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Effect of storage media on human periodontal ligament cell apoptosis

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Abstract – The ability of storage media to preserve periodontal ligament (PDL) cell vitality has been previously evaluated. However, the mechanisms by which different storage conditions alter the functional status of PDL cells have not been determined. The purpose of the present study was to investigate, *in vitro*, the level of programed cell death or apoptosis in a population of PDL cells following storage under different conditions. Primary human PDL cells were plated into 24-well-culture plates and allowed to attach for 24 h. Cells were then exposed for 1 h to milk, Hank's balanced salt solution (HBSS), Soft Wear contact lens solution or Gatorade at room temperature or on ice. Culture medium was used as a negative control. Apoptosis was evaluated at 24, 48, and 72 h after treatment on quadruplicate samples by using the ST 160 ApopTag Fluorescein Direct *In Situ* Detection Kit. The total number of cells and the total number of apoptotic cells were counted. The results indicated that at 24 and 72 h, PDL treated with Gatorade and the contact lens solution displayed the highest percentages of apoptotic cells when compared with the other treatment groups at room temperature. Overall, cells treated on ice showed significantly lower levels of apoptosis when compared with treatments at room temperature. In conclusion, the results indicated that apoptosis plays a major role in cell death in cells treated with Gatorade and contact lens solutions in comparison to other storage solutions and that storage on ice can inhibit programed cell death.

Tooth avulsion is a fairly common and complicated injury that involves damage to the supporting tissues of the tooth as well as the dental pulp (1). The healing pattern of an avulsed tooth after replantation in the alveolar socket will depend upon the healing potential of each cellular component of the tissues involved. Furthermore, it will depend on replantation management and patient-specific factors (1). Minimal damage to the periodontal ligament (PDL) is critical for regeneration of the attachment apparatus and to protect the root from resorption (2, 3).

Extraoral time and storage conditions are the most crucial factors in determining the viability of the remaining PDL cells, and thus the prognosis for the avulsed tooth (4). Studies have shown that an avulsed tooth can be replanted without complications after 1–3 h of being placed in suitable storage conditions (2, 5, 6). An ideal storage medium would be one that is capable of preserving the viability, mitogenicity, and clonogenic capacity of the damaged PDL in order to facilitate repopulation of the denuded root surface thereby preventing further root resorption (7). The storage medium should have a physiological osmolality and pH and should be maintained at an appropriate temperature to allow optimal cell growth or survival. Finally, the ideal storage media should be readily available for use in emergency situations.

Many studies have investigated the efficacy of various storage media for maintaining the vitality of the PDL cells following tooth avulsion. Milk has proven to be superior to saliva in maintaining vitality, both *in vitro* and *in vivo* (8–14). Its superiority has been attributed to the physiologic osmolality of milk (8). Hank's balanced salt solution (HBSS) is a sterile, non-toxic, pH balanced, isotonic salt solution commonly used for the irrigation of wounds and as a standard cell culture medium (CM). A commercially available tooth preservation system using HBSS as the storage medium, Save-A-Tooth™, has been developed (Save-A-Tooth Inc., Pottstown, PA, USA). However, this system is not yet available in pharmacies or drug stores. In an effort to find additional solutions that would be readily available for use, Gatorade® (Gatorade, Chicago, IL, USA) and contact lens solutions have been investigated for their ability and suitability to serve as a storage medium for avulsed teeth (15–18). In addition to storage media, a reduced storage temperature has been shown to have a positive effect on maintaining cell viability (18).

Cell death by necrosis typically occurs after severe cellular injury but most cells in the body die through energy dependent form of cell death termed apoptosis (19, 20). Apoptosis plays a complementary but opposite role to mitosis in the regulation of animal cell populations. During the adult life, apoptosis is of central

importance for elimination of unwanted cells with potentially harmful mutations (21). Apoptosis can result when the environment becomes so hostile that a cell can no longer maintain homeostasis. This process is different from necrosis where cell death is not programed and often results in a functional deficit for the host; apoptosis on the other hand, can provide for maintenance or enhancement of function (22). The apoptotic threshold of a cell will depend on the ratios and relative abundances of different positive and negative regulators (19).

