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Descriptive Morphology of the Structure and Functionality of the Fibrin Network of Advanced Platelet Rich Fibrin (A-PRF) Membranes with and without Ozone used for Root Coverage

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Abstract

Introduction

Advanced platelet-rich fibrin (A-PRF) membranes are widely used in regenerative dentistry due to their ability to promote tissue healing. However, studies evaluating the effects of ozone treatment on the structure and functionality of A-PRF remain scarce. This study aims to analyze the descriptive morphology of fibrin networks in A-PRF membranes with and without ozone treatment for root coverage applications.

Objective

To evaluate the descriptive morphology of the structure and functionality of the fibrin network of A-PRF membranes with and without ozone used for root coverage.

Methods

A-PRF was obtained from 10 patients with Cairo type 1 gingival recession, following the protocol of Miron & Choukroun (2018). Blood samples (60 mL) were collected via venipuncture, centrifuged at 1300 rpm for 14 minutes, and separated into A-PRF membranes. Ozone gas (5 mcg per 5 mL) was applied to five 10 mL blood tubes before centrifugation. After processing, two membranes from each patient (one ozonized and one non-ozonized) were histologically analyzed.

Results

Histological evaluation of the 18 analyzed membranes (10 non-ozonized, 8 ozonized) revealed that ozone-treated A-PRF membranes exhibited a denser and more homogeneous fibrin network compared to untreated membranes. This structural improvement may contribute to enhanced initial support in root coverage procedures, stabilizing the fibrin clot and promoting tissue regeneration.

Conclusion

The results suggest that ozone treatment positively influences the structural characteristics of A-PRF membranes, improving fibrin network density and uniformity.

Keywords: A-PRF (Advanced Platelet-Rich Fibrin), Ozone, Fibrin Network, Root Coverage, Histological Analysis

Introduction

Ozone, also known as triatomic oxygen or trioxygen, is a natural compound formed by three oxygen atoms under the influence of ultraviolet rays [1]. Ozone therapy, which has been used since the 1800s, was used during the First World War by doctors who recognised the antibacterial properties of this agent. Due to the scarcity of medical resources, these professionals applied ozone directly to infected wounds, noting not only its effectiveness in fighting infection, but also its effects on blood circulation and its anti-inflammatory properties [2].

Ozone can be administered in various forms, such as gas, water and oil [3]. In Brazil, Law 14.648 of 2023 authorised ozone therapy as a complementary procedure [4]. According to the National Health Surveillance Agency (ANVISA), ozone has oxidising and bactericidal properties and can be used in dental and aesthetic procedures. The indications for its safe and effective use include the treatment of dental caries, acting as an antimicrobial agent, in the disinfection phase of the root canal

system, in surgery, aiding in the tissue repair process, in the aesthetic area, used to aid in the cleaning and asepsis of the skin and in periodontics, in the prevention and treatment of inflammatory and infectious conditions affecting periodontal tissues [5]. It has also been used to stimulate collagen production and increase gingival thickness when combined with platelet-rich fibrin (PRF) in the regeneration of periodontal tissues [6].

Autologous platelet concentrates (APC) have emerged as a potential regenerative material, being used in isolation or with other techniques. APC is fundamental in soft tissue healing, due to the release of growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), insulin growth factor (IGF), platelet-derived growth factor-AB (PDGF-AB) and interleukin-1β (IL-1β). These growth factors play an important role in promoting the repair and regeneration of the tissues surrounding the teeth, both soft and hard, while helping to reduce inflammation, pain and discomfort. They are categorised into three generations: Platelet-rich plasma (PRP), platelet-rich fibrin (PRF) and concentrated growth factors (CGF). The first generation, PRP, is obtained by centrifuging to concentrate the platelets and is accompanied by the use of additional chemical products and is not used very often. The second generation, PRF, introduced by CHOUKROUN *et al*. 2006 [7], is a concentrate of fibrin and platelets, which is obtained by centrifugation, but without the use of chemicals. PRF is classified into subtypes, such as L-PRF (platelet-rich fibrin with leucocytes), A-PRF (advanced platelet-rich fibrin) and PRF prepared with titanium, differentiated according to the speed and duration of centrifugation. The third generation, CGF, involves obtaining the concentrate by varying the centrifugation speed, without the addition of chemical products. Both PRP and PRF have been used in soft tissue reconstruction in cases of gingival recessions (RG). However, in A-PRF, neutrophilic granulocytes are present within the clot, which is beneficial and optimises the functionality of monocytes/macrophages and lymphocytes, thus contributing to the support and integration of the regenerated tissue [8].

