Serum-Containing Medium Effect on Isolation Rate of Dental Pulp Cells from Cryopreserved Intact Deciduous Teeth

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Objectives: *To isolate cells from pulp of intact cryopreserved deciduous teeth. The null hypothesis raised here is to find no difference in the establishment of cell culture after cryopreservation (1) using culture medium supplemented with different concentrations of fetal bovine serum (FBS); and (2) between teeth with different stages of physiological root resorption.* ***Study design:*** *Intact deciduous teeth with different root resorption stages were cryopreserved using FBS and Dimethyl Sulfoxide (DMSO) medium (9:1) in a progressive freezing process, by placing the samples in the refrigerator (4°C/60 min) and subsequently transferring them to a -80°C freezer (controlled device -1°C/min/24 hours), and finally into liquid nitrogen (-196°C/30 days). After the thawing process, the cell isolation was performed by enzymatic digestion (type I collagenase). The cells were re-suspended into the culture medium with 10% (G1) or 20% (G2) of FBS. Microscopic analysis was performed after 30 days to visualize the cell attachment.* ***Results:*** *The culture establishment rate was higher in G2 (75%) than G1 (12.5%) (p=0.041). There was no difference between the different stages of root resorption.* ***Conclusions:*** *It was possible to establish cell cultures from the pulp of intact cryopreserved deciduous teeth. The use of 20% FBS after thawing improved the culture rate.*

Key words: *Deciduous teeth; Cryopreservation; Fetal Bovine Serum.*

INTRODUCTION

Stem cells from deciduous human teeth exhibit a high proliferation rate and differentiation potential in multiple lineage, which promotes their potential use in research and pre-clinical trials[1,2]. The option for these cells is also attractive because their obtainment causes minimal discomfort to the donor once deciduous teeth are physiologically exfoliated and pulp tissue, a stem cell niche, is usually discarded[3]. The exfoliation process occurs usually between 5 and 13 years of age, thus, after this period it is no longer possible to obtain these cells.

The culture and expansion of stem cells after their isolation would be one method to maintain them for a long-term period. However, there are some complications, such as the decrease in the ability of differentiation, senescence, induced by consecutive passages[4], the possibility of genetic alterations[5] and the economic cost involved[6]. Thus, cryopreservation is presented as an alternative to maintain the viability of these cells until their use is required, allowing for long-term storage and safety for clinical application[5].

Recent studies suggest that the cryopreservation of hDPCS (human dental pulp stem cells) is viable[7]. These hDPSC maintain the surface markers unaltered and they also have proliferation and differentiation potential[4,8,9], even when cryopreserved for long periods[5,10]. Moreover, when the initial isolation processing is not possible, some studies have demonstrated the freezing capacity of the whole permanent tooth for future isolation of hDPSCs[11-14], showing the culture rates in a range of 20-70%.

Most of these studies utilized a culture medium supplemented with 15-20% of fetal bovine serum (FBS) [4,5,9,13,14] or fetal calf serum (FCS) [8,10] for the culture and expansion of the cells subjected to cryopreservation. Animal serum is an important additive for cell growth and essential for the early stages of isolation[15]. However, the use of lower concentrations or the absence of animal serum in the culture medium would be ideal because it can transmit zoonosis and it contains unspecific growth factors, reducing the possibilities for clinical application[16].

The hDPSCs from deciduous teeth have the advantage of presenting higher proliferation rates and higher differentiation potential when compared to hDPSCs from permanent teeth[17]. However, few studies have reported the cryopreservation viability of these cells[4,18] and there is still an absence in the literature regarding the cryopreservation of intact deciduous teeth without initial isolation processing.

The purpose of this study was to isolate cells from cryopreserved

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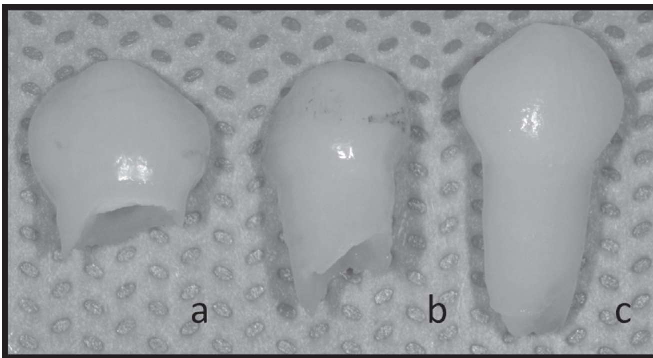
deciduous teeth and test the null hypothesis that there is no difference in the establishment of cell culture after cryopreservation of intact deciduous teeth using: (1) culture medium supplemented with different concentrations of FBS and (2) teeth at different root resorption stages.

MATERIALS AND METHOD

Sound deciduous teeth (n=16) with absence of trauma and no clinical or radiographic signs of pulpal disease, presenting different root resorption stages (Figure 1), were collected from patients aged from 7 to 13. Before tooth extraction, the parents were fully informed about the procedure and destination of the tooth, and signed a consent form approved by the Ethics Committee of Federal University of Rio Grande do Sul (n. 20865). The tooth extraction decision was part of the orthodontic treatment plan design for the patients by their dentists who were not related to this research.

