ACTA ODONTOLOGICA SCANDINAVICA, 2016
VOL. 74, NO. 8, 598–604
http://dx.doi.org/10.1080/00016357.2016.1224919

ORIGINAL ARTICLE

Effect of a cryopreservation protocol on the proliferation of stem cells from human exfoliated deciduous teeth

Fernanda Ginani[a], Diego Moura Soares[b], Luciana Maria Rabêlo[c], Hugo Alexandre Oliveira Rocha[c], Lélia Batista de Souza[a] and Carlos Augusto Galvão Barboza[a,d]

[a]Postgraduate Program in Oral Pathology, Federal University of Rio Grande do Norte, Natal, Brazil; [b]Department of Dentistry, Federal University of Rio Grande do Norte, Natal, Brazil; [c]Postgraduate Program in Biochemistry, Federal University of Rio Grande do Norte, Natal, Brazil; [d]Postgraduate Program in Structural and Functional Biology, Federal University of Rio Grande do Norte, Natal, Brazil

ABSTRACT
Objective: The aim of the present study was to evaluate the influence of a cryopreservation protocol on the proliferation and viability of stem cells from human exfoliated deciduous teeth (SHEDs).
Materials and methods: Cells from the pulp of three deciduous teeth were isolated and characterized to confirm their stem cell nature. In second passage, part of the cells were submitted to normal conditions of cell culture (Control group), while part of the cells were maintained in 10% DMSO diluted in foetal bovine serum and submitted to the following cryopreservation protocol: 2 h at 4 °C, 18 h at −20 °C and then at −80 °C for two intervals (30 days – Cryopreservation I; and 180 days Cryopreservation II). Cell proliferation and cell cycle were evaluated at intervals of 24, 48 and 72 h after plating, and apoptosis-related events were analyzed at 72 h.
Results: All groups exhibited an increase in the number of cells, and no significant differences between the cryopreserved and control groups were observed ($p > .05$). The distribution of cells in the cell cycle phases was consistent with cell proliferation, and the percentage of viable cells was higher than 99% in all groups, indicating that cell viability was not affected by the cryopreservation protocol throughout the experiment.
Conclusion: The proposed cryopreservation protocol is adequate for the storage of SHED, permitting their use in future experimental studies.

ARTICLE HISTORY
Received 4 April 2016
Revised 6 July 2016
Accepted 8 August 2016
Published online 29 August 2016

KEYWORDS
Cell culture; dental pulp; stem cells; cryopreservation; cell proliferation

Introduction

Human deciduous teeth have been described as a promising source of mesenchymal stem cells. These cells are able to differentiate *in vitro* into different cell lineages and could be used in different clinical applications, including dental tissue engineering,[1] bone defect repair [2] and even the treatment of neural damage and degenerative diseases,[3,4] a fact highlighting the importance of further studies in this area.

The isolation of stem cells from exfoliated deciduous teeth has no ethical or legal implications since this tissue is considered 'disposable' and readily accessible in young patients.[5] The fact that children usually lose 20 deciduous teeth throughout childhood offers multiple opportunities for the preservation of these stem cells, in contrast to other tissue sources such as umbilical cord blood.[6]

The potential of clinical application of stem cells derived from the pulp of exfoliated deciduous teeth (SHEDs) is also attributed to the non-significant immunogenicity of these cells, thus permitting their use in allogenic transplants without immunosuppressors.[7,8] These cells are therefore considered important for the creation of cell banks, which require the preservation of cells for long periods of time.[9–11]

A safe approach to store SHEDs for subsequent use are cryopreservation techniques, which cease the biological functions of living tissues at an ultra-low temperature, generally about −196 °C.[12] The identification of simple, efficient and safe cryopreservation protocols for each type of cell, especially altering the maintenance temperature of cells, is important to reduce the costs of the process and to permit the installation of easily accessible stem cell banks.[13]

In this respect, it is extremely important to determine whether the cryopreservation protocol used affects the viability and proliferative capacity of cells. Therefore, the objective of the present study was to evaluate *in vitro* the influence of a cryopreservation protocol on the proliferation and viability of SHEDs.

