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ORIGINAL ARTICLE

Application of Three Types of Scaffolds in Pulp Regeneration for Permanent Mature Teeth with Periapical Lesions: A Randomized Controlled Trial

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ABSTRACT

Objective: This study aimed to evaluate pulp regeneration by comparing the application of native chitosan-based scaffolds with enzymatically modified chitosan-based scaffolds in mature teeth with apical lesions, using clinical and radiographic assessments.

Methods: The eligibility criteria for this study were participants aged between 15–45 years, free from systemic diseases and with necrotic mature single-rooted teeth with periapical lesions. The teeth were equally and randomly allocated into three groups (1:1:1 allocation): Group A received treatment with a Blood Clot (BC) scaffold; Group B with a combination of Native Chitosan and Blood Clot (NCS+BC) scaffold; and Group C with Enzymatically-Modified Chitosan and Blood Clot (EMCS+BC) scaffold. Clinical procedures were performed over two appointments. During the first appointment, canals underwent standardized mechanical and chemical preparation, followed by a modified triple antibiotic paste application, then sealed with glass ionomer cement. After three weeks, the antibiotic paste was removed. Subsequently, the regenerative procedure was conducted based on the group assignment. Participants were monitored at one, three, six-, and twelve months post-treatment to evaluate the treated teeth clinically and radiographically, focusing on the status of periapical lesions and tooth sensibility through cold testing. Statistical analysis included the Kruskal-Wallis and Mann-Whitney U tests to determine significant differences in healing degrees among the three groups over time. Additionally, the Chi-square test was used to assess significant differences in tooth sensibility frequencies during the cold test across the groups.

Results: Thirty teeth from twenty-four participants were included. There were no significant differences in the frequencies of healing degrees among the three studied groups (BC, NCS+BC, EMCS+BC) after one, three, and twelve months. The degree of healing after six months in the EMCS+BC group was higher than in other groups, and there were no statistically significant differences in the frequencies of healing degrees after six months between the NCS+BC group and BC group. The frequencies of tooth sensibility in the cold test among the three studied groups (BC, NCS+BC, EMCS+BC) were significantly different after six and twelve months. The tooth sensibility in the BC group was smaller than that of both the NCS+BC group and EMCS+BC group, and there were no statistically significant differences in the frequencies of tooth sensibility between the NCS+BC group and EMCS+BC group.

Conclusion: The application of the EMCS+BC scaffold demonstrates superior outcomes in pulp regeneration after six months, with a higher degree of healing observed compared to the NCS+BC and BC groups. There were no statistically significant differences at one month, three months, and twelve months. Additionally, tooth sensitivity was more pronounced in the EMCS+BC and NCS+BC groups.

Keywords: Blood clot, chitosan, mature teeth, scaffolds, tissue regeneration

This research is part of the PHD study of Aliaa Alshahhoud approved by the Local Research Ethics Committee of the Faculty of Dentistry (UDDS-1189-25092018/SRC-74).

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HIGHLIGHTS

- This study compared the use of three types of scaffolds (blood clot, native chitosan and blood clot, and enzymatically-modified chitosan and blood Clot) in the success of regenerative endodontic treatment for mature necrotic permanent teeth with periapical lesions.
- The study found that the degree of healing after six months in the group using enzymatically modified chitosan combined with a blood clot was higher than in both the native chitosan combined with a blood clot group and the blood clot group alone, with a statistically significant difference.
- The study found that tooth sensitivity in the groups using enzymatically modified chitosan and native chitosan combined with blood clot scaffolds was higher than in the group using the blood clot scaffold alone.

INTRODUCTION

Dental pulp infection occurs due to caries or trauma, which requires removing infected tissues in the necrotic tooth and replacing them with inert obturation materials. This traditional endodontic treatment has been known for many years as a good and practical technique (1).

Regarding the success of traditional endodontic treatments, there is a relatively high failure rate of up to 14%. This failure often occurred due to the inability to achieve an effective seal and the presence of post-treatment bacterial contamination, or the persistence of chronic disease conditions. These issues are primarily attributed to the anatomical complexity of the root canals, which leaves certain areas unprepared and difficult to access for proper sterilization (2).

The removal of dental pulp diminishes the mechanical integrity of the tooth due to the extensive preparation required for the pulp chamber, leading to a significant loss of dental tissue. Additionally, the sensory and defensive functions of the pulp are lost, rendering the tooth more susceptible to infections. This increased vulnerability is attributed to the death of odontoblasts and the absence of dental pulp, which normally provides defense against various pathological factors (2).

Modern science has recently focused on biological endodontic treatments as alternatives to traditional methods, utilizing tissue engineering techniques. Tissue engineering is the science of designing and creating regenerative tissue that mimics the original tissue, aiming to replace diseased or damaged tissue (3).

The first attempt to generate pulp tissue was made by Nygaard Østby in 1961, involving the over-preparation of the apical foramen to induce blood clot formation and partial filling with gutta-percha to permit tissue growth into the remaining root canal space. In necrotic canal cases, a 4% formaldehyde solution was used for disinfection. Histological studies of these cases revealed the deposition of mineralized tissue along the root canal, as well as the formation of connective tissue (4).

Achieving optimal results for regenerative endodontic procedures (REPs) depends on four important principles: using a disinfection protocol that disinfects the root canal effectively to create a microenvironment leading to the proliferation and differentiation of stem cells, using a biocompatible scaffold that provides chemical stability and mechanical resistance to newly formed tissue, using growth factors to boost proliferation and differentiation, and applying chemotactic factors to facilitate the organization migration of stem cells into the root canal area (5).

Tissue engineering applications represent an effective strategy, particularly in pulp regeneration. For optimal efficacy, this approach necessitates biological scaffolds that mimic the extracellular matrix and possess properties that maintain the vitality of stem cells during implantation. These scaffolds should support cell growth and differentiation, promote adhesion, and accelerate the migration of endogenous stem cells, thereby contributing to the generation of functional dental pulp (6). Scaffolds are classified depending on their origin into natural biological scaffolds (for example, blood clot, platelet-rich plasma, platelet-rich fibrin, collagen, chitosan, demineralized dentine, and silk-based scaffolds), and synthetic scaffolds: such as bioactive ceramics scaffolds, polymeric scaffolds, and peptide scaffolds (5).

Furthermore, several studies indicate that chitosan-based scaffolds are valuable natural biomaterials possessing distinctive characteristics and a wide range of biomedical applications (7).

Chitosan is a copolymer of glucosamine and N-acetylglucosamine units linked by 1–4 glucosidic bonds. It is a deacetylated derivative of chitin. This polysaccharide is a porous material with several characteristics that render it a highly suitable substance for biomedical applications including anticholesterolemic and antimicrobial activity, biodegradability, biocompatibility, non-carcinogenicity, hemostatic potential, fungistatic activity, accentuated affinity to proteins, and the ability to promote cell adhesion, proliferation, and differentiation (8).

Chitosan-based scaffolds have been found to promote the proliferation, migration, and differentiation of dental pulp stem cells and mesenchymal stem cells *in vivo* and *in vitro* (9, 10). However, the poor mechanical properties and the high swelling tendency of chitosan, which lead to easy deformation, pose significant limitations to its use in biomedical applications (11). To address these limitations and enhance cell adhesion, more than one study have explored chemical modifications of chitosan to optimize its physicochemical and mechanical properties, including its hydrophobicity and surface texture (12, 13). More recently, enzymatic methods for chitosan modification have emerged as a promising alternative to traditional chemical techniques, which are often toxic, environmentally harmful, and nonspecific (14). However, the enzymatically modified chitosan with phenol is non-toxic to mesenchymal stem cells and it has much higher antioxidant activity compared to novel chitosan (15).

