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Influence of the Angle of Periodontal Intrabony Defects on Blood Clots: A Confocal Microscopy Study

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Abstract: Intrabony defects can be the result of parodontitis. In this study we aimed to examine how clot stability is affected by different infrabony defect angles and superficial treatments in regenerative surgery. Methods: Thirty single-root extracted teeth were cut to obtain a section from each tooth. This section was placed in an artificial model containing an infrabony defect with three walls in order to achieve 10 models at angles of 25°, 37°, and 50°. Five root samples for every angle were not subjected any treatment (NT) and five were subjected to root conditioning with a neutral pH solution of ethylenediaminetetraacetic acid (EDTA) at 24%, applied for 2 min. Venous blood was put into the artificial models containing the root sections, and these were placed in an incubator at a constant temperature of 37 °C for 2 h. Samples were analyzed by laser confocal microscopy. Results: All samples exhibited signs of retraction. The EDTA group exhibited a plateau trend for infrabony defects, while in the NT group, there was a statistically significant difference between clot retraction and the increase in defect amplitude. Conclusion: The greater the angle of the infraosseous defect, the higher the rate of clot retraction. This showed that EDTA could reduce the influence of the angle of the infraosseous defect.

Keywords: intrabony defect; blood clot; EDTA; confocal microscopy

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Citation: Bevilacqua, L.; Patatti, S.; Macrì, A.; Del Lupo, V.; Porrelli, D.; Maglione, M. Influence of the Angle of Periodontal Intrabony Defects on Blood Clots: A Confocal Microscopy Study. *Appl. Sci.* **2024**, *14*, 10306. https://doi.org/10.3390/app142210306

Academic Editors: Iole Vozza and Bruno Chrcanovic

Received: 11 July 2024
Revised: 21 October 2024
Accepted: 7 November 2024
Published: 9 November 2024

1. Introduction

Periodontitis is a chronic immune-mediated inflammatory oral disease that progressively destroys the tooth-supporting apparatus (periodontium). Patients, once diagnosed with periodontitis, should be treated according to a pre-established stepwise approach to therapy. The goal of non-surgical treatment is reduce the bacterial load to a threshold where the host response can defeat it; however, the deeper the probing depth (PD), the more challenging it is to approach the most apical portions of the pocket and the efficacy of the non-surgical therapy might decrease [1–3]. Citterio 2022 [4] concluded that non-surgical periodontal therapy (NST) was able to reduce PD ≥ 5 mm from 28% to 11.7%, so that NST was able to eliminate, at 12 months, 2/3 of the baseline pockets, achieving 90% of sites with PD ≤ 4 mm. Furthermore, it was seen that non-surgical therapy left a mean number of 7.33 sites with PD ≥ 6 mm—sites that must face further surgical therapy if the patient wants to reach periodontal stability. Therefore, according to the 2020 EFP Guidelines, it is recommended to treat teeth with residual deep pockets associated with infrabony defects with periodontal regenerative surgery, which aims to increase the periodontal attachment and bone level, which may result in pocket depth decrease and increased stability. Cortellini and Tonetti [5] have shown how regeneration therapy is effective in the treatment of one-, two-, and three-wall intrabony defects; the morphology of the defect is important because it is one of the factors that determines the stability of the blood clot, which is fundamental for regeneration [6]. Even though it seemed that deeper intrabony defects could heal with more clinical attachment gain than shallower ones, Cortellini showed that they had the same potential for regeneration, since the percentage of clinical attachment level (CAL) gain related to the baseline defect depth was similar [7]. Indeed, an acute angle has a greater potential for regeneration because it creates a narrower area for the creation and stability of the coagulum and wider defects have been associated with reduced amounts of clinical attachment level and bone gain at 1 year [8]. Therefore, a wider angle has less regenerative potential and this might be due to the reduced capacity to create a self-maintaining space for the coagulum, and to the possible collapse of the membrane [9]. The problem concerning this study was the low number of samples, and therefore, the reduced power to detect any possible statistical inconsistency for both angle and clot retraction. Different surgical techniques have been introduced and more preservation of interdental tissue can be achieved to maintain clot stability; a wide defect which is non-supporting can be compensated for by the use of biomaterials such as bone substitutes and membranes.

