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Research Article

Combination of Platelet-rich Fibrin and Stromal Vascular Fraction from Adipose Tissue Enhances the Wound Healing in Sprague Dawley Rats

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ABSTRACT

Chronic non-healing ulcer is defined as a persistent defect in the skin for the period of more than six weeks and does not exhibit any tendency to heal following three or more months. Platelet-rich fibrin (PRF) and stromal vascular fraction (SVF) cells obtained from adipose tissue have been widely reported for diabetic wound heling. In this study, the efficacy of the SVF combined with PRF which contains abundant growth factors, for wound healing was evaluated using an animal model. PRF from venous blood was prepared from 8 donors and SVF from lipoaspirate were harvested from a single donor. Three groups were created (i) SVF + PRF as treatment group; (ii) Povidone Iodine (PI) - as standard of care; (iii) negative control group - allowing natural wound healing. Dorsal full thickness wounds were created in 40 male Sprague-Dawley (SD) diabetic rats. The SVF+PRF groups were subdivided into 3 groups having 40,000, one and two million cells per dose per application. On day seven of this study, rats were euthanized and wounds were analyzed microscopically and macroscopically. Wounds closed faster in the SVF + PRF group than in the control group or PI groups, with less inflammation, prominent signs of re-epithelization and blood vessels. The combination of SVF and PRF may provide an additive stimulatory effect to support angiogenesis and accelerate the wound healing process; accordingly. Our results suggest that the combination of SVF and PRF enhances wound healing.

Introduction

Diabetic foot infections have been an increasingly common problem globally due to the sustained rise in new incidences of diabetes and the increasing body weight of diabetic patients.[1,2] Diabetic foot ulcer (DFU) complications are the main reason for diabetes-related hospitalizations and lower extremity amputations.[3]

Wound healing is a highly complex process that remains a major challenge in modern medicine. Among the factors contributing to these non-healing conditions, impairment of cytokine production and reduced vascularization play crucial roles.[3]

Adipose tissue is one of the most accessible tissues by mild operation and the only tissue in the human body that can be removed without leaving a functional defect. A vast amount of the stromal vascular fraction (SVF) in adipose and connective tissues can be easily obtained from patients using conventional liposuction and isolation methods.[4] The SVF consists of a heterogeneous mesenchymal population of cells that includes not only adipose stromal, hematopoietic stem and progenitor

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cells but also endothelial cells, erythrocytes, fibroblasts, lymphocytes, monocyte/macrophages and pericytes.[5,6]

SVF has been shown to promote angiogenesis, partially through secretion of various growth factors (GFs) such as vascular endothelial growth factor (VEGF), the presence of endothelial progenitor cells (EPCs) and the supportive role of ASC with pericytic properties.[7-9]

In the past decade, various studies have demonstrated that the therapeutic use of platelet-rich plasma (PRP) was successful as a basic sealing and hemostatic therapeutic tool in plastic surgery, especially in oral, hand, and aesthetic surgery.[10-12] However, many limitations in PRP therapy, such as the association of anti-coagulants, have the benefits and clinical outcomes of PRP use in treating chronic ulcers, resulting in the inhibition of the wound healing process.[13,14] Thus, platelet-rich fibrin (PRF) was developed as an easy to prepare, nontoxic, and biocompatible platelet-based therapeutic alternative.[15,16] Also known as "Glue Therapy", PRF used as a cell scaffold was proposed as an effective approach to promote and repair wound healing.[17]

