

Histologic evaluation of apical pulp of immature apex following extraction, surface treatment, and replantation in different storage media in dogs

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Abstract – Aim: This study was designed to evaluate the apical pulp of open apex teeth following extraction, surface treatment, and preservation in different storage media in dogs. **Materials and methods:** Four healthy male dogs were included in this prospective animal study. In the first phase of extraction, 30 teeth were extracted atraumatically from dogs (incisors and premolars) and then randomly divided into three groups based on different types of storage media: (i) milk, (ii) HBSS, and (iii) dry storage. In milk and HBSS groups, teeth were contaminated by dog's saliva and then kept in milk and HBSS solution, respectively, for 2 h. In the third group, teeth were stored in a dry storage for 2 h. Before replantation, all teeth were soaked in 10% doxycycline. After 2 months, in the second phase of extraction, all experimental teeth were extracted (40 teeth including control group). Three longitudinal sections (3–4 mm thickness) were stained with H&E and investigated under optical microscope. Histologic evaluations were performed, and data were analyzed using Kruskal–Wallis and Fisher's exact test ($\alpha = 0.05$). **Results:** There was a significant difference between the milk and control groups regarding the presence of the odontoblastic layer and resorption ($P = 0.04$). Also, there was a significant difference between dry storage and control group in the presence of the odontoblastic layer ($P = 0.02$). There was no statistically significant difference in other histologic factors between different groups ($P > 0.05$). It was revealed that there is no significant difference between different groups regarding the intensity ($P = 0.06$) and the type of the inflammation ($P = 0.24$). Also, the hyperemia showed no significant difference between different groups ($P = 0.51$). **Conclusion:** It was concluded that HBSS and milk are appropriate storage media. Also, it was shown that 2 h dry storage probably does not threaten the vitality of the pulp cells.

Avulsion is a common traumatic event which is characterized by 'complete displacement of a tooth out of its socket' (1). It has been shown that the resilient structure of the surrounding bone does not resist extrusive forces, and (2) this may explain the high incidence of maxillary centrals avulsion in 7- to 14- old children (3, 4).

The vitality of the PDL cells, regenerative capacity of dental pulp cells, and prognosis of the avulsed teeth depend on different factors. The width of the root canal, extra oral time, chemical properties of storage media, and management of the teeth before replantation are factors that affect the prognosis of the avulsed teeth. Petrovic et al. (5) confirmed that combination of

delayed replantation and unfavorable storage media threatens the prognosis of the avulsed teeth.

An ideal storage media should preserve the viability, mitogenicity, and clonogenic capacity of the damaged PDL cells (6). Various storage media have been suggested in the literature during the extra oral time. Milk, Hank's balanced salt solution (HBSS), saliva, saline solution, and many other media were evaluated and used with different degree of success (7). There is a consensus among clinicians that the ability of the storage media to support cells vitality is more important than the extra oral time in preventing root resorption (8).

Following avulsion, the neurovascular supply is severed and the periodontal ligament is ruptured. In the best condition, pulp will be regenerated and the healthy PDL cells will be reorganized (9). Pulp infection and necrosis are common after avulsion events. The consequence of pulpal necrosis is more serious in immature teeth as it halts the normal process of root development, which results in open apex teeth with thin dentinal walls.

Pulp revascularization is an ideal outcome of tooth avulsion. Following the pulp revascularization, the healthy pulp prevents the subsequent infection–resorption cycle and ensures root development which leads to a thick dentinal wall, which is resistant to fracture (9). It has been confirmed that pulp revascularization ability is favored in open apex teeth, preserved in an appropriate storage media with <45 min extra oral time (10, 11). Also, Cvek et al. (12, 13) demonstrated that the presence or absence of the microorganism in the pulpal lumen affects the pulp revascularization ability. Even under optimum conditions including short extra oral time and the absence of gross contamination, pulp necrosis is expected in 40% of replanted avulsed teeth (13, 14). Consequently, it was suggested that microorganisms' penetration into the pulp canal space prevents the pulp revascularization. Therefore, antibiotics were proposed to enhance the pulp regeneration ability. Cvek (13) suggested that the rate of the microorganism and inflammatory reactions can be diminished by topical treatment of the exposed root with doxycycline before replantation.

