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Mixed effects of long-term frozen storage on cord tissue stem cells

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Abstract

Background aims. Cord tissue (CT) storage is promoted as an opportunity to preserve a source of mesenchymal stromal cells (MSCs) for future use. We analyzed maximal MSC yields from fresh and frozen CT including functional capacity after long-term cryopreservation as a means of assessing potential utility. *Methods.* CT was evaluated immediately upon harvest or frozen and banked for 5 years before analysis. Upon thawing, cell viability and yield were determined, as were growth characteristics and the ability to differentiate into various tissues. After thawing, enzymatic digestion of CT to release MSCs resulted in poor cell recoveries and few viable cells, requiring explant cultures to recover sufficient cell numbers for analysis. Upon expansion of surviving cells, fluorescence-activated cell sorter analysis showed the cells to be MSCs based on phenotype (CD34−, 45−, 44+, 90+, 105+) and function (ability to form adipocytes and osteoblasts). Frozen CT, however, exhibited decreased plating efficiency, increased doubling times but near equivalent maximum cell expansion, compared with fresh CT. *Conclusions.* Poor cell yields and recoveries, along with slower growth characteristics, make frozen CT a less-than-optimal choice for MSC banking, despite good functional recovery. In addition, because the amount of fresh CT available at birth is limited and total MSC yields are low, even fresh CT-MSC requires extensive *in vitro* expansion before clinical use, which limits it application.

Key Words: *banking, cord tissue, cryopreservation, mesenchymal stromal cells, regenerative medicine, stem cells, tissue engineering*

Introduction

Mesenchymal stromal cells (MSCs) are cells with multi-lineage potential that hold great promise for regenerative medicine. Various clinical and preclinical studies have shown that these cells can be successfully used for the treatment of various diseases and disorders (1,2). Extensive studies have shown MSC differentiation into adipocytes, osteoblasts, chondrocytes, muscles and neurons (3–5). MSCs are most commonly obtained from bone marrow or adipose tissue (i.e., fat) based on ease of collection and MSC yields. Human cord tissue (CT) obtained from the umbilical cord after the birth of a child, is a novel MSC source that might be used for cell-based therapies because this tissue is easily collected and abundantly available. However, the MSCs are bound up in the tissue and must be isolated before use. Different methods have been reported for successful isolation of MSCs from CT, including enzymatic (6,7) and non-enzymatic digestion (8–10), with resultant release of cells with multi-lineage differentiation potential (6,10–13). However, it should be noted that there are federal restrictions on the processing of CT for clinical use. Any methodology that uses a more than minimally manipulated approach (e.g., any enzymatic digestion method) is regulated under the human cells, tissues and cellular and tissue-based products guidelines of the U.S. Food and Drug Administration (FDA) and requires an IND (investigational new drug) application (under 12 CFR 1271 regulations). These same restrictions would also apply to CT banking and subsequent thawing for clinical use.

CT must be collected at the time of birth to be useful because this is the only time it is available. Thus, CT banking has been promoted as a potential MSC source for future use if stored frozen even for extended periods of time. To date no study has examined the effects of long-term cryopreservation on the utility of CT-MSCs isolated from thawed cord tissue for use in regenerative medicine and tissue engineering. In this study, we evaluated total MSC yield from freshly harvested CT along with determinations of MSC growth and functional capacity. These results were compared with results obtained from long-term (5-year) frozen CT samples that were thawed and analyzed in a similar fashion (i.e., phenotype, plating efficiency, growth characteristics and differentiation

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capacity). It was observed that fresh CT was a poor source of MSCs based on cell yield per gram of tissue compared with other MSC sources (e.g., adipose tissue), although these cells could be expanded extensively *in vitro*. Thawed CT-MSCs retained the phenotype and differentiation capability observed with fresh CT-MSC. However, upon thawing and enzymatic digestion to isolate MSC, most cells died, resulting in poor viable cell yields. Sufficient cells for analysis could only be obtained with explant cultures, which revealed MSCs that had lower plating efficiency and longer doubling times. However, the frozen CT-MSCs could be expanded extensively and could be differentiated into various tissues.