Given the importance of this material in the adhesion and proliferation of stem cells, and the fact that the available information is limited only to the results of laboratory studies (14, 15), and since laboratory studies are far from clinical reality, it was necessary to investigate the effectiveness of this material in clinical application, especially in the field of pulp regeneration.

The potential of enzymatically modified chitosan with phenolic compounds for dental pulp regeneration has not yet been documented in the existing literature. Therefore, this study is considered one of the first clinical studies to evaluate its effects on pulp formation and its efficacy in healing periapical lesions. The null hypothesis suggests that the three mentioned scaffolds have similar effects on the regenerative treatment of necrotic teeth with periapical lesions.

MATERIALS AND METHODS

Trial Design, Settings, and Ethical Approval

This double-blinded randomized clinical trial used a three-arm parallel group with a 1:1:1 allocation ratio, designed to compare several scaffolds (Blood Clot, Native Chitosan, and Enzymatically Modified Chitosan) in terms of their efficacy in pulp regeneration by clinical and radiographic evaluation, undertaken from January 2019 to May 2023 at the Endodontic Department of the Faculty of Dentistry, Damascus University. This study adhered to the ethical guidelines of the Declaration of Helsinki and received ethical approval from the Local Research Ethics Committee of the Damascus University Faculty of Dentistry (approval no: UDDS-1189-25092018/SRC-74). The project was funded by Damascus University (funder no. 501100020595) and retrospectively registered at the ISRCTN registry under ID number ISRCTN44531236.

Sample Size Calculation

Based on a previous study (16), the sample size for the current investigation was calculated using G* Power 3.1.9.4 (Heinrich-Heine-Universität, Düsseldorf, Germany). For the ANOVA analysis, a sample size of 10 participants per group was determined, yielding a total of 30 participants across the 3 groups. This configuration provided an effect size (f) of 0.4, based on changes in the size of periapical lesions following regeneration procedures, with a power of 80% and a significance level of 0.05.

Eligibility Criteria

The eligibility criteria were consistent with those used in previous research (17, 18). During the study recruitment period, participants aged 15–45 years were referred to the Endodontic Department clinics at Damascus University due to apical lesions in their teeth from outpatient clinics. The principal investigator (A.A.) examined the participants, reviewed their medical and dental histories, and these participants would be assessed for inclusion criteria as follows: individuals with necrotic single-rooted permanent teeth with mature roots who were willing to attend work sessions and follow-ups, and with periapical lesions of less than 10 mm assessed radiographically. The potential participants would then be assessed for the exclusion criteria as follows: the presence of systemic diseases compromising immune function, pregnancy, history of major surgeries (e.g., cardiac, kidney transplantation, hemodialysis), drug allergies, severely decayed or fractured teeth that could not be isolated with a rubber dam, teeth requiring post and core restorations, previous root canal treatment, root fractures, external or internal resorption, developmental anomalies, generalized chronic periodontitis, draining sinuses, calcified canals, or canals with significant curvature.

Randomization

The included teeth were randomly allocated into three groups following a parallel design. On January 1, 2019, a random sequence was generated by Y. A. T. using www.random.org to assign participants to one of three treatment groups: Group A received the Blood Clot scaffold, Group B received the Native Chitosan combined with Blood Clot scaffold, and Group C received the Enzymatically-Modified Chitosan combined with Blood Clot scaffold. To ensure randomization, 30 numbered sheets of paper were individually placed in opaque envelopes, and each patient selected an envelope at the start of their second visit. The number in the envelope determined the regenerative protocol they would undergo. If the patient had more than one included tooth, they were asked to draw more than one envelope, corresponding to the number of teeth included.

Blinding

As this was an interventional study, it was not applicable to blind the treating clinician to the type of scaffold used during treatment. However, the participants were completely blinded. The treatment outcomes were assessed by two trained researchers, not involved in the treatment of the participants, who were calibrated to the evaluation criteria and blinded to the scaffold type used.

Pre-operative Assessment

The participants were initially examined for eligibility at a baseline visit, and the tooth sensibility was tested to confirm the negative tooth response using the cold test, before testing the sensation of the treated tooth, other adjacent vital teeth and non-vital if present were tested, to establish a reference response and for comparison, and adjacent vital teeth responded positively to cold test.

Periapical radiographs were taken to confirm the completion of the apices and the presence of a periapical lesion.

The trial was introduced to participants, after explaining detailed treatment procedures, potential outcomes, complications, and follow-up periods, and the participants were asked to sign a printed informed consent that explained the aim and details of the study.

Interventions

The American Association of Endodontists AAE protocol was applied for endodontic regeneration procedures and the clinical work was done in two visits (AAE 2021) (19).

First Visit

Local anesthesia was performed by using 2% lidocaine with adrenaline (Adrecain Dental, Avenzor, Damascus, Syria). Isolation was performed with a rubber dam, and the treated

Figure 1. Isolation with rubber dam and opening of the pulp chamber

Figure 2. Modified triple antibiotic paste inside the root canal

tooth surface was wiped with a piece of gauze moistened with 5.25% sodium hypochlorite (Shahbadend, Aleppo, Syria). The access cavity was prepared by a sterilized round diamond bur (Lusterdent, Zhengzhou, Henan, China) at high speed under a copious water spray (Fig. 1).

The pulp tissue was removed by a barbed broach size 25# (Shin File, Shinhung, Korea). Subsequently, the patency of the root canal was checked using a #15 K-file (Mani, Tochigi, Japan), then the working length was determined clinically using an electronic apex locator (E-PEX pro; Eighteeth, Changzhou, Jiangsu Province, China) and confirmed radiographically with an intraoral periapical radiograph using a digital sensor (Nanopix1; Eighteeth, Changzhou, Jiangsu Province, China) and a radiography device (X-MIND-De Gotzen-Fagnano Olona-Italy) with the exposure dose 0.16 µSv.

Mechanical instrumentation of the canal was performed using rotary files (Fanta System, Fanta-Dental, Shanghai, China), with a rotary device (E-Connect Pro, Eighteeth, Changzhou, Jiangsu Province, China). The apical foramen was prepared to a size of #35, meaning that the measurement of the apical foramen after the preparation was 0.35 mm (20).

Subsequently, chemical debridement was done using 2.5% sodium hypochlorite solution (Shahbadend, Aleppo, Syria) at a rate of 20 ml using irrigant tips with side openings (Endo-Top; Fanta-Dental, Shanghai, China), and the tip was inserted 1 mm shorter than the working length, the canal was dried with paper points (Topdent, Shanghai, China) and then washed with 20 ml of physiological saline solution, the canal was then dried with paper points (Topdent, Shanghai, China).

Low concentration 5 mg/ml of modified triple antibiotic paste containing (metronidazole (Alpha, pharmaceutical industries, Aleppo, Syria), ciprofloxacin (Alpha, pharmaceutical industries, Aleppo, Syria), and cefaclor (ELSaad Pharma, Aleppo, Syria), in the ratio 1:1:1 by weight) was prepared in a creamy consistency using propylene glycol and macrogol ointment taken in 1:1 ratio by weight (17). Subsequently, it was placed inside the canal to the cementoenamel junction level by using a lentulo spiral (Lusterdent, Zhengzhou, Henan, China), The pulp chamber was cleaned of any traces of dressing (Fig. 2) and the access cavity was sealed by glass ionomer cement (GC FUJI IX, Tokyo, JAPAN). Participants were recalled after 3 weeks (19).