Cells undergoing apoptosis are characterized by distinctive morphological and biochemical features that are exclusive of this mode of cell death (23). The most remarkable biochemical event in apoptosis is the DNA cleavage between nucleosomes, producing multiple fragments (24). Evaluation of apoptosis by means of DNA fragmentation in individual fixed, cells or in tissue sections can be accomplished by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique. The TUNEL method consists of labeling the 3′-hydroxyl ends of DNA fragments with an enzyme and a labeled nucleotide. DNA from apoptotic cells is detected by addition of the labeled nucleotide (i.e., dUTP); normal or proliferative nuclei containing unfragmented DNA do not stain with this method. The major advantage of using the TUNEL technique is its ability to quantitatively assess apoptotic cell populations (25).

The aim of this study is to investigate, *in vitro*, the level of programed cell death or apoptosis in a population of PDL cells recovered after storage in different conditions which could be one factor influencing the outcome of healing in replantation.

Materials and methods

Preparation of PDL cell cultures

Existing and unidentifiable primary human PDL samples isolated from freshly extracted human teeth as described by Sigalas et al. (18) were used in all experiments. A 1 ml frozen vial was rapidly thawed in a water bath at 37°C. The cell solution was then transferred to a 12 ml tube containing 5 ml of prewarmed PDL growth media consisting of αMEM (Gibco™ Invitrogen Corporation, Grand Island, NY, USA) and 10% fetal bovine serum (FBS, Equitech-Bio Inc., Kerrville, TX, USA). The tube was centrifuged for 10 min at 500 ***g*** at 20–25°C in a Sorvall RT6000. The supernatant was removed and the cell pellet was resuspended in 10 ml of fresh prewarmed growth media. Five ml of the cell suspension and 5 ml of fresh prewarmed media were added to each of two 100 mm plates (Costar®; Corning, NY, USA). The plates were incubated at 37°C in a 5% CO_2 environment overnight. The medium was replaced with prewarmed CM and thereafter was replaced every other day. This was repeated until the cells reached about 90% confluence, or until ready to passage. For each experiment 5×10^4 cells were plated per well. The plates were then transferred to an incubator at 37°C with 5% CO_2 and the cells were allowed to attach to the plate overnight.

Exposure of PDL cultures to different solutions

After 24 h the CM was removed and 2 ml of the five different experimental solutions, either at room temperature or on ice (4°C) were added for 1 h. The experimental storage solutions used in the experiment were: (i) milk (Schepps 2% reduced fat milk, fortified with vitamins A&D; Schepps Dairy, Dallas, TX, USA); (ii) HBSS (Invitrogen; Carlsbad, CA, USA); (iii) CM (αMEM + 10% FBS); (iv) Gatorade Thirst Quencher/Lemon-Lime (Gatorade Co.); and (v) contact lens solution (Soft Wear®; CIBA Vision Co., Duluth, GA, USA). Four wells for each temperature and for each observation period were examined for each of the five different storage solutions.

After 1 h of exposure at room temperature and on ice, the storage solutions were removed, each well was rinsed three times with 2 ml of sterile 1 × PBS (VWR/EM Science, West Choster, PA, USA), 2 ml of prewarmed CM was added to each well and the plates were all returned to the incubator.