Methods

Collection, processing and cryopreservation of CT

CT ($N = 5$ fresh and 5 frozen/thawed tissues) was obtained from full-term deliveries from the university hospital. Five-inch tissue pieces were cut from the umbilical cord with sterilized scissors and processed within 24 hours. Segments were prepared for cryopreservation as described subsequently. All samples were obtained with written consent from the donors according to the instructions from the Institution Review Board at the University of Arizona. All samples were collected and frozen in 2008 and thawed 5 years later in 2013. Not all data were obtained with all 5 CT samples, as noted in the figure legends.

Specifically, the CT segment was cleaned and sterilized with alcohol and betadine. It was then cut with sterile scissors, and a 4- to 5-inch segment was placed into a sterile container with transport buffer. The transport buffer contains phosphate-buffered saline (PBS) with 1% fetal bovine serum (FBS), penicillin-streptomycin, gentamycin and amphotericin. The sample was transported at room temperature within 24 hours to the laboratory. Upon receipt, the CT sample was washed in PBS to remove residual blood, followed by a 70% ethanol wash and a final sterile saline wash. The CT was then cut into small 5-mm ringlets or minced into small pieces using a sterile scalpel. The CT pieces were then placed in PBS containing 20% cord plasma and 10% dimethyl sulfoxide (final) for 30 min at 4°C on a rocking platform. Samples were frozen in 4.5-cc cryovials (~1.0–1.5 g total/cryovial) using a controlled rate freezer to −180°C. All samples were stored in the liquid phase of a liquid nitrogen dewar.

CT was thawed at room temperature for 30 seconds, followed by a complete thaw in a 37°C water bath (~2 min). The tissue was washed extensively with complete media at 4°C on a rocking platform to remove dimethyl sulfoxide for 20–30 min. Finally, a wash in PBS containing 20% cord plasma was used, followed by re-suspension in PBS/20% cord plasma for analysis and culture.

Trypan blue exclusion assay

Cells were mixed with trypan blue (1:1) to determine the number of viable cells. The percentage of viable cells was calculated by dividing the number of trypan blue–negative cells by the total number of cells examined, multiplied by 100.

Explant culture and expansion of MSCs

MSCs from CT were isolated using a non-enzymatic digestion as previously described (11–14). Briefly, CT was washed extensively with PBS containing penicillin and streptomycin in a 100-mm petri plate. The tissue was minced into fine pieces that were placed into a 25-cm^2 culture flask. Three milliliters of complete medium was added to the flask, and the pieces were distributed evenly. After 4–6 days, the pieces were removed and cultured in a new flask. In 10–14 days, cell colonies were observed. The cells from both flasks were harvested using trypsin-ethylenediaminetetraacetic acid and pooled. A total of 25,000 cells were cultured in each subsequent 25-cm^2 culture flask in complete medium. Complete medium consisted of MEM supplemented with 10% FBS and 1% each of non-essential amino acids, sodium pyruvate, L-glutamine and streptomycin/penicillin antibiotics.

Cell surface antigen profile of CT-derived cells

Cell surface antigen expression was evaluated by flow cytometry. Freshly isolated MSC or thawed cells at passage 1 were harvested by treatment with 0.05% trypsin/ethylenediaminetetraacetic acid, re-suspended in PBS and counted. One hundred thousand cells were incubated with the following primary antibodies: CD34 phycoerythrin–, CD44– allophycocyanin, CD45– fluorescein isothiocyanate, CD90–Alexa Fluor 700 (AF-700) and CD105-allophycocyanin for 30 min at 4°C. Samples were analyzed using an LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) using FACS DIVA software (BD Biosciences). Unstained cells were used to establish flow cytometer settings. Debris and auto-fluorescence were removed by using forward scatter. At least 10,000 gated events were used for each analysis.

Cell yields and recoveries

MSCs from fresh and thawed cord tissues were obtained by enzymatic digestion as follows (14). The tissue was digested with 0.1% type IV collagenase