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Delayed replantation of rat teeth after use of reconstituted powdered milk as a storage medium

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Abstract – Minimal extraoral dry storage period and moist storage for the avulsed tooth are identified as key steps for the treatment protocol of tooth replantation. Among the possible moist storage media, bovine milk has stood out because of its capacity of preserving the integrity of the periodontal ligament (PDL) fibers. This condition has attracted the attention to investigate the use of powdered milk, which is one of the presentation forms of bovine milk, as a feasible storage medium in cases of delayed tooth replantation. The aim of this study was to evaluate the healing process after delayed replantation of rat teeth stored in reconstituted powdered milk and long shelf-life (ultra high temperature) whole milk. Forty maxillary right rat incisors were assigned to four groups ($n = 10$): group I – the teeth were extracted and immediately replanted into theirs sockets; group II – the teeth were stored for 60 min in 200 ml of freshly reconstituted powdered milk; group III – the teeth were stored for 60 min in 200 ml of long shelf-life whole milk; group IV – the teeth were kept dry for the same time. All procedures were performed at room temperature. Next, the root canals of teeth in groups II, III, and IV were instrumented, filled with a calcium hydroxide-based paste, and replanted into their sockets. All animals received systemic antibiotic therapy and were killed by anesthetic overdose 60 days after replantation. The pieces containing the replanted teeth were removed, fixed, decalcified, and paraffin-embedded. Semi-serial 6-μm-thick sections were obtained and stained with hematoxylin and eosin for histomorphological analysis. There was statistically significant difference ($P < 0.05$) between groups I and IV regarding the presence of replacement resorption and PDL remnants on root surface. The powdered milk and long shelf-life whole milk presented similar results to each other and may be indicated as storage media for avulsed teeth.

In spite of its low frequency compared to other traumatic injuries, tooth avulsion is a reality among the events that can lead to tooth loss. Sporting activities, high-risk works as well as the increasingly widespread use of automotive vehicles have contributed to increase the incidence of this type of dental trauma. Tooth avulsion is a complex traumatic injury characterized by the rupture of the neurovascular bundle and periodontal ligament (PDL) exposing the tooth to the outer environment (1).

The best treatment option for an avulsed permanent tooth is its immediate replantation into the socket, even if the conditions are not so favorable (1). The maintenance of PDL vitality is extremely important for a good prognosis (2) because the presence of necrotic PDL remnants results in the development of root resorption, which may lead to loss of the replanted tooth (3, 4).

Therefore, it is generally well accepted that replantation is the treatment of choice for an avulsed tooth, which can yield, at least temporarily, the reestablishment of esthetics, and function (1). However, in spite of its recognized therapeutic value, in practice, immediate replantation rarely occurs due to factors such as the presence of extensive life-threatening injuries, complex damage to the recipient site or lack of knowledge of replantation procedures (3–5).

Under these conditions, the best results are obtained when the extra-alveolar time does not exceed 5 min (5, 6). The longer the exposure of an avulsed tooth to dry storage, the worse the prognosis of replantation. In order to minimize the adverse effects of avulsion, various storage media have been investigated for their ability to maintain the cells viable, and are considered crucial for preservation of PDL cell vitality. In fact, the ability of the storage/transport medium to support cell viability may be even more important to tooth replantation than the extraoral period (3, 7, 8).

The storage medium should be capable of preserving cell vitality, adherence capacity and clonogenicity (9), and should be readily available at the moment of avulsion to allow its rapid access. It should have

physiologically compatible pH and osmolarity and should not cause any cell damage (10–12). Among the several types of studied storage media are Viaspan, Hank's solution (13, 14), tap water (15), saliva (4), saline (16), and bovine milk (4, 17, 18).

