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Research Article

Evaluation of Healing Effect of Stromal Vascular Fraction and Platelet Rich Plasma Application on Ulcerative Ear Wound in Diabetic Rabbit

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ABSTRACT

A debilitating complication of diabetes mellitus is diabetic ulcers, which leads to increased overall morbidity in patients. The high growth factor content in Platelet Rich Plasma (PRP) makes it a widely used intervention for the treatment of diabetic foot ulcers. The topical application of Stromal Vascular Fraction (SVF) could possibly enhance wound healing. This study aims at evaluating the efficacy of combining SVF and PRP on wound healing in diabetic rabbit model. Diabetes was induced in New Zealand white rabbits by intravenous injection of 125 mg/Kg Alloxan. After two weeks of alloxan, three 6 mm diameter, full thickness excision wounds were made, on inner side of the right ear pinna. The animals after induction were allocated into 4 groups with [8 Diabetic (treated with SVF+PRP), 4 Diabetic (treated with 10% Povidone Iodine (PI)), 1 non-diabetic (treated with SVF+PRP), 1 non diabetic (treated with 10% PI)]. The effect of combined therapy was evaluated by assessing wound margin closure rate, histo-pathological evaluation, and inflammatory cell infiltration, epithelization of ulcerative region, neo-vascularization, and fibrosis. We observed that the rate of wound closure is enhanced in wounds treated with SVF+PRP as compared to the PI solution. Wound closure and healthy healing were demonstrated by histo-pathological analysis. The analysis clearly indicates that the healing process of PI treated animals is slower than that of SVF + PRP treated animals. In conclusion, based on wound healing assessment and histo-pathological examination, the diabetic rabbits treated with SVF + PRP exhibited early development of granulation tissue and early signs of wound closure as compared to diabetic animals with normal PI dressing.

Introduction

Comprising 10% of the total body mass, the skin is the largest organ of vertebrates and is crucial for defense and survival. Each injury induces loss of the integrity of the skin resulting in functional imbalance, possibly accompanied by disability or even death.[1] Diabetes mellitus is responsible for delayed or impaired wound healing, leading to chronic ulcer formation. Chronic wounds represent a relevant clinical and socioeconomic burden. Diabetes is a chronic disease that involves approximately 350 million people (6.5%) worldwide. The annual incidence of foot ulcers among people with diabetes is estimated at between 1% and 4.1%, and the annual incidence of amputation is 0.21–1.37%.[2,3] The amputation rate is 15–70 times higher in diabetic individuals than in general.[4,5]

Povidone-iodine (PI) is an antimicrobial agent that has been used in a wide range of wound-healing applications for over 60 years. It has broad-spectrum antimicrobial activities (against bacteria, viruses, and fungi), anti-inflammatory properties, and a well-established safety profile.[6,7] Some reports have suggested that PI may interfere with wound healing; however, in vivo studies have demonstrated that PI does not disturb wound healing, especially when lower doses are used (<5% PVP-I).[8]

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Local ulcer wound care with dressing and repeated debridement of necrotic tissue is a typical clinical treatment of diabetic ulcers. However, the results are not encouraging, and about 14–20% of the patients with diabetic ulcers still get amputated. Several methods have been developed for diabetic ulcer wound healing based on inflammation or growth factors.[9,10] Various treatments have been developed to treat diabetic ulcers. However, most of these focus on one facet of wound healing like inflammation. Diabetic ulcers have a multifactorial aetiology, and therefore the current treatment strategies focused on one facet would have limited efficacy. Stem cells and growth factors have recently been reported to provide a comprehensive solution by addressing the multiple factors involved during the diabetic healing process. Growth factors, or cytokines, are biologically active polypeptides responsible for modifying the growth, differentiation and metabolism of target cells.[11] Growth factors can act by both paracrine and autocrine mechanisms and bring about cellular communication via binding to specific cell surface receptors with the resultant induction of a complex cascade of signal transduction pathways. Numerous studies have also reported increased expression of various growth factors, including EGF, KGF, TGF-beta1, VEGF, and PDGF.[12,13] These growth factors contribute to the repair, regeneration, and neovascularization in diabetic wounds.

