Placenta 36 (2015) 1192–1203

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

# Comparative studies of mesenchymal stem cells derived from different cord tissue compartments – The influence of cryopreservation and growth media

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ARTICLE INFO

*Article history:*
Received 11 June 2015
Received in revised form
24 August 2015
Accepted 25 August 2015

*Keywords:*
Umbilical cord tissue cryopreservation
MSCs differentiation
Adipogenesis
Chondrogenesis
Osteogenesis

ABSTRACT

*Introduction:* We have identified some critical aspects concerning umbilical cord tissue mesenchymal stem cells: the lack of standards for cell isolation, expansion and cryopreservation, the lack of unanimous opinions upon their multilineage differentiation potential and the existence of very few results related to the functional characterization of the cells isolated from cryopreserved umbilical cord tissue. Umbilical cord tissue cryopreservation appears to be the optimal solution for umbilical cord tissue mesenchymal stem cells storage for future clinical use. Umbilical cord tissue cryopreservation allows mesenchymal stem cells isolation before expected use, according with the specific clinical applications, by different customized isolation and expansion protocols agreed by cell therapy institutions.
*Methods:* Using an optimized protocol for umbilical cord tissue cryopreservation in autologous cord blood plasma, isolation explant method and growth media supplemented with FBS or human serum, we performed comparative studies with respect to the characteristics of mesenchymal stem cells (MSC) isolated from different compartments of the same umbilical cord tissue such as Wharton's jelly, vein, arteries, before cryopreservation (pre freeze) and after cryopreservation (post thaw).
*Results:* Expression of histochemical and immunohistochemical markers as well as electron microscopy observations revealed similar adipogenic, chondrogenic and osteogenic differentiation capacity for cells isolated from pre freeze and corresponding post thaw tissue fragments of Wharton's jelly, vein or arteries of the same umbilical cord tissue, regardless growth media used for cells isolation and expansion.
*Discussion:* Our efficient umbilical cord tissue cryopreservation protocol is reliable for clinical applicability of mesenchymal stem cells that could next be isolated and expanded in compliance with future accepted standards.



## 1. Introduction

Although currently umbilical cord tissue mesenchymal stem cells (MSCs) have been assessed in preclinical and clinical therapies ranging from regenerative medicine to immunological or hematopoietic support, laboratories around the world lack an internationally standardized practice for cells isolation, expansion and cryopreservation [1,2]. Thus, controversial results were identified regarding MSCs properties, but it was not determined whether the functional characteristics were caused by different isolation and expansion method or were due to their different cord tissue compartments source [3–5]. For potential therapeutic applications, some authors considered that MSCs must be isolated from whole umbilical cord tissue [2,6–9], Wharton's jelly region [10–13], or umbilical cord tissue vessels [14], using different growth media.

*Abbreviations:* A, Arteries; DMSO, Dimethyl Sulfoxide; FBS, Fetal Bovine Serum; HS, Human serum; MSCs, Mesenchymal stem cells; PAS, Periodic Acid Schiff; PBS, Phosphates buffered saline; PT, Post thaw; PF, Pre freeze; V, Vein; WJ, Wharton's jelly.

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http://dx.doi.org/10.1016/j.placenta.2015.08.011
0143-4004/

If some authors highlighted the multilineage differentiation inability [15,16], other authors considered that umbilical cord tissue MSCs represents a mixed population and only some populations derived from specific umbilical cord tissue compartment manifest this potential [17–21], meanwhile other authors showed that umbilical cord tissue MSCs can performed easily multilineage differentiation [2,4,6,7,22]. Nevertheless, is of great interest for clinical therapies to know whether differentiation potential of isolated cell populations is dependent on their location in the umbilical cord tissue.

For future intended use, it appear worthwhile that umbilical cord tissue MSCs may be safety stored and delivered decades later to a patient [6,23] but there are no directives in this regard. At the moment, besides different umbilical cord tissue compartment approach, two main different directions were remarked in order to preserve MSCs: the cryopreservation of isolated/expanded MSCs or the cryopreservation of umbilical cord tissue fragments. Some authors considered that banking of isolated cells instead of tissue might be of greater value for future clinical application, since is allow storage of fully characterized and controlled, ready-to-use, product [2,6,11,13,14]. Regardless of the success achieved in cryopreservation storage of MSCs at laboratory scale, many important technical and medical issues remain with respect to the production and storage of these cells for clinical applications [24]. In the context of a lack of standardized guidelines of these cells for clinical applications, it seems that umbilical cord tissue cryopreservation appears to be the optimal solution for umbilical cord tissue MSCs storage for future clinical use because the cryopreservation of the previously isolated and expanded cells using unstandardized protocols can be considered non compliant in relation to future accepted standards.

