of endothelial cells.

Tr-ELC induced from HWJSC may fulfill the requirements for use in tissue engineering protocols applied to the vascular system at the viability and microanalytical levels.

Key words: Tissue engineering, Wharton stem cells, Endothelial-like cells, X-ray microanalysis

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Introduction

The gold standard conduit used to clinically replace diseased small-diameter arteries and veins is still autologous vascular tissue (DiMuzio et al., 2006). However, new creative combinations of scaffolds and stem cells are being used to elaborate artificial vascular conduits by tissue engineering (Lanni et al., 2001; Mitchell and Niklason, 2003; DiMuzio and Tulenki, 2007; L´Hereux et al., 2007; Wu et al., 2007a; Ku and Park, 2010). Although the results obtained with the use of native vascular grafts are normally very good, harvesting this kind of tissue is not always available from the patient and the drawbacks and problems associated to their use make the search for alternative substitutes based on tissue engineering strategies (Ravi et al., 2010).

Development of an efficient vascular substitute by tissue engineering is strongly dependent on the availability of an appropriate biomaterial able to

reproduce the functional properties of the vascular wall, and an optimal source of cells to be seeded on the biomaterial. These cells should provide structural boundaries to the circulating blood in the interior surface of the scaffold and serve as a selectively permeable thromboresistant surface (Michiels, 2003).

Due to the limited capability of human endothelial cells to grow and proliferate in culture (Rodríguez-Morata et al., 2008), recent works focused on the use of transdifferentiated endothelial-like cells (Tr-ELC) for the generation of artificial vascular conduits. In most cases, cells are obtained from different stem cell sources, including mesenchymal stem cells, which are easy to obtain and maintain in culture (DiMuzio and Tulenki, 2007; Wu et al., 2007b; Kim and von Recum, 2008; Wang et al., 2009; Alaminos et al., 2010; Shen et al., 2013; Kang et al., 2014). Particularly, human umbilical cord Wharton's jelly stem cells (HWJSC) have high telomerase activity and low expression of class I and II major histocompatibility complex antigens which make them promising candidates for vascular tissue engineering protocols (Mitchell and Niklason, 2003; Sarugaser et al., 2005; Lupatov et al., 2006; Lund et al., 2007; Wu et al., 2007b; Garzón et al., 2013, 2014).

One of the main factors influencing the success of therapies based on the use of bioengineered vascular substitutes is cell viability (Sánchez-Quevedo et al., 2007; Alaminos et al., 2007a, 2010; Rodríguez-Morata et al., 2008; Marañés Gálvez et al., 2011; Garzón et al., 2012b; Martín-Piedra et al., 2013; Rodríguez et al., 2013a,b). This is especially important in the case of vascular endothelial cells, since cell seeding and attaching to the scaffold is one of the limiting factors of this technology. Using a cell source with high cell viability could contribute to prevent cell loss once the vascular substitutes are implanted in vivo or subjected to pulsatile forces in bioreactors (Villalona et al., 2010). However, cell viability of Tr-ELC used for vascular tissue engineering has not been evaluated to this date.

In this context, one of the most accurate methods to determine cell viability of cell populations used for tissue engineering is quantitative electron-probe X-ray microanalysis (EPXMA) (Warley and Gupta, 1991; Warley, 1993, 1997). This non-destructive method allows the efficient determination of the intracellular concentrations of the most important elements found in the cell to predict cell viability with higher sensibility and specificity than most methods commonly used for the same purpose. In addition, cell death can be detected in very early stages and therefore selection of the most appropriate cells can be performed (Garzón et al., 2012a,b; Martín-Piedra et al., 2013, 2014).

The aim of this study was to evaluate cell viability of Tr-ELC by using for the first time EPXMA, not only to accurately determine the viability of transdifferentiated endothelial cells, but also to establish the microanalytical pattern of these cells for their use in vascular tissue engineering protocols.

Materials and methods

Cell isolation and culture

Four human umbilical cords were obtained at the University Hospital Príncipe de Asturias (Alcalá de Henares, Madrid, Spain). Cords were preserved at 4ºC in Eagle's Minimum Essential Medium (MEM) supplemented with penicillin, streptomycin and antimycotics (Sigma-Aldrich, St. Louis, MO) and processed within 24h. All patients included in the study provided informed consent for the use of the umbilical cords, and the local research and ethical committees approved this study.

To obtain endothelial cells from the human umbilical vein (HUVEC), the method of Jaffe was used (Jaffe et al., 1973). In brief, a 0.1% collagenase I solution was instilled into the umbilical vein for 15 minutes at 37°C, and detached cells were collected by centrifugation. HUVEC cells were cultured in Medium 199 plus (Gibco-BRL/Invitrogen) supplemented with 20% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 2 mM/l of L-glutamine, 1.5% of antibiotic-antimycotic solution and 1.5% of human platelet-derived endothelial cell growth factor (ECGF) (Sigma-Aldrich).

To obtain human umbilical cord Wharton's jelly stem cells (HWJSC), the blood vessels and the amniotic epithelium of the umbilical cord were surgically dissected and separated. The remaining tissue -the Wharton's jelly- was fragmented and digested in 0.1% collagenase I (Gibco-BRL/Invitrogen) at 37°C for 4 h. Isolated HWJSC were harvested by centrifugation and cultured in Amniomax culture medium (Gibco-BRL/Invitrogen).

