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Microscopic evaluation of the effect of different storage media on the periodontal ligament of surgically extracted human teeth

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Abstract – The objective of this study was to microscopically evaluate the human periodontal ligament adhered to extracted teeth, after extra-alveolar period of 1 h using, as storage media, pasteurized milk (group I), chicken egg white (group II) and artificial saliva (group III). Forty intact premolars were selected, with indication of tooth extraction for orthodontic reasons. After the extraction of 30 teeth, they were maintained dried on a gauze at room temperature for 10 min, and then immersed in the selected storage media. After the established time, the teeth were washed with saline solution and placed in 10.0% buffered formalin. Ten teeth were extracted and immediately immersed in 10.0% neutral formalin (group IV). Thereafter, they were submitted to histological processing. After fixation and decalcification, the specimens were cut at the cervical, medium and apical thirds, inserted in paraffin and serially sectioned, with 6-μm thickness. They were stained by hematoxylin–eosin and analyzed under light microscopy. According to the results of quantitative analysis, there was no statistically significant difference in the number of cells per mm^2 between groups I, II and III. The qualitative analysis showed similar results in relation to the organization of collagen fibers and the number of cells in groups I and II, but group III displayed a higher disorganization of the collagen fibers and also a higher reduction in the number of cells. Based on these results, it was concluded that the quality of periodontal ligament was affected by the storage media, when compared with the control group. There was a statistically significant difference in the number of cells per mm^2 between the control group and groups I, II and III. There was no significant statistical difference in the number of cells per mm^2 between groups I, II and III.

Studies of Andreasen (1985) demonstrated that 30% of children suffer from some type of trauma to the primary dentition up to 7 years, and 22% to the permanent dentition up to 14 years, considering only the anterior teeth (2). Thus, more than 50% of children aged up to 14 years suffer from some type of trauma, varying from a simple enamel fracture to tooth avulsion. Nearly 15% of traumatic lesions result in avulsion, especially at the age range from 7 to 10 years.

Immediate reimplantation is one of the factors that contribute most to the repair of the periodontal ligament. When the tooth is reimplanted immediately after avulsion, the rate of repair of the periodontal ligament ranges from 85.0 to 97.0% (4).

Among the postoperative alterations observed in late tooth reimplantations, the literature agrees that cementodentinal resorption is the most significant, not only owing to its constancy, but also because it is one of the greatest causes for failure (10). Clinical and experimental studies have demonstrated that resorptions are directly related to the storage medium and extra-alveolar time (3).

Notwithstanding, immediate reimplantation is the ideal treatment for the re-establishment of the supply of nutrients to the cells in the periodontal ligament on the root surface, in some situations reimplantation may be delayed. When this occurs, the tooth should be stored in a humid medium to keep the viability of the cells in the periodontal ligament until treatment may be performed (12).

Several studies demonstrated that saliva is a better storage medium than dry storage for short extra-alveolar times (4, 5). The clinical utilization of saliva as a storage medium, despite its availability, has the disadvantage of the risk of swallowing of the avulsed tooth, besides the activity of microorganisms and salivary enzymes.

As it is hypotonic and contaminated, storage in tap water is demonstrated to be as harmful for the cells in the periodontal ligament as dry storage. The saline solution has achieved good outcomes in short periods; however, it

may not be available at the place where accidents occur, which limits its utilization (7).

Storage media, such as contact lenses solutions, rehydration fluids, plastic films and alcohol, were unable to preserve the vitality of cells in the periodontal ligament (4, 13, 16).

Comparisons between milk and saliva for the evaluation of the viability of cells in the periodontal ligament have demonstrated that milk is better than saliva (5, 6, 8). For these authors, milk may be used a storage medium for longer extraoral periods (up to 6 h) because it also contains nutritive substances, such as amino acids and carbohydrates; furthermore, milk is pasteurized.

Even though milk has been mentioned as an alternative storage medium in experimental *in vitro* studies or animal studies, its implications in clinical terms have not been observed so far (4).

Experimental studies were also conducted on rats with utilization of clear gelatin, more or less diluted in water (17) and coconut water solution (1) as alternative means for storage of avulsed teeth. However, the results were inferior when compared with bovine milk.

