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Umbilical cord tissue cryopreservation: a short review

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Abstract

In this review we present current evidence on the possibility of umbilical cord tissue cryopreservation for subsequent clinical use. Protocols for obtaining umbilical cord-derived vessels, Wharton's jelly-based grafts, multipotent stromal cells, and other biomedical products from cryopreserved umbilical cords are highlighted, and their prospective clinical applications are discussed. Examination of recent literature indicates we should expect high demand for cryopreservation of umbilical cord tissues in the near future.

Keywords: Umbilical cord, Tissue cryopreservation, Cryoprotectants, Vessels, Graft, Mesenchymal stem cells

Background

In 1974, umbilical cord blood (UCB) was reported to be a source of hematopoietic stem and progenitor cells [1], and in 1988, the first transplantation of cryopreserved UCB to an infant with Fanconi anemia, an inherited bone marrow disease, was performed in France [2]. Over the next 30 years, numerous studies were published demonstrating the regenerative potential of particular UCB-derived cell populations, and a global network of public and private biobanks of UCB was established [3, 4].

For many years, the solid tissues of umbilical cord (UC) were treated as a valueless medical waste. The past decade, however, has been notable for intensive development of biomedical products on the basis of UC tissues—for example, UC-derived mesenchymal stem cells (MSCs), which can be obtained from total UC or its dissected compartments (perivascular, intervascular, subamniotic zones of Wharton's jelly, and subendothelial layer of blood vessels). With their high proliferative potential, karyotype and phenotype stability, differentiation plasticity, paracrine activity, and immunomodulatory properties, UC-derived MSCs can claim the title of the new "gold standard", ousting the renowned bone marrow-derived MSCs [5–7]. Other examples of UC-derived biomedical products are decellularized UC vessels used as grafts for vascular surgery [8–10] and Wharton's jelly-derived extracellular matrix for wound healing [11].

The main disadvantage of UC as a tissue source is its transientness: it is only available during a short time period immediately postpartum. An effective solution to this problem may be provided by its careful cryopreservation with all efforts made to protect the useful components (cells, stromal matrices, specialized tissues) during storage. This short review presents current evidence on the possibilities of UC tissue cryopreservation, which would allow the use of its particular components in cell therapy and regenerative medicine.

Cryopreservation of UC-derived vessels

Surgical reconstruction of small vessels involves autologous transplantation as a gold standard, but this is not always affordable [9]. Decellularized umbilical vessels of appropriate diameter and considerable length without branches represent a suitable material for vascular prostheses [8–10, 12–15]. This makes the effective cryopreservation of UC-derived vessels highly important for vascular surgery. Experiments show that although cryopreservation of the UC vessels significantly affects the subsequent decellularization efficacy (which may be attributed to condensation of extracellular matrix during freezing), it has no influence on their mechanical properties such as stiffness, burst pressure, and suture retention strength [12].

The protocols for cryopreservation of UC blood vessels as a biomaterial for allogeneic transplantation do not imply the preservation of the cellular component. For this reason, the cryopreservation medium is composed of saline with no cryoprotectants. It is used in > 20 volume excess to the volume of fresh material at a cooling rate of 1 °

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Arutyunyan *et al. Stem Cell Research & Therapy* (2018) 9:236

Page 2 of 7

C/min, with subsequent storage at − 20℃ [12]. In the case of cryopreservation of UC blood vessels for autologous transplantation, preservation of the living cells in the blood vessel walls would make sense. However, no appropriate investigation of cell survival during UC vessel cryostorage with cryoprotectants has been done [15]. At the same time, the possible utility of the UC blood vessel-derived scaffolds is not confined to vascular surgery, but may be extended to tissue engineering options for nerve [16], periodontal tissue [17], and musculoskeletal soft tissue [18] regeneration.

Cryopreservation of Wharton's jelly
UC stroma contains a unique gelatinous substance which is missing in the human body after birth. It is called Wharton's jelly (WJ) after Thomas Wharton (1614–1673), an English physician and anatomist. WJ protects the blood vessels (two umbilical arteries and one umbilical vein) from clumping and also ensures cord flexibility. It is a rich reservoir of growth factors and contains significant amounts of extracellular matrix components such as collagen (types I, III, IV, and V), hyaluronic acid, and several sulfated glycosaminoglycans [5]. Such an attractive combination of biomechanical and biochemical features makes WJ an important candidate material for medical applications. For example, a biomimetic spongy scaffold, which had been produced from decellularized WJ by using a freeze-drying technique, was shown to improve the attachment, penetration, and growth of fibroblasts and speed up wound healing processes [11].