To induce the transdifferentiation of HWJSC to the endothelial phenotype (Tr-ELC), Amniomax culture medium was removed from the HWJSC cultures and the culture medium used for the HUVEC cells was added as previously reported (Alaminos et al., 2010)

In all cases, cells were maintained at 37°C in a humidified incubator with 5% CO_2 and the culture medium was changed once every 3 days.

Phenotypical analysis of HWJSC and Tr-ELC

To analyze the phenotypical pattern of the different cell cultures, we used a contrast light microscope on cells growing in culture flasks. Then, cells were fixed in 4% formaldehyde and stained with haematoxylin and eosin and morphology was analyzed with a light microscope (Nikon Eclipse i90). Finally, cell phenotype was examined by using a scanning electron microscope (SEM). Cells were first fixed in 2.5% glutaraldehyde, critical-point dried and gold-coated. Then, a Quanta FEI scanning electron microscope was used to determine cell morphology.

To investigate the endothelial differentiation process of the HWJSC to Tr-ELC, expression of the endothelial

marker CD31 was analyzed. Cells cultured on culture chambers were first fixed in 4% paraformaldehyde and washed in PBS. After treatment with diluted H_2O_2 and preincubation in 3% BSA, antigen retrieval was performed with 0.1% triton X-100 and anti-CD31 antibodies were used at a 1:100 dilution. Incubation with the primary antibody was carried out overnight at 4°C. Cells were washed in PBS and incubated with secondary anti-mouse antibodies for 1h and with extravidin-alkaline phosphatase for 90min. CD31 detection was revealed with a Fast Red detection kit (Sigma–Aldrich). Positive expression of CD31 was finally detected by using a light microscope.

Quantitative Electron Probe X-Ray Microanalysis (EPXMA)

For quantitative electron probe X-ray microanalysis, HUVEC, HWJSC and Tr-ELC cells were subcultured on gold grids covered with a thin layer of pioloform (polyvinyl butyral) (Ted Pella, Redding, CA) following previously published protocols (Alaminos, 2007a,b; Sánchez-Quevedo et al., 2007; Rodríguez-Morata et al., 2008; Martín-Piedra et al., 2013, 2014). Cells were seeded at a density of 5,000 per grid and cultured in the medium were the cell cultures were established. After 24 h of culture, support grids containing the cells were washed in ice-cold distilled water for 5 sec to remove the extracellular medium. After washing, excess water was drained from the surface and the grids were subsequently plunge-frozen in liquid nitrogen. After cryofixation, the grids were placed in a precooled aluminum specimen holder at liquid nitrogen temperature and freeze-dried at decreasing temperatures for 24 h in an E5300 Polaron (Polaron Equipment Ltd, Watford, UK) freeze-drier apparatus equipped with a vacuum rotatory pump system. Freeze-dried gold grids were carbon-coated in a high-vacuum coating system and microanalyzed within 6 h.

Electron probe X-ray microanalysis of the specimens was performed by using a Philips XL30 scanning electron microscope (Eindhoven, The Netherlands) equipped with an EDAX DX-4 microanalytical system and a solid-state backscattered electron detector. The microanalytical conditions were as follows: tilt angle 0°, take-off angle 35°, and working distance 10 mm. The acceleration voltage was 10 kV. All spectra were collected in the spot mode at 10,000 X (equivalent to 50 nm spot diameter) for 200 sec live time, and the number of counts per second recorded by the detector was around 500. All determinations were performed on the central area of the cell nucleus. To determine total ion content, we used the peak-to-local-background (P/B) ratio method with reference to standards composed of 20% dextran containing known amounts of inorganic salts (Crespo et al., 1993; López-Escámez et al., 1993, 1994b; López-Escámez and Campos, 1994a; Campos et al., 1994; Sánchez-Quevedo et al., 1998). In this study we quantified the ionic content of 30 cells corresponding to each cell type -HUVEC, HWJSC and Tr-ELC-.

Statistical study

Average values and standard deviations were obtained for each intracellular element in each cell population -HUVEC, HWJSC and Tr-ELC-. To establish the statistical significance of differences between HWJSC and Tr-ELC, between HWJSC and HUVEC and between Tr-ELC and HUVEC regarding ionic cell contents, we used the statistical non-parametric test of Mann-Whitney. For all tests, a two-sided $p<0.05$ was considered statistically significant. All analyses were performed with the same statistical software (SPSS 15.0).

Results

HWJSC isolation and transdifferentiation

The methods and techniques used in this work allowed us to efficiently isolate and culture HWJSC. These cells showed the typical spindle-shape mesenchymal phenotype (Fig. 1) and fulfilled all the requirements of mesenchymal stem cells (expression of undifferentiation markers and multilineage differentiation potential).

After induction to the endothelial lineage, cells showed a characteristic morphological change, with cells becoming more polygonal and less elongated than control HWJSC (Fig. 1).

To confirm the endothelial transdifferentiation of these cells, immunohistochemistry for the endothelial marker CD31 was carried out. As shown in Fig. 2, our results showed some cells with positive expression of this marker.

Assessment of cell viability by electron probe X-ray microanalysis

Individual microanalytical spectra were obtained for each cell type by using electron probe X-ray

Table 1. Intracellular concentrations of all major elements found in HWJSC, HUVEC and Tr-ELC cell cultures and K/Na ratio as determined by quantitative electron-probe X-ray microanalysis.