However, studies demonstrating the association between PRF membranes and ozone are still scarce, so little is known about the effectiveness of this association. ANITUA *et al*. 2015 [9], reported two forms of ozonation, either by continuous flow of ozone or by constant volume (by syringe), for the complete evaluation of ozonated PRGF, including its composition (growth factors), its biomechanics (properties of the fibrin clot) and biological properties (proliferative effects on primary oral cells) and observed that low doses of ozone did not modify the properties and results of PRGF, while higher doses altered the fibrin coagulation process and induced a destructive effect on morphogenic and growth factors, reducing or inhibiting their biological potential.

Objective

To evaluate the descriptive morphology of the structure and functionality of the fibrina network of A-PRF membranes with and without ozone used for root coverage.

Methodology

Ethics Committee

The study was approved by the Human Research Ethics Committee (CEP) of the Universidade Estadual do Oeste do Paraná - UNIOESTE (Cascavel, Paraná, Brazil) under protocol number 6.275.772.

Recruitment

Participants who volunteered for the study were recruited through adverts on social networks and submitted to an assessment by an examiner who was aware of the eligibility criteria. Those who met the inclusion criteria and none of the exclusion criteria were invited to take part in the research. Each person was informed verbally and in writing about the nature of the study and the procedures involved. In addition, the signing of the Informed Consent Form was required for the procedures to begin.

Sample calculation

The sample calculation was based on probability distributions from the t-test family (Wilcoxon and Mann-Whitney tests for comparing two groups). The effect size used of 0.9, type 1 error (α) of 0.05, power of analysis (β error) of 0.9 resulted in a minimum of 7 patients, 1 ozonised membrane and 1 non-ozonised membrane from each patient. However, this study used a minimum of 8 membranes per group. The sample calculation was carried out using the Bioestat@ programme - version 5.3 (Instituto de Desenvolvimento Sustentável Mamirauá, AM, Brazil).

Eligibility criteria

Patients with Cairo RT1 gingival recession were included in the study. The teeth had to be healthy, with all sites showing a probing depth of less than or equal to 3mm, with a bleeding index of less than or equal to 5%, without gingival inflammation and free of caries.

Patients with a history of systemic disease, drug use, pregnancy or lactation, pathogenic occlusal interferences, carious lesions and previous surgery at the recession site were excluded**.**

All subjects were involved in a Basic Periodontal Treatment Programme and follow-up to eliminate the possible etiological factor and prevent recurrence. This programme included manual instrumentation with Gracey 5/6, 7/8, 11/12 and 13/14 periodontal curettes (Hu-Friedy, Chicago, IL, USA), as well as possible occlusal adjustments, awareness-raising regarding eating habits and oral hygiene guidance (brushing using the modified Bass technique with a soft brush and flossing). All patients received periodontal maintenance therapy.

Membrane preparation: Protocol for obtaining autologous platelet-rich fibrin and membrane ozonation

The protocol for obtaining A-PRF was according to Miron & Choukroun (2018). The sequence of the process for obtaining A-PRF was divided into three stages [10]:

Stage 1: Venipuncture and blood collection.
Stage 2: Cell separation (centrifugation).
Stage 3: Preparation of the A-PRF membranes.