Decellularized allografts are regularly used in clinical practice, particularly in ophthalmology and wound treatment [19]. One of the most common allografts is based on the amniotic membrane, which consists of a monolayer of simple epithelium with a thick basement membrane and the underlying avascular stromal region. This graft is obtained by dehydration or, alternatively, by freezing, which better protects the tissue architecture and the biologically active molecules of the extracellular matrix [19]. Allograft material of similar structure on the basis of WJ from cryopreserved UCs was introduced in 2014. The content of high molecular weight hyaluronic acid (which is suggested to be the key isoform of hyaluronic acid responsible for the therapeutic properties) after thawing is reportedly higher in WJ than in the amniotic membrane. Moreover, extracts of UC tissue, but not amniotic membrane, promote anti-inflammatory cytokine IL-10 expression and a decrease in pro-inflammatory cytokine IL-12 expression in the macrophage cell line RAW264.7. This result indicates that the UC allografts have certain advantages [19].

Similar to the case of vascular tissue, cryopreservation of UC for obtaining the WJ matrices does not imply the preservation of the cellular component. For this reason, the fresh tissue is simply cooled to − 80 °C without cryoprotectants [19]. Grafts obtained on the basis of cryopreserved WJ have already proven to be effective in the treatment of spina bifida [20], complex lower extremity ulcers with exposed bone, tendon, muscle, and/or joint capsule, as well as multiple comorbidities including diabetes, ischemia, and underlying osteomyelitis [21–23].

Cryopreservation of the UC cellular component
Initiation of pregnancy with sperm that had been stored on dry ice for a short while was first done in 1953. The subsequent introduction of liquid nitrogen for the long-term cryostorage of sperm in the early 1960s substantially contributed to the efficacy of the approach [24]. Contemporary cryotechnologies allow the long-term preservation of cells both in suspensions and within whole tissue fragments (e.g., whole adipose tissue [25, 26], dental follicle tissue [27], bone marrow fragments [28], testicular [29] and ovarian [30] tissues), from which cells can be successfully isolated after thawing. Compared with the storage of isolated cells, the storage of unprocessed tissues has a number of advantages: minimization of time, labor, and material expenses; storage of cells in their natural environments; future possibilities of cell isolation and expansion in accordance with as yet unknown future standards.

Full-scale experimental studies of UC tissue cryopreservation started about 10 years ago. Several types of UC cells, including epithelial and endothelial cells, are valuable for regenerative medicine and tissue engineering and can be cultured [31–33]. Quite recently, an effective method for human umbilical vein endothelial cell (HUVEC) cryopreservation was reported [34]; importantly, the stage of cell culturing and expansion before transfer of the samples to the biobank is omitted in this procedure. Briefly, primary endothelium pellets, which are isolated from UC by enzymatic digestion, are frozen and placed in a liquid nitrogen freezer for long-term storage followed by fast thawing at + 37 °C. With this protocol, 14 viable HUVEC cultures have been successfully obtained from 17 primary endothelial pellets, which is an 82% success rate. The authors consider this approach helpful in improving the efficiency and logistics of biobanking, especially when processing large collections of endothelial samples [34].

However, the majority of such studies are predominantly focused on the isolation of MSCs from cryopreserved UC tissue. It is important to note that it is hypothesized that the therapeutic potential of MSCs substantially reduces during cryostorage (the so-called "cryo stun effect"), which explains the multiple failures of clinical trials utilizing cell transplants immediately after thawing [35]. In this regard, cryopreservation of

Arutyunyan *et al. Stem Cell Research & Therapy* (2018) 9:236

Page 3 of 7

whole UC tissue for subsequent isolation and expansion of MSCs for experimental or clinical purposes represents a strategy of choice.

