EFFECT OF STORAGE CONDITIONS ON PERIODONTAL HEALING MECHANISM AFTER TOOTH REPLANTATION

（再植後の歯根周囲組織の治癒に関わる保存状態の影響）

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ABSTRACT

Background: Periodontal ligament (PDL) healing and long term prognosis of replanted avulsed teeth should rely on several factors including length of time under dry condition and type of the storage medium. A number of storage media have been investigated as to their ability to maintain the viability of the PDL cells and thus to permit longer extra-alveolar periods prior to replantation of avulsed teeth.

Aim: The aim of the present *in vivo* study was to evaluate the effect of storage conditions (dry and milk) on periodontal healing of avulsed teeth and analyze its effect on delayed replanted teeth.

Design: Twelve freshly extracted maxillary single-rooted incisor and mandibular premolar teeth were extracted from the Beagle dogs and bench-dried at the room temperature for 45 and 60 minutes (n=3 each) as control. The experimental teeth immersed for 45 and 60 minutes in whole bovine milk (n=3 each). Following these two storage periods, teeth were replanted into the washed sockets. Untill 8 weeks, 7 times of micro-CT were achieved. After 8 weeks dogs were sacrificed, specimens processed to 4-μm thick serially-sections for histopathologic examination and morphometric assessments. Thus, the proportions of the roots that exhibited signs of surface resorption (SR), replacement resorption (RR) i.e. ankylosis and normal PDL were noted.

Results: Root surface exhibited clear sign of surface resorption at 4 and 8 weeks after the dry storage for 60 weeks and 45 weeks, respectively. In milk storage group, 45 min. group shows a good PDL-bone transition, and PDL exhibits parallel fibers in periodontal space. Two storage groups of 60 min. showed surface resorption, and bone formation was observed in close contact to the root, characterizing dental ankylosis (replacement resorption), in which dry storage was more severe**.** Resorption in dry group was significantly higher that the milk group. In 60 min. groups, again a significantly higher resorption was found with dry groups than the other groups. For all groups, 60 min. storage were showed significantly higher resorption than 45 min. (p<0.05).

Conclusions: Based on the results of this study, milk storage was found to be a suitable medium for a period of less than one hour. Surface resorption has occurred of the tooth replantation when stored dry conditions in dogs under these experimental conditions.

Introduction

Tooth avulsion is a complex traumatic injury that results in the complete displacement of a tooth from its alveolar socket to the outer environment**.** It can also affect the pulp and periodontal ligament (PDL) tissues as well as dental hard tissues, alveolar bone and gingival mucosa [1,2)].

The occurrence of complete avulsion ranges from 1 to 16% of all traumatic injuries to the permanent dentition [3)]. Extraoral factors such as mechanical trauma to the PDL, dehydration, and PDL cell viability, in particular, aggravate the prognosis [4)]. The viability of PDL cells is dependent on the duration of extra-alveolar time, storage media of the tooth, and preservation of the root portion; all of these factors are critical for the prognosis of dental replantation [3,5)]. The most important factor among these is the length of time under dry condition because it directly affects the viability of PDL cells remaining on the root surface [6)]. Wound healing normally results from the coexistence between PDL fibroblasts and the osteoblasts from the socket wall. If the damage is minor and not sustained by infection, healing will take place in adjacent, non-injured PDL cells [7)]. If there were a severe PDL damage at the time of injury [7)] or prolonged extra-alveolar desiccation of PDL cells**,** ankylosis and replacement resorption might ensue [8)].

In vivo and *in vitro* researches suggest the use of different storage media to maintain PDL cell viability and improve the prognosis for tooth replantation. These storage media include milk, Hank's balanced salt solution (HBSS) saliva, emdogain propolis, Viaspan, egg albumin, epigallocatechin-3-gallate (EGCG) and some cell-culture media [8-11)].

Until 1970s, saliva had been recommended as the most suitable storage medium, but there were no studies where saliva was compared to other possible storage media. First research for comparison the storage effect of milk with that of saliva on human PDL cells was reported by Blomlöf and Otteskog in 1980 [12)]. Further researches supported that brief storage in saliva followed by subsequent storage in milk was better than storage in saliva only with respect to number of viable PDL cells in the following years [11)]. Milk itself is very easily accessible storage medium, which offers a suitable environment for PDL cells.

Dry storage of avulsed teeth leads to death of PDL cells attached to the root. In this context, the activities of cells derived from PDL play a crucial role. The PDL contains several cell populations including fibroblasts, cementoblasts, osteoblastic and osteoclastic cells**,** and mesenchymal cells. Thus, this complex process of PDL regeneration requires the PDL cells proliferation, differentiation and colonization of the wound area[13]. It is, therefore, suggested that further research on improving periodontal tissue healing after replantation should concentrate on preservation of vital PDL by use of PDL cell proliferation and differentiation.

Although the underlying mechanism of this ankylosis remains to be un-clarified, clinical and experimental studies have shown that PDL viability is the most important factor in its prevention [14,15]. The presence of necrotic PDL remnants on the root surface may trigger an inflammatory root resorption process, which is the major cause of replantation failure and tooth loss [16]. The first reaction after replantation of a tooth with a damaged root surface is an inflammation. Dead and dying PDL cells, along with other debris on the root surface, can initiate the inflammatory response that includes osteoclast-mediated root resorption in proportion to the initial damage [17].

