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Potential of platelet rich fibrin in extracted socket healing of Sprague Dawley rat model through acceleration of wound contraction, protein and collagen content.

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Abstract

Healing of extraction socket is a complex process, that involves both soft and hard tissue healing remodeling. Unlike other tissues, wounds of oral tissues are more prone for delayed healing due to constant movement of the oral tissues that disturb the formation of connective tissue. Also, exposure to various food materials provide a nutrient rich environment that enhance the pathogenic infection which leads to abscess, alveolar osteitis or dry socket especially after extraction. Platelet rich fibrin (PRF) is a naturally derived biomaterial, with considerable advantages since it is autologous, easy and less time consuming preparation, cost-effective, non-cytotoxic, biocompatible and biodegradable. Moreover, it is an efficient biomaterial with anti-inflammatory property. *In-vivo* physiological response of the tissues to injury is a complex interaction and cannot be replicated via *In-vitro* model. Hence, the present study aimed to investigate the potential of PRF in acceleration of protein, collagen content and also in wound contraction in the extracted socket tissue of Sprague Dawley rat model. The results of structure analysis showed that, PRF composed of nano structured fibrin nodules with rough surface. The content of the PRF, significantly increased the total protein and collagen content of the extracted socket tissue in a dose dependent manner. Also, the lowest concentration, efficiently reduce the swelling and increased the wound contraction. The data suggested that, the beneficial effect of

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concentration or quantity-based platelet rich fibrin application may raise the possibility of new approach as complementary treatment beside conventional therapy.

Key words

Platelet rich fibrin, Collagen, Protein, Extraction socket, Wound contraction

Introduction

Healing of extraction socket is a complex process, that involves both soft and hard tissue remodeling. However, normal wound healing is a primary molecular process that consists of three phases: inflammation, proliferation and remodeling. A cascade of events such as inflammation, angiogenesis, granulation tissue, reepithelization, extracellular matrix formation and osteogenesis occur in a highly orchestrated manner (Marsell et al., 2011). Inflammatory phase begins with the invasion of phagocytic neutrophils and results in the release of chemotactic factor that further attract and recruit neutrophils to the wound bed. During proliferation, contribution of new blood vessels (angiogenesis), inflammatory cells and immature fibroblasts forms granulation tissue that replace the fibrin clot (Laurens et al., 2006). The final phase of wound healing is remodeling, characterized by replacement of granulation tissue with the mature extracellular connective tissue matrix. Subsequently, epithelial cells from the periphery of the wound margins migrate across the wound bed and re-epithelization takes place. Flux of fibroblasts reorganize the collagen fibers whereas, myofibroblasts regulate connective tissue synthesis. Furthermore, myofibroblasts also cause contraction of the adjacent wound edges thus favors in reduction of the wound size (Van De Water et al., 2013). In the late stage of

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remodeling, replacement of the connective tissue by immature fibrillar bone occurs, which is then replaced by mature bone.

Any dysregulation in these phases can cause delay in wound healing and may lead to chronic wound. Unlike other tissues, oral tissues are more prone for delayed healing due to constant movement of oral tissues that disturbs the formation of connective tissue. Moreover, exposure to various food materials, provides a nutrient rich environment that enhance the pathogenic infection which leads to abscess, alveolar osteitis or dry socket (Mamoun., 2018). This might leads to loss of bone volume and are insufficient to support implants and prosthesis especially after extraction. For the past two decades, various methods such as debridement, irrigation, antibiotics, proteolytic enzymes, growth factors and bone substitutes are being used to counteract the unwanted postoperative sequele and to hasten the healing further. But, each of these methods has their own drawbacks and was not possible to demonstrate that one technique is superior compared to others (Schnutenhaus et al 2020). Nowadays, various research in the field of dentistry have mainly focused in selecting appropriate biomaterial of natural origin to speed up the healing and to regenerate the tissues in a natural way.