Despite numerous studies regarding the efficacy of different storage media in preserving the pulp vitality, none of the currently used media is ideal (7). As most published studies have investigated the effect of different storage media on PDL cells vitality (7, 15, 16) and no study has evaluated the apical pulp of open apex teeth, this study was designed to histologically assess the apical pulp of open apex teeth following extraction, preservation in different storage media, and surface treatment with doxycycline in dogs.

Materials and methods

This experimental prospective study was approved by the animal department of Torabinejad Dental Research Center and local ethical committee of Isfahan University of Medical Science (#390044).

Four Iranian healthy male dogs aged 7–9 months and weighing 10–15 kg were selected. As dogs were in the intertransitory period of the mixed dentition, periodic radiographs were taken to evaluate the root length of permanent teeth. Finally, 40 open apex permanent teeth with more than 1- to 2- mm apical foramen diameter, without caries or periodontal problems, and completion of 1/2 to 2/3 of roots length were included. In this study, 10 teeth were extracted in each dog (three incisors, first premolar, and second premolar in each jaw). It was decided to keep the canine and carnassial teeth, which are essential for their nutrition.

Under aseptic condition, dogs were sedated with 0.02 mg kg⁻¹ Acepromazine and then 10 mg kg⁻¹ Ketamine (Ketamine HCL; Alfasan, Woerden, Holland),

and 2 mg kg⁻¹ xylazine injections were used to anesthetize dogs. Dogs were maintained under general anesthesia using 5% Halothane (Halothane, Bp; Nicholas Piramal India Limited, India) and N₂O.

After the general anesthesia, 30 teeth were randomly extracted atraumatically in the first phase of extraction and randomly divided into three groups (10 teeth in each group), and 10 remained teeth were extracted in the second phase of extraction as control group:

- 1 Milk
- 2 HBSS
- 3 Dry storage media (gauze pack)

In the milk and HBSS groups, teeth were contaminated by dog's saliva and then kept in milk (Pakco, Tehran, Iran) and HBSS solution (Save-A-Tooth; Merck co, Darmstadt, Germany), respectively, for 2 h. Then, teeth were soaked in 10% doxycycline solution (10 capsules in which each contain 100 mg doxycycline were mixed with 10 cc distilled water in a dark room) for 10 min. Teeth were repositioned and splinted by a flexible wire (≠06) and resin composite (Kerr, Orange, CA, USA).

In the third group, teeth were stored in room environment in a gauze pack for 2 h and then were soaked in 10% doxycycline for 10 min after being contaminated by dog's saliva. Teeth were replanted and splinted like other groups.

Animals were fed on soft diets following the surgery. Penicillin–streptomycin vials were injected two times a day for 3 days to prevent any postoperative infections. The splints were removed after 1 week, and dogs were monitored for 2 months to minimize any potential trauma to the replanted teeth.

After 2 months, in the second phase of the extraction, all included teeth (40 teeth including control group) were atraumatically luxated by an elevator and extracted by a proper forceps. The control teeth were evaluated without any interventions. Then, all teeth were soaked in 10% formalin and cells fixed for 24 h. After 12 weeks, all specimens were calcified using EDTA. Three longitudinal sections (3–4 mm thickness) were prepared for each tooth by paraffin blocks. The paraffin sections were processed and stained by hematoxylin and eosin (H&E) and then were investigated blindly under an optical microscope (×100, ×40; Nikon E400, Tokyo, Japan) by an oral pathologist. Teeth preparation flowchart is summarized in Fig. 1.

Histologic surveys

Histologic evaluations were performed in three different categories:

- A Cellular inflammatory reaction, including the type and intensity of the inflammation.
- B Pulpal soft tissue changes, including hyperemia, necrosis, and odontoblastic layer condition.
- C Pulpal hard tissue changes (dentinal bridge formation), including location, continuity, and thickness of the dentinal bridge.