Tap water is considered unsuitable because it is hypotonic and causes rapid cell lysis (15). Although saliva is more effective than tap water, it is more hypotonic than milk and contains bacteria thus being a potential source for bacterial contamination (19). Saline yields better results than saliva and can maintain PDL vitality for up to 30 min (16). Bovine milk, in turn, has produced good results in previous studies (8, 11, 19) with reports of up to 6 h of storage (18). Although other storage media, like Viaspan and Hank's solution, can maintain PDL cell integrity for much longer periods (13, 18), the advantage of milk is its availability at scene of an accident.

Among the forms of presentation of bovine milk, the regular pasteurized milk has the advantage of undergoing a pasteurization process for bacterial elimination. However, it has a shorter shelf-life and requires refrigeration (7). The long shelf-life (ultra high temperature; UHT) whole milk has the same advantages as those of the regular pasteurized milk, but it has an extended shelf-life and does not need special storage conditions such as refrigeration (7). Several *in vitro* (4, 9, 11, 13, 19) and *in vivo* (18, 20, 21) studies have demonstrated the feasibility of using whole bovine milk as an interim storage/transport medium for avulsed teeth. More recently, an *in vitro* study has demonstrated that powdered milk may be a favorable storage medium with respect to the maintenance of viable PDL cells (8).

Therefore, it is opportune to investigate this product *in vivo*, simulating the clinical conditions of tooth replantation. The trauma resulting from extraction, the possible contamination of the root surface and tooth handling during root canal filling, and tooth repositioning into the socket are aspects found clinically in cases of replantation that could interfere with the results. Therefore, the aim of this study is to evaluate the healing process after replantation of rat teeth stored in reconstituted powdered milk and long shelf-life whole milk.

Material and methods

The research proposal was reviewed by the Ethics in Animal Research Committee of the School of Dentistry of Araçatuba (São Paulo State University, Brazil) and the study design was approved. Forty male Wistar rats (*Rattus norvegicus albinus*), weighing 250–300 g were used in this study. The rats were obtained and maintained in animal care facility of the School of Dentistry of Araçatuba (São Paulo State University, Brazil) and were fed ground solid ration and water *ad libitum*.

The animals received an intramuscular injection of xylazine chloride (Anasedan; AgriBrands do Brasil Ltda., Jacareí, SP, Brazil; 0.03 ml 100 g^{-1} body weight) to attain muscular relaxation and were then anesthetized with ketamine chloride (Dopalen; AgriBrands do Brasil Ltda.) at a dose of 0.07 ml 100 g^{-1} body weight. After anesthesia, asepsis of the anterior maxilla was performed with iodine polyvinylpyrrolidone (Riodeine; Ind. Farmacêutica Rioquímica Ltda., Rio de Janeiro, RJ, Brazil), followed by syndesmotomy, luxation and non-traumatic extraction of the maxillary right incisor of all animals using custom-made instruments (22).

The extracted teeth were randomly assigned to four groups, according to the following treatments: group I – the teeth were immediately replanted into their sockets without root canal therapy; group II – the teeth were immersed in 200 ml of freshly reconstituted powdered milk (Leite Ninho; Nestlé Brasil Ltda, Ibiá, MG, Brazil) prepared according to the manufacturer's instructions (2 full tablespoons dissolved in 200 ml of previously boiled tap water); group III – the teeth were immersed in 200 ml of UHT whole milk (Parmalat Brasil S.A. Indústria de Alimentos, Sta Helena de Goiás, GO, Brazil); group IV – the teeth were kept dry on a workbench. Except for group I, in which the extraoral time was < 1 min, all teeth were maintained in their respective storage media for 60 min at room temperature (22°C).

The extracted teeth of groups II, III, and IV had their dental papilla and enamel organ removed with a #15 scalpel blade (Embramac Exp. e Imp., Campinas, SP, Brazil) and the pulp tissue was extirpated through a retrograde route with a slightly curved #35 Hedstrom file (25 mm; Sybron Kerr Corporation, Orange, CA, USA). The canals were irrigated with saline (Ariston Ind. Quím. e Farm. Ltda, São Paulo, SP, Brazil), fluid aspiration was performed with a 25×6 needle coupled to a disposable syringe, and the root canals were dried with absorbent paper points (Dentsply Ind. e Com. Ltda., Petrópolis, RJ, Brazil).