Biomaterial is a substance, that is engineered in a specific shape, either naturally or artificially. When implanted or injected in the extracted socket, it interacts with the host tissue and produces a desirable outcome of natural healing. Subsequently, micro environmental cues caused by these materials modulate the cell response of the innate immune system (Mariani et al., 2019). Also, the surface chemistry of the biomaterial governs the cell types and has an influence over their phenotypes. Biomaterials of natural origin that have anti-inflammatory properties can act as a suitable carrier for anti-inflammatory therapeutics. The ideal biomaterial should possess a 3D structured architecture and should be biocompatible enough to produce

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negligible immune reaction. Such biomaterials, enhance cell attachment and migration, make diffusion and proliferation of vital cells on to the surface of the scaffold before lay down of new extracellular matrix (ECM), until the damaged tissue is repaired (Hortensius and Harley., 2016).

Platelet rich fibrin (PRF) is a natural biomaterial that consists of well-organized fibrin network. The ideal 3D scaffold architecture of PRF, entraps platelets and leukocytes with in the fibrin marix. In comparison to synthetic scaffolds, PRF is considerably advantageous since it is readily available, autologous, less time consuming and easy preparation, cost-effective, non-cytotoxic, biocompatible and biodegradable. Studies have demonstrated that, PRF has anti-inflammatory, osteogenic and angiogenic activity and thus act as an efficient biomaterial (Dohan Ehrenfest et al., 2009). During wound repair, the *In-vivo* physiological response of the tissues to injury is complex interaction and cannot be replicated via *In-vitro* model and it requires a more efficient strategy. Hence, the present study aimed to investigate the efficacy of PRF in the extracted socket healing via acceleration of wound contraction, protein and collagen content using *In-vivo* Sprague Dawley rat model.

Materials and Methods

Experimental Animal

The *In-vivo* study was approved by the Institutional Animal Ethical committee, Rajah Muthiah medical college and Hospital, Chidambaram, Tamil Nadu, India. All experimental protocols were performed and observed according to internationally accepted principles for laboratory animal use and care. Male Sprague-Dawley rats (250-300 g) were acquired from National Animal Resource Facility for Bio-Medical Research and National Centre for Laboratory Animal Sciences, Tarnaka, Hyderabad, India. The rats were housed in polypropylene cages in the experimental central animal house, with a 12: 12h light/ dark cycle under 25˚C of controlled

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temperature, and 55- 60% of relative humidity. Animals were provided standard rodent pellet diet and water ad libitum and were allowed to acclimatize for two weeks before the surgical procedure. The animals were selected randomly and were divided into four groups (Group 1 control without PRF, group 2 (treated with 100µl of PRF), group 3 (treated with 200µl of PRF) and group 4 (treated with 400µl of PRF)).

.**Preparation of Platelet-Rich Fibrin (PRF)**.

The injectable form of PRF was prepared according to a previously published protocol (Jasmine et al., 2020). Peripheral blood 3 mL was collected from the rats and immediately placed in a preprogrammed centrifuge. As per the protocol, centrifugation was performed. The collected liquid portion was injectable form of PRF and was used immediately as before gelation.

Analysis of PRF topography using Field Emission Scanning Electron Microscopy (FE-SEM)

The freshly prepared PRF was fixed with buffered 2.5% glutaraldehyde (pH 7.2) for 1 h and post fixed with osmium tetroxide. The fixed sample was dehydrated with ascending series of ethanol for 15 minutes each. The dehydrated sample was sputter coated with platinum. The specimens were observed and photographed digitally using JSM – 7600 Field Emission Scanning Electron Microscope (Jeol, Japan) operating at 5kV. Using higher magnification, the average fibrin diameter of PRF was measured.

Surgical procedure

Standard surgical procedure was followed in the entire study (Ge et al., 2012; Luciana et al., 2002)**.** The animals were anaesthetized with a local infiltration of 2% lignocaine. The anaesthetized animals were placed in the operation theater and the maxillary right incisor was

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gently luxated using cutting edge enamel hatchet as elevators after elevation of the surrounding gingiva and was extracted with a curved artery forceps. The symmetrical wound of each rat was covered with respective quantity of PRF in the study group and without PRF in the control group. Immediately after surgery, single oral dose of diclofenac (10 mg/Kg) was given. Animals were monitored daily to assess food and water intake, general behavior and body weight. Postoperatively, for 1 to 5 days, swelling and wound contraction were analyzed in all the groups. The total protein and collagen content were analyzed at 7, 14 and 28th days.