The evaluation was performed based on the following scales:

- A Inflammation:

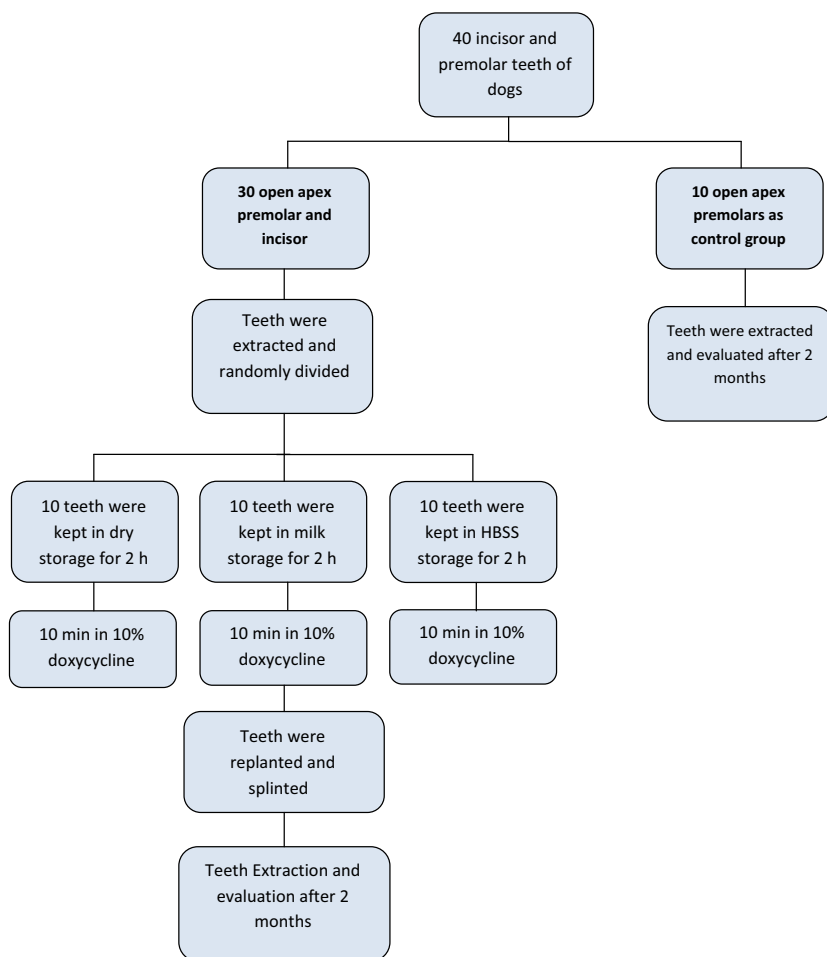


Fig. 1. Flowchart of experimental and control groups.

- a) Type:
 - 0) Without inflammation
 - 1) Acute inflammation
 - 2) Chronic inflammation
 - 3) Combination of acute and chronic inflammation
- b) Intensity:
 - 1) 0–30 inflammatory cells (mild)
 - 2) 30–60 inflammatory cells (moderate)
 - 3) More than 60 inflammatory cells (severe)
- B Soft tissue changes:
 - a) Hyperemia:
 - 1) 1–3 blood vessels (mild)
 - 2) 3–5 blood vessels (moderate)
 - 3) More than 5 blood vessels (severe)
 - b) Necrosis:
 - 0) Without necrosis
 - 1) Sign of necrosis
 - c) Odontoblastic layer cell:
 - 0) No odontoblastic layer cells
 - 1) Presence of odontoblastic layer cells
- C Calcification:
 - 0) No cell calcification
 - 1) Presence of calcification
- D Resorption:
 - 0) No resorption

- 1) Presence of resorption
- E Dentin thickening:
 - 0) No thickening
 - 1) Thickening

Statistical analysis of the results was performed using Kruskal–Wallis and Fisher's exact test by SPSS software version 15 (SPSS Inc, Chicago, IL, USA) ($\alpha = 0.05$).

Results

From the 40 included teeth, 6 teeth were damaged during extraction or section preparation and discarded from the study. Finally, six teeth in milk, 10 teeth in HBSS, eight teeth in dry storage, and 10 teeth in control groups remained for histologic evaluations.

The photomicrographs of teeth in different groups are presented in Fig. 2. The distribution of the histologic observations in different groups has been shown in Table 1 (qualitative–nominal variable).