Second Visit

After three weeks, the safety of the temporary restoration was confirmed and the initial treatment was evaluated to ensure no signs or symptoms. If it was present, the triple antibiotic paste was applied again, and if it was not present, local anesthesia was administered with 3% mepivacaine without a vasoconstrictor (Avocaine Dental 3%; Avenzor, Damascus, Syria), and then the glass ionomer cement has been removed and the tooth was re-accessed under rubber dam isolation.

The antibiotic paste was removed by irrigation with 20 ml physiological saline solution followed by 10 ml of 17% EDTA solution (Metabiomed, Chungcheongbuk, Korea) and left for one minute within the canal, and the final irrigation was done using 20 ml physiological saline solution, the root canal was dried with paper points and the regenerative procedure was done according to the group to which each patient belonged.

1. Group A: Blood clot scaffold (BC)

In this group, bleeding was induced inside the root canal by gentle over-instrumentation 2–3 mm beyond the apical foramen using a pre-curved #25 K-file (Mani, Tochigi, Japan). It was gently twisted 2 to 3 revolutions clockwise and then also counterclockwise. After obtaining clear bleeding in the canal that reaches the level of the cementoenamel junction, a small cotton pellet soaked with saline is placed in the coronal third of the canal for 10 minutes to allow the blood clot to form (21) (Fig. 3).

2. Group B: Native chitosan with blood clot scaffold (NCS+BC)

In this group, bleeding was induced inside the root canal in the same way that had been done or group A, then, native chitosan with high molecular weight (310,000–375,000 kDa) and a degree

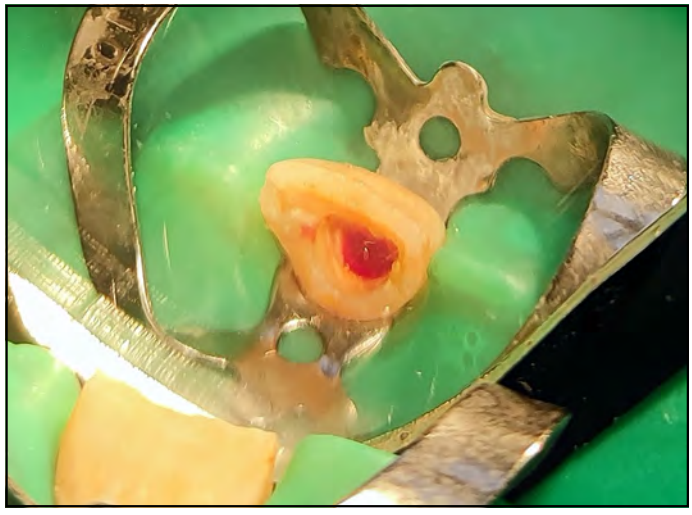

Figure 3. Induction of intracanal bleeding and blood clot formation

of deacetylation greater than 95% (Sigma Aldrich-St. Louis, MO, USA) was applied as a scaffold for pulp regeneration. Native chitosan was introduced in powder form (Fig. 4a) and was dissolved to obtain chitosan gel in the Department of Biology, Faculty of Science, X University. Chitosan gel was injected into the root canal by inserting the injection tip into the middle of the pulp canal. Subsequently, it was injected slowly with gradual withdrawal until the canal was filled. It was then mixed with the blood clot by using the #25 K-file until they were homogenous (22).

Preparation of chitosan gel

Diluted acetic acid at a concentration of 2% was obtained by dissolving 2 ml of acetic acid (Sigma Aldrich-St. Louis, MO, USA) in 100 ml of distilled water. After that, 300 mg of chitosan powder was dissolved in 15 ml of previously prepared 2% diluted acetic acid and stirred for twenty-four hours using a magnetic stirrer (Stuart, CNG Instruments Sdn Bhd, Shah Alam, Selangor, Malaysia) at a rate of 1200 rpm at room temperature, then the pH of the chitosan was adjusted by adding sodium hydroxide solution NaOH (N4) to reach a pH of 5.5–6, which was measured using a pH meter (3510 PH Meter, Cole-Parmer Instrument co, Vernon Hills, Illinois, USA) (14). The materials were sterilized with ultraviolet light and kept in the refrigerator until application (Fig. 4b).

3. Group C: Enzymatically modified chitosan with blood clot scaffold (EMCS+BC)

In this group, bleeding was induced inside the root canal in the same way that had been done for group A, then, the enzymatically modified chitosan which has been modified in the Department of Biology, Faculty of Science, Damascus University, was injected into the root canal and mixed with the blood clot by using k-file size 25# until they were homogenous (22).

Modifying Chitosan

Native high molecular weight chitosan 310000–375000 kDa with a degree of deacetylation greater than 95% was used from (Sigma Aldrich-St. Louis, MO, USA) company.

The chitosan was cleaned of impurities to provide pure chitosan. A chitosan solution was prepared at 1% V/W by dissolving chitosan powder in acetic acid at a concentration of 1% V/V with continuous stirring for 24 hours at a temperature of 25˚.

After that, the chitosan was purified using micron membranes with porosity (0.22µm) under vacuum (Unisart, SARTORIUS, Gottingen, Germany). The acidity of the purified chitosan was adjusted with sodium aqueous solution NaOH (N4) to reach a pH of 8. Then the prepared chitosan was washed with sterile water and dried with a special desiccator. Then the dried chitosan was ground and preserved at a heat of 4oC to be modified.

The chitosan was modified through chemical grafting between the NH2 amino groups of chitosan and the enzymatic oxidation products of catechin) Sigma Aldrich-St. Louis, MO, USA), which were obtained by oxidizing catechin with an oxidation reaction catalyzed by the laccase enzyme Suberase® (Sigma Aldrich-St. Louis, MO, USA), which is a fungal enzyme extracted from the fungus Trametes Versicolor. The grafting process was carried out in an aqueous medium at a temperature of 30˚ under atmospheric conditions, where 1g of Chitosan particles was mixed with 5 mm of Catechin and 45 ml of a phosphate buffer in the processor. The reaction was started by adding 0.13 ml of the laccase enzyme Suberase® and placed under continuous stirring for 4 hours.

