Evaluation of hemostatic agents containing aluminum chloride: From *in vitro* studies to clinical outcomes in endodontic microsurgery

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Yae Lim Kim

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Abstract

Evaluation of hemostatic agents containing aluminum chloride: From *in vitro* studies to clinical outcomes in endodontic microsurgery

The aims of this study were (1) to evaluate cytotoxicity of aluminum chloride containing hemostatic agents, Expasyl (Pierre Rolland, Merignac, France) and Traxodent (Premier Dental, Plymouth, PA, USA), on human osteoblasts (HOBs, Promocell, Hidelberg, Germany), (2) to investigate their hemostatic efficacy and tissue reaction in standardized bone defects of rabbit calvaria, and (3) to assess their effects on outcome of apical surgery in a randomized controlled trial.

In *in vitro* assessment, cell viability of HOBs cultured in high and low concentration of material extract medium was evaluated by Cell Counting Kit-8 (CCK-8). The expression levels of osteogenic specific gene alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) and inflammatory specific gene interleukin (IL)-6, IL-8, and transforming growth factor (TGF)-β were analyzed by Quantitative Real-Time Polymerase Chain Reaction (RT-PCR).

In *in vivo* study, six round bone defects were surgically created in the calvaria of six New Zealand rabbits. The defects in each rabbit were randomly assigned to one of the following 6 groups: control, epinephrine impregnated cotton with pressure, Expasyl with curettage and saline irrigation, Expasyl with saline irrigation only, Traxodent with curettage and saline irrigation, and Traxodent with saline irrigation only. All hemostatic agents were applied for 2 minutes and removed accordingly. Three examiners evaluated and scored the amount of bleeding prior to and following the application of hemostatic agents by analyzing photographs taken during surgical procedures. Hemostatic efficacy was then determined by the difference in mean initial and final bleeding score. Histological analysis was conducted on the samples using two staining methods: Hematoxylin and eosin (HE) and Masson-Goldner trichrome. These techniques were employed to assess tissue responses and bone regeneration in relation to hemostatic agents applied.

A clinical investigation was structured as a randomized controlled trial with three parallel groups depending on hemostatic agents used. Endodontic microsurgery procedures were performed in adherence to the Yonsei protocol described in a previous study and operated by three operators. Hemorrhage control was evaluated as adequate or inadequate by the operator and two independent examiners. The participants were scheduled for recall visits at 6 and 12 months and the outcomes were assessed based on radiographic and clinical measures at 12-month follow-up.

Cell viability of HOBs cultured in Expasyl or Traxodent significantly decreased with a time interval from 2 minutes to 1 hour. The expression of TGF-B significantly decreased and the expression of IL-8 significantly increased in the Expasyl group only. Both RUNX-2 and OCN expressions were significantly reduced in the Expaysl and Traxodent group. Expasyl proved to be most efficient in bleeding control, but it also induced the most inflammation and caused delay in bone healing. Traxodent was relatively easily removed and did not interfere with bone healing; however, it did not exhibit a statistically significant hemostatic effect when compared to the control defects. In the clinical trial, 83 patients were evaluated at 12-month follow-up and the overall success rate of endodontic microsurgery was 91.6%. Statistical analysis revealed no significant difference in surgery outcome depending on the types of hemostatic agents used. The results of this study suggest that Expasyl can be used as additional hemostatic agent if adequate hemostasis is not achieved with epinephrine alone during apical surgery; however, due to its cytotoxicity and potential to cause adverse tissue reactions, Expasyl should be removed as thoroughly as possible after application. Although the main composition of Traxodent is also 15% aluminum chloride, it was not as efficient in bleeding control; therefore, Traxodent is not an appropriate substitute for Expasyl as a hemostatic agent in periapical surgery.

Keywords: endodontic surgery; periapical surgery; hemostasis; hemostatic agents; aluminum chloride; Expasyl; Traxodent

I. Introduction

Apical surgery is a reliable treatment option in cases with persistent periapical lesions where nonsurgical retreatment is not feasible. Introduction and continued development of microsurgical principles and root-end filling materials resulted in improved success rate of apical surgery over time, which have approached 90% or above (von Arx, 2010). Among several prognostic factors in apical surgery, the quality of the retrograde filling is one of intra-operative factors that may influence the outcome of treatment (Friedman, 2005). Hermetic sealing of the root canal is critical as the main objective of surgery is to promote apical healing by confining irritants within the root canal. In order to achieve the tight seal of the root canal system, hemostasis is a crucial step in apical surgery for assessment of resected root surface and placement and setting of root-end filling material (Kim & Kratchman, 2006). Failure to achieve adequate hemostasis can impede visibility in the surgical site, extend operating time, diminish the quality of surgical procedures, and increase intraoperative and postsurgical bleeding, and swelling (Witherspoon & Gutmann, 1996).

Many hemostatic agents have been used to control hemorrhage in apical surgery, including bone wax (Seldon, 1970), calcium sulfate (Scarano et al., 2012), collagen-based products (Eliyas et al., 2014; Witherspoon & Gutmann, 1996), ferric sulfate (Lemon et al., 1993; Vickers et al., 2002), vasoconstrictors (e.g. epinephrine) (Menendez-Nieto et al., 2016; Vickers et al., 2002; Vy et al., 2004), aluminum chloride (Menendez-Nieto et al., 2016; Penarrocha-Oltra et al., 2019; Penarrocha-Oltra et al., 2020), and electrocauterization (Penarrocha-Oltra et al., 2019). Kim et al. (1997) suggested use of epinephrine pellet with pressure for 2 minutes to achieve hemostasis with additional use of ferric sulfate on small bleeding sites after initial hemostasis. Ferric sulfate has been found to be an effective hemostatic agent with its ability to coagulate blood; however, its extended use and incomplete removal from the surgical site can result in significant inflammation and delayed healing (Jeansonne et al., 1993).

Von Arx et al. (2006) proposed a novel application for Expasyl (Pierre Rolland, Merignac, France), which is a paste originally designed for gingival retraction (Dederichs et al., 2019), as hemostatic agent in periapical surgery. In rabbit calvarial defect model, von Arx studied the hemostatic capability and associated tissue responses of bone wax, aluminum chloride, and ferric sulfate. Expasyl proved to be the most efficient in controlling hemorrhage but

inflammatory and foreign body tissue reaction was caused by remaining paste in the bone defect (Jensen et al., 2010; von Arx et al., 2006). Expasyl is composed of two key components that contribute to its hemostatic efficacy: kaolin, a type of white clay, is responsible for maintaining the consistency of the paste to provide mechanical pressure while aluminum chloride enhances its hemostatic effect. High viscosity of Expasyl makes it a good gingival retraction paste when used as an alternative to gingival cord to capture a detailed impression of the preparation margin. In case of its use as hemostatic agent in bone cavity, however, consistency of the paste makes it difficult to remove which can result in prolonged inflammatory and foreign body tissue reaction. Jensen et al. (2010) suggested removing the outermost layer in the bone defect using a rotating instrument to reduce the adverse tissue reaction caused by remaining material. Use of a rotary instrument may be effective in removing Expasyl after the application, but it can lead to unnecessary bone loss.

Traxodent (Premier Dental, Plymouth, PA, USA) is also an aluminum chloride containing paste used for cordless gingival retraction in prosthetic and restorative dentistry (Dederichs et al., 2019). According to the manufacturer, Traxodent is a soft paste that can be rinsed without leaving a residue due to its low viscosity. It could be used as an alternative to Expasyl in apical surgery, as its main hemostatic component is also 15% aluminum chloride, but with a physical property that makes it easier to remove from the bone cavity. If Traxodent shows similar hemostatic efficacy to Expasyl, it could be a more desirable hemostatic agent. The aim of this study was to evaluate cytotoxicity of Expasyl and Traxodent, on human osteoblasts, which might have adverse effect on bone healing after apical surgery. Furthermore, their hemostatic efficacy and tissue reactions were evaluated in standardized rabbit calvarial bone defects. Finally, the influence of aforementioned hemostatic agents on the outcome of periapical surgery was evaluated in a randomized controlled trial.

II. Materials and methods

1. *in vitro* study: cytotoxicity of different hemostatic agents on human osteoblasts

1.1. Preparation of conditioned medium

To evaluate the short-term effect, Expasyl and Traxodent conditioned medium were prepared in sterile conditions by combining them with complete medium (DMEM, Gibco, Grand Island, NY, USA) at a high concentration of 200 μg/ml. Prepared medium were incubated for 24 hours in an incubator and filtered using 0.20 μm filters (Minisart; Sartorius Stedim Biotech, Goettingen, Germany). For making the low concentration condition medium to observe the long-term effect, the high concentration medium was diluted by adding complete medium to the concentration of 5 μg/ml.

Table 1. Chemical composition of materials used in this study

Material	Composition	Manufacturer
Bosmin solution	Epinephrine, sodium chloride, sodium sulfite, 35% hydrochloric acid, chlorobutanol, distilled water	Jeil Pharmaceutical Co., LTD; Seoul, Korea
Expasyl	Aluminum chloride hexahydrate 15%, kaolin 70%	Acteon Group - Pierre Rolland; Merignac, France
Traxodent	Aluminum chloride 15%, silicon dioxide, potassium nitrate, potassium sorbate	Premier Dental; Plymouth, PA, USA

1.2. Cell viability test

Human osteoblasts (HOBs, Promocell, Hidelberg, Germany) were seeded in a 96-well plate at a density of 1.5 x 10^4 cells per cell. Following a 24-hour period for cell adhesion, the culture medium was replaced with conditioned medium. Cell viability was analyzed by Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) following the manufacturer's instructions at various time points to evaluate both immediate and prolonged effects. Measurements were made at 2 minutes, 10 minutes and 1 hour post-exposure to assess the short-term effect and at 1 day, 3 days, and 7 days to evaluate the long-term effect. A spectrophotometer (VersaMaxMultiplate Reader, Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the plates at 450 nm.

1.3. Inflammatory and osteogenic gene expression and osteogenesis evaluation

HOBs were cultured in a 6-well plate (SPL) at an initial seeding density of 1 X 10^5 cells per well in a complete medium (DMEM; Gibco) until they proliferated to 80% to 90% confluence. The cells were then incubated for 3, 7, and 14 days in the osteogenic induction medium, which is a complete medium supplemented with 100 nM Dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 1.8 mM KH_2PO_4 (Sigma-Aldrich), 10mM B-Glycerol phosphate (Sigma-Aldrich), and 100uM L-ascorbic acid 2-phosphate (Sigma-Aldrich). Cells maintained in basal medium were used as a control and conditioned medium was replaced at 2-day intervals.