Materials and methods

Subjects

The study was approved by the Ethics Committee of the Federal University of Rio Grande do Norte (CEP/UFRN, Protocol No. 232/2011). Three deciduous teeth in the final

CONTACT Carlos Augusto Galvão Barboza cbarboza@cb.ufrn.br Postgraduate Program in Structural and Functional Biology, Federal University of Rio Grande do Norte, Avenida Salgado Filho, 3000, Campus Universitário, CEP 59078-970 Natal, RN, Brazil

stage of exfoliation or with an indication for extraction were obtained from children aged 6–10 years and used for the isolation of SHEDs.

Cell culture

After extraction, each tooth was immediately stored in a Falcon tube containing 5 mL alpha-MEM medium on ice. In a laminar flow cabinet, the teeth were washed three times for 10 min each with a solution consisting of alpha-MEM medium supplemented with 10,000 IU/mL penicillin, 10,000 μg/mL streptomycin, 100 mg/mL gentamicin and 250 μg/mL amphotericin B in order to eliminate possible contamination.

For the isolation of SHEDs, the pulp tissue was carefully removed by curettage, and the extract was submitted to enzymatic digestion with 3 mg/mL collagenase I (Gibco, Grand Island, NY) and 4 mg/mL dispase (Gibco, Grand Island, NY) for 1 h at 37 °C. The cells obtained from each sample were cultured in 35-mm diameter tissue culture dishes (TPP, Switzerland) containing alpha-MEM medium supplemented with 15% foetal bovine serum (FBS) at 37 °C in a 5% CO2 atmosphere until they had reached 70–90% confluence. The medium was changed every 3 days.

In the first passage (P1), the cells were analyzed to confirm their stem cell nature, according to the criteria of The International Society for Cellular Therapy established by Dominici et al. [14] First, an aliquot of cells was evaluated by flow cytometry using the Human MSC Analysis Kit (BD Stemflow™, San Diego, CA) to evaluate surface markers of mesenchymal stem cells (CD90-FITC; CD73-APC; CD105 PerCP-Cy5.5) and MSC negative cocktail (CD45, CD34, CD11b, CD19, HLA-DR). The same analysis was also performed after the cryopreservation intervals in order to identify whether cryopreserved cells maintain their properties as MSCs. Additionally, the multilineage differentiation potential of SHEDs was confirmed by culturing the cells in osteogenic and adipogenic differentiation media (StemPro® Differentiation Kits, Invitrogen, Carlsbad, CA) for up to 21 days.

Study design

After characterization, SHEDs were divided into three groups: control, immediate culture of SHEDs; cryopreserved I, cryopreservation of SHEDs for 30 days and subsequent culture as described for the control group; cryopreserved II, cryopreservation of SHEDs for 180 days and subsequent culture as described for the control group.

The cells of each group were evaluated regarding their proliferative capacity and to identify apoptosis- and cell cycle-related events, as shown in Figure 1.

Cryopreservation

In the second-passage (P2), the cells of each tooth were counted and divided into three aliquots of 1×10^6 cells; two of them were submitted to cryopreservation and the third one was submitted to normal conditions of cell culture (control group). The SHEDs were immersed in FBS with 10% dimethyl sulphoxide (DMSO) in 2-mL cryo-vials and submitted to the following cryopreservation protocol: 2 h at 4 °C, 18 h at −20 °C and then maintained at −80 °C. These cells were cryopreserved for 30 and 180 days. After this period, the cells were thawed and submitted to the same cell culture protocol as described for the control group.

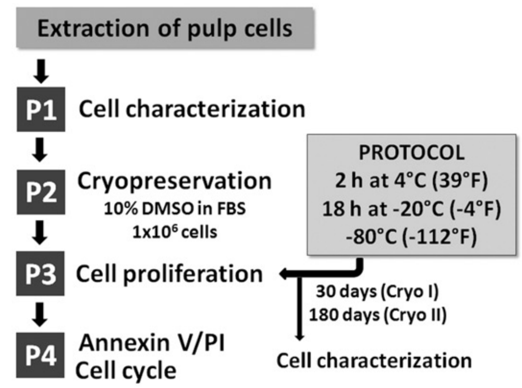

Figure 1. Experimental design. P1, first passage; P2, second passage; P3, third passage; P4, fourth passage.

The cryo-vials containing the SHEDs were thawed in a water bath at 37 °C. The cells obtained were washed carefully to remove the cryoprotectant and then centrifuged at 1200 rpm for 8 min. The supernatant was aspirated, and the cells were resuspended in cultured medium and incubated.

