JOURNAL OF COMPLEMENTARY MEDICINE RESEARCH
Journal of Complementary Medicine Research, ISSN: 2146-8397 Vol. 14, No. 2, 2023 (pp.104-109)
RESEARCH ARTICLE
WWW.JOCMR.COM

EDTA/EMD as root chemical agents on enhancing the attachment of gingival fibroblast: *In vitro* study

Baher K. Felemban[1], Walaa F. Babgi[2], Ahmed M. H. Bagasi[3], Osama M. Felemban[4], Ehdaa A. AlTurkistani[2], Salwa A. Aldahlawi[1], Abdel-Rahman Youssef [5,6]

[1]*Department of Basic and Clinical Oral Sciences, Division of Periodontology, Faculty of Dental medicine, Umm Al-Qura University, Makkah, Saudi Arabia*

[2]*Resident, Saudi Board of Periodontic, Umm Al-Qura University, Makkah, Saudi Arabia*

[3]*General Dentist, Private Sector, Makkah, Saudi Arabia*

[4]*Pediatric Dentistry Department, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia*

[5]*Department of Basic and Clinical Oral Sciences, Division of Basic Medical sciences, Faculty of Dental medicine, Umm Al-Qura University, Makkah, Saudi Arabia.*

[6]*Department of Microbiology and Immunology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.*

ABSTRACT

Background: Since chronic periodontitis has destroyed the root surface, it is challenging for fibroblast cells to bind to the root during root covering therapy. The ability of the gingival fibroblast to deposit collagen and regain attachment depends on the root surface microstructure; thus, the surface must be treated to be more compatible with gingiva cells.

Aim of Study: The study aimed to compare the efficiency of EDTA/EMD root conditioning agents and the capability to modify the root surface at the microstructure grade to deliver a fundamental surface to stimulate fibroblast adherence.

Materials and methods: Healthy extracted teeth were collected; a total of samples (n=30) have been split into a control group and four test groups, each with six samples. The test groups have been conditioned with either HA, 24% EDTA, EMD, or EDTA/EMD for two different time intervals. Fibroblast cells were planted on each sample and then incubated for 72 hours. Cell density in groups was determined using the MTT assay.

Results: Adhesion of viable fibroblast to the root surface showed variable results with varied materials and application times. Generally, it was higher in the longer-time duration groups except for HA. The most favorable result was observed in the EDTA plus EMD group, followed by the EDTA group when applied for 4 minutes. The least favorable result is with HA after 2 minutes of application.

Conclusion: EDTA plus EMD group display remarkable root surface modifications that induce fibroblast adhesion to the surface compared to other groups, as they showed a synergistic effect when applied to the same sample.

How to cite this article: Felemban BK, Babgi WF, Bagasi AM, Felemban OM, Al-Turkistani EA, Al-Dahlawi SA, Youssef AM. EDTA/EMD as root chemical agents on enhancing the attachment of gingival fibroblast. *In vitro* study. Journal of Complementary Medicine Research, Vol. 14, No. 2, 2023 (pp. 104-109)

INTRODUCTION

The most common oral disease is periodontitis which destroys the tooth-supporting tissue (Pihlstrom et al., 2005a). Destructive periodontal diseases develop pathological alteration in the periodontal tissue, including alveolar bone resorption, destruction of connective tissue attachment, and apical displacement of the junctional epithelium (LOWENGUTH & BLIEDEN, 1993). The pathogenesis of periodontitis involves the production of various bacterial enzymes and metabolites; these products tempt structural, physical, and biological changes on the root surface. These changes impair cell adhesion to the root surface (Polson & Proye, 1983; ROCHA et al., 2015). The damaged root surface by periodontal disease is permineralized and covered by contaminants bacterial toxins, and other biologically active substances (J. P. Blomlöf et al., 1996).