Immediately after extraction, the teeth were placed in a flask with transport medium containing DMEM (Dulbecco's modified Eagle medium – Sigma- Aldrich, St. Louis, MO) / HEPES, 10% fetal bovine serum (Laborclin, Pinhais, Brazil), 100U/mL penicillin, 100mg/mL streptomycin and 0.45mg/mL gentamicin (Gibco, Grand Island, NY) and transported to the laboratory within 6 hours. Under sterile conditions, the teeth were removed from the flask and cleaned with gauze and 0.12% chlorexidine solution. The teeth were randomized into two groups: 10% FBS (supplemented medium containing 10% FBS for culture after cryopreservation) or 20% FBS (supplemented medium containing 20% FBS for culture after cryopreservation).

Figure 1. Root resorption stages according to groups: a) total root resorption; b) 1/3 root remaining and c) 2/3 root remaining.



Freezing

The deciduous teeth were displaced in a 2mL vial containing 1.5 mL cryoprotectant solution of 10% dimetylsulfoxide and 10% FBS (1:9), and kept in a 4°C environment for 60 minutes. Following, the vials were subjected to a dump-freeze method consisting of a vial suspension in an isopropanol bath (Mr. Frosty – NALGENE TM Cryo 10°C freezer Container, Cat. No 5100-0001) within a -80°C mechanical freezer for a -1C/min cooling rate for 24 hours. The following day, the vials were placed into -196°C liquid nitrogen.

Thawing

After 30 days of cryopreservation, the vials were recovered and partially thawed for less than 1 minute in a 37°C water bath. Once out of the vial, the teeth were washed with cold culture medium supplemented with 10 or 20% FBS (DMEM/HEPES, 10 or 20% FBS, 1 or 2% antibiotics) according to the groups.

Isolation and Cell Culture

For both groups, all the pulp tissue was removed from the dentin walls with a dentin excavator, placed in buffer containing 0.2% type I collagenase solution (Gibco, Grand Island, NY) for 60 minutes in a 37°C bath and centrifuged (10 min, 800 G). The remaining pellet was re-suspended and seeded onto a 12 well plate. The first medium change occurred after 72 hours and successively every 3 to 4 days. The identification of an agglomerate of cells within a period of 30 days was considered criteria for the success of the culture establishment. When the cells were not present after 30 days, the culture was inactivated and registered as a failure.

Statistical Analysis

The chi-square test was used to verify statistical differences in the establishment of the culture between serum groups and between resorption root stages. The statistical analysis was performed using the Statistical Package for Social Sciences version 20.0 (SPSS Inc., Chicago, IL, USA, 2011) at 5% significance level.

RESULTS

The success rate in establishing cell cultures was statistically significantly higher (p= 0.041) in 20% FBS group (Table 1). Figure 2 shows a microscopic view (40x) of the cells growing in culture after cryopreservation of the deciduous teeth.

Table 2 shows no differences in the culture rate, according to the different resorption stages of the deciduous teeth.

Table 1. Establishment of cell culture according to concentration of Fetal Bovine Serum in the culture medium.

	Culture success				
	no	**yes**	**total**	**%**	***p***
10% FBS	7	1	8	12.5	0.041
20% FBS	2	6	8	75	
Total	9	7	16		

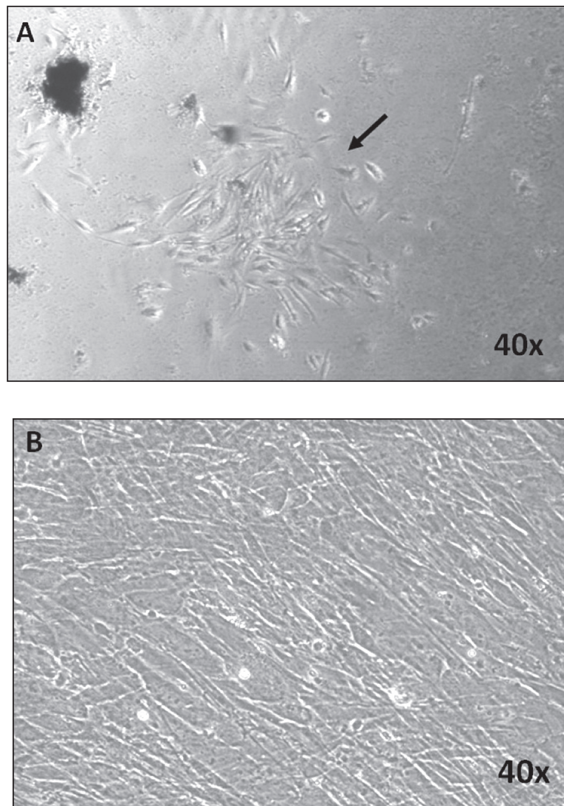
Fisher's exact test (p<0.05)

Table 2. Cell culture establishment regarding the root resorption stage of deciduous teeth.