Despite significant advances in medical care and nutrition, there is a growing need to develop novel strategies to improve cutaneous wound healing. The medical field is rapidly advancing towards developing low or non-invasive procedures and accelerated treatments that can achieve reduced morbidity and a good functional recovery in our patients to improve their quality of life. In the last few years, these simple and cost-efficient procedures have potentially impacted economic costs for standard general medical treatments.[14]

Cell-based therapy is an attractive approach for the treatment of difficult non-healing wounds.[15] This field of regenerative medicine focuses primarily on stem cells, which are specialized cells that can self-renew and differentiate into multiple cell types.[16] Adult multipotent stem cells are crucial for physiological tissue renewal and regeneration after injury. Mesenchymal stem cells (MSCs) have been isolated from various sites, including bone marrow, adipose tissue, and amniotic fluid.[17-19] Earlier reported studies have shown that different types of stem cells, such as bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived stem cells (ASCs), are capable of enhancing wound healing in experimental diabetic models.[20-23] BM-MSCs have demonstrated several properties *in vitro* that can promote tissue repair, including the production of multiple growth factors, cytokines, collagens, and matrix metalloproteinases[19-24] and the ability to promote the migration of other skin cells, such as keratinocytes.[25] Although BM-MSCs have demonstrated several properties that promote wound healing, the preparations of BM-MSCs are time-consuming. ASCs are abundant in fatty tissue and have a multilineage mesenchymal potential similar to BM-MSCs. Previous studies have indicated that harvesting and culturing MSCs from adipose tissue is easier than collecting bone marrow cells.[22,26] ASCs represent an alternative source of multipotent stem cells with characteristics similar to BM-MSCs.[27] ASCs are easier to isolate and are relatively abundant, making these cells a potential source for wound repair and regeneration.[19-21]

Platelet-rich plasma (PRP), which provides an abundance of nutrients and offers a suitable microenvironment for ASCs to promote proliferation and migration, is a good candidate for enhancing the wound healing effect of ASCs due to its unique composition.[28,29] PRP is an autologous product of blood plasma with a high platelet concentration and contains various growth factors and cytokines, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF).[30] Although more evidence of the efficacy of PRP alone as a treatment for wound healing is needed, the positive impact of PRP on chronic wounds has been proven in many studies.[31] More importantly, PRP can promote the proliferation and migration of ASCs, which indicates that it has a potential synergistic regenerative effect on chronic wounds.[32-34] The use of growth factors to promote cutaneous wound healing has existed since the 1940s, and they can be applied in a wide range of ways, either by traditional topical or intralesional administration or by using specific scaffolds or even gene therapy.[35]

Animal and human trials have reported successful PRP clinical applications for chronic skin ulcers,[36,37] acute cutaneous wounds,[38-40] burns and plastic and cosmetic surgery.[41,42]

Recently, stem cells, especially adipose-derived stem cells (ADSCs) that have multidirectional differentiation potential and an ability to secrete various growth factors and inflammation factors,[43,44] have become important tools in transplantation therapy to promote the healing of refractory wounds.[45] Several research teams have shown that ADSCs can effectively promote the healing of refractory wounds.[46,47] However, isolation of ADSCs requires in vitro cell culture and expansion and can take from days to weeks. These steps increase the possibility of infection by microorganisms, such as bacteria, and thus, can be difficult for the clinical application of ADSC-based cytotherapy.[48] At the same time, studies have found that non-expanded stromal vascular fraction (SVF) derived from digestion, filtration, and centrifugation of mature fat cells contains a variety of fat cells and mesenchymal stem cells (MSCs) that opens up new directions for the treatment of difficult wounds.[49] Although SVFs help treat

many diseases, it is currently not commonly used in the clinic.[50]