Periodontal therapy aims to regenerate the lost periodontium; this goal cannot be achieved if the diseased root surface is not treated and prepared properly for regeneration (Polson & Caton, 1982). It has been shown that periodontal healing is impaired in the presence of endotoxin in the root cementum, and removal of these toxins is a prerequisite, providing the root surface biocompatible for regeneration (J. P. Blomlöf et al., 1997).

KEYWORDS:
HA: hyaluronic acid; EDTA: Ethylenediaminetetraacetic acid; EMD: Enamel Matrix Derivative; MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).
ARTICLE HISTORY:
Received : Dec 28, 2022
Accepted : Jan 17, 2023
Published: Feb 20, 2023
DOI:
10.5455/jcmr.2023.14.02.16

The conventional periodontal treatment of periodonti- tis-affected roots is mechanical instrumentation that does not completely remove the contaminated surface and results in smear layer formation (Bittencourt et al., 2007; J. Blomlöf et al., 1997). This smear layer is amorphous and composed of organic and inorganic debris that hinders periodontal healing (ROCHA et al., 2015). Different adjunctive therapies have been introduced to compensate for the limitations of mechanical instrumentation; chemical root conditioning is one of the most used adjunctive treatments (Silva et al., 2016).

The attachment of fibrine clots to the root surface after root surface alteration by root conditioning material was evaluated using an electron microscope; the study showed that conditioning agents enhance the fibrin network's adhesion to the root surface (Subramanian et al., 2017). The effectsof several root conditioning agents as an adjunct to scaling and root planing were analyzed. These studies revealed that they equally removed the smear layer; however, the effecton dentinal tubules was different (Nanda et al., 2014). The time of application is a critical modification factor affecting dentinal tubule patency (Torkzaban& Seyedzadeh, 2016).

A recent study evaluated the outcome of using different conditioning materials as adjuncts to SRP on fibroblast attachment to the surface of the root; the study concluded that SRP alone, followed by conditioned surface agents, resulted in significantly higher fibroblast attachment (Babgi et al., 2021).

The new trend in periodontal regeneration is the EMD, which is a commercial product of enamel matrix derivatives (Gestrelius et al., 2000). Multiple studies revealed that EMD promotes periodontal ligament cell adhesion, proliferation, matrix production, and cell growth and enhances the production of growth factors (Narani et al., 2007). These properties increase their use in periodontal regeneration (Barekatain et al., 2014).

There is still controversy between the studies regarding the efficiency and the need for root surface treatment.

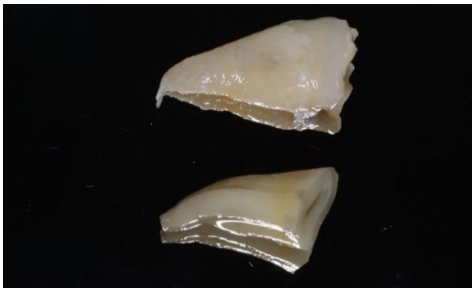

Fig. 1: Crown decapitation display.

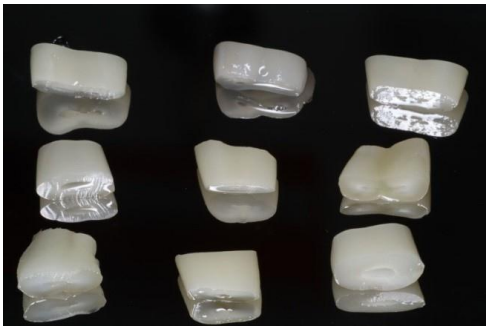

Fig. 2: Display experiment samples.

AIM

To evaluate the reflection of treated root surface alteration with root conditioning materials on fibroblast cell adhesion.

MATERIAL AND METHODS

Experimental design

Single-rooted teeth (n = 18) have been assigned into 5 groups: the control, the untreated group, and four groups treated with different conditioning materials (HA, EDTA, EMD, and EDTA with EMD). In a subsequent treatment, primary gingival fibroblasts were seeded on top of root samples and the sustainability of the cells attached to each root was measured by MTT assay.

