Evaluation of low-intensity pulsed ultrasound on reducing replacement root resorption of replanted teeth in dogs

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DEDICATION (감사의 말)

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마지막으로, 늘 사랑과 믿음으로 지켜봐 주시고 물심양면 아낌없이 지지해주신 사랑하는 부모님과 큰삼촌에게 가장 깊은 감사의 말씀을 드립니다.

더욱 성장하여 그 마음에 보답하는 사람이 되도록 노력하겠습니다.

2021년 12월 성새미 올림

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Evaluation of low-intensity pulsed ultrasound on reducing replacement root resorption of replanted teeth in dogs

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Tooth avulsion is considered one of the most serious types of dental injuries. Most replanted teeth demonstrate root resorption after traumatic avulsion, including surface root resorption, inflammatory root resorption and replacement resorption. Typically, extensive replacement resorption is a major problem encountered, even after appropriate treatments of avulsed tooth followed by tooth replantation, resin-wire-splinting and root canal treatment. Previous studies show low-intensity pulsed ultrasound accelerates bone and periodontal healing. This study aimed to investigate the effects of low-intensity pulsed ultrasound (LIPUS) on replacement root resorption after replantation of avulsed teeth stored in dry condition in dogs.

A total of 73 premolar roots from 4 male mongrel dogs were intentionally avulsed with forceps after root canal treatment and was divided into four groups. —HN, HL, DN, and DL—according to storage conditions and whether or not they received LIPUS treatment. H and D represents HBSS contained groups and dry groups. LIPUS treated groups are marked as L, and non-treated groups as N. 38 roots were kept in Hanks' Balanced Salt Solution for 30 minutes (HN and HL groups), whereas the remaining 35 roots were left to dry in the air for an hour (DN and DL groups) prior to replantation. Following replantation, the roots in the HL and DL groups (21 and 18 roots, respectively) received a 20-minute daily LIPUS treatment for 2 weeks. The animals were euthanized 4 weeks after the operation. Micro-computed tomography images were acquired for each root and the amount of replacement root resorption was measured three-dimensionally. Histological assessments were also carried out.

According to the resorption area measured by micro-CT, there was significantly less replacement root resorption for the roots in the DL group , which teeth received a two-week daily LIPUS treatment after being dried and replaced, compared to the DN group ($p < 0.01$), which were also dried and replanted but lacked LIPUS treatment (25.35% and 39.46%, respectively). Histologic findings in the DN group demonstrated evident replacement root resorption, whereas the DL group revealed less resorption compared to the DN group. Both HBSS storage groups (HL 1.02% and HN 1.11%) had significantly reduced surface resorption compared to the DN group (3.61%), regardless of LIPUS exposure ($p < 0.01$ and $p < 0.05$, respectively). In addition, the DL group (2.30%) demonstrated no significant differences in surface root resorption compared to the HL or HN groups. Overall degree of surface resorption was minimal and confined to the cementum.

Within the limitations, these results suggest that LIPUS treatment could attenuate the replacement resorption of avulsed teeth stored in dry condition, thereby improving their prognosis.

Key words: ankylosis; low intensity pulsed ultrasound; periodontal healing; periodontal ligament; replantation; replacement resorption; root resorption

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1. INTRODUCTION

Tooth avulsion implies complete displacement of the tooth out of its socket (World Health Organization, 1994). Tooth avulsion affects 0.5 to 16% of traumatic dental injuries in permanent dentitions and is considered to be the most serious type of them (Andreasen, Andreasen, and Andersson, 2018). While tooth replantation is the indicated treatment for some of the avulsions, both pulp and periodontal tissues suffer from extensive damage during the extra-alveolar period. Healing reactions are dependent upon various factors such as storage media, length of the extra-alveolar period, root development, and contamination of the root surface (Petrovic et al., 2010; Andreasen et al., 1995b, 1995a).

External root resorption is seen as a major complication encountered after replantation because of periodontal damage. A meta-analysis on the incidence of root resorption after the replantation of avulsed teeth showed that the incidence of surface, inflammatory, and replacement root resorption was 13.3%, 23.2%, and 51.0%, respectively (Souza et al., 2018).

Surface root resorption, or repair-related resorption, can be seen in a normal healing process and considered as a favorable type of periodontal healing. It is self-limiting, not progressive, and shows repair with new cementum. In this case, most resorption lacunae are superficial and confined to the cementum. Histologically, this type of healing shows localized areas along the root surface with superficial resorption lacunae repaired by new cementum, presumably healed by periodontal ligament-derived cells. This type of resorption lacunae with similar histologic morphology are also reported on non-traumatized root surfaces, with a frequency as high as 90% of all teeth examined (Henry and Weinmann, 1951).

Inflammatory root resorption, or infection-related resorption, is associated with necrosis and infection of pulp. In contrast to surface root resorption, inflammatory resorption is histologically characterized by bowl-shaped resorption cavities in cementum and dentin associated with inflammatory changes of adjacent tissue. When injuries to the periodontal ligament or cementum induce small resorption cavities on the root surface, these resorption cavities expose dentinal tubules. This may become a typical problem when toxins from infected necrotic pulp tissue penetrate along the dentinal tubules to the periodontal tissues, which will initiate inflammatory response. This consequently intensifies the resorption process of the root surface, which may progress very rapidly (Andreasen, 1981e).

Replacement root resorption, or ankylosis-related resorption, appears as a fusion between the root surface and alveolar bone. Histologically it can be verified 2 weeks after replantation of avulsed tooth (Andreasen, 1980b). Specifically, it appears to be related to the absence of a vital periodontal ligament cover of the root surface (Andreasen, 1981c; Andreasen, 1981d; Lindskog et al., 1985). While progressive resorption gradually resorbs the entire root, transient resorption induces temporary ankylosis which later disappears. Extensive drying of the tooth of removal of entire periodontal ligament always results in progressive ankylosis (Andreasen, 1981d). It is assumed that adjacent bone marrow cells with osteogenic potential repopulates the damaged periodontal ligament space and form ankylosis (Line, Polson, and Zander, 1974).

Root resorption after replantation of avulsed teeth have been the subject of numerous experimental studies. A series of histological analyses of avulsed human teeth showed that almost half of the periodontal ligament was lost after replantation (Haas et al., 2008). This indicates serious healing complications that may result in replacement root resorption being commonly observed in avulsed teeth after replantation. This has been confirmed by experimental replantation in dogs (Yamada et al., 1999b), monkeys (Andreasen, 1980a; Nasjleti et al., 1975), and humans (Breivik and Kvam, 1987).

Many studies were conducted to improve regenerative responses and healing of periodontal tissues to minimize replacement root resorption after replantation of avulsed teeth. This includes handling of avulsed tooth at the site of accident, critical extraoral dry time period, periodontal ligament cell damage, storage media, chemical treatment of root surface before tooth replacement and oral medication. Animal studies have shown that storage in milk or saliva has almost the same effect as storage in saline. A commercial tissue culture medium (Viaspan) could also be used, according to *in vitro* experiments. Nevertheless, avulsed teeth should always be replanted as soon as possible (Udoye, 2012). Dry extraoral periods exceeding 1 hour leads to periodontal ligament cell death, followed by extensive root resorption (Andreasen, 1995). Mechanical cleansing or sterilization of root surface should also be avoided due to same reasons. Current trauma guidelines recommend systemic antibiotic therapy for tetanus prophylaxis and prevent microbial infection (Fouad, 2020). A recent study on human permanent teeth found no significant difference in outcome after topical antibiotic treatment before tooth replantation (Tsilingaridis, 2015). Until now a reliable therapeutic protocol for teeth with severe periodontal ligament damage has not yet been universally established.