The pioneering studies in this field were unsuccessful as no MSC cultures were obtained from WJ samples cryopreserved for 1 week, 1 month, or 6 months in liquid nitrogen, despite cryoprotection with 10% dimethyl sulfoxide (DMSO) and 5% glycerol [36]. Both DMSO and glycerol are renowned cryoprotectants used to prevent cell damage during freezing of cell culture stocks by interrupting the intracellular formation of ice crystals. Nevertheless, preservation of living cells in a WJ sample prepared with 1.5 M DMSO and 0.1 M sucrose by slow freezing (but not by vitrification) was demonstrated in 2012 [37]. Even more convincing data on obtaining MSCs from UC tissues after storage in liquid nitrogen were published in 2013; these MSCs were phenotypically and functionally identical to those obtained from fresh tissues [38]. Several scientific groups have reported their success throughout 2014–2018, suggesting a variety of protocols for the cryopreservation of UC tissue. The results are presented in Table 1.

As can be seen from Table 1, the majority of effective protocols utilize DMSO as the main cryoprotectant. With its small molecular weight of 78.13 g/mol, DMSO is capable of penetrating into the cell via the plasma membrane, preventing the formation of ice crystals by stable hydrogen bonding with water molecules. DMSO has been successfully used for the cryopreservation of cell cultures since 1959; however, it is now being replaced with DMSO-free standards for cryopreservation media. This is primarily because of the rather high toxicity of DMSO for both the cells and their recipients. DMSO is toxic at temperatures above 4 °C even at low concentrations (about 1% is enough), and with an increase in temperature it quickly decomposes into a mixture of toxic products with the distinctive odor of dimethyl sulfide. Moreover, it is impossible to remove it by washing, even with the use of a specialized system for washing cell transplants. The reasonable alternative is a medium supplemented with a cocktail of non-penetrating cryoprotectants (e.g., glucose, sucrose, galactose, or trehalose) and intracellular cryoprotectants (e.g., ethylene glycol, propylene glycol (1,2-propanediol), glycerol, formamide, methanol, and butanediol) [39–41]. According to pilot studies, this cocktail provides better tissue preservation than DMSO [41, 42]. Product lines of the leading biotech companies are now complemented with DMSO-free cryopreservation media, e.g., CryoSOfree™ DMSO-free Cryopreservation Medium by Merck, STEM-CELLBANKER® DMSO Free - GMP grade by AMSBIO, and ReproCryo DMSO Free Cryopreservation Medium by Stemgent.

Another problem which critically limits the clinical applicability of cryopreserved UC tissue-derived MSCs is the presence of xeno proteins in the cryopreservation medium, which usually contains up to 90% by volume of fetal calf serum. The use of xeno-free media significantly increases the efficacy of MCS isolation from cryopreserved UC samples [43, 44], whereas subsequent growing of the cells in xeno-free conditions facilitates a substantial all-round improvement in their properties (reduced apoptosis and immunogenicity, enhanced proliferation, increased secretion of hepatocyte growth factor and prostaglandin E2) [45, 46]. It is plausible that the proposed replacement of calf serum with autologous serum or suitable pharmacological substances (e.g., human serum albumin) will eventually prevail. In our opinion, the xeno-free standard for UC tissue cryopreservation should be introduced as soon as possible.

Current prospects of UC tissue banking

Transplantation of UC-derived MSCs is a subject of increasing interest. More than a hundred clinical trials have been currently announced by the FDA (http://www.clinicaltrials.gov/). UC-derived MSCs are intended for the treatment of cardiovascular, liver, and skeletal muscle failures, autoimmune and neurological disorders, and many other diseases [5–7]. In addition, several clinical trials of WJ-based allografts obtained from cryopreserved material (e.g., NEOX®CORD 1 K by AMNIOX Medical, Inc.), sponsored by biotech companies, are currently in progress. It is of no surprise, therefore, that numerous cryobanks, previously engaged in UC blood storage, now offer UC tissue cryopreservation and storage services. The first ten search results on the query *umbilical cord tissue cryobanking* include five banks located in the USA (Cryo-Cell, ViaCord, CariCord, Alpha-Cord, and New England Cord Blood Bank), two in the UK (Cells4Life and Future Health Biobank), two in Australia (CellCare and CryoSite), and one in South Africa (Cryo-Save). Whole UC tissue preservation has such important advantages as the total in situ preservation of all cell types and the relatively low costs of the procedure (about 2.5 times lower than the costs of UC blood cryopreservation). In our opinion, the optimal solution can be provided by banking of UC blood as a source of hematopoietic stem cells simultaneously with UC tissues as a source of autologous grafts or neonatal MSCs for autologous transplantation.