Stage 1 was carried out before the surgical procedure for root coverage began. A trained professional (nurse) venipunctured 60 ml of blood. The venipuncture protocol followed the recommendations of the Ministry of Health, with a Vacuum Collection device, following the steps: Threading the needle into the adapter (cannon). Adjusting the tourniquet and choosing the vein; antisepsis of the collection site with cotton wool moistened in 70% alcohol. Performing the puncture. Insert the tube into the holder,

pressing it as far as it will go; release the tourniquet as soon as the blood flows into the tube; instruct the patient to press the punctured part with cotton wool, keeping the arm extended without bending it.

In Stage 2, the 6 tubes of 10 mL of blood each were taken immediately to the centrifuge (Montserrat FibrinFUGE25) (figure 1A). The fibrin membranes were obtained by centrifuging at approximately 1300 rpm for 14 minutes (figure 1B). At the end of the centrifugation, the tubes were collected and the fibrin was not removed immediately. It was left for at least 30 minutes before being used in the surgical bed.

In Stage 3, the kit for making stainless steel PRF membranes (Intra-Luck®) was used. The centrifuged intermediate portion, the fibrin clot, was separated from the red cell portion and the platelet-poor plasma (figure 1C) and placed in the stainless steel box, with the compressive lid in place, without tightening it. The weight itself (130g) was enough to compress the clot and obtain the membranes (figure 1D), without damaging the cellular structures present in the fibrin mesh.

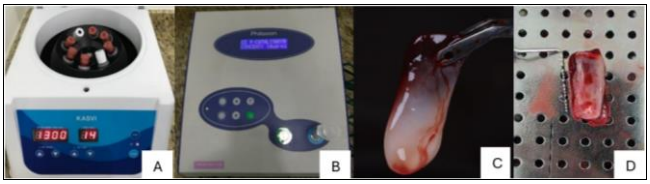

Fig 1: A. Centrifuge configured according to the protocol followed; **B**. Ozone generator; **C**. A-PRF membranes after centrifugation. **D**. Membrane in the stainless steel box and the compression cap in place, without tightening it

Ozone gas was produced from medical oxygen using an ozone generator (Philozon, Balneário Camboriú, Santa Catarina, Brazil) (figure 1B). Subsequently, the 5 tubes of 10 mL each of blood were ozonated with 5mL at a concentration of 5mcg of ozone gas and 1 tube was not ozonated and immediately centrifuged as described in Step 2 of the Miron & Choukroun protocol (2018). Of these 6 tubes, 4 ozonised tubes were used in another study and 2 tubes, 1 ozonised and 1 non-ozonised, were used in this study.

After obtaining the membranes according to Step 3 of the Miron & Choukroun (2018) protocol, the two membranes made from each of the 2 tubes were placed on strips of cardboard to keep them aligned and flat, and immersed in a 10% buffered formaldehyde solution.

A total of 18 membranes were used in this study, 10 without ozone and 8 with ozone, due to the loss of 2 membranes with ozone during histological processing.

Histological Analysis of A-PRF Membranes

Histological processing

Immediately after obtaining the membranes after centrifugation, they were placed on strips of cardboard, in order to keep them aligned and flat, and immersed in a 10% buffered formaldehyde solution. The pieces were labelled (group 1: A-PRF with ozone; and group 2: A-PRF without ozone) and sent to the UNIOESTE Dental Pathology Laboratory (LabPAT), where automatic histological processing was carried out for approximately 12 hours (Automatic tissue processor, Leica Microsystems® TP1020, Nussloch, Germany). This was followed by the inclusion of the pieces in a horizontal direction (lying on the bottom of the cassette) and the paraffin blocks were obtained (Purified Paraffin, code 1228, lot 1008459, Vetec Química Fina, Rio de Janeiro, Brazil) (Figure 2). The blocks were cut using a semi-automatic microtome (Hestion®, ERM3000, Daintree Scientific, St. Helens, CHEMISTRY) and 5µm histological sections were obtained from each for histological analysis, taking care that each block was deepened until the entire length of the membrane was included in the section. The sections were mounted on histological slides and stained using the Hematoxylin and Eosin histochemical technique for subsequent histological analysis (Figure 3).

Descriptive morphological analysis

All the slides, stained using the Hematoxylin and Eosin technique, were evaluated under a binocular optical microscope (Leica Microsystems® DM 500, Nussloch, Germany), using a 100x objective with immersion oil, and a comparative descriptive morphological analysis of the fibrin network formed in the A-PRF membranes with and without ozone was carried out.

Fig 2: A. Storage of the A-PRF membranes in 10% formaldehyde; B. Histological processing; C. Inclusion of the pieces

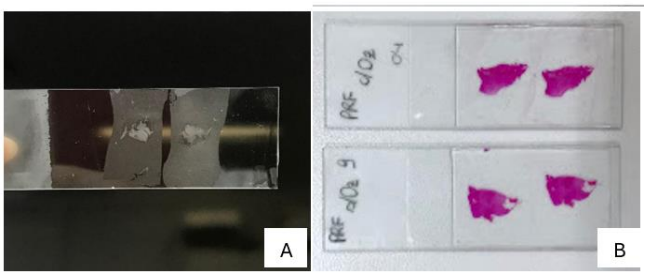

Fig 3: A. Histological section; **B.** Histological slides stained with A-PRF with and without ozone

Results

In the A-PRF membranes without ozone, fibrin networks composed of branched, thin and interconnected fibrils were observed. The density of the network varied, with more compact areas, with a higher concentration of fibrin, and less dense areas. On the histological slides, the fibrin was pink due to its affinity for eosin. In addition, platelets and leukocytes were found interspersed with the fibrin network.

Of the A-PRF slides without ozone analyzed (n=10, 100%), 8 (80%) showed similar dimensional characteristics, with a fibrin network made up of fine, compact and interconnected fibers (Figure 4A), concentrated in certain regions. The remaining two (20%) showed greater thickness and spacing between them (Figure 4B).

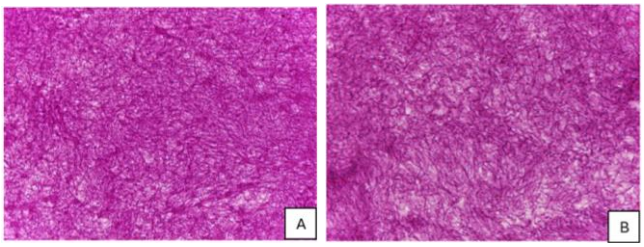

Fig 4: Representative photomicrographs of the histological slides of A-PRF without ozone (A, B; H&E 1000x)

After analyzing all the A-PRF membranes without ozone, the membranes with ozone were analyzed. The A-PRF membranes with ozone (n=8, 100%) showed a thicker and more widely spaced fibrin network, providing greater clarity in the visualization of the fibrillar structure (Figure 5A and B).

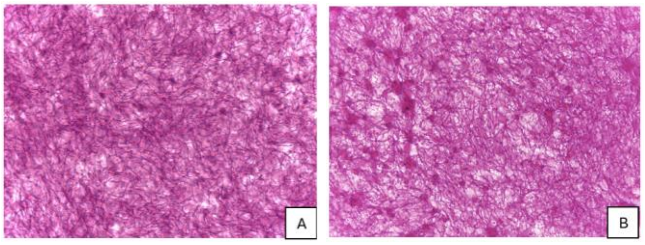

Fig 5: Representative photomicrographs of the histological slides of A-PRF with ozone (A, B; H&E 1000x)

Discussion

The main histological differences observed between the A-PRF membranes without ozone (Figure 6A) and with ozone (Figure 6B) include the presence of a denser and more homogeneous fibrin network in the membranes treated with ozone. This characteristic may provide greater stability to the clot, favoring faster stopping of bleeding, as well as the formation of a more compact and resistant clot, which offers better initial support for tissue repair [11].