PRF is a platelet concentrate collected on a single fibrin membrane containing all the favorable constituents for healing. The scientific rationale behind the use of platelet preparations lies in the fact that platelets serve as a reservoir for numerous growth factors that are known to play a crucial role in hard-tissue and soft-tissue healing processes.[18] Intact platelets contained within a fibrin matrix release a relatively constant concentration of growth factors over 7 days, stimulating human osteoblastic proliferation. An effect on neo angiogenesis also has been shown through histology. In a membrane form, the platelets can be used as a fibrin bandage that serves as a matrix to accelerate the healing of wound edges.[19] Platelets are considered to play an important role in the healing process of wounds, as they release GFs upon activation following their pulverization on the wound bed within the first hour.[20] These GFs contribute to the repair of injured tissues through promoting angiogenesis, synthesis of extracellular matrix components like collagen, laminin, and integrin, and interestingly via inducing a re-epithelization of the injured site.[10,21] In this study, we investigate the wound healing effect of PRF formed using thrombin and fibrinogen along with SVF, used as topical application and to compare with the control non-treated group and standard dressing group using povidone iodine (PI) application in Sprague Dawley (SD) rats with wounds created on their dorsal region. We propose our protocol as a potentially new cost-effective therapeutic approach with better and faster outcomes than traditional chronic wound healing treatments.

Materials and Methods

Ethics Statement and Animals

All procedures complied with the CPCSEA guideline. The IAEC approval number for this study is Project Proposal No.: Form B / 13 / 2015-16.

Total forty adult male SD rats were allotted for the study. At time of streptozotocin intraperitoneal injection rats weighing 200–250 gm with ages 9–11weeks. Animals were housed at temperature of 22 ± 3°C, relative humidity 30 to 70%, and 12 hours light–dark cycle with free access to standard food and RO water *ad libitum*.

For induction of diabetes, streptozotocin (STZ) was prepared freshly in 0.01M cold citrate buffer and administered to overnight fasted rats as a single dose of 40 mg/kg body weight via intraperitoneal route. Animals blood glucose levels were monitored after 72 hours of STZ injection, using ACCU-CHEK INSTANT GLUCOMETER (Roche diagnostic Ltd). Animals with fasting blood glucose levels more than 400 mg/dL were considered as diabetic and were selected for the study.

The diabetic rats were kept under observation for one month before wound creation to ensure the development of chronic diabetes. Insulin injections were given at 5–10 IU per/ kg by sub cutaneous route daily to control the higher blood glucose levels (blood glucose level was maintained between 400–700 mg/dL).

All animals (n = 40) after one month of diabetes induction received general anesthesia via an intramuscular injection of a combination of ketamine hydrochloride (75 mg/kg) and xylazine (10 mg/kg). After anaesthesia, the dorsal region was shaved and 70% isopropyl alcohol was applied over the shaved area. Full thick-ness wound over shaved area was created with the help of an autoclave-sterilized razor-sharp metal punch, a 6.0 mm^2 fragment of skin was removed until the exposure of the dorsal muscle fascia. Wounds were cleaned with PI to avoid bacterial infection. No PI was applied after that. Oral analgesic, paracetamol at a dose of 500 mg/L in a drinking water bottle was provided for two days to relieve the pain due to wound creation.

Human Lipoaspirate Samples

The study was conducted following the Institutional review board of Wockhardt Hospitals, Mumbai, India (study number WHIRB/04-WH/WCRM/Autologous/ ATSVF – CALA /2011). Human adipose tissue samples were obtained with written informed consent from individuals undergoing elective cosmetic surgery at the department of plastic surgery, Wockhardt Hospitals. Lipoaspirate tissue was obtained from abdomen region by the ultrasound assisted tumescent lipoaspiration technique.

Study Design

The study was conducted in SD rats weighing around 250–300 g at the start. Total 40 rats were divided in to five group (8 animals/group) *viz,* Control (G1), PI treatment (G2) and three test treatment groups (thrombin and fibrinogen along with SVF cells per cm^2) with 40000 (G3A),1.0 million (G3B) and 2.0 million cell (G3C) (Table 1). Four, full thickness excision wounds were created using 6 mm diameter biopsy punch on the dorsal side of each

Table 1: Study design, allocation of number of animals in different dose groups

Dose Group No.	*Group Details*	*Treatment details*	*Animal*	
			Total No.	*I.D No.*
G1	Control	No treatment	8	1-8
G2	Standard of care	Topical application of Povidone iodine	8	9-16
G3	G3A	40,000 cells/ cm^2	8	17-24
	G3 B	1.0 million cells/ cm^2	8	25-32
	G3 C	2.0 million cells/cm^2	8	33-40

rat. The animals from treatment group were applied with the mixture of PRF and SVF to form a film on the wound surface. Four consecutive applications were given at daily interval (initiated on Day 0 ending on Day 3). Animals from control group were allowed to heal naturally, and animals from standard of care group were applied with PI locally on the wound surface.