Specimen collection and preparation

From private dental clinics, single-rooted teeth (n = 18) that had been removed for orthodontic treatment have been collected. The crown of each tooth was removed (Figure 1) using new disc burs, and the roots were sectioned horizontally into nearly equal parts (Figure 2) to obtain thirty samples. The roots were cleaned with distilled water, sterilized, and stored until the time of the experiment.

Cell culture

Healthy patients at Umm -Al Qura Dental Teaching Hospital signed consent for periodontal surgery. The keratinized soft tissue obtained from the surgical site has been stored ina normal saline container and delivered to a laboratory for fibroblast cell extraction. The collected specimen underwent phosphate-buffered saline washing before being incubated in dispase 1 mg/mL (Sigma, USA) for 12 hours at 4 °C. Further, the specimen was sliced into minor pieces, dissected to attain the connective tissue, cultivated in a full cell growth medium in a 25 mL flask, and kept at 37 °C in a humidified environment of 5% CO_2. Dulbecco Modified Eagle Medium (DMEM, Gibco Thermo Scientific, USA) is used as the growth medium, and it is supplemented with 10% fetal bovine serum (HyClone Thermo Scientific, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, USA), and 2.5 µg/mL amphotericin B (Gibco Thermo Scientific, USA).

Cell viability and MTT assay

Five groups of root specimens were created. The control group has not received chemical therapy. The remaining

Fig. 3: Illustration of the sample root conditioning procedure.

sample's surfaces were treated with various root conditioning agents: hydrant BG: HA Gel is a cross-linked (1.6%) and natural (0.2%) Hyaluronic Acid gel (REGEDENT AG. Zürich. Switzerland), 24% trisodium EDTA gel (biodinamica-Portugal) (Figure 3), EMD (Strauman, Sweden) and EDTA followed by EMD. The chemical agents were applied using a micro-brush in a rubbing motion for different duration, HA for 1 and 2 minutes, and the remaining agents were applied for 2 and 4 minutes. The samples were rinsed with saline and dried. The sixth passage of primary gingival were placed ina 500 µl complete growth medium at a density of 2 × 104 cells/well (Figure 4) after root treatment. The plate was incubated for 72 hours to allow the cells to connect to the roots. Following the incubation period, each sample with root and connected cells was transported to a new well of a 48-well plate, and an MTT (3-(4,5-dimethylthi- azol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was performed to evaluate the viability of fibroblasts connected to the surface of the root (Talebi-Ardakani et al., 2017).

MTT assay

DMEM medium with 0.5 mg/ml MTT (ThermoFisher Scientific, USA) 500 µl/well in a 48-well plate to settle the samples with attached fibroblast cells for 3 hours at 37°C. Following incubation, the medium was withdrawn, and the formazan crystals were dissolved by adding a DMSO:isopropanol (1:1) solubilization solution for 30 minutes. Employing a spectrophotometric microplate reader (SpectroStar Nano, BMG Lab) at a wavelength of 570 nm, the solution has been transported to a 96-well plate at a rate of 100 µl/well, and the optical density (OD) of each well has been calculated.

Statistical analysis

Six samples from each group were used to assess the cell viability, and the median (SEM) is reported as the experiment's findings. For statistical analysis, GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA) was utilized. A difference is significant if $P \leq 0.05$.

RESULTS

The study examined the adhesion of human gingival fibroblasts to healthy root surfaces treated with various conditioning agents.

As shown in (Figure 5), HA application for one minute revealed fibroblast adhesion with a median of (1.04) and (0.62) for two minutes. EDTA material application for two minutes showed a median of (0.75) and a median of (1.31) for four minutes. EMD material revealed a median of (1.03) for 2 minutes and (1.07) for four minutes. EDTA with EMD revealed a median of (0.73) for two minutes and (1.56) for 4 minutes.

For both short and long application times, there wasn't a statistically significant difference in fibroblast adhesion among the control and tested groups. However, there is a statistically significant difference in fibroblast adhesion among two different time applications of the same root conditioning material; HA (p=0.004), EDTA (p=0.026), and EDTA with EMD group (p=0.002).