Next, the root canals were filled with a calcium hydroxide (Calcium Hydroxid Fur Analyse, Criedel, De Rainag Seelge, Hannover, Germany) and propyleneglycol-based paste via retrograde route using lentulo drills (25 mm; Sybron Kerr) at low speed. Thereafter, the teeth were replanted in their respective sockets after alveolar curettage and irrigation with saline. No type of retention was used.

After replantation, all animals received a single intramuscular dose of benzathine G penicillin 20 000 IU (Fontoura Wyeth S.A., São Paulo, SP, Brazil). Sixty days after replantation, the rats were sacrificed by anesthetic overdose with sodium thiopental (Cristália Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil). The right maxilla containing the replanted tooth was separated from the left maxilla at the midline using a #15 scalpel blade (Embramac Exp. e Imp.). A cut with straight scissors was made on the region distal to the third molar and the pieces containing the replanted teeth were removed, fixed in 10% formalin for 24 h, and decalcified in 4.13% EDTA solution, pH 7.0. After decalcification, the specimens were embedded in paraffin in a horizontal position in such a way that the cuts were initiated in the mesial surface of the tooth. Longitudinal semi-serial 6-μm-thick sections were obtained (one microscopic slide per specimen) and stained with hematoxylin and eosin for histologic and histomorphometric analyses.

The middle third was used for histomorphometric analysis. For measurement of the root area affected by

the resorption process, the images of the longitudinal root sections were divided into three thirds (cervical, middle and apical) using a compass, a ruler, and a fine pen. The middle third was selected for the measurements because this region is not damaged by the surgical procedures. The cervical and apical thirds, on the other hand, are affected by the grasping action of the forceps and cutting action of the scalpel blade during tooth extraction and dental papilla removal, respectively.

Images were captured using a digital video camera (JVC TK-1270 Color Video Camera, Tokyo, Japan) coupled to a Carl Zeiss microscope (Axiolab, Thornwood, NY, USA) and connected to a microcomputer and using Microsoft VidCap video capture software (Microsoft Corp., Redmond, WA, USA). Two image captures (720 × 480 pixels) were made to cover the entire middle third. These images were first recorded as figures (TIF 24) and further joined using an image-editing software (Corel Photo Paint 10; Corel Corporation, Ottawa, ON, Canada). ImageLab 2001 software (Diracom Bio Informática, Vargem Grande do Sul, SP, Brazil) was used for delimitation of the areas of root resorption. The percentage of resorbed dentin was calculated from the dentin area delimited along the entire third, according to each type of root resorption. For analysis of the complete repair (intact cementum and PDL) and the areas of ankylosis, the root surface perimeter corresponding to each type of repair was considered. The obtained data were recorded in pixels and entered in the Excel software (Microsoft Corp.) for statistical analysis.

Statistical analysis

For the analysis of the complete repair, the data normality was verified. The significance of differences between groups in relation to this parameter was determined by an ANOVA, followed by a *post hoc* Tukey's test when the ANOVA suggested a significant difference between groups ($P < 0.05$).

In order to evaluate the ankylosis as well as the inflammatory, surface, and replacement resorption, the data were transformed into scores. This transformation was made in order to allow the use of an appropriate statistical method as many specimens presented no root resorption or ankylosis, and therefore, their values were zero. For statistical analysis, scores from 1 to 4 were attributed to surface resorption, inflammatory resorption, replacement resorption and ankylosis, one being the best result, four being the worst result, and two and three occupying intermediate positions. *Root resorption area*: the areas of inflammatory and replacement root resorption were measured in representative slides. A 4-point scoring system was used, as follows: 1 – no resorption; 2 – 0.1–50% of the area with resorption; 3 – 51–99% of the area with resorption; 4 – 100% of the area with resorption. For *ankylosed area*: the perimeter of the ankylosed areas was measured in representative slides. A 4-point scoring system was used, as follows: 1 – absence of ankylosis; 2 – small areas of ankylosis; 3 – 1/3 of the root is ankylosed; 4 – > 1/3 of the root is ankylosed.