Another aspect, about which little is still known, is the chemical treatment of the root surface. In the past, various chemical conditioning agents have been proposed as topical treatments of the exposed root surface as adjunctive therapy during periodontal treatment [10,11]. The most widely used are CA (citric acid), PA (phosphoric acid), and EDTA (ethylenediaminetetraacetic acid). Of these, EDTA, thanks to its neutral pH and its ability to remove the smear layer and widen the dentin tubule, has been commonly used in periodontal therapy in different concentrations from 12% to 24%, without damaging biological structures [10–12]. Until now, no precise standardization protocols has been proposed for the use of EDTA, and there is a need to understand the best way to promote proper fibrin clot formation to enhance healing capacity, which is a determining factor in early wound healing. The early phase of the healing is crucial in maintaining the interface between the coagulum and the radicular surface in order to achieve a successful regeneration [13]. Therefore, it is important to maintain space in the defect for the coagulum. Good wound closure is the primary intention, and minimally invasive approaches have been shown to give enough stability to the tissue to prevent it from collapsing and to create an adequate space for the coagulum to be stable, hence reducing the negative effects of wide and less-containing defects.

Thus, the present study aimed to evaluate, in vitro, how different angle of infrabony defects affected the contraction of the clot in periodontal bone regeneration. Secondly, we investigated the influence of root conditioning, using EDTA, on clot contraction. The null hypothesis was that the angle and the conditioning would not influence the adhesion of blood elements to the root surface.

2. Materials and Methods

2.1. Specimen Collection

Thirty single-root teeth extracted for periodontal disease and with an interproximal CAL $\geq$ 6 mm (measured intraoperatively) were collected from surgical patients after obtaining patients' informed consent under a protocol approved by the institutional review board of the University of Trieste (report number 84, 13 November 2017). Teeth affected by root caries or having root-conservative restoration or endodontic treatment were deliberately excluded.

2.2. Specimen Preparation

The freshly extracted teeth were cleaned with 0.9% NaCl solution for 1 min and underwent a manual scaling and root planing (SRP) procedure. Teeth were then cut by a blade microtome in order to obtain, for each tooth, a section at least 6 mm high, 2 mm wide, and 4 mm deep.

To recreate, in vitro, the clinical condition of an infrabony defect associated with the tooth, 30 models were made by three-dimensional printing using a fused deposition modeling (FDM) technique.

Each model thus obtained had 3 walls in order to simulate the most-contained intrabony defect. Models were designed to hold the tooth section and mime different angles of intrabony defects by changing the inclination of one of the internal walls of the structure, in order to achieve 10 models at angles of 25°, 37°, or 50°.

One tooth section and a laboratory coverslip were fixed into each model using cyanoacrylate-based adhesive to allow analysis of the sample.

The 30 assembled samples were divided into two groups of 15 samples, composed of 5 samples with a 25 ° angle, 5 samples with a 37 ° angle, and 5 samples with a 50 ° angle. The root surfaces of the samples in the first group did not undergo any treatment (NT = untreated), while the teeth of the second group of samples were subjected to root conditioning with a neutral pH solution of ethylenediaminetetraacetic acid (EDTA) at 24% (Pref-Gel, Straumann) applied for 2 min then rinsed and dried (Figure 1).