As the frequency of total data is <40, Fisher's exact test was performed for qualitative variables. Table 2 shows the results of Fisher's exact test between different groups. Based on Table 2, there is a significant difference between the milk and control groups regarding the presence of the odontoblastic layer and resorption

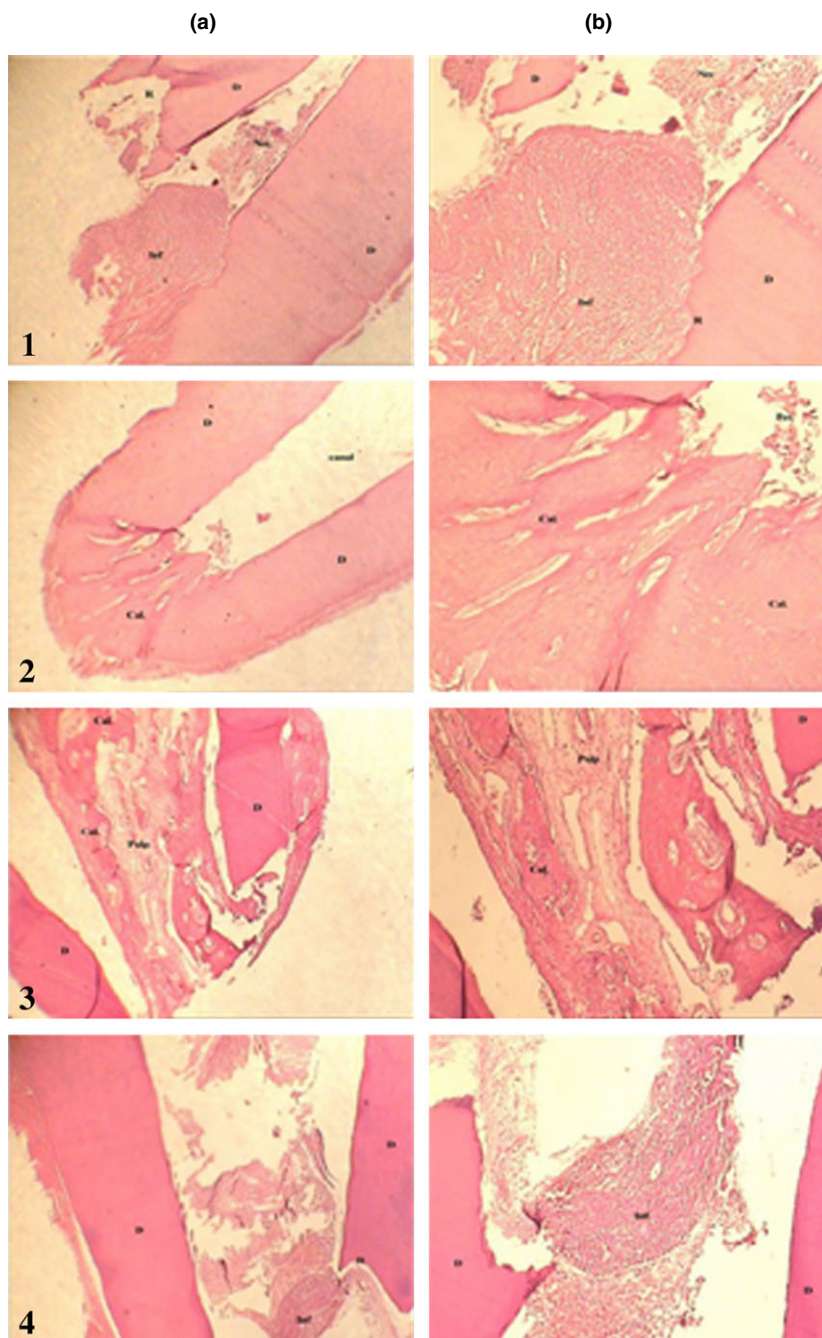


Fig. 2. Photomicrographs of open apex teeth in milk media (1), control group (2), HBSS media (3), and dry storage media (4). H&E; original magnification (a) $\times 40$ (b) $\times 100$ (D: dentin, Cal: calcification, R: resorption, Inf: inflammation).