The use of ultrasound for medical applications has been investigated for decades (Ensminger and Bond, 2011). Low-intensity pulsed ultrasound (LIPUS) is a specific type of ultrasound that delivers impulses at a low-intensity and outputs pulsed waves. The effectiveness of LIPUS treatments is mostly ensured by non-thermal effects, including acoustic cavitation and biological signaling (Lin et al., 2016). LIPUS treatment has been mostly used in the field of orthopedics. It has been shown that LIPUS enhances and accelerates fracture healing through increased bone formation in cases of delayed and/or impaired bone healing, and significantly decreases period of bone healing (Schandelmaier et al., 2017; Rutten et al., 2016). Its applications for orthopedic treatment have been approved by the U.S. Food and Drug Administration (FDA) in 1994 and 2000 (Rubin et al., 2001). Although the impact of LIPUS in the osteogenic responses of the bone has been well documented, elucidating the clinical effectiveness of LIPUS in orofacial regions warrants further study. Soft tissue healing and significant decrease in areas of root resorption lacunae have been reported to this date (Khanna et al., 2009; Rego et al., 2011). It was found that the application of LIPUS for sinus augmentation promotes new bone formation in rabbits (Takebe et al., 2014). A pilot study demonstrated enhanced alveolar bone healing of extraction sockets (Kang et al., 2016), while another showed the prevention of bisphosphonate-related osteonecrosis of the jaw, both of which were carried out in a rat model (Hidaka et al., 2019). Recently, it was found that LIPUS treatment not only enhances the migration of periodontal ligament stem cells in vitro (Wang, Li, et al., 2018), but also accelerates 2-wall alveolar bone defects in canines, in addition to inhibiting root resorption after luxation and immediate replantation in rats (Rego et al., 2011; Shirakata et al., 2020). LIPUS treatment studies have also yielded positive results in orthodontics, especially with orthodontically induced inflammatory root resorption (OIRR). LIPUS treatment minimized

OIRR caused by torque in ovariectomized osteoporotic rats (Dahhas et al., 2016) and in healthy human patients (Raza et al., 2016). Although the exact mechanism by which LIPUS minimized OIRR is not fully understood, it is shown that LIPUS regulates osteoclast differentiation in OIRR (Liu et al., 2012), and increase the thickness of predentin, cementum and bone remodeling increased in rat models (Alshihah et al., 2020) and beagles (Al-Daghreer et al., 2014). However, no study to date has evaluated the effects of LIPUS in animals other than rat models, or on root replantation after avulsion solely dependent on periodontal healing, excluding the inflammatory effects due to infected pulp tissues, which would more accurately reflect the effects of LIPUS on replacement root resorption of avulsed teeth.

Thus, this study aimed to investigate the effects of LIPUS on replacement root resorption after replantation of avulsed dog teeth stored with different conditions and periods, excluding the adverse effects from necrotic pulp. It was hypothesized that replacement root resorption of LIPUS treated roots is reduced compared to those receiving no supplementary treatment after replantation.

2. MATERIALS AND METHODS

2.1. Animal experiments

Four healthy male mongrel dogs, 10–14 months of age and weighing approximately 25–30 kg, were included in this study. The animals were housed and monitored daily for the duration of the study in the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Seoul, Republic of Korea. They were allowed a week for acclimation prior to the experiment. They were kept in individual cages at 22°C with relative humidity of 50%, and a 12-hour light/dark cycle. Approximately 500 g of solid food (Purina; Nestle SA, Vevey, Switzerland) was provided to each animal every day, and water was available ad libitum during the experimental period. Experiments were carried out in compliance with the ARRIVE guidelines, approved by the Yonsei University Health System Institutional Animal Care and Use Committee, Seoul, Republic of Korea (Approval No. 2019-0068). Informed consent was obtained from the owners and all experiments were performed in accordance with relevant guidelines and regulations.

2.2. Surgical procedures

Premolar teeth of each animal were included for the experiment. The roots were divided into four groups according to storage conditions before replantation and whether or not they received a LIPUS treatment after replantation. The roots in each group were evenly distributed among the four dogs, such that each group equally included the right and left, maxillary and mandibular premolar roots. Congenital missing, impacted, or root-fractured teeth were excluded from the study. H and D represents HBSS contained and dried groups after replantation. LIPUS treated groups are marked as L, non-treated groups as N. Thirty-eight roots were kept in Hanks' Balanced Salt Solution (HBSS) for 30 minutes (HN and HL groups), whereas the remaining 35 roots were left to dry in the air for an hour (DN and DL groups) before the replantation. The roots in the HL and DL groups received a daily LIPUS treatment after the replantation (Table 1). Detailed information of root distribution and excluded number of roots is available in Supplementary Table 1.

Table 1. Allocation of roots for each group.

	HBSS storage	**Dry storage**	**Total**
LIPUS treatment	21 (HL)	18 (DL)	39
No LIPUS treatment	17 (HN)	17 (DN)	34
Total	38	35	73

