Tooth Storage, Dental Pulp Stem Cell Isolation, and Clinical Scale Expansion without Animal Serum

Emily J. Eubanks, Susan A. Tarle,* and Darnell Kaigler, DDS, MS, PhD*†*

Abstract

Introduction: Dental pulp stem cells (DPSCs) have therapeutic potential for dentin and dental pulp regeneration. For regenerative approaches to gain clinical acceptance, protocols are needed to determine feasible ways to store teeth, isolate DPSCs, and expand them to clinical scale numbers. **Methods:** In this study, 32 third molars were obtained from patients and immediately placed in saline or tissue culture medium followed by overnight storage at 4°C or immediate isolation of DPSCs. Upon isolation, cells were expanded in medium containing either fetal bovine serum (FBS) or human serum (HS). Cell proliferation (population doubling time [PDT]), cell surface marker expression, and multipotency were compared between DPSCs in FBS and DPSCs in HS. **Results:** The time frame of storage and storage medium did not affect the ability to isolate DPSCs. However, using HS instead of FBS in the initial isolation of DPSCs significantly decreased ($P < .01$) the isolation success rate from 89% (FBS) to 23% (HS). Yet, incorporating fibronectin in the DPSC initial isolation (using HS) significantly ($P < .01$) increased the isolation success rate to 83%. Interestingly, it was found that the proliferation rate was significantly ($P < .05$) higher for DPSCs in HS (PDT = 1.59 ± 0.46) than that for DPSCs in FBS (PDT = 2.84 ± 2.5). Finally, there was no difference in the expression of CD73, CD90, CD105, or multipotency (as measured by osteogenic, adipogenic, and chondrogenic differentiation) between DPSCs in FBS and DPSCs in HS. **Conclusions:** These findings show a clinically feasible method of storing third molars for the isolation of DPSCs. Additionally, DPSCs can be isolated and expanded to clinical scale numbers in media devoid of FBS and still maintain their phenotypic properties. *(J Endod 2014;40:652–657)*

Key Words

Cell therapy, dental pulp, dental pulp stem cells, human serum, regeneration, saline, tooth storage

From the *Department of Periodontics and Oral Medicine, University of Michigan, Ann Arbor, Michigan; and †Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan.

Address requests for reprints to Dr Darnell Kaigler, Department of Periodontics and Oral Medicine, University of Michigan, 1011 North University Avenue, Ann Arbor, MI 48109. E-mail address: dkaigler@umich.edu

0099-2399/$ - see front matter

http://dx.doi.org/10.1016/j.joen.2014.01.005

In the context of regenerative therapies, dental stem cells hold great promise for cell therapy approaches in dentistry; studies have already shown encouraging results in their ability to regenerate bone and periodontal tissues (1–5). Specifically, the identification of dental pulp stem cells (DPSCs) provides promise for regenerating lost dental pulp and tooth tissues because of their ability to differentiate into dentin and pulplike tissue (6–12). Current methodologies for tooth storage, cell isolation, and expansion of DPSCs have limitations regarding their use therapeutically (13). First, if DPSCs are to be used clinically, a feasible way to store teeth after extraction needs to be established. Second, most current protocols for the expansion of these cells incorporate animal serum (14, 15). Again, from a therapeutic standpoint, the use of animal serum should be reduced or eliminated from clinical cell therapy protocols in order for these approaches to meet safety and regulatory guidelines.

There have been a number of cryopreservation protocols evaluated for DPSC storage after their isolation (8, 16–19). Yet, one of the practical challenges for cell therapies using DPSCs is the potential degradation of the pulp tissue between the time of tooth extraction and DPSC isolation and/or cryopreservation. It has been shown that these cells can remain viable for up to 5 days after extraction (20). Although these are important findings, the storage of teeth and subsequent isolation and the storage of dental stem cells are not very practical in a standard dental practice setting because of the lack of available reagents and solutions (ie, tissue culture medium) needed for storage. The feasibility of storage and expansion of these cells would be enhanced if there was a readily available storage solution into which clinicians could place the teeth until DPSC isolation could occur. There are currently companies attempting to address this issue with proprietary tooth storage solutions for DPSC "banking" (www.store-a-tooth.com). Nonetheless, further studies are clearly needed to examine the feasibility and safety of dental stem cell banking for therapeutic indications. Efficient, generalizable, user-friendly, predictable, and safe processes need to be developed in order to store teeth extracted in the dental office. Thus, identifying an optimum storage medium, determining the length of time cells remain viable in this medium, and establishing standardized cell isolation and expansion protocols are of great importance. The hypothesis underlying the proposed study is that DPSCs can be isolated from extracted teeth that have been stored in a medium devoid of animal serum. Additionally, after DPSC isolation, these cells can be expanded to clinical scale numbers without ever being exposed to animal serum; media containing human serum (HS) is used instead.

Methods

Tooth Storage and DPSC Isolation

Thirty-two third molars were obtained from tooth extractions from patients ranging from ages 15–22 years. Teeth were placed in either a sterile saline solution ($n = 25$) or alpha minimum essential medium (α-MEM) containing 15% fetal bovine serum (FBS) ($n = 7$). From these teeth, DPSCs were isolated immediately ($n = 16$) or stored for 24 hours at 4°C before being isolated ($n = 9$). The isolation of DPSCs was performed as previously described (7). Briefly, the crown of the tooth was cut just above the cementoenamel junction to open up the contents of the pulp. The pulp cells in the chamber and canals were cleaned out using various instruments, avoiding nerve tissue, and placed in Iscove modified Dulbecco medium (IMDM) without serum. After isolation, the cell suspension was placed in a conical tube and centrifuged at 1600 rpm for 5 minutes at room temperature. The supernatant was

aspirated, and the pellet was resuspended in a Dispase II–Collagenase solution. The solution was placed at 37°C for 60 minutes, inverting the tube at 15-minute intervals.

IMDM was added to the cells before the suspension was centrifuged at room temperature for 5 minutes at 1600 rpm. The cell pellet was resuspended in IMDM without serum. This cell suspension was placed in a T25 tissue culture flask in 1 of the following 3 conditions: containing 15% FBS + α-MEM ($n = 30$), containing 15% human serum (HS) + α-MEM ($n = 20$), or containing 15% HS + α-MEM, which had a fibronectin (FN) coating on the bottom of the flask ($n = 17$).

Cell Proliferation Population Doubling Time

Cells counts were performed at each passage, and the population doubling time (PDT) was calculated and compared between conditions. In order to determine PDT, the following calculation was used: $\frac{(\#\text{ days from } P_0 \text{ to } P_1)(\log 2)}{(\log P_1 - \log P_0)}$, where P_0 is the number of cells at the initial passage and P_1 is the number of cells at the next passage.

Cell Surface Marker Expression

Flow cytometry to determine the expression levels of the cell surface markers CD90, CD73, and CD105 was performed. DPSCs were harvested from T150 flasks, washed, and aliquoted equally into tubes. Cells were first incubated with a blocking solution containing CD16/CD32 at 4°C for 10 minutes followed by washing. Cells were then incubated with the specific antibodies conjugated with fluorochromes (Biolegends, San Diego, CA) at 4°C for 30 minutes. After washing, these cells were analyzed on a Beckman Coulter MoFlo flow cytometer.