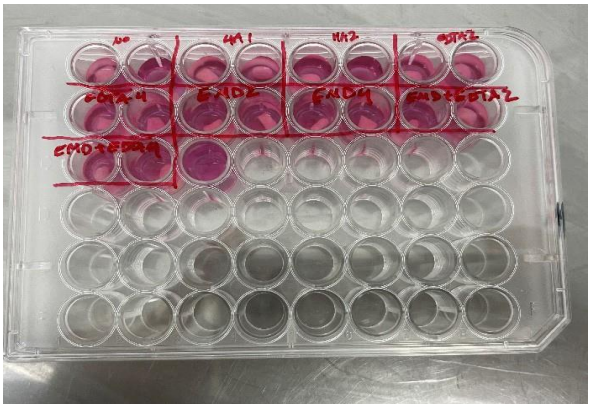

Fig. 4: Display the culture media containing fibroblast cells to be attached to exeriment samples.

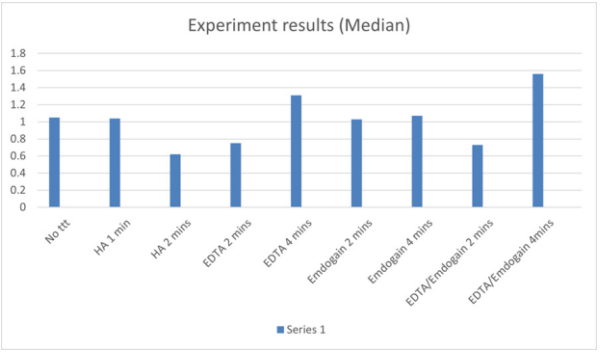

Fig. 5: The findings of an experiment are analyzed in a simplistic manner.

DISCUSSIONS

The study aims to evaluate the ability of fibroblast adhesion consecutive to the microstructure modification of the cementum layer. The healthy root surface with no micro-structure damage is believed to be the most compatiblesurface for gingival fibroblast adhesion. This surface was utilized in this experiment in the control group and comparedto a modified surface with different materials. Three different root conditioning materials were applied to the root surface. Each material is used for two different periods (short and long material application time).

HA for 1 minute resulted in more viable fibroblast attachment than other groups, excluding the control in the short-time application class, as shown in table (1). However, the control groups declared more fibroblast cells' devotion on the root surface in comparison with the HA 1-minute group, although the amount of cell attachment discrepancy could not reach a statistically significant level among the two groups. This result concurs with a recent enhancement after a 1-minute application of HA (Babgi et al., 2021). When HA application time increased to two minutes, the number of fibroblast cells attached to the root surface was significantly reduced to the lowest compared to all groups. These findings imply that surface damage occurs following prolonged root surface contact with HA, resulting in less compatible adhesion between the surface and fibroblasts.

Based on human research, HA is a promising substance due to its potent action on fibroblasts and other cells to improve root surface soft tissue adhesion. HA is a natural polysaccharide in the extracellular matrix of different body tissues, like periodontal

Table 1: Comparative analysis of Fibroblast adhesion to modified surfaces

	Median (min-max)	Control	HA	EDTA	EMD	EDTA and EMD
Short time	Median (min-max)	1.05 (0.63 - 1.56)	1.04 (0.99 - 1.23)	0.75 (0.76 - 1.19)	1.03 (0.92 - 1.27)	0.73 (0.31 - 1.22)
	p-value †		1.00	1.00	1.00	0.150
Long time	Median (min-max)	1.05 (0.63 - 1.56)	0.62 (0.40 - 1.01)	1.31 (0.97 - 1.64)	1.07 (0.84 - 1.48)	1.56 (1.39 - 1.92)
	p-value †		0.150	0.150	0.749	0.150
	p-value ‡		0.004*	0.026*	1.00	0.002*