Wound contraction and swelling reduction

The wound contraction in each rat from all the groups were measured and compared between control and treated groups. The wound area was measured using digital vernier caliper. The frequency of swelling was measured using a flexible tape starts from the corner of mouth of one side and run over the skin of the upper jaw and goes through the corner of the other side and over the skin of the lower jaw and ends in the same starting point.

Estimation of protein

The 100 mg of tissue was homogenized with ice cold distilled water. To precipitate the protein, the tissue sample was mixed with 5 mL trichloro acetic acid (TAC) and kept in ice bath for 30 minutes. Then the tubes were centrifuged at 5000 rpm for 15 minutes. The pellet was washed with TCA and then with absolute alcohol. The lipid free samples were resuspended in TCA and kept at 90˚C for 15 minutes (Porat et al., 1980). After incubation the protein quantity was determined by the Bradford protein estimation method (Kruger., 1994).

Estimation of collagen

Finely, minced tissue was treated with 15 mL of acetone for 6 h and was repeated twice. After, removing acetone, extraction was performed by adding 15 mL of ether and incubated for 12 h.

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The collected residues were dried to constant weight at 108˚C. Each dried tissue (10 mg) sample was placed in a 15 mL screw cap tubes with 4 mL of water and autoclaved for 3 hours at 120˚C. The residues were washed twice with sterile distilled water by centrifugation. The excess water in the residue was removed by evaporation. For collagen extraction, 1 mL of 6N HCl was added to each test tube and autoclaved for 3 hours at 120˚C. After autoclaving the hydrolysate was neutralized by 1N NaOH and filtered. The optical density of each sample was measured at 540nm. Using hydroxyproline standard graph, the collagen level of the samples was quantified (Cissell et al., 2017).

Statistics

The obtained results were expressed as a mean ± standard deviation (SD), and the data were analyzed using SPSS (IBM Corporation, USA) software and Microsoft Office Excel, by performing One-way analysis of variance and t-test. Comparisons were made between untreated control group and treated groups and the P value ≤ 0.05 is considered as statistically significant.

Results and Discussion

Topography of PRF by Scanning electron microscopy (SEM)

Topography analysis is a key factor to understand the structural properties of biomaterials. In the present study, SEM micrograph revealed the topography of rat PRF. Figure 1 shows fibrin network of PRF matrix composed of aggregated form of fibrins with nanopores. In the matrix, fibrins are arranged in the form of nanostructured nodules with rough surface. Studies revealed that, scaffolds with rough surface exhibit significant physio-chemical reaction at the implanted site (Vielreicher et al., 201). Indeed, roughness of the scaffolds modulate the biological response of the tissues at the wound area and has a direct influence on phenotype