The purpose of the present study was to assess the effect of storage conditions (dry and whole bovine milk) in maintaining the viability of human PDL cells on delayed replanted teeth by means of histopathologic examination and morphometric assessments.

Materials and Methods

Beagle dogs (age: 6 years, body weight 10.9±2.3 kg; total of 8 dogs) were purchased from Japan SLC (Shizuoka, Japan). The dogs were allowed free access to food and water *ad libitum* at all times and were maintained on a 12 h light/dark cycle (lights on 8:00 to 20:00) at 23±1℃, humidity 60±10% environment for a period of 1 month before use. All beagle dogs were maintained and used in accordance with the guidelines of the Care and Use of Laboratory Animals of Nihon University School of Dentistry at Matsudo (05-0003).

Surgical procedure

All dogs were injected intravenously injection with sodium pentobarbital (Somnopentyl®, Kyoritsu Seiyaku, Tokyo, Japan) at a dose of 25 mg/kg. Non−carious, closed apex and periodontally sound maxillary incisor and mandibular first and second

premolar teeth were atraumatically extracted from both jaws and randomly stored in dry at the room temperature and immersed in a flask containing 10 mL of whole bovine milk for 45 and 60 minutes. Following storage periods, sockets washed and the excess on the root surface was gently wiped away with gauze.

Animal study groups

After extraction, the teeth were divided into 4 groups (n=3) according to the experimental extra alveolar period and specific treatment as follows:

Group Ⅰ（GⅠ:Teeth were bench-dried for 45 min.）

Group Ⅱ（GⅡ:Teeth were bench-dried for 60 min.）

Group Ⅲ（GⅢ:Teeth were stored in whole bovine milk for 45 min）

Group Ⅳ（GⅣ:Teeth were stored in whole bovine milk for 60 min).

After the pre-determined storage time, socket washed by saline irrigation and the teeth were gently replanted in their original sockets and splinted with filled light-cured resin (Beautifil flow, Shofu, Japan) functionally for 15 days. The animals were given on commercial solid diet (DS-A, Oriental Yeast Co., Ltd, Japan), and was maintained on soft diet, mixed with the same portion of water (50% water) for the period of splinting. After surgery, each Beagle dogs received an intramuscular injection of sodium ampicillin (Viccillin®, Meiji, Tokyo, Japan) at a dose of 20 mg/kg and was then returned to its cage and allowed to move freely.

Micro-CT examination

The quantitative imageology analysis of newly-grown bone was performed using an *in vivo* Micro-CT system (Rigaku-mCT®, Tokyo, Japan). Beagle dog maxilla and mandible were scanned using Micro-CT with an X-ray source of 90 kV/50 μA at 0, 1, 2, 4, 6 and 8 weeks after surgery. During the period of imaging, the animals were given general anesthesia in a manner similar to above and restrained on a fixed base, so as not to affect the images by body movement. Each Beagle dogs were set on the object stage and imaging was performed on the sample over a full 360^{o} rotation with an exposure time of 17 sec. An isotropic resolution of 130×130×130 μm voxel size was selected, which displayed the micro-structure of beagle dog mandible bone.

Histological analysis (Specimen processing)

The dogs were sacrificed by ketamine hydrochloride (Ketalar® Daiichi-sankyo, Japan) 8 weeks after the operation. After animal's upper and lower jaws were removed, the tissue

blocks (including teeth, bone and soft tissue) fixed with 10% phosphate-buffered formalin for 20 days, decalcified in K-CX® (FALMA, Japan) for 2 weeks, and then trimmed, dehydrated and embedded in paraffin. Serial sections (4 μm) were obtained in a mesial-distal surface for histopathologic and morphometric analyses examination. The sections were stained with haematoxylin and eosin (H&E), followed by taking photographs histometric measurements were then conducted using an Image Command 7098 (version 1.60) (Olympus Optical Co. Ltd, Tokyo, Japan). Seven sections were obtained in each tooth. Twenty one sections were used in each groups.

Characteristics of the PDL, alveolar bone, cementum, and dentin were observed in addition to the occurrence of resorption using according to the Andreasen criteria[18].
The following points were noted:
1) Normal periodontium (PDL)
2) Surface resorption (SR)
3) Replacement resorption (RR) i.e. ankylosis

Morphometric analysis

At the intersections between the horizontal lines and the peripheral contour of the root surface were analyzed and classified as either normal or absorption. Areas of surface resorption, replacement resorption and total area of resorbed root dentin and cementum were considered for analysis. The total root dentin area and the area of resorbed root dentin were measured in square millimeters, and were converted to percentages for statistical analysis. The Image-Pro Plus 3.0 (Media Cybernetics) morphometric program was used to calculate the peripheral contour of the root surface. The areas of total 21 sections with seven serial sections of 4μm thick in each tooth were measured by the program

Statistical analysis

The percentage of each histological classification for each root and each treatment group was calculated. NCSS (Number Cruncher Statistical System) 2007&PASS 2008 Statistical Software (Utah, USA) were employed. All results were expressed as means ± S.D. Data was analyzed using Kruskal-Wallis followed by Mann-Whitney U tests for pair wise comparisons. Differences were considered to be significant for $p<0.05$.