Despite the existence of storage media of better quality, such as Hank's balanced salt solution (HBSS) and Viaspan, the lack of availability of these products at the place and moment of accident makes their recommendation questionable.

According to Rozenfarb et al. (19), other options are required as substitutes to bovine milk when it is not available. These authors found similar results compared with milk when using chicken egg albumin for the preservation of human skin fibroblasts.

A few studies (9, 19, 20) have addressed the utilization of chicken egg white as the storage medium for avulsed teeth, as it is easily available. In 1995, Andreasen indicated saliva as the best storage medium (4).

The aim of this study was to microscopically evaluate the human periodontal ligament adhered to the extracted tooth, after an extra-alveolar period of 1 h, by utilizing pasteurized bovine milk, chicken egg white and artificial saliva as storage media.

Materials and methods

Clinical procedures

This study was conducted on patients with indicated extraction of maxillary and mandibular premolars for orthodontic reasons, with one or two roots and vital pulp, completely formed roots and periodontal integrity. Patients attended the Surgery Clinic of the Dental School at Federal University of Goiás.

After clinical procedures of extraoral and intraoral antisepsis, the tooth was anesthetized and syndesmotomy was performed, followed by extraction with forceps, as less traumatic as possible, to avoid the occurrence of root and bone fracture. After extraction, suture was performed and the patients received routine postoperative recommendations.

After extraction, the teeth were dry stored for 10 min on a sterilized gauze, at room temperature, and then held by their crowns with the aid of pliers and put in a labeled plastic flask containing 30.0 ml of the storage medium to be tested.

This led to the achievement of four study groups with 10 teeth each, as follows:

Group I – Commercial pasteurized milk (whole milk Parmalat, São Paulo, SP, Brazil)

Group II – Chicken egg white (Granja Saito, large eggs, Bela Vista de Goiás, GO, Brazil)

Group III – Artificial saliva (Apothicário Pharmacy, Araçatuba, SP, Brazil)

Group IV – Control group, comprising extraction and immediate placement in a recipient with 10% neutral formalin.

After a pre-established period of 60 min and holding the teeth by their crowns with the aid of pliers, the teeth in groups I, II and III were washed with saline solution (0.9% saline solution; In Halex Istar, Indústria Brasileira, Goiânia, GO, Brazil). Then, they were immediately transferred to individual plastic flasks containing 30.0 ml of 10% neutral formalin for at least 48 h. Thereafter, they were sent to the Experimental Surgery Laboratory of the Surgery and Integrated Clinic Department of Araçatuba Dental School, São Paulo State University, for processing.

Laboratory processing

After fixation in 10% neutral formalin, the specimens were washed in tap water and then submitted to the demineralization process in ethylenediaminetetra-acetic acid (EDTA; Dinâmica Reagentes Analíticos, Rio de Janeiro, Brazil). The demineralization process was maintained for 60 days, after which the root presented a rubber-like consistency, without resistance to sectioning by a microtome blade.

After the decalcification process, teeth were sectioned in three parts, for the achievement of three transverse sections. These sections allowed approximate division of the teeth in cervical, middle and apical thirds. Thereafter, the thirds were submitted to dehydration, diaphanization and immersion in paraffin, for later inclusion in paraffin blocks. Semiserial sections, 6 μm in thickness, were achieved with the aid of a microtome. The sections were stained with hematoxylin and eosin for light microscopy assessment.

Three samples of each tooth, adding up to 30 samples per group, were evaluated on a light microscope (Carl Zeiss, Göttingen, Germany) with 20× and 40× magnification. Analysis of the results was descriptive and quantitative. The histopathologic phenomena were described, and all events were reported and related according to the group.

One section of each third was submitted to quantitative analysis, with the selection of three fields for the counting of cells per mm^2. The number of cells in the periodontal ligament was quantified by morphometric analysis, by using a light microscope containing a square reticule (Carl Zeiss 4740680000000).