	HWJSC	HUVEC	Tr-ELC
Na	126.82±40.19	112.11±35.4	45.61±20.3
Mg	20.99±3.81	32.23±11.11	21.4±5.71
P	277.61±37.05	306.33±70.72	232.4±45.28
S	40.95±13.45	82.66±15.22	46.25±20.15
Cl	199.88±36.71	267.47±55.11	169±43.74
K	302.6±101.14	452.36±65.12	429±71.11
Ca	8.53±9.64	16.79±10.92	16.24±14.12
K/Na	2.39	4.04	9.41

For each element, the average ± standard deviation is shown.

microanalysis. In each spectrum, peaks corresponding to the different elements existing at the intracellular level were observed (Fig. 3). Intracellular concentrations of each major element are shown in Table 1, showing that the K/Na ratio was 2.39 in HWJSC, 4.04 in HUVEC and 9.41 in Tr-ELC.

The statistical analysis (Table 2) demonstrated that the intracellular concentrations of Mg, P, S, Cl, K and Ca were significantly higher in HUVEC than in HWJSC, although no differences were found for Na. When HWJSC were compared with Tr-ELC, we found a significant increase in the concentrations of S and Ca and a significant decrease of Na in the transdifferentiated cell types. Finally, the comparison of HUVEC and Tr-

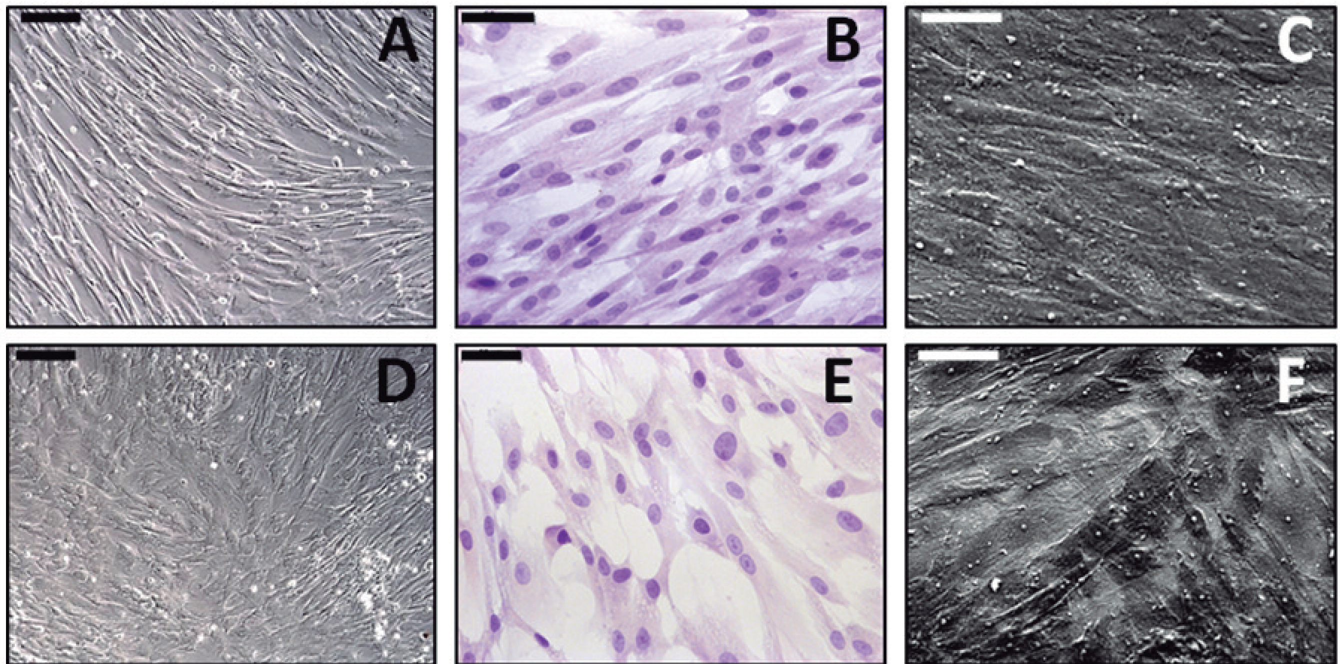

Fig. 1. Phenotypical analysis of HWJSC **(A, B and C**) and Tr-ELC **(D, E and F**) using contrast light microscopy **(A, D)**, haematoxylin-eosin staining **(B, E)** and scanning electron microscopy **(C, F)**. Scale bar: 50 μm.