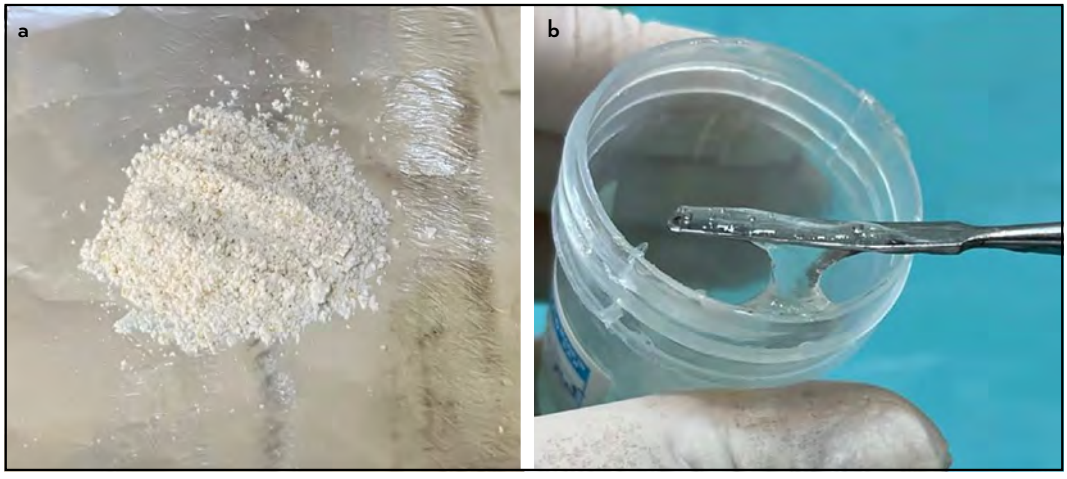

Figure 4. Native Chitosan; (a) powder form, and (b) gel form

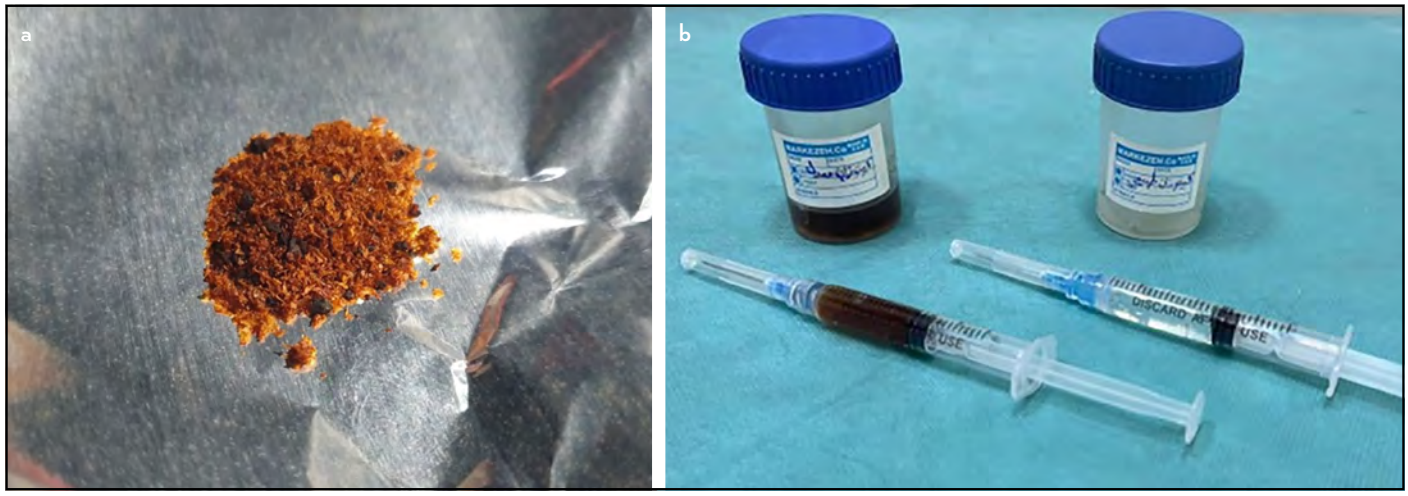

Figure 5. Enzymatically modified chitosan; (a) powder form, and (b) gel form

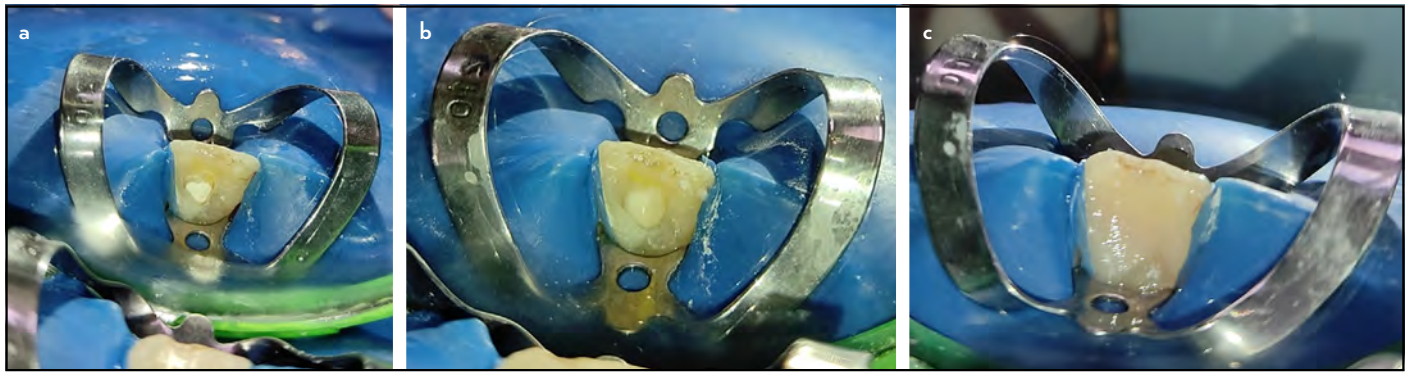

Figure 6. Stages after scaffolds application; (a) application of white mineral trioxide agregate, (b) application of glass ionomer cement, and (c) the final restoration with composite

The reaction was then stopped by purifying the reaction mixture through micronized membranes with a porosity (0.22µm) under vacuum (Unisart, SARTORIUS, Gottingen, Germany). The resulting chitosan was then washed well with a phosphate buffer, methanol, ethanol, and finally an acetone solution to remove all catechin and enzyme molecules from the modified chitosan. In addition, the resulting chitosan was chemically treated with an HCL/ethanol solution (50:50) and KOH/ethanol solution (50:50) at room temperature until color stability was achieved (14) (Fig. 5a).

The enzymatically modified chitosan was prepared in gel form using the same method as native chitosan gel. (Fig 5b).

Coronal Seal Procedures

After applying the scaffolds to the experimental groups, white MTA material (MTA Cem; Nexobio, Chungcheongbuk, Korea) was applied. It was mixed with distilled water according to the manufacturer's instructions and applied using the MAP system (MAP system, Medesy, Maniago, Italy) within the root canal orifices (23) (Fig. 6a). A layer of Glass Ionomer cement (GC FUJI IX, Tokyo, JAPAN) was applied to the MTA material (Fig. 6b), it was left to wait for five minutes, and then the teeth were restored with a composite resin material (Tetric N-Ceram, Ivoclar Vivadent, Schaan, Liechtenstein) (19) (Fig. 6c).

Follow-up

The participants were followed up at one, three, six, and twelve-month intervals to evaluate treated teeth clinically by exa1mination of the presence of spontaneous or palpable pain, soft tissue swelling, or sinus tract, and radiographically to assess the condition of the periapical lesion (Figs. 7, 8).

The sensibility test was conducted using ethyl chloride (Pulp Spray, Cerkamed Medical Company, Staloea Wola, Poland) after isolating the tooth to be tested. A piece of cotton soaked in it was placed on the buccal surface for 15 seconds. Before testing the sensation of the treated tooth, other adjacent vital teeth and non-vital if present were tested, to establish a reference response and for comparison.

Outcomes Measurements

The clinical and radiological follow-up results were recorded during the follow-up periods, where they were evaluated by two specialists who were blinded to the treatment group to which the patient was assigned. The certified degrees for periapical lesion healing and the corresponding value given for each degree were as follows:

0. **Failure:** The size of the lesion has increased radiologically or the existing lesion has not changed in size with the presence of symptoms or signs.