The expression levels of osteogenic specific gene alkaline phosphatase (ALP), osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) and inflammatory specific gene interleukin (IL)-6, IL-8, and transforming growth factor (TGF)-β were analyzed by Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). The mRNA expression levels of ALP, OCN, RUNX2, IL-6, IL-8 and TGF-β were quantified using the β-actin gene as endogenous control for normalization. Extraction of mRNA was conducted using the RNeasy mini kit (Qiagen) and reverse transcription into cDNA was performed with 1000 ng RNA using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific) following the manufacturer's instructions. Then, qPCR was performed with the QuantStudio 3 system (Applied Biosystems, Foster City, CA, USA). The expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Following a 7-day period of osteogenic induction, alkaline phosphatase (ALP) staining

was performed using the ALP staining Kit (Sigma-Aldrich). In addition, after 14 days of induction, the amount of mineralization was evaluated by Alizarin Red staining with Alizarin Red S solution (ACROS, Gyeonggi-do, Korea) according to the manufacturer's instructions.

1.4. Statistical analysis

Each experimental procedure was conducted three times, with the resulting data expressed as means accompanied by their corresponding standard deviations (SD). Differences among groups were analyzed with Kruskal-Wallis test using the SPSS Statistical Software version 29 (IBM Corp., Armonk, NY, USA), and a P value < 0.05 was considered statistically significant.

2. *in vivo* study: evaluation of hemostatic efficacy and tissue reactions in rabbit calvarial defects

2.1. Study design

Six round defects measuring 5 mm in diameter and 1.5 mm in depth were produced in the calvaria of rabbit, three on each side of the sagittal suture. The six defects received one of the following treatments in a randomized sequence to minimize potential bias from the anatomical positioning of bone defects on hemostatic effects. One group of three animals was sacrificed 3 weeks after the surgery, and a second group of three animals was sacrificed 12 weeks after the surgery.

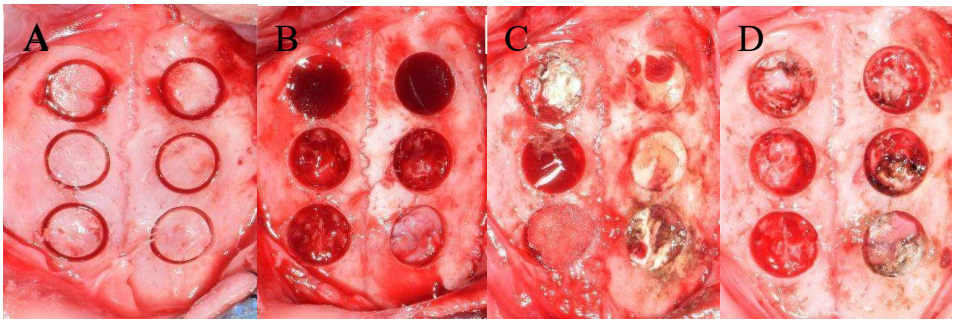

Figure 1. Six standardized defects in rabbit calvarium. (A) After circular defects were drilled with a bone trephine. (B) Before application of hemostatic agents. (C) After placement of hemostatic agents. (D) After removal of hemostatic agents

- Control: no hemostatic agent was placed
- Epinephrine: epinephrine (Bosmin solution) cotton pellet was placed in the bone defect with pressure for 2 minutes and the pellet was removed.
- Expasyl and saline irrigation only: Expasyl was placed into the bone defect and flushed with the adjacent cortex using a spatula. After 2 minutes, the paste was removed with saline irrigation only.
- Expasyl and curettage: Expasyl was placed into the bone defect and flushed with the adjacent cortex using a spatula. After 2 minutes, the paste was removed with a dental curette and saline irrigation.
- Traxodent and saline irrigation only: Traxodent was placed into the bone defect and flushed with the adjacent cortex using a spatula. After 2 minutes, the paste was removed with saline irrigation only.
- Traxodent and curettage: Traxodent was placed into the bone defect and flushed with the adjacent cortex using a spatula. After 2 minutes, the paste was removed with a dental curette and saline irrigation.

2.2. Surgical procedure

Approval of the study design was granted by the authorities of the Department of Laboratory Animal Resources, Yonsei Biomedical Research Institute, Yonsei University College of Medicine (approval number 2018-0109). The research was conducted in six mature New Zealand white rabbits at least 16 weeks old and weighing between 3.5 and 4 kg.

The surgical procedure in this study was determined based on previous studies (Jensen et al., 2010; von Arx et al., 2006). All procedures were performed under general anesthesia. The animals were medicated with ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg) by intramuscular injection into the hindleg. Each animal was administered meloxicam (0.3 mg/kg) and enrofloxacin (5 mg/kg) subcutaneously. Postoperatively, the animals were given the same analgesics and antibiotics once a day subcutaneously for 3 days.

The calvarial area was shaved and the skin was disinfected using a povidone-iodine solution (Betadine®). A midline cranial incision was made after the subcutaneous injection of 2% lidocaine with 1:100,000 epinephrine for local anesthesia and the periosteal flap was reflected to expose the calvarium. Six round bone defects (diameter 5mm, depth 1.5mm)

were drilled into the outer cortical bone with a trephine bur under copious saline irrigation. Drilling was done with extra caution to avoid perforation of the inner cortex which would lead to exposure of the dura mater. Three bony defects on each side of the sagittal suture were created after removing the outer cortical bone plates with surgical curette.

One group of three animals was sacrificed after 3 weeks, and a second group of three animals after 12 weeks. Following the sedation with alfaxalone (6 mg/kg, IM) and xylazine (5 mg/kg, IM), a cannula was inserted into the lateral ear vein. After additional administration of alfaxalone (3 mg/kg, IV), death was induced with potassium chloride (250 mg/kg). After a skin incision, the calvarium specimens were retrieved with an oscillating autopsy saw and immediately immersed in a 4% formaldehyde solution.

2.3. Visual analysis of hemorrhage control

Three evaluators independently examined the photos taken prior to and following the application of hemostatic agents to give each defect bleeding score. Bleeding intensity at each site was evaluated using a 0-7 scale, where 0 indicated no bleeding and 7 represented severe hemorrhage, as established by von Arx et al. (2006). Hemostatic effect was then determined by the average difference between the initial and final bleeding score.

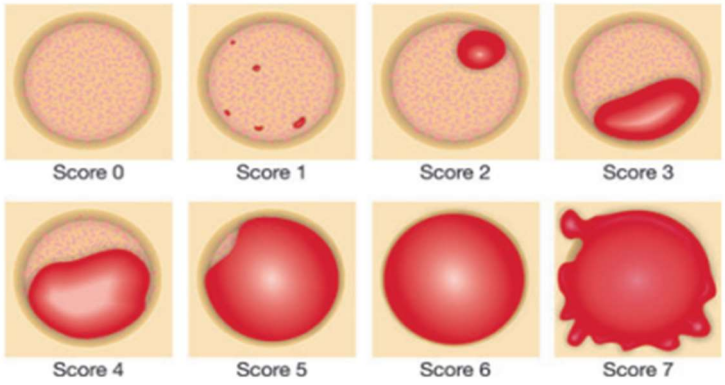

Figure 2. Schematic illustration used for visual assessment of bleeding (von Arx et al., 2006).

2.4. Histological analysis

Specimens were decalcified in 5% formic acid for 2 weeks and embedded in paraffin wax. Embedded specimens were serially sectioned along 5-mm diameter round defect at 5-μm-thick sections. Mayer's hematoxylin and eosin (H&E, Cancerdiagnostics, Durham, NC, USA) and Masson-Goldner trichrome (Biognost, Zagreb, Croatia) were used for staining, and the slides were observed under 4x to 10x magnification with a light microscope (OlympusBX43) for qualitative analysis.

2.5. Statistical analysis

Commercially available software (SPSS version 29, IBM Corp., Armonk, NY, USA) was used for the statistical analysis and the results were expressed as means and standard deviation for each experimental group. The Kruskal-Wallis test was used to determine whether there are any statistically significant differences between means of reduction in bleeding score depending on the hemostatic agent. Additionally, Mann-Whitney U test was performed to detect differences between hemostatic agents. Fleiss Kappa values were calculated to evaluate inter-rater agreement on initial and final bleeding score. A P-value < 0.05 was considered statistically significant.

3. Clinical study

3.1. Subject enrollment and inclusion/exclusion criteria

The study design was approved by the Institutional Review Board of Yonsei University Dental Hospital (IRB number: 2-2018-0017). Patients were recruited from the Department of Conservative Dentistry at College of Dentistry, Yonsei University, Seoul, Korea between July 2018 and May 2020. After screening, patients were thoroughly informed about the study and those who consented to participate were asked to fill out a written consent.

Inclusion criteria for this study were periapical lesions measuring less than 10mm in diameter and involving a single tooth. Criteria for exclusion were patients with anticoagulant therapy, apicomarginal defects or probing depth greater than 6mm, teeth with vertical root fracture, or incomplete protocols.

3.2. Sample size calculation

The required sample size was determined using G power software (Universität Düsseldorf, Düsseldorf, Germany, version 3.1.7). For an effect size of 0.3, significance level 5%, and statistical power 85%, a sample size of 111 patients was estimated. Considering dropout rate of 20%, the target sample size was 140 patients.

3.3. Randomization and hemostasis evaluation

Allocation to epinephrine, Expasyl, or Traxodent was made based on a randomization list created using randomization.com website. The treatment group allocation was withheld from the operators until the hemostasis step during surgery. The surgeon evaluated the adequacy of hemostasis during surgery and documented it as adequate or inadequate. Based on the studies by Vy et al. (2004) and Azargoon et al. (2011), adequate hemostasis was defined as complete bleeding control, providing a hemorrhage-free operating field, and inadequate hemostasis was defined as mild, sporadic bleeding that continued after application of the material. Two independent blinded examiners later evaluated hemostatic efficacy and recorded the value by watching the video recorded by the camera installed on a dental microscope.

3.4. Surgical procedure

Surgical procedures were performed by three operators according to the Yonsei protocol described in a previous study (Song & Kim, 2012). All operations were carried out under surgical microscope (OPMI PICO; Carl Zeiss, Göttingen, Germany) except incision, flap reflection, and suture. Patients were prescribed preoperative anti-inflammatory drugs and antibiotics: oral amoxicillin (250 mg) and ibuprofen (400 mg) three times daily.

Infiltrative local anesthesia was accomplished with 2% lidocaine with 1:800;000 epinephrine. Following the incision and flap reflection, osteotomy was performed to expose the periradicular area. After removing granulation tissue, root was resected approximately 2 to 3 mm from the apex with no or minimal bevel angle using a no.170 tapered fissure bur under abundant sterile distilled water irrigation. The resected root surfaces were inspected under 20x to 26x magnification with micromirrors (ObturaSpartan, Fenton, MO, USA) after methylene blue staining to evaluate the quality of resection and search for any overlooked anatomic details such as fins and isthmuses. Retrograde cavity was then prepared to a 3-mm depth using KIS ultrasonic tips (ObturaSpartan).