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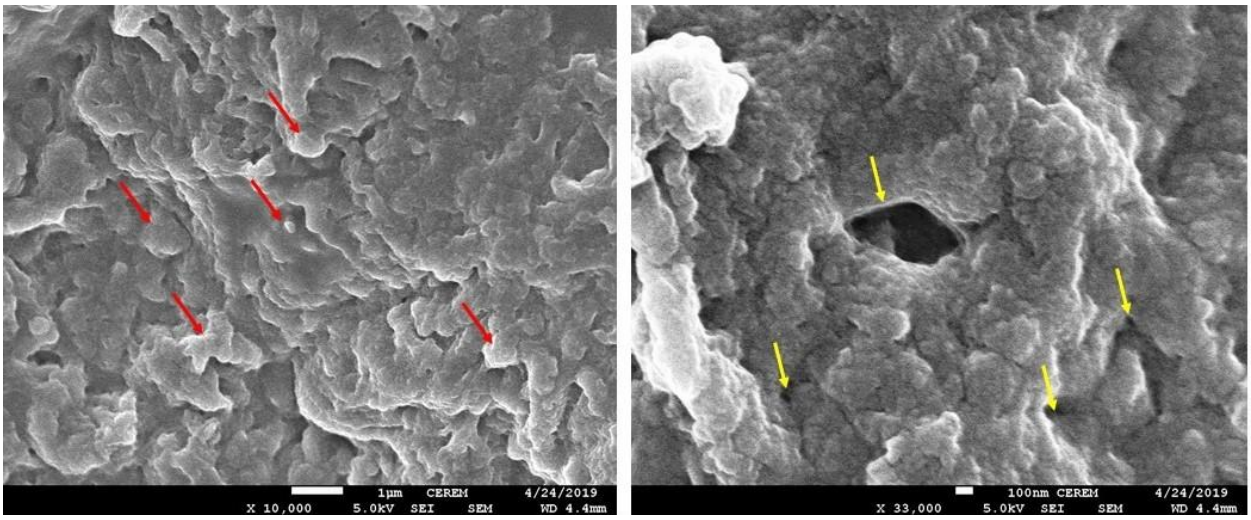
expression. Also, it enhances the cell adhesion and provides cell signals via adhesion receptors, mainly integrins (Hatano et al., 1999). The cells adhered on to the scaffolds govern and regulate the cellular response and enhance the release of bioactive compounds that are essential for cell proliferation and differentiation (Langeberg and Scott., 2015). The cell content (platelets and leucocytes) of PRF matrix does not seem to be on the surface, hence it might get entrapped in the inner core of the fibrin matrix. The compact arrangement of nanostructured fibrin nodule of the PRF matrix shows the structural properties like homing suitability, cellular attractability and durability. This structure-based scaffold properties strongly influence the healing potential of PRF at the wound area.

Figure 1. Topography of Platelet rich fibrin (injectable form) by field emission Scanning electron microscopy. Yellow arrows – Nano pores, Red arrows – Nano nodules of Fibrin



Total protein and collagen content

Table 1 shows the total protein and collagen content in the extracted socket tissue of rats in PRF treated groups and untreated control. On $7^{th,}$ 14^{th} and 28^{th} days, two to three-fold increase in the total protein content in all the study groups were observed and was significantly higher compared

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to control. Effective wound healing is determined by the total protein content. Moreover, it is especially crucial for the formation and maturation of tissues (Russell., 2001). In PRF matrix, the fibrin provides a source for the amino acids that enhance the socket healing. PRF increases the non-collagenous protein content in a dose dependent manner. However, protein plays an essential role in each phase of wound healing. Protein is the source of nitrogen containing compounds and are essential for the synthesis of amino acids, cell proliferation, new blood vessel formation, fibroblast proliferation, immune cells functioning, tissue remodeling, wound contraction and the formation of structural proteins (Harris and Fraser., 2004). During proliferative phase, the proliferation of fibroblasts and synthesis of collagen depend on the tissue protein content (Stechmiller., 2012). During injury, the demand for protein increased up to 250% and caloric demand up to 50% to maintain the natural healing potential (Breslow et al., 1993). The results of collagen content analysis (hydroxyproline) also follows similar pattern to those observed in the protein analysis. The continuous increase in collagen content was observed at $7^{th,}$ 14^{th} and 28^{th} days in all the study groups. Measuring the new collagen formation is important in the study of wound healing in animal model. Collagen is the major connective tissue component especially in the extracted socket. The extracted socket healing depends on the precise metabolism, regulation, deposition and subsequent maturation of collagen. Some studies have shown that the production and deposition of collagen during initial stage of healing are essential for new tissue formation and wound contraction (Frade et al., 2001). Thus, collagen plays a vital role in maintaining and repairing the structural integrity of socket tissues and its function (Yung-Kai and Che-Yung., 2010). However, the remarkable increase of collagen content in the PRF treated socket tissue confirms the pro-acceleration of not only the synthesis of collagen but also

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the cross linking of collagen. Our results further declare that, PRF application in the extracted socket provides a direct and instant availability of fibrin structure that promotes, prompt release of growth factors during the physiological events of extracted socket healing.

