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Preparation of canine platelet rich fibrin membrane and its characterisation using light microscopy and scanning electron microscopy

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Abstract

The current study was undertaken to analyse the cellular components and three-dimensional organisation of "platelet rich fibrin" membrane (PRFM), an autologous blood derived biomaterial. Ten millilitres of whole blood collected from six canine patients which was then centrifuged at 542.4G (3200 rpm for 10 minutes). The PRF clot thus obtained was pressed to form the platelet rich fibrin membrane (PRFM). The structural analysis and cell composition of PRFM was analysed using scanning electron microscopy (SEM) and histopathology. Evaluation with SEM revealed clearly organised fibrin in parallel strands and the cells were embedded in this compact fibrin matrix. Histopathological examination showed that the leukocyte cytoplasm stained a darker pink along with RBCs, the fibrin matrix seemed homogenous in light pink and platelet aggregates were dark blue/violet. According to the study, the majority of the platelets and leukocytes from the blood sample were present in the PRFM, and their distribution followed a characteristic three-dimensional pattern with the fibrin strands.

Keywords: *PRF, SEM, dogs, keratitis*

The field of tissue engineering and regenerative medicine has experienced significant growth, leading to promising outcomes in laboratory research and clinical implementation of bioengineered structures for wound repair. However, early tissue engineering approaches to wound regeneration mostly focused on scaffolds, growth factors, or cells; as a result, the wound healing process was not perfected, leading to aberrant scar formation, poor vascularization, and sensory loss (Berthiaume *et. al*., 2011). Products connected to platelets have been created for application in contemporary regenerative medicine. Choukron and coworkers in 2001 employed platelet-rich fibrin (PRF), a second-generation platelet derivative, for the first time (Choukroun *et al*., 2001). Platelets are essential for wound healing. Platelet-derived growth factors are essential for the proliferative stage of healing, which includes fibroplasia, reepithelialization, and neovascularisation (Stadelmann *et al*., 1998). As a naturally occurring bioscaffold, PRF offers a biophysical and biochemical environment that supports tissue regeneration and healing. In addition to adhesive proteins like fibrinogen,

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fibronectin, vitronectin, and thrombospondin-1, it also contains platelets, leukocytes, and cytokines (Choukron *et al*., 2006). Compared to platelet rich plasma (PRP), PRF is less expensive, easier to prepare and use and does not change biochemically (Giannini *et al*., 2015).

By encouraging physiologic neoangiogenesis, the fibrin arrangement creates a natural framework that is crucial to the healing processes. Additionally, the PRF clot's leukocyte concentration directly promotes tissue remodelling and regeneration (Ozer and Colak, 2019). In human medicine, PRF is being utilised more and more to treat wounds of many causes, including burns and diabetes (Shreyas *et al*., 2017). The success of in vitro and in vivo investigations is adding to the body of evidence demonstrating its efficacy. Interest in employing PRF in veterinary medicine has increased as a result of this clinical success as well as other desirable qualities such being autologous and being easy and inexpensive to make.

The majority of research on platelet concentrates centres on the quantities of growth factors and platelets; fibrin structure and mechanical qualities are frequently disregarded. On the other hand, the mechanical properties of the membrane and the biology of all fibrin-based biomaterials are directly influenced by the architecture of fibrin. Thus, additional research is required to examine the properties of each of these biomaterials. The current work used histology and scanning electron microscopy for the structural analysis of the platelet rich fibrin membrane (PRFM) in dogs.

Materials and methods

Preparation of the canine PRF membrane

Ten millilitres of fresh blood was drawn from the saphenous vein into two 10mL tubes without anticoagulants using a scalp vein (20 G) catheter from six dogs, immediately after induction of anaesthesia for treatment of ulcerative keratitis. The tubes were immediately centrifuged at 542.5 G (3200 rpm for 10 min) (Demir *et al*., 2022). Following centrifugation, red blood cells accumulated at the bottom of the tube, platelet-rich fibrin gel at the middle and platelet-poor plasma (PPP) were accumulated at the top of the tube. Using k-wire, the fibrin gel along with the RBC clot below were gently removed from the tube and the platelet rich fibrin membrane was prepared by pressing the fibrin gel (**Fig. 1**).