Recently, several authors evaluated the alternative to cryopreserve the umbilical cord tissue fragments for extended periods of time, but, controversial results were obtained concerning the revitalized capacity of frozen/thaw tissue fragments in order to cell isolation and very few results about the differentiation potential of these cells are available [8,9,12,13,25].

For the first time, applying an optimized protocol for umbilical cord tissue cryopreservation in autologous cord blood plasma, we compared multiple characteristics of the cells isolated from distinctive compartments of the same umbilical cord tissue (Wharton's jelly, vein, arteries), for the both pre freeze and corresponding post thaw tissue status, using two different growth media.

## 2. Methods

### 2.1. Umbilical cord collection, processing and cryopreservation

Umbilical cord blood and tissue were obtained after delivery of normal term babies (n = 10) with written consent of the parents. After umbilical cord blood collection previously described [26], a portion of umbilical cord (length 20 cm) was placed into sterile container with phosphates buffered saline (PBS) supplemented with 1% penicillin/streptomycin 10.000 U/10.000 μg/ml and 1% amphotericin B 250 μg/ml (Gibco). The collection container was kept at 4 °C and was brought to the laboratory for processing and cryopreservation within 48 h.

A selected umbilical cord segment of 10–12 cm was repeatedly washed and umbilical vessels were canulated with PBS supplemented with 1% antibiotics to removed the traces of contaminant blood cells. Using an aseptic technique, the segment was dissected longitudinally and split open to expose the umbilical vessels that were removed. Thus, three different compartments of umbilical cord tissue were individualized: Wharton's jelly (WJ), vein (V) and arteries (A). The each individualized tissue compartment was minced in 0.5 $cm^2$ fragments, obtaining pre freeze (PF) tissue fragments that were meant for both pre freeze cultures initiation and cryopreservation.

For cryopreservation, the fragments were immersed in PBS solution and centrifuged at 1000 RPM for 5 min. Following removal of the supernatant fraction, autologous umbilical cord blood plasma was added in a volumetric ratio of 1:2 (1 volume tissue pellet and 2 volumes umbilical cord blood plasma). The each final tissue suspension was treated with 10% DMSO (Cryo Sure-DEX40, 55% w/v Dimethyl Sulfoxide USP Grade, 5% w/v Dextran; WAK-Chemie Medical GmbH) and was dispersed in 2 ml cryovials (Greiner Bio One) in order to cryopreserve. The cryopreservation was perform by slow freezing using the controlled rate freezer CBS 2100, Custom BioGenic Systems until −90 °C. The cryovials were stored in liquid nitrogen tank at −195 °C for one month. The post thaw fragments (PT) were obtained by immersing the vials in water bath for 2 min at 37 °C.

### 2.2. Umbilical cord blood plasma

Autologous umbilical cord blood plasma used for cord tissue cryopreservation was obtained after umbilical cord blood volume reduction by hidroxietilamidon (10% HES Fresenius Pharma, Austria) sedimentation in a standardized banking process as has been previously described [27].

### 2.3. Isolation and culture of mesenchymal stem cells

For each cord tissue compartment (WJ, V, A) of the same cord tissue, pre freeze and post thaw, were isolated MSCs using explant method. Briefly, fragments of 0.5 $cm^2$ were placed in culture plate CELLSTAR-100 mm (Greiner Bio One). For comparative studies, tow different growth media were used: Dulbecco's modified Eagle's medium (DMEM, High glucose w/Glutamax I, Gibco Life Technologies) supplemented with 20% FBS Superior (Biochrom AG, EU approved), 1% penicillin/streptomycin 10.000 U/10.000 μg/ml (Biochrom AG) and Dulbecco's modified Eagle's medium (DMEM, High glucose w/Glutamax I, Gibco Life Technologies) supplemented with 20% poolled allogeneic human serum (HS), 1% penicillin/streptomycin 10.000 U/10.000 μg/ml (Biochrom). The plates were incubated at 37 °C in a humidified atmosphere with 5% $CO_2$, with twice of week growth medium replace. When adherent cells reach 60% confluency, the cord fragments were removed from culture and cells were harversted with 0.25% trypsin (Trypsin-EDTA Gibco). For further expansion, the tryipsinezed cells were replated at a density of 1–1.7 × $10^4$ cells/$cm^2$. Up to 6 passages were done for each cultures condition. The cells were observed with inverted microscope Motic AE 21 equipped with digital camera.

### 2.4. Proliferative potential

Harvested cells were counted using hemocytometer and population doubling was obtained for each passage according to the formula: PD = log2 (Nt/N0), where N0 is the initial cell number and Nt is the harvested cell number. The population double time was obtained according to the formula: PDT = T/PD where T is the time for each passage.

### 2.5. Immunophenotyping analysis

Cells harvested from every passage were incubated at room temperature for 20 min in the dark with 7-aminoactinomycin D (7-AAD) and following mouse anti-human antibodies, which are