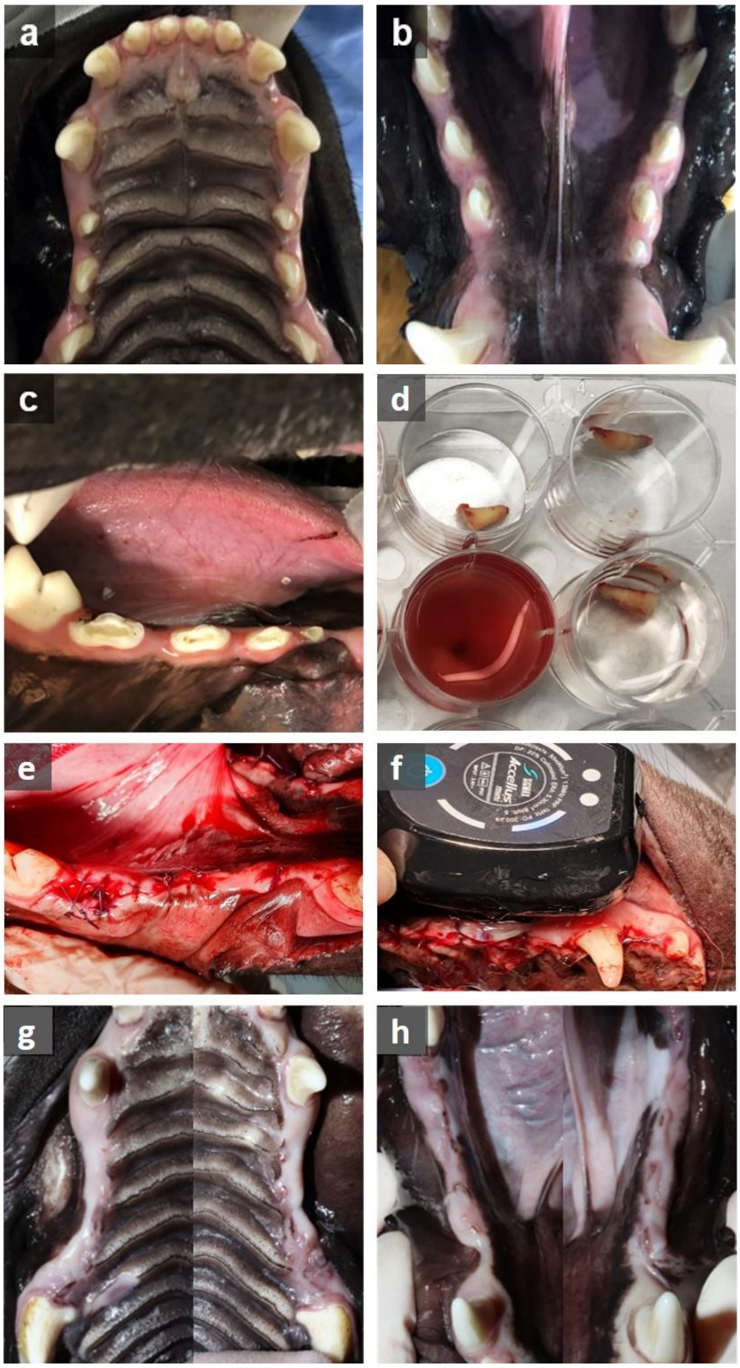
a
b
c
d
e
f
g
h
Accellus

Figure 1 Clinical photographs during experimental periods. (a, b) Pre-operative photographs of maxilla and mandible. (c) After root canal treatment. Access opening is filled with RMGI material. (d) After extraction, roots were decoronated. Each root is placed separately in an aseptic 16-well plate as designated. DL, DN group roots are left dry, while HL, HN roots are submerged in HBSS. In this figure, only a part of the plate is shown. (e) After replantation. Replanted roots were covered by gingiva and sutured. (f) Daily 20 minute LIPUS treatment for 14 days under intravenous general anesthesia. (g, h) Intraoral photos of maxilla and mandible, 4 weeks after the operation.

Three experienced dental clinicians performed all surgical procedures under general and local anesthesia under aseptic routines. General anesthesia was induced by alfaxalone (Alfaxan; Jurox Pty Ltd., Rutherford, NSW, Australia), medetomidine hydrochloride (Tomidine; Provet Ltd., Istanbul, Turkey) intravenously and was maintained by 2% isoflurane (Ifran Liq; Hana Pharm Co Ltd., Republic of Korea) in conjunction with pure oxygen by inhalation.

Preoperative root canal treatments were performed aseptically on all experimental teeth to minimize the effects of pulpal infection as a stimulus for external root resorption. After occlusal reduction and access opening with sterile diamond bur, coronal flaring was carried out using gates-glidden drills (Dentsply Maillefer, Baillagues, Switzerland). The working length was determined using size #20 stainless steel k-type hand instruments (Dentsply Maillefer, Baillagues, Switzerland) and a periapical radiograph. Nickel-titanium rotary instruments (Profile; Dentsply Maillefer, Baillagues, Switzerland) and k-type hand instruments were used for pulp extirpation and root canal preparation to apical delta. The apical enlargements of root canals were accomplished up to size #50-70 with k-type hand instruments. After the shaping of root canal was finished, final irrigation was performed using 5 mL of 2.5% NaOCl solution using a syringe with a 30-gauge needle. Lastly, the root canals were dried with sterile paper points and obturated with Vitapex paste (J Morita, Tokyo, Japan). Occlusal access holes and canal orifices were filled with resin-modified glass ionomer (Fuji II LC; GC, Tokyo, Japan) (Figure 1a-c). Intentional avulsion was performed as atraumatically as possible with forceps (Physics Forceps; GoldenDent, Detroit, MI, USA) and gentle bucco-lingual luxation forces. After extraction, two-rooted premolars were hemisectioned into two single roots. The crown portion of the tooth was cut at cervical or furcation level with a high-speed diamond bur and removed to avoid occlusal forces and complete coverage by gingiva during healing periods. Each root was placed in its prepared designated space in a 16-well plate. For the HN and HL groups, each well was filled with 2.5 mL of HBSS, ensuring that the root was completely submerged in the solution for 30 minutes. For the DN and DL groups, the root was swiftly air dried and then placed in an empty well to dry for an hour. After the given time, the roots were replaced in the socket of their own position and sutured with 4-0 Coated Vicryl (Ethicon Inc, Sommerville, NJ, USA) such that each root was adequately covered with gingiva (Figure 2). Periapical radiographs were taken before the operation, after the root canal treatment, and after the replantation to confirm each procedure, respectively (Figure 2a-c. Figure 3a-c, Figure 4a-c and Figure 5a-c). After the surgical procedures, the animals were administered ketorolac thromethamine (Keromin inj; Hana Pharma Co Ltd., Republic of Korea), Cefazolin sodium (30 mg/kg, Chonkundang, Republic of Korea), Meloxicam (0.2 mg/kg, Boehringer Ingelheim, Greece) for a week.

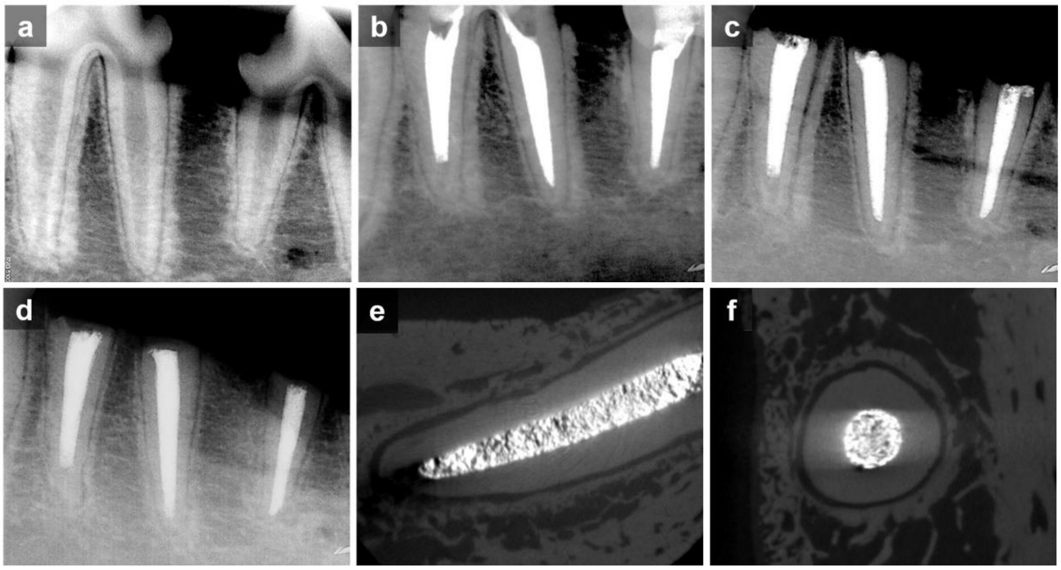

Figure 2. Representative serial radiographs and micro-CT images of roots in the HL group. **(a)** Pre-operation. **(b)** After root canal treatment. **(c)** After replantation. **(d-f)** 4 weeks after the operation. Note that the roots were surrounded by intact lamina dura.

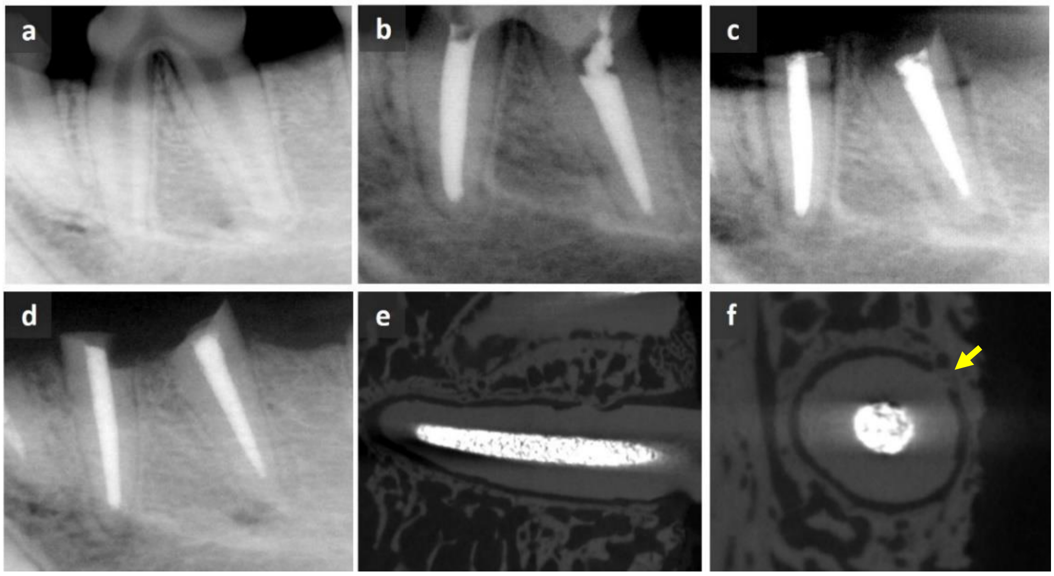

Figure 3. Representative serial radiographs and micro-CT images of roots in the HN group. **(a)** Pre-operation. **(b)** After root canal treatment. **(c)** After replantation. **(d-f)** 4 weeks after the operation. Most parts of the roots are surrounded by intact lamina dura, while partial replacement root resorption is seen (arrow).