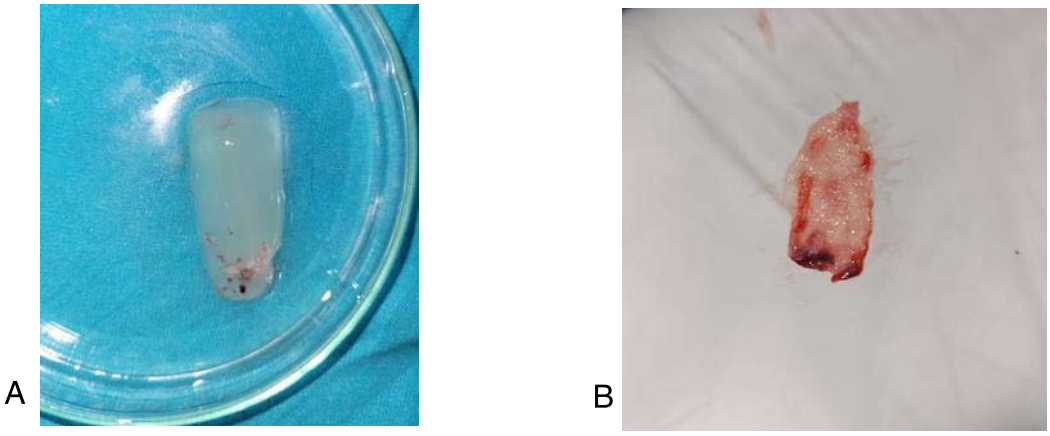


Fig. 1. A. PRF clot after separation B. PRF membrane after preparation

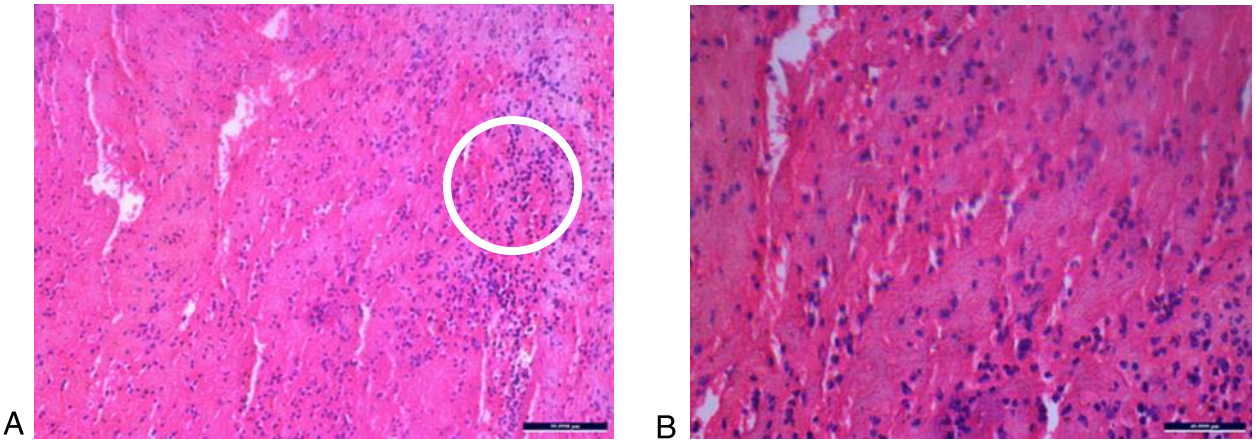


Fig. 2. Presence of leukocytes and platelet aggregates which are stained deep blue to violet in colour indicated by white circles (Light microscopy: original magnification: A. 20X, B. 40X)