Differentiation Staining

For multipotent potential, osteogenic, adipogenic, and chondrogenic pathways were evaluated using von Kossa, alcian blue, and oil red O stains. Cells were plated in 12-well plates at 30,000 cells/well and cultured for 4 weeks with the appropriate media being replaced every 2–3 days. Cells were fixed to the plates using 10% formalin for osteogenic and adipogenic induction and cold methanol for chondrogenic induction. For von Kossa staining, 5% silver nitrate was added to each well and incubated in the dark for 30 minutes at room temperature. After this incubation period, wells were washed and then exposed to ultraviolet light for 30 minutes. Sodium thiosulfate (1%) was added to each well and allowed to sit for 3–4 minutes at room temperature. Oil red O stain and alcian blue stain were added to the appropriate wells and allowed to incubate at room temperature for 30 minutes. To the wells with alcian blue stain, after the incubation period, the stain was removed, and 0.1 N hydrochloric acid was used to wash the wells. Staining of the cells was recorded by photography.

Statistical Analysis

Statistical analysis was performed with the use of Instat software (GraphPad Software, San Diego, CA). All data were reported as mean ± standard deviation unless otherwise noted. Statistically significant differences were determined by 2-tailed Student *t* tests, and statistical significance was defined as $P < .05$.

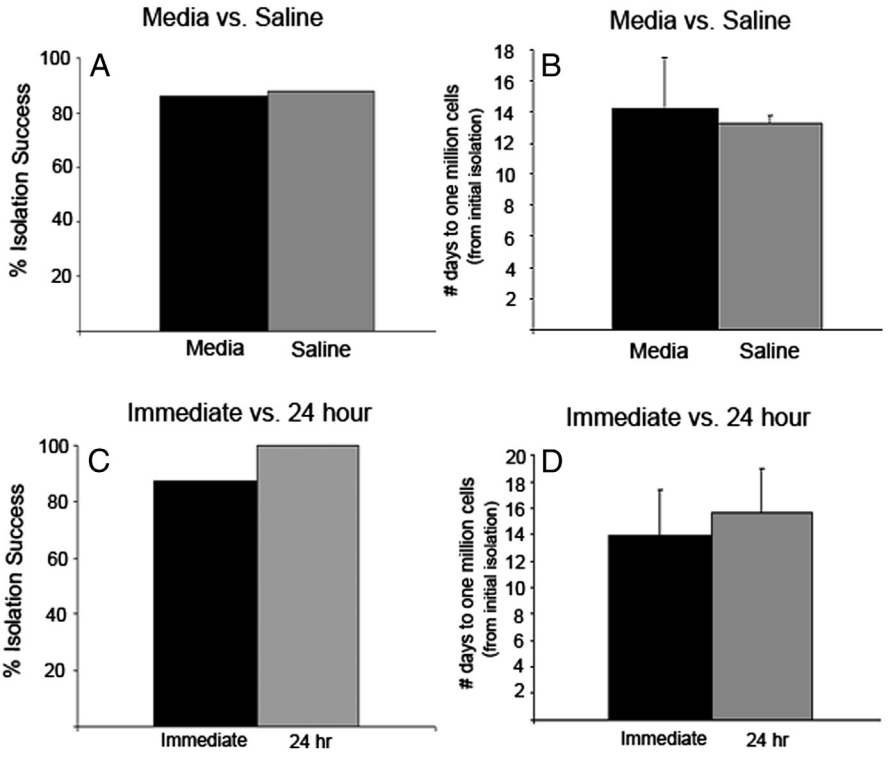

Figure 1. The effect of tooth storage conditions on the ability to isolate DPSCs. (*A*) The percentage of teeth that yielded DPSCs was compared between teeth stored in media containing FBS ($n = 7$) versus those stored in sterile saline ($n = 25$) before DPSC isolation. (*B*) The number of days it took to yield 1 million cells was compared between teeth stored in media containing FBS versus those stored in sterile saline. (*C*) The percentage of teeth that yielded DPSCs was compared between teeth stored in saline overnight at 4°C ($n = 9$) versus those in which DPSCs were isolated immediately after tooth extraction ($n = 16$). (*D*) The number of days it took to yield 1 million cells was compared between teeth stored overnight in saline at 4°C versus those in which DPSCs were isolated immediately after tooth extraction.