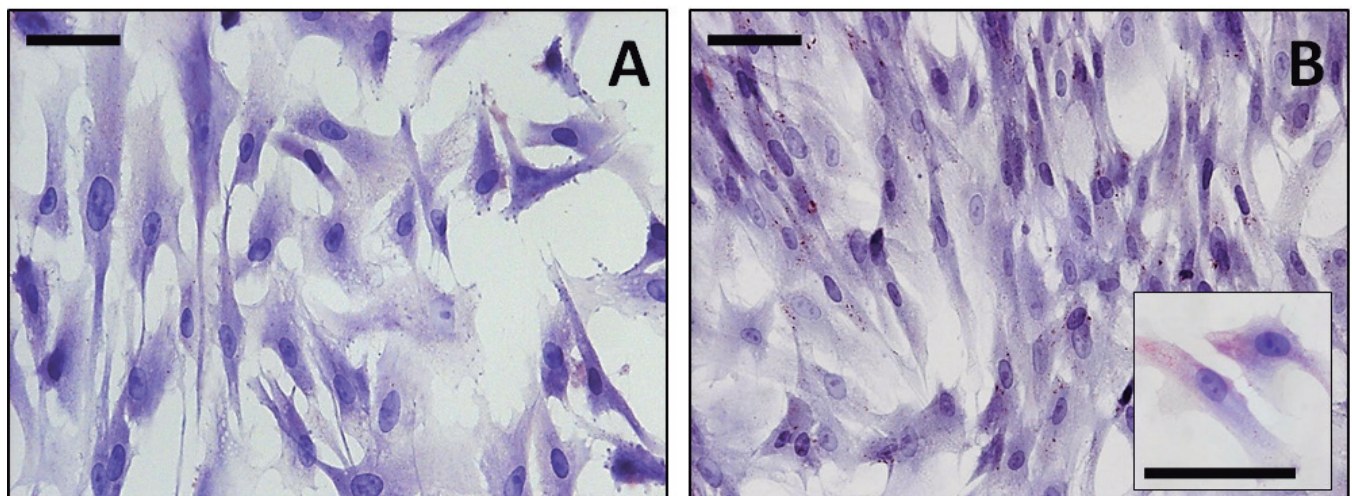

Fig. 2. CD31 immunohistochemical analysis of HWJSC **(A)** and Tr-ELC **(B)**. Positive signal can be detected as red spots. Scale bar: 50 μm.

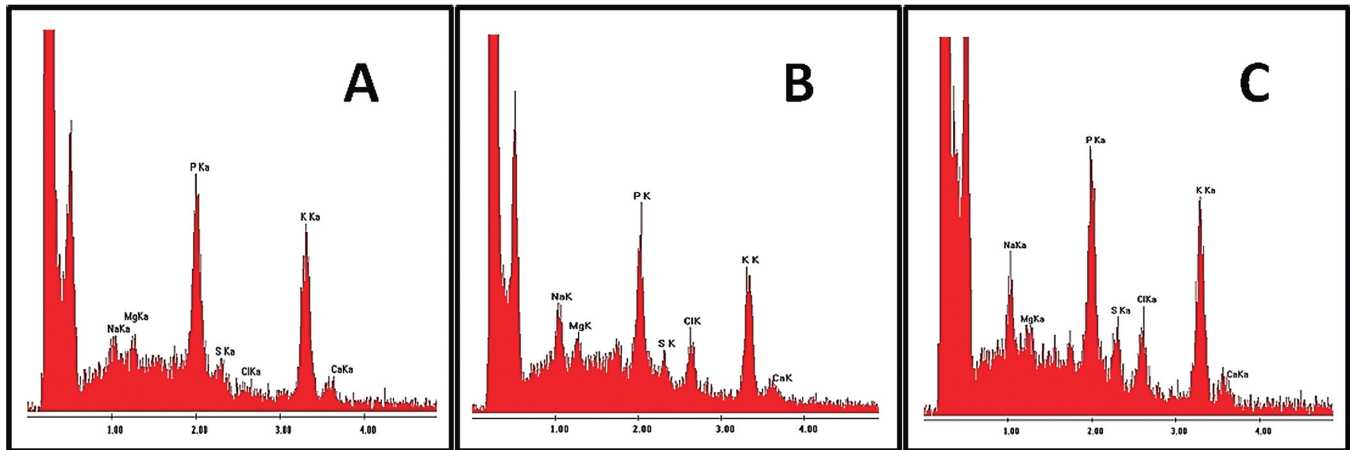

Fig. 3. Microanalytical spectra corresponding to HUVEC **(A)**, HWJSC **(B)**, and Tr-ELC (**C**).

Table 2. Results of the statistical comparison of intracellular elements for the three cell types analyzed in this work (HWJSC, HUVEC and Tr-ELC).

	[Na]	[Mg]	[P]	[S]	[Cl]	[K]	[Ca]
HWJSC vs. HUVEC	0.348	0.000*	0.002*	0.000*	0.004*	0.001*	0.018*
HWJSC vs. Tr-ELC	0.003*	0.277	0.762	0.022*	0.174	0.070	0.031*
HUVEC vs. Tr-ELC	0.010*	0.000*	0.002*	0.001*	0.000*	0.017*	0.605

Values correspond to p values for the Mann-Whitney statistical test. Statistically significant p values ($p<0.05$) are highlighted with asterisks (*).

ELC revealed that the intracellular contents of Na, Mg, P, S, Cl and K were significantly higher in HUVEC, with no differences for the concentrations of Ca.

Discussion

The limited availability and the problems associated with obtaining autologous healthy vessels for bypass grafting procedures has led to the fabrication of different models of artificial arteries and veins by tissue engineering using various and innovative scaffolds (Ravi et al., 2009). In addition to the efficient development of a bioengineered vascular conduit, it is necessary to generate a luminal surface of endothelial cells whose structure is as similar as possible to the natural lining of blood vessels (Bujan et al., 2004). In this milieu, transdifferentiated endothelial-like cells (Tr-ELC) have been recently suggested and applied as candidates for coating bioengineered vascular scaffolds.

In our work, we selected HWJSC as a source of cells for endothelial transdifferentiation. These cells were used not only because they have previously shown efficient phenotypical and genetic endothelial differentiation potential, but also due to their accessibility, painless procedures to donors and immunological properties (Lu et al., 2006; Alaminos et al., 2010). In addition, we demonstrated that these cells used for endothelial transdifferentiation showed typical markers of HWJSC and had differentiation potential to the osteogenic, chondrogenic, and adipogenic lineages (Alaminos et al., 2010).