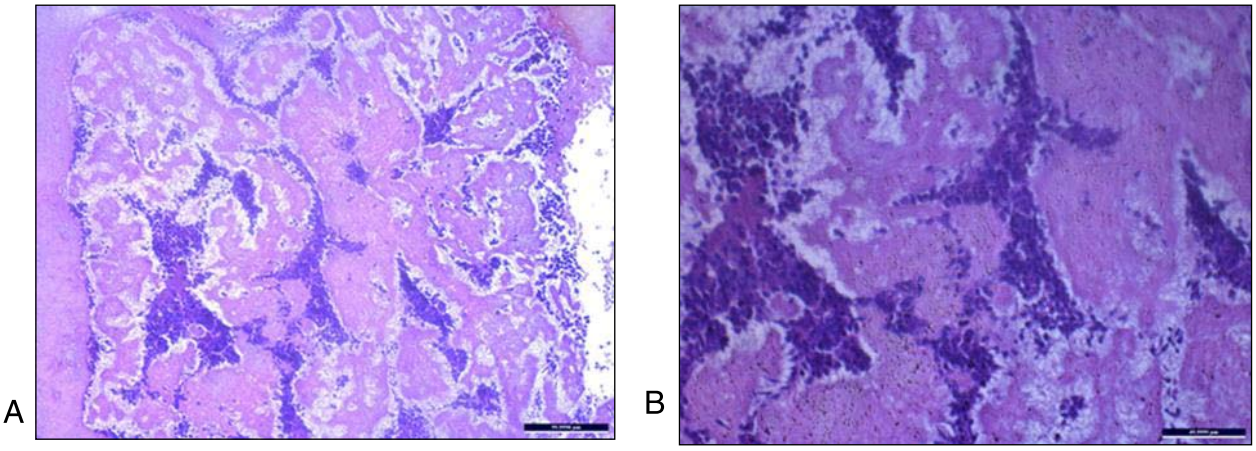


Fig. 3. Presence of leukocytes and platelet aggregates which are stained deep blue to violet in colour which is concentrated towards the periphery of membrane due to pressing of PRF clot (Light microscopy: original magnification: A. 20X, B. 40X)

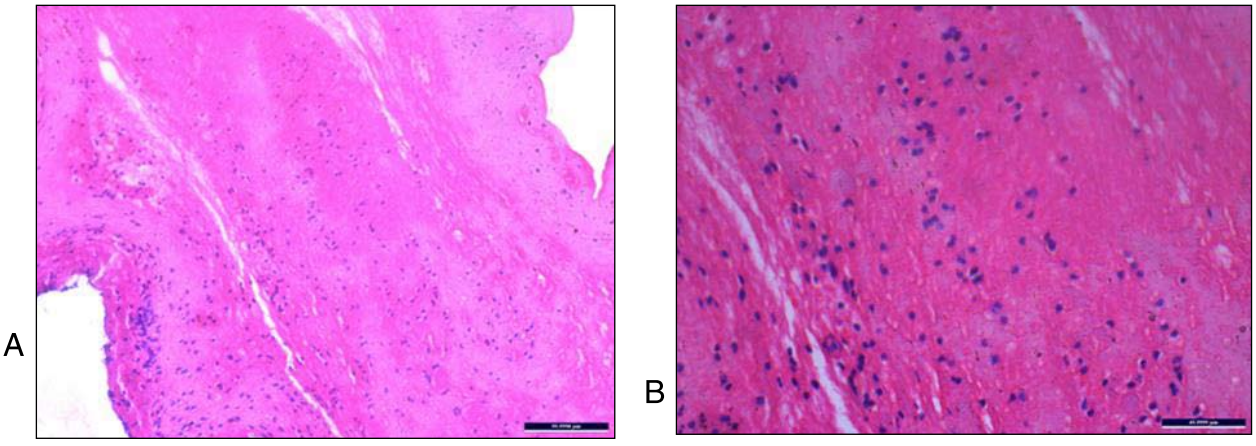


Fig. 4. Light-microscopic analysis of the PRF membrane: The hematoxylin and eosin stainings were not sufficient to correctly distinguish the various cell bodies trapped in the fibrin matrix. PRFM membrane stained uniformly pale pink. Cell bodies-stained deep blue or violet in colour clot (Light microscopy: original magnification: A. 20X, B. 40X)

Histologic Procedures for SEM Evaluation

A morphologic evaluation of the PRF clot and membrane was performed with a scanning electron microscope (TESCAN VEGA-3-LMU, Czech Republic) at Central Instrumentation Lab at College of Veterinary and Animal Sciences. All the six PRF membranes were fixed in 2.5% glutaraldehyde for 24 hours and treated for dessication using ascending grades of isopropyl alcohol (50%, 60%, 70%, 80%, 90% and 100% each for 30 minutes). The specimens were sputter coated with 20 nm gold and subsequently examined in a scanning electron microscope. Photographs were taken at 7 kV using 4000 to 6000 magnifications. Scanning electron microscopy was used for the identification of the cell bodies trapped in the matrix (leukocytes, platelets, and RBCs) and to analyse the overall architecture of the fibrin network.