Results

Micro-CT findings

Images were obtained using a micro-CT device (R_mCT®) at 90 kv and 50 μA at 0, 1, 2, 4, 6 and 8 weeks after surgery. The scan time is 17 seconds. The image reconstruction was carried out on personal computer using specially designed I-View® (J.Morita,Japan). R_mCT® findings for the specimens are shown in Figures 5-8.

A clear surface resorption image was not seen in the micro-CT view for dry 45 minutes dry storage (GI) and 45 minutes milk storage groups (G III). In the micro CT view, the surface resorption image was seen from 4 weeks after the operation (marked with arrows) for 60 minutes dry storage group (G II). The surface resorption image was also seen from 8 weeks after the operation (marked with arrow) for 60 minutes milk storage group (G IV).

Histopathologic features

The descriptive histopathologic features for each group were realized as follows:

GI (extra-alveolar dry period of 45 min.):

In this group, no curious abnormalities were observed on the root surface. Periodontal ligament (PDL) was almost well-organized between the scattered, thin cementum and new bone formation. Bone formation was incomplete and irregular shape in a newly formed loosely connective tissue composed of fibroblasts and capillaries (Figure 1).

GII (extra-alveolar dry period of 60 min.):

This group was the most severely affected by root resorption. In all cases, the root was damaged in various degrees. Surface resorption was observed seriously in the middle and apical thirds. This group also presented the surface resorption, especially in the apical thirds. The PDL was poorly organized in many specimens. In some areas, an incomplete newly formed, curvilinear shape of alveolar bone tissue was presented in loosely arranged fibrous tissue (Figure 2).

GIII (immersed in milk, period of 45 min):

This group presented intact root surface, thin to moderate cementum layer, well-organized PDL and alveolar bone. There were well structured parallel collagen fibers reattaching to the cementum and alveolar bone. Namely, no signs of resorption and abnormalities were observed in a major part of the root surface (Figure 3).

GIV (immersed in milk, period of 60 min):
A remarkable finding in this group was small surface resorption on the root surface. The PDL was better organized in areas where the cementum layer preserved than where it was absent. In latter areas, collagen fibers were poorly structured and revascularization was more distinct. In a few samples, small portions of alveolar bone tissue appeared juxtaposed cementum, characterizing dental ankylosis (Figure 4).

Morphometric analysis

The total root dentin area and the area of resorbed root dentin were measured in square millimeters, and were converted to percentages for statistical analysis. At the intersections between the mesial-distal surface and the peripheral contour of the root surface were analyzed and classified as either normal or resorbed condition (Figure 9).

In summary, microphotographs revealed areas of surface resorption which was observed at root surface of replanted tooth 8 weeks after dry storage. Root surface exhibited indications of surface resorption as 0.11±0.01 for dry storage and 0.05±0.01 for milk storage for 45 min. In milk storage group, 45 min. group shows good PDL-bone transition, PDL exhibits parallel fibers in periodontal space. 60 min. storage group shows surface resorption areas as 0.38±0.01 for dry storage and 0.15±0.01 for milk storage groups and bone formation was observed in close contact to the root, characterizing dental ankylosis (replacement resorption). Figure 10 shows that present means (%) and standart deviations of the area of resorbed root dentin according to total resorption.
Statistically difference between the groups in 45 min of dry and milk groups was observed ($p<0.05$). Resorption in dry group was significantly higher that the milk group. In 60 min. groups, again dry group's significantly higher resorption was found than the other groups. For all groups, 60 min. storage were showed significantly higher resorption than 45 min. ($p<0.05$).

Discussion

In cases of tooth avulsions, the primary goal was to preserve the vitality of the periodontal ligament (PDL) cells attached to the root surface, until appropriate treatment can be performed [2)]. This may bring about a favorable reattachment of the periodontal ligament. The maintenance of PDL vitality is extremely important for a good prognosis because the presence of necrotic PDL remnants results in the development of root resorption, which may lead to loss of the replanted tooth [2,19)].

The best treatment option for an avulsed permanent tooth is its immediate replantation into the socket, even if the conditions are not so favorable. In case of delayed replantation, the tooth should be stored in a medium that maintains periodontal ligament cell viability, until a definitive dental treatment can be accomplished. Some solutions that are readily available at the moment of an accident or are easily accessible have been evaluated as storage media for avulsed teeth. Despite its adequate osmolarity, saline is capable of preserving PDL cell vitality only for a short period of time[20)]. Saliva is a better interim storage medium for avulsed teeth than tap water, but it has lower osmolarity than that of milk and may present bacterial products. Bovine milk has presented the best results among the storage media that are most commonly found at the site of an accident [11)]. Researchers have made many attempts to find storage media that are able to preserve PDL cell viability and prevent unpredictable sequel of inflammatory root resorption or replacement resorption post replantation. So far several materials including milk, normal saline, saliva, HBSS have been also recommended and each possesses certain advantages and disadvantages [1)].

Although Blomlöf et al.[14)] and Trope and Friedman [21)] recommend milk as an excellent storing solution, milk can not revive the degenerated cells. An avulsed tooth which has remained in a dry medium and later has been put into milk before reimplantation, will probably have as undesirable prognosis as that which has been into a dry medium and has undergone reimplantation [14)]. It has been well established that two major sequel leading to failure post-replanation of inflammatory root resorption and replacement resorption. During 45 minutes storage time, incidence of resorption area was found 0.05±0.01; while storage in milk for 60 minutes showed only 0.15±0.01. Many studies have examined the critical dry times before irreparable damage to the PDL cells has occurred. Andreasen and Hjorting-Hansen[22)] showed that teeth replanted within 30 min had a better success rate than those that were extraoral for longer period of time prior to

the replantation. In this study, milk storage group showed higher incidents of PDL healing and in parallel with previous studies, was found to be a suitable storage medium for a period of 45 and 60 minutes. Previous studies also recommended milk as suitable storage medium for up to 6 h considering its availability at most accident sites [11,14,20].