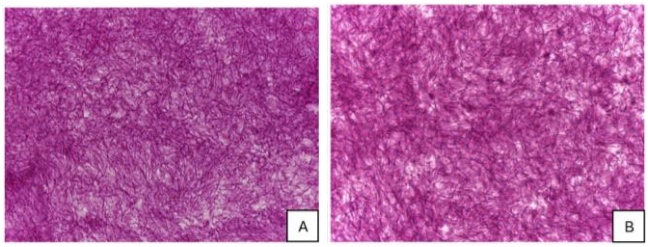

Fig 6: A. Representative photomicrograph of the histological slides of A-PRF without ozone (H&E 1000x); **B**. Representative photomicrograph of the histological slides of A-PRF with ozone (H&E 1000x)

In addition, ozonized membranes present a lower risk of the wound reopening and greater protection against infections, as well as more effective control of inflammation. This benefit stems from ozone's antibacterial and anti-inflammatory properties and its ability to improve blood circulation [2].

The results of this study indicate that ozonation of A-PRF membranes can have a positive impact on the structure and functionality of fibrin, favoring clot stability and enhancing the tissue regeneration process. Histological analysis revealed that the membranes treated with ozone have a denser and more homogeneous fibrin network compared to the untreated membranes, which may contribute to better initial support in root coverage. The greater density of the clot can lead to better stopping of bleeding and the formation of a more resistant framework for cell proliferation [12].

A comparative analysis of A-PRF membranes with and without ozone revealed differences in the thickness and organization of the fibrin network. While membranes without ozone had a more irregular and porous mesh, those treated with ozone had a more compact and well-organized structure. The proximity between fibrin and leukocytes is essential for the release of cytokines and growth factors [13]. This finding corroborates previous studies which have shown that controlled doses of ozone can optimize fibrin formation without compromising its biological integrity [9]. It is possible that this change in fibrillar structure occurs due to oxidation caused by ozone. Controlled oxidation induced by ozone can increase antimicrobial properties and stimulate the release of bioactive factors, contributing positively to tissue regeneration processes [14].

However, the influence of ozone on the growth factors present in PRF is still a matter of debate in the literature. Some studies suggest that high doses of ozone can alter the bioactivity of these factors, impacting on their functionality [15]. Therefore, it is essential that future research assesses the optimal exposure time and concentration of ozone to maximize its positive effects without compromising the biocompatibility of the membranes.

Furthermore, although the results are promising, some limitations of this study should be considered. The sample size was small, and the analysis was carried out *in vitro*, not taking into account the biological and mechanical factors present in a clinical environment. Long-term clinical studies are needed to validate the safety and efficiency of this approach in dental practice.

Finally, the findings of this study reinforce the potential of ozone as a promising agent in regenerative dentistry, especially when associated with biomaterials such as A-PRF. The application of this technique could represent a significant advance in the treatment of gingival recessions and other conditions that require stimulation of tissue healing.

Conclusion

The results of this study indicate that ozonation of advanced platelet-rich fibrin membranes (A-PRF) has positive effects on the structure and functionality of fibrin networks, optimizing their characteristics for root-coating applications. Histological analysis revealed that ozone-treated membranes have a denser and more homogeneous fibrin network, which may favor clot stability, reduce the risk of infection and provide better initial support for tissue repair.

In addition, ozone's antibacterial, anti-inflammatory and blood circulation-enhancing properties have demonstrated potential clinical benefits, highlighting its relevance as an adjuvant in the use of regenerative materials in dentistry. Nevertheless, further studies are needed to validate these findings in different clinical contexts and to more comprehensively explore the efficacy and safety of this approach.

Therefore, this study contributes to understanding the impact of ozone therapy on dental biomaterials, providing a basis for future research and strengthening the applicability of this technique in regenerative practices.

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