Hemostasis of the bone cavity in epinephrine group was performed by applying sterile

cotton pellets saturated with 0.1% epinephrine (Bosmin; Jeil Inc, Seoul, Korea) with pressure for 2 minutes. In Expasyl group, Expasyl was applied to the bony crypt using an applicator gun provided by the manufacturer. After 2 minutes, Expasyl was removed using a curette accompanied by copious sterile saline irrigation to minimize the amount of hemostatic material left in the bony crypt. In Traxodent group, Traxodent was applied with its bendable syringe tip and left in the cavity for 2 minutes and removed with a curette and sterile saline irrigation as well. The root-end cavities were incrementally filled with RetroMTA and primary wound closure was accomplished with 5-0 monofilament sutures.

3.5. Follow-up and outcome assessment

Outcome was evaluated based on clinical and radiographic findings. On recall visits, clinical examination was conducted to evaluate any abnormalities or symptoms such as mobility, pain, periodontal pocket formation, sinus tract formation, swelling, tenderness to palpation or percussion, or subjective discomfort. The radiographs taken at 12 months follow up were assessed by two independent examiners using the criteria by Molven et al. (1987). The observations of radiographs were classified into complete healing, incomplete healing (scar tissue), uncertain healing, or unsatisfactory healing (failures).

The criteria for success included the absence of clinical signs and/or symptoms and radiographic evidence of complete or incomplete healing. Failure was defined as any clinical signs and/or symptoms or radiographic evidence of uncertain or unsatisfactory healing.

3.6. Statistical analysis

Hemostatic efficacy was analyzed using Kruskal-Wallis test and the difference between each group was compared with Mann-Whitney U test. A Fisher's Exact test was conducted to analyze and compare the success rate of endodontic microsurgery depending on the hemostatic agent used (epinephrine, Expasyl, or Traxodent). Fleiss Kappa values were calculated to evaluate inter-rater agreement on adequacy of hemostasis during surgery. SPSS Statistical Software version 29 (IBM Corp.,Armonk, NY, USA) was used and a P-value $< .05$ was considered significant.

III. Results

1. *in vitro* study

1.1. Cell viability test

Cell viabilities of HOBs cultured with high concentration of material extract conditioned medium to evaluate short-term effects of each material are shown in Figure 3. Cell viability decreased as incubation time increased from 2 minutes, 10 minutes, to 1 hour in both Expasyl and Traxodent groups. After an hour of incubation in conditioned medium for both groups, cell viability optical dentistry was close to 0, meaning almost no cells were viable. In other words, there was a linear correlation between longer time intervals and increased cytotoxity for Expaysl and Traxodent in short-term effect. After 1 day, 3 days, and 7 days of incubation, however, no statistically significant difference was observed between control, Expasyl, and Traxodent.

1.2. Inflammatory and osteogenic gene expression and osteogenesis evaluation

1.2.1. Inflammatory gene expression

Expression of inflammatory cytokines, IL-6, IL-8, and TGF-β, is shown in Figure 4. The expression of TGF-β significantly decreased and the expression of IL-8 significantly increased in the Expasyl group only. There was no significant difference in expression of IL-6 cytokine in either group.

1.2.2. Osteogenic gene expression

To analyze the effect of hemostatic agents on osteogenic activity in human osteoblasts, ALP, RUNX-2 and OCN expressions were measured. Both RUNX-2 and OCN expressions were significantly reduced in the Expaysl and Traxodent group (Figure 5). There was no significant difference in ALP expression, which matched the result of ALP staining (Figure 6).

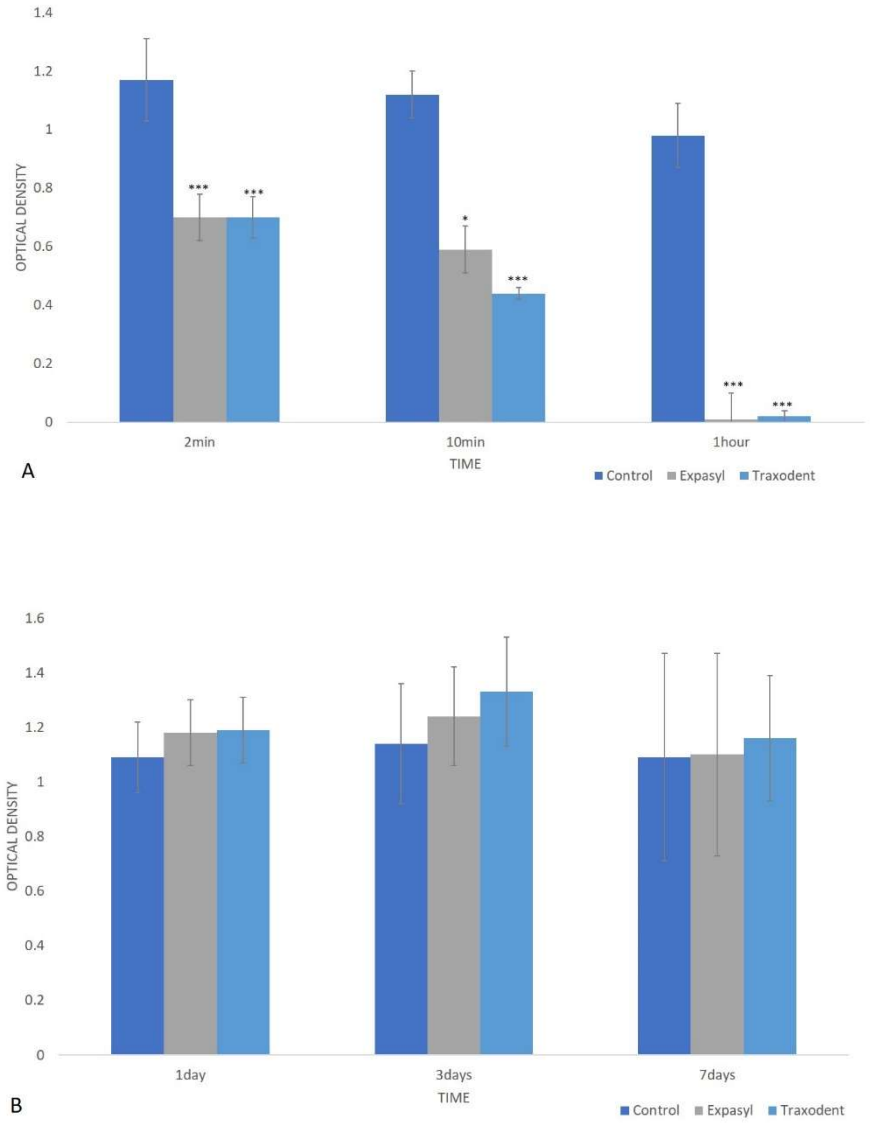

Figure 3. Cell viability of human osteoblasts cultured with (A) high concentration of material extract conditioned medium and (B) low concentration of material extract conditioned medium. Absorbance values were measured at 450nm using the Cell Counting Kit-8 assay. Cell viability significantly decreased in both Expasyl and Traxodent groups from 2 minutes, 10 minutes, and 1-hour of incubation. (* $p < .05$, *** $p < .001$)

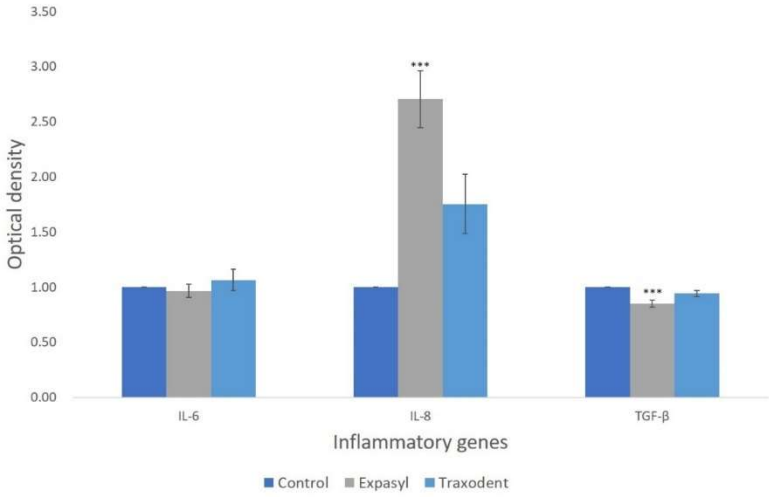

Figure 4. Human osteoblast inflammatory gene expression. There was no significant difference between the groups in IL-6 expression. Expression of IL-8 was significantly increased in Expasyl group and TGF β was significantly decreased in Expasyl group. (*** p< .001)

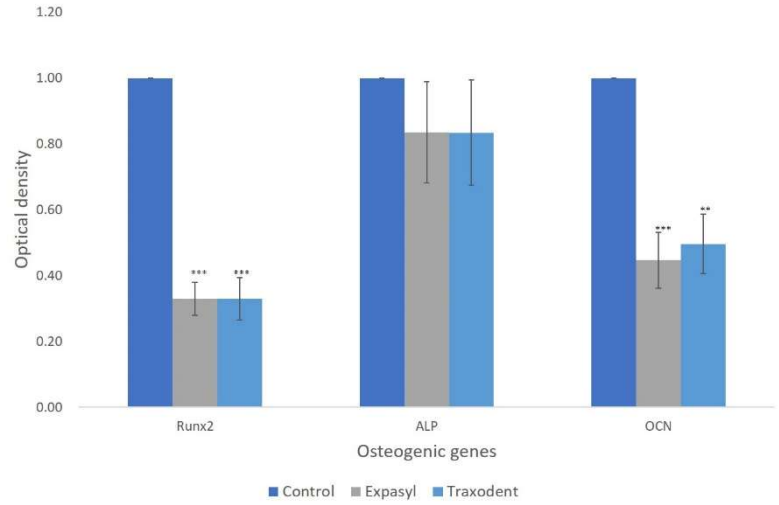

Figure 5. Human osteoblast osteogenic gene expression. Expression of RUNX2 and OCN was significantly decreased in Expasyl and Traxodent groups. No significant difference was observed in expression of ALP. (** p < .01, *** p< .001)

1.2.3. ALP and ARS staining

After 7 days of HOB incubation in material extract medium, ALP staining was performed. As shown in Figure 6, relatively less area is stained for HOBs cultured in Expasyl and Traxodent extract medium compared to the positive control. The difference, however, is not as pronounced as in ARS staining conducted after 14 days of incubation (Figure 7). HOBs cultured with Expasyl and Traxodent extract medium displayed significantly decreased ARS-stained area compared to the positive control. The result of the quantitative analysis of ARS staining is shown in Figure 8. The optical density values of Expasyl and Traxodent group were significantly decreased, which indicates decreased formation of mineral deposits.