† p-value using Mann Whitney U test (comparison to the control)
‡ p-value using Mann Whitney U test (comparison of short to a long time interval)
* Significant difference between HA, EDTA, and EDTA with EMD (Short and long-time application)

ligaments (Dahiya & Kamal, 2013). These experiments suggested that HA could be used in the therapy of periodontal disease owing to its anti-inflammatory, anti-edematous, and anti-bacterial impacts (Pihlstrom et al., 2005b). HA is described as having unique structural properties bonded to the protein expressed in the Extra-cellular matrix and collagenous fibers (Oksala et al., 1995) responsible for mediating cell adhesion, motility, migration, and proliferation (Oksala et al., 1995). In addition, HA has various biological functions involved in the wound-healing phase, including revascularization and re-epithelialization, which have been documented in vivo experiments. In contrast to the aforementioned studies, that further left the HA biomodification agent on the roots, the current research washed the HA off the roots after application, which may reduce ~~the~~ benefits of the HA, nullify the biological contribution effect, and preserve only the temporary indirect impact of the material on the root surface topography. In the study, Root surface treatment using HA without having the natural molecular benefits created an undesirable modification, which could harm fibroblast adhesion ability.

The application of the material in EDTA groups continued for 2 and 4 minutes for surface treatment. Fibroblast adherence in EDTA 2 minutes application indicates the lowest capability of the cells to attach to the root surface compared to all groups in short material application time, except EDTA/EMD 2 minutes application. Nonetheless, the 4 minutes of application of EDTA showed the highest amount of fibroblast cells attachment to healthy root surfaces than all other groups, excluding EDTA/EMD 4 minutes application. The evidence suggested that time duration contact between the root surface and EDTA material is critical to establish sufficient surface modification to gain the maximum fibroblast cell attachment. Other studies have the same conclusion; 4 minutes of EDTA application resulted in better fibroblast behavior involving cell adherence and growth (Gamal & Mailhot, 2003; Torkzaban& Seyedzadeh, 2016).

The microscopic modification that occurred through the application of EDTA might contribute to intensifying the ability of collagen deposition and cell adhesion. The EDTA demineralized the inter-tubular matrix to expose enormous dentinal tubules and unsealed tubule orifices on the surface to maintain a secure connection to the cells (Pihlstrom et al., 2005b).

Similarly, EMD was applied for 2 minutes and 4 minutes as root surface treatment. The quantity of cell attachment is slightly higher in EMD 4 minutes than in the control and EMD 2 minutes groups. For EMD, the application time factor has a minor effect on the root surface. Although EMD has a biological influence on cells (Aimetti et al., 2017; al Machot et al., 2014; Fileto Mazzonetto et al., 2021; Windisch et al., 2022), this impact was eliminated in the study to retain the modification effect alone on the root surface. EMD was able to reconstruct the surfaces to be convenient for fibroblast and attached to dentin or cementum, relatively, to match the healthy root surface.

EDTA and EMD were applied to the same root surface individually, raising the fibroblast attachment heavily in EDTA/EMD for 4 minutes compared to all other groups. In this group, each material contacted the root surface for 4 minutes on separate occasions, resulting in more attached fibroblast than 2 minutes module with a statistically significant difference. In addition, the total number of cells adhering to the root module is higher in EDTA/EMD 4 minutes compared to the control group; however, it made no statistically significant difference. EDTA and EMD were applied for 4 minutes to the same root module to observe the fibroblast adhesiveness behavior; the load of the cells to move toward the model and adhere to the root surface were extensively high. The microstructure histology development reaches an extraordinary surface texture to create the foundation for fibroblast attachment.

The EDTA effect on dentin surface has been studied microscopically (Shreehari et al., 2016) and was the most efficient material in exposing the collagen and dentinal tubules additionally to smear layer elimination. This property of EDTA augments the effect of other materials, such asEMD, in this study by providing a more favorable surface for fibroblast adhesion.