Our results first confirmed the transdifferentiation potential of HWJSC to the endothelial lineage as previously demonstrated by our research group (Alaminos et al., 2010). This was first proved at the phenotypical level, with Tr-ELC showing a clear modification of their morphology, becoming more similar to a simple epithelium consisting of flattened polygonal cells as in the case of HUVEC. In contrast, control non-induced HWJSC were elongated or spindle-shaped, and their morphology was similar to other types of mesenchymal cells. Then, the process of trans-differentiation was demonstrated by the presence of the typical endothelial cell marker CD31. CD31 is a major constituent of the endothelial intercellular junctions and plays an important role in endothelial cell physiology. All these results suggest that endothelial phenotype is present in Tr-ELC (Alaminos et al., 2010) and that these cells could have clinical usefulness.

To confirm the possible in vivo application of these cells, cell viability was analyzed in transdifferentiated cells. Among the array of methods used to evaluate cell viability in cells kept in culture, quantitative electron-probe X-ray microanalysis was found to be the most accurate, sensitive and specific method in several cell types (Garzón et al., 2012a,b, 2013; Martín-Piedra et al., 2013, 2014). Furthermore, the use of EPXMA allows detection of cells undergoing early stages of apoptosis or necrosis that other techniques are not capable of detecting. In addition, the ionic changes that occur in cultured cells can act as very early markers of cell death and are detectable in cells before morphological and structural changes appear (Fernández-Segura et al.,

1996, 1997, 1999a,b; Rodríguez et al., 2011, 2013).

Although EPXMA has been applied to evaluate cell viability of HUVEC and HWJSCs (Rodríguez-Morata et al., 2008; Garzón et al., 2012b, 2013), this is the first time that this crucial parameter has been analyzed in Tr-ELC in order to determine whether these trans-differentiated cells could be appropriate for tissue engineered protocols.

Most studies confirm that the intracellular concentration of sodium and potassium and, especially, the K/Na ratio, is the best and most accurate microanalytical indicator of cell viability (Roomans, 2002a,b; Salido et al., 2004; Vilches et al., 2004; Alaminos et al., 2007b, 2008, 2010; Sánchez-Quevedo et al., 2007; Fernández-Segura and Warley, 2008).

The normal intracellular concentrations of K and Na are strictly maintained and controlled in viable cells by the function of a specific transmembrane K/Na pump (Alaminos et al., 2008). Cell survival is highly dependent on the integrity and functionality of this system, since the transmembrane Na-K gradient is very important for macromolecule synthesis and cell function (Falciola et al., 1994). In our study, we found that the sodium concentrations were similar for both HWJSC cells and HUVEC, while the Na levels were significantly lower in Tr-ELC. This marked intracellular Na concentration decrease in Tr-ELC contributes to the high K/Na levels found in these cells. Regarding the potassium concentrations, the lowest levels were found in HWJSC and the highest levels in HUVEC. These differences were statistically significant when HUVEC cells were compared to Tr-ELC and HWJSC cells. No statistically significant differences were found between HWJSC and Tr-ELC cells for intracellular potassium. This finding shows that the transdifferentiation process from HWJSC to Tr-ELC did not significantly reduce the intracellular potassium concentration, thus contributing to maintain the K/Na ratio and, therefore, cell viability.

In addition, previous reports demonstrated that sodium and potassium concentrations are directly involved in controlling cell volume (Zhang and Roomans, 1998; Becker et al., 2005). According to these reports, when the cell volume decreases, a compensatory volume increase mechanism is stimulated, and water is transferred into the cell concomitantly with Na and Cl. On the other hand, when the cell volume increases a mechanism of regulatory volume decrease is also stimulated, by which water is expelled out of the cell with a simultaneous decrease in the intracellular concentrations of Cl and K followed by an increase in Ca (Zhang and Roomans, 1998; Becker et al., 2005; Alaminos et al., 2008). In our work, calcium levels were similar for HUVEC and Tr-ELC, being significantly lower in HWJSC. This could be related to the phenotypic changes observed during the trans-differentiation process of HWJSC and, therefore, with the process of flattening of these cells to resemble the endothelial nature of HUVEC.

When we analyzed the intracellular concentration of chlorine, we demonstrated that the lowest levels were found in Tr-ELC, which were similar to HWJSC and significantly lower than HUVEC chlorine contents. The decrease of chlorine is an early indicator of apoptosis that normally coincides with a decrease in potassium and a sodium increase (Skeeper et al., 1999; Alaminos et al., 2007b). Determination of chlorine concentrations is fundamental to assess the viability of cells of a particular cell population. Maintenance of the chlorine levels, along with the K/Na ratio found in transdifferentiated cells allows us to confirm the high viability of Tr-ELC.

In relation to phosphorus, we found that the concentration of this element did not significantly vary after induction of the transdifferentiation process in HWJSC. It is known that the decrease in phosphorus concentrations correlates with cell mass, concentration of intracellular organic constituents, nucleic acid content and the phosphorylation level of the cell. Therefore, alterations of the contents of phosphorous may indicate serious structural cell damage (Roomans, 2002a,b). In our results, the fact that the levels of this element did not significantly vary in Tr-ELC confirms that the transdifferentiation process did not seriously affect the cells at the structural level.

One of the elements that is directly associated to the presence of sulphated glycosaminoglycans and proteoglycans at the cellular level is sulfur (Sánchez-Quevedo et al., 1989; Roomans, 2002b). In our study, we found that transdifferentiation of cells to the Tr-ELC lineage is able to significantly increase the presence of this element in the cells, and this could be related to a higher production of sulphated glycosaminoglycans and proteoglycans and, therefore, with the acquisition of certain levels of differentiation and maturation.