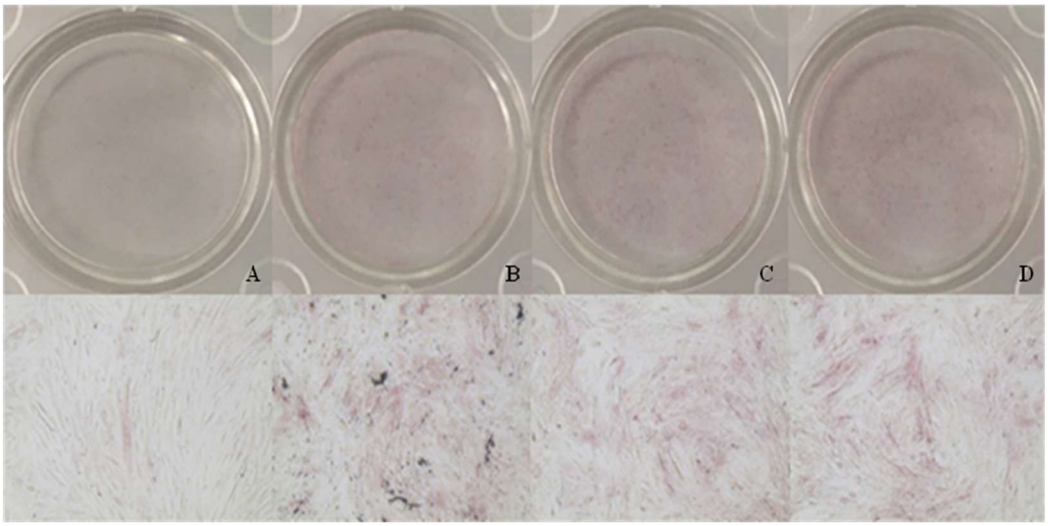

Figure 6. ALP staining of human osteoblasts cultured with material extract medium. ALP staining was performed after 7 days. (A) Negative control, (B) Positive control, (C) Expasyl, (D) Traxodent. No remarkable difference is observed in (C) Expasyl and (D) Traxodent groups compared to control group.

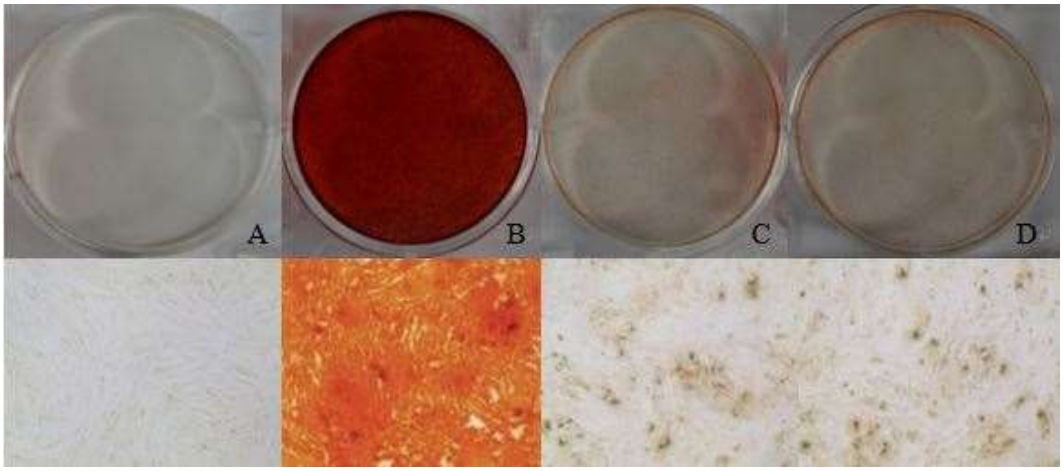

Figure 7. ARS staining of human osteoblasts cultured with material extract medium. ARS staining was performed after 14 days. (A) Negative control, (B) Positive control, (C) Expasyl, (D) Traxodent. Less mineral deposits are observed in (C) Expasyl and (D) Traxodent groups compared to (B) positive control.

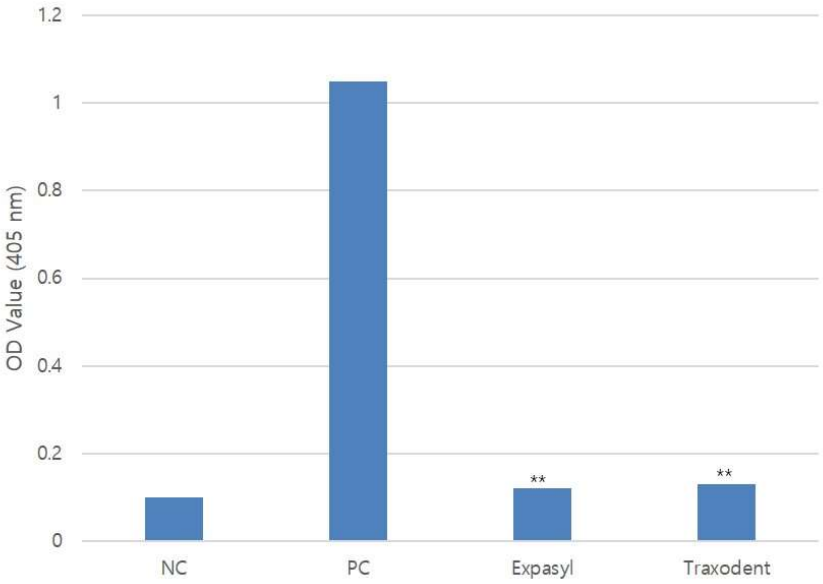

Figure 8. ARS quantification. Both Expasyl and Traxodent show statistically significant decrease in optical density (** $p < .01$).

2. *in vivo* study

2.1. Histological analysis

In all defects regardless of hemostatic agents used, woven bone formation was evident after 3 weeks of healing period (Figure 9). Within the intertrabecular spaces of new woven bone, fat cells, stromal cells, and blood vessels could be identified. Foreign body reactions with multinucleated giant cells were visible both in defects where Expasyl or Traxodent was used for hemostasis. In Traxodent groups, there was no noticeable difference in the amount of woven bone formation between defects where only saline irrigation was used to remove the hemostatic agent versus where a surgical curette was additionally used for mechanical removal. On the other hand, lower density of new woven bone formation was observed in defects where Expasyl was removed only by saline irrigation compared to the defects cleaned by curettage along with saline irrigation. Difference in amount of new woven bone formation was no longer observed after 12 weeks of healing period (Figure 10).

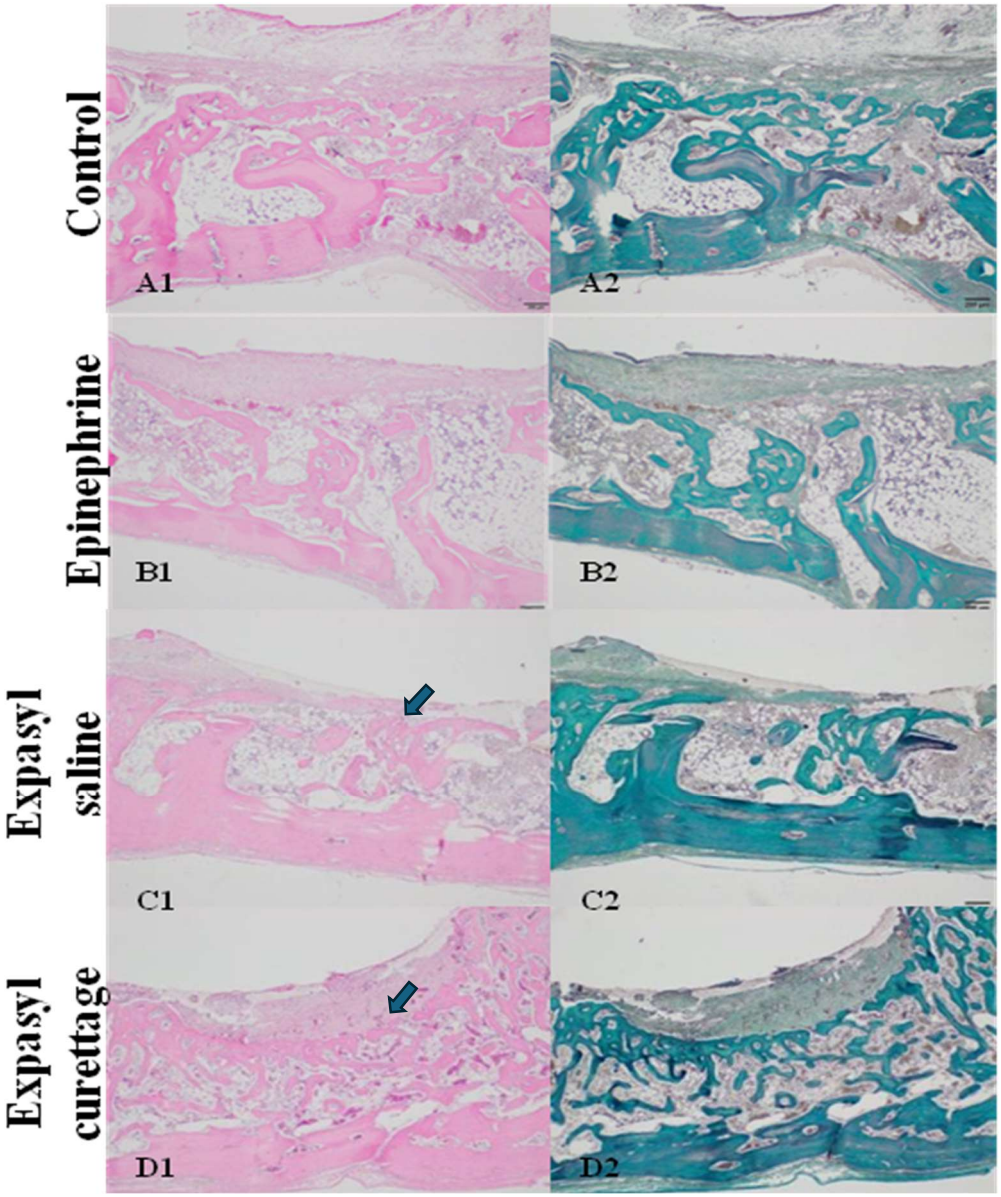

Figure 9. Hematoxylin-eosin staining and Masson-Goldner trichrome staining evaluation under low magnification at 3 weeks. (A1, A2) Control. (B1, B2) Epinephrine group. (C1, C2) Expasyl removed by saline irrigation only group. (D1, D2) Expasyl removed with curettage and saline irrigation group. Area indicated with an arrow in C1 and D1 shows difference in density of a newly formed woven bone.