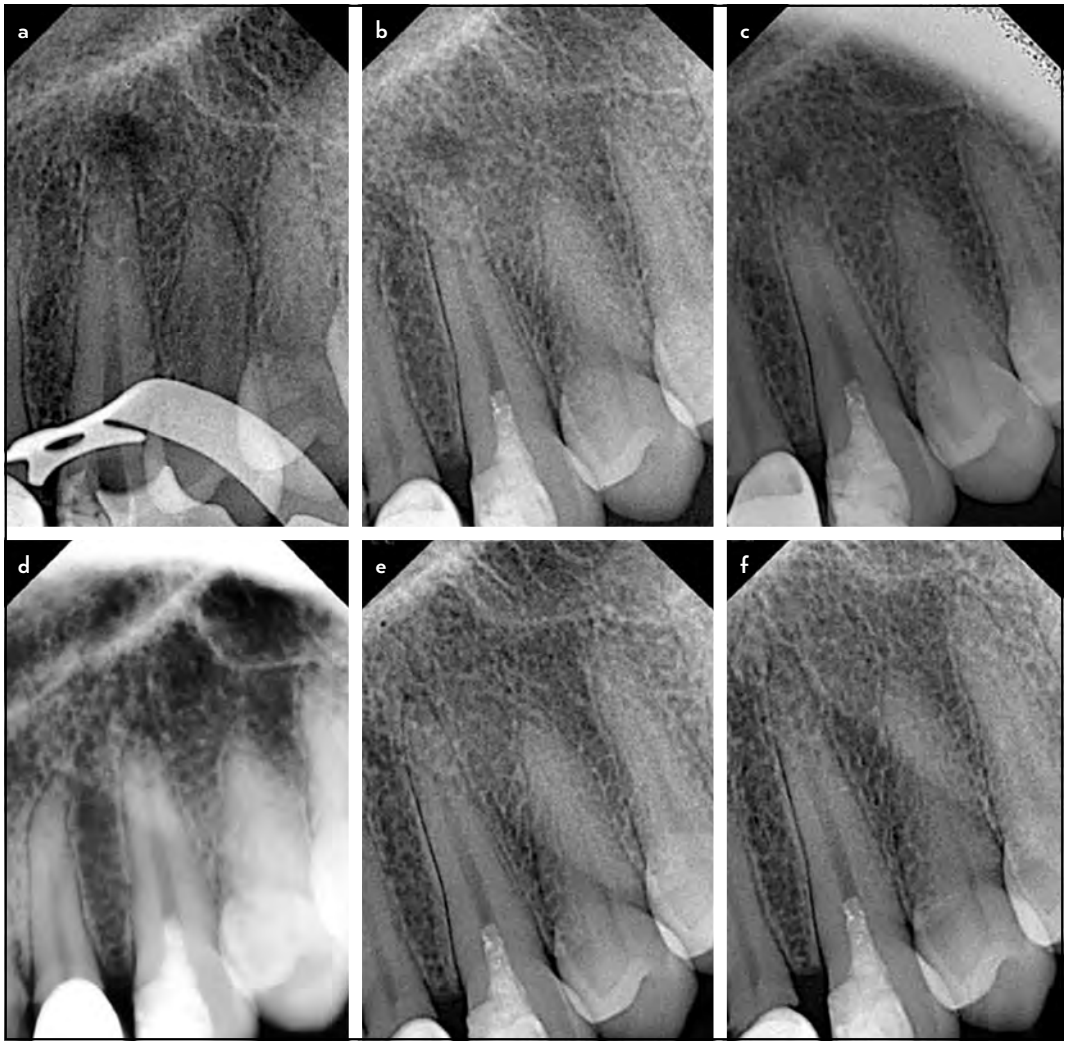

Figure 7. A case periapical radiographs: (a) Preoperative radiograph of tooth #23 shows Periapical radiolucent lesion at the apex of root. (b) A postoperative radiograph after REPs with (BC) scaffold alone. (c) One-month follow-up radiograph. (d) Three-month follow-up radiograph. (e) Six-month follow-up radiograph, (c, d, e) shows decreasing the size of periapical lesion and evidence of healing of periapical lesion. (f) Twelve-month follow-up radiograph, periapical lesion show healing

REPs: Regenerative endodontic procedures, BC: Blood clot

1. **Doubt:** The size of the lesion remains the same radiographically with no symptoms or signs.
2. **Healing:** The radiographic evidence indicates a decrease in the lesion size without complete disappearance, and the tooth is clinically healthy without symptoms or signs.
3. **Success:** The radiographic evidence confirms that the lesion has completely disappeared and the tooth is clinically healthy without any symptoms or signs.

Evaluation of sensibility test:

The results of the cold test by ethyl chloride were recorded as follows:

- Response.
- No response.

Notably, the primary and tertiary goals established by the American Association of Endodontists for assessing treatment success were adopted to evaluate the outcomes of regenerative treatments performed on the samples in this study (19).

Statistical Analysis

The analytical statistical study of the current research data has been completed using (SPSS – Version 13.0) program, Kruskal-Wallis test was conducted to study the significant differences in the frequencies of healing degrees among the three studied groups (BC, NCS+BC, EMCS+BC) according to the period studied when statistically significant differences were found, pairwise comparisons were conducted using the Mann-Whitney U test among the three groups according to the period studied to determine which of these studied groups significantly differs from the others at a confidence level of 95%.

A chi-square test was performed to study the significant differences in the frequencies of tooth sensibility in the cold test among the three groups (BC, NCS+BC, EMCS+BC) according to the period studied at a confidence level of 95%.