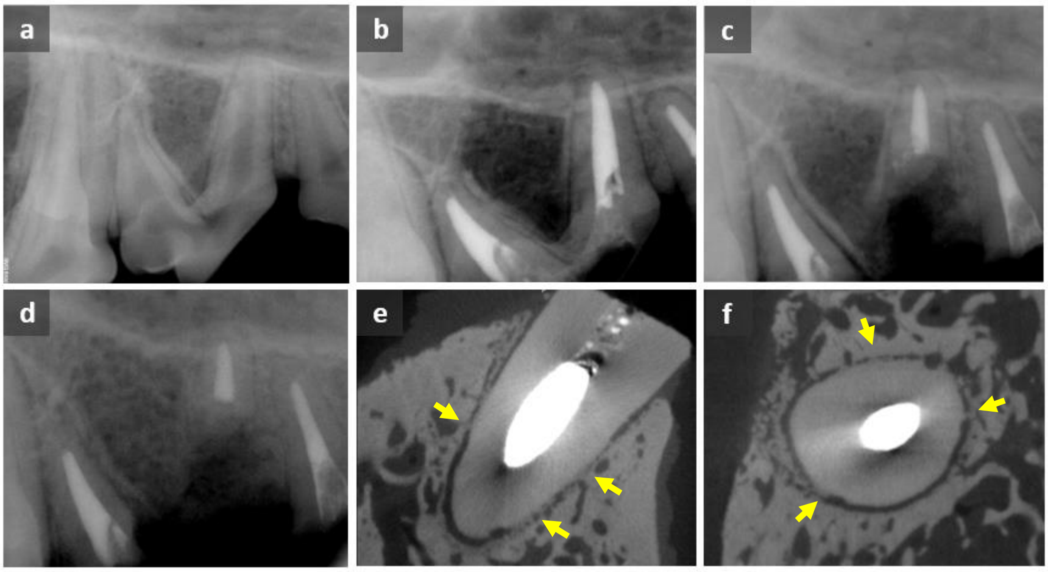

Figure 4. Representative serial radiographs and micro-CT images of roots in the DL group. **(a)** Pre-operation. **(b)** After root canal treatment. **(c)** After replantation. **(d-f)** 4 weeks after the operation. Sparse replacement root resorption pointed by arrows.

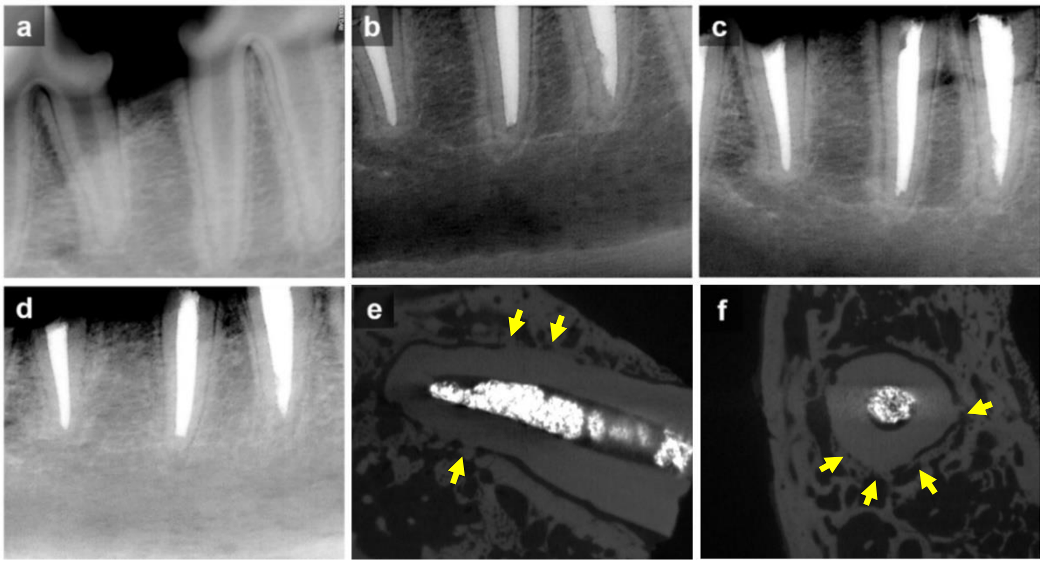

Figure 5. Representative serial radiographs and micro-CT images of roots in the DN group. **(a)** Pre-operation. **(b)** After root canal treatment. **(c)** After replantation. **(d-f)** 4 weeks after the operation. Note that the lamina dura became indistinct and the evidence of replacement root resorption was seen in the micro-CT images (arrows).

2.3. Low-intensity pulsed ultrasound (LIPUS) exposure

After replantation, the roots in the HL and DL groups received LIPUS treatment starting from the day of surgical procedure for 2 weeks (Figure 1f). The LIPUS instrument used in this study (Accellus Mini; Nippon Sigmax Co, Tokyo, Japan) consisted of a circular transducer with a diameter of 26 mm and an effective radiating area of 5.3 cm^2. The pulsed ultrasound signal had a 1.5 MHz frequency, 30 mW/cm^2 ultrasound intensity, and 1 kHz pulse repetition rate. For daily ultrasonic exposure, intravenous general anesthesia was delivered to each animal. The transducer was placed in contact with the buccal gingiva, in the region corresponding to the designated quadrant. A single practitioner located the device on the buccal mucosa to cover the targeted area. A coupling gel was kept constantly in place to optimize penetration of the ultrasound energy into the tissues. The ultrasound was used for 20 minutes daily for 14 consecutive days.