Histologic Procedures for Light-Microscopy

For histopathological evaluation using light microscopy, PRF membranes were collected in 10 per cent formalin and dehydrated in increasing gradients of alcohol (70%, 80% and 90% for one hour each and in three grades of absolute alcohol for two hours each) and immersed in two grades of chloroform (I and II) for one and half hour each. After complete dehydration, it was impregnated in wax for one and half hour each for embedding in paraffin wax to form blocks. For each PRFM, a series of 20 successive sections of 4-5 microns were prepared in the long axis of the membrane. The sections were then treated with water bath at 70 ºC and was transferred to a slide coated with egg albumin. These sections were stained using haematoxylin and eosin and observed under light microscopy at 20x and 40x magnifications**.**

Results and discussion

A very practical biomaterial for regenerative therapy has been emerged in the form of PRFM among the numerous other forms of platelet concentrates (Kawase, 2015). Additionally, its versatility as a matrix scaffold as well as its greater capability for tissue regeneration than platelet-rich plasma" (PRP) has enhanced its appeal (Bai *et al*., 2017; Marrelli and Tatullo, 2013). Unlike other platelet concentrate subtypes, PRFM has been manufactured

onsite according to the patient needs and used right away for regenerative therapy. The biologic activity of the fibrin molecule alone may be responsible for the significant cicatricial capability of the PRFM, and the gradual polymerisation mode offered the PRFM a particularly beneficial physiologic design to aid in the healing process (Anitua *et al*., 2004). Even while platelets and leukocyte cytokines were crucial to the biological activity of this biomaterial, the fibrin strands that surrounded them unquestionably acted as the deciding factor in the true therapeutic potential of the PRFM (Dohan *et al*., 2009). Autologous PRF membrane-based regeneration therapy has been proven to be an effective treatment modality for deep stromal corneal ulcers by providing good functional and cosmetic results along with adequate ocular support (Ajin *et al*., 2024).

Light-Microscopic Study

Under light microscopy, the PRF clot was seen to consist of two primary parts: a red section towards the end of the clot that was full with RBC, and a yellow fibrin component that made up the clot's main body. The platelet aggregates showed dark blue or violet whereas the fibrin matrix seemed homogenous in light pink when stained with haematoxylin and eosin and similar findings were observed by Akshata *et al*. (2021) in her study (**Fig. 2**). Leukocyte cytoplasm and red blood cells were more difficult to see because of their darker pink colour. The platelet aggregates were stained dark blue with haematoxylin, which was consistent with the findings of the current investigation (**Fig. 3 & Fig. 4**). As such, it was quite challenging to separate them from the platelet aggregates. In the transition layer, platelet aggregates, leukocytes and RBC were mixed

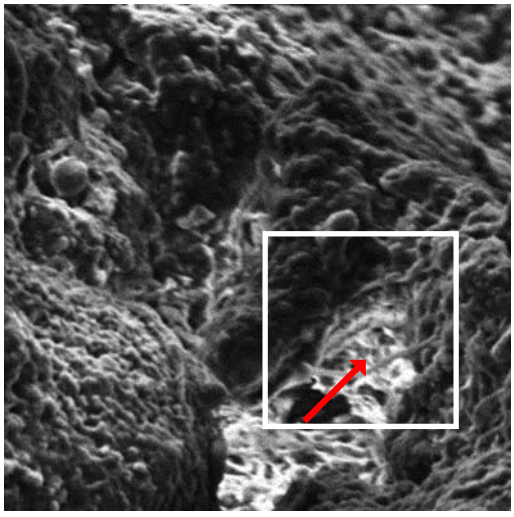


Fig. 5. Fibrin bundles were organized in very thick parallel bundles and compactly arranged (Indicated by red arrow)