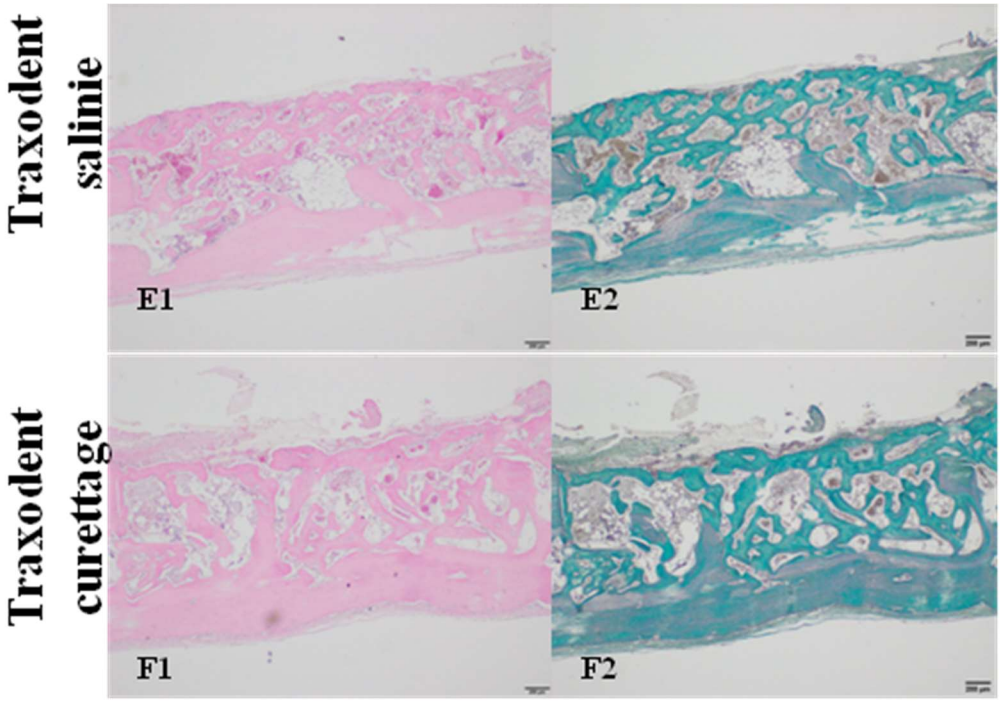

Figure 9 continued. (E1, E2) Traxodent removed by saline irrigation only group. (F1, F2) Traxodent removed with curettage and saline irrigation group.

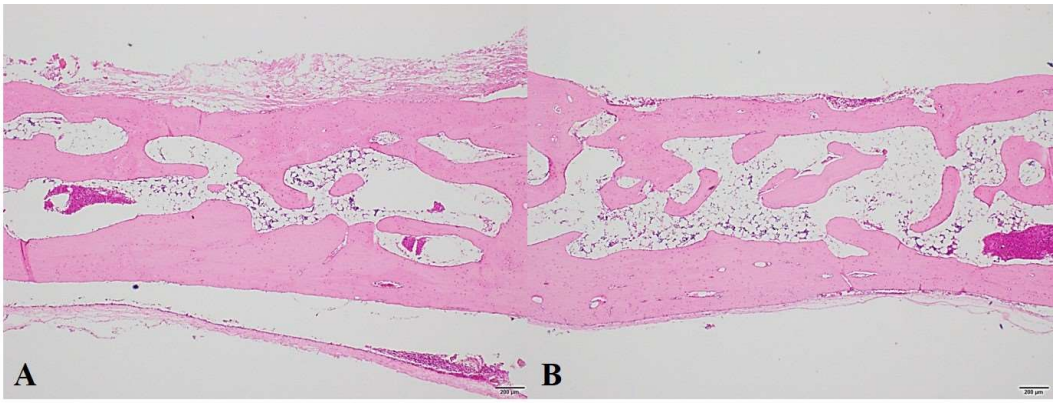

Figure 10. Hematoxylin-eosin staining evaluation under low magnification at 12 weeks. No remarkable difference is observed in bone formation between A) Expasyl removed by saline irrigation only and B) Expasyl removed with curettage and saline irrigation group.

2.2. Hemostatic effect

The visual analysis of the hemostatic effect showed the biggest reduction in bleeding score in the Expasyl groups as shown in Table 2. Epinerphine and Traxodent showed similar hemostatic effect. Significant difference in reduction in bleeding score was observed only between Expasyl and other groups. Fleiss' kappa showed that there was moderate agreement between the three evaluators' judgements for both initial and final bleeding score, k=.524 (95% CI, .451 to .596), $p < 0.001$ and k=.471 (95% CI, .389 to .553), $p < 0.001$, respectively.

Table 2. Mean bleeding scores and mean bleeding reduction

Treatment	Initial bleeding score	Final bleeding score	Calculated bleeding reduction
Control	4.61 (±0.92)	4.11 (±1.53)	0.50 (±1.46)
Epinephrine	3.17 (±1.92)	2.44 (±1.20)	0.72 (±1.64)
Expasyl with curettage	3.94 (±1.11)	1.5 (±0.71)	2.44 (±1.58)
Expasyl with irrigation only	4.0 (±2.22)	1.83 (±0.98)	2.17 (±1.65)
Traxodent with curettage	4.22 (±1.80)	3.44 (±2.04)	0.78 (±2.13)
Traxodent with irrigation only	4.06 (±1.63)	3.39 (±1.54)	0.67 (±1.97)

3. Clinical study

Among 140 patients assessed for eligibility, 9 patients disagreed to participate in this study, 3 patients were excluded because of vertical root fracture, 8 patients because of apicomarginal defect, 2 patients because of lesion size greater than 10 mm, and 4 patients because of incomplete protocols. Of 114 patients included in the study, 28 patients were lost to follow-up and 3 patients had their treated teeth extracted due to persistent symptoms. Total of 83 patients were examined clinically and radiographically at the 12-month follow-up. Descriptive statistics of patients according to the hemostatic agents are listed in table 3.

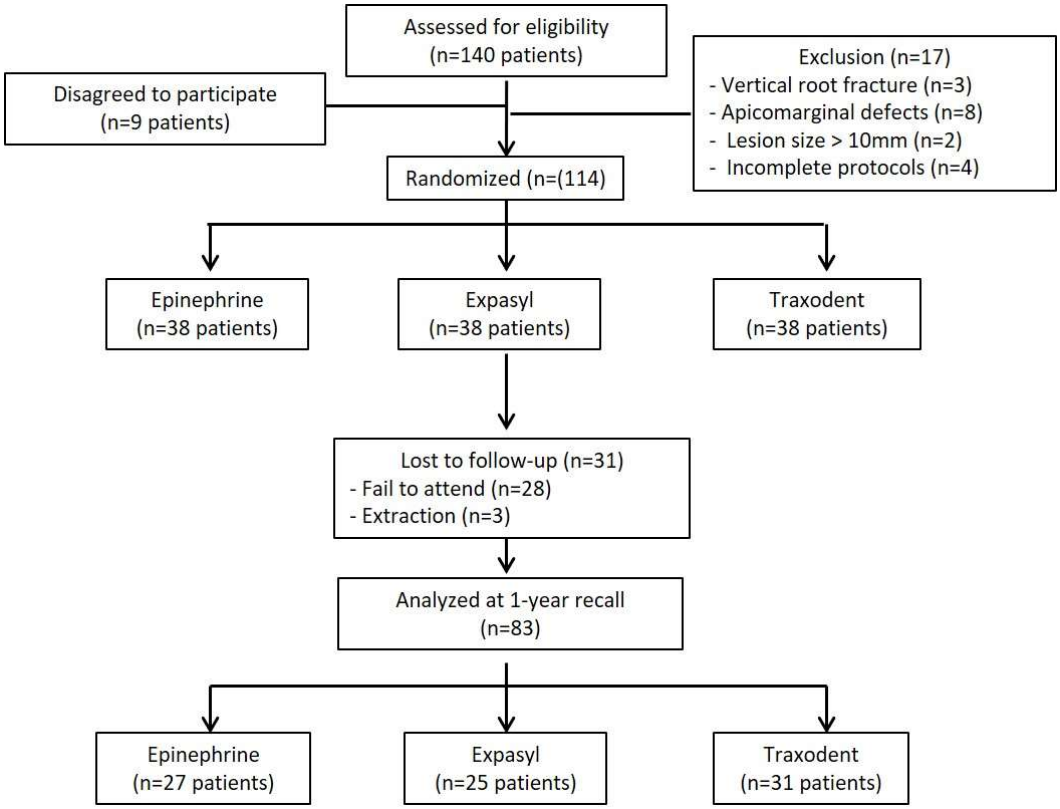

Figure 11. Flow diagram illustrating the procedure of the clinical trial.

Table 3. Descriptive Statistics of Patients Included in the Study

	Epinephrine (n=27) (32.5%)		Expasyl (n=25) (30.1%)		Traxodent (n=31) (37.3%)		Total (n=83)	
	n	%	n	%	n	%	n	%
Sex								
Male	8	30.8	6	24.0	9	29.0	23	27.7
Female	19	69.2	19	76.0	22	71.0	60	72.3
Age (y)								
20-29	7	25.9	10	40.0	9	29.0	26	31.3
30-39	9	33.3	9	36.0	10	32.3	28	33.7
40-49	6	22.2	5	20.0	7	22.6	18	21.7
50-59	1	3.7	1	4.0	3	9.7	5	6.0
>60	4	14.8	0	0	2	6.5	6	7.2
Tooth type								
Maxillary anterior	13	48.1	14	56.0	16	51.6	43	51.8
Premolar	5	18.5	5	20.0	10	32.3	21	24.1
Molar	6	22.2	2	8.0	1	3.2	8	10.8
Mandibular anterior	1	3.7	2	8.0	0	0	3	3.6
Premolar	1	3.7	0	0.0	1	3.2	2	2.4
Molar	1	3.7	2	8.0	3	9.7	6	7.2

Effective hemostasis was established in 19 of 27 cases (70.4%) in epinephrine group, in 24 of 25 cases (96.0%) in Expasyl group, and 22 of 31 cases (71.0%) in Traxodent group. A statistically significant difference in hemostatic efficacy was present between the groups (P = .039). The radiographic images of cases included in this study corresponding to each healing category of the Molven criteria are shown in figures 12 through 15. Assessed based on both clinical and radiographic measures, overall success rate at 12-month follow-up was 91.6%, with a success rate of 92.6% for epinephrine, 96.8% for Traxodent, and 88.0% for Expasyl. The statistical analysis of the success rates did not show significant difference between the groups (P = .516). Fleiss' kappa showed that there was high agreement between the three evaluators' judgements for hemostatic efficacy, k=.767 (95% CI, .642 to .891), p < 0.001.