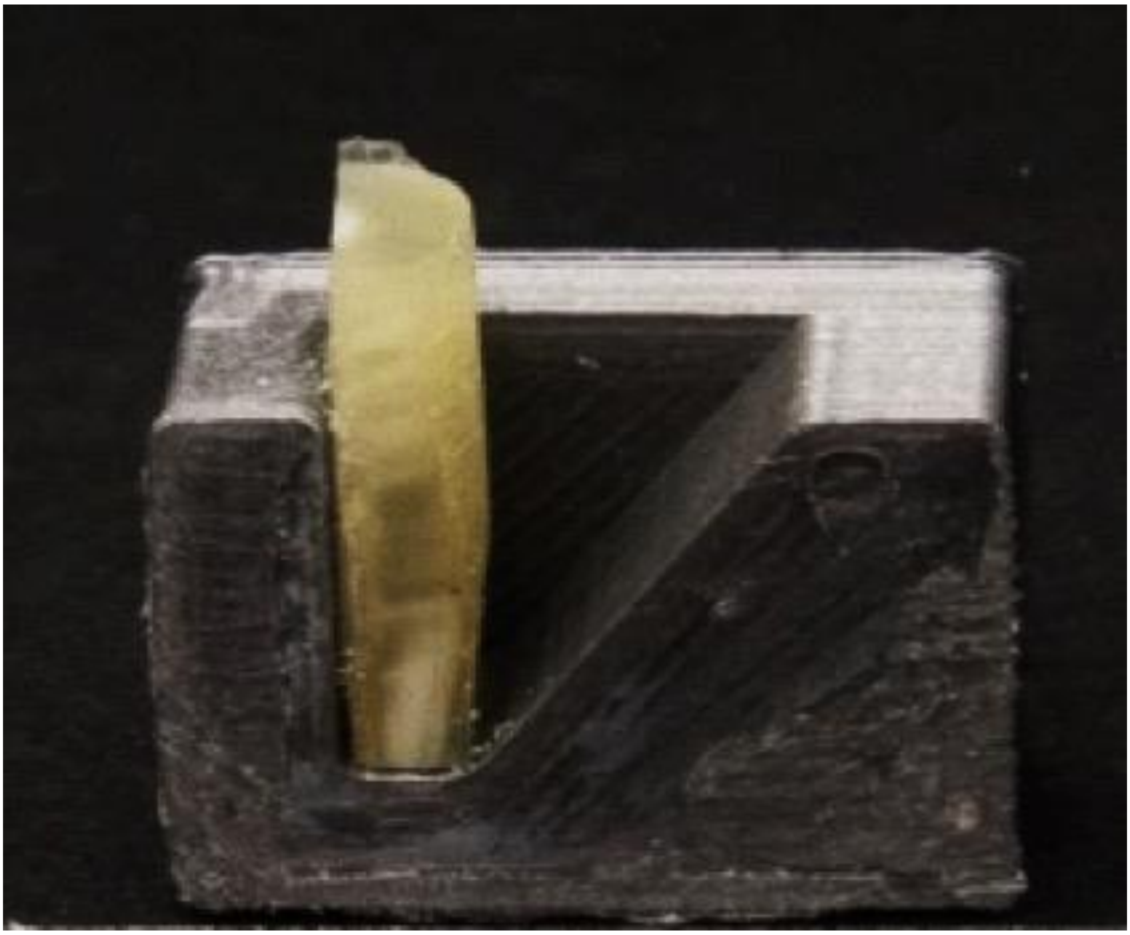


Figure 1. Sample with 37° angle.

Venous blood (temperature 37°) was taken from a 25-year-old healthy donor with a normal coagulation profile and transferred into the samples using a syringe in order to fill them entirely up to the top. Afterwards, the samples were placed in an incubator at a constant temperature of 37 °C for 2 h.

2.3. Confocal Microscopy Evaluation

FITC and TRITC fluorochromes (Merk Life Science S.r.l., Milan, Italy) were used to stain the samples. Fluorescent samples were analyzed using a Nikon C1si confocal microscope, using 4× and 10× Plan Fluor objectives. The FITC fluorochrome was analyzed using an argon laser (488 nm), collecting the fluorescence with an acquisition channel of 500–530 nm. The TRITC fluorochrome was analyzed using a solid-state laser (561 nm) collecting the fluorescence with an acquisition channel of 565–615 nm.