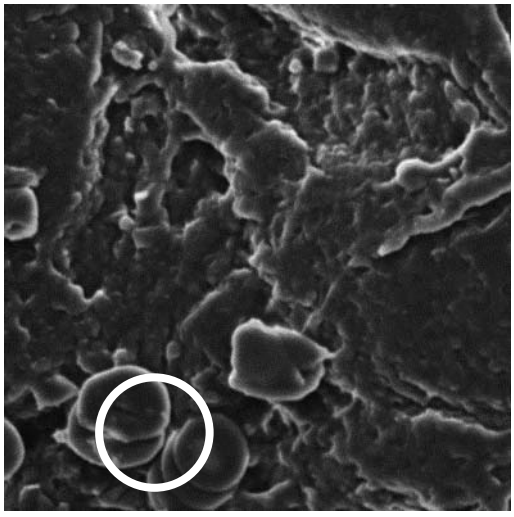
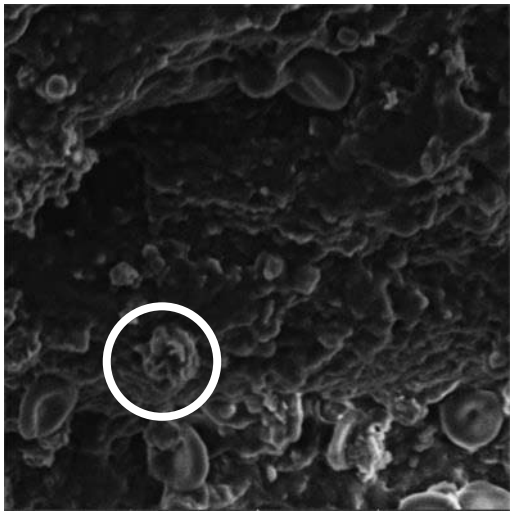


Fig. 6 . Presence of RBCs indicated by white circles located mostly towards the border of white and red clot (SEM: original magnification: 5000X)



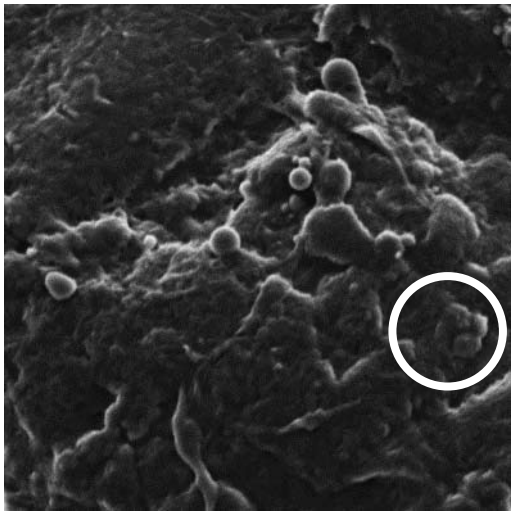


Fig. 7. Platelets were often enmeshed in the fibrin network and sometimes appeared as aggregates (white circles) that were easily identified (SEM: original magnification: 5000X)

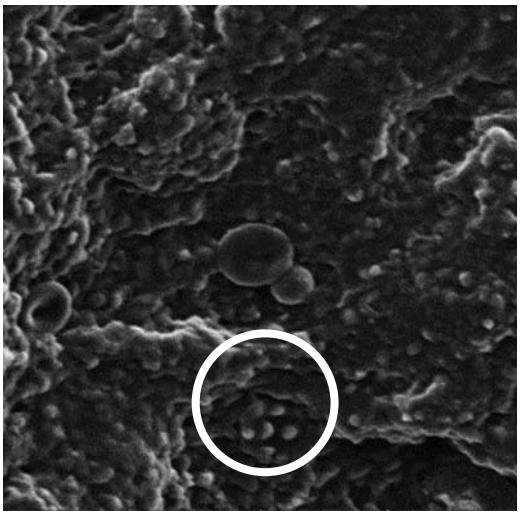


Fig. 8. Leukocytes appeared like spherical structures with an irregular surface (white circles)

together which was in accordance with the observations made by Dohan *et al*. (2009). The fibrin strands in PRFM were larger, more branched and had a more organised architecture, as revealed by light microscopy which was in accordance with the observations made by Aggour *et al*. (2017). However, these favourable characteristics were not evident through the entire membrane as some fibres were deformed compared to their natural shape. This arrangement could contribute to the increased elastic modulus and similar observations were also recorded in the present study.