($P = 0.04$). The odontoblastic layer was observed in all specimens of the control group, and there was no sign of resorption in the control group. Also, there was a significant difference between dry storage and control groups in the presence of the odontoblastic layer ($P = 0.02$). Other histologic factors showed no statistically significant differences between different groups ($P > 0.05$).

Table 3 shows the distribution of the histologic changes in different groups (qualitative–ordinal variable). The Kruskal–Wallis test was performed to analyze the ordinal variables. It was revealed that there is no significant difference between different groups regarding the intensity, type of the inflammation, and hyperemia ($P = 0.06$, $P = 0.24$, and $P = 0.51$, respectively).

Discussion

Properties of different storage media can affect the rate of root resorption and pulp healing (17, 18). Milk, tap water, HBSS, saliva, isotonic saline, and egg white have been suggested to preserve the avulsed teeth until the replantation (15, 19). Our study revealed that there is no significant difference in most of the histologic observations between different groups.

During the study, six teeth were discarded as they were damaged during the extraction or section preparation. We investigated the effects of milk and HBSS storage media on the vitality of pulp cells. Jabarifar et al. (20) confirmed that the reproducibility of the

Table 1. Distribution of the histologic observations in different groups (*N* = number of teeth in each group)

Storage media	HBSS	Milk	Dry storage	Control
Histologic criteria				
Presence of calcification	<i>N</i> = 3	<i>N</i> = 2	<i>N</i> = 3	<i>N</i> = 6
Presence of resorption	<i>N</i> = 3	<i>N</i> = 3	<i>N</i> = 3	0
Presence of dentin thickening	<i>N</i> = 6	<i>N</i> = 3	<i>N</i> = 5	<i>N</i> = 8
Presence of odontoblastic layer	<i>N</i> = 8	<i>N</i> = 3	<i>N</i> = 4	<i>N</i> = 9
Presence of necrosis	<i>N</i> = 2	<i>N</i> = 2	<i>N</i> = 2	0

pulp cells is comparable in milk and HBSS storage media after 1, 3, and 6 h. The superiority of the milk storage media over saliva, HBSS, and normal saline, in preserving the vitality of periodontal ligament cells, has been shown in different studies (21, 22). Patel et al. (23) found no significant difference in the vitality of the cells remaining on the root surface, between teeth kept in milk and saline storage media. Huang et al. (24) concluded that milk is an appropriate short-term storage media. Also, it was suggested that cold milk might increase the recovery rate and the vitality of the cells (25–27). In the present study, there was no significant difference in the histologic findings between milk and HBSS groups. For instance, the rate of resorption and necrosis was comparable between two groups. It has been shown that milk demonstrates superior physiological properties including PH and osmolality over other storage media (21, 23). Also, desirable histologic responses in the milk group can be attributed to the nutritional substances such as amino acids, carbohydrates, and vitamins in the milk (6, 21, 28–30). It should be mentioned that the milk used in the present study was pasteurized which decreases the potentially harmful bacteria (6, 29) and enzymes (6, 21). However, the number of teeth in the milk group was less than other groups as some of them were excluded from the study following injuries during extraction or preparation, and this could have affected the result of the study.

There was no significant difference in the evaluated histologic factors between HBSS and control groups in this study. HBSS is recommended by the American Association of Endodontists as an ideal storage media for preserving avulsed teeth (31). The superiority of HBSS over milk and water in preserving cells vitality as an ideal storage media has been confirmed (7, 15, 32). Also, it was proposed that the storage time may

Table 3. Distribution of the histologic observations in different groups (qualitative–ordinal variable)