Table 4. Hemostatic Efficacy According to Hemostatic Agent Used

	Total n (%)	Epinephrine n (%)	Expasyl n (%)	Traxodent n (%)
Total	83 (100.0)	27 (100.0)	25 (100.0)	31 (100.0)
Adequate	65 (78.3)	19 (70.4)	24 (96.0)	22 (71.0)
Inadequate	18 (21.7)	8 (29.6)	1 (4.0)	9 (29.0)

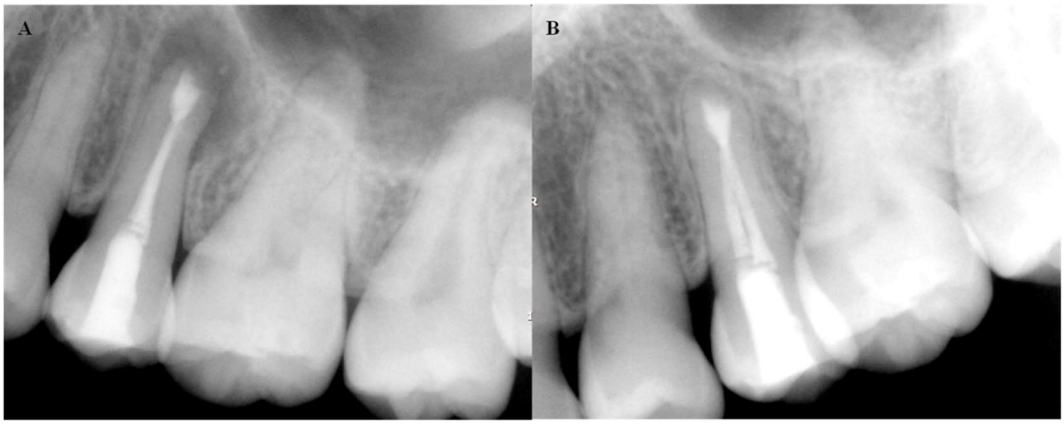

Figure 12. Maxillary second premolar radiographically assessed as complete healing 1 year after apical microsurgery. (A) Postoperative and (B) 1-year follow-up.

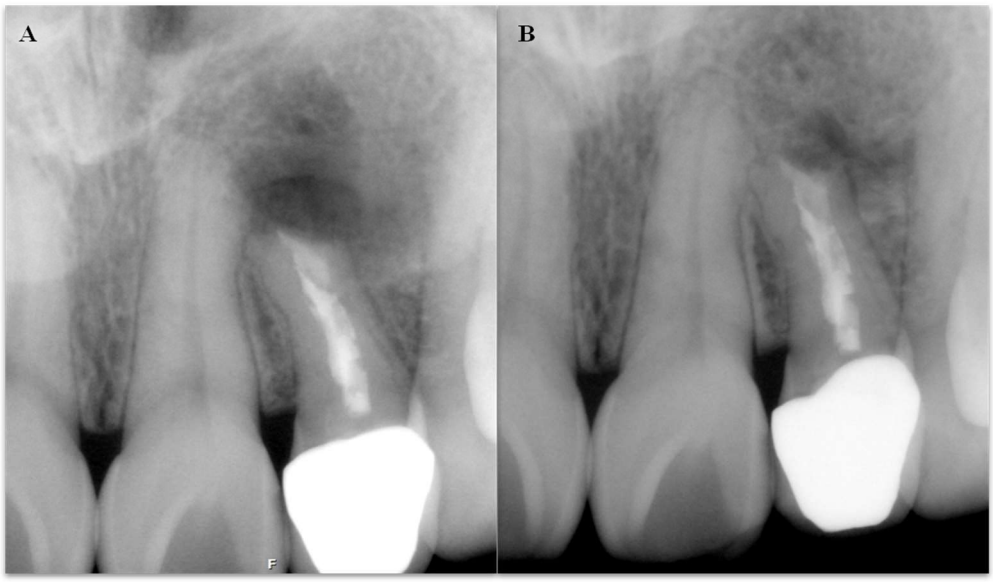

Figure 13. Maxillary lateral incisor radiographically assessed as incomplete healing 1 year after apical microsurgery. (A) Postoperative and (B) 1-year follow-up.

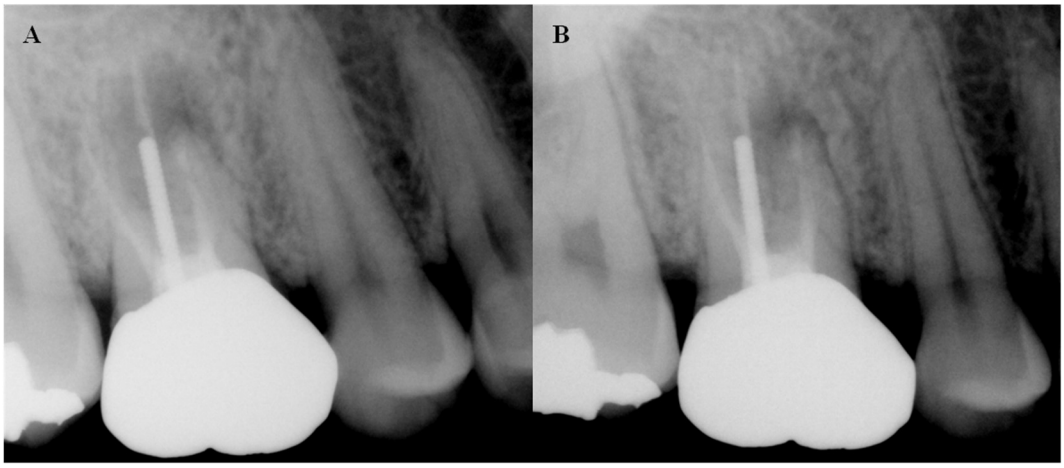

Figure 14. Maxillary first molar radiographically assessed as uncertain healing 1 year after apical microsurgery. (A) Postoperative and (B) 1-year follow-up.

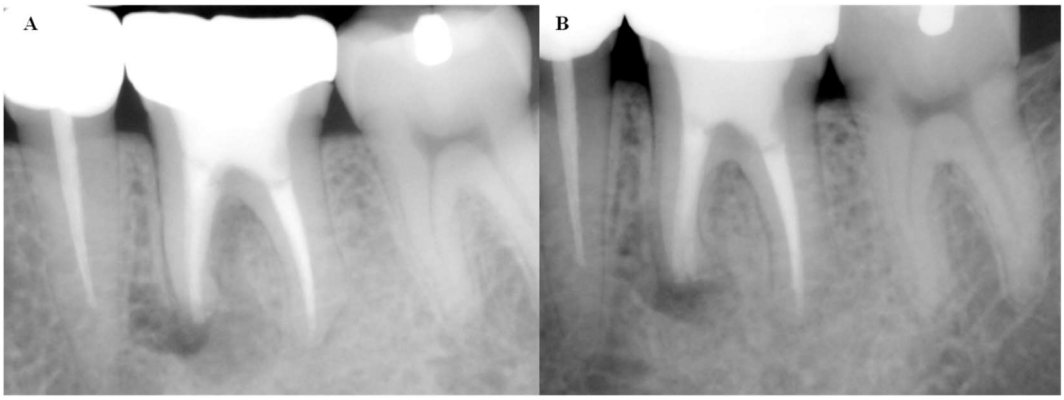

Figure 15. Mandibular first molar radiographically assessed as unsatisfactory healing 1 year after apical microsurgery. (A) Postoperative and (B) 1-year follow-up.

Table 5. Summary of Treatment Outcome by Radiologic Criteria per Hemostatic Agent Used

	Epinephrine (n=26)		Expasyl (n=26)		Traxodent (n=31)		Total (n=83)	
Category of healing	n	%	n	%	n	%	n	%
Complete	24	92.3	21	84.0	30	96.8	75	90.4
Incomplete	1	3.8	2	8.0	1	3.2	4	4.8
Uncertain	2	7.7	1	4.0	0	0	3	3.6
Unsatisfactory	0	0	1	4.0	0	0	1	1.2

IV. Discussion

The aim of this study was to evaluate two different types of aluminum chloride containing hemostatic agents for their cytotoxicity *in vitro*, hemostatic efficacy and tissue reactions *in vivo*, and effect on outcome of apical surgery in clinical trials. Few studies have evaluated the cytotoxic effects of aluminum chloride on human gingival fibroblasts *in vitro* since the products such as Expasyl was initially developed as cordless gingival retraction material to be used in prosthetic and restorative dentistry. Labban et al. (2019) and Nowakowska et al. (2021) showed that Expasyl has cytotoxicity on human gingival fibroblast. When Expasyl is used as a hemostatic agent in periapical surgery, it comes in direct contact with a bone rather than gingiva. However, there is limited information on the effect of Expasyl or other aluminum chloride containing hemostatic agents on human osteoblasts. *In vitro* assessment in this study evaluated the cytotoxic effect of Expasyl and Traxodent on HOBs using CCK-8, which is a colorimetric assay for the assessment of cell viability. Two different dilutions of conditioned medium were prepared to assess the short-term and the long-term effect of hemostatic agents. HOBs were cultured in a high concentration medium for 2 minutes, 10 minutes, and 1 hour to replicate the clinical condition where remnants of hemostatic materials are left in the bone cavity after the application. Considering the dilution of remaining material by blood over time, low concentration material extract medium was used to culture HOBs for 1 day, 3 days, and 7 days to evaluate the long-term effect. Statistically significant decrease in cell viability was observed with increasing time interval, from 2 minutes to 1 hour, for the short-term effect. Almost no viable cells were observed in the 1-hour group. Contrarily, there was no statistically significant difference in cell viability in low concentration of material extract medium. Based on these results, it can be inferred that hemostatic agents left in the bone cavity show highest toxicity within an hour and become less toxic over time as their concentration decreases in clinical condition. Although the cell toxicity may decrease over time, it is advisable to minimize the initial inflammatory response to reduce the negative effect on bone healing. Therefore, an effort needs to be made to remove the hemostatic materials thoroughly after their application in a bone cavity to reduce the amount of residues during apical surgery.

Expression of inflammatory cytokines, IL-6, IL-8 and TGF-β, were measured to evaluate the inflammatory response of HOBs. IL-8 is a cytokine secreted by osteoblasts that promotes RANKL-induced osteoclastogenesis (Amarasekara et al., 2018) and its expression was significantly increased in Expasyl group. Expression of TGF-β, which is a good indicator of osteoblast proliferation (Li et al., 2003), was significantly reduced in

HOBs cultured in Expasyl extract medium. There was no significant difference in expression of IL-6 (Amarasekara et al., 2018), which is also an osteoclastogenic cytokine, compared to the control. Bone maintains homeostasis through continuous and balanced formation and resorption of bone (Li et al., 2003). Normal remodeling process can be disrupted by change in osteoblast and/or osteoclast activity, which can result in a net bone loss. In response to Expasyl and Traxodent, the secretion of inflammatory cytokines has shifted in a direction that increases osteoclast and decreases osteoblast activity, which would result in disturbed regulation of bone homeostasis.

Three osteogenic markers, ALP, OCN, and RUNX2, were evaluated in this study to assess the influence of Expasyl and Traoxdent on osteogenic potential of HOBs. ALP expression is an early indicator of osteogenesis (Darjanki et al., 2023) and OCN is a differentiation marker mainly in the later stage and is detected after mineralization (Ikegame et al., 2019). RUNX2 is a key transcription factor associated with osteoblast differentiation (Nakamura et al., 2020). Expression of OCN and RUNX2 was significantly decreased in both Expaysl and Traxodent group while the ALP expression was not affected by either hemostatic agent. The result of RT-PCR was consistent with that of ALP staining as no remarkable difference could be seen between the positive control, Expasyl, and Traxodent group. ARS staining showed decreased stains of mineral deposits and optical density values were close to that of negative control for both Expasyl and Traxodent group. It can be interpreted that the osteogenic induction was reduced, resulting in decrease in mineralization.