Analysis of cell proliferation

Third-passage (P3) SHEDs of each group were cultured in 24-well plates at a density of 3×10^4 cells/well. The Trypan blue exclusion method was used for the analysis of cell proliferation in the different groups. For this purpose, the number of cells adhered to the plastic surface of four wells ($n = 4$) per pulp at 24, 48 and 72 h after plating was determined.

Analysis of apoptosis and cell cycle

The effects of the cryopreservation protocol on apoptosis were evaluated by flow cytometry using the FITC/Annexin V Dead Cell Apoptosis Kit with FITC annexin and propidium iodide (PI) (Invitrogen Corp., Carlsbad, CA). For this purpose, the cells were cultured in triplicate in 6-well plates at a density of 2×10^5 cells/well. After 24 and 72 h of culture, the cells were trypsinized, collected and washed with ice-cold PBS. The supernatant was discarded, and the cells were resuspended in 200 μL 1X binding buffer. Next, 3 μL annexin V-FITC and 1 μL (100 μg/mL) PI were added. The cells were incubated for 15 min at room temperature protected from light. After incubation, 400 μL 1X binding buffer for annexin V was added, and the cells were analyzed in a flow cytometer, measuring the fluorescence emitted at 530 and 575 nm.

For evaluation of the effects of the cryopreservation protocol on the cell cycle, the cells were incubated with 2% paraformaldehyde for 30 min, washed with ice-cold PBS and permeabilized with 0.01% saponin for 15 min. The cells were incubated with 10 μL RNAse (4 mg/mL) for 30 min at 37 °C. Next, 5 μL PI (25 mg/mL) and 200 μL ice-cold PBS were added to the cells and cell cycle analysis was performed in a flow cytometer (585/42 nm).

Statistical analysis

Differences between groups at each time point were analyzed by the Kruskal–Wallis and Mann–Whitney tests, considering a level of significance of 5% ($p < .05$).

Results

In flow cytometry, the cells revealed positive staining for surface markers of mesenchymal stem cells (CD90, CD73, CD105) and negative staining for markers of haematopoietic cells (CD45, CD34, CD11b, CD19, HLA-DR) in the first passage (P1) and after 30 and 180 days of cryopreservation (Figure 2). Light microscopy analysis showed the deposition of mineralized matrix stained with von Kossa stain and lipid vesicles stained with Oil Red O, corresponding to the differentiation of osteoblastic and adipose cells, respectively (Figure 3).

All groups exhibited an upward shift of cell proliferation (Figure 4). No significant differences were observed between the cryopreserved and control (non-cryopreserved) groups ($p > .05$).

With respect to cell viability analyzed by the Trypan blue exclusion method, Table 1 shows no significant changes in the percentage of viable cells in the different groups