SVF Preparation

The protocol regarded by Zuk *et al.*[22] is still regarded as the most widely used method for isolation of SVF. Briefly, 100 ml lipoaspirate sample Adipose tissue is harvested by liposuction and washed in phosphate buffered saline (PBS) to remove blood remnants. The resulting adipose tissue was enzymatically digested with collagenase Type 1 (GIBCO cat no. 17100017), centrifuged at 300xg for 10 mins. The SVF pellet was washed three times with PBS, and RBCs were removed by ACK (ammonium-chloride-potassium) RBC lysis solution. The SVF isolated cells were cryogenically frozen in liquid nitrogen until further used.

Once isolated, characterization of the cell composition of freshly isolated SVF has been accomplished through flow cytometry (Canto II Flow Cytometer, Backman Coulter, USA) which allows the identification of the surface marker expression of the cells in vitro.

The following fluorochrome-labeled monoclonal antibodies were used for SVF analysis:

CD31-FITC, CD34-PE, and CD45-FITC, CD 73 -PE, CD 90 – PE, CD 105-PE, CD 146-FITC, CD 271-PE, HLA-ABC - PE-Cy^{TM}5, HLA-DR -FITC. Viability was assessed using 7-Amino-Actinomycin D (7-AAD).

PRF Preparation

Whole blood was collected from healthy human volunteers. Briefly, 8 ml of blood was collected by venepuncture in a ACD vacutainer (BD 364606) for obtaining plasma, and 5 mL of whole blood was collected in a BD SST vacutainer tube (BD 367986) for the preparation of serum. The clotting cascade requiring the conversion of fibrinogen to fibrin was catalyzed by thrombin in the presence of calcium. The thrombin and fibrinogen were obtained from human plasma and serum. The resulting clot was used as a haemostatic agent, wound cover, and scaffold for the added SVF.

Analysis of Growth Factor from PRF

The presence of growth factors was confirmed by performing enzyme-linked immunosorbent assay (ELISA) using commercial kits available from R&D Systems. The PRF sample was assayed for platelet-derived growth factor (PDGF), transforming growth factor- beta (TGF-β), vascular endothelial growth factor (VEGF), using respective ELISA kits. Additionally, the level of p-Selectin was also analyzed to determine the level of platelet activation in the pooled sample.

Treatment of Wound with PRF and SVF

Day of wound creation was considered Day 0, and animals were randomly allocated to the control group, standard of care group and PRF+SVF treatment group. Each group was comprised of 8 animals. The animals from the treatment of group were applied with the mixture of PRF and SVF to form a film on the wound surface, the cell number was calculated and applied as described in Table 1. Four consecutive treatments were given at daily interval (initiated on Day 0 and ending on Day 3). Animals from control group did not receive any application and wounds were allowed to heal naturally. The animals from standard of care group were applied with PI locally on the wound surface.

Measurement of Wound Contraction

Wound margin was traced on transparent plastic sheet after wound creation by using transparent paper and the area was measured by graph paper. Wound contraction was measured on day 0, 4 and 7.

The epithelialization time was measured from the initial day (Day 0) to the day when the scab fell off from the wound surface exclusive of leaving a healed wound behind.

Histopathological Examination

Specimens were fixed in 10% neutral buffered formalin and processed to embed in paraffin according to the routine histoprocessing technique. Subsequently, 4 sections were made from each animal one from each wound area and stained with hematoxylin-eosin (HE – basic staining. The semi-quantitative method was used to evaluate histological processes and structures like re-epithelization, PMNL (polymorphonuclear leucocytes), fibroblasts, new vessels, and new collagen.