Data obtained in the different groups were analyzed statistically by Kruskal–Wallis test and multiple comparisons were done by Dunn's test. Significance level was set at $\alpha = 5\%$.

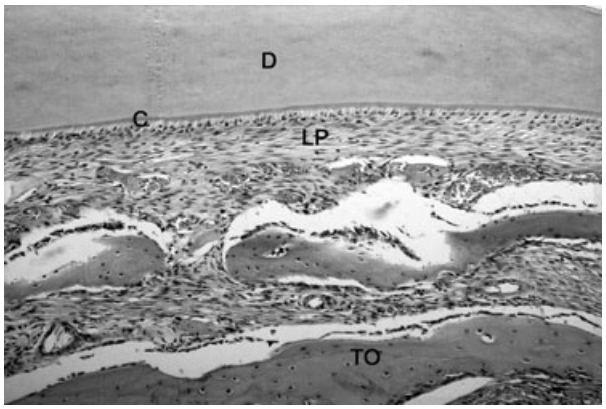

Fig. 1. Group I (immediate replantation) – root surface with intact dentin (D) and cementum (C) covered by periodontal ligament (PDL). Bone tissue (BT). H.E. 63×.

Results

The animals endured adequately all experimental procedures. During the course of the study, only one tooth was lost (group 1 – immediate replantation). The results of group I were characterized by the great extension of non-resorbed root surface, in which the cementum was intact and covered by a connective tissue rich in collagen fibers and fibroblasts (Fig. 1). This repair pattern was present in 89.4% of the analyzed area. In some areas, the fibers were arranged parallel to the root surface, while in many others, the oblique fiber arrangement suggested reattachment to root surface. In five specimens, these characteristics were present throughout the extension of the analyzed area. Inflammatory root resorption was not present and in only one specimen a small ankylosed area was observed, characterized by the presence of bone tissue juxtaposed to the cemental surface. Replacement resorption was not a frequent event, being observed in only three specimens, with shallow involvement of root surface. In the affected areas, the resorbed cementodentinal tissue was replaced by bone tissue. In some cases, resorption cells were close to dentin.

In group II, a large intact root surface area was also observed (71.7%) (Fig. 2). In seven specimens, this repair pattern was present in an extension > 80% of the analyzed area. Some regions of more superficial root resorption were found in four specimens. In these points, the resorbed dentin was repaired by a cementum-like mineralized tissue which, in turn, was covered by connective tissue (Fig. 3). Inflammatory root resorption was present in five specimens of this group, in which the resorbed areas were filled with connective tissue exhibiting an inflammatory cell infiltrated with lymphocytes, histiocytes, and, in some cases, neutrophils. Ankylosed areas were not found, but replacement resorption was observed in six specimens. As observed in group 1, this group also exhibited small-sized and shallow resorbed areas.

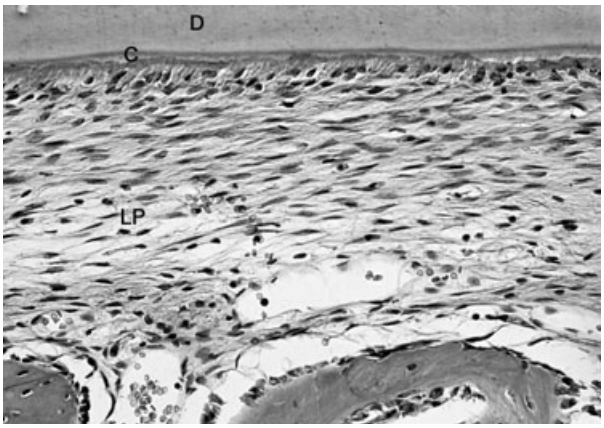

Fig. 2. Group II (powdered milk). Root surface with intact cementum (C) layer covered by periodontal ligament (PDL). Note the oblique fiber arrangement suggesting reattachment. Dentin (D). H.E. 160×.