The mean number of cells in the periodontal ligament per mm^2 and respective standard deviations, observed on each third for each group, were assessed and statistically

analyzed by analysis of variance (ANOVA) followed by the Tukey test. The significance level adopted was 0.05.

Results

Qualitative analysis

Group I – pasteurized bovine milk
The teeth presented periodontal ligament with clear collagen fibers with initially perpendicular orientation. Following this, they presented curved orientation, involving bundles of capillaries and venules. The periodontal ligament presented preserved cellularity, with scattered areas with reduced number of fibroblasts. These alterations were observed in discontinuous areas and occurred at all levels submitted to analysis (Fig. 1 – middle third). When reduced cellularity was visible, the bundles of collagen fibers also lost their distinctness and orientation. The blood vessels, in turn, were dilated; yet the endothelium was preserved.

Group II – chicken egg white
The teeth presented periodontal ligament with clear and perpendicularly directed bundles of collagen fibers, which subsequently became tangential to the tooth surface. Simultaneously, they were curved involving bundles of capillaries and venules. The connective tissue of the periodontal ligament presented a reduction in the number of cells in some areas. These fields with greater cellularity occurred at all thirds analyzed (Fig. 2 – middle third). The bundles of collagen fibers were disorganized. The blood vessels were preserved, occasionally dilated and exhibited reduced number of endothelial cells.

Group III – artificial saliva
The teeth presented ligament with clear and perpendicularly directed bundles of collagen fibers, which became tangential and parallel, with areas of disorganization and spaces between the fibers. The connective tissue exhibited expressive reduction in cellularity, and the areas of reduced cellularity were discontinuous and presented variable intensity, at all thirds analyzed (Fig. 3 – middle third). Some fields exhibited *ghosts* of cells. At these areas, the bundles of collagen fibers lost their distinctness and were less preserved. The blood vessels presented variable width and were dilated in some cases, with reduced number of endothelial cells.

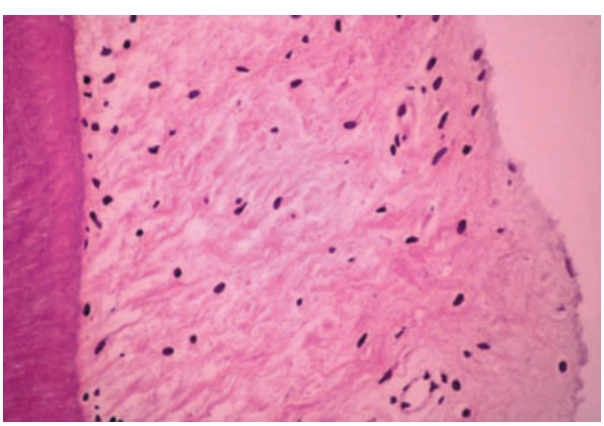

Fig. 1. Middle third – remnants of periodontal ligament adhered to the cementum. Reduced cellularity and mild loss of distinctness of collagen fibers (Hematoxylin and Eosin (HE)- 20×).

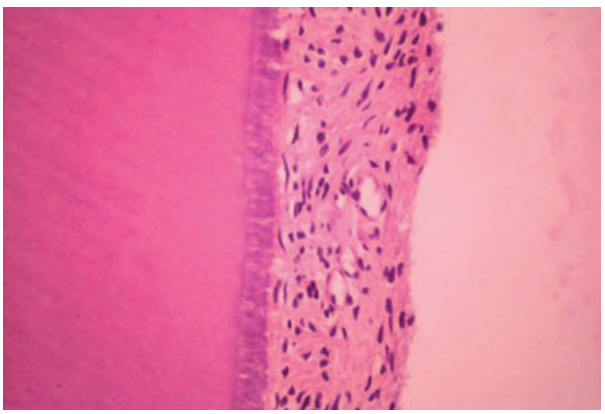

Fig. 2. Middle third – remnants of periodontal ligament adhered to the cementum. Reduced number of fibroblasts and collagen fibers around capillaries and venules (HE-20×).