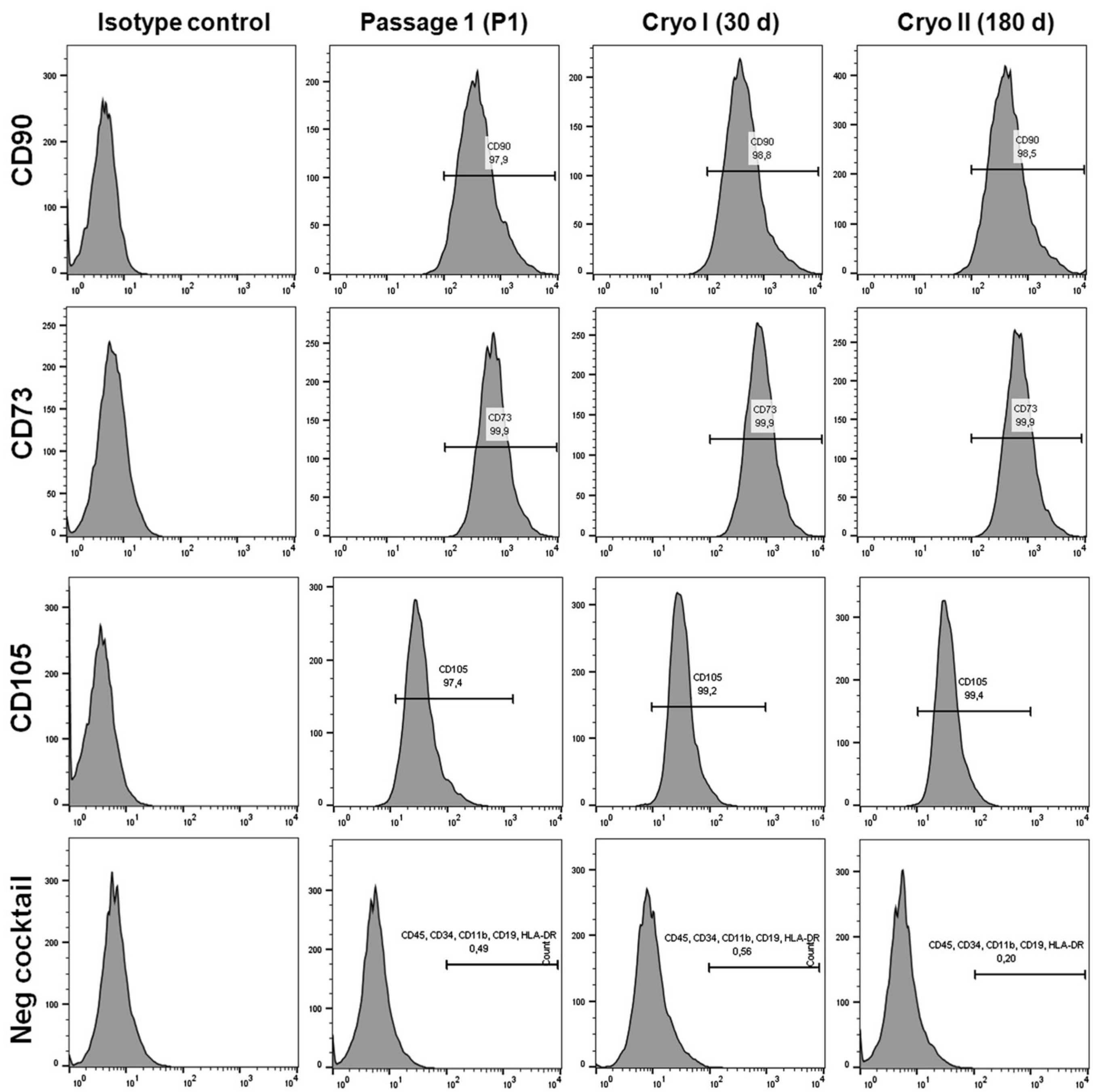

Figure 2. Flow cytometric analysis of the expression of positive and negative mesenchymal stem cell markers on SHEDs.

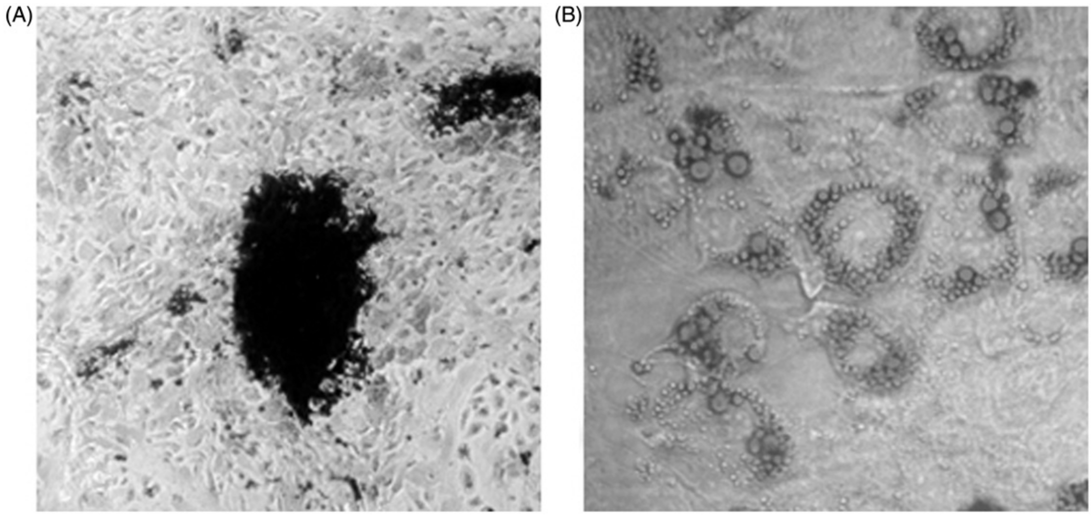

Figure 3. Photomicrograph of SHEDs subjected to osteogenic (A) and adipogenic differentiation (B). Light microscopy, A: von Kossa stain, original magnification 40×; B: Oil Red stain, original magnification 100×.

