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Preparation and analysis of bovine platelet rich fibrin gel with special emphasis on histology and scanning electron microscopy#

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Abstract

Platelet rich fibrin (PRF) has gained recognition as a regenerative biomaterial with potential applications in soft tissue healing. The study was aimed to prepare bovine origin platelet rich fibrin gel and characterisation by histology and scanning electron microscopy. PRF gel was prepared from venous blood collected from cross-bred postpartum dairy cattle centrifuged at 3200 rpm for 12 minutes. Histology and scanning electron microscopy revealed a compact fibrin network and the cells were distributed within PRF gel. The findings could provide a comprehensive understanding of cellular and matrix composition of bovine PRF gel, essential for its effective clinical application.

Keywords: *Platelet rich fibrin gel, histological analysis, scanning electron microscopy, bovine PRF*

Platelet rich fibrin (PRF) gel represents a second-generation platelet containing biomaterial that follows the first-generation platelet rich plasma. Platelet rich fibrin contains growth factors, fibrin, leukocytes and cytokines similar to platelet rich plasma but at very high concentrations.

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Platelet rich fibrin greatly simplifies the process of synthesis compared to the former platelet rich plasma with the single step centrifugation and without the need of addition of anticoagulants. PRF fulfils the beneficial therapeutic effects of platelet rich plasma by acting as a scaffold by providing a supporting framework (Kang *et al*., 2011). Platelet rich fibrin has been effectively used in wound healing due to the delivery of growth factors (Bilgen *et al*., 2021). Platelet rich fibrin gel traps platelets and their associated cytokines within the fibrin network thus promoting tissue regeneration (Civinini *et al*., 2011).

The original concept of PRF gel was proposed by Choukroun *et al*. (2006) in France, owing to its use in oral and maxillofacial surgeries. Platelet rich fibrin gel has been used as an adjunct to various therapeutic procedures in veterinary surgery for improved wound angiogenesis in porcine wound models (Roy *et al*., 2011) and in the healing of deep corneal ulcer in dogs (Demir *et al*., 2022), for early epithelialisation. Soares *et al.* (2021) used PRF gel for grafting of chronic skin wounds in feline patients. Analysis of PRF using histology was initially done in human sample by Ehrenfest *et al.* (2010). Based on the available literature, studies had not been yet reported on the preparation and analysis of bovine platelet rich fibrin gel with scanning electron microscopy and histology. Hence, this work was conducted as a pilot study in the field of bovine regenerative medicine and therapy, and observations made from the study are pioneering and were compared with humans, rabbit and other species due to scarce reference. The preparation of bovine platelet rich fibrin gel and its analysis can serve as a basis for the advancement of research in the field of bovine regenerative medicine aiding the veterinarians practicing in the field conditions and thereby providing the cost-effective therapy for farmers. Hence, the study was carried out to prepare and analyse platelet-rich fibrin (PRF) gel derived from bovine blood for regenerative therapy in veterinary patients.

Materials and methods

Preparation of bovine PRF gel

Present study was carried out in eight apparently healthy crossbred dairy cattle housed in University Livestock Farm and Fodder Research and Development Scheme, Mannuthy. The blood samples were analysed in an automated haematology analyser to assess the baseline values of platelet count in each animal. Animals with platelet counts above 2.5 $\times 10^5/\mu L$ were selected for the preparation of PRF gel. Fifteen millilitres of venous blood was aseptically collected from the jugular vein of cattle in sterile syringes and transferred into 15 ml centrifuge tubes without any anticoagulants added. The tubes were immediately centrifuged at 3200 rpm for 12 minutes in a centrifuge.

After centrifugation, platelet-poor plasma (PPP) was accumulated at the top, platelet-rich fibrin gel in the middle and red blood cells at the bottom of the tube. Platelet-poor plasma was discarded and PRF gel in the