In the short material application model, all groups revealed that there were fewer cells adhering to the root surface than in the control group, concluding that the times selected for each material had an unfavorable effect or the time duration was not adequate to obtain the microstructure modification to enhance the adhesion compared to the unmodified root surface. In contrast, the length of substance application increased; all chemicals, with the exception of HA, strengthened the link between cells and root surface, particularly in the EDTA and EDTA/EMD groups. The materials demand sufficient time to achieve fundamental histological alteration to affect cell attitude toward the root surface. In the study, HA was the

only material that damaged the root surface structure; the discovery was noticed by low cell attachment level after 2 minutes of material application. Moreover, the other materials escalate the cell attachment more than the control group. Therefore, the opportunity was given to these materials to alter the surface structure by expanding the time of contact between materials and the root surface, ultimately promoting cell motion and conjunction with the modified surface.

The inconsistency between the study findings and earlier studies could be elucidated by differences in sample size, application methods, washing time, and the type of cells used for attachments.

CONCLUSION

Within the constraints of the research, it is shown that root surface conditioning using EDTA/EMD for 4 minutes is preferable and may be applied in periodontal regenerative therapy. Future studies overcoming the study limitations are necessary to confirm these findings.

Summary:

EDTA/EMD root surface conditioning material for 4 mins displays a microstructure surface modification compatible with fibroblast cells.

Ethical Approval:

The Um Al-Qura University's local biological and medical ethics committee approved the experiment.

Financial support: Self-funding, no grant.

Conflict of interest:

The authors declare that there is no conflict of interest related to the paper's publication.

Informed consent:

Formal written consent forms were obtained from patients prior to sample collection.

Authors Contributions:

BF: Idea initiation, literature review, study design, providing study material, supervision, data interpretation, manuscript writing, review, and editing. **WB:** data collecting, Literature review, manuscript writing, review, and editing. **AB, EA:** data collection. **OF:** statistical analysis. **AY:** provide study materials. Handling cell extraction and culture. **SA:** provide study materials. All authors read and approved the final study manuscript.