Apoptosis assays

To determine the levels of apoptosis by TUNEL, an ApopTag® Fluorescein *In Situ* Apoptosis Detection Kit (CHEMICON® International Inc., Temecula, CA, USA) was used at 24, 48, and 72 h following exposure of the cells to the test solutions. The TUNEL technique consists of labeling the free 3′-hydroxyl ends of a DNA fragment by enzymatically adding a nucleotide (i.e., digoxigenin-dUTP) covalently attached to digoxigenin using terminal deoxynucleotidyl transferase (TdT). Detection of the digoxigenin molecule covalently attached to the nucleotide can be accomplished by using an anti-digoxigenin antibody conjugated to fluorescein, which fluoresces green. Thus, DNA-breaks that are labeled by TdT with the digoxigenin-dUTP are tagged with the fluorescent antibody causing apoptotic cells to fluoresce green (Fig. 1a and d). After the apoptotic assay was complete, the plates were counterstained by using propidium iodide (PI) (red labeled cells, Fig. 1b and e) (Sigma-Aldrich, St Louis, MO, USA) to obtain total remaining cell counts for each group after each treatment and to calculate the ratio of apoptotic cells to the total remaining cell number. Double labeling of apoptotic and PI staining confirmed that the PI labeled cells were apoptotic (yellow labeled cells, Fig. 1c and f). Normal or active nuclei did not fluoresce green with this method (red cells, Fig. 1c and f). A Leica DM IRBE inverted fluorescent confocal microscope (Exton, PA, USA) was used to capture cell images and to count the cells.

To determine the total number of cells per well and the total number of apoptotic cells, the following protocol was used. The total number of PI labeled cells was counted for two randomly selected confocal fields and the total number fluorescence positive apoptotic cells within those fields was also counted. The confocal field diameter was 2.32 mm and the diameter of a well on the 24 well plate was 15 mm. To calculate the total number of cells in a single well, the average cell counts

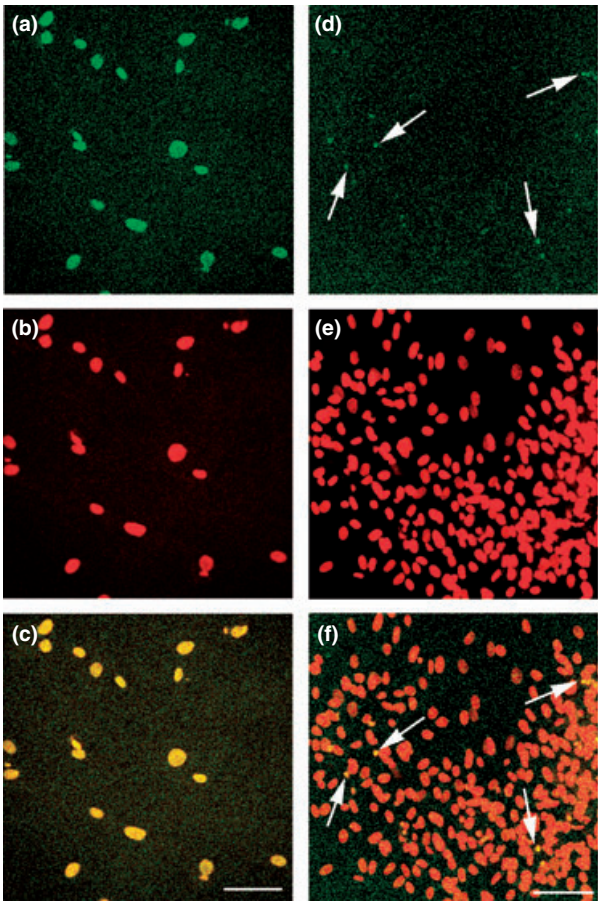

Fig. 1. Periodontal ligament (PDL) treated for 1 h with Gatorade or culture medium (CM) at room temperature followed by culturing in αMEM and 10% fetal bovine serum at 37°C for 24 h. In panels a through c PDL cells were exposed to Gatorade and in panels d through f PDL cells were exposed to CM at room temperature. (a and d) Fluorescein labeled apoptotic nuclei (green). (b and e) Propidium iodine stained cell nuclei (red). (c) Composite of panels a and b. (f) Composite of panels d and e. Yellow nuclei represent propidium iodide labeled cells undergoing apoptosis. Note: In panels d and f arrows are points at flurescein (green) or fluorescein/propidium idodine double labeled cells (yellow), respectively. Size bar = 75 μm.

from the random confocal fields was multiplied by 41.76, which is the well area divided by the confocal field area.

Calculations and statistical analysis

The percent of apoptotic cells per well was calculated by dividing the number of green fluorescent apoptotic cells (green labeled cells, Fig. 1a and d) in a well by the number of total PI labeled cells (red labeled cells, Fig. 1b and e) and multiplied by 100. The percentage of PI labeled cells (i.e., percent remaining cells after treatment) was calculated by dividing the number of red fluorescent PI stained nuclei by the number of total cells plated. The data were analyzed and graphed by using the GRAPHPAD PRISM® (VERSION 4) SOFTWARE. STATISTICAL SIGNIFICANCE was determined using a two-way ANOVA and a Bonferroni *post hoc* test.

Results

Room temperature

After exposure to the five different experimental solutions at room temperature, the Gatorade group showed the highest percentage of apoptotic cells after 24 h; 100% of PI labeled cells. Apoptosis in the Gatorade treated group was significantly different ($P < 0.001$) from all other groups at 24 and 72 h (Fig. 2a and c). The percentage of apoptotic cells in the contact lens solution group was 42% of PI labeled cells and this was significantly higher ($P < 0.01$) when compared with the milk, HBSS and CM groups at 24 and 72 h (Fig. 2a and c). Milk, HBSS and CM all had a low percentage of apoptotic cells and no significant difference ($P > 0.05$) was found among these treatment groups for each

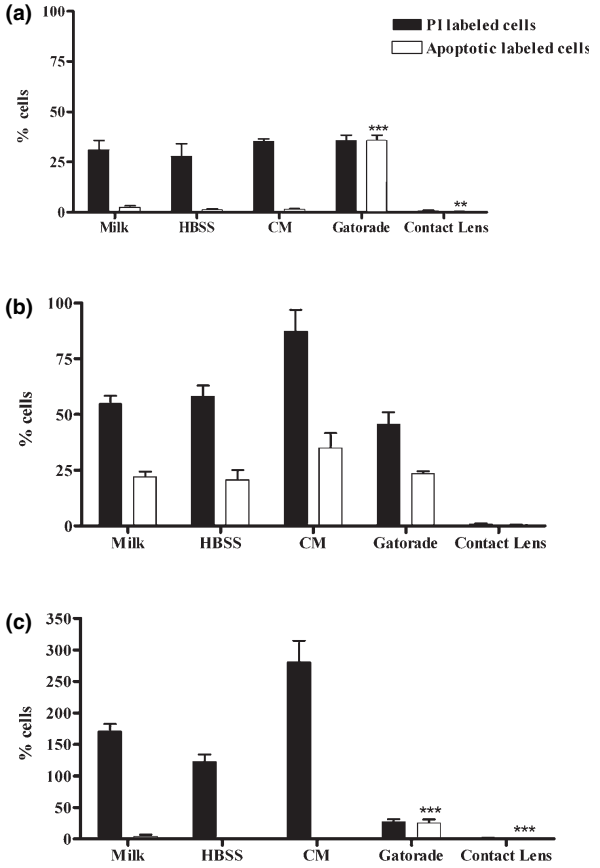

Fig. 2. Histograms showing the percentage of total propidium iodide (PI) labeled cells and percentage of total apoptotic cells after exposure for 1 h to the different experimental solutions at room temperature followed by culturing in αMEM and 10% fetal bovine serum at 37°C for (a) 24, (b) 48, and (c) 72 h. The percentage of total apoptotic cells was calculated by dividing the number of apoptotic labeled cells by the number of PI labeled cells and multiplying by 100 = % cells (open bars). The percentage of total PI labeled cells was calculated by dividing the number of PI labeled cells by the total plated cells and multiplying by 100 = % cells (solid bars). Note: The percentage scale on panel c is larger than in panels a and b. $^{**}P < 0.01$, $^{***}P < 0.001$ percentage apoptotic cells as compared to other treatment groups.

observation period (Fig. 2). On the other hand, a decrease in the percentage of apoptotic cells was observed at 48 h, for both the Gatorade and the contact lens group (Fig. 2b).