For each sample, the instrument acquired a series of consecutive scans for a distance of 100 microns from the outside to the inside of the sample. These were then exported as a two-dimensional image merging the two channels.

On the two-dimensional images, the following measures were detected using ImageJ v1.54 software in order to investigate the contraction of the clot in the various experimental groups: H-concavity (height of the concavity created by the retraction of the clot in its most coronal part), S-retraction (surface resulting from the retraction of the clot in its most coronal part), and S-clot (surface of the retracted clot). S-retraction/S-clot × 100 (the percentage ratio between S-retraction and S-clot) was calculated and statistical analysis of the results was performed (Figure 2).

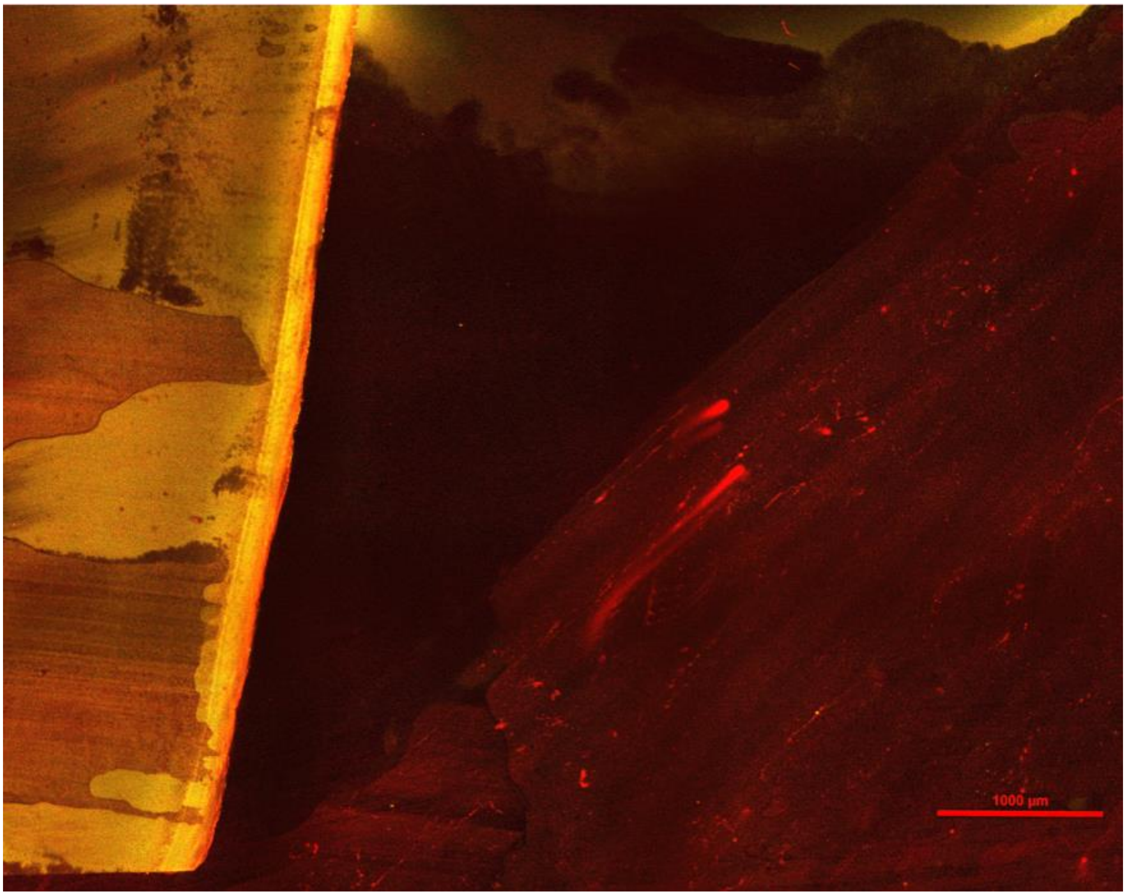


Figure 2. Clot analyzed by laser confocal microscopy and measures detected for each sample.