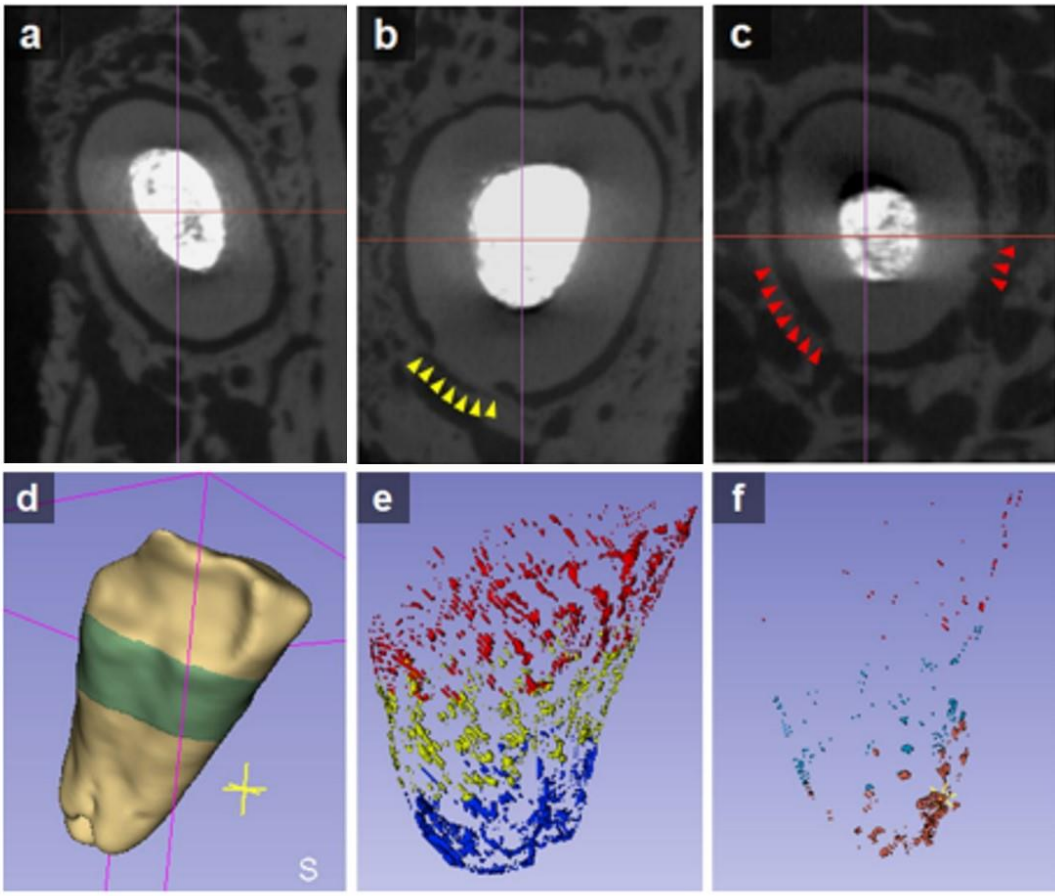

Figure 6. Representative micro-CT images and 3D reconstruction for quantitative measurement of the amount of root resorption. The root embedded in the alveolar bone alone was included. **(a)** Normal root surface without any root resorption. Regular and continuous radiolucent areas between the root surface and the alveolar bone, indicating PDL space, can be seen. **(b)** Replacement root resorption presenting direct connection between the root surface and the alveolar bone without interposition of a radiolucent area (yellow arrowheads). **(c)** Surface root resorption presenting craters or holes in the root surface (red arrowheads) **(d)** Visualization of the root by 3D reconstruction. **(e)** Distribution of replacement root resorption. **(f)** Distribution of surface root resorption.

2.4. Three-dimensional (3D) analysis

Four weeks following the operation (Figure 1g, h), the dogs were euthanized under general anesthesia using an overdose of potassium. After taking periapical radiographs for all roots (Figure 2d, Figure 3d, Figure 4d, Figure 5d), both maxillary and mandibular premolar areas were removed en bloc with the surrounding hard tissues and fixed in 10% buffered paraformaldehyde.

The blocks were attached in a custom attachment and scanned by a micro-computed tomography (micro-CT) imaging system (Quantum GX; PerkinElmer, Hopkinton, MA, USA) at an isotropic resolution of 40 μm. The images of each sample were quantitatively assessed with regard to both replacement root resorption and surface root resorption. In each coronal cross-section image (Figure 6a), absence of radiolucent area between alveolar bone and root surface were measured for replacement root resorption (Figure 6b) and distribution of craters or holes in root surface were measured for surface root resorption (Figure 6c) using MeshLab (Cignoni et al., 2008) 2020 (https://www.meshlab.net/). Images of each specimen were reconstructed with 3D Slicer (Fedorov et al., 2012) 4.11.0 (https://www.slicer.org/) that provided transaxial, coronal, and sagittal cross sections and visualization, including volume rendering of the samples (Figure 6d). The areas of replacement and surface root resorption were reconstructed three-dimensionally (Figure 6e and 6f), and the percentage of replacement and surface root resorption to whole root surface were calculated respectively.

2.5. Histologic evaluation

The specimen blocks were decalcified in 10% ethylenediaminetetraacetic acid solution (pH 7.4) at 4 °C for 4 weeks embedded in paraffin. Serial, 3μm-thick sagittal sections of selected roots were cut including the surrounding tissue. For histological analyses, the sections were stained with hematoxylin and eosin (H & E) and observed under light microscopy.

2.6 Statistical analysis

The percentages of surface and replacement root resorption measured from micro CT were compared among the four groups using a one-way analysis of variance followed by a Tukey's post-hoc test. All statistical analyses were performed under a 95% confidence level using the SPSS 25 (IBM Corp, Somers, NY, USA) software program.

3. RESULTS

3.1. Postoperative clinical observations

Postoperative clinical healing was uneventful at all sites. Pus discharge from the gingiva was noticed in some experimental teeth with buccal bone fracture at 2 weeks, which were excluded. No visible adverse reactions, including suppuration, abscess formation or increased tooth mobility, were observed in all roots after 4 weeks.

3.2. Amounts of root resorption

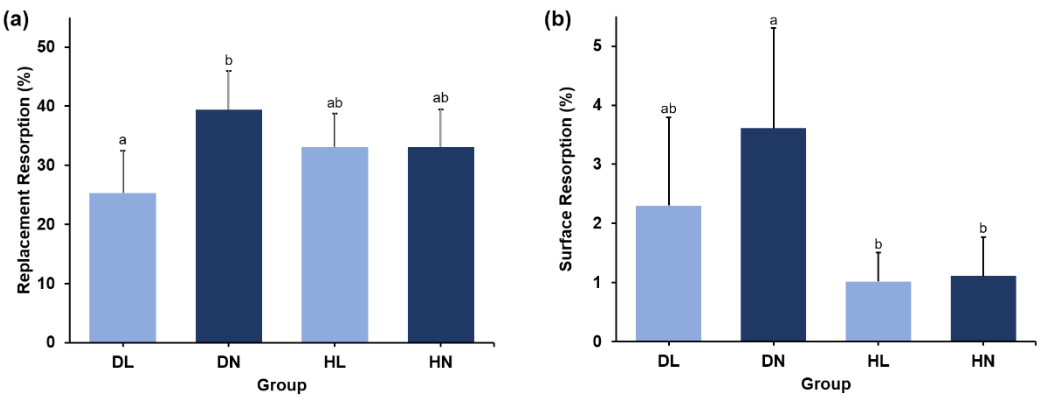

Figure 7. Percentage of root resorption area measured from µCT in each group. The percentages of resorption areas were compared among the four groups using a one-way analysis of variance followed by a Tukey's post-hoc test. The data are presented as the mean ±SD of each group. **(a)** Replacement resorption. **(b)** Surface resorption. Different alphabets (a, b) indicate significant difference among the groups ($p < 0.05$).

In dry storage conditions, there was significant less replacement root resorption in the LIPUS-treated group (DL) compared to the non-treated group (DN) ($p < 0.01$). Neither the DN nor the DL group was significantly different from the HL or HN group. However, the roots in the DN group tended to have the largest percent of replacement resorption, while those in the DL group had the least (Figure 7a). The representative radiographs and micro-CT images of roots in the HL, HN, DL and DN groups are shown in Figure 2, Figure 3, Figure 4 and Figure 5, respectively.

Both HBSS storage groups (HL and HN) had significantly reduced surface resorption compared to the DN group, regardless of LIPUS exposure ($p < 0.01$ and $p < 0.05$, respectively). There were no significant differences between the HBSS treated groups. Within the dry storage groups (DL and DN), the roots in the DL group showed decreased surface resorption compared to those in the DN group. However, there was no statistically significant difference. In addition, the DL group demonstrated no significant differences in surface root resorption compared to the HL or HN groups (Figure 7b).