Results

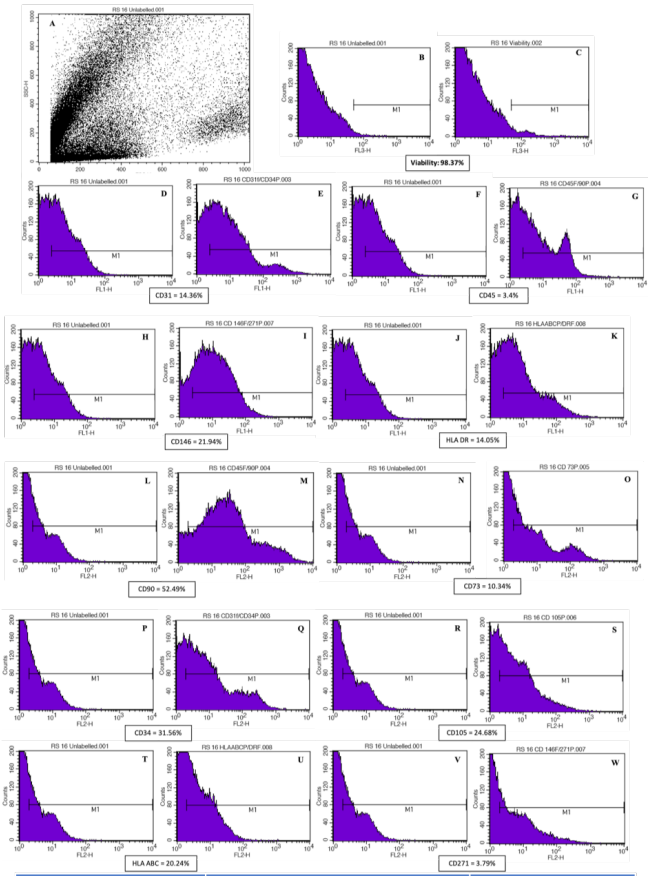
After enzymatic dissociation, the SVF isolated was analyzed for the cell yield, viability and surface markers. Sterility testing was also performed to ensure that the treatment of animal wounds would be free from any microbial or fungal contamination from the isolated SVF. Endotoxin levels were determined to ensure that these cells are following standards applicable for topical applications Table 2.

Viability was assessed using 7AAD staining shown in Fig. 1, B&C. Cells were then analyzed for the presence surface markers CD31, 45, CD34, CD73, CD146, CD271, HLA ABC and DR.

SVF is positive for the markers for mesenchymal stem cells such as CD 73, 90 and 105. Our results also demonstrate that these markers are expressed as a high percentage (Fig. 1). The SVF also expresses CD 34 (31.56%) but the pan hematopoietic marker CD45 had a comparatively lower expression (3.4%). Our data also shows the presence of CD 146 (21.94%). CD146 is expressed endothelial cells and plays a key role in vessel functions. The CD 34 expression along with the high CD 90 expression could represent a more "primitive" population of cells. The presence of CD 146 could explain the results in the wound closure of SVF+PRF treated animals.

The presence of CD 271 (3.79%) also confirms the presence of a mesenchymal stem cell like population. The high percentage of CD 73, CD 90 and CD 105 surface markers indicate that the SVF is a rich source of stem cells, since mesenchymal stem cells are predominantly characterised by these markers.