Santos et al. [23] had reported that the maintenance of avulsed teeth in dry storage for one hour should be avoided to prevent unfavorable results regarding the incidence of root resorption after replantation based on the histopathologic examination. However, in one hour, severe surface resorption was observed in the root surface with poor PDL organization. In case of milk storage for one hour, changed the PDL changed to better organized in areas where the cementum layer preserved than where it was absent. In a few samples RR was seen which characterizing dental ankylosis. Although these events are not the favorable healing, this will happen slowly with no loss of the alveolar ridge height, which is important for future prosthesis planning.

Recent researches have shown that storing the tooth in an environment that closely resembles the original socket environment is the most important factor for maintaining vitality. This environment is one that has the proper osmolality (cell pressure), pH, nutritional metabolites and glucose. The value of pH and the osmolality of the storing environments must be physiologic, for both interfere in the surviving of cells of the periodontal ligament. The cellular growth may occur between 290 and 330 m osmol /kg and the pH must be between 7.2 and 7.4, but growth may occur between 6.6 and 7.8 [24].

In a study done by Blomlöf et al.[12] with whole milk, PDL cells survived for only 1 h, probably because of the low pH milk (4.2–4.5). In an *in vitro* study by Rozenfarb et al.[24], viability of skin fibroblasts in several culture media including milk, egg albumen, saliva and MEM (minimal essential medium; a culture medium) was investigated. They found no significant difference amongst MEM, egg albumen and milk, although all were superior to saliva. They reported that the osmolarity of the MEM, milk and egg albumen ranged from 251 to 298 m osmol/ kg, whereas the saliva was hypotonic, with an osmolarity of 73 m osmol/ kg, which could explain the difference observations[24].

In another study by Hiltz and Trope [25], although the vitality of human lip fibroblasts in milk maintained a high percentage after 6 h (68.2%), Viaspan was the most effective storage medium at all observation periods with 37.6% cell survival after 168 h storage. Recently green tea extract (GTE) was found effective in maintaining the viability of

human PDL cells is similar to that of HBSS and higher than that of milk [26]. Green tea extracts (GTEs) contain catechin (epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin-3-gallate), which is one of the polyphenols from Green Tea. It has been reported that GTEs have remarkable anti-inflammatory, anti-oxidant, and anti-carcinogenic effects[27] and showed the best ability for storage of an avulsed tooth although the osmolality and pH of GTE was not ideal.

In milk storage group, 45 min. group shows good PDL-bone transition, PDL exhibits parallel fibers in periodontal space. The group of 60 min. storage shows surface resorption areas, and bone formation was observed in close contact to the root, characterizing dental ankylosis (RR)**.** Our results indicated that milk storage is effective in maintaining PDL cell viability for replantation in one hour and it could preserve the viability over 85%.

HBSS is a widely used standard solution recommended by the International Association of Dental Traumatology as a storage medium for avulsed tooth[21]. The osmolality and pH of HBSS are 270 to 290 m osmol/ kg and 7.2, respectively. Although HBSS has the ability to provide long-term preservation of PDL cells[21], it is not yet available in pharmacies or drug stores at the scene of an accident. So, milk is known as the appropriate storage medium for avulsed tooth because it is easy to get in the event of an accident and able to maintain PDL cells. In addition, milk has low bacterial content, a physiologically compatible pH and osmolality, and it provides some essential nutrients, and growth factors to cells[23]. However, instead of milk storage, keeping in dry is ineffective in maintaining PDL cell viability because it leads to rapid death of PDL cells and high incidence of bacterial contamination.

As a conclusion, the ideal treatment of choice at the time of avulsion should be immediate replantation, in order to reestablish the natural nutrient supply to the periodontal ligament cells. This would also minimize further damage and enhance the healing process. Unfortunately, there may be situations where there may be a delay in replantation. Under such circumstances, the tooth should be stored in a medium such as milk that maintains periodontal ligament cell viability, until a definitive dental treatment can be accomplished.

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Legend

Fig.1 Group Ⅰ (Dry 45min.)

Obvious root resorption was not seen at 8 weeks after replantation in Group 1.

Fig.2 Group Ⅱ (Dry 60min.)

The microphotograph of reimplanted tooth at 8 weeks after replantation was shown in areas of SR observed at root surface. *(H&E, original magnification x200).*

Fig.3 GroupⅢ(Milk 45min.)

Forty five min storage in milk group shows a good PDL-bone transition, PDL exhibits parallel fibers in periodontal space.

Fig.4 GroupⅣ(Milk 60min.)

Sixty min storage in milk group shows SR areas, bone formation was observed in close contact to the root *(H&E, original magnification x200).*

Fig.5 Micro CT (Group Ⅰ (Dry 45min.))

Slight surface resorption image was seen at 6 weeks after replantaion in the micro CT view for dry 45 group.

Fig.6 Micro CT(Group Ⅱ (Dry 60min.))