Table 2 . Percentage of resorption area measured from micro CT

	Replacement resorption		**Surface resorption**	
	Mean (%)	±SD	Mean (%)	±SD
DL	25.35	14.7	2.30	2.99
DN	39.46	12.99	3.61	3.39
HL	33.11	11.35	1.02	0.98
HN	33.13	12.67	1.11	1.32

3.3. Descriptive histological results

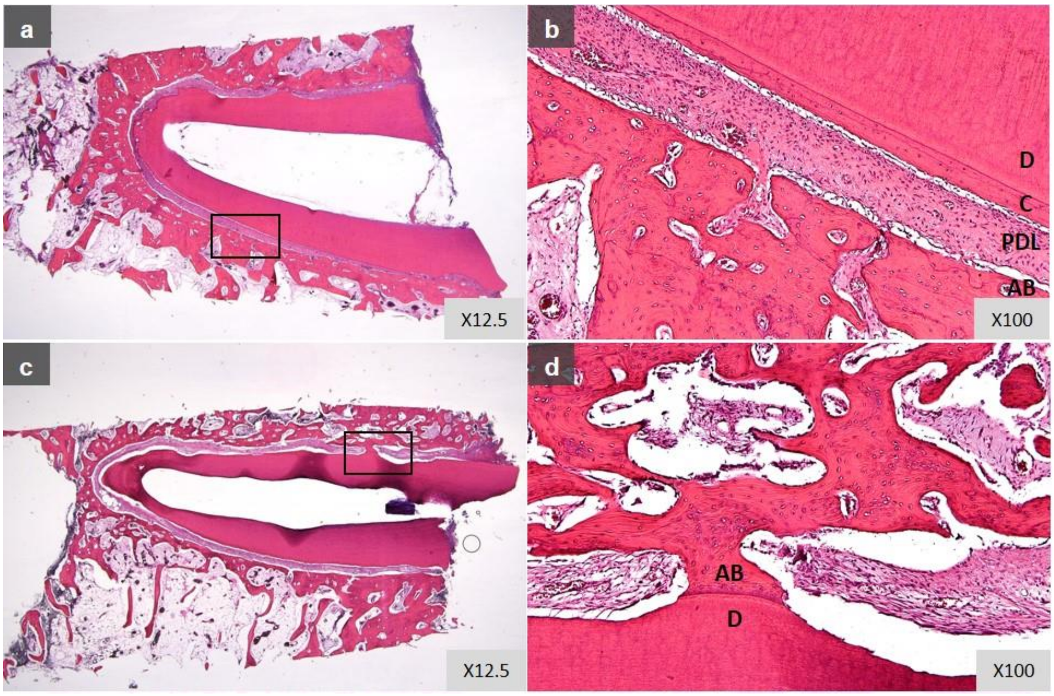

Figure 8. Histologic overview of sections of the replanted roots (HL, HN). **(a, b)** A root in the HL group, pointing to minimal root resorption. Note the normal periodontium organized PDL with collagen fibers and cementum layer. **(c, d)** A section of root in the HN group, showing localized fusion of alveolar bone and dentine, indicating replacement root resorption.

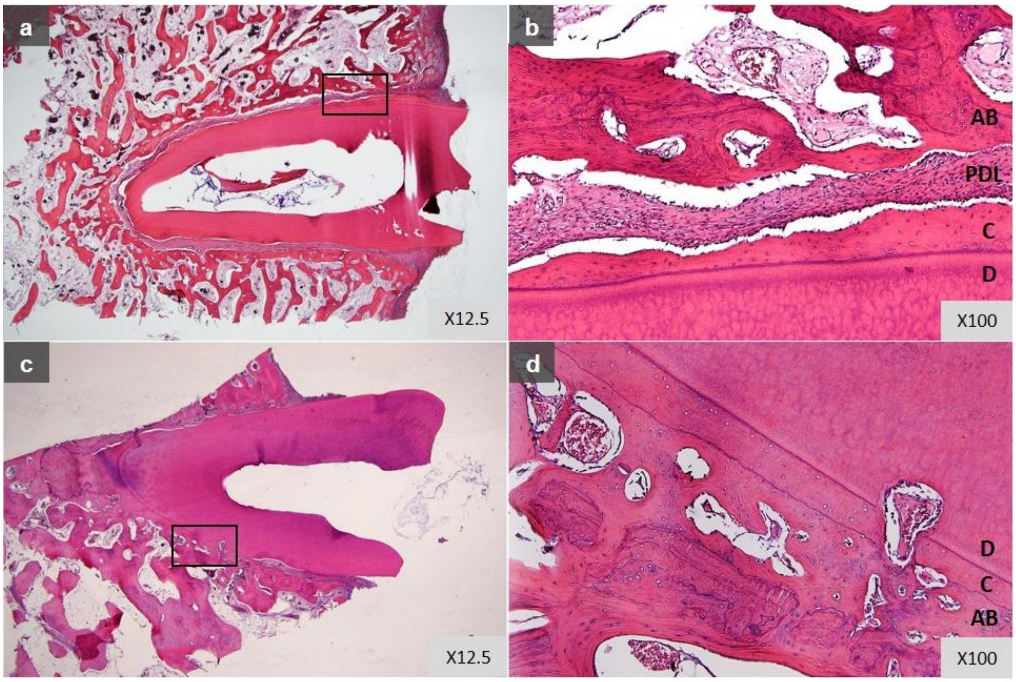

Figure 9. Histologic overview of sections of the replanted roots (DL, DN). **(a,b)** A root in the DL group, showing surface root resorption confined to cementum. The dentin shown is intact. **(c, d)** A root in the DN group, showing extensive PDL loss and fusion of cementum and alveolar bone.

Cross sections were evaluated for healing according to the criteria of Andreasen (Andreasen and Andersson 2011a). For most roots in the HL and HN groups, the cementum integrity could be observed with periodontal ligament (PDL) space preserved with relatively uniform thickness, where the root was clearly separated from the alveolar bone (Figure 8). Meanwhile, the DL group revealed a substantially inhibited development of severe root resorption lacunae compared to the DN group. Histological findings in the DN group demonstrated evident replacement root resorption, where the cementum of the root and alveolar bone was connected, with no clear border in between (Figure 9). Similar degree of slight surface resorption was shown in all groups, corresponding to the micro-CT analysis results.

4. DISCUSSION

This study focused on the effects of LIPUS treatment on replacement root resorption of avulsed teeth after replantation with different storage conditions and periods in dog models. Tooth avulsion situation of dog molars is simulated by extraction. In previous studies, the most frequent animal model for avulsion studies are dogs and monkeys, since wound-healing responses appears to be similar to humans (Cunha et al., 2002; Trope et al., 1995; Troope, Hupp, and Mesaros, 1997; Nethander, Skoglund, and Kahnberg, 2003; Andreasen and Andersson, 2011b). The PDL response of rats molars has been examined for trauma, but their intensive bone response to injury differs from that of human (Andreasen and Skouoaard, 1972). Previously conducted in vivo studies on effect of LIPUS on root resorption after avulsion have utilized rats. This study selected fully developed mongrel dogs for results resembling that of humans. Moreover, pulp extirpation and filling with root canal filling material followed by coronal obturation was carried out since immediate root canal treatment is recommended in the clinic in actual situations. This also allows more accurate experimental outcome, since toxin from the necrotic pulp which cause inflammatory root resorption is eliminated and thereby only surface resorption and replacement root resorption can be taken into account. Even though compressive damage of periodontal tissue may defer to that of real trauma and intentional extraction, it is considered neglectable in this study because the most crucial factor to periodontal ligament survival is the degree of its extraoral dehydration.