2.4. Statistical Analysis

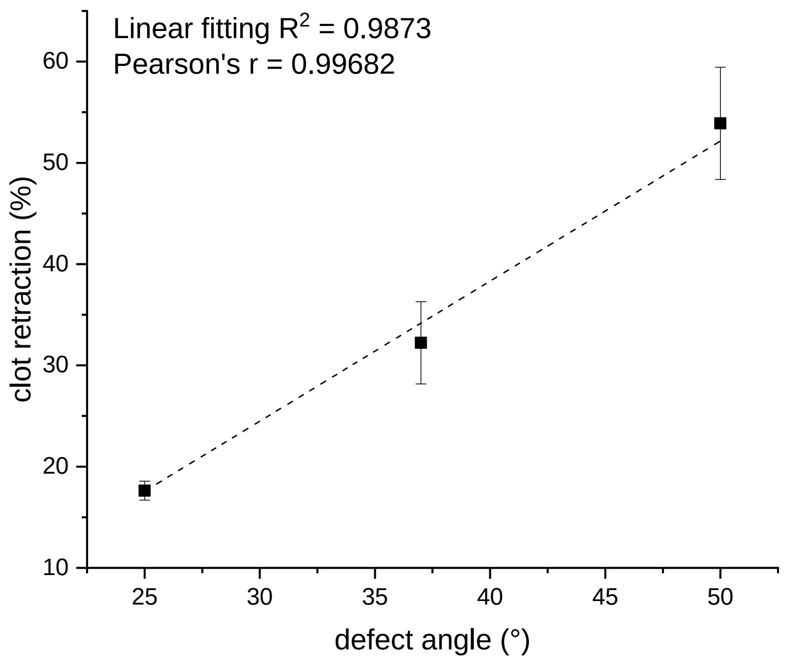
The statistical analysis was conducted using Origin Pro 2018 software. The normality of the data was assessed using the Kolmogorov–Smirnov test, and the homogeneity of variances was tested using Levene's test. Differences in values between groups and subgroups were evaluated using ANOVA corrected with Tukey's post-hoc test. Statistical significance was pre-set at $p = 0.05$

3. Results

All samples exhibited signs of retraction, which means the formation of a concavity in the most coronal portion of the clot with separation of the serum component. According to an initial visual examination under a confocal microscope, lower retraction was observed in the samples treated with EDTA. Analyzing Table 1, the non-treated group (NT) showed that the height of the concavity formed by the clot (H-concavity) remained almost unchanged with varying defect angles. Another important finding from Table 1 is that the largest coronal area formed by clot retraction (S-retraction) exhibited an increase in the mean value with an increase in the defect angle; it transitioned from 1.12 mm^2 in the 25° angle, to 2.57 mm^2 in the 37° angle, reaching 5.40 mm^2 in the 50° angle, confirming that the greater the defect angle, the greater the clot retraction. Our study results show that the area relative to the retracted clot (S-coagulum) increased along with the angle amplitude (Table 1 and Scheme 1), showing a statistically significant relationship between clot retraction and the increase in defect amplitude.

Table 1. Average measurements and comparison values related to the NT and EDTA survey group.