		Culture success		
		yes	**total**	***p***
Root resorption stage	2/3 root remaining	3	5	*0.66*
	1/3 root remaining	2	5	
	Total root resorbtion	2	6	
	Total	7	16	

Pearson's Chi-square (p>0.05)

Figure 2. A) Microscopic view (40x) of cell attachment (arrow) from intact cryopreserved deciduous tooth. B) Microscopic view (40x) of a confluence culture.



DISCUSSION

The dental pulp of deciduous tooth is a niche of stem cells and therefore, a potential source for tissue engineering[2]. Usually, children lose their primary teeth when the permanent successor tooth starts the eruption process and this cell niche is then discarded[3].

The results of this study have demonstrated the capacity of the establishment of cell culture from human intact deciduous teeth subjected to cryopreservation for 30 days. The success rate for the establishment of the culture varied according to the concentration of FBS used in the culture medium, rejecting the first null hypothesis. The different stages of root resorption of the deciduous teeth demonstrated no difference in cell culture establishment, so the second null hypothesis was confirmed.

Although the isolation of stem cells from pulp of deciduous teeth is related to the resorption process[19], it seems that the different stages of resorption considered in this study did not affect the culture rate after cryopreservation. However, alterations of cell activities, such as proliferation and differentiation potential, may occur, and were not evaluated in this study.

When the Stem Cells from Human Exfoliated Deciduous teeth (SHED) were evaluated, Zhu and colleagues (2013) observed higher proliferation and mineralization potential in the final stage of root resorption compared to the initial stage. This data could be justified by the changes in the microenvironment, due to the resorption process as well as the activation of the stem cells during the repair process, which occurs in the final stage.

The 20% FBS group showed a 75% cell culture rate, corroborating with other studies in which permanent teeth were cryopreserved intact[11-13], whereas 10% FBS group only showed a 12.5% cell culture rate, six times less. Chen and colleagues (2010) also reported only 20% of cell culture from cryopreserved permanent teeth; however the sample was comprised of teeth with pathological root resorption, inflammation, and periodontitis.

During the cryopreservation process, the formation of ice crystals in the cell cytoplasm leads to osmotic imbalance and possible dehydration[21]. In order to prevent this phenomenon, cryoprotectants are added to the freezing media[8]. When the pulp is frozen inside the hard tissue of the tooth, insulation occurs, which is caused by the dentin and enamel layers[6] and the cryoprotectant only penetrates through the apical foramen or the area of resorption. In this case, dehydration can occur even with the use of cryoprotectants and a medium with more supplements (20% FBS) can support the cell growth.

Recent study demonstrated cell encapsulation and thawing techniques as an alternative to optimize the viability of cells from the pulp of deciduous teeth after cryopreservation[18]. However, when there is no time for initial processing, which is important when considering the natural exfoliation of the deciduous teeth, freezing the whole tooth may be an alternative for storage of the cells but it has not yet been studied.

Another technique demonstrated by Giuventú and colleagues (2011), utilized the laser to create micro-channels on non-exfoliated deciduous teeth roots. These teeth were subjected to freezing for 10 days at -80°C and showed a higher isolation success rate compared to the control (no laser). However, it is important to search for a method that does not require any prior processing for cryopreservation in liquid nitrogen (-196°C). Moreover, the use of laser to improve the establishment of cell culture involves time and high cost.

Thus, the use of the supplementation like recommended by the initial studies of characterization of dental pulp stem cell , with animal serum in higher concentrations (20% FBS)[1,22], is a way for optimizing cell isolation from intact cryopreserved deciduous teeth and can be used as an improvement in the process. The idea of banking stem cells for research from cryopreserved deciduous teeth may be considered because of the success rate of 75% in isolation and the recoverability of cells in all root resorption stages.

However, a significant number of stem cells are necessary for therapeutic purposes and in this study the proliferative capacity and differentiation potential of these cultures were not evaluated. Moreover, although stem cells from dental pulp cultured with animal serum have been used successfully in humans[23], there is a risk of immune response to animal products[15].

According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, for any cells to be characterized as mesenchymal stem cells, the following criteria are necessary: (1) adherence to plastic, (2) presence of surface markers for mesenchymal cells (CD73, CD90 and CD105), and (3) differentiate into osteoblasts, chondroblasts and adipocytes[24]. The present study is limited to the isolation of cells with plastic adherence similar to the stem cells from deciduous teeth isolated and characterized by a previous study from this laboratory[19]. Thus, future studies are required for the immunophenotypic characterization of cells from pulp of intact cryopreserved deciduous teeth, in relation to viability, differentiation potential and proliferation rate.

CONCLUSION

- It was possible to establish cell culture from intact cryopreserved teeth when using culture medium supplemented with 20% FBS.
- The establishment of pulp cell culture was not influenced by different stages of root resorption in deciduous teeth. Additional experiments will be required to establish the identity and usefulness of the cells obtained by this method.

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