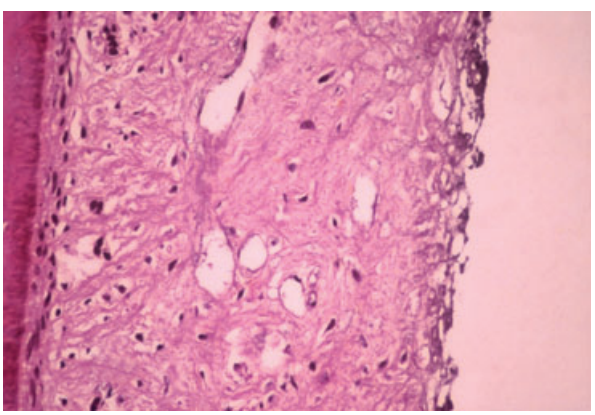

Fig. 3. Middle third – remnants of periodontal ligament adhered to the cementum. Expressive reduction in the number of fibroblasts, endothelial cells and important disorganization of collagen fibers (HE-20×).

Group IV – control
The teeth presented preserved the periodontal ligament. From the cementum, the bundles of collagen fibers were clear and perpendicularly directed. Following this, they were curved, involving bundles of capillaries and venules. The connective tissue presented a large number of cells, rich in fibroblasts, at all thirds analyzed (Fig. 4 – middle third), and the blood vessels presented clear endothelium.

Quantitative analysis

Tables 1–3 present the mean, standard deviation, number of specimens, ANOVA and the Tukey test related to quantitative evaluation of the number of cells per mm^2, in the periodontal ligament of each group, provided by the different storage media.

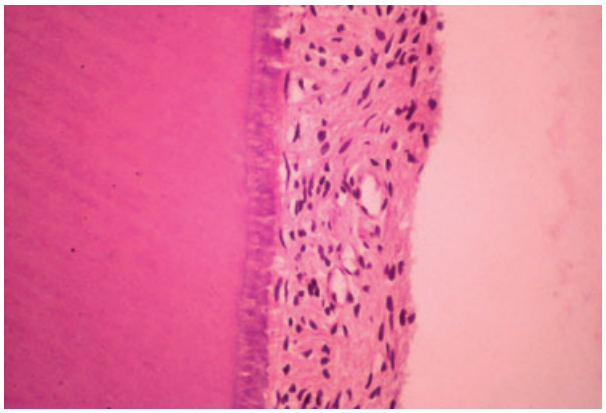

Fig. 4. Middle third – remnants of periodontal ligament adhered to the cementum. Preserved fibroblasts and endothelial cells. Presence of blood vessels with normal characteristics (HE-20×).

Table 1. Mean, standard deviation and number of samples related to quantitative evaluation of the number of cells per mm^2 of the periodontal ligament for each group, provided by the different storage media

Group	Mean	Standard deviation	Number
Group I	2227.13000	827.879000	30
Group II	2373.93000	767.887000	30
Group III	2140.30000	652.676000	30
Group IV	3330.57000	1216.331000	30

Discussion

This study was conducted on patients with indicated extraction of premolars for orthodontic purposes, in order to evaluate the microscopic aspect of the human periodontal ligament after storage in different media. Andreasen (1981) considers that procedures experimentally employed for extraction are not directly related to the trauma by avulsion, as the periodontal ligament suffers more damage during extraction than after avulsion (3). To assess the damages caused by extraction, a control group was used, in which the teeth were extracted and immediately immersed in 10% neutral formalin (group IV – control).

In the specimens in the control group, the bundles of collagen fibers were clear and organized. The periodontal ligament was preserved, exhibiting a large number of cells and blood vessels with normal characteristics.

According to the methodology employed in the present study, the results demonstrated differences between the microscopic events described in the periodontal ligament, when the tooth was stored in pasteurized bovine milk (group I) and in chicken egg white (group II) compared with artificial saliva (group III). Even though alterations were observed in the organization of bundles of collagen fibers, besides reduced cellularity when teeth were stored in artificial saliva, this difference was not statistically significant as to the number of cells per mm^2, between groups I, II and III, yet it was significant when compared with the control group.

The present results disagree with the findings of Patil et al. (18) and Lekic et al. (14), who employed a similar methodology, yet did not find statistically significant difference between the number of cells in the periodontal ligament of teeth stored in milk for 2 h and the control group.