Experiment	Days	Group 1 (Control)	Group 2	Group 3	Group 4
Total Protein (mg/100mg of tissue)	7th Day	9.73±0.41	16.58±1.24***	22.78±3.77***	29.72±1.06***
	14th Day	12.60±0.80	21.72±0.57***	26.38±1.15***	32.03±0.43***
	28th Day	14.70±1.18	27.18±0.62***	28.54±0.49***	34.20±0.91***
Collagen (mg/100mg of tissue)	7th Day	4.09±0.49	7.99±0.25***	8.51±0.71***	9.54±0.29***
	14th Day	4.68±0.12	9.03±0.49***	9.35±0.13***	9.85±0.10***
	28th Day	5.34±0.55	9.51±0.34***	9.98±0.18***	11.38±2.16***

Table 1. Potential of Platelet rich fibrin in acceleration of protein and collagen content in the extracted socket tissues

Values are expressed as mean ± SD in six animals of each group $p < 0.001$ highly significant compared to control.

Swelling reduction potential of PRF

Damage to the tissues during surgical procedure is the main cause for postoperative swelling other than infection (Arta et al 2011). Moreover, few hours after surgery, swelling is more pronounced and starts to decline thereafter for few days (Hanz et al 1985). Reduction of swelling

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is a sign that shows a decrease in the inflammatory response of the damaged tissue in order to begin the healing cascade (Chen et al., 2017). In the current study, reduction in swelling was analyzed in all the groups for 5 postoperative days. The percentage of reduction in swelling during the five subsequent days for group 1 to 4 are depicted in the figure 2. Among all treated groups, the lowest concentration of PRF (group 2) exhibits significant reduction in swelling postoperatively from day 1 to 5 (range from 61.9 to 8.8%), followed by group 3 (range from 61.8 to 11.8%). Astonishingly, in group 4, the swelling level (range from 93.7 to 28.8%) was significantly higher than control group (range from 80.3 to 32.5%). This might be due to, increased platelets, leucocytes and growth factors concentration when the concentration of PRF increases that might have resulted in stress induced alterations. However, decrease in PRF concentration results in reduction in cell contents like platelets and leucocytes. A quick response of phagocytic cleanup by neutrophils via digestive enzyme that infiltrate the injured tissue and further clearance of the dead cells by monocytes (Jayakodi et al., 2012) shifts the macrophage phenotype from M1 towards healing phenotype M2. Hence, significant reduction in swelling was observed in group 2 and group3. Swelling reduction results revealed a shift from inflammatory response to anti-inflammatory response, thus confirms the speed up of healing process by PRF at lower concentration compared to control and group 4.

Figure 2. Graphical representation of swelling reduction in percentage on various days in the control and treated groups. Values are expressed as mean ± SD in six animals of each group p <0.001 highly significant compared to control.