Ice

After exposure on ice to the five different experimental solutions, the Gatorade group showed the highest percentage of apoptotic cells after 24 h with 99% of PI labeled cells being apoptotic (Fig. 3a), consistent with the room temperature data. As a percentage of total cells this finding was significantly higher ($P < 0.001$) than all the other groups (Fig. 3a). There were no significant differences among the milk, HBSS, CM and contact lens solution groups ($P > 0.05$; Fig. 3). At 48 and 72 h, the Gatorade group had a significantly higher ($P < 0.001$) percentage of apoptotic cells when compared with all other groups (Fig. 3b and c, respectively). No significant differences were found when the remaining groups were compared with each other ($P > 0.05$; Fig. 3).

Room temperature vs ice

When the PDL cells were exposed to the different experimental solutions on ice, there was a decreasing trend in the overall percentage of apoptotic cells (Fig. 4). At 24 h, the percentage of apoptotic cells in the contact lens solution group on ice was significantly lower ($P < 0.001$) when compared with contact lens solution at room temperature (Fig. 4a). At 48 h, the milk, HBSS, CM and contact lens solution treatment groups showed a significant decrease in the percentage of apoptotic cells when treated on ice, compared with the same treatment groups at room temperature (Fig. 4b). On the other hand, the Gatorade group showed a significant decrease

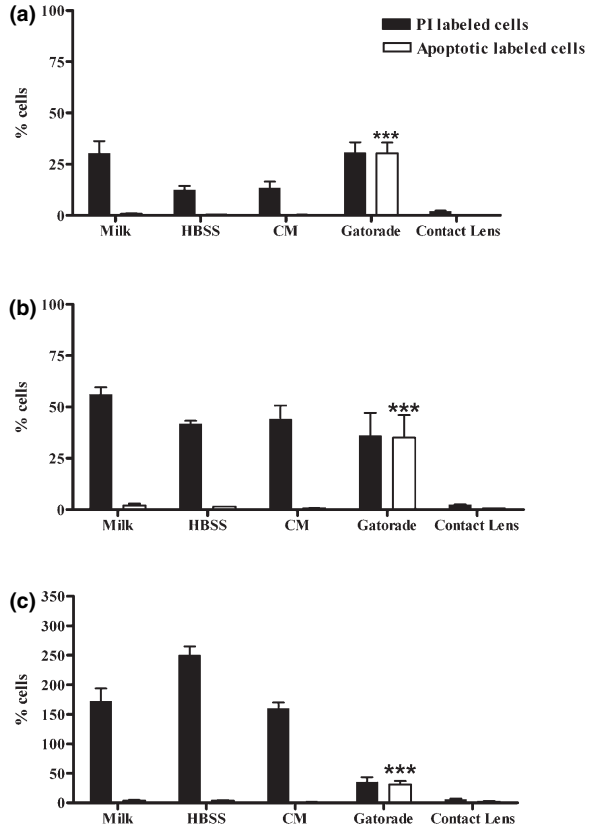

Fig. 3. Histograms showing the percentage of total propidium iodide (PI) labeled cells and percentage of total apoptotic cells after exposure for 1 h to the different experimental solutions on ice followed by culturing in αMEM and 10% fetal bovine serum at 37°C for (a) 24, (b) 48, and (c) 72 h. The percentage of total apoptotic cells was calculated by dividing the number of apoptotic labeled cells by the number of PI labeled cells and multiplying by 100 = % cells (open bars). The percentage of total PI labeled cells was calculated by dividing the number of PI labeled cells by the total plated cells and multiplying by 100 = % cells (solid bars). Note: The percentage scale on panel c is larger than in panels a and b. ***$P < 0.001$ indicates the percentage of apoptotic cells in the Gatorade group as compared to all other treatment groups.

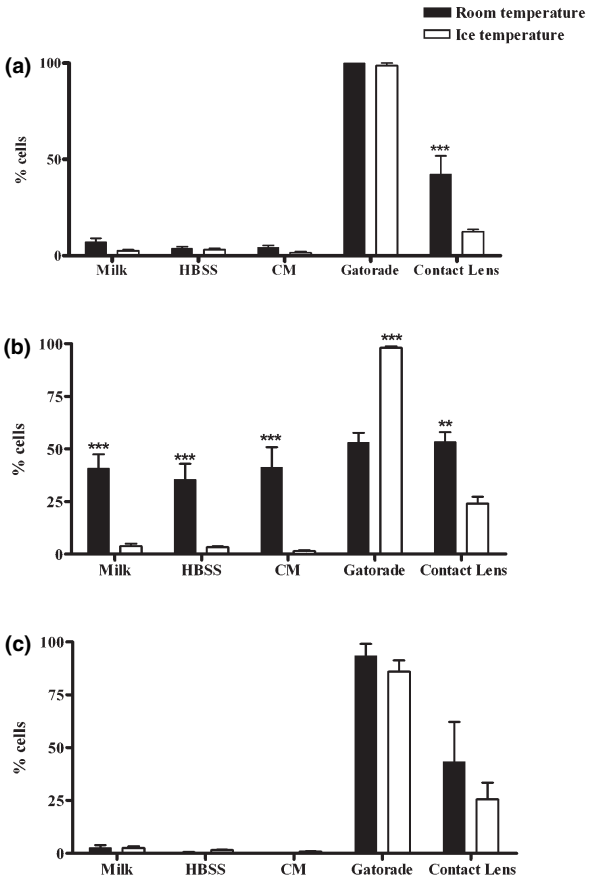

Fig. 4. Histograms showing the percentage of total propidium iodide (PI) labeled cells and percentage of total apoptotic cells after exposure for 1 h to the different experimental solutions at room temperature and on ice followed by culturing in αMEM and 10% fetal bovine serum at 37°C for (a) 24, (b) 48, and (c) 72 h. The percentage of total apoptotic cells was calculated by dividing the number of apoptotic labeled cells by the number of PI labeled cells and multiplying by 100 = % cells (open bars). The percentage of total PI labeled cells was calculated by dividing the number of PI labeled cells by the total plated cells and multiplying by 100 = % cells (solid bars). **$P < 0.01$, ***$P < 0.001$ room temperature as compared to treatment on ice.

($P < 0.001$) at 48 h in number of apoptotic cells when treated at room temperature compared with treatment on ice (Fig. 4b). At 72 h, no significant differences were found when treatment at room temperature were compared with treatment on ice ($P > 0.05$, Fig. 4c).

A reduced level of PI labeled cells vs total cells plated was observed for up to 48 h (Figs 2 and 3) indicating that the number of PI labeled cells attached to the plate was reduced versus the number of cells originally plated after treatment. This finding suggests that the cells did not attach to the plate after treatment. In contrast, after 72 h in culture the number of PI labeled cells was greater than the total cells plated for most treatment groups suggesting that the cells proliferated to greater numbers than what was plated after this amount of time in culture.

Discussion

This study focused on apoptotic cell death to evaluate PDL damage following avulsion. Two main processes involved in cell death are necrosis and apoptosis. Apoptotic cell death in PDL as a result of tooth avulsion has not yet been investigated. The present study was conducted in order to determine the role of apoptosis in PDL cell death after storage in five different storage media for 1 h both at room temperature and on ice.