In the natural wound healing process, blood plays an important role in promoting tissue regeneration by providing a variety of cells, growth factors, cytokines,



Surface marker	Antibody	% Positive
CD 31	FITC Mouse Anti-Human	14.36%
CD 34	PE Mouse Anti-Human	31.56%
CD 45	FITC Mouse Anti-Human	3.40%
CD 73	PE Mouse Anti-Human	10.34%
CD 90	PE Mouse Anti-Human	52.49%
CD 105	PE Mouse Anti-Human	24.68%
CD 146	FITC Mouse Anti-Human	21.94%
CD 271	PE Mouse Anti-Human	3.79%
HLA ABC	PE-Cy™5 Mouse Anti-Human	20.24%
HLA DR	FITC Mouse Anti-Human	14.05%

Fig. 1: Surface marker analysis and viability of isolated SVF. forward and side scatter of SVF (A), unlabeled cells in FL1-H (D,F,H) unlabeled cells FL2-H (L,N,P,R,V) unlabeled cells FL3-H (B), labelled cells FL1-H (E,G,I,K) labeled cells FL2-H (M,O,Q,S,W)) and labeled cells FL3-H (C). summary tabulation of surface markers.

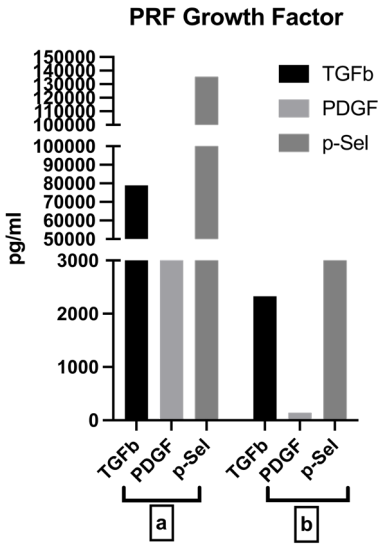


Fig. 2: Analysis of platelet rich fibrin growth factors: p-selectin is a marker for platelet activation. a- whole blood platelets activated by fibrinogen and thrombin to form PRF, high levels of growth factor consistent with high levels of p-selectin indicating platelet activation and release of growth factors. b- baseline growth factor and p-selectin level in collected non activated platelets.

Table 2: SVF isolated from 100 mL of lipoaspirate, suspended in 16 mL of freezing mixture, 16 vials were frozen as 1ml aliquots. Each vial contained around 420x106 SVF cells. These cells were plated to test for sterility and also tested negative by mycoplasma PCR.

SVF Parameter Assessed	*Observed / Calculated Values*
Cell Count per ml	415.8×10^6
Volume of Cell Suspension	16.2ml
Sterility by Nutrient Agar	No growth observed
Sterility by Sabouraud Agar	No growth observed
Endotoxin Using LAL Kit	< 0.0625 EU/ml
Mycoplasma by PCR	Not detected