Surface resorption image was seen from 4 weeks after the operation (arrow) in the micro CT view for dry 60min.

Fig.7 Micro CT(GroupⅢ(Milk 45min.))

Surface resorption image was not seen in the micro CT view for the milk storage 45 min.

Fig.8 MicroCT(GroupⅣ(Milk 60min.))

Slight surface resorption image was seen from 6 weeks after the replantation and obvious surface resorption was seen at 8 weeks after the operation (arrow) for the milk storage 60 min.

Fig.9 Microscopic and Image pro plus used on the root surface (resorption/root surface measured)

At the intersections between the horizontal lines and the peripheral contour of the root surface were analyzed and classified as either normal or resorbed.

Areas of surface root resorption, replacement resorption, and total area of resorbed root were considered for analysis. The total root surface area and the area of resorbed root area were measured in square millimeters, and were converted to percentages for statistical analysis.

Fig.10 Total resorption area

Fig.10 shows that present means (%) and standard deviations of the area of total resorbed root area.

Statistically difference between the groups of Group 1 and Group 3 in 45 min were observed. Resorption in dry group was significantly higher that the milk group in 45 min and 60 min. In 60 min. groups, again dry group's significantly higher resorption was found than the other groups. For all groups, 60 min. storage were showed significantly higher resorption than 45 min.

Fig.11 Surface resorption area

Fig.11 shows that present means (%) and standard deviations of the area of surface resorbed root area.

Statistically difference between the groups of Group 1 and Group 3 in 45 min were observed. Resorption in dry group was significantly higher that the milk group in 45 min and 60 min. In 60 min. groups, dry group's significantly higher resorption was found than the other groups. For all groups, 60 min. storage were showed significantly higher resorption than 45 min.

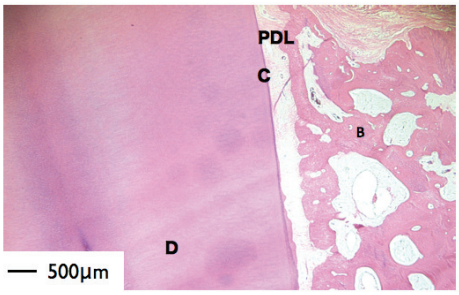
Fig.12 Replacement resorption area

Fig.12 shows that present means (%) and standard deviations of the area of replacement resorbed root area.

Statistically difference between the groups of Group 1 and Group 3 in 45 min were observed. Resorption in dry group was significantly higher that the milk group in 45 min and 60 min. In 60 min. groups, dry group's significantly higher resorption was found than the other groups. For all groups, 60 min. storage were showed significantly higher resorption than 45 min

Table 1 Micro CT findings

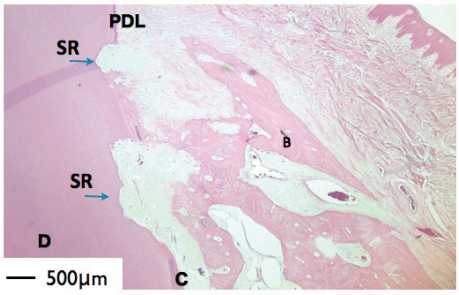
In micro CT images, slight root resortion was seen at 6 and 8 weeks after the replantation in Group Ⅰ. In Group Ⅱ, obvious root resorption was seen at 4, 6, 8 weeks after the replantation. In Group Ⅲ, no root resorption was seen until 8 weeks after the replantation. In Group Ⅳ, slight root resortion was seen at 6 weeks and obvious resorption was seen at 8 weeks.



Group Ⅰ (Dry 45min.)

PDL : Periodontal Ligament
B : Bone
C : Cementum
D : Dentine

Fig.1 Group Ⅰ Dry 45min.



Group Ⅱ (Dry 60min.)

PDL : Periodontal Ligament
B : Bone
C : Cementum
D : Dentine
SR: Surface resorption

Fig.2 Group Ⅱ Dry 60min.

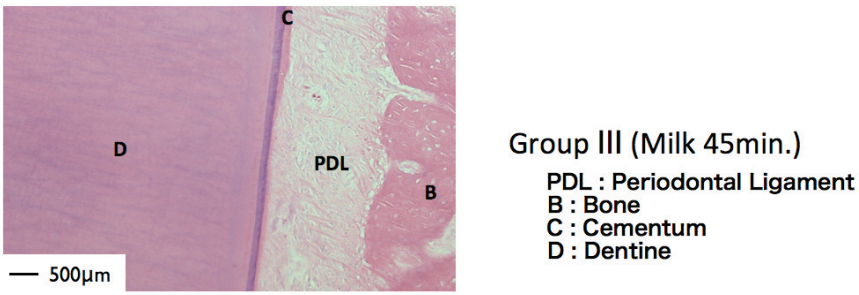


Fig.3 Group Ⅲ Milk 45min.

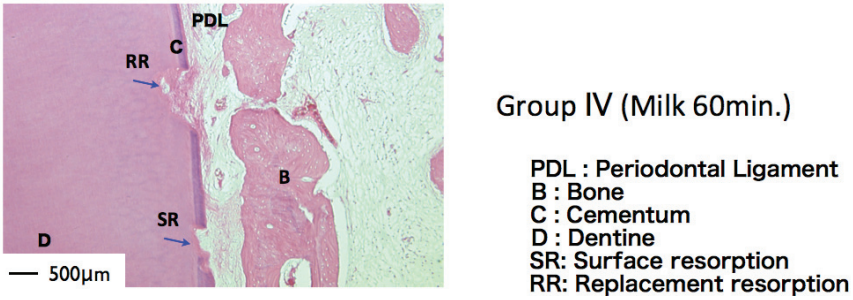


Fig.4 Group Ⅳ Milk 60min.