Apoptosis in Gatorade

Our results indicate that apoptosis played a major role in cell death when the PDL cells were exposed to Gatorade for 1 h regardless of the temperature. At 24 and 72 h after treatment at room temperature and on ice, nearly all the cells were found to be apoptotic. Thus, it appeared that Gatorade may have an apoptosis triggering effect in human PDL cells. It has been proposed that mild membrane damage caused by injurious agents might allow an influx of calcium, sufficient to trigger apoptosis, but insufficient to extensively activate phospholipases with resultant necrosis (26). It is possible that mild membrane damage might have been caused by the low pH of Gatorade (pH 3) which is not conducive to cell growth or survival (27). An earlier study investigated the osmolality of Gatorade and the authors found it to be higher than that reported by the Exercise Physiology Laboratory at the Gatorade Sports Science Institute. According to the manufacturer, Gatorade has a physiological osmolality ranging from 280 to 360 mOsm l^{-1} however; Harkacz et al. found Gatorade to be hypertonic (407 mOsm l^{-1}) (15). This finding could have caused the cells to shrink (or lose water) when exposed to the Gatorade, which is an important change during apoptotic cell death.

An interesting finding was that after 48 h at room temperature only 53% of the PI labeled cells were apoptotic in the Gatorade group. This finding is inconsistent with our results from the 24- and 72-h periods, where the apoptotic levels reached 100% and 93%, respectively. This result was consistent for all four replicates at 48 h room temperature. This obscure result could have been because of a bacterial contamination passed to the replicate wells for the 48 h time point such that the bacteria killed the cells by a necrotic process before Gatorade treatment severely impacted the cell death process observed and altered the percent values obtained. The validity of this explanation is not clear as an obvious bacterial contamination was not evident in the 48 h treatment wells.

The level of apoptosis found in the contact solution treatment group was higher when compared with the CM, HBSS or milk groups at both experimental temperatures. However, this finding was only significant when the cells were stored at room temperature. Our results concerning contact lens solution are in agreement with previous studies (16). At all times and regardless of the temperature, very few cells were recovered from the contact lens solution, suggesting that the preservatives in these solutions might have caused the cells to become detached from the culture wells or induced the cells to die because of a different cell death process, i.e., necrosis. Thus, assuming necrosis as the cause for the lack of PI labeled cells, the data are consistent with contact lens solution as being pro-necrotic in contrast to Gatorade which is pro-apoptotic. Based on these results and within the limitations of our study, contact lens solutions are not recommended as a temporary storage medium for the avulsed tooth.

Apoptosis and healing

Cell death by apoptosis differs from necrosis in that the former does not elicit an inflammatory reaction in the surrounding tissues (20, 28). Apoptosis may be preferable in cases of tooth avulsion because the establishment of an inflammatory reaction by necrosis in the surrounding tissues could lead to resorption. It is well known that cementum is more resistant to resorption than bone or dentin (29, 30), and it is likely that cementoclasts require prior activation or persistent triggering by external factors such as bacterial products, inflammatory cytokines, infection and/or necrotic debris before resorption can ensue (31). All these factors are normally found when the vitality of the periodontal membrane has been lost prior to replantation (32). But in contrast to apoptosis, recruited macrophages and inflammatory cells release increased levels of cytokines and activating factors in response to necrosis (20). Activating factors such as osteoclast activating factor (OAF) and prostaglandins attract and activate cementoclasts that are responsible for resorbing cementum (33). Increased cementum resorption could reduce the healing prognosis. Therefore, subtle cell death by apoptosis lacks this inflammatory response, because a reduced inflammatory reaction following replantation may result in less resorption and better prognosis.

Studies focused on apoptosis of PDL fibroblasts are relevant to the endodontic field because cell survival is critical to successful tissue regeneration upon replacement of an avulsed tooth. Factors impacting PDL survival and potentially endodontic treatment are dental materials, bacteria, and trauma (34). For example, dental polymers and root end filling materials (35, 36), bacterial components (37) and compression of periodontal cells

(38) have been shown to induce apoptosis in PDL. Moreover, after trauma expression of serine proteases could potentially induce apoptosis of PDL fibroblasts (39). In contrast, molecules expressed in PDL fibroblasts such as insulin – like growth factor-IGF-1 (40) and secreted frizzled-related protein -SFRP1 (41) have been found to reduce apoptosis in PDL cells. Future research studying molecules and signaling mechanisms preventing or causing cell death could be of value to find pharmacological agents that would modulate cell death process and improve replantation prognosis.

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