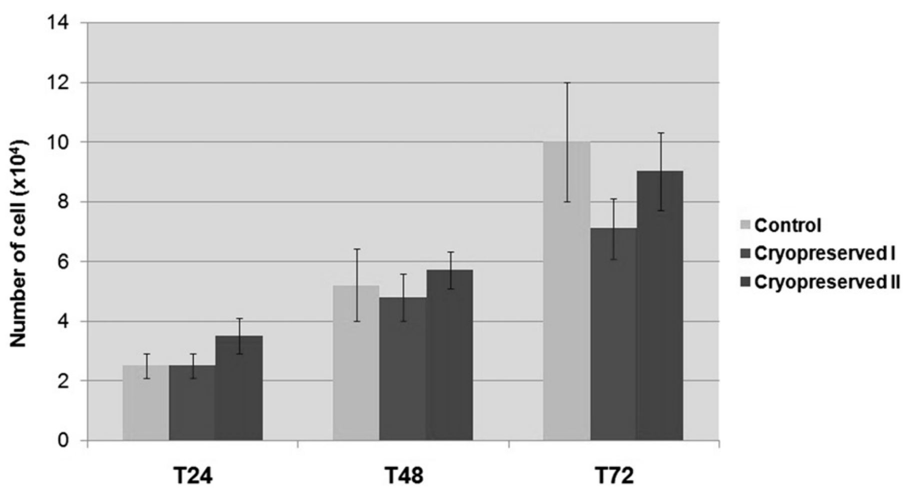

Figure 4. Number of SHEDs submitted or not to cryopreservation over time.

Table 1. Percentage of SHED viability in the groups studied at the different time points.

	Viability (%)		
	T24	T48	T72
Control	100	100	97.7
Cryopreserved I	98.8	99.3	99.5
Cryopreserved II	99.2	98.7	98.7

throughout the experiment. Flow cytometry revealed a percentage of viable cells higher than 99% in all groups (Figure 5), indicating that cell viability was not affected by the cryopreservation protocol throughout the experiment.

Figure 6 illustrates the percentage of cells in each cell cycle phase. The results were consistent with cell proliferation, with most cells in the three groups being in the S and G2/M phases at the three time points analyzed.

Discussion

The necessity to maintain live cells for a long time without compromising their functions led to the development of cryopreservation techniques. Most cryopreservation protocols for mesenchymal stem cells reported in the literature store cells at a temperature of −196 °C (liquid nitrogen).[15–17] However, this procedure is associated with high maintenance costs and is not accessible to all research laboratories. A simpler, more accessible and low-cost protocol was used in the present study. In this protocol, SHEDs stored at a temperature of −80 °C did not show changes in their proliferative capacity or viability.

Analysis of cell proliferation in the experimental groups studied (cryopreserved I and cryopreserved II) showed an increase in the number of cells at the three time points (24, 48 and 72 h), confirming that SHEDs maintained their proliferative capacity when submitted to the cryopreservation protocol proposed. These results are consistent with those reported in the literature,[18–22] indicating that the process of freezing and thawing exerts no negative influence on the proliferation of mesenchymal stem cells. A number of studies have demonstrated that stem cells isolated from teeth, including the periodontal ligament, dental pulp or apical papilla, can be successfully cryopreserved and maintain their viability after thawing.[18,23,24]