Angle	H-Concavity (mm)		*p* Values	S-Retraction (mm^2)		*p* Values	S-Clot (mm^2)		*p* Values	S-Retraction/S-Clot		*p* Values
	NT	EDTA		NT	EDTA		NT	EDTA		NT	EDTA	
25°	4.85 ± 0.32	5.13 ± 0.15	0.14	1.12 ± 0.06	0.57 ± 0.09	0.012	6.33 ± 0.29	8.14 ± 0.64	0.012	18 ± 1	7 ± 1	0.012
37°	4.50 ± 0.26	5.13 ± 0.07	0.012	2.57 ± 0.33	1.10 ± 0.13	0.012	8.04 ± 1.15	9.65 ± 0.38	0.012	32 ± 4	11 ± 1	0.012
50°	4.52 ± 0.16	5.27 ± 0.13	0.012	5.50 ± 0.51	1.21 ± 0.25	0.022	9.79 ± 0.38	14.86 ± 1.71	0.012	54 ± 6	8 ± 2	0.012



Scheme 1. Linear fitting of the mean percentage S-retraction/S-clot values.

In the EDTA group, H-concavity data were similar to those of the untreated group. Regarding S-retraction, data showed an increase from the average value of 0.57 mm^2 in the 25-degree samples, 1.10 mm^2 in the 37-degree samples, and finally, 1.21 mm^2 in the 50-degree samples. Similar results were seen for the average values of S-coagulum. The percentage ratio of S-retraction/S-coagulum, however, yielded similar results among the different angles and did not show a progressively increasing trend (Table 1).

Comparing the results of the two main groups, there was a statistically significant difference. On average ($p < 0.05$), the height of the concavity due to clot retraction (H-concavity) was higher in the samples treated with EDTA compared with the NT group (Table 1).

Another observation arising from the comparison of the two groups was that, in the samples subjected to root conditioning, the surface of the clot retraction (S-retraction) was smaller compared with that of the NT group. The percentage ratio of S-retraction/S-coagulum showed similar values among different angles, specifically an average of 7% in the 25-degree samples, 11% in the 37-degree samples, and 8% in the 50-degree samples. Consequently, these data lacked the linear fitting observed in the corresponding NT group.

4. Discussion

Analysis of our data led us to suspect that there was an increase in the S-retraction/S-clot ratio with the increasing angle. This was likely due to the fact that as the defect angle increased, the blood volume was greater, resulting in a stronger clot contraction. Our results show that the area relative to the retracted clot (S-coagulum) increased along with the angle amplitude, ranging from 1.12 mm^2 in the 25-sample set, to 2.57 mm^2 in the 37-sample set, up to 5.50 mm^2 in the 50-sample set. An important aspect to obtaining an improvement in clinical and radiographic outcomes 12 months post-regenerative-surgery is the type of the defect. Nibali, in a systematic review, underlined how it is fundamental to have deeper, narrower defects and contenitive defects [14].

To date, there has been no literature available on the influence of the infraosseous defect angle and the gap between the clot and the root. For this reason, in our study, we explored how various infraosseous defects may affect clot stability and how EDTA can influence the stability between the root and the clot.

Root surfaces exposed to the oral cavity change their mineral density and are contaminated by bacteria and their products, so one of the aims in periodontal therapy is to decontaminate the infected root surface, which can inhibit periodontal tissue reattachment [15,16].

It has been demonstrated that scaling and root planing are not able to fully decontaminate the infected root. Moreover, this can produce a compact smear layer covering the instrumented surface, which inhibits the attachment of the blood elements from the periodontal wound and fibroblast migration [17,18] crucial for the early wound-healing events.

These factors have become the rationale for the use of demineralizing agents in periodontal therapy, due to their potential to neutralize the effects of endotoxins from periodontal pathogens, remove the smear layer, and promote the exposure of dentin collagen fibrils and proteoglycans which represent the substrate for the linkage and maintaining a proper fibrin clot [12,19].

Despite the promising results of several in vitro studies, there is still controversy in the literature regarding the benefits of these agents, largely caused by lack of standardization that has reduced the observational quality of relevant studies [19–22].

The analysis of our results revealed that in both the NT (non-treated) and EDTA groups the depth of the concavity after clot retraction did not appear to be influenced by the width of the defect. In the experimental group treated with EDTA, values of the S-retraction/S-clot ratio were found to be numerically similar across different angles. Therefore, it can be inferred that root conditioning appears to reduce the influence that the defect's width has on clot stability. On the contrary, analysis in the NT groups showed an increase in clot retraction along the root surface in proportion to the angle width.

Futhermore, in recent literature, Stefanini [23] explains how chemical conditioning increased the biocompatibility of the treated surfaces. The use of EDTA to condition the radicular surface could result in a better adhesion and organization of the blood. This is due to the fact that, using EDTA, the clot has a greater interaction between the collagen structure of the dentin and fibrin clot weave was recorded. EDTA helps to remove the smear layer and exposes the underlying dentin substrate. In this way, it promotes the connection between the fibrils and the fibrin; therefore, the healing process could be more predictable when the mechanical debridement is associated with etching of the root surface.

Our results are in line with the hypotheses deduced from previous studies. Therefore, in the experimental group treated with EDTA, the values of the percentage ratio between the retraction surface and the clot surface were numerically similar across different angles.

It can be speculated that root conditioning with EDTA appears to reduce the influence that the defect width has on clot stability.

In the EDTA-treated group, a lesser clot retraction was observed, which could have been a result of EDTA's chelating action on various cations, influencing the coagulation cascade. The ions released by EDTA's chelating action activate certain enzymes such as factor VIII, factor X, and the prothrombin complex, leading to a reduced availability and exhibiting an anticoagulant effect. While this could potentially lead to tissue repair alteration, the protocol for EDTA use in regeneration surgery—of application for 2 min and effective rinsing for another 2 min—has not shown any delays in tissue repair; on the contrary, it has demonstrated benefits in terms of clot stability.

To date, the data found in the literature have not been able to establish the real effectiveness of EDTA in promoting and accelerating the adhesion of blood cells to the exposed root [24]. Moreover, EDTA, with its amine groups with lone-pair electrons that chelate calcium, can inhibit the coagulation cascade [25,26] if not completely removed from the root surface.

Moreover, according to Porrelli [27] a cleansed root surface, free from a smear layer and demineralized by EDTA, would lead to better coagulum adaptation and less contraction in that area, increasing the regenerative potential of the defect. However, we cannot exclude the possibility of a greater contraction on the surfaces of the coagulum that were not analyzed using the techniques we employed, such as the areas in contact with the walls of the PLA (polylactic acid) model, or that the contraction was not detectable through confocal microscopy. During experimentation, in fact, using higher magnifications with confocal microscopy, it was not possible to observe a gap between the clot and the root. This is likely due to greater fluorescence of the dental element compared with the clot, creating an overlap of unreadable images under the microscope.

Thus, we can speculate that the blood-clot contraction was correlated with the dimension of the angle, although using EDTA, the blood contraction was reduced.

5. Conclusions

In conclusion, the results of this preliminary study confirm the crucial role of blood contraction in regenerative therapy. In this study we underline how increased width of the infrabony defect leads to a greater contraction of the clot, and the additional use of chemical decontamination, treating the root surface with EDTA, could delay the clot contraction despite of the angle's width.

Strengths and Limitations

Additional in vitro investigations with an increased number of specimens, as well as clinical studies, are needed to validate the procedure further.

Furthermore, considering the use of computerized microtomography with three-dimensional analysis could provide a more thorough examination of tooth–clot–infrabony-defect interactions.

Author Contributions: Conceptualization: L.B.; formal analysis: S.P.; investigation: S.P. and A.M.; methodology: D.P.; project administration: M.M.; writing—original draft: V.D.L.; writing—review and editing: V.D.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was approved by the institutional review board of the University of Trieste (report number 84, 13 November 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors do not have any financial interests, either directly or indirectly, in the products or information listed in the paper. The study was self-supported by the authors.

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