In relation to the intracellular concentration of magnesium, there are no statistical differences between HWJSC and Tr-ELC. It has been shown that a decrease in the concentration of magnesium is correlated with a reduction in cellular ATP concentrations and DNA replication (Buja et al., 1985; Di Francesco et al., 1998). Our results indicate that these important intracellular processes could be maintained and are not significantly affected in the process of HWJSC transdifferentiation.

In summary, all these results obtained by quantitative electron-probe X-ray microanalysis demonstrate that HWJSC have adequate differentiation potential to the vascular endothelial lineage with high levels of cell viability. The high K/Na ratio in Tr-ELC, in association with the maintenance of the intracellular levels of chlorine, phosphorous and magnesium, with an increase of the calcium and sulfur ensure that transdifferentiated cells fulfill all the cell viability requirements for use in tissue engineering protocols applied to the vascular system.

References

Alaminos M., Garzón I., Sánchez-Quevedo M.C., Moreu G., González-Andrades M., Fernández-Montoya A. and Campos A. (2007a).

Time-course study of histological and genetic patterns of differentiation in human engineered oral mucosa. J. Tissue Eng. Regen. Med. 1, 350-359.

Alaminos M., Sánchez-Quevedo M.C., Muñoz-Avila J.I., García J.M., Crespo P.V., González-Andrades M. and Campos A. (2007b). Evaluation of the viability of cultured corneal endothelial cells by quantitative electron probe X-ray microanalysis. J. Cell Physiol. 211, 692-698.

Alaminos M., González-Andrades M., Muñoz-Avila J.I., Garzón I., Sánchez-Quevedo M.C. and Campos A. (2008). Volumetric and ionic regulation during the in vitro development of a corneal endothelial barrier. Exp. Eye Res. 86, 758-769.

Alaminos M., Perez-Kohler B., Garzón I., García-Honduvilla N., Romer B., Campos A. Bujan J. (2010). Transdifferentiation potentiality of Human Wharton's Jelly Stem Cells towards vascular endothelial cells. J. Cell Physiol. 223, 640-647.

Becker D., Blasé C., Bereiter-Hahn J. and Jendrach M. (2005). TRPV4 exhibits a functional role in cell-volume regulation. J. Cell Sci. 118, 2435-2440.

Buja L.M., Hagler H.K., Parsons D., Chien K., Reynolds R.C. and Willerson J.T. (1985). Alterations of ultrastructure and elemental composition in cultured neonatal rat cardiac myocytes after metabolic inhibition with iodoacetic acid. Lab. Invest. 53, 397-411.

Bujan J., Garcia-Honduvilla N. and Bellon J.M. (2004). Engineering conduits to resemble natural vascular tissue. Biotechnol. Appl. Biochem. 39,17-27.

Campos A., López-Escámez J.A., Crespo P.V., Cañizares F.J. and Baeyens J.M. (1994). Gentamicin ototoxicity in otoconia: quantitative electron probe X-ray microanalysis. Acta Otolaryngol. 114, 18-23.

Crespo P.V., López-Escámez J.A., Cañizares F.J. and Campos A. (1993). X-ray microanalytical determination of P, S and K concentrations in the gelatinous membrane of the utricle. Acta Otolaryngol. 113, 176-180.

Di Francesco A., Desnoyer R.W., Covacci V., Wolf F.I., Romani A., Cittadini A. and Bond M. (1998). Changes in magnesium content and subcellular distribution during retinoic acid-induced differentiation of HL60 cells. Arch. Biochem. Biophys. 360,149-157.

DiMuzio P. and Tulenki T. (2007). Tissue engineering applications to vascular bypass graft development: the use of adipose-derived stem cells. J. Vasc. Surg. 45, 99-103.

DiMuzio P., Fischer L., McIlhenny S., DiMatteo C., Golesorhki N., Grabo D., Tarola N., Mericli A., Shapiro I. and Tulenko T. (2006). Development of a tissue-engineered bypass graft seeded with stem cells. Vascular 14, 338-342.

Falciola J., Volet B., Anner R.M., Moosmayer M., Lacotte D. and Anner B.M. (1994). Role of cell membrane Na,K-ATPase for survival of human lymphocytes in vitro. Biosci. Rep. 14, 189-204.

Fernández-Segura E. and Warley A. (2008). Electron probe X-ray microanalysis for the study of cell physiology. Methods Cell Biol. 88,19-43.

Fernández-Segura E, García J.M. and Campos A. (1996). Topographic distribution of CD18 integrin on human neutrophils as related to shape changes and movement induced by chemotactic peptide and phorbol esters. Cell Immunol. 171, 120-125.

Fernández-Segura E., Cañizares F.J., Cubero M.A., Revelles F. and Campos A. (1997). Electron probe X-ray microanalysis of cultured epithelial tumour cells with scanning electron microscopy. Cell Biol Int. 21, 665-669.

Fernández-Segura E., Cañizares F.J., Cubero M.A., Campos A. and Warley A. (1999a). A procedure to prepare cultured cells in suspension for electron probe X-ray microanalysis: application to scanning and transmission electron microscopy. J. Microsc. 196, 19-25.

Fernández-Segura E., Cañizares F.J., Cubero M.A., Warley A. and Campos A. (1999b). Changes in elemental content during apoptotic cell death studied by electron probe X-ray microanalysis. Exp. Cell Res. 253, 454-462.