Storage media	HBSS	Milk	Dry storage	Control
Histologic surveys				
Hyperemia	Mild: <i>N</i> = 7	Mild: <i>N</i> = 4	Mild: <i>N</i> = 4	Mild: <i>N</i> = 8
	Moderate: <i>N</i> = 3	Moderate: <i>N</i> = 1	Moderate: <i>N</i> = 3	Severe: <i>N</i> = 1
	Severe: <i>N</i> = 1	Severe: <i>N</i> = 1	Severe: <i>N</i> = 1	
Intensity of inflammation	Mild: <i>N</i> = 8	Mild: <i>N</i> = 2	Mild: <i>N</i> = 4	Mild: <i>N</i> = 9
	Moderate: <i>N</i> = 1	Moderate: <i>N</i> = 4	Moderate: <i>N</i> = 2	
	Severe: <i>N</i> = 2		Severe: <i>N</i> = 2	
Type of inflammation	Grade 0: <i>N</i> = 7	Grade 0: <i>N</i> = 2	Grade 0: <i>N</i> = 3	Grade 0: <i>N</i> = 7
	Grade 2: <i>N</i> = 2	Grade 2: <i>N</i> = 3	Grade 2: <i>N</i> = 2	Grade 1: <i>N</i> = 1
	Grade 3: <i>N</i> = 2	Grade 3: <i>N</i> = 1	Grade 3: <i>N</i> = 3	Grade 3: <i>N</i> = 1

be increased by adding IGF-1 and PDGF-BB growth factors to HBSS (33). Our results are in agreement with the mentioned studies as it was shown that HBSS is a suitable storage media. HBSS is a standard, biocompatible solution (34, 35). HBSS demonstrates that balanced PH and osmolality equal to 7.2 and 320 mosmol kg⁻¹, respectively (34, 36), which are optimal for cell growth (28, 37). This solution contains glucose, magnesium, and calcium ions that make HBSS capable to reconstitute the depleted cells (34, 36). In the present study, there was no significant difference between milk and HBSS storage media regarding the pulp's histologic responses. This finding is in agreement with Ashkenazi (6) and Mario studies (38). Considering these facts, HBSS can be regarded as an appropriate storage media.

There are conflicting results regarding the superiority of different storage media. Some studies consider milk as an appropriate storage media (21–24), while others demonstrate HBSS as superiority (15, 32, 33, 39). However, some studies reported that there is no significant differences between these two storage media (20, 40). Different conditions of storage media and variable histologic factors assessed in different studies may explain these contradictory results. The temperature of the storage media, extra oral time, and condition of the tooth apex can highly affect the efficacy of storage

Table 2. Fisher's exact test between different groups of the study

	Calcification	Restoration	Thickness	Odontoblastic layer	Necrosis
Milk–HBSS	1.00	0.60	1.00	0.60	0.58
Milk–control	0.31	0.04*	0.20	0.04*	0.143
Milk–dry storage	1.00	1.00	1.00	1.00	1.00
HBSS–control	0.17	0.21	0.15	0.21	0.47
HBSS–dry storage	1.00	1.00	1.00	0.37	1.00
Dry storage–control	0.34	0.08	0.52	0.02*	0.20

*A significant difference was observed ($\alpha = 0.05$).

media (5, 41) as Andreasen (42) reported high failure rate after replantation of closed apex teeth.

Teeth in the dry storage group were kept out of the alveolar socket for 2 h during our study and then replanted. No significant differences were observed between this group and other groups regarding the histologic responses, while it was reported that the dry storage time should not exceed 30 min and cells lose their vitality after 45 min (41).

It should be mentioned that all teeth in experimental groups were soaked in 10% doxycycline solution for 10 min. The use of doxycycline solution may explain the insignificant differences between different groups. Surface treatment with doxycycline solution prior to replantation can enhance the revascularization rate (13, 14). The histologic changes in our study may be attributed to the antimicrobial effect of doxycycline which diminishes the rate of microorganism in the pulpal lumen (9).

Most of the reviewed studies have surveyed the effect of storage media on the vitality of PDL cells, and there are limited studies regarding the efficacy of storage media in preserving the pulp vitality. Therefore, it is not reasonable to completely compare the result of our study with other surveys. As a result, it is recommended to investigate the effect of different storage media on the pulp cells in further researches.

Based on the results of the present study, it can be concluded that

- 1 HBSS and milk showed no significant difference in the histologic responses of pulp cells compared to control group, and they can be used as an ideal storage media prior to tooth replantation.
- 2 Previously, avulsed teeth with more than 2 h extra oral time had little chance of success; however, in the present study, there was no significant difference between dry storage and control groups regarding the pulp's histologic findings.
- 3 The use of doxycycline prior to replantation can improve the pulp vitality and prevent the pulp necrosis.

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