In the opinion of Blomlöf & Otteskog (5) and Blomlöf et al. (7), the time does not influence the amount of cells in the periodontal ligament of monkeys' and humans' teeth, up to 6 h, when stored in bovine milk.

The results of descriptive microscopic analysis revealed that the periodontal ligament of teeth stored in chicken egg white (group II) presented clear bundles of collagen fibers as that stored in bovine milk. However, some specimens in the chicken egg white group displayed areas of disorganization. Fields with reduced cellularity were found at all thirds analyzed; however, there was no statistically significant difference when compared with groups I and III, but a statistically significant difference was observed when compared with the control group.

As stated by Carvalho Jr. (9), experiments on extracted human teeth stored in chicken egg white for more extended periods of time may indicate if this is a favorable or unfavorable medium.

Velasco-Bohórquez (20) measured the pH of chicken egg white and achieved a value of 9.38. For this investigator, the increase in root resorptions observed

Table 3. Individual comparisons, after application of the Tukey test, between specimens of different groups as to the quantitative evaluation of the cells in the periodontal ligament, provided by the different storage media

Comparison	Difference	Critical value	Significance
Group I × group II	-146.800	600.290	NS
Group I × group III	86.830	600.290	NS
Group I × group IV	-1103.430	600.290	Significant
Group II × group III	233.630	600.290	NS
Group II × group IV	-956.630	600.290	Significant
Group III × group IV	-1190.270	600.290	Significant

NS, non-significant.
Significance level: 5.0%.

Table 2. Analysis of variance related to quantitative evaluation of the cells in the periodontal ligament between groups, provided by the different storage media

Source of variation	Sum of squares	Degrees of freedom	Mean square	'*F*	Probability
Between groups	27 248 415.0	3	9 082 804.99	11.4232	$P = 0.00000128$
Residue	92 233 907.0	116	795 119.888		$P < 0.05$
Total	119 482 322	119			

in a study on rats' teeth after storage in chicken egg white for 2 h may be attributed to the pH, and also to the large amount of proteins that might act as foreign body.

The opinion that chicken egg white might be an ideal storage medium for avulsed teeth, when investigated, displays quantitative and qualitative outcomes that may interfere with the preservation of the vitality of cells in the periodontal ligament. The qualitative factor would be related to the shell, air chamber, white and yolk, which may be altered from formation to consumption of the egg. The possible influencing factors are feeding, age, care with the chickens, metabolism, acquired diseases and application of vaccines. Environmental factors, such as temperature, relative air humidity and care in storage also influence the quality of the egg (11).

Despite the lack of statistically significant difference in the number of cells between the artificial saliva group (group III), the bovine milk group (group I) and the chicken egg white group (group II), microscopically, there were larger areas of disorganization of bundles of collagen fibers and remarkable reduction in the number of cells when compared with groups I and II.

The results found in the present study are in agreement with the findings of Velasco-Bohórquez (20), who stated that artificial saliva might interfere with the organization of the bundles of collagen fibers. This could be attributed to the following: (i) the lesion in the periodontal ligament seems to be related to the components of saliva; (ii) it is a hypotonic solution with osmolality of 73.3 $mOsm^{-1}$ kg^{-1}; and (iii) it has a slightly acidic pH of 6.3. This pH is different from the pH of 7.1 observed in human saliva (6, 15).

In the present study, even though there was no statistically significant difference in the number of cells per mm^2 between the study groups, from a microscopic standpoint, descriptive analyses revealed alterations in the organization of bundles of collagen fibers in the periodontal ligament. Thus, it is implied that there was a difference in the quality of the periodontal ligament between groups whose teeth were stored in pasteurized bovine milk, chicken egg white and artificial saliva, and evidently in the control group.

Conclusion

Considering the present results and experimental conditions, it can be concluded that the quality of the periodontal ligament was affected by the storage media when compared with the control group. There was a statistically significant difference in the number of cells per mm^2 between the control group (group IV) and groups I, II and III, while there was no statistically significant difference in the number of cells per mm^2 between groups I, II and III.

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