3D quantitative measurements of replacement root resorption and surface root resorption were carried out by micro-CT images to assess the effects of LIPUS on the healing of cementum and periodontal ligaments after a given trauma. In most previous studies, the amounts of root resorption were histologically measured (Andreasen and Andersson, 2011a). Histological measurements help estimate the proportion of the root resorption area, but one should keep in mind that root resorption is not evenly distributed on the root surface and may preferentially affect corner surfaces (Andreasen, 1987; Andersson et al., 1987). If a single axial section is used as a reference of resorption, one section direction might show only little root resorption, whereas another section may demonstrate much more resorption.

Recently, 3D images of the microarchitecture of bone specimens from different species and body parts have been obtained by using micro-CT. This produces high resolution images by micro-CT and digitally reconstructs the tooth and surrounding alveolar bone, allowing for the quantitative assessment of root surface defects or bony attachments (Sasai et al., 2014; Wang, Qiu, et al., 2018). Nevins et al., showed that micro-CT can be used for the 3D quantitative assessment of alveolar bone (Nevins et al., 2005). De Paula Reis et al., showed, when compared to histologic analyses, that micro-CT in in vivo studies can be a promising method for the identification of the different stages of tooth resorption and repair, in addition to the evaluation of the total extension of the periodontium (de Paula Reis et al., 2014). In this study, replacement root resorption, fusion between alveolar bone and root surface, and surface root resorption, craters or holes in root surface, were measured in each coronal cross-section image. The root resorption areas were reconstructed three-dimensionally and the percentage of resorption area to whole root surface were calculated. To the best of our knowledge, this is the first published report utilizing micro-CT to calculate the total surface area of a root to identify specific pathological parameters.

Dry storage for 60 minutes was a factor to increase damage to the periodontal ligament covering the root surface, according to the International Association of Dental Traumatology (IADT) guidelines for the management of avulsed permanent teeth where 60 minutes of extra-oral drying time is suggested as a criterion in making treatment plans and prognosis (Fouad et al., 2020). The length of dry extra-alveolar storage period regarded as one of the strongest factors on PDL healing after tooth avulsion, affecting the survival of the PDL cells along the root surface. The guidelines indicate that after an extra-alveolar dry time of 30 minutes, most PDL cells are non-viable (Barbizam et al., 2015; Andreasen 1981a). Andreasen et al. reported increasing rate of ankylosis after delayed replantation over 5 minutes (Andreasen, Borum, and Andreasen 1995). Maslamani et al., said that when the extra-oral dry time exceeds 60 minutes, most PDL cells do not survive, and surface root resorption or dentoalveolar ankylosis of the tooth can progress further (Maslamani et al., 2016).

This study's results showed that the surface resorption of the DN group was significantly higher than that of the HN and HL group ($p < 0.05$ and $p < 0.01$, respectively), possibly because of the low survival rates of PDL cells on the root surfaces, similar to those of previous studies (Andreasen, Borum, and Andreasen, 1995; Ehnevid et al., 1993). In contrast, the DL group did not show any significant differences, whether with DN or with

HN or HL. These results imply that LIPUS may positively affect regeneration of the periodontal tissue after intense avulsion conditions. However, since preventive root canal treatment measures were taken, the overall degree of surface resorption was minimal because of its self-limiting characteristics demonstrating repair with new cementum (Andreasen and Hjorting-Hansen, 1966; Andreasen and Andreasen, 1992). Most resorption lacunae are superficial and confined to the cementum, which is consistent with previous studies.

On the other hand, replacement resorption yielded results that are more definite. A significant reduction in replacement root resorption was found in the DL group compared to the DN group ($p < 0.01$). The HN and HL yielded similar outcomes. These results also implied that LIPUS might improve the recovery of cementum and periodontal tissue while necrotic PDL has undergone inflammatory change. These results are consistent with those of previous studies which showed that LIPUS accelerates the healing of resorption areas with the reparative cementum of orthodontic patients (El-Bialy, El-Shamy, and Graber, 2004) and stimulates the cementum regeneration of periodontal defects in canine models (Wang, Qiu, et al., 2018). Furthermore, several in vitro studies demonstrate that LIPUS has an anabolic effect on human periodontal ligament cells by promoting mature cementoblasts (Inubushi et al., 2008) and accelerating the differentiation of immature cementoblasts (Dalla-Bona et al., 2006). It has also been shown that LIPUS has the potential to accelerate endogenous periodontal mesenchymal stem cell recruitment for periodontal tissue regeneration on a molecular level (Wang, Li, et al., 2018). They also demonstrated the inhibitory effect of a 21-day LIPUS application on root resorption using an experimental model of tooth replantation involving luxation and the immediate replacement of maxillary first molars in rats (Rego et al., 2011).

After replantation, connective tissue cells proliferate, obliterating the gap in of damaged periodontal ligament, followed by epithelium reattachment on day 7. First superficial osteoclast attack on the root surface can be seen (Andreasen, 1980c). Resorption activity can be recognized along the root surface after 2 weeks, while the split line in the periodontal ligament is healed and collagen fibers extend from the cementum surface to the alveolar bone. The fusion of the alveolar bone and the root surface can be found histologically 2 weeks after replantation (Andreasen, 1980b). In bacterial free, ideal conditions, complete regeneration of the periodontal ligament usually takes about 4 weeks, including full recoverage of the nerve supply (Mandel and Viidik, 1989; Yamada et al., 1999a). Two weeks of LIPUS exposure aims to improve the first two phases of healing and interfere with root resorption process. A total of four weeks was given for full gingival recovery.

Until now, both laboratory and clinical trials have demonstrated that ultrasound can stimulate tissue repair and wound healing (Dyson, 1968; Ter Haar, 2007b). LIPUS exposure accelerates the inflammatory phase, consequently showing anti-inflammatory effect (Iashchenko, Ostapiak, and Semenov, 1994; Mukai et al., 2005b). LIPUS has also been shown to enhance collagen synthesis by fibroblasts in the proliferative stage (Mortimer and Dyson, 1988).

Fibroblasts are responsible for the formation, maintenance and remodeling of PDL fibers, and comprise a very active cell renewal system. It had been shown that the half-life of collagen in the PDL of rats is about 6-9 days (Orlowski, 1978), and is more rapid in PDL than in that of gingiva, pulp of other connective tissues (Kameyama, 1975). It has been shown that LIPUS exposure accelerates collagen synthesis by fibroblasts (Mortimer, 1988; Inubushi, 2008) thus contributing to accelerated healing of PDL.

Observations on the OPG/RANK/RANKL system in the PDL supports the hypothesis that PDL cells act as a barrier that prevents resorption of the roots by inhibiting the resorbing cells. Preosteoblasts express active signal molecules, RANKL (receptor activator of nuclear factor κB ligand), which activate mononuclear osteoclast progenitor cells when the receptor (RANK) attach to their surface. The osteoclast progenitor cells differentiate and merge, forming osteoclasts, which potentially may attack the root surface Osteoprotegerin (OPG), a soluble molecule able to bind and cover both the RANKL and the soluble sRANKL, is able to give protection to the root surface. The OPG/RANK/RANKL signaling pathway is able to both protect against or activate osteoclastic activity, and recent studies suggest this as a possible explanation for the protective action of the hard tissue covering cells (i.e. odontoblasts, cementoblasts and PDL cells). The OPG/RANKL ratio level can be upregulated or downregulated by growth factors, hormones and inflammatory factors which may explain root or bone resorption (Hofbauer, 2000; Bucay, 1998; Fukushima, 2003; Wise, 2004). LIPUS treatment has been shown to lower mRNA expression of TNF-α (Tumor Necrosis Factor α), which in turn lowers

RANKL mRNA expression, consequently effecting activation of osteoclast and cementoclast cells *in vitro* and *in vivo* (Rego, 2011). Also, it has been shown that LIPUS attenuates root resorption induced by orthodontic tooth movement by altering OPG/RANKL ratio and suppress cementoclastogenesis (Inubushi, 2013). LIPUS induced the formation of a precementum layer, thicker cementum and reparative cellular cementum in orthodontically induced root resorption in dogs (Al-Daghreer, 2014).