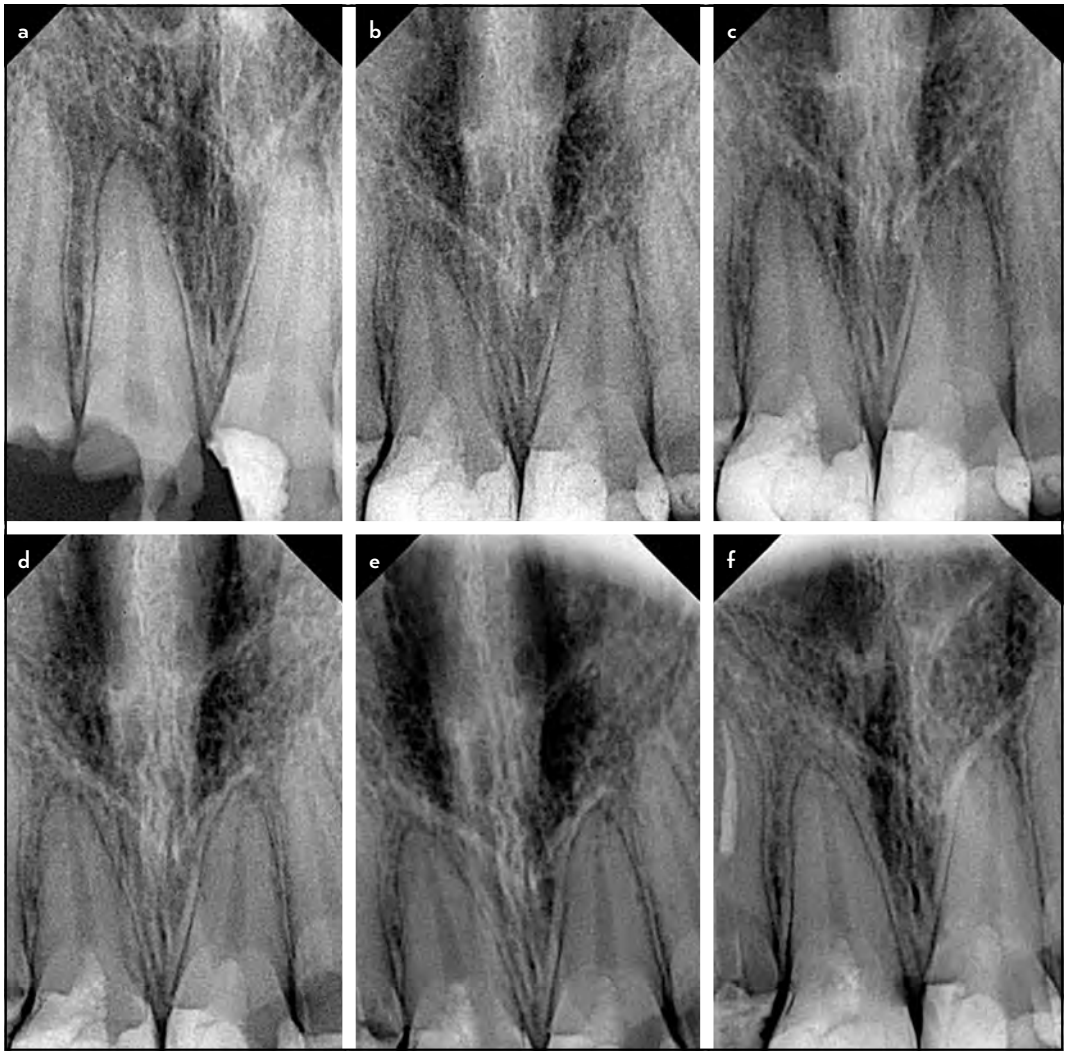

Figure 8. Two cases periapical radiographs: (a) Preoperative radiograph of tooth #11 and #21 shows Periapical radiolucent lesions at the apices of roots. (b) A postoperative radiograph after REPs with (EMCS+BC) scaffold for tooth #11, and (NCS+BC) scaffold for tooth #21. (c) One-month follow-up radiograph. (d) Three-month follow-up radiograph. (e) six-month follow-up radiograph, (c, d, e) shows decreasing the size of periapical lesions and evidence of healing of periapical lesions. (f) Twelve-month follow-up radiograph, periapical lesion show healing

REPs: Regenerative endodontic procedures, NCS+BC: Native chitosan and blood clot, EMCS: Enzymatically-modified chitosan

RESULTS

Thirty teeth from twenty-four participants (7 males and 17 females) aged between 15 and 45 years ($\bar{X}$=29.5) were included in the study. No significant differences were reported between the groups regarding the age (p=0.932) and gender (p=0.366) of the treated participant, indicating that the allocation of participants into study groups was randomized.

Age and gender distributions across groups and statistical tests' results for their comparison among groups are presented in Table 1 and Table 2 respectively. The flow chart of the participants was described in Figure 9.

- ***Healing degree of periapical lesions:***

  Table 3 summarizes the healing degree of periapical lesions frequency and the Kruskal-Wallis test results after 1, 3, 6, and 12 months of treatment in the groups.

  There were no significant differences in the frequencies of healing degrees among the groups (BC, NCS+BC, and EMCS+BC) after one month, three months, and twelve months. However, the Kruskal-Wallis test showed significant differences in the frequencies of healing degrees among the groups (BC, NCS+BC, and EMCS+BC) only after six months. Therefore, the Mann-Whitney U test was utilized to study the pairwise comparisons among groups at six months (Table 4).

  The degree of healing after six months in the EMCS+BC group was higher than those of both the NCS+BC group and the BC group. Moreover, there were no statistically significant differences in the frequencies of healing degrees after six months between the NCS+BC group and BC group.

- ***Tooth sensibility in the cold test:***

  Table 5 summarizes the treated teeth sensitivity response/no response frequency, and the Kruskal-Wallis test results after 1, 3, 6, and 12 months of treatment.

  There were no significant differences in the frequencies of tooth sensibility in the cold test among the three studied

TABLE 1. Descriptive and analytic statistics of age distribution across groups

Scaffold	Mean	SD	Min	Max	p*
BC	29.7	9.3	15	45	0.932
NCS+BC	29.5	9.3	17	45	
EMCS+BC	29.7	9.1	17	45	

*: One-Way ANOVA. SD: Standard deviation, Min: Minimum, Max: Maximum, NCS+BC: Native chitosan and blood clot, EMCS: Enzymatically-modified chitosan

TABLE 2. Descriptive and analytic statistics of gender distribution across groups

Scaffold	Sex		Total	p*
	Male	Female		
BC	2	8	10	0.366
NCS+BC	3	7	10	
EMCS+BC	2	8	10	

*: Chi-square test. NCS+BC: Native chitosan and blood clot, EMCS: Enzymatically-modified chitosan

groups (BC, NCS+BC, and EMCS+BC) after one and three months because there was no tooth sensation in the cold test after one month and after three months for all teeth regardless of the scaffold applied. However, there were significant differences in the frequency of tooth sensibility in the cold test among the three studied groups (BC, NCS+BC, and EMCS+BC) after six and twelve months (Table 6).

The tooth sensibility in the BC group was smaller than that of both the NCS+BC group and EMCS+BC group, and there were no statistically significant differences in the frequencies of tooth sensibility between the NCS+BC group and EMCS+BC group.

DISCUSSION

Regenerative pulp therapy is divided into two essential techniques: Cell-Based strategy and Cell-Homing strategy. The latter is based on the host-existing endogenous stem cells from dental papilla recruitment and is considered one of the least complex and most clinically applicable techniques. A review is currently directed toward understanding and developing tissue regeneration using a cell-homing strategy to generate tissues more similar to dental pulp in form and function (24).

A rigorous methodology was followed in the current study to investigate the efficacy of three scaffolds in the context of regenerative endodontic treatment: blood clot, neutral chitosan with blood clot, and enzymatically modified chitosan with blood clot. Root canal debridement involved extensive irrigation with a 2.5% concentration of sodium hypochlorite, following AAE recommendations to maximize disinfection and support the survival of SCAP (19).

Special irrigant tips with closed ends and side vents with irrigating tips positioned about 1 mm from the root end minimize the possibility of extrusion of irrigants into the periapical space and minimize cytotoxicity to stem cells in the apical tissues (19).

The size of the apical foramen was prepared at 0.35 mm, with the aim to obtain the minimum apical diameter needed for endodontic regeneration of mature teeth. Laureys et al. (25) showed that the smallest apical diameter teeth ranging between 0.24 and 0.53 mm did not prevent revascularisation and demonstrated new tissue formation. Shah and Logani (21) and Saoud et al. (26) reported clinical and radiographic healing of periapical lesions related to mature teeth after apical enlargement until file sizes of 30 and 35 respectively. Subsequently, a modified triple antibiotic paste consisting of Ciprofloxacin, Metronidazole, and Cefaclor was applied to eliminate the effect of tooth discolouration resulting from the use of Minocycline, and it was placed inside the canal to the level of the cementoenamel junction to minimize crown staining (19). Moreover, a low concentration of triple antibiotic paste was applied at 5 mg/ml to reduce cytotoxicity to stem cells in the apical tissues (19). Then, irrigation with 17% EDTA was performed to induce the release of growth factors through surface demineralization of the dentine, facilitating enhanced MSC attachment and growth (27). Moreover, the canals underwent thorough rinsing with a physiological saline solution to reduce potential direct toxicity on the stem cells (28). In the second session, local anesthesia was given without a vasoconstrictor so as not to affect bleeding and induction of blood clots (29).