The stromal vascular fraction (SVF) obtained by collagenase digestion and centrifugal separation contains a variety of biologically active cells.[51] A subgroup of SVF cells has many characteristics similar to mesenchymal stem cells from bone marrow sources and are named "adipose-derived stem cells (ADSCs)".[52] They are an ideal source of stem cells.[51] SVFs collected according to a normal operation method can promote the survival of transplanted fat and treat the refractory wound. In animal tests, SVF promoted angiogenesis, increased vascular density, and improved blood supply to the ischemic myocardium. It is well known that the purity and number of stem cells obtained by the direct separation method are relatively low, and it takes a long time to purify and cultivate them in vitro. These cells may become infected by microorganisms such as bacteria during this process. These factors weaken the potential of ADSC-based cell therapy. In contrast to ADSCs, SVF can be isolated in real-time and contains sufficient cells to eliminate the need for in vitro amplification. Therefore, a combination of PRP isolated from the patient's blood and SVF obtained from a minor surgical procedure to obtain adipose tissue could be an ideal locally applied treatment for difficult to heal diabetic wounds.[53]

Our study aims to determine an autologous treatment approach for healing diabetic ulcers, which could be developed as a treatment modality for diabetic ulcers. Keeping in mind that both PRP and SVFs have demonstrated their ability to heal diabetic wounds, there are very few reports on the combined use of PRP and SVF to treat diabetic wounds.

Materials and Methods

Ethics Statement and Animals

The Wockhardt animal test facility is registered with an Indian statutory authority, a Committee for Control and Supervision of Experiments on Animals (CPCSEA). The wockhardt scientific team is committed to enhancing animal welfare and ensuring that the studies are conducted to cause minimal suffering or distress to the animals consistent with the scientific objectives. All procedures complied with the CPCSEA guideline. Before the study started, this study protocol was discussed, explained and approved by Wockhardt Institutional Animal Ethics Committee (IAEC).

The IAEC approval number for this study is Project Proposal No.: Form B / 011/ 2013-14.

New Zealand white rabbits were selected for this study because rabbit ear has been widely used as an ischemic wound model.[54] A 6-mm punch biopsy down through the cartilage will create a full-thickness wound that lacks a vascular base and has a very limited lateral vascular supply. Because the dermis of the rabbit ear is firmly attached to the cartilage, the avascular wound bed cannot close by contraction and, instead, heals via epithelization and granulation tissue formation.

For this study 20 New Zealand white rabbits were selected. During the experiment, the animals were housed in an environment-controlled room with temperature, and relative humidity maintained at 24 ± 2°C and 30 to 70%, respectively. Photoperiod was controlled to give 12 hours light and 12 hours dark cycles in the experimental room. Each rabbit was housed individually in standard, stainless steel cages providing pelleted feed and drinking water in a polycarbonate bottle with a stainless steel sipper nozzle. All animals had free access to RO-treated water and a standard pelleted laboratory animal diet (Nav Maharashtra Chakan Oil Mills, Pune).

Induction and Maintenance of Diabetes

All rabbits were administered with Alloxan (Sigma Aldrich, USA] at the dose of 125 mg/kg by slow intravenous injection via ear vein. Animals have fasted overnight before dosing. For each animal, the dose solution was prepared freshly by dissolving the required quantity of Alloxan in normal saline to achieve a concentration of 50 mg/mL, and it was injected immediately within 3-5 minutes. After Alloxan injection, 1% dextrose was provided in a drinking water bottle for first 48 hours to avoid hypoglycemia. Blood glucose levels (BGL) were monitored and recorded twice daily in the first two weeks using blood glucose testing strips (Accu-check, Roche Diagnostics). After two weeks, BGL were stable and were monitored once daily throughout the experimentation. Animals were maintained with BGL between 200–400 mg/dL. Hypoglycemic animals were treated with 5% DNS intravenously as needed, whereas hyperglycemic (>400mg/dl) animals were treated with insulin (Wosulin) subcutaneously to maintain the BGL between 200–400 mg/dL. The insulin dose was adjusted to decrease 100 mg/dL per 1-IU of Wosulin. Normal rabbits were allocated in the control group, and their blood glucose levels were monitored simultaneously with alloxan-induced animals, to ensure induction of diabetes.