REFERENCES

1. Pihlstrom BL, Michalowicz BS.Johnson NW. Periodontal diseases. Lancet. 2005; 366(9499):1809–20.
2. Lowenguth RA, Blieden TM. Periodontal regeneration: root surface demineralization. Periodontol. 2000, 1993;1(1):54–68.
3. Polson AM, Proye MP. Fibrin linkage: a precursor for new attachment. J Periodontol. 1983;54(3):141–7.
4. Rocha F, Zandim-Barcelos Dl, Rossa Junior C, et al. The smear layer created by scaling and root planing is physiologically eliminated in a biphasic process. Brazil Oral Res. 2015; 29:1.
5. Blomlöf LB, Lindskog SF. Smear removal Blomlöf and collagen exposure after non-surgical root planing followed by etching with an EDTA gel preparation. Journal of Periodontol. 1996; 67(9):841–5.
6. Polson AM, Caton J. Factors influencing periodontal repair and regeneration. J Periodontol. 1997; 53(10):617–25.
7. Blomlöf JP, Blomlöf LB, Lindskog SF. Smear layer formedby different root planing modalities and its removal byan ethylenediaminetetraacetic acid gel preparation. Int J Periodontics & Restorative Dent. 1997; 17(3):242–9.
8. Blomlöf J, Blomlöf L, Lindskog S. Effect of different concentrations of EDTA on smear removal and collagen exposure in periodontitis-affected root surfaces. J Clin Periodontol. 1997; 24(8):534–7.
9. Bittencourt S, Ribeiro Edel P, Sallum EA, et al. Root surface biomodification with EDTA for the treatment of gingival recession with a semilunar coronally repositioned flap. J Periodontol. 2007; 78(9):1695–701.
10. Silva AC, Moura CCG, Ferreira JA, et al. Biological Effects of a Root Conditioning Treatment on Periodontally Affected Teeth - An In Vitro Analysis. Brazil Dent J. 2016; 27:160–8.
11. Subramanian S, Appukuttan D, Tadepalli A, et al. Root Conditioning with Citric Acid and Ethylenediaminetetraacetic Acid and their Effect on Fibrin Clot Adhesion to Dentin-A Scanning Electron Microscopic Study. J Clin Diagnostic Res. 2017; 11(8):Zc82-zc85.
12. Nanda T, Jain S, Kaur H, et al. Root conditioning in periodontology - Revisited. J Nat Sci, Biol Med. 2014;5(2):356–8.
13. Torkzaban P, Seyedzadeh SS. The Effect of Four Commonly Used Root Conditioner Agents in Different Time Periods Applied on Periodontally Diseased and Healthy Teeth. J Dent Biomater. 2016; 3(2):241–7.
14. Babgi W, Alhajaji M, Al-Mehmadi L, et al. Effect of root conditioning agents hyaluronic acid, EDTA and chlorhexidine on the attachment of human gingival fibroblasts to healthy root surface. Saudi Dent J. 2021; 33(6):342-347
15. Gestrelius S, Lyngstadaas SP, Hammarström L. Emdogain--periodontal regeneration based on biomimicry. Clin Oral Investigat. 2000; 4(2):120–5.
16. Narani N, Owen GR, Häkkinen L, et al. Enamel matrix proteins bind to wound matrix proteins and regulate their cell-adhesive properties. Eur J Oral Sci. 2007; 115(4):288–95.
17. Barekatain M, Mafi M, Amini S, et al. Emdogain effect on gingival fibroblast adhesion in bioabsorbable and non-resorbable barrier membranes: An in vitro study. Dent Res J. 2014;11(4):429–35.
18. Talebi-Ardakani MR, Torshabi M, Karami E,et al. Comparison of Er:YAG Laser and Hand Instrumentation on the Attachment of Cultured Human Gingival Fibroblasts to Periodontally Involved Root Surfaces. J Lasers Med Sci. 2017; 8(Suppl 1):S51-s55.
19. Dahiya P, Kamal R. Hyaluronic Acid: a boon in periodontal therapy. North Am J Med Sci. 2013; 5(5):309–15.
20. Oksala O, Salo T, Tammi R, et al. Expression of proteoglycans and hyaluronan during wound healing. J Histochem Cytochem. 1995; 43(2):125–35.
21. Gamal A, Mailhot J. The effects of EDTA gel conditioning exposure time on periodontitis-affected human root surfaces: surface topography and PDL cell adhesion. Int Acad Periodontol. 2003; 5:11–22.
22. Fileto Mazzonetto AL, Casarin RCV, Santamaria MP, et al. Clinical, radiographic, and patient-centered outcomes after use of enamel matrix proteins for the treatment of intrabony defects in patients with aggressive periodontitis: A 12-month multicenter clinical trial. J Periodontol. (2019; 92(7):995–1006.
23. Al Machot E, Hoffmann T, Lorenz K, et al. Clinical Study Clinical Outcomes after Treatment of Periodontal Intrabony Defects with Nanocrystalline Hydroxyapatite (Ostim) or Enamel Matrix Derivatives (Emdogain): A Randomized Controlled Clinical Trial. BioMed Res Int. 2014; 2014:786353. doi: 10.1155/2014/786353.
24. Aimetti M, Ferrarotti F, Mariani GM, et al. A novel flapless approach versus minimally invasive surgery in periodontal regenerationwith enamel matrix derivative proteins: a 24-month randomized controlled clinical trial. Clin Oral Investigat. 2017; 21(1):327–37.

25. Windisch P, Iorio-Siciliano V, Palkovics D et al. The role of surgical flap design (minimally invasive flap vs. extended flap with papilla preservation) on the healing of intrabony defects treated with an enamel matrix derivative: a 12-month two-center randomized controlled clinical trial. Clin Oral Investigat. 2022; 26(2):1811–21.

26. Shreehari AK, Darekar HS, Borthakur R. A comparative analysis of root surface biomodification with ethylene diamine tetra acetic acid and tetracycline hydrochloride: An in vitro scanning electron microscopic study. Med J Armed Forces India. 2016; 72(2):145–51.