Chitosan was applied as a scaffold in dental pulp regeneration due to its distinctive properties as an antimicrobial and antifungal scaffold, with biocompatibility and biodegradability that support cell growth and proliferation (30). Despite the widespread use of chitosan in various applications, it has some disadvantages such as its low solubility in neutral environments, resulting in a weakened ability to interact effectively. Additionally, its weak physical properties, including brittleness and stiffness from the strong hydrogen bonds between its molecules (31). Kim et al. (32) highlighted that the chitosan alone application did not yield favorable results in cell adhesion, proliferation, and differentiation. Hence, the chitosan modification to alter its properties aimed at achieving better results. However, not all chitosan derivatives delivered the desired outcomes in dental pulp regeneration. Palma et al. (33) noted that the addition of hyaluronic acid or pectin to chitosan did not improve dental pulp regeneration in dogs' teeth. Although Ducret et al. (10) also explained that the chitosan gel application with fibrin provided some benefits through the antimicrobial properties of chitosan, there was no clear improvement in cell adhesion and proliferation properties. On the other hand, enzymatic modification of chitosan presented several distinctive properties, such as increased adhesion, proliferation, and differentiation of stem cells, along with enhanced antimicrobial efficacy. This was experimentally demonstrated by Aljawish et al. (15, 34).

To date, there is no previous clinical study for the application of enzymatically modified chitosan in pulp regeneration techniques or even to compare it with native chitosan scaffolds and blood clots to determine which is better in pulp regeneration, hence the aim of the current study.

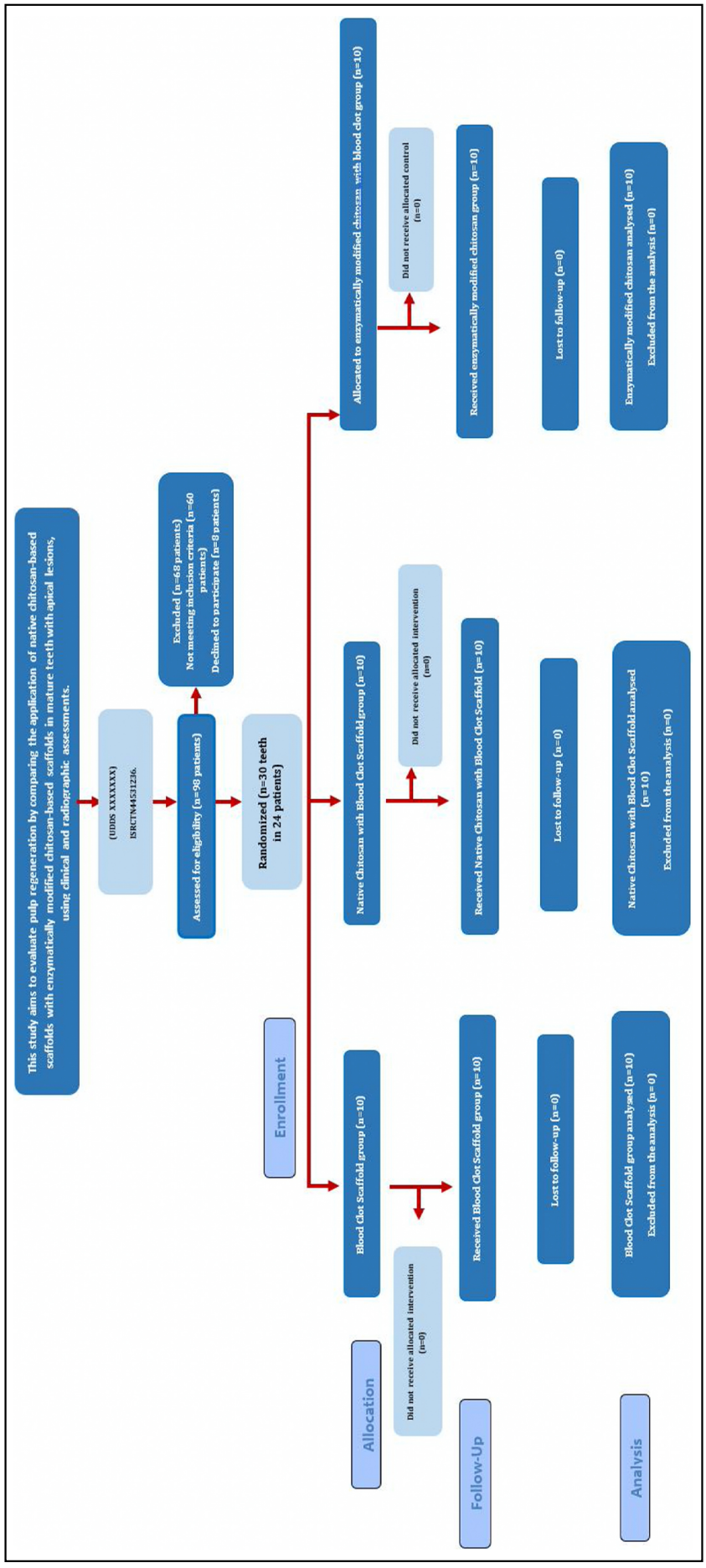

Figure 9. Consort 2010 flowchart

The studied scaffolds were applied in gel form due to their ability to be used more effectively, allowing access to all the complicated and relatively small details of the pulp canal, and enabling greater cell migration (35).

It is worth noting that native chitosan and enzymatically modified chitosan scaffolds were applied in combination with a blood clot to take advantage of the synergistic action resulting from the combination of the scaffolds with the blood clot allowing the migration and adhesion of stem cells that are attracted to the pulp canal after the induction of bleeding from the apical end (36). Finally, MTA was chosen because it has been used in the majority of the published cases, while other barrier materials, although newly introduced, are still undergoing testing to assess their effectiveness in REPs (27). Moreover, MTA was positioned beneath the cemento–enamel junction to prevent coronal staining (37).

Patient selection and follow-up are key considerations in clinical studies. Conveniently, all participants in this study resided close to the research facility, facilitating ease of follow-up. Moreover, as part of the inclusion criteria, patients signed an informed consent form, agreeing to attend radiographic follow-up appointments. This approach ensured full participation, with no dropouts from the sample.

The treatment success and failure criteria, adopted from the American Association of Endodontists, aimed to achieve three goals: elimination of symptoms and evidence of bony healing (primary), increased root wall thickness and/or root length (secondary but desirable), and a positive response to sensibility testing, potentially indicating more organized vital pulp tissue (tertiary) (19).

The results were analyzed according to the primary and tertiary objectives of the REPs.