Previous studies have evaluated hemostatic effect and tissue reaction of various hemostatic agents such as bone wax, ferric sulphate (Stasis, Belport Co, Camarillo, CA, USA), and aluminum chloride-containing paste (Expasyl, Pierre Rolland, Merignac, France). Of these studies, *in vivo* animal model used by von Arx et al. (2006) was used as the basis for *in vivo* part of this study. Von Arx et al. (2006) reported that Expasyl alone or in combination with Stasis was most efficient in bleeding control in bone defects of rabbit calvaria. Results of present study also showed that Expasyl was the most effective hemostatic agent compared to Traxodent and epinephrine. Removal method of the hemostatic agent, either saline irrigation only or mechanical removal with curette and saline irrigation, did not affect the hemostatic effect of Expasyl. Since the main composition of Traxodent, 15% aluminum chloride, is the same as that of Expasyl, it was expected that Traxodent would show a similar hemostatic effect as Expasyl. However, Traxodent not only showed less reduction in bleeding score compared to Expasyl, but also demonstrated lower hemostatic effect than epinephrine. Although both Expasyl and Traxodent contain 15%

aluminum chloride, the consistency of two pastes is quite different. According to the manufacturer, Expasyl undergoes a physical dehydration process and becomes more dry and rigid after setting. A study conducted by Dederichs et al. (2019) comparing pressure exerted by different cordless gingival displacement materials reported that Expasyl generated pressure that is significantly greater than other materials tested in their study. Such physical property of Expasyl provides mechanical pressure on the bleeding site in addition to its chemical reactions including blood protein precipitation and blood vessel constriction. Traxodent, on the other hand, is described by the manufacturer as a soft paste that has strong affinity to aqueous solutions. Due to its low viscosity, Traxodent does not generate as much pressure as Expasyl on application sites. According to Dederichs et al. (2019), pressure generated by Traxodent and Expasyl was 82.74 ± 29.29 kPa and 524.35 ± 113.88 kPa, respectively. Thus, Traxodent relies more on its chemical component, aluminum chloride, for hemostatic effect.

In the histologic analysis using H&E and Masson-Goldner trichrome staining, adverse tissue reactions were observed in defects where Expasyl or Traxodent was used. In this study, two methods were used to remove the hemostatic agents after their application in bone defects: saline irrigation only or mechanical removal by curettage followed by saline irrigation. Jensen et al. (2010) suggested cleaning the surgical site with a bone curette or freshening the walls of the bony crypt with a rotating instrument to avoid leaving residues of Expasyl in cancellous bone. Because use of a rotary instrument could result in unintended bone removal, a surgical curette was used in this study to clean the bony crypt. Traxodent was easily removed from the bone defect with saline irrigation whereas Expasyl was visible even after cleaning with a surgical curette. Histologic analysis of the specimens after 3 weeks of healing revealed delayed bone healing when Expasyl was removed only with saline irrigation. Less amount of woven bone formation was observed compared to defects where Expaysl was removed with a surgical curette followed by saline irrigation. On the other hand, there was no remarkable difference in bone healing between two groups with different cleaning methods for Traxodent.

Several studies have evaluated the efficacy of different hemostatic agents in periapical surgery or the relationship between the type of hemostatic agents used and the treatment outcome, but very few studies have assessed both. Von Arx et al. (2007) used Expasyl and/or ferric sulfate to obtain hemostasis during periapical surgery of 194 human teeth and reported a success rate of 90.2%. Menendez et al (2016) reported significantly greater hemostatic efficacy of aluminum chloride (72.5%) compared to epinephrine group (52.1%).

Neither study, however, analyzed the influence of the hemostatic agent upon the outcome of surgery. Penarrocha-Diago et al. (2013) reported in their retrospective study that there was no significant difference in outcome of periapical surgery between two hemostatic agents: Expasyl and small sterile dressings saturated with anesthetic solution with adrenaline as vasoconstrictor at a concentration of 1:100,000. In this case, efficacy of each hemostatic agent was not studied.

Present study evaluated not only the efficacy of hemostatic agents but also assessed their impact on outcome of periapical surgery. Expasyl demonstrated significantly superior hemostatic efficacy, and this finding is consistent with the results reported by Menendez et al. (2013). Outcome assessment was performed using clinical and radiographic examinations at 12 months post-surgery because the minimum duration of follow-up needed to determine the outcome of periapical surgery has been established as 12 months (Friedman, 2005; Menendez-Nieto et al., 2016; Molven et al., 1987). 1-year success rate of periapical surgery has been reported as 91.6% by Tsesis et al. (2009) and 91.4% by von Arx et al. (2010) in their meta-analysis, which coincides with the result of this study (91.6%). Results of Fisher's exact test ($p = 0.516$) indicate no statistically significant association between the type of hemostatic agent used and the success rate of periapical surgery. Although a well-controlled randomized controlled trial can provide strong evidence of causation, it is often challenging to attain the sample size requirements due to strict eligibility criteria and patients' failure to attend the recall visits. Relatively small sample size of this clinical study provides limited validity of results and therefore, meta-analysis that pool data from RCTs with similar designs can provide more reliable suggestions for clinical practice in the future.

V. Conclusion

Both Expasyl and Traxodent showed cytotoxicity in human osteoblasts and seemed to interfere with osteogenesis in cellular level, based on changes in expression level of inflammatory and osteogenic genes: IL-6, IL-8, TGF-β, ALP, OCN, and RUNX-2. In the rabbit calvarial model, bleeding reduction was most prominent in defects where Expasyl was used. The low-viscosity Traxodent was easier to remove compared to Expasyl and minimal residues were observed in the defects. However, the observed hemostatic effect of Traxodent fell short of the anticipated outcomes. Histological analysis demonstrated inflammatory response in both Expasyl and Traxodent groups, but delayed osseous healing

was observed only in Expasyl groups. Overall success rate of endodontic microsurgery in this study was 91.6%, similar to success rates reported by previous meta-analysis (von Arx et al., 2010, Tsesis et al., 2009). There was no significant difference in clinical outcomes based on hemostatic agents used. Within the limitations of this study, it can be concluded that Expasyl is a more effective hemostatic agent compared to epinephrine and Traxodent. Although it does have cytotoxicity on human osteoblasts and induces adverse tissue reactions, Expasyl does not significantly compromise surgical outcomes when used in clinical settings. Based on these findings, it can be proposed that epinephrine should be used as the primary hemostatic agent in apical surgery since it is the most biocompatible material. However, when adequate hemostasis is not achieved by epinephrine alone, the additional use of Expasyl can be recommended as an adjunctive measure. Because of the limited number of randomized controlled trials comparing hemostatic agents in periapical surgery, additional research will be needed to provide more robust evidence regarding the efficacy of hemostatic agents and their influence on surgical outcomes.

References

1. Amarasekara, D., Yun, H., Kim, S., Lee, N., Kim, H., & Rho, J. (2018). Regulation of osteoclast differentiation by cytokine network. *Immune Netw, 18*(1), e8.
2. von Arx, T., Jensen, S.S., & Hänni, S. (2007). Clinical and radiographic assessment of various predictors for healing outcome 1 year after periapical surgery. *J Endod, 33*(2), 123-128.
3. von Arx, T., Jensen, S.S., Hanni, S., & Schenk, R.K. (2006). Haemostatic agents in periradicular surgery: an experimental study of their efficacy and tissue reactions. *International Endodontic Journal*, *39*(10), 800–808.
4. von Arx, T., Peñarrocha, M., & Jensen, S. (2010). Prognostic factors in apical surgery with root-end filling: a meta-analysis. *J Endod, 36*(6), 957-973.
5. Azargoon, H., Williams, B.J., Solomon, E.S., Kessler, H.P., He, J.N., & Spears, R. (2011). Assessment of hemostatic efficacy and osseous wound healing using HemCon dental dressing. *J Endod, 37*(6), 807–811.
6. Cle-Ovejero, A. & Valmaseda-Castellon, E. (2016). Hemostatic agents in apical surgery. A systematic review. *JouMed Oral Patol Oral Cir Bucal, 21*(5), 652-657.
7. Darjanki, C., Prahasanti, C., Fitria, E., Kusumawardani, B., Wijaksana, I. & Aljunaid, M. (2023). RUNX2 and ALP expression in osteoblast cells exposed by PMMA-Hap combination: An *in vitro* study. *Journal of Oral Biology and Craniofacial Research, 13*(2), 277-282.
8. Dederichs, M., Fahmy, M., Kuepper, H., & Guentsch, A. (2019). Comparison of gingival retraction materials using a new gingival sulcus model. *Journal of Prosthodontics, 28*(7), 784-789.
9. Eliyas, S., Vere, J., Ali, Z., & Harris, I. (2014). Micro-surgical endodontics. *Br Dent J, 216*(4), 169–177.
10. Friedman, S. (2005). The prognosis and expected outcome of apical surgery. *Endod Topics, 11*(1), 219-262.
11. Ikegame, M., Ejiri, S., & Okamura, H. (2019). Expression of non-collagenous bone matrix proteins in osteoblasts stimulated by mechanical stretching in the cranial suture of neonatal mice. *J Histochem Cytochem, 67*(2),107-116.
12. Jensen, S.S., Yazdi, P.M., Hjørting-Hansen, E, Bosshardt, D.D., & von Arx, T. (2010). Haemostatic effect and tissue reactions of methods and agents used for haemorrhage control in apical surgery. *Int Endod J, 43*(1), 57–63.
13. Jesslén, P., Zetterqvist, L., & Heimdahl, A. (1995). Long-term results of amalgam versus glass ionomer cement as apical sealant after apicectomy. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 79*(1), 101-3.
14. Khater, A.G.A., Al-hamed, F.S., Safwat, E.M., Hamouda, M. M. A., Shehata, M. S. A., & Scarano, A. (2021). Efficacy of hemostatic agents in endodontic surgery: A protocol of systematic review and network meta-analysis. *J Evid Based Dent Pract, 21*(3), 1-13.