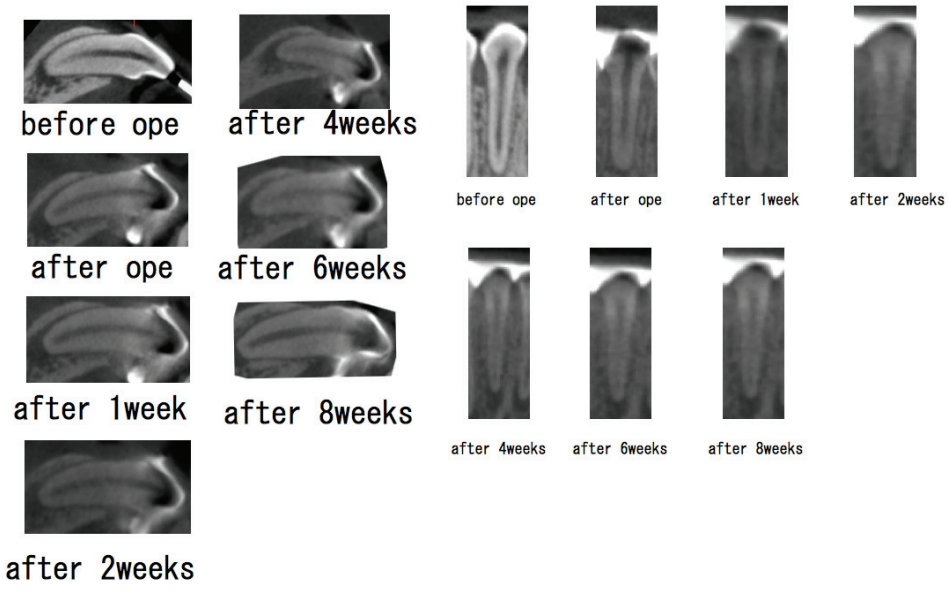


Fig.5 Micro CT (Group Ⅰ (Dry 45min.))

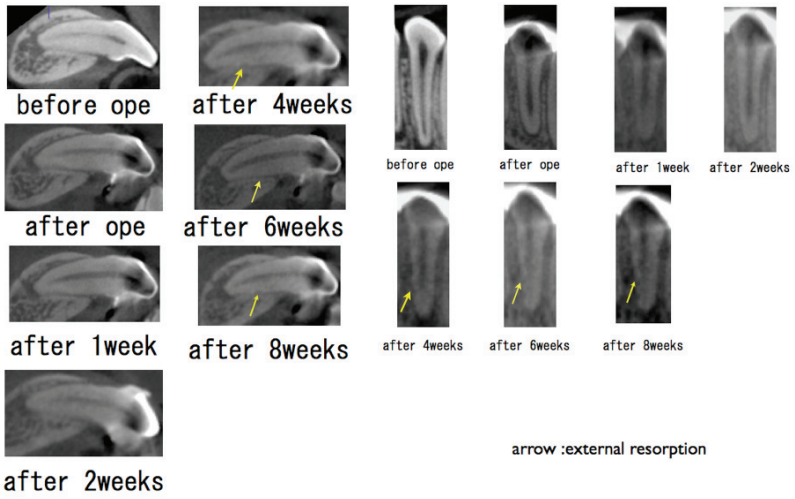


Fig.6 Micro CT (Group Ⅱ (Dry 60min.))

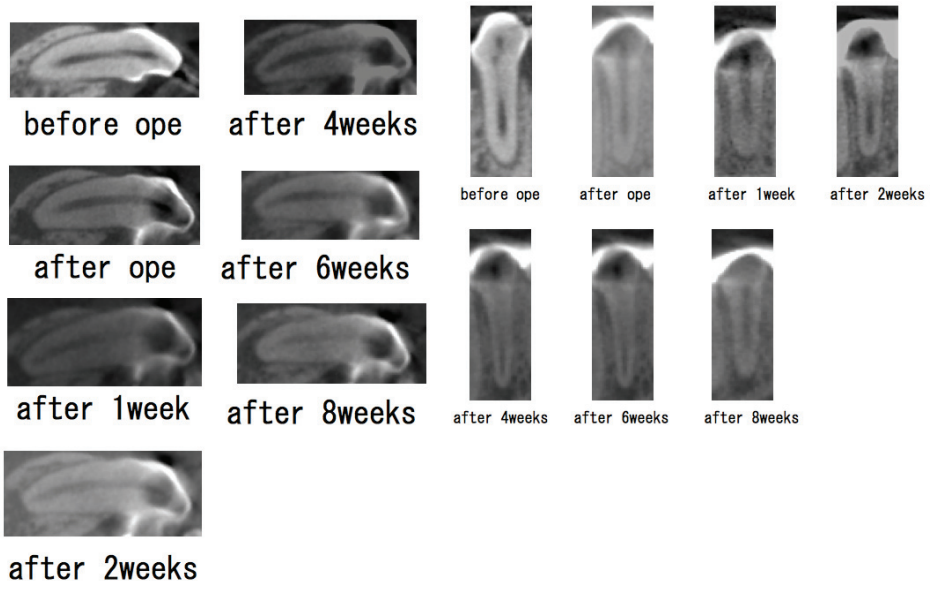


Fig.7 Micro CT (GroupⅢ(Milk 45min.))