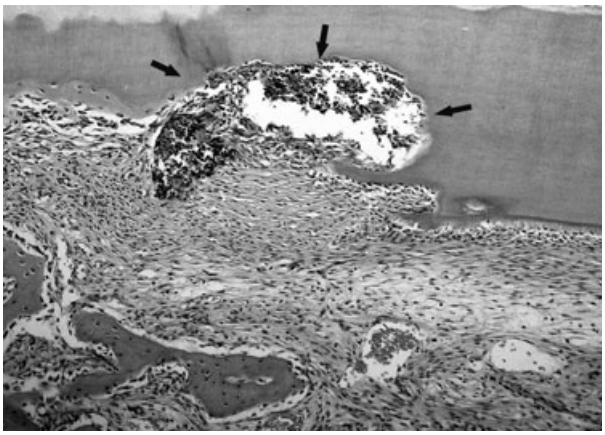

Fig. 4. Group III [long shelf-life (UHT) milk] – area of inflammatory root resorption (arrow). Inflammatory infiltrate present in the connective tissue of the resorbed area – H.E. 63×.

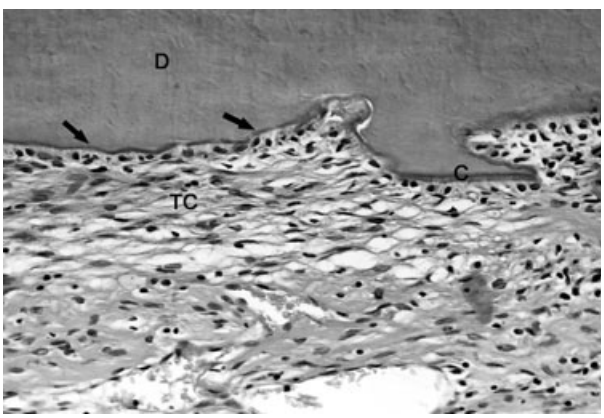

Fig. 3 Group II (powdered milk). Area of root resorption repaired by cementum-like mineralized tissue (arrow). The collagen fiber bundles of the connective tissue are arranged parallel to the root surface – H.E. 160×.

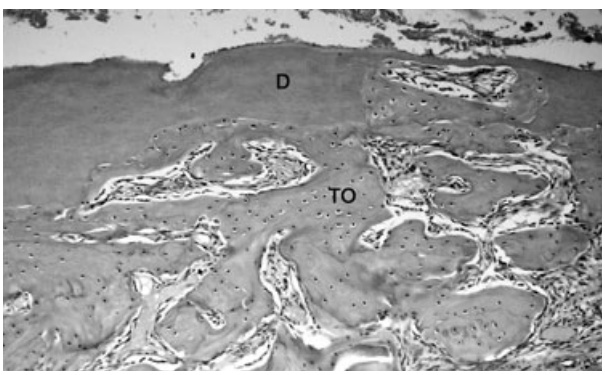

Fig. 5. Group IV (dry storage). Extensive and deep root surface area resorbed and replaced by bone tissue (BT). Dentin (D). H.E. 63×.

In group III, the integrity of root surface was observed in 62.7% of the analyzed area, in the same way as observed in group II. There were extensive areas of complete healing in six specimens. No ankylosed areas were observed and few cases of inflammatory resorption were identified (Fig. 4), being present in only three specimens in small-sized and shallow areas. Although this group presented more cases of replacement resorption (eight) in comparison to groups I and II, the resorbed areas were neither extensive nor deep.