15. Kim, S. & Kratchman, S. (2006). Modern endodontic surgery concepts and practice: a review. *J Endod, 32*(3), 601–623.
16. Kim, S. & Rethnam, S. (1997). Hemostasis in endodontic microsurgery. *Dent Clin North Am, 41*(3), 499-511.
17. Labban, N., AlOtaibi, H., Mokeem, A., AlJameel, M., & AlRasheed. T. (2018). The direct cytotoxic effects of different hemostatic agents on human gingival fibroblasts. *Journal of Prosthodontics, 28*(4), 896-901.
18. Lemon, R.R., Steele, P.J., & Jeansonne, BG. (1993). Ferric sulfate hemostasis:effect on osseous wound healing. Left in situ for maximum exposure. *J Endod, 19*(4), 170–173.
19. Li, J., Chang, W., Lin, J., Ruaan, R., Liu, H., & Sun, J. (2003). Cytokine release from osteoblasts in response to ultrasound stimulation. *Biomaterials, 24*(13), 2379-2385.
20. Lustmann, J., Friedman, S., & Shaharabany, V. (1991). Relation of pre- and intraoperative factors to prognosis of posterior apical surgery. *J Endod, 17*(5), 239-241.
21. Menendez-Nieto, I., Cervera-Ballester, J., Maestre-Ferrin, L., Blaya-Tarraga, J., Penarrocha-Otra, D., & Penarrocha-Diago, M. (2016). Hemostatic agents in periapical surgery: a randomized study of gauze impregnated in epinephrine versus aluminum chloride. *J Endod, 42*(11),1583-1587.
22. Molven, O., Halse, A., & Grung, B.. (1987). Observer strategy and the radiographic classification of healing after endodontic surgery. *Int J Oral Maxillofac Surg, 16*(4), 432–9.
23. Nakamura, T., Takahashi, A., Kasahara, M., Yamaguchi, A., & Azuma, T. (2020). Tissue-nonspecific alkaline phosphatase promotes the osteogenic differentiation of osteoprogentor cells. *Biochemical and Biophysical Research Communications, 524*(3),702-709.
24. Nowakowkska, D., Kulbacka, J., Wezgowiec, J., Szewczyk, A., Baczynska, D., Zietek, M., Wieckiewicz, W., & Saczko, J. (2021). Biological response induced in primary human gingival fibroblasts upon exposure to various types of injectable astringent retraction agents. Materials, 14(8), 1-13.
25. Penarrocha-Diago, M., Maestre-Ferrin, L., Penarrocha-Oltra, D., & von Arx, T. (2013). Influence of hemostatic agents upon the outcome of periapical surgery: Dressings with anesthetic and vasoconstrictor or aluminum chloride. *Med Oral Patol Oral y Cir Bucal, 18*(2), 272-278.
26. Penarrocha-Oltra, D., Menendez-Nieto, I., Cervera-Ballester, J., Maestre-Ferrin, L., Penarrocha-Diago, M., & Penarrocha-Diago, M. (2019) Aluminum Chloride versus Electrocauterization in Periapical Surgery: A Randomized Controlled Trial. J Endod. 2019;45:89–93.
27. Penarrocha-Oltra D, Soto-Penaloza D, Penarrocha-Diago M, Cervera-Ballester J, von Arx T, Penarrocha-Diago M. Aluminum Chloride versus Electrocauterization in Periapical Surgery: A Randomized Controlled Trial. *J Endod, 45*(2), 970-976.
28. Penarrocha-Oltra, D., Soto-Penaloza, D., Penarrocha-Diago, M.,Cervera-Ballester, J., Cabanes-Gumbau, G., & Penarrocha-Diago, M. (2020). Hemostatic agents in endodontic

surgery of maxillary molars. A randomized controlled pilot study of polytetrafluoroethy-lene (PTFE) strips as an adjunct to epinephrine impregnated gauze versus aluminum chloride. *Med Oral Patol Oral Cir Bucal, 25*(5), 634-643.

29. Rubinstein, R.A. & Kim, S. (2002). Long-term follow-up of cases considered healed one year after apical microsurgery. *J Endod, 28*(5), 378-83.
30. Rud, J., Andreasen, J.O., & Jensen, J.E. (1972). A follow-up study of 1,000 cases treated by endodontic surgery. *Int J Oral Surg, 1*(4), 215-28.
31. Sauveur, G., Roth, F., Sobel, M., & Boucher, Y. (1999). The control of haemorrhage at the operative site during periradicular surgery. *Int Endod J, 32*(3), 225–228.
32. Scarano, A., Artese. L., Piattelli, A., Carinci, F., Mancino, C., & Iezzi, G. (2012). Hemostasis control in endodontic surgery: a comparative study of calcium sulfate versus gauzes and versus ferric sulfate. *J Endod, 38*(1), 20–3.
33. Schlund, M., Depeyre, A., Ranganath, S., Marchandise, P., Ferri, J., & Chai, F. (2022). Rabbit Calvarial and mandibular critical-sized bone defects as an experimental model for the evaluation of craniofacial bone tissue regeneration. *J. Stomatol Oral Maxillofac Surg, 123*(6), 601-609.
34. Selden, H.S. Bone wax as an effective hemostat in periapical surgery. *Oral Surg Oral Med Oral Pathol, 29*(2), 262–264.
35. Song, M., Jung, I.Y., Lee, S.J., Lee, C.Y., & Kim E. (2011). Prognostic factors for clinical outcomes in endodontic microsurgery: a retrospective study. *J Endod, 37*(7), 927–33.
36. Tsesis, I., Faivishevsky, V., Kfir, A., & Rosen, E. (2009). Outcome of surgical endodontic treatment performed by a modern technique: a metaanalysis of literature. *J Endod, 35*(11), 1505-11.
37. Vickers, F.J., Baumgartner, J.C., & Marshall, G. (2002). Hemostatic efficacy and cardiovascular effects of agents used during endodontic surgery. *J Endod, 28*(4), 322–3.
38. Vy, C.H., Baumgartner, J.C., & Marshall, J.G. (2004). Cardiovascular effects and efficacy of a hemostatic agent in periradicular surgery. *J Endod, 30*(6), 379–83.
39. Wang, N., Knight, K., & Dao, T. (2004). Treatment outcome in endodontics – the toronto study. Phases I and II: apical surgery. *J Endod, 30*(11), 751-761.
40. Witherspoon, D.E. & Gutmann, J.L. (1996). Haemostasis in periradicular surgery. Int Endod J, 29(3), 135–49.
41. Xu, J., Yu, L., Liu, F., Wan, L., & Deng Z. (2023). The effect of cytokines on osteoblasts and osteoclasts in bone remodeling in osteoporosis: a review. *Frontiers in Immunology, 14*, 1-16.

Abstract (In Korean)

미세 치근단 수술에서 사용되는 알루미늄 염화물 성분의 지혈제 평가 : *in vitro* 연구, 동물 연구, 무작위 배정 연구

치근단절제술의 성공적인 결과를 위해서는 적절한 밀도의 근단부 충전이 필수적이다. 절제된 치근의 표면을 확인하고 재료를 오염없이 충전하기 위해서는 수술 부위의 효과적인 지혈이 중요하다. 본 연구의 목적은 1) 알루미늄 염화물을 함유한 지혈제인 Expasyl (Pierre Rolland, Merignac, France)과 Traxodent(Premier Dental, Plymouth, PA, USA)의 인간 조골세포(HOBs, Promocell, Hidelberg, Germany)에 대한 세포독성을 평가하고, 2) 토끼 두개골의 표준화된 골 결손부에서 이들의 지혈 효과와 조직 반응을 조사하며, 3) 무작위 배정 연구를 통해 치근단 수술의 결과에 미치는 영향을 평가하는 것이다. 세포 실험에서는 고농도 및 저농도의 재료 추출 배지에서 배양된 HOB 의 세포 생존율을 Cell Counting Kit-8 (CCK 8) 로 측정하였다. 골형성 특이 유전자인 알칼리인산분해효소 (ALP), 오스테오칼신 (OCN), Runt-related transcription factor 2 (RUNX2) 와 염증 특이 유전자인 인터루킨 ((IL)-6, IL-8, 그리고 형질전환 성장인자(TGF)-β의 발현 수준을 실시간 중합효소 연쇄반응(RT-PCR)으로 분석하였다.

동물 실험에서는 6마리의 뉴질랜드 토끼의 두개골에 6개의 표준화된 골 결손 (직경 5mm, 깊이 1.5mm)을 형성하고, 각 결손부는 다음 6개 그룹 중 하나에 무작위로 배정되었다: 대조군, 에피네프린, Expasyl 적용 후 소파술과 생리식염수 세척을 시행한 그룹, Expasyl 적용 후 생리식염수로만 세척한 그룹, Traxodent 적용 후 소파술과 생리식염수 세척을 시행한 그룹, Traxodent적용 후 생리식염수로만 세척한 그룹. 모든 지혈제는 2분간 적용 후 속해있는 그룹에 따라 해당되는 방법으로 제거되었다. 지혈제 적용 전후의 출혈량은 수술 중 촬영된 사진을 바탕으로 3명의 검사자가 평가하고 점수화 하였으며, 지혈효과는 초기와 최종 출혈 점수의 평균 차이로 결정되었다. 헤마톡실린-에오신(HE) 염색과 마손 삼색 염색 두 가지 방법을 사용하여 분석한 조직 표본을 통해 사용된 지혈제와 관련된 조직 반응과 골 재생을 평가하였다.

임상 연구는 사용된 지혈제에 따라 세 개의 평행 그룹으로 구성된 무작위 대조 시험으로 구성되었다. 미세치근단 수술 절차는 이전 연구에서 보고된 연세 프로토콜에 따라 세 명의 술자에 의해 진행되었다. 사용된 지혈제의 지혈 효과는 술자와 두 명의 독립적인 검사자에

의해 적절 또는 부적절로 평가되었다. 환자들은 수술 후 6개월과 12개월에 내원하여 방사선 및 임상적 검사 결과를 기반으로 평가되었다.

세포 실험 결과 Expasyl 또는 Traxodent 에서 배양된 HOB 의 세포 생존율은 2분, 10분, 1시간으로 갈수록 유의하게 감소하였다. Expasyl 그룹에서만 TGF-B 발현은 유의하게 감소했고 IL-8 발현은 유의하게 증가하였다. RUNX-2 와 OCN 발현은 Expasyl 과 Traxodent 그룹 모두에서 유의하게 감소하였다.

동물 실험 결과 Expasyl 은 출혈 조절에 가장 효과적이었지만, 가장 많은 염증을 유발하고 골 치유를 지연시켰다. Traxodent 는 상대적으로 쉽게 제거되고 골 치유를 방해하지 않았지만, 대조군 결손에 비해 유의한 지혈 효과를 보이지 않았다.

임상 실험에서 총 83명의 환자가 술 후 12개월에 평가되었고 미세치근단 수술의 전체 성공률은 91.6% 였다. 통계 분석 결과, 사용된 지혈제 유형에 따른 수술 결과의 유의한 차이는 나타나지 않았다.

이 연구 결과는 Expasyl 이 미세치근단 수술 중 epinephrine 만으로 충분한 지혈을 얻을 수 없을 때 추가적인 지혈제로 사용될 수 있음을 시사하지만, 세포독성과 부작용을 일으킬 가능성 때문에 적용 후 가능한 철저히 제거해야 함을 나타낸다. Traxodent 의 경우 주요 성분은 Expasyl 과 동의함에도 불구하고 지혈 효과가 충분하지 않은 것으로 나타났다.

핵심되는 말 : 미세치근단 수술; 치근단절제술; 무작위 배정 연구; 알루미늄 염화물; 지혈제; Expasyl; Traxodent