Fat Collection and Ulcerative Ear Wound Creation

At the time of fat collection and ear wound creation, the animals were anesthetized by intramuscular injection of Xylazin (5 mg/kg) and Ketamine combination (35 mg/kg). With the help of biopsy punch, three parallel excision wounds were made of approximately 6 mm diameter on the inner side of right ear pinna at the epidermis and dermis level. Small incision (approximately 3 inches) was made on dorsal thoracic area and about 3–6-gram fat was excised by blunt dissection. After weighing, the fat was transferred into a petri dish containing phosphate buffer saline (PBS). The incision was closed with silk sutures.

Isolation of Stromal Vascular Fraction (SVF)

Approximate 3–6 g of excised fat was transferred in a petri dish and teased using forceps and incision blade, and 20 mg of collagenase (MP biomedical USA, Cat No. 195109, lot No. 9690K) dissolved in 2 ml of 1X PBS was added. The fat was allowed to incubate at 37°C for digestion for a period of 1hour with intermittent mixing. After digestion, this solution containing digested fat was centrifuged at 300g for 10 minutes. The cell pellets were aspirated using a 20 ml syringe with 18G needle from the bottom of the tube. Cell pellets from individual animals were pooled and washed with 1X PBS by centrifugation at 300g for 10 minutes thrice. This pooled SVF pellet was divided in 3 parts. One part was used for application on the day of ear wound creation (Day 0), while other 2 parts were preserved at -70°C for application on Day 2 and Day 6. The isolated SVF was plated on Potato Dextrose Agar plates and Tryptic Soya Agar plates and incubated at 37°C for 72 hours., plates were assessed for microbial/fungal growth every 24 hours.

Preparation of Platelet Rich Plasma and Activation of Platelets

Approximate 7 ml of blood was collected in a centrifuge tube containing anticoagulant (Acid citrate dextrose Solution A) and centrifuged at 1550 rpm for 15 minutes. The straw color plasma was separated in a fresh tube and centrifuged at 3400 rpm for 5 minutes. The supernatant was discarded, leaving only 0.9 plasma ml. The platelets pellet was mixed with 0.9 mL plasma, and 350 µL of calcium chloride was added and kept at 37°C till a clot was observed. This PRP was divided in 3 parts, each containing 300 µL. One part was used for application on the day of wound creation (Day 0) while the other 2 parts were preserved at -20°C for application at Day 2 and Day 6.

Study Design

Two groups of rabbits were created. The first group was intravenously administered alloxan as described above. In this group, two weeks after the induction of diabetes, the BGL was stable. These diabetic rabbits were randomly allocated to different groups (Table 1). Another group of rabbits was created as a non-diabetic group. The diabetic group was further divided into 2 groups; Group 1 and 2. Group 1 was treated with SVF+PRP, and group 2 was treated with PI. The non-diabetic group of animals was also divided into 2 groups; Group 3 and 4. Group 3 animals received topical PI application and Group 4 animals received topical SVF+PRP. Fig. 1 is a schematic of the overall study plan.

Application of SVF + PRP on the Wound Surface

The SVF+PRP was applied on the collagen sheet (Kollagen–D™, Manufactured, Eucare Pharmaceuticals (P) Ltd, Chennai, Batch No. KGD 9E108) was cut according to the wound size and kept at 37ºC for proper absorption. Post absorption, collagen sheet was applied on the ulcerative wound on the ear of rabbits in groups G1 and G4, after cleaning the wounds with povidone iodine (PI). This collagen sheet was held in place with surgical elastic tape for 24 hours and then removed. This dressing and application were done on days 0, 2 and 6. The wound was cleaned daily with normal saline in rabbits from all the groups followed by applying povidone iodine.

Wound Healing Assessment

Ear wounds were assessed for healing status parameters such as color, presence of scab and granulation tissue on days 0, 2, 6, 8, 10, 13. Photographs were taken on same day.

Necropsy and Tissue Collection

On Day 13, all animals were subjected to necropsy and detailed gross pathology evaluation.