SEM Evaluation

The PRF membrane surface under SEM evaluation displayed the gauze thread print at low magnification. Since fibrin functions as a physiological adhesive, the fibrin clot's compression into a membrane produced an extremely compact matrix. The fibrin was evidently arranged into parallel strands at higher magnification, giving the impression that it was thick and dense. The fibres in the PRF membrane were organised compactly, and the bundles were parallel in arrangement (**Fig. 5**). The cells were embedded in this compact fibrin matrix. This resulted from the compression method used to create the membrane, and the results agreed with those of Dohan *et al*. (2009). Clusters of RBC were visible in the red portion of the clot due to their distinctive morphology, or the biconcave shape of the cells (**Fig. 6**). Thick fibrin threads and a few strewn RBCs made up the border between the RBC clot and the yellow clot, which stood in for the buffy coat region. It seemed like the fibrin network was developed. Additionally, it contained fibrin and platelets that underwent severe clotting and aggregation to create massive, dense clusters (**Fig. 7**). Each of these results was consistent with the observations of Akshata *et al*. (2021). Platelets and leukocytes were projected into producing fibrin clots in the Choukroun's PRF process, which was essentially based on a mechanical concept. The procedure looked to be quite stable, even with considerably changing production parameters (Dohan *et al*., 2009). The PRF clot was created by a centrifugal natural polymerisation process, and it seemed that over the course of around seven days, the fibrin architecture of this clot allowed growth factors and matrix glycoproteins to be released gradually (Dohan *et al*., 2006). The PRF membrane examination showed that the fibrin strands were squeezed and attached to one another, indicating the compression of the fibrin matrix. This result has a substantial therapeutic significance because of the large number of leukocytes implanted within each membrane and the fact that small lymphocytes were particularly effective in reducing inflammatory responses (**Fig. 8**). Dohan *et al*. (2009) also discovered comparable findings. Platelets lost all of their original shape due to clotting and aggregation processes. Consequently, only a substantial aggregate of platelet-fibrin polymers was able to be differentiated from non-activated platelets (discoid bodies). This study's results were consistent with those of Akshata *et al*. (2021). Preliminary trials also showed that the membrane thus produced was also effective in the treatment of ulcerative keratitis in dogs (**Fig. 9**).

Conclusion

The majority of the platelets and leukocytes from the drawn blood sample were present in the PRF membrane, and the centrifugation procedure produced a three-dimensional pattern in the distribution of these cells. This study demonstrated that the extended aggregation

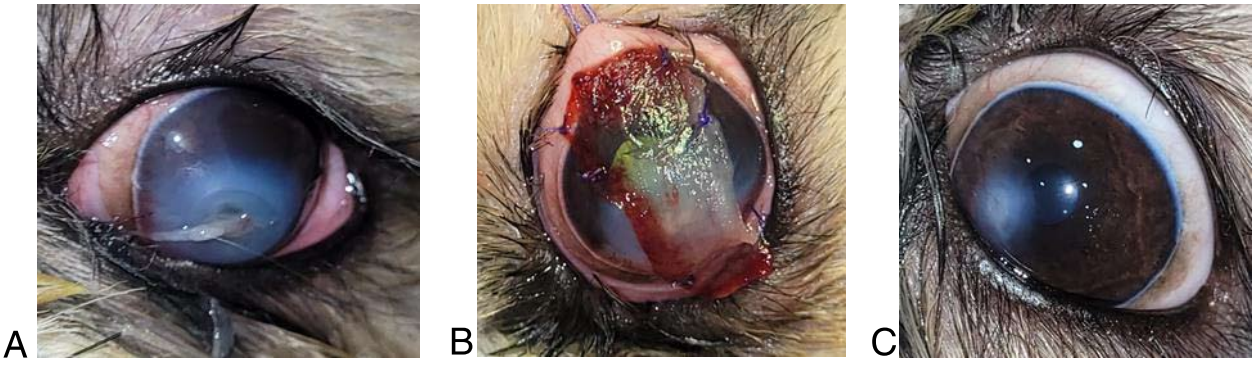


Fig. 9. PRFM grafting for treatment of canine corneal ulcer: A. Day 0 B. Day of Surgery C. Day 28 post surgery

and clotting process resulted in an alteration of platelet morphology and a concentration of cells towards the membrane's periphery. The PRF clot was pressed to form the membrane.

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Conflict of interest

The authors declare that they have no conflict of interest.