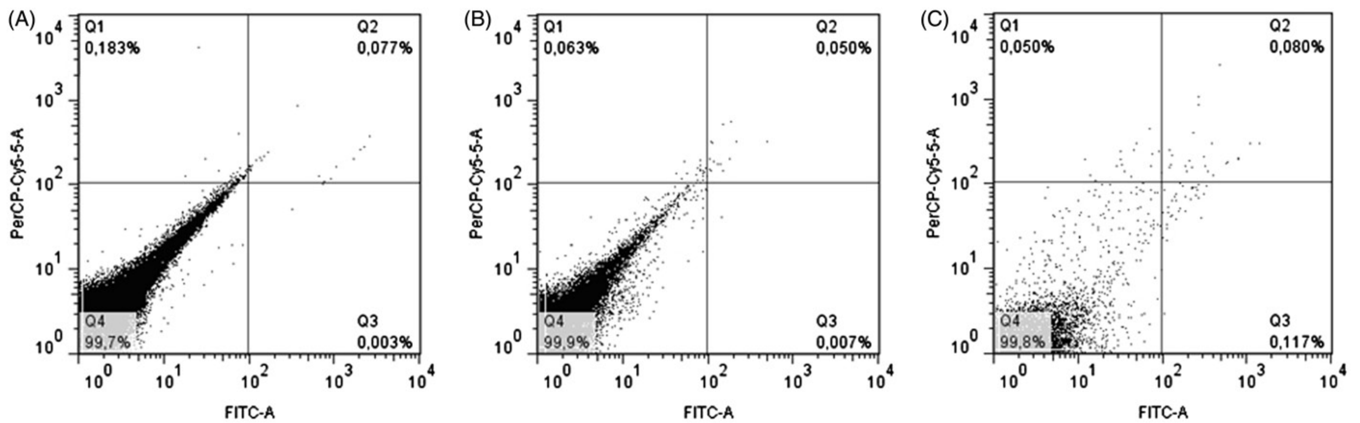

Figure 5. Immunostaining of SHEDs with annexin V/PI. (A) Control; (B) cryopreserved I; (C) cryopreserved II. Q1:annexin V negative/PI positive; Q2: annexin V positive/PI positive; Q3:annexin V positive/PI negative; Q4: annexin V negative/PI negative.

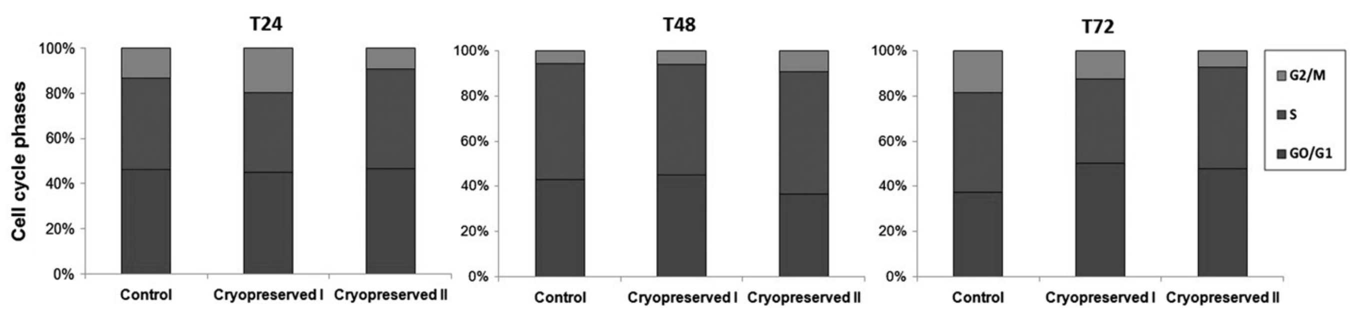

Figure 6. Distribution of cells in the different cell cycle phase over time.

Woods et al. [18] have frozen dental pulp stem cells from permanent teeth for 1 week, 1 month and 6 months at temperatures of −196 °C (liquid nitrogen) and −85 °C, and observed no difference in the proliferation rate or differentiation capacity of the cells between the groups studied at any of the time points. These findings suggest that cells expanded in culture can be stored at −80/−85 °C for at least 6 months and probably for longer periods of time without causing cell damage, a fact also demonstrated in this study.

In the present study, two different durations of the cryopreservation protocol were evaluated. The interval of 30 days was chosen based on recent studies,[13,18,21] and a longer duration (180 days) to observe the effects of the protocol for longer durations of cryopreservation. Previous studies report different durations ranging from 1 day to longer time intervals.[15,25] Our results agree with those reported in the literature, showing no significant differences in the proliferation or viability of cryopreserved cells when compared to non-cryopreserved cells.

It is possible that the duration of cryopreservation affects cell viability; however, shorter periods such as the one used in the present study or longer periods [25–28] such as those tested by Ma et al. [22] did not alter cell proliferation. These authors observed that SHEDs obtained from pulp tissues cryopreserved for more than 2 years (25–30 months) maintained their stem cell characteristics such as self-renewal capacity, multipotency, capacity to regenerate tissues *in vitro* and *in vivo* immunomodulatory effect.[22]

We chose to cryopreserve isolated pulp stem cells, since unfavourable results in terms of cell yield have been reported when cryopreservation is performed using intact deciduous teeth. Ji et al. [26] observed that cryopreservation for 3–9 months reduced cell activity and cell viability. Studying pulp stem cells obtained from intact deciduous teeth cryopreserved for 7 days; Lindemann et al. [27] found no differences in the immunophenotypic characteristics or differentiation capacity of the cells when compared to control; however, morphological alterations and changes in the proliferation potential were observed.