In group IV, root surface was more severely affected by resorption, but few cases of ankylosis were observed. Some resorbed areas were repaired by a connective tissue rich in ill-organized collagen fibers and fibroblasts. The complete repair were found in 51.0% of the root surface. In most specimens, the resorbed areas were filled with bone tissue and, in several points, resorption cells were found close to dentin. In some cases, almost the entire dentinal structure was replaced by bone tissue (Fig. 5). Few areas of ankylosis were found and inflammatory resorption was identified in only two specimens, though involving a great extension of dentinal tissue.

Table 1. Number of teeth, mean (in pixels) and standard deviations among the groups regarding the complete repair

Groups	No. teeth	Mean (SD)
Immediate	9	969.69 (222.28)
Powdered milk	10	476.49 (179.23)
UHT milk	10	443.74 (201.69)
Dry storage	10	351.83 (239.78)

Statistical analysis

Table 1 depicts the number of teeth, means, and standard deviations among the groups regarding the occurrence of complete repair. In this case, there was statistically significant difference ($P < 0.05$) between groups I (immediate replantation) and II (powdered milk); groups I and III (UHT milk); groups I and IV (delayed replantation) (Table 2).

Table 2. Tukey test for individual comparisons among the four groups regarding the complete repair

Comparison	*P* value*
Immediate × powdered milk	<0.05
Immediate × UHT milk	<0.05
Immediate × dry storage	<0.05
Dry storage × powdered milk	>0.05
Dry storage × UHT milk	>0.05
UHT milk × powdered milk	>0.05

*Statistically significant at 5%.

There was no statistically significant difference ($P < 0.05$) among the groups with respect to inflammatory root resorption, surface root resorption and ankylosis (Table 3). Regarding replacement resorption, there was statistically significant difference ($P < 0.05$) only between groups I (immediate replantation) and IV (dry storage + delayed replantation).

Discussion

In this study, in order to investigate the repair process in replanted teeth, the middle root third was selected, and standardized because the cervical and apical thirds may be affected by the surgical (23, 24) and endodontic (24) procedures, respectively, which could interfere with the results.

In a replanted tooth, the maintenance of the viability of the cemental PDL and cementoblasts is mandatory to the occurrence of fiber reattachment under normal conditions (25). When these structures are damaged due to trauma or desiccation of root surface, three types of resorption might occur. If the damaged area is small, there may be surface resorption, which occurs in the absence of a significant inflammatory process. In case of contamination of the area, inflammatory resorption may develop (15). In the absence of contaminants and if the lesion involves a great extension of root surface there may be ankylosis or replacement resorption (25).

In the present study, the low inflammatory resorption rates observed in all groups are consistent with the findings of previous studies (26) and demonstrates that the protocol adopted for controlling this type of resorption, comprising endodontic treatment, calcium hydroxide-based intracanal dressing (3, 16, 27), and systemic antibiotic therapy (15) was effective.

In the group in which root canal therapy was not performed, the good results are justified by the accomplishment of immediate replantation. This type of replantation preserves the PDL vitality (3) and, in teeth with large foramina, allows pulp tissue revascularization (5, 28), which reduces the influence of a necrotic pulp tissue or degenerated PDL acting as stimuli to inflammatory resorption (3). Even the lack of excessive extraoral handling of the teeth during endodontic treatment might have contributed to the low ankylosis, and replacement resorption rates observed in this group (3, 24). The histological findings confirmed that immediate replantation is the best treatment option as the extraoral time is minimum and no storage medium is required (3, 5).