References

Aggour, R. L., Sabry, H. A. and Hassan, G. S. 2017. In vitro evaluation of mechanical and structural properties of leukocyte-platelet-rich fibrin and platelet-rich fibrin matrix. *Egyptian dental J*. **63:** 1531-1539.

Ajin S. K, Venugopal, S. K., Ramankutty, S., Philip, L. M., John martin, K.D. and Prasanna, K.S. 2024. Management of deep corneal ulcer in a dog using autologous platelet rich fibrin (PRF) membrane. *J. Vet. Anim. Sci*. **55**:312-316.

Akshata, K. R., Ranganath, V., Nichani, A. S., Shetty, P. R., Bhat, P. and Vinay, B. V. 2021. Evaluation of composition and architecture of the platelet rich fibrin: An observational study. *Ann. Romanian Soc. Cell Biol*. **25**: 7330-7340.

Anitua, E., Andia, I., Ardanza, B., Nurden, P. and Nurden, A. T. 2004. Autologous platelets as a source of proteins for healing and tissue regeneration. *J. Thromb. Haemost*. **91**: 4–15.

Bai,M. Y., Wang, C. W., Wang, J. Y., Lin, M. F. and Chan, W. P. 2017. Three-dimensional structure and cytokine distribution of platelet-rich fibrin. *Clinics.* **72**: 116–124.

Berthiaume, F., Maguire, T., J. and Yarmush, M., L. 2011. Tissue engineering and regenerative medicine: history, progress, and challenges. *Annu. Rev. Chem. Biomol. Eng.* **2**:403-430.

Choukroun, J., Adda, F., Schoeffler C. and Vervelle, A. 2001. An opportunity in perioimplantology: The PRF. *Implantodontie*. **42**: 55-62.

Choukroun, J., Diss, A., Simonpieri, A., Girard, M., O., Schoeffler, C. and Dohan S., L. 2006. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part V: histologic evaluations of PRF effects on bone allograft maturation in sinus lift. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod*. **101**:299–303.

Demir, A., Erdikmen, D.O., Sevim, Z.T. and Altundag, Y. 2022. The use of an autologous platelet-rich fibrin (PRF) membrane for the treatment of deep corneal ulcers in dogs. *Veterinarski. Arhiv*. **92**: .443-458.

Dohan, D. M., Choukroun, J., Diss, A., Dohan, S. L., Dohan, A. J., Mouhyi, J. and Gogly, B. 2006. Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part II: platelet-related biologic features. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **101**: 45-50.

Dohan, E. D. M., de Peppo, G. M., Doglioli, P. and Sammartino, G. 2009. Slow release of growth factors and thrombospondin-1 in Choukroun's plateletrich fibrin (PRF): a gold standard to achieve for all surgical platelet concentrates technologies. *Growth Factors*. **27**: 63-69.

Giannini, S., Cielo, A., Bonanome, L., Rastelli, C., Derla, C., Corpaci, F. and Falisi, G. 2015. Comparison between PRP, PRGF and PRF: lights and shadows in three similar but different protocols. *Eur. Rev. Med. Pharmacol. Sci*. ***19***: 927-930.

Kawase, T. 2015. Platelet-rich plasma and its derivatives as promising bioactive materials for regenerative medicine: Basic principles and concepts underlying recent advances. *Odontology*. **103**: 126–135.

Marrelli, M. and Tatullo, M. 2013. Influence of PRF in the healing of bone and gingival tissues. Clinical and histological evaluations. *Eur. Rev. Med. Pharmacol. Sci*. **17**: 1958–1962.

Ozer, K. and Colak, O. 2019. Leucocyte-and platelet-rich fibrin as a rescue therapy for small-to-medium-sized complex wounds of the lower extremities. Burns & Trauma. **7**: 1-9.

Stadelmann, W., K., Digenis, A., G. and Tobin, G., R. 1998. Physiology and healing dynamics of chronic cutaneous wounds. *Am. J. Surg*. **176**:26-38.

Shreyas, N., S., Agrwal, S., Agrwal, C., Karki S. and Khare, S. 2017. Efficacy of autologous platelet-rich fibrin in chronic cutaneous ulcer: a randomized controlled trial. IP *Indian J. Clin. Exp. Dermatol*. **3**:172–81. ■