Recent studies shows evidence that LIPUS induce early cementoblastic differentiation of human immature cementoblasts from the PDL by increasing alkaline phosphatase (ALP) activity, accelerating the repair of root resorption (Inobushi, 2008). Cementoblastic differentiation is also induced by enhanced prostaglandin E_2 production through the activation of EP2/EP4 receptor pathway by LIPUS (Rego, 2010).

To exclude any potential factors that may influence or aggravate root resorption other than storage conditions, preoperative root canal treatment was performed and filled with calcium hydroxide paste to minimize the progression of inflammatory root resorption due to infection of necrotic pulp (Andreasen, 1981b). The presence of bacteria in the root canal and dentinal tubules has been shown to interfere with normal periodontal healing process, including new deposition of cementum and reformation of PDL fibers (Birn, 1966). In addition, decoronized roots were covered with gingiva as excessive initial force might cause severe root and bone resorption (Kiyokawa et al., 2017; Mine et al., 2005). The lack of a negative control was based on the fact that an ideal storage medium would preserve the teeth in an environment that is similar to that of an immediate replantation (i.e. the positive control). It is also based on the extensive literature showing higher levels of replacement resorption for teeth that have been kept dry (Panzarini et al., 2013; Andreasen, 1980a). HBSS has been shown to be the best storage medium during the extra-oral phase (Sigalas et al., 2004).

All experimental roots were intended for submucosal replacement and healing, some roots showed partial exposal of coronal part of roots to the oral cavity. No insufficient apical or coronal periodontal healing was observed, but transmucosal healing may be of different biology compared to submucosal healing. Furthermore, the periodontal healing response according to difference of maxilla and mandible alveolar bone should also be performed in future studies.

5. CONCLUSION

Within the limitations of this animal study, both the 3D quantitative measurements of micro-CT and the histopathological examination demonstrated that LIPUS stimulation helped prevent the replacement resorption of avulsed teeth after delayed replantation. These results highlight the therapeutic implications of ultrasound stimulation in periodontal regeneration, suggesting the potential of LIPUS to inhibit dentoalveolar ankylosis. Additional studies are warranted to evaluate and capitalize on its full potential in the orofacial area. This animal study demonstrates one aspect of the application between therapeutic ultrasound and dental research. Considering that the ultrasound is actively used in medical fields, it could certainly contribute to the advancement of dental research.

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SUPPLEMENTARY TABLE

Supplementary table 1. Allocation and number of roots included before operation and after 4 weeks. Congenital missing, root fracture or buccal bone fracture during tooth extraction were excluded from initially assigned experimental groups. Pre-operative (pre) numbers indicate initially allocated root numbers while post-operative (post) numbers are the final number of roots used for resorption area measurements.

		dog #1		dog #2		dog #3		dog #4		total	
		pre	post	pre	post	pre	post	pre	post	pre	post
HL	Mx.	3	3	4	1	0	0	2	2	9	6
	Mn.	3	0	4	3	7	6	4	4	18	15
	Sum	6	3	8	4	7	6	6	6	27	21
HN	Mx.	2	2	3	3	5	4	2	0	12	9
	Mn.	4	3	3	3	0	0	4	2	11	8
	Sum	6	5	6	6	5	4	6	2	23	17
DL	Mx.	2	1	1	1	0	0	3	2	6	4
	Mn.	4	4	3	3	7	4	3	3	17	14
	Sum	6	5	4	4	7	4	6	5	23	18
DN	Mx.	3	3	2	0	5	5	3	3	13	11
	Mn.	3	1	4	4	0	0	3	1	10	6
	Sum	6	4	6	4	5	5	6	4	23	17

ABSTRACT (KOREAN)

탈구 및 재식 된 개 치아에서 저강도 진동 초음파의 대치성 치근 흡수의 저해 효과에 대한 평가

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치아 완전 탈구는 여러 치아 외상 중 가장 심각한 것에 속한다. 대부분의 재식 된 치아는 외상성 탈 구 이후 치근 표면흡수, 염증성 치근 흡수 또는 대치성 치근 흡수를 일으킨다. 그 중에서도 광범위한 대치성 치근 흡수는 치아 예후에 아주 심각한 영향을 미치는데, 이것은 탈구된 치아의 적절한 구강위 처치 및 재식, 레진 강선 고정 그리고 근관치료 후에도 발생한다. 저강도 진동 초음파는 뼈와 치주조직의 치유를 촉진한다는 사실이 이전의 여러 연구들에 의하여 밝혀졌다. 따라서 본 연구에서 우리는 완전탈구된 후 건조된 개의 치아에서, 저강도 진동 초음파가 손상된 치주인대 세포로 인해 발생하는 대치성 치근 흡수에 미치는 영향을 알아 보고자 한다.

본 연구는 약 10개월령, 25kg의 잡견 4마리의 소구치 73개를 이용하였다.
소구치들은 건조 유무와 저강도 진동 초음파 적용 유무에 따라 총 4개의 군으로

분류되었는데(HN, HL, DN, DL), 상악과 하악, 좌우가 균등하게 배정되었다. 우선 소구치들을 근관치료 후 겸자로 발치하였고, 각 정해진 군에 따라 Hanks' balanced salt solution 에 보관하거나 60분간 건조되도록 방치하였다. 이후 치아를 원위치에 재식하고, 정해진 그룹에 따라 2주간 매일 20분씩 저강도 진동 초음파를 적용하였다. 이후 2주간의 회복기가 지난 후 동물들을 희생하여 치아가 포함된 치조골을 채취, 미세 전산화 단층촬영과 조직학적 분석을 시행하였다. 대치성 치근 흡수 면적은 미세 전산화 단층촬영을 통해 3차원적으로 분석하였다.

건조 후 재식 된 치아 군에서, 저강도 진동 초음파 처치를 받은 치아군(DL)이 처치를 받지 않은 치아군(DN)보다 통계적으로 유의하게 적은 대치성 치근 흡수를 나타냈다($p < 0.01$).

조직학적 분석에서도 DN 군은 명확한 대치성 치근 흡수를 보인 반면, DL 군은 상대적으로 적은 대치성 치근 흡수를 나타내었다.

표면 흡수의 경우, 건조된 후 저강도 진동 초음파 처치를 받지 않은 군(DN)에서 HBSS에 보관된 그룹(HL, HN)에 비해 통계적으로 유의하게 더 많이 진행된 경향을 보였다. 그러나 표면흡수는 전체적으로 매우 낮은 비율을 보였으며 백악질에 국한되었고, 이는 발거 이전에 예방적으로 진행된 근관 치료로 인한 것으로 보인다. 이러한 결과는 기존에 진행된 다른 연구들과 일치한다.

본 연구에 따르면 탈구 후 장시간 구강 외에서 건조 된 치아에서, 구강 내 재식 후 저강도 진동 초음파 적용을 하는 것이 추후 해당 외상치의 대치성 치근 흡수를 줄일 것으로 보이며, 따라서 더 좋은 예후를 보일 것으로 예상된다.

핵심 되는 말: 대치성 치근 흡수, 저강도 진동 초음파, 치근 유착, 치근 흡수, 치아 재식, 치주인대, 치주 치유