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"COMPARATIVE EVALUATION OF STRUCTURAL DIFFERENCES OF L-PRF AND A-PRF+-A SEM ANALYSIS" "STRUCTURAL DIFFERENCES OF L-PRF AND A-PRF+"

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ABSTRACT:

INTRODUCTION:
Platelet-rich fibrin (PRF), a second-generation platelet concentrate containing platelets and leukocytes in a fibrin membrane, has shown promise as a therapeutic tool for tissue repair. However, there is still ongoing debate regarding the superiority of different types of platelet concentrates, in promoting periodontal regeneration.
AIM: The aim of this study is to compare and assess the differences in the Structure of L-PRF and A-PRF+ with and without the addition of antibiotics using Scanning Electron Microscopy analysis.
MATERIAL & METHODS:
Blood was collected from 20 subjects and 0.5 ml of antibiotics were added to respective tubes and centrifuged as per protocol to obtain L-PRF and A-PRF+ following which they were made into respective membranes. The samples were further processed for SEM analysis.
RESULTS:
A-PRF + membranes had thicker density fibrin network when compared to L-PRF membranes. The distribution of leukocytes and platelets were found to be more homologous in A-PRF+ membranes when compared to L-PRF membranes. With addition of antibiotics more thicker fibrin networks were obtained.
CONCLUSION:
Within the limitations of the study,there were structural differences in PRF membranes .Further studies are essential for more conclusive results.

KEY WORDS: PRF, SCANNING ELECTRON MICROSCOPY, LEUKOCYTES, FIBRIN NETWORK

1. INTRODUCTION

Periodontal disease is characterized by the destruction of soft connective tissues, bone loss, and loss of connective tissue attachment to cementum. If left untreated, these changes can lead to tooth loss. Restoring the lost structures and their function is the goal of periodontal regeneration. Bioactive surgical additives have been developed to regulate inflammation and enhance the healing process(Prakash et al 2011). Platelet-rich fibrin (PRF), a second-generation platelet concentrate containing platelets and leukocytes in a fibrin membrane, has shown promise as a therapeutic tool for tissue repair. However, there is still ongoing debate regarding the superiority of different types of platelet concentrates, such as L-PRF, A-PRF, A-PRF+, i-PRF, t-PRF, H-PRF, C-PRF, e-PRF, and Bio-PRF, in promoting periodontal regeneration. Further research is needed to determine their comparative effectiveness in this context.
Leukocyte-platelet-rich fibrin (L-PRF) was introduced in the early 2000s to enhance wound healing and tissue regeneration after intraoral surgical procedures. L-PRF is composed of a dense fibrin matrix that incorporates platelets and leukocytes, releasing cytokines and growth

factors crucial for the healing process(Dohan et al 2006 and 2010). L-PRF is centrifuged at 2700 rpm for 12 minutes and has been reported to release higher levels of growth factors over 10 days compared to other platelet concentrates(Kobayashi et al 2016).
Advanced platelet rich fibrin+ (A-PRF+) was introduced in 2014 as a new concept for cell-based tissue engineering. It involves a low-speed centrifugation at 1300 rpm for 8 minutes. A-PRF+ has a more homogenous platelet distribution in its distal part compared to standard PRF. It contains more neutrophils, aiding in monocyte/macrophage differentiation. A-PRF+ also has higher platelet content and significantly higher levels of growth factors such as TGF-β, PDGF, VEGF, and chemotactic molecules than original PRF. The lower centrifugation speed results in increased growth factor concentration and neoangiogenic potential(Ghanaati et al 2014).
The aim of this study is to compare and assess the differences in the Structure of L-PRF and A-PRF+ with and without the addition of antibiotics using Scanning Electron Microscopy analysis.

2. MATERIAL AND METHODS

A total of 20 healthy volunteers were included in the study. The type of study design was in vitro conducted for a period of six months (July 2022–December 2022). The study design and consent forms for all procedures performed with the study subjects were approved by the institutional ethical committee -CSICDSR/IEC/0212/2021.
PRF PREPARATION
In two successive visits, blood samples were collected from 20 healthy volunteers in glass test tubes without anticoagulants. The tubes were immediately centrifuged after the incorporation of antibiotics for preparation of L-PRF (Leukocyte Platelet Rich Fibrin) and A-PRF+ (Advanced Platelet Rich Fibrin+).
Visit 1: 10 ml for L-PRF
Visit 2: 10 ml for A-PRF+
PROTOCOL FOR CENTRIFUGATION The centrifuge used is REMI-4C with a rotor capacity of 8x15 ml tubes. It has a digital speed indicator and a 0-60 minutes digital countdown timer.
L-PRF -For the preparation of L-PRF, the blood collected was centrifuged at 2,700 rpm for 12 minutes at room temperature using a fixed-angle centrifuge.
A-PRF+- For the preparation of A-PRF+, the blood collected was centrifuged at 1300 rpm for 8 minutes at room temperature using a fixed-angle centrifuge.
ANTIBIOTICS INCORPORATION IN PRF
Prior to centrifugation, blood samples collected were equally divided into 8 calibrated test tubes (2.5 ml of blood in each test tube) and 0.5 ml of antibiotics were added. The following antibiotics were added.
1. Amoxicillin-Penicillin group of antibiotics which is commonly prescribed in periodontal infections.
2. Ofloxacin- Quinolone group of drugs to which Aggregatibacter actinomycetocomitans are more susceptible.
3. Gentamicin-Aminoglycoside group which can be prescribed to patients allergic to penicillin group.

The following samples were obtained.

1.	L-PRF
2.	L-PRF and amoxicillin
3.	L-PRF and ofloxacin
4.	L-PRF and gentamicin
5.	A-PRF+

6.	A-PRF+ and amoxicillin
7.	A-PRF+ and ofloxacin
8.	A-PRF+ and gentamicin

PROCEDURE FOR SEM ANALYSIS

A total of eight PRF samples are used, with four samples from L-PRF (Leukocyte-Rich Platelet-Rich Fibrin) membranes and four samples from A-PRF$_+$ (Advanced Platelet-Rich Fibrin) membranes.A step by step processing is as follows(Sam et al 2015):

1.Fixation: The PRF samples are fixed in a 2% glutaraldehyde-containing phosphate-buffered saline (PBS) solution for 4 hours. Glutaraldehyde is a common fixative used in microscopy to preserve cellular structures.

2.Dehydration: After fixation, the samples undergo a series of ethanol dehydration steps. The samples are sequentially immersed in 50%, 70%, 80%, 90%, and 100% ethanol solutions, with each step lasting 30 minutes. This process gradually removes water from the samples.

3.Drying: To facilitate complete removal of ethanol and water, the samples are treated with 100% hexamethyl disilazane (HMDS) for 10 minutes.

4.Washing and Aeration: Excess HMDS is washed off the samples, and they are left to aerate overnight. This step allows any residual drying agent to evaporate and ensures the samples are ready for SEM observation.

5.Mounting: The dried PRF samples are mounted onto stubs. Stubs are small metal or plastic platforms that hold the samples securely during SEM observation.

6.Sputter-coating: To enhance conductivity and prevent charging effects during SEM imaging, the samples are coated with a thin layer of gold or another conductive material using a sputter-coater. This coating ensures better image quality and resolution.

The prepared samples are observed using a scanning electron microscope (SEM) at an accelerating voltage of 30kV. The SEM uses a focused beam of electrons to scan the sample surface, generating high-resolution images. Different magnifications can be employed to examine various levels of detail.

3. RESULTS

The SEM evaluation of the PRF membrane revealed a dense network of thick fibrin fibers and thin fibrillae, giving it a distinctive 3D appearance. The upper portion of the membrane appeared clear, indicating the absence of cell bodies and primarily consisting of the fibrin matrix. In contrast, the lower portion exhibited several platelets, leukocytes, and a smaller number of red blood cells embedded within the fibrous network. Platelets were either embedded within the fibers or visible as aggregates, suggesting their involvement in membrane formation.

L-PRF and A-PRF+ MEMBRANE:

In the L-PRF membrane, the thin fibrin fibers were strongly polymerized with the matrix .The distribution of leukocytes were not found in the central portion of the membranes which reveals the concentration of leukocytes in the buffy coat area.(FIGURE 1 & 3)

In contrast, in the A-PRF+ membrane, the thick fibrin fibers were loosely polymerized with the fibrin matrix . All the visible cell bodies were trapped between the fibrin matrix, and a few leukocytes were localized on the textured surface on the central portion of the membrane which signifies the uniform distribution of cells in A-PRF+ membranes.(FIGURE 2& 4)

ANTIBIOTICS INCORPORATED MEMBRANES:

When density of the fibers were assessed it was found that the density of fibrin network were found to be more denser with incorporation of antibiotics than plain PRF

membranes(FIGURES 5-8) .The density was more with addition of gentamycin.(FIGURES 9& 10).

4. DISCUSSION

The rationale for utilizing PRF membrane stems from the fact that platelet α granules contain various growth factors (GFs) that play essential roles in the repair of both hard and soft tissues. These GFs include platelet-derived growth factors, transforming growth factor-β, vascular endothelial growth factor, and epidermal growth factor[7]. PRF acts as a fibrin biomaterial with a specific molecular structure facilitated by a low thrombin concentration. This structure serves as an optimal matrix for the migration of endothelial cells and fibroblasts. It promotes rapid angiogenesis (formation of new blood vessels) and facilitates the remodeling of fibrin, leading to the development of a more robust connective tissue.

The SEM evaluation of the platelet-rich fibrin (PRF) membrane in this study aimed to examine its surface morphology and identify the trapped cell types, providing insights into its biological properties. In the junction between the red and yellow parts of the fibrin clot (buffy coat area), spherical structures with irregular surfaces were observed, which could potentially be identified as leukocytes in L-PRF membranes. In A-PRF+ membranes, spherical structures were evenly distributed throughout the membrane. Additionally, a dense aggregate of activated platelets was observed on a mature fibrin background. Beyond the buffy coat area, numerous areas showed dense clusters of platelets resulting from extensive aggregation and clotting. The morphology of the platelets was significantly altered by the aggregation and clotting processes, making it impossible to identify non-activated platelets with a discoid shape. Instead, only large aggregates of platelet-fibrin polymers were observed. This is in accordance to the study done by Sam et al. where there was distribution of cells in buffy coat area in L-PRF membrabes.When density of fibrin network were compared, PRF with addition of antibiotics had comparatively thicker fibrin networks which signifies the enmeshment of antibiotics within the fibrin network and sustained release of antibiotics over time(Su et al 2009).

Kawasaki et al. conducted a study with thrombin-activated platelet-rich plasma (PRP) and obtained similar results, demonstrating the contribution of platelets to the structural rigidity of the fibrin network(Kawasaki et al 2004). The presence of dense clusters of platelets along the buffy coat area and yellow part of the PRF membrane confirms the entrapment of platelets within the fibrin clot. This entrapment is beneficial for the wound healing process as platelets serve as a rich source of growth factors (GFs).

Our findings align with the results reported by Dohan et al., who also observed an increase in platelets and leukocytes along the buffy coat area(Dohan et al 2010). It may be essential to preserve a small layer of red blood cells (RBCs) at the end of the PRF clot to maximize the collection of platelets and leukocytes. SEM examination of the PRF membrane additionally revealed condensed and interconnected fibrin strands, which may be attributed to the compressive forces acting on the fibrin matrix. These findings shed light on the structural characteristics and cellular components of PRF, providing insights into its potential mechanisms of action in tissue regeneration. The SEM analysis revealed spherical leukocytes with irregular surfaces; besides, A-PRF+ had thicker diameter fibers and a more condensed matrix as compared with L-PRF which is in contrast to the study done by Arunima et al where there was thicker L-PRF fibers than A-PRF membranes(Arunima et al 2021).

The volume of antibiotics to be added is a crucial factor to decide. The use of antibiotics exceeding Minimal inhibitory concentration (MIC) may lead to impaired wound healing and cytotoxic effects on various cells (Antoci, Adams, Hickok, Shapiro and Parvizi,2007). Polak et al studied the formation and integrity of PRF by the addition of different concentrations of antibiotics (0.5ml, 1ml, 2ml) and concluded that 0.5 ml had no interference in the formation as

well as integrity of PRF(Polak et al 2019).On addition of antibiotics the diameter of the fibers were found to be larger when compared with plain PRF .Since this the first study which assessed the structural differences in PRF membranes with addition of antibiotics ,comparison with previous studies is not possible .

5. CONCLUSION

Within the limitations of the study, differences in the distribution of leukocytes and fiber density between L-PRF and A-PRF+ membranes were identified. It was also observed that adding antibiotics resulted in a denser fibrin network. These findings suggest the potential use of PRF with antibiotics as a local drug delivery system. However, further studies with larger sample sizes are needed to obtain more conclusive results.
CONFLICT OF INTEREST :NIL

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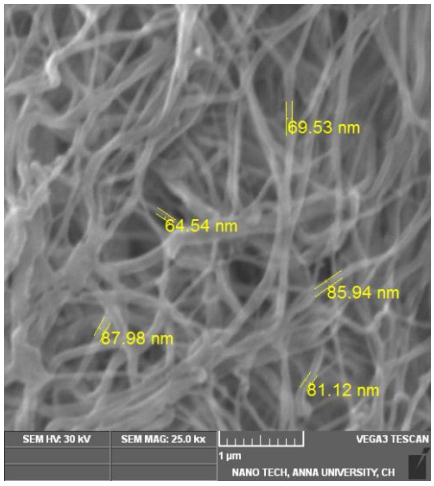

FIGURE 1: L-PRF

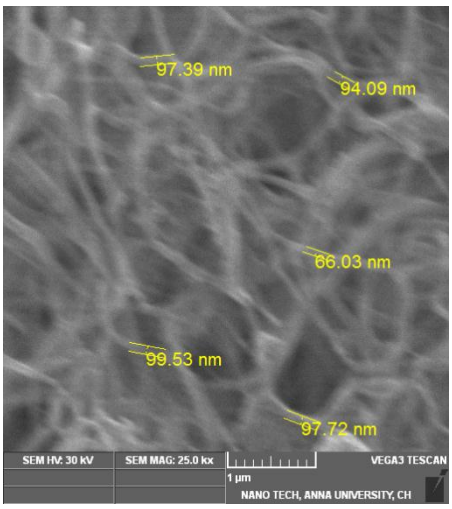

FIGURE 2: A-PRF+

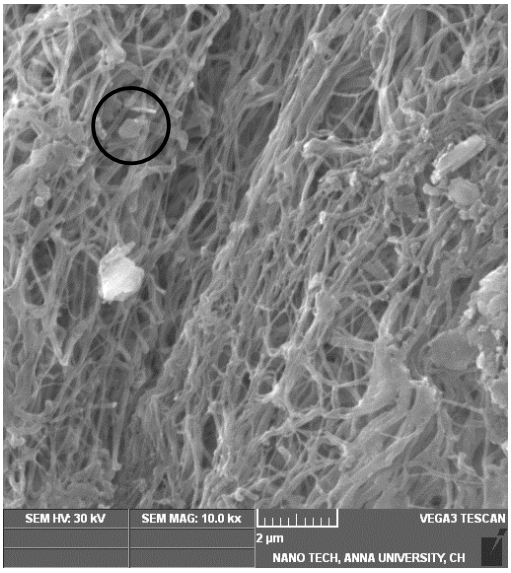

FIGURE 3: BUFFY COAT AREA -SCANTY LEUKOCYTES in L-PRF

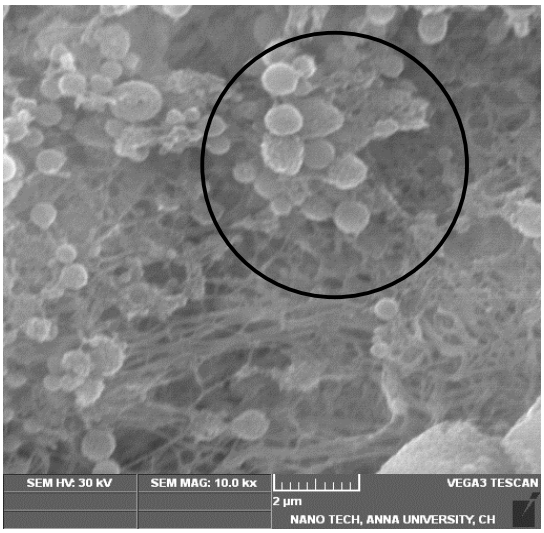

FIGURE 4: BUFFY COAT AREA -HOMOGENOUS DISTRIBUTION OF LEUKOCYTES IN A-PRF+

ANTIBIOTICS INCORPORATED PRF MEMBRANES
L-PRF WITH AMOXICILLIN

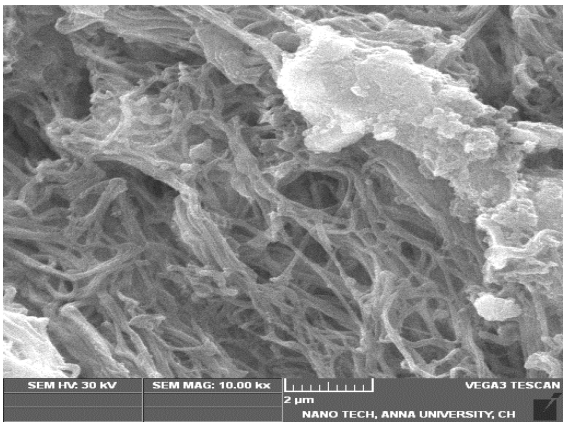

FIGURE 5 a:PLATELET AGGREGATES

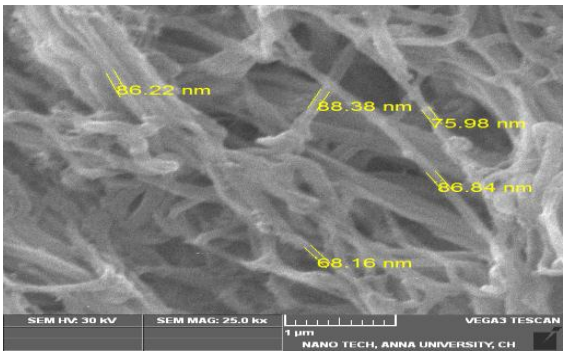

FIGURE 5b: FIBRIN NETWORK DENSITY

A-PRF+ WITH AMOXICILLIN

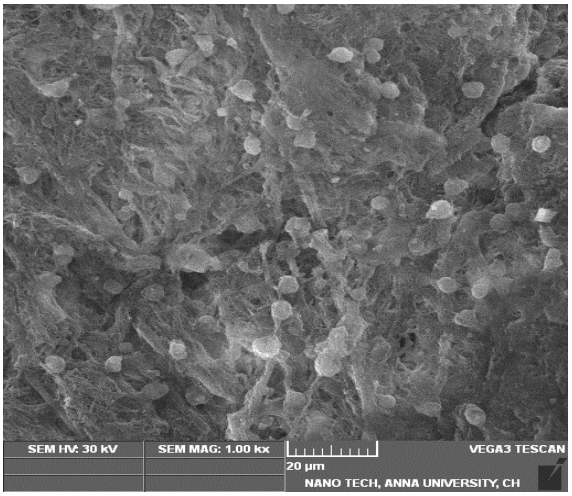

FIGURE 6 a:DISTRIBUTION OF LEUKOCYTES

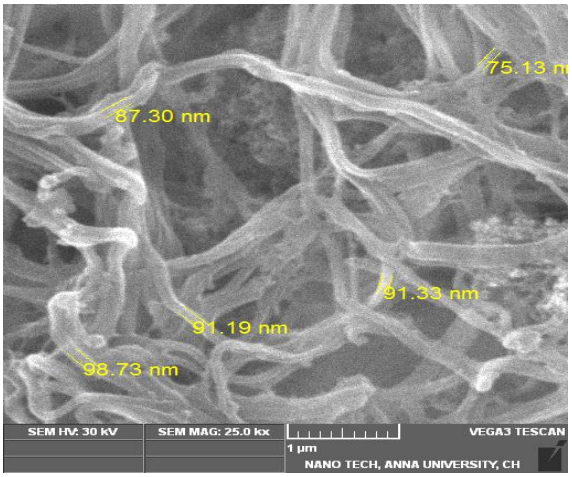

FIGURE 6b: FIBRIN NETWORK DENSITY

PRF WITH OFLOXACIN

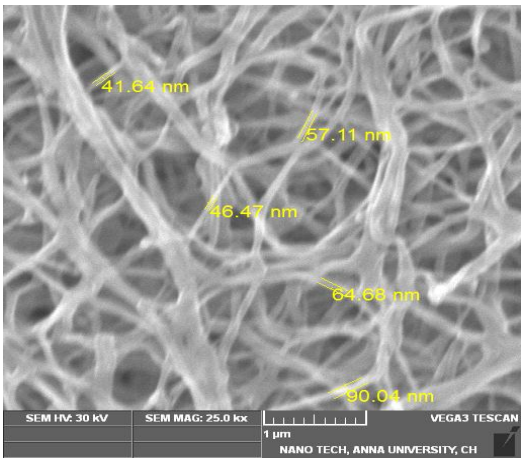

FIGURE 7: L-PRF WITH OFLOXACIN

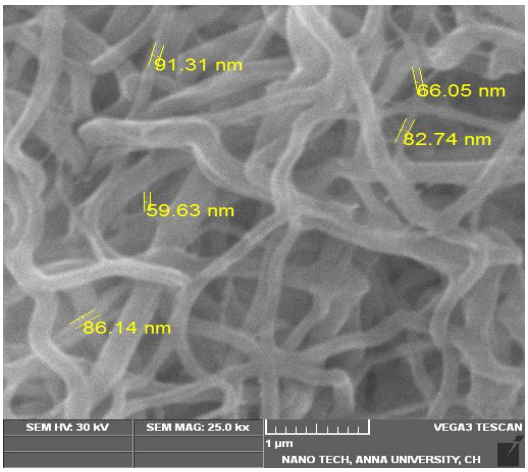

FIGURE 8: A-PRF+ WITH OFLOXACIN

PRF WITH GENTAMYCIN

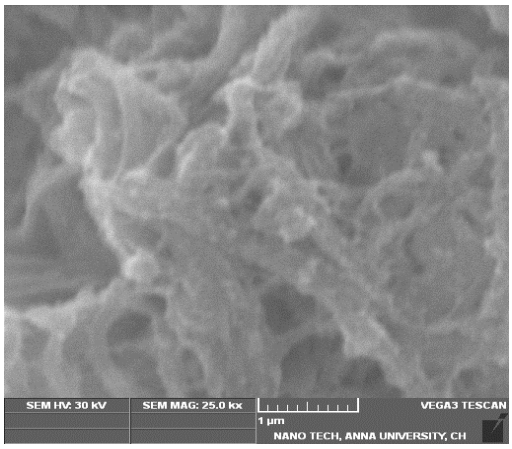

FIGURE 9:L-PRF WITH GENTAMYCIN

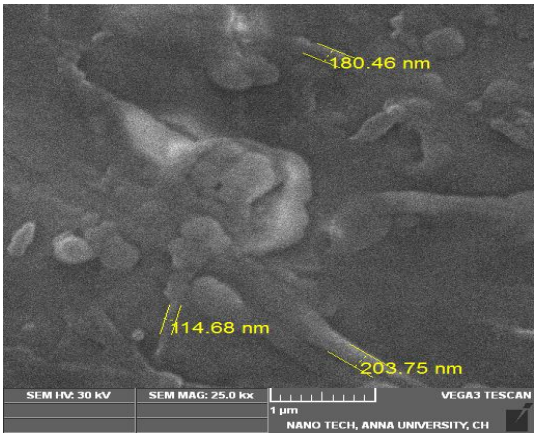

FIGURE 10: A-PRF+ WITH GENTAMYCIN