Conclusions

Examination of recent literature indicates we should expect high demand for cryopreservation of human UC tissues in the near future. The choice of a protocol for cryopreservation depends on the task—preservation of blood vessels, WJ, or the cellular component. The efficacy of obtaining living cells from thawed UC tissues is largely influenced by the composition of the cryoprotectant

Arutyunyan et al. Stem Cell Research & Therapy (2018) 9:236

Page 4 of 7

Table 1 Results of experimental studies on obtaining MSCs from cryopreserved UC tissues

Cryopreservation protocol (cryoprotectant; freezing; storage; thawing)	Method used for obtaining primary cultures	First migratory cells from tissue fragments or first cell colonies	MSC phenotype	Differentiating potential of MSCs (in vitro)	Other findings	Reference
10% DMSO; controlled freezing to −180 °C; storage in liquid nitrogen for 5 years; thawing at room temperature for 30 s, followed by a complete thaw in a 37°C water bath	Explants	After 10–14 days of culture	CD44+, CD90+, CD105+, CD34−, CD45−	Adipogenic, osteogenic	MSCs isolated from thawed tissue displayed lower plating efficiency, along with a prolonged cell doubling time and fewer total cell doublings, compared with MSCs from fresh tissue	[47]
10% DMSO + 0.2/0.5 M sucrose; freezing to −80 °C (1 °C/min); storage in liquid nitrogen for 5–29 days; thawing in a 37 °C water bath	Explants	—	CD73+, CD90+, CD34−	Adipogenic, chondrogenic	It took longer to obtain MSCs from cryopreserved UC explants compared with the corresponding fresh explants from the same donor. Cryostorage time influenced neither the terms of MSC outgrowth nor their mean population doubling time	[43]
10% DMSO; programmed freezing to −90 °C; storage in liquid nitrogen for 2–3 months; thawing in a 37°C water bath	Explants pre-incubated in collagenase IV solution for 30–45 min on ice and covered with a stainless steel mesh to protect the tissue from floating	After 7–9 days of culture	CD90+, CD105+, CD73+, CD13+, CD29+, CD44+, CD54+, CD117+, CD71+, CD146+, HLA−ABC+, CD34−, CD45−, HLA-DR−, CD309/VEGF R2/KDR−	Adipogenic, osteogenic	MSC cultures were obtained from all examined tissue samples irrespective of the time of incubation with the cryoprotectant (5–60 min). Proliferative activity of MSCs isolated from fresh and frozen biological material did not differ	[48]
10% DMSO (Cryo Sure-DEX40, 55% w/v DMSO + 5% w/v Dextran); slow freezing to −90 °C; storage in liquid nitrogen for 1 month; thawing in a 37°C water bath	Explants	After 10–14 days of culture	CD90+, CD105+, CD34−, CD45−	Adipogenic, chondrogenic, osteogenic	MSCs from cryopreserved UC explants and corresponding fresh explants from the same donor showed similar adipogenic, chondrogenic, and osteogenic in vitro differentiation capacities regardless of growth media used for their isolation and expansion. The time required to reach 60% confluence for the post-thaw cultures was longer. No difference in doubling population time was observed for the cells derived from pre-freeze tissues vs post-thaw tissues	[49]
STEM-CELLBANKER (contains DMSO and anhydrous dextrose); freezing to −80 °C (2 °C/min); storage in liquid nitrogen for 2 weeks; thawing in a 37°C water bath	Explants covered with a stainless steel mesh to protect the tissue from floating	—	CD73+, CD105+, CD90+, CD44+, HLA-ABC+, CD45−, CD34−, CD14−, CD19−, HLA-DR−	Adipogenic, chondrogenic	Cells derived from UCs after cryostorage retained their immunosuppressive properties, as assessed by allogeneic mixed lymphocyte reactions, and their potential to differentiate into adipocytes and chondrocytes was comparable with cells derived	[44]

Arutyunyan et al. Stem Cell Research & Therapy (2018) 9:236

Page 5 of 7

Table 1 Results of experimental studies on obtaining MSCs from cryopreserved UC tissues (Continued)