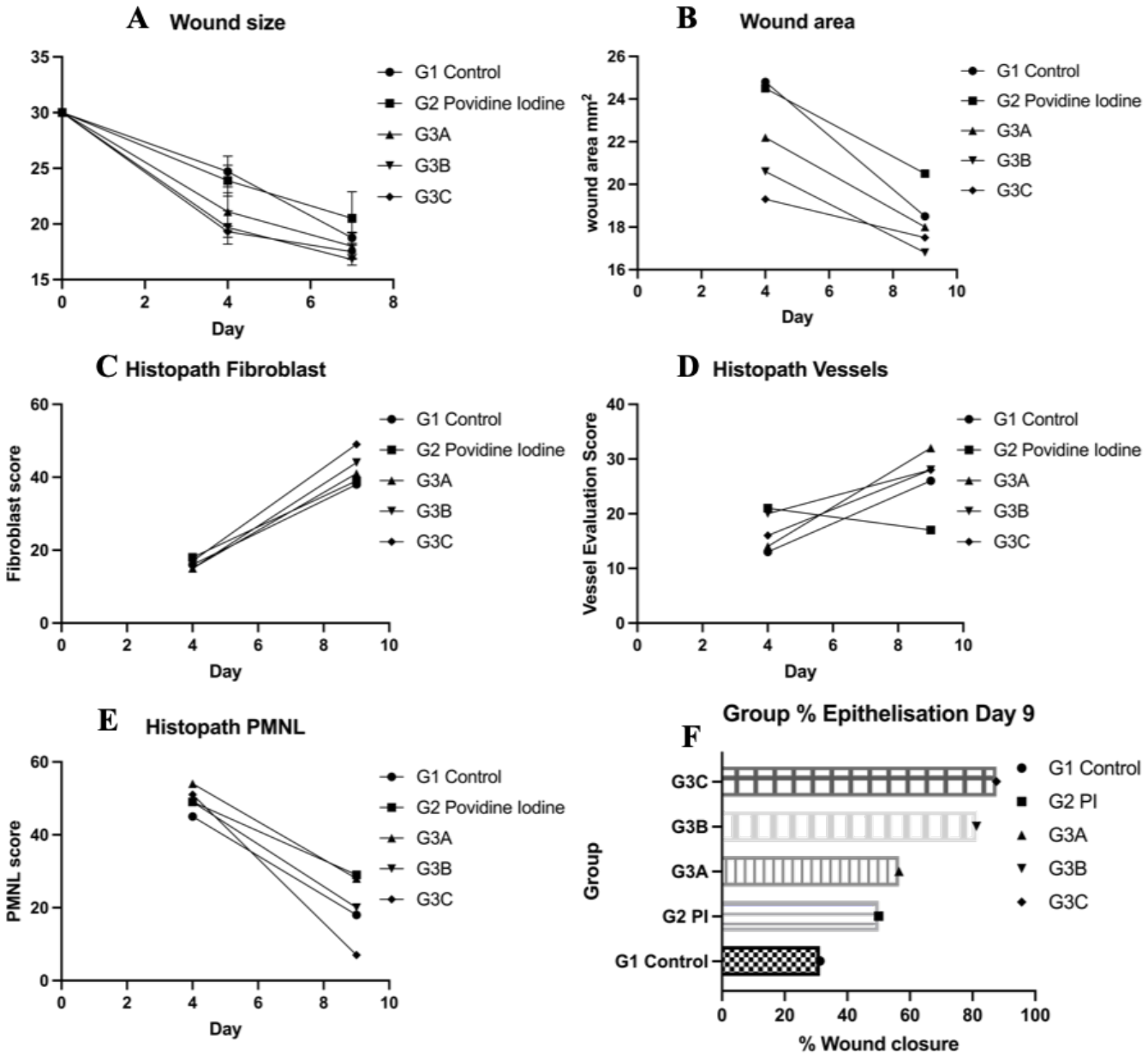


Fig. 3: A. wound size assessment over Days 0, 4 and 7. there is a significant difference in wound closure between the G3 treatment groups compared to control. notably there is a graded dose response with enhanced wound closure observed in the high dose group.
B. wound area histopathology scores on day 4 and day 9.
C. histopathology scores: fibroblast on day 4 and day 9.
D. histopathology scores: vessels on day 4 and day 9.
E. histopathology scores: PMNL on day 4 and day 9. group 3 C having PRF with $2x10^6$ cells/cm^2 shows the best scores indicating healthy and faster healing compared to the untreated and PI treated controls. all PRF+SVF treatments demonstrated faster healing.
F. eepithelization as observed on various days. treatment group G3A,B and C showed enhanced epithelization compared to the control. there is a notable graded response as the SVF cell concentration is increased.

and coagulation factors. Ultra-physiological doses of platelets (platelet-rich plasma) were originally developed to increase the number of platelets at the defective site, but healing was thought to be non-optimal, but additional additives were required. Hence, a second-generation concentrate called fibrin (PRF), rich in platelets has been developed. PRF secretes several growth factors, including transformed growth factor β1 (TGFβ1), platelet growth factor (PDGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor I (IGF1). We report high levels of PDGF, TGF-beta were released after platelet activation. Platelet activation was reported by the elevation of p-selectin after adding the acting agents of fibrinogen and thrombin. This release profile can be

attributed to improving angiogenesis, cell behaviour, and tissue regeneration (Fig. 2).

Wound contraction was measured on days 0, 4 and 7. The rate of contraction of the control group, PI treatment group and test treatment group wounds are shown in (Fig. 3 A&B). On day 4, the percentage contraction was 21.1, 19.1 and 19.3 in test treatment groups, G3A, G3B and G3C groups respectively. These percentages were higher than the control group (17.5). The results revealed that wounds from test treatment with stem cell resulted in faster contraction of the wound when compared to that of control and PI treatment group.

The epithelialization time was counted from the initial day (Day 0) to the day when the scab fell off from the wound surface exclusive of leaving a healed wound behind. On Day 8 and 9, the epithelization status of wounds was recorded. The number of wounds with complete epithelization was found to be significantly higher in all three treatment groups compared to the control and PI treatment groups (Fig. 3 F). Total epithelization percentages were 87, 81 and 56 in test treatment groups G3A, G3B and G3A, respectively. Whereas epithelization percentages in control group (G1) and PI treatment group (G2) were 31.5 and 50, respectively. There was no significant difference between one million stem cell concentration and two million stem cell concentration groups.

On day 4 of wound creation, histopathological scores for epithelization, PMNL, presence of fibroblast and vessels of control group, PI group and stem cell treated groups (all 3 concentrations) did not differ significantly (Fig. 3 C, D, E). On Day 9 of wound creation, the epithelization and fibroblast score of two million cells treatment group (G3 C) exhibited a significantly higher score than all other groups. All the remaining groups (viz, control, PI, and SVF + PRF treatment with 40,000 and one million cells/ application) showed similar epithelization and fibroblast score by and large. This indicated that 2 million cells treated group showed rapid epithelization and healing capacity than all other groups on day 9. Histopathological score for PMNL on day 9 was not significantly different for control, PI, and 40,000 and one million cell treatment group. The PMNL score of two million cells treatment group was significantly lower than all other groups indicating that the inflammation procedure at wound site has been superseded by granulation and healing procedure.

Discussion

The results revealed that wound from PRF & SVF treatment resulted in faster contraction of wound when compared to that of control and PI treatment group. The wounds with complete epithelization were found to be significantly higher in all three test treatment groups compared to the control and PI treatment groups. In Histopathological examination, two million cells treated group showed rapid epithelization and wound closure than all other groups on day 9. Based on the scores of wound area, epithelization time, wound contraction percentage, histopathological scoring of epithelization, PMNL and fibroblast, it is concluded that two million cells per application site showed better wound healing potential under the present experimental conditions in SD rat.

Wound healing is a complex process coordinated by numerous molecular events leading to the closure of the wound. CD 146 has been demonstrated as a biomarker on vascular endothelium, which is involved in angiogenesis, underlying that CD 146 may play a critical role in vascular endothelial cell activity and revascularization. PDGF and the help of TGF-β play an important role in augmenting the inflammatory response and tissue debridement. TGF-β1 is important in inflammation, angiogenesis, re-epithelialization, and connective tissue regeneration. The faster healing rates and wound closure can be attributed to high levels of TGF-beta and PDGF in the PRF as well as presence of cells in SVF that are positive for surface markers such as CD 146, which are expressed by Endothelial Progenitor cells and plays a key role in angiogenesis. In addition to CD 146, we demonstrated a mixed population of cells in SVF positive for surface marker such as CD 73, CD 90, CD 105, associated with MSCs and CD 34 associated with HSCs. Revascularization also occurred in the SVF + PRF group, as indicated by the presence of blood vessels in these treatment groups, unlike in the control group. The number of blood vessels was higher in the SVF + PRP group than in the SVF or PRP group. This data demonstrated that PRF and SVF combined therapy significantly accelerated healing of wounds induced experimentally in diabetic rats. This could be attributed to the combined therapeutic effect of SVF and growth factors from PRF in enhancing angiogenesis and triggering epidermal cell proliferation and wound closure.

This research gives insight into the application of combining PRF with SVF for faster wound healing. This combination may provide an additive stimulatory effect to support angiogenesis owing to high expression of CD146 in the SVF, and accelerate the wound healing process; accordingly, this combination is a potential alternative to PI treatment.

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