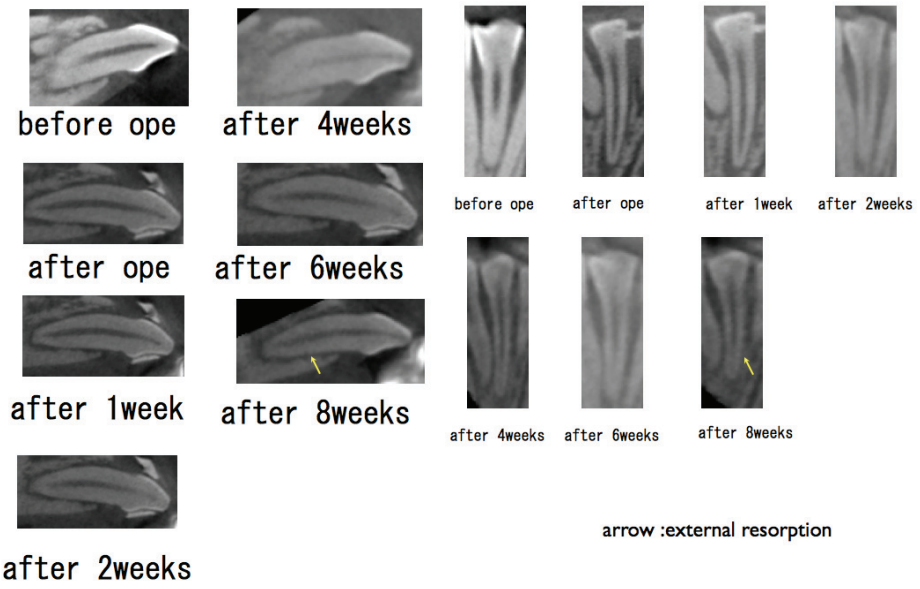


Fig.8 Micro CT (GroupⅣ(Milk60min.))

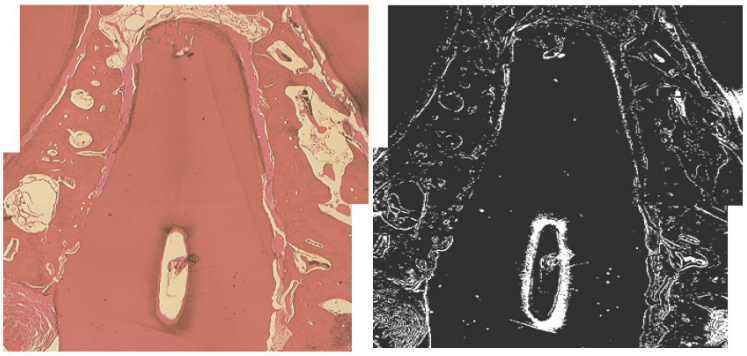


Fig.9 Microscopic and Image pro plus used on the root surface (resorption/root surface measured)

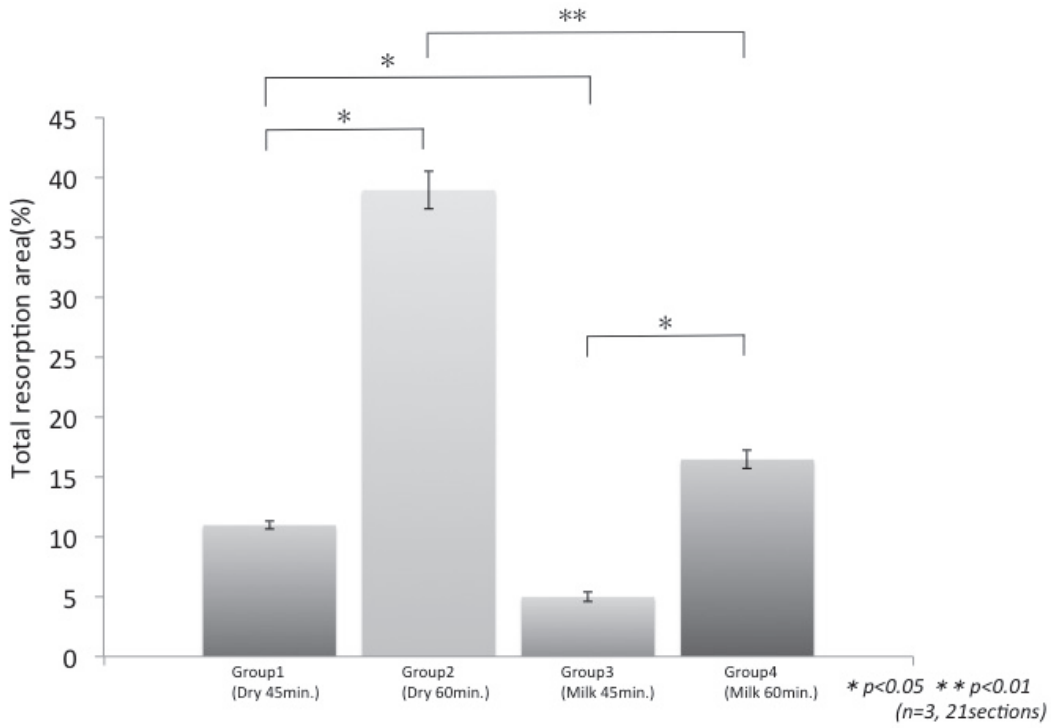


Fig.10 Total resorption area

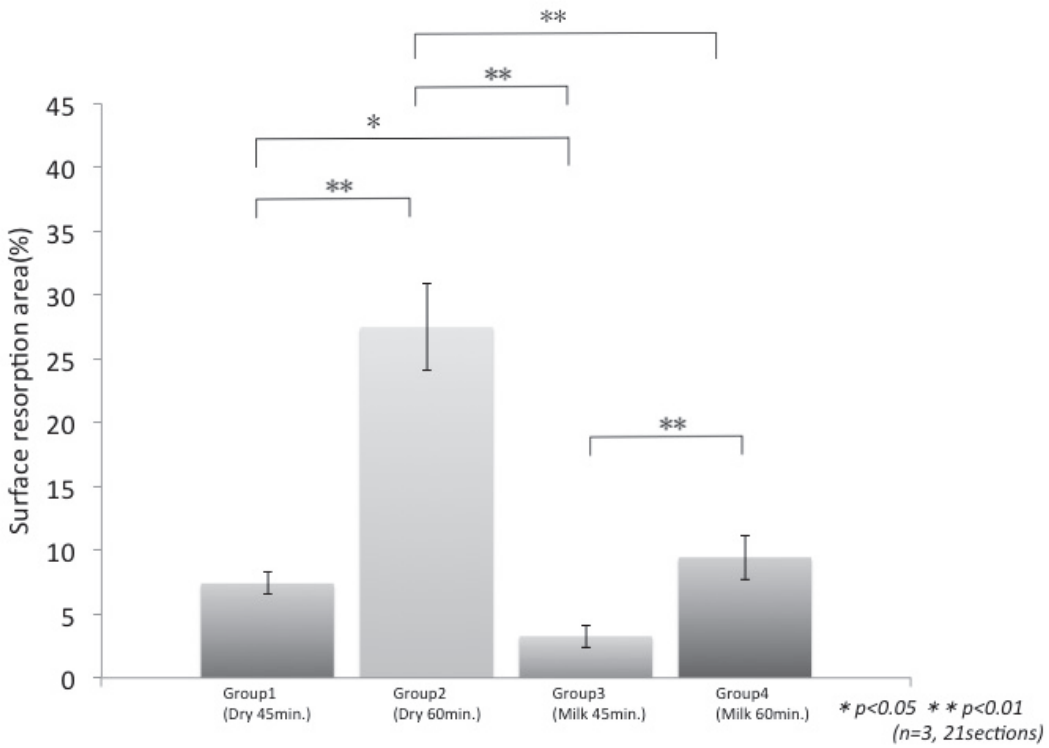


Fig.11 Surface resorption area

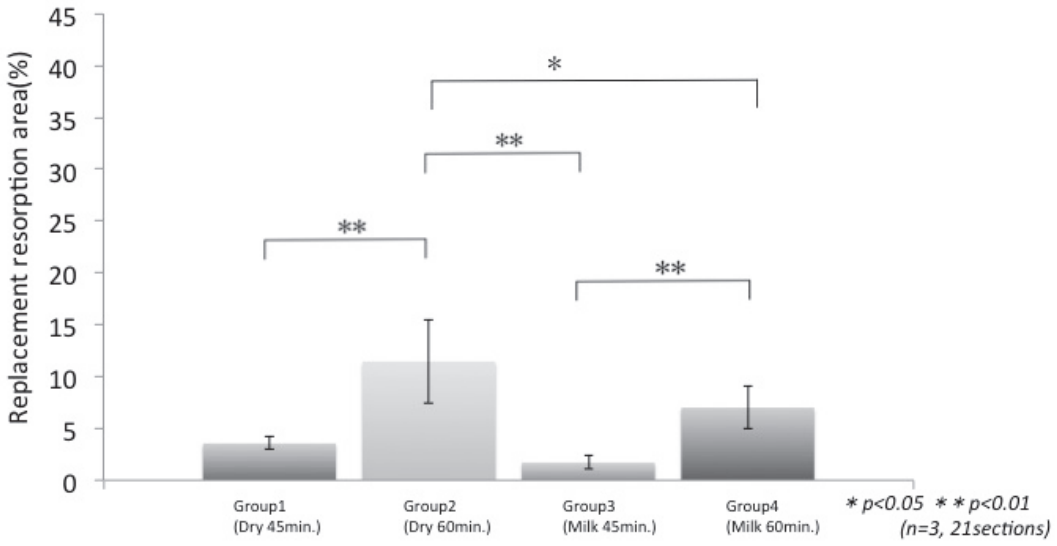


Fig.12 Replacement resorption area

Table.1 Micro CT findings

	after 1 week	after 2 weeks	after 4 weeks	after 6 weeks	after 8 weeks
Group I (Dry45min.)	(−)	(−)	(−)	(±)	(±)
Group II (Dry60min.)	(−)	(−)	(+)	(+)	(+)
Group III (Milk45min.)	(−)	(−)	(−)	(−)	(−)
Group IV (Milk60min.)	(−)	(−)	(−)	(±)	(+)