The results showed that the degree of healing after six months in the EMCS+BC group was higher than those of both the NCS+BC group and BC group, and there were no statistically significant differences in the frequencies of healing degrees after six months between the NCS+BC group and BC group. There were also no significant differences in the frequencies of healing degrees among the groups (BC, NCS+BC, EMCS+BC) after twelve months. This is attributed to the importance of enzymatically modified chitosan in increasing stem cell adhesion, proliferation, and differentiation, thus, faster tissue

TABLE 3. Descriptive statistics of the healing degree of periapical lesions in groups and the p-values of significance testing

Studied period	Scaffold	Number	Periapical lesions healing degrees				Mean rank	Chi-square value	p^
			Failure	Doubt	Healing	Healed			
After 1 month	BC	10	0	6	4	0	16.00	0.278	0.870
	NCS+BC	10	0	7	3	0	14.50		
	EMCS+BC	10	0	6	4	0	16.00		
After 3 months	BC	10	0	3	7	0	15.50	0.921	0.631
	NCS+BC	10	0	4	6	0	14.00		
	EMCS+BC	10	0	2	8	0	17.00		
After 6 months	BC	10	0	3	7	0	12.45	7.466	0.024*
	NCS+BC	10	0	2	8	0	13.80		
	EMCS+BC	10	0	0	7	3	20.25		
After 12 months	BC	10	0	1	3	6	14.85	0.455	0.797
	NCS+BC	10	0	1	3	6	14.85		
	EMCS+BC	10	0	0	3	7	16.80		

^: Kruskal-Wallis test, *: Significant difference. NCS+BC: Native chitosan and blood clot, EMCS: Enzymatically-modified chitosan

TABLE 4. The pairwise comparison to examine the significance of healing degrees among the three studied groups

Time period	Group I	Group J	U-value	p^
After 6 months	EMCS+BC group	NCS+BC group	28.0	0.028*
		BC Group	24.5	0.017*
	NCS+BC group	BC Group	45.0	0.615

^: Mann-Whitney U test, *: Significant difference. EMCS+BC: Enzymatically-modified chitosan and blood clot, NCS: Native chitosan

regeneration and faster healing of periapical lesions. This was proven in the study of Aljawish et al. (15), who assessed the growth of stem cells on chitosan films and compared them with enzymatically modified chitosan, and concluded that increasing the hydrophobic property and increasing surface roughness of the enzymatically modified chitosan, increases cell adhesion and proliferation.

The EMCS+BC group demonstrated superior healing degrees after six months of treatment. This may contribute to a higher rate of patient satisfaction with endodontic therapy overall, as patients tend to feel better in a shorter period of time. Additionally, a quicker resolution of dental issues allows the patient to regain full function of their teeth sooner, which is particularly important for essential functions such as eating and speaking. It is also anticipated that endodontic problems resolved within a six-month timeframe may lead to better long-term stability.

The tooth sensibility in the BC group was smaller than those of both the NCS+BC group and EMCS+BC group, and this is attributed to the synergistic effect resulting from combining the scaffolds with the blood clot, which helps stabilize the blood clot. After the hybrid scaffold stabilizes, the cells begin to secrete the extracellular matrix that contains growth factors, the third important element in pulp regeneration, which increases cell differentiation and generation of more organized pulp tissue (38).

As previous studies have indicated one of the most important disadvantages of a blood clot when applied as a scaffold in pulp regeneration is its instability and rapid dissolution, which reduces its effectiveness in pulp regeneration using Cell-Homing technology (39).

Also, this synergistic effect in the NCS+BC group and EMCS+BC group increased the number of stem cells and the amount of growth factors that help the differentiation of these cells.

The results of the current study were consistent with the results of Moreria et al. (36), where it was demonstrated that applying native chitosan alone and without participating with the blood clot did not give the desired results in pulp regeneration compared to the hybrid scaffold that combines between chitosan and a blood clot.

The tooth sensibility was positive in 60% of the cases in the NCS+BC group and EMCS+BC group. The results of the current study were consistent with the results of Youssef et al. (40), where it was reported that tooth sensibility was positive in a percentage of 50%. However, the current study disagreed with Saoud et al. (20), as it was reported that the tooth sensibility was negative in all cases, this matter is attributed to different techniques and instruments used that might have influenced the results.

Treatment complications are always a potential risk, and regenerative procedures are no exception. A review study highlighted that a frequent complication in clinical trials utilizing cell homing is calcification and eventual obliteration of the root canal space over time. This not only constitutes a failed regenerative treatment but can also complicate future pulpectomy procedures, potentially lowering their success rate (24). Fortunately, no such complications were encountered during the one-year follow-up of the studied sample.

The limitations of this study include that it was retrospectively registered, and it was not applied to a histological study using immunological stains that help identify the nature of the new tissues and formed cells more accurately than accurately determine the tissues formed after the application of scaffolds with cases of teeth with periapical lesions.

TABLE 5. Descriptive statistics of the teeth sensibility of periapical lesions in groups and the p-values of significance testing

Studied period	Scaffold	Number	Teeth sensibility		Chi-Square value	p^
			No response	Response		
After 1 month	BC	10	10	0	0	1.000
	NCS+BC	10	10	0		
	EMCS+BC	10	10	0		
After 3 months	BC	10	10	0	0	1.000
	NCS+BC	10	10	0		
	EMCS+BC	10	10	0		
After 6 months	BC	10	9	1	5.833	0.049*
	NCS+BC	10	5	5		
	EMCS+BC	10	4	6		
After 12 months	BC	10	9	1	6.787	0.033*
	NCS+BC	10	4	6		
	EMCS+BC	10	4	6		

^: Kruskal-Wallis test, *: Significant difference. NCS+BC: Native chitosan and blood clot, EMCS: Enzymatically-modified chitosan

TABLE 6. The pairwise comparison to examine the significance of teeth sensibility among the three studied groups

Time period	Group I	Group J	Chi-Square value	p^
After 6 months	EMCS+BC group	NCS+BC group	0.202	0.653
		BC group	5.495	0.019*
	NCS+BC group	BC group	3.810	0.051
After 12 months	EMCS+BC group	NCS+BC group	0	1.000
		BC group	5.495	0.019*
	NCS+BC group	BC group	5.495	0.019*

^: Chi-Square test, *: Significant difference. EMCS+BC: Enzymatically-modified chitosan and blood clot, NCS: Native chitosan

It is suggested to conduct further histological studies using immunological stains on the application of enzymatically modified chitosan alone or in combination with a blood clot with cases of teeth with or without periapical lesions, and it is suggested to conduct further clinical and radiological studies on the application of enzymatically modified chitosan alone or in combination with a blood clot and their effect on pulp regeneration in vital cases without periapical lesions in mature and immature teeth. A longer follow-up period is also recommended to monitor the regenerative process.

CONCLUSION

Based on the data of the current study, we conclude the following:

- Using enzymatically modified chitosan in combination with a blood clot as a scaffold for pulp regeneration gives excellent results.
- The degree of healing in enzymatically modified chitosan in combination with a blood clot group was higher than that of native chitosan in combination with a blood clot group and blood clot group alone with a significant difference at six months. However, there were no significant difference at one month, three months, and twelve months.
- The tooth sensibility in enzymatically modified chitosan and native chitosan with blood clot scaffolds is higher than in the blood clot group alone.
- There were no statistically significant differences in the frequencies of tooth sensibility between the NCS+BC group and the EMCS+BC group.
- The use of the enzymatically modified chitosan with a blood clot as a scaffold for pulp regeneration is recommended, especially in the Cell-Homing technology.

Disclosures

Ethics Committee Approval: The study was approved by the Faculty of Dentistry Damascus University Local Research Ethics Committee (no: UDDS-1189-25092018/SRC-74, date: 25/09/2018).

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