Among the different parameters of cryopreservation, the cryoprotective agent can also significantly affect the survival rate and proliferation potential of cells after thawing. In the present study, DMSO was chosen since it is widely used for the cryopreservation of different cell types.[16,25] Our experiments showed that DMSO provided favourable results in terms of cell viability and proliferative capacity after cryopreservation of SHEDs for 30 and 180 days. This agent has been associated to favourable results in terms of maintain cell functions after cryopreservation of cells for a period of 30 days.[13,18] Ding et al. [23] compared three cryoprotective agents (10% DMSO plus 90% FBS, 10% glycerol plus 90% FBS and 10% ethylene glycol plus 90% FBS) for the cryopreservation of stem cells from human apical papilla for a period of 6 months, and found no significant differences in cell viability, colony-forming efficiency and cell proliferation. In addition, Woods et al. [18] showed that DMSO at concentrations of 0.5, 1.0 and 1.5 M yielded better results in terms of cell viability than propyleneglycol or ethylene glycol at the same concentrations.

The freezing rate is another crucial factor that affects cell viability after cryopreservation, and its effect should be determined for each specific cell in order to prevent the formation of intracellular ice crystals.[28] The standard protocol established by Vasconcelos et al. [13] for other cell types in culture was adopted in this study: 2 h at 4 °C, 18 h at −20 °C and subsequent storage at −80 °C for 30 and 180 days. This protocol was found to be effective in maintaining the viability of SHEDs after thawing.

Xu et al., [12] using human mesenchymal stem cells, evaluated the osmotic effects and effects of heat shock caused by the cryopreservation procedures on post-thaw cell viability and recovery. The results showed a significant decrease in cell viability when different freezing rates (1, 5 and 10 °C/min) were used. The rate of 1 °C/min provided the best results in terms of the maintenance of cell morphology and integrity, as well as cell recovery after cryopreservation. A protocol consisting of a gradual and slow decrease in freezing temperature was therefore used in the present study.

Analysis of apoptosis-related events by flow cytometry showed that most cells continued to be viable after 30 and 180 days of cryopreservation. Papaccio et al. [25] investigated the influence of cryopreservation of dental pulp stem cells for long periods of time (2 years). The authors observed no death of cryopreserved cells due to apoptosis, although some cells had undergone necrosis as a result of the formation of intracellular ice. The latter fact was not observed in the present study since no PI-stained cells were identified. This marker interacts with DNA, but is unable to cross the plasma membrane. Positive staining indicates necrosis or the final stage of apoptosis.

Taken together, the present results demonstrate that the cryopreservation protocol proposed is adequate for the storage of dental pulp stem cells derived from human deciduous teeth, permitting their use in future experimental studies. The proposed protocol is simple, more accessible and low-cost, what makes it accessible to all research laboratories. Further studies are needed to evaluate the differentiation potential of these cryopreserved cells and their integration capacity with biomaterials in order to permit their use in *in vivo* experiments and future clinical applications in tissue regeneration therapies.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Notes on contributors

Fernanda Ginani, MT, MSc, is a PhD-student. She has long experience working with human dental stem cells.

Diego Moura Soares, DDS, MSc, is a PhD-student. He has experience in pediatric dentistry and cell culture.

Luciana Maria Rabêlo, BSc, MSc, is a PhD-student. She has experience in biochemistry and protein chemistry.

Hugo Alexandre Oliveira Rocha, BPharm, MSc, PhD, is an Associate Professor. His research interest includes biotechnology, biochemistry, cell culture, and extracellular matrix biology.

Lélia Batista de Souza, DDS, MSc, PhD, is a Professor. Her research interest is oral pathology, focusing on odontogenic tumours, oral cancer, and salivary gland neoplasms.

Carlos Augusto Galvão Barboza, DDS, MSc, PhD, is an Associate Professor. His research interest is craniofacial biology and tissue regeneration, with focus on stem cell biology, biomaterials, and laser therapy.