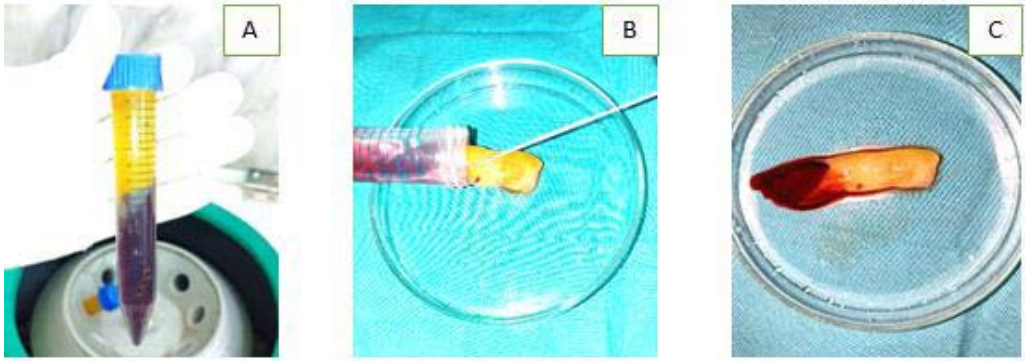

Fig. 1. Steps in the generation of PRF. (A) After centrifugation of whole blood in a glass tube, three layers will be visible (B) PRF was decanted using a sterile tweezer (C) PRF clot

middle part was gently removed from the tube using forceps and transferred to sterile Petri dish. Red blood cell clots below the PRF gel were removed with sterile scissors.

Procedure for Histology (H & E staining)

The representative sample of PRF gel obtained from selected animals was fixed in 10 per cent formalin. This was followed by dehydration using ascending grades of isopropyl alcohol (70 percent, 80 percent and 90 percent) one hour each followed by absolute alcohol-I, II and III, each for 2 hours. The sample was immersed in two grades of chloroform (I and II) for cleaning and impregnated in wax (grades I and II). Processed tissues were embedded in paraffin wax to make slide blocks. The paraffin box was then loaded on a microtome and sections of about 4-5 microns were cut. The sections in wax were then placed on water bath at 70 ºC and then transferred to a slide which was already coated with egg albumin. A series of successive 4-5 µm sections were performed according to the long axis of the clot. These sections were stained using haematoxylin and eosin (Feldman and Wolfe, 2014). Air dried sections were viewed under light microscope at 20X and 40X magnifications. The fibrin network and cellular distribution of PRF gel were evaluated.

Procedure for scanning electron microscopy

A single representative PRF gel sample was subjected to scanning electron microscopy (SEM). The PRF gel was fixed in 2.5 per cent glutaraldehyde for one hour and treated for desiccation using ascending grades of isopropyl alcohol (50, 60, 70, 80, 90 and 100 per cent, respectively) with 30 minutes in each grade. PRF gel was cut at each end and specimens were sputter coated with 20 nm gold and analysed by scanning electron microscopy. Photographs were taken at 7 kV using 5000X magnification.

Results and discussion

Gross morphology of PRF gel

Different centrifugation force and time combinations were reported by various researchers for the preparation of PRF from human patients. Raja and Naidu (2008) used 2700 rpm for 12 minutes while Wu *et al*. (2012) used 3000 rpm for a duration of 12 minutes for the preparation of human PRF. Ardakani *et al*. (2016) obtained PRF gel following 3200 rpm for 12 minutes protocol for human PRFM preparation. On centrifugation at 3200 rpm for 12 minutes, bovine PRF gel appeared as a compact, flexible and elastic gel, stable at room temperature (25°C) whereas centrifugation at 3000 rpm for 10 minutes yielded loose and viscous gel at room temperature. Gutierrez *et al*. (2021) obtained human PRF with similar findings at the same centrifugal force and time combination of 3200 rpm and 12 minutes. The authors also found that lower centrifugal force-time combinations of 2700 and 3000 rpm at eight and ten minutes, respectively yielded smaller clots with reduced density with greater amount of exudate compared with the protocol opted in present study.

The morphological appearance of PRF gel revealed two distinct components: a yellow fibrin portion with a gel-like consistency and RBC portion at the end of the gel (Fig.2). Wang *et al*. (2019) obtained similar morphologic features in rabbit PRF gel prepared with 3000 rpm-10 minutes centrifugation protocol. Due to its flexible nature, PRF clots were frequently used to fill cavities in plastic surgery procedures (Charrier *et al*., 2008). The thickness of PRF may differ depending on variables like the speed of centrifugation, duration, and the equipment utilised (Ehrenfest *et al*., 2018).

Fig. 2. PRF gel obtained after removal of RBC clot.