Garzón I., Alfonso-Rodríguez C.A., Martínez-Gómez C., Carriel V., Martín-Piedra M.A., Fernández-Valadés R., Sánchez-Quevedo M.C. and Alaminos M. (2014). Expression of epithelial markers by human umbilical cord stem cells. A topographical analysis. Placenta 35, 994-1000.

Garzón I., Carriel V., Marín-Fernández A.B., Oliveira A.C., Garrido-Gómez J., Campos A., Sánchez-Quevedo M.C. and Alaminos M. (2012a). A combined approach for the assessment of cell viability and cell functionality of human fibrochondrocytes for use in tissue engineering. PLoS ONE 7, e51961.

Garzón I., Perez-Kohler B., Garrido-Gómez J., Carriel V., Nieto-Aguilar R., Martín-Piedra M.A., García-Honduvilla N., Buján J., Campos A. and Alaminos M. (2012b). Evaluation of the cell viability of human Wharton's jelly stem cells for use in cell therapy. Tissue Eng. Part C Methods 18, 408-419.

Garzón I., Miyake J., González-Andrades M., Carmona R., Carda C., Sánchez-Quevedo M del C., Campos A. and Alaminos M. (2013). Wharton's jelly stem cells: a novel cell source for oral mucosa and skin epithelia regeneration. Stem Cells Transl. Med. 2, 625-632.

Jaffe E., Nachman R., Becker C. and Minick CR. (1973). Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745-2756.

Kang B.J., Lee S.H., Kweon O.K. and Cho J.Y. (2014). Differentiation of canine adipose tissue-derived mesenchymal stem cells towards endothelial progenitor cells. Am. J. Vet. Res. 75, 685-691.

Kim S. and von Recum H. (2008). Endothelial stem cells and precursors for tissue engineering:cell source, differentiation, selection, and application. Tissue Eng. Part B Rev. 14, 133-147.

Ku S.H. and Park C.B. (2010). Human endothelial cell growth on mussel-inspired nanofiber scaffold for vascular tissue engineering. Biomaterials 31, 9431-9437.

Lanni G., Napolitano M.M., Scala A. and De Rosa P. (2001). "Tube-guided" implant of bioprosthetic shunt for hemodialysis. Am. J. Surg. 181, 50-51.

López-Escámez J.A. and Campos A. (1994). Standards for X-ray microanalysis of calcified structures. Scanning Microsc. 8, 171-185.

López-Escámez J.A., Crespo P.V., Cañizares F.J. and Campos A. (1993). Standards for quantification of elements in the otolithic membrane by electron probe X-ray microanalysis: calibration curves and electron beam sensitivity. J. Microsc. 171, 215-222.

López-Escámez J.A., Cañizares F.J., Crespo P.V., Baeyens J.M. and Campos A. (1994). Electron probe microanalysis of gentamicin-induced changes on ionic composition of the vestibular gelatinous membrane. Hear. Res. 1, 60-66.

L'Heureux N., McAllister T.N. and de la Fuente L.M. (2007). Tissue-engineered blood vessel for adult arterial revascularization. N. Engl. J. Med. 357,1451-1453.

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. and 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 R.D., Wang S., Lu B., Girman S., Holmes T., Sauve Y., Messina D.J., Harris I.R., Kihm A.J., Harmon A.M., Chin F.Y., Gosiewska A. and Mistry S.K. (2007). Cells isolated from umbilical cord tissue rescue photoreceptors and visual functions in a rodent model of retinal disease. Stem Cells 25, 602-611.

Lupatov A.Y., Karalkin P.A., Suzdal'tseva Y.G., Burunova V.V., Yarygin V.N. and Yarygin K.N. (2006). Cytofluorometric analysis of phenotypes of human bone marrow and umbilical fibroblast-like cells. Bull. Exp. Biol. Med. 142, 521-526.

Marañés Gálvez C., Liceras Liceras E., Alaminos M., Fernández Valadés R., Ruiz Montes A.M., Garzón I., Sánchez-Quevedo M.C. and Campos A. (2011). Generation of a substitute for human oral mucosa and verification of its viability by tissue-engineering. Cir. Pediatr. 24, 13-18.

Martín-Piedra M.A., Garzón I., Oliveira A.C., Alfonso-Rodríguez C.A., Sánchez-Quevedo M.C., Campos A. and Alaminos M. (2013). Average cell viability levels of human dental pulp stem cells: an accurate combinatorial index for quality control in tissue engineering. Cytotherapy 15,507-518.

Martín-Piedra M.A., Garzón I., Oliveira A.C., Alfonso-Rodríguez C.A., Carriel V., Scionti G. and Alaminos M. (2014). Cell viability and proliferation capability of long-term human dental pulp stem cell cultures. Cytotherapy 16, 266-277.

Michiels C. (2003). Endothelial cell functions. J. Cell Physiol. 196, 430-443.

Mitchell S.H. and Niklason L. (2003). Requirements for growing tissue-engineered vascular grafts. Cardiovasc. Pathol.12, 59-64.

Ravi S., Qu Z. and Chaikof E.L. (2009). Polymeric materials for tissue engineering of arterial substitutes. Vascular 17, 45-54.

Ravi S., Qu Z. and Chaikof E.L. (2010). Biomaterials for vascular tissue engineering. Regen. Med. 5, 107-120.