Cryopreservation protocol (cryoprotectant; freezing; storage; thawing)	Method used for obtaining primary cultures	First migratory cells from tissue fragments or first cell colonies	MSC phenotype	Differentiating potential of MSCs (in vitro)	Other findings	Reference
					from fresh UC tissue	
Recovery™ Cell Culture Freezing Medium (contains 10% DMSO); programmed freezing to −150 °C; storage in liquid nitrogen for 1 month; thawing in a 37 °C water bath	Post-thaw WJ was syringed through an 18 G needle a few times to loosen the gelatinous material and dislodge the stem cells; explants for entire cord segments	After 6 days of culture for cell suspensions, after 12 days of culture for explants	CD29+, CD44+, CD73+, CD90+, CD105+, HLA-ABC+, CD14−, CD34−, CD45−, HLA-DR−	Chondrogenic, osteogenic	Freezing of freshly dissected WJ worked better than freezing of entire UC segments in terms of higher MSC viability and proliferation rates, lower numbers of annexin-V-positive and sub-G1 cells, and enhanced osteogenic and chondrogenic in vitro differentiation	[50]
10% DMSO or 0.05 M glucose + 0.05 M sucrose + 1.5 M ethylene glycol cocktail; conventional freezing to −80 °C (1 °C/min) or programmed freezing to −140 °C; storage in liquid nitrogen for 3 months; thawing in a 37 °C water bath	Enzymatic digestion with DPBS containing 1 mg/ml collagenase type I at 37 °C for 15 min	After 5 days of culture	CD73+, CD90+, CD105+, CD34−, CD45−	Adipogenic, chondrogenic, osteogenic, hepatogenic	A new cocktail cryoprotectant ensured better preservation of WJ tissue than DMSO. Proliferation capacities of the cells isolated from fresh and programmed freezing WJ samples were comparable, proliferation capacity of the cells from conventional freezing WJ samples was significantly lower. MSCs from the conventional freezing samples showed upregulated expression of pro-apoptotic factors (BAX, p53, and p21) and downregulated expression of anti-apoptotic factor (BCL2), compared with MSCs from the fresh and programmed freezing samples	[42]
10% DMS; freezing to −80 °C (1 °C/min); storage in liquid nitrogen for 3 days	Explants or "conditioned explants" (cultured for 2 weeks allowing adaptation to the culture conditions prior to freezing)	After 14 days of culture for explants, after 2–8 days of culture for "conditioned explants"	CD73+, CD90+, CD105+, CD14−, CD31−, CD34−, CD45−	Adipogenic, chondrogenic, osteogenic	Cryopreserved "conditioned explants" could be repeatedly cryopreserved to show repeated outgrowth MSCs with the same properties for at least four freeze/thaw/explant culture cycles. Increasing the number of freeze/thaw/explant culture cycles to 7 and 10 associated with significant decrease in proliferation capacity	[51]
CryoStor CS10 (contains 10% DMSO); freezing to −80 °C; storage in liquid nitrogen for 1 month; rapid thawing at 37 °C	Explants (tissue pieces were allowed to rest without medium for 10 min to ensure adherence to the culture plate)	After 14 days of culture	CD73+, CD90+, CD105+, CD34−, CD45−, CD14−, CD19−, HLA-DR−	–	Two newly developed approaches for quantitative evaluation of UC tissue as a cell source were suggested (a standardized explant approach and a metabolic activity assay)	[52]

Arutyunyan *et al. Stem Cell Research & Therapy* (2018) 9:236

Page 6 of 7

medium, freezing mode, and protocol used for cell isolation. Although few data are available on the survival of endothelial or epithelial cells in cryopreserved UC tissues, some current protocols allow MSCs to be obtained from UC tissues after cryostorage that are phenotypically and functionally identical to those obtained from fresh tissues.

Abbreviations
DMSO: Dimethyl sulfoxide; MSC: Mesenchymal stem cell; UC: Umbilical cord; UCB: Umbilical cord blood

Availability of data and materials
Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions
IA and TF wrote the text and GS conceived of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare that they have no competing interests.

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Arutyunyan *et al. Stem Cell Research & Therapy* (2018) 9:236

Page 7 of 7

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