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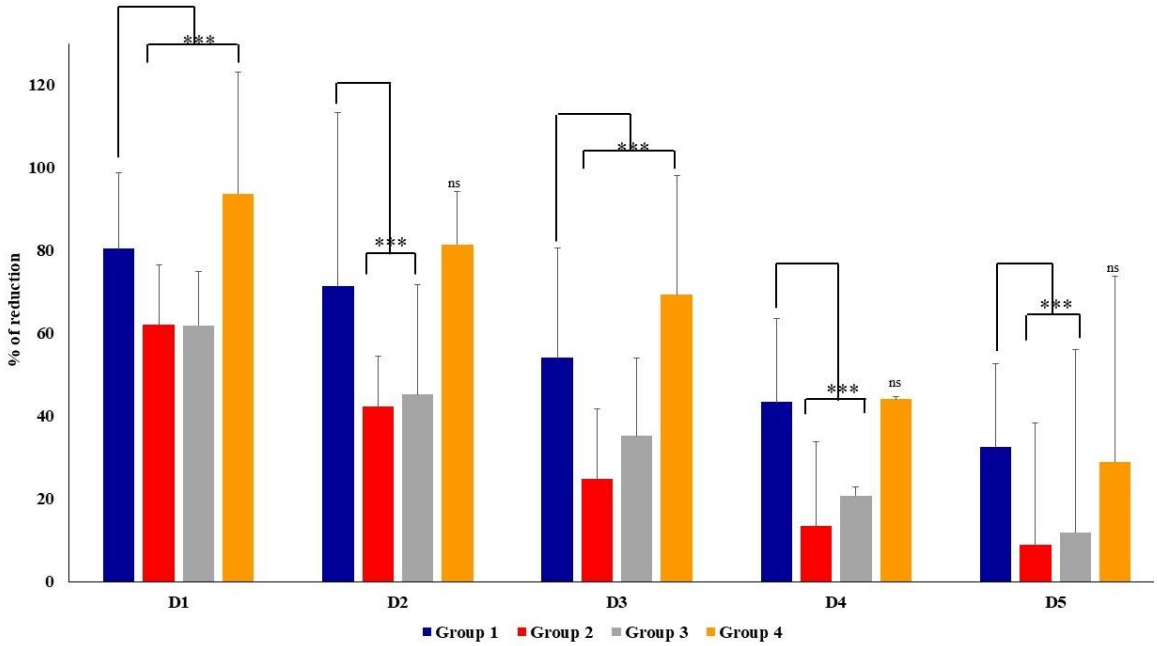

Wound contraction potential of PRF

Wound contraction is the reduction of wound size over a specific period of time, in an attempt to close the defect after the creation of the wound. Moreover, it is an effective means to bring about closure of wounds especially in oral tissues like extraction socket which often expose with constant movement that results in disruption of connective tissue formation (Gauglitz et al 2011). The gradual reduction of the socket area was measured in millimeter, using digital Vernier caliper from the postoperative day 1 to 5 and the results and photographs of control and treated groups were shown in fugue 3. Group 2 shows steady and sharp decrease in the wound size from 9 mm to 5 mm on 1 to 5 postoperative days, whereas group 3 shows a gradual decrease from 9 mm to 5 mm. Hence, a slight variation was observed between these two groups. Group 1 (range from 9.6 mm to 7.6 mm) and 4 (range from 9.4 mm to 6.9 mm) exhibit similar wound contraction result. All the obtained results were statistically significant. The differentiation of fibroblasts into myofibroblasts and their contractile activity contribute to the contraction of wound (Wipff et al 2007). In addition, differentiation of fibroblasts into myofibroblasts

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phenotype is mainly based on their adhesion to the connective tissue matrix and stimulation by transforming growth factor beta (TGF-β) (Justin and Yella 2012). A combined effect of epithelial migration across the margins as well as the wound contraction along the wound bed results in a better approximation of the wound. The lower concentration PRF groups (group 2 and 3) initiate the proliferative stage effectively that results in differentiation of fibroblasts into myofibroblasts phenotype. Hence, faster wound contraction in these groups.

Figure 3 a. Graphical representation of wound contraction on various days in control and treated groups. Photographical image of wound contraction b. Before extraction, c. Group 1 (Extracted control), d. Group 2 (Treated with 100µl of PRF (injectable form)), e. Group 3 (Treated with 200µl of PRF), f. Group 4 (Treated with 400µl of PRF).

Conclusion

The present study demonstrated the potential of platelet rich fibrin in the extracted socket healing of Sprague Dawley rat model. The structure of platelet rich fibrin composed of

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nanofibrin nodules with rough surface that can significantly increase the total protein and collagen content of the extracted socket tissue. Moreover, lower concentration of PRF efficiently reduces the swelling and increases the wound contraction during the initial phase of healing. The beneficial effect of concentration or quantity-based platelet rich fibrin application may raise the possibility of new approach as complementary treatment beside conventional therapy.

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