Rodríguez I.A., López-González G., Rodríguez M.A., Campos-Sánchez F. and Alaminos M. (2011). Biological evaluation of 2-hydroxyethylmethacrylate (HEMA) toxicity in human gingival fibroblasts with histochemical X-ray microanalysis. J. Adhes. Dent. 13, 375-381.

Rodríguez M.A., López-López M.T., Durán J.D., Alaminos M., Campos A. and Rodriguez I.A. (2013a). Cryopreservation of an artificial human oral mucosa stroma. A viability and rheological study. Cryobiology 67, 355-362.

Rodríguez I.A., Ferrara C.A., Campos-Sánchez F., Alaminos M., Echevarría J.U. and Campos A. (2013b). An in vitro biocompatibility study of conventional and resin-modified glass ionomer cements. J. Adhes. Dent. 15, 541-546.

Rodríguez-Morata A., Garzón I., Alaminos M., García-Honduvilla N., Sánchez-Quevedo M.C., Bujan J. and Campos A. (2008). Cell viability and prostacyclin release in cultured human umbilical vein endothelial cells. Ann. Vasc. Surg. 22, 440-448.

Roomans G.M. (2002a). X-ray microanalysis of epithelial cells in culture. Methods Mol. Biol. 188, 273-289.

Roomans G.M. (2002b). Application of X-ray microanalysis to the study of cell physiology in cells attached to biomaterials. Eur. Cell Mater. 3, 1-8.

Salido M., Vilches J. and Roomans G.M. (2004). Changes in elemental concentrations in LNCaP cells are associated with a protective effect of neuropeptides on etoposide-induced apoptosis. Cell Biol. Int. 28, 397-402.

Sánchez-Quevedo M.C., Crespo P.V., García J.M. and Campos A. (1989). X-ray microanalytical histochemistry of human circumpulpar and mantle dentine. Bone Miner. 6, 323-329.

Sánchez-Quevedo M.C., Nieto-Albano O.H., García J.M., Gómez de Ferraris M.E. and Campos A. (1998). Electron probe microanalysis of permanent human enamel and dentine. A methodological and quantitative study. Histol. Histopathol. 13,109-113.

Sánchez-Quevedo M.C., Alaminos M., Capitan L.M., Moreu G., Garzón I., Crespo P.V. and Campos A. (2007). Histological and histochemical evaluation of human oral mucosa constructs developed by tissue engineering. Histol. Histopathol. 22, 631-640.

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

Shen W.C., Liang C.J., Wu V.C., Wang S.H., Young G.H., Lai I.R., Chien C.L., Wang S.M., Wu K.D. and Chen Y.L. (2013). Endothelial progenitor cells derived from Wharton's jelly of the umbilical cord reduces ischemia-induced hind limb injury in diabetic mice by inducing HIF-1α/IL-8 expression. Stem Cells Dev. 22, 1408-1418.

Skepper J.N., Karydis I., Garnett M.R., Hegyi L., Hardwick S.J., Warley A., Mitchinson M.J. and Cary N.R. (1999). Changes in elemental concentrations are associated with early stages of apoptosis in human monocyte-macrophages exposed to oxidized low-density lipoprotein: an X-ray microanalytical study. J. Pathol.188,100-106.

Vilches J., Salido, M., Fernández-Segura E, and Roomans G.M. (2004). Neuropeptides, apoptosis and ion changes in prostate cancer. Methods of study and recent developments. Histol. Histopathol 19, 951-961.

Villalona G.A., Udelsman B., Duncan D.R., McGillicuddy E., Sawh-Martinez R.F., Hibino N., Painter C., Mirensky T., Erickson B., Shinoka T. and Breuer C.K. (2010). Cell-seeding techniques in vascular tissue engineering. Tissue Eng. Part B Rev. 16, 341-350.

Wang S.H., Lin S.J., Chen Y.H., Lin F.Y., Shih J.C., Wu C.C., Wu H.L. and Chen Y.L. (2009). Late outgrowth endothelial cells derived from Wharton jelly in human umbilical cord reduce neointimal formation after vascular injury: involvement of pigment epithelium-derived factor. Arterioscler. Thromb. Vasc. Biol. 29, 816-822.

Warley A. (1993). Quantitative X-ray microanalysis of thin sections in biology: appraisal and interpretation of results. In: X-ray microanalysis in biology. Experimental techniques and applications. Sigee D.C, Morgan A.J, Summer A.T. and Warley A. (eds). Cambridge University Press. Cambridge. pp 47-57.

Warley A. (1997). X-ray microanalysis for biologists. Portland Press. London.

Warley A. and Gupta B.L. (1991). Quantitative biological X-ray microanalysis. In: Electron microscopy of tissues, cells and organelles: A practical approach. Harris J.R (ed.). IRL Oxford University Press. Oxford. pp 243-281.

Wu H.C., Wang T.W., Kang P.L., Tsuang Y.H., Sun J.S. and Lin F.H. (2007a). Coculture of endothelial and smooth muscle cells on a collagen membrane in the development of a small-diameter vascular graft. Biomaterials 28,1385-1392.

Wu K.H., Zhou B., Lu S.H., Feng B., Yang S.G., Du W.T., Gu D.S., Han Z.C. and Liu Y.L. (2007b). In vitro and in vivo differentiation of human umbilical cord derived stem cells into endothelial cells. J. Cell Biochem. 100: 608-616.

Zhang W. and Roomans G.M. (1998). Volume-induced chloride transport in HT29 cells studied by X-ray microanalysis. Microsc. Res. Tech. 40, 72-78.

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