In the other groups, storage time of 60 min was employed to simulate the most commonly observed condition in clinical practice with respect to the extra-alveolar time of replanted teeth (5, 29). It was observed that a long dry storage time as that used in the present study (group IV) is deleterious to the avulsed tooth, which is in accordance with the results of studies published elsewhere (3, 5, 18). Replacement root resorption was more frequent in this group. This relationship has been described and demonstrates that PDL repair depends on the existence of viable cell remnants adjacent to the injured area (3). In this case, the vitality of the cell layer closest to the cementum, the cementoblasts, plays a key role on the repair process (30). Compared to dry storage, storage in milk produced conditions that allowed these cells being less affected by the external medium, as previously reported (4). The properties such as physiologically compatible pH (31, 32) and osmolarity (19, 33), presence of nutrients (9, 11), and growth factors (8) provide to bovine milk characteristics that make it an adequate interim storage medium for exarticulated teeth.

The findings of the present study regarding the use of long shelf-life whole milk differed from those of a previous study using monkey's teeth (20). In that study, the repair by PDL fiber reattachment occurred in 92% of the analyzed root surface areas of the teeth stored in whole milk for 1 h and replanted thereafter. When the storage time was increased to 3 h, this type of repair was observed in 85% of the analyzed root surface areas (20). Another study using storage times of 2 and 6 h in whole milk reported repair by PDL fiber reattachment in 90% and 79% of the areas, respectively (21). The higher percent values recorded in those studies compared to the present experiment may be attributed to the type of

Table 3. Frequency of scores among the groups regarding the inflammatory resorption, replacement resorption, surface resorption and ankylosis

	Inflammatory resorption				Replacement resorption				Surface resorption				Ankylosis			
Scores	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3	G4
1	9	5	3	8	6	4	2	2	9	6	8	10	8	10	10	10
2		5	7		3	6	8	5		4	2		1			
3				2				3								
4																
N	9	10	10	10	9	10	10	10	9	10	10	10	9	10	10	10

G1, group 1; G2, group 2; G3, group 3; G4, group 4.

animal model used. As the rat has a more accelerated metabolism than that of the monkey (34), its PDL could be more vulnerable to storage medium and time variations.

Regarding the powdered milk, Pearson et al. (8), in a recent *in vitro* study to determine the efficacy of several milk substitutes compared to whole milk in maintaining the viability of human PDL cell cultures, have demonstrated that reconstituted powdered milk and whole milk had similar capacity of preserving PDL cell viability after 1-h storage. Likewise, in the present study, there was no statistically significant difference between both types of milk used as storage media. Even though, it was observed that the powdered milk preserved the integrity of root surface in 72% of the analyzed area. When the avulsed teeth were stored in whole milk or kept dry, this value shifted to 62% and 51%, respectively.

Although it is acknowledged that more favorable results could be obtained using milk at a temperature close to 4°C (18, 31), the rationale for using the milk at room temperature was that refrigeration is not always available at the site of accident. On the other hand, knowing that PDL repair occurs within 2–4 weeks after replantation (35), the values reached 60 days after replantation suggest the feasibility of using reconstituted powdered milk as a storage medium as, even with immediate replantation, the percent intact (non-resorbed) root surface area was 89.40%. It should also be mentioned that the powdered milk has the same characteristics as that of the long shelf-life (UHT) whole milk, as it is a lyophilized form of this product. Additionally, precautions, such as the use of boiled water for reconstitution of powdered milk, minimize the likelihood of bacterial contamination. This product has an extended shelf-life, ease of transportation, and does not require any special conservation like refrigeration, which are important aspects to be considered as far as the accessibility and availability at the scene of an accident are concerned.

Conclusions

Under the tested experimental conditions and according to the analytical criteria adopted in this study it may concluded that:

- The reconstituted powdered milk and long shelf-life (UHT) whole milk presented similar results to each other, and may thus be used as interim storage media for avulsed teeth.
- Immediate tooth replantation yielded the best results and should be the treatment of choice, whenever possible.
- The maintenance of avulsed teeth in dry storage for periods as long as 1 h should be avoided to prevent unfavorable results regarding the incidence of root resorption after replantation.

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