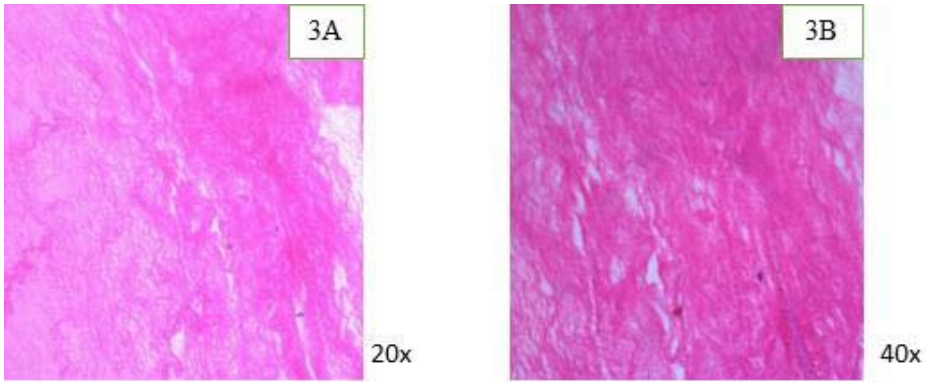

Fig. 3. (A &B) Photomicrograph of platelet-rich fibrin clot (20 x and 40 x). Dotted circles revealed densely arranged fibrin matrix with sparse leukocytes. (H & E staining)

Bovine PRF gel was thick yellow coloured compared to pale yellow in rabbit (Wang *et al*., 2019). A pale yellow PRF clot has been extracted by Bai *et al*. (2018) from human blood and noted that the human PRF clot appeared to be more yellowish in comparison to rabbit PRF. Thick yellow colour of bovine PRF might be attributed to the higher carotenoids in bovine plasma (Yang *et al.*, 1992).

Histology - H & E staining

On microscopic examination, PRF gel revealed densely arranged fibrin strands with minimum interfibrous space and a uniform light pink colour, but platelets and leukocytes were not evident due to thickness of PRF gel (Fig 3. A & B). Several studies in PRF membranes had reported that leukocytes were trapped within the fibrin matrix, imparting a dark blue colour, while platelets were distributed as small, scattered spots that resemble fibrin like structures (Kang *et al*., 2011; Jasmine *et al*., 2021). The density and composition of the fibrin matrix were recognised as crucial factors in a platelet concentrate (Ehrenfest *et al*., 2009). According to Ehrenfest *et al*. (2010), it was difficult to distinguish cellular components like platelets from leukocytes in PRF on histology which might be due to the thickness of the PRF gel. The fibrin gel was thicker near buffy coat region, owing to a higher concentration of cells, creating a scaffold in which these cells were densely arranged.

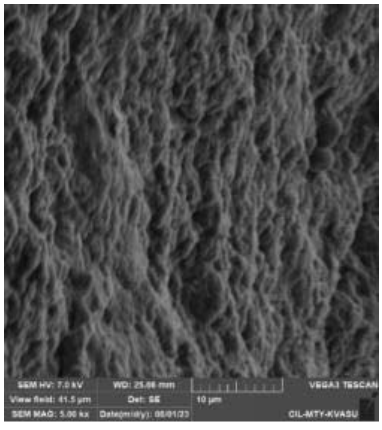

Fig. 4. SEM image of PRF gel. White circle denotes compact fibrin strands which are polymerised and adhered to each other. Dotted circle denotes erythrocytes trapped in PRF gel

Scanning electron microscopy

The scanning electron microscopical analysis showed compact fibrin network with denser and irregular surface morphology in platelet rich fibrin gel with sparse erythrocytes. Sam *et al*. (2015) similarly identified cellular elements like leukocytes with platelets entrapped in mature fibrin background. Fibrin strands were strongly polymerised and adhered to each other and was similar to the findings of Ehrenfest *et al*. (2018)

Conclusion

Centrifugation of 15 ml of bovine venous blood at 3200 rpm for 12 minutes yielded yellow, dense, and stable PRF gel. Histological analysis of PRF gel revealed pink fibrin strands and on scanning electron microscopical analysis, thick and dense fibrin was observed. Hence further studies should be

conducted to analyse composition of bovine PRF with transmission electron microscopy.

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Conflict of interest

The authors declared that they have no conflict of interest.

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