Funding

Fundação de Apoio à Pesquisa do Estado do Rio Grande do Norte (FAPERN) [PPP-IV/2012]

References

[1] Miura M, Gronthos S, Zhao M, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA. 2003;100:5807–5812.
[2] Zheng Y, Liu Y, Zhang CM, et al. Stem cells from deciduous tooth repair mandibular defect in swine. J Dent Res. 2009;88:249–254.
[3] Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009;88:792–806.
[4] Morsczeck C, Völlner F, Saugspier M, et al. Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SHED) after neural differentiation in vitro. Clin Oral Investig. 2010;14:433–440.
[5] Cordeiro MM, Dong Z, Kaneko T, et al. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod. 2008;34:962–969.
[6] Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. Trends Cell Biol. 2010;20:715–722.
[7] Pierdomenico L, Bonsi L, Calvitti M, et al. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation. 2005;80:836–842.
[8] Noël D, Djouad F, Bouffi C, et al. Multipotent mesenchymal stromal cells and immune tolerance. Leuk Lymphoma. 2007;48:1283–1289.
[9] Arora V, Arora P, Munshi AK. Banking stem cells from human exfoliated deciduous teeth (SHED): saving for the future. J Clin Pediatr Dent. 2009;33:289–294.
[10] Petrovic V, Stefanovic V. Dental tissue-new source for stem cells. ScientificWorldJournal. 2009;9:1167–1177.
[11] Sloan AJ, Waddington RJ. Dental pulp stem cells: what, where, how? Int J Paediatr Dent. 2009;19:61–70.
[12] Xu X, Liu Y, Cui Z, et al. Effects of osmotic and cold shock on adherent human mesenchymal stem cells during cryopreservation. J Biotechnol. 2012;162:224–231.
[13] Vasconcelos RG, Ribeiro RA, Vasconcelos MG, et al. In vitro comparative analysis of cryopreservation of undifferentiated mesenchymal cells derived from human periodontal ligament. Cell Tissue Bank. 2012;13:461–469.
[14] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–317.
[15] Gonda K, Shigeura T, Sato T, et al. Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. Plast Reconstr Surg. 2008;121:401–410.
[16] Martinello T, Bronzini I, Maccatrozzo L, et al. Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation. Res Vet Sci. 2011;91:18–24.
[17] Miyamoto Y, Oishi K, Yukawa H, et al. Cryopreservation of human adipose tissue-derived stem/progenitor cells using the silk protein sericin. Cell Transplant. 2012;21:617–622.
[18] Woods EJ, Perry BC, Hockema JJ, et al. Optimized cryopreservation method for human dental pulp-derived stem cells and their tissues of origin for banking and clinical use. Cryobiology. 2009;59:150–157.
[19] Oh YH, Che ZM, Hong JC, et al. Cryopreservation of human teeth for future organization of a tooth bank – a preliminary study. Cryobiology. 2005;51:322–329.
[20] Perry BC, Zhou D, Wu X, et al. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use. Tissue Eng Part C Methods. 2008;14:149–156.

[21] Temmerman L, Dermaut LR, De Mil M, et al. Influence of cryopreservation on human periodontal ligament cells in vitro. Cell Tissue Bank. 2008;9:11–18.

[22] Ma L, Makino Y, Yamaza H, et al. Cryopreserved dental pulp tissues of exfoliated deciduous teeth is a feasible stem cell resource for regenerative medicine. PLoS One. 2012;7:e51777.

[23] Ding G, Wang W, Liu Y, et al. Effect of cryopreservation on biological and immunological properties of stem cells from apical papilla. J Cell Physiol. 2010;223:415–422.

[24] Temmerman L, Beele H, Dermaut LR, et al. Influence of cryopreservation on the pulpal tissue of immature third molars in vitro. Cell Tissue Bank. 2010;11:281–289.

[25] Papaccio G, Graziano A, d'Aquino R, et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol. 2006;208:319–325.

[26] Ji EH, Song JS, Kim SO, et al. Viability of pulp stromal cells in cryopreserved deciduous teeth. Cell Tissue Bank. 2014;15:67–74.

[27] Lindemann D, Werle SB, Steffens D, et al. Effects of cryopreservation on the characteristics of dental pulp stem cells of intact deciduous teeth. Arch Oral Biol. 2014;59:970–976.

[28] Hubel A. Parameters of cell freezing: implications for the cryopreservation of stem cells. Transfus Med Rev. 1997;11:224–233.