Animals were fasted overnight before necropsy.

Animals were euthanized by an overdose of thiopentone sodium by intravenous injection. The cranial, thoracic, and visceral cavities were opened and examined macroscopically. Tissues from ear wound, liver, kidney, spleen, lung, heart, pancreas and brain were collected and preserved in 10% neutral buffered formalin.

Histopathological Assessment

Collected tissues were processed, embedded in paraffin wax, sectioned at 5 µ thickness and stained with hematoxylin and eosin method. Sections from ear wound tissue were additionally subjected to Masson's trichrome staining to

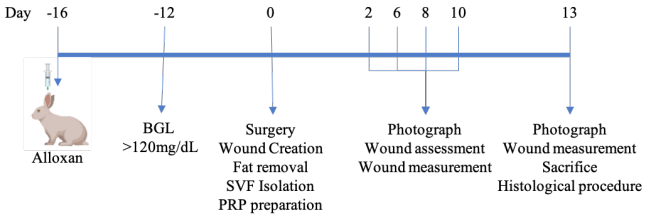

Fig. 1: Schematic representation of time points of the study design.

Table 1: Study design and animal groups

Dose group	*Type of animal / Treatment*	*(Animal Identification Number)/ Number of animals*
G1	Diabetic/Treated with SVF+PRP	(Animal No 1, 2, 4, 5, 6, 7, 8,15)/8
G2	Diabetic/10% PI application	(Animal No 9-12)/4
G3	Non-Diabetic/10% PI application	(Animal No 13)/1
G4	Non-Diabetic/ Treated with SVF+PRP	(Animal No. 14)/1
Total number of animals		14

further understand the healing status at the cellular level. The collected tissues were screened for histopathological alterations. The histopathological grading of wound lesion was done as per method described.[55]

Results

Induction of Diabetes

The blood glucose levels were estimated from administering Alloxan monohydrate from Day -16 (induction of diabetes) to Day 12. Glucose (mg/dL) was significantly increased in the animals treated with alloxan. On successfully determining the glucose level to be above normal (~90 mg/dL) animals were considered for the study. The induction of diabetes with alloxan demonstrated animals with high glucose levels in their blood (Fig. 2). Animals with glucose levels consistently above 120 mg/dL were considered for the study. Blood glucose analysis (Fig. 3) demonstrates that diabetes was successfully induced and high blood glucose levels were maintained in Group 1 and 2. There was slight reduction in body weight in all diabetic animals. Few animals showed slight bodyweight reduction, but this weight loss was not consistently observed in all animals within the same treatment group, therefore it was not considered as an effect of the test item (Fig. 4).

Fat Collection Weight and SVF Count Isolated

The SVF obtained was divided into 3 aliquots for the three dosing schedules on day 0, 2 and 6. A0092K13/14 and A0092K13/15 were not operated for fat collection. Fig. 5 shows the SVF cell count from fat (grams) from an individual animal.

The cells used to treat these animals were obtained from the G2 animal. Table 2 gives the number of cells used at every dosing schedule.

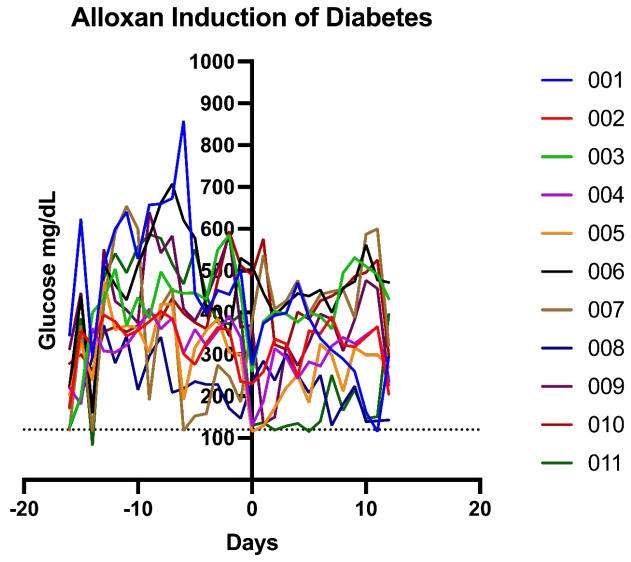

Fig. 2: Induction of diabetes with alloxan. alloxan was administered at Day -16, blood glucose levels were monitored every day.

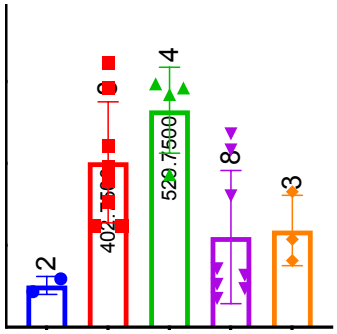

Fig. 3: Blood glucose levels of animals from various groups at day 0 and day 13 (day of necropsy).

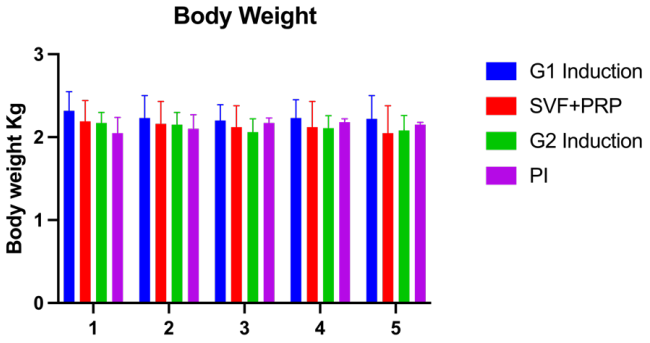

Fig. 4: Body weights of animals during induction phase and treatment phase.

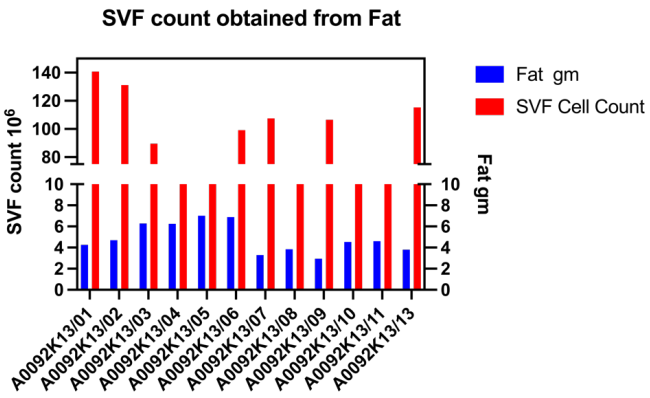

Fig. 5: Count of SVF cells obtained from fat (grams) of individual animals.

Table 2: Number of cells used at every dosing schedule

	Cell Count (10^6 cells)		
Animal No.	*1st Dose*	*2nd Dose*	*3rd Dose*
A0092K13/01	43.6	37.2	35.8
A0092K13/02	40.3	36.8	34.2
A0092K13/03	26.2	22.8	21.3
A0092K13/04	21.8	18.6	17.6
A0092K13/05	20.3	17.1	16.1
A0092K13/06	30.4	25.8	24.1
A0092K13/07	31.2	26.8	24.9
A0092K13/08	19.1	16.8	15.62

SVF + PRP Treatment Enhanced Wound Healing

The overall results indicated that the wound size was reduced in the SVF+PRP treated animals compared to the 10% PI. This result indicated that SVF +PRP treatment via topical application could accelerate diabetic wound healing (Fig. 6).

Ear wound was assessed for healing status parameters such as color, presence of scab and granulation tissue on days 0, 2, 6, 8, 10, 13. Photographs were taken on each day (Fig. 7). Based on healing assessment of the ear, the diabetic animals treated with SVF+PRP exhibited early development of granulation tissue and early signs of wound closure as compared to diabetic animals with routine dressing.

The wound size was markedly reduced in the SVF+PRP treated groups compared to the 10% PI treatment groups. Treatment groups 1 and 4 (SVF+PRP) showed the fastest wound closure rate assessed over 13 days. G1 (Group 1 – diabetic rabbits treated with SVF+PRP), G2 (Group 2 – diabetic rabbits treated with 10%PI), G3 (Group 3 – Non-Diabetic animal treated with 10%PI), G4 (Group 4 – Non diabetic animal treated with SVF+PRP).

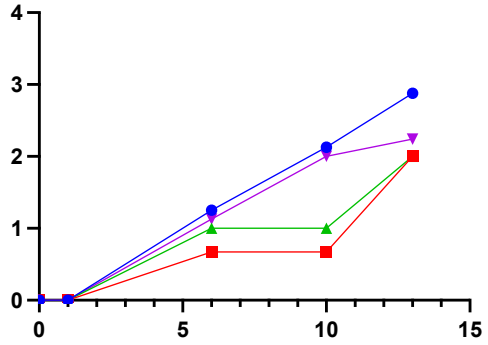

Fig. 6: Wound margin closure in different groups after SVF + PRP application on wound

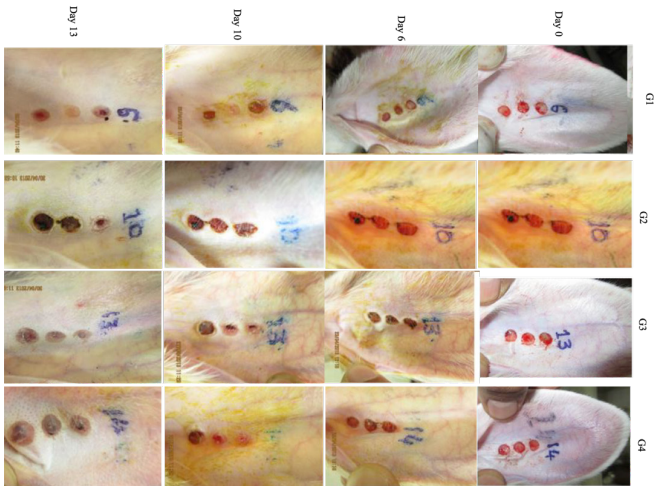

Fig. 7: Representative gross appearance of wound healing for each treatment on day 0, 6, 10, and 13 post-wound.

Histopathological Evaluation

The intensity and presence of epithelization and fibrotic changes at ulcerative wound sites were much more in diabetic animals treated with SVF and PRP than that of PI treated animals (Fig. 7). The epithelial changes such as pyknotic epithelium, acanthosis and parakeratosis were more intense in diabetic SVF+PRP treated animals than PI treated animals. On the contrary, PI treated diabetic animals showed more intense inflammatory changes and neovascularization at the wound site than SVF+PRP treated animals. This variation clearly indicates that the healing process of PI-treated animals is slower than that of SVF and PRP-treated animals, thus exhibiting the beneficiary effect of the therapy.

Other visceral organs viz, liver, spleen, kidney, heart, brain, and lungs did not show any significant findings except a few spontaneous lesions unrelated to the treatment.

Atrophy of pancreatic islets of Langerhans was noted in diabetic animals, which was an expected effect of alloxan.

Photomicrograph of a skin section from "Normal" ear pinna (Fig. 8-a). Photomicrograph of a skin section from the non-diabetic PI treated group showing unhealed ulcer (Fig. 8-b). Photomicrograph of a skin section from Diabetic SVF+PRP treated group showing healed ulcer. Marked fibrosis, complete epithelialization, less vascularization, thickened epithelial layer, and advanced healing stage (Fig. 8-c). Photomicrograph of a skin section from Diabetic PI treated group showing unhealed ulcer. Incomplete closure of epithelial layer, marked inflammatory infiltration, more vascularization indicates early stages than SVF+PRP treated animals (Fig. 8-d).

Assessment of Ear Wound Healing

The assessment of ear wound healing was carried out on day 0, 2, 6, 8, 10, 13 after SVF and PRP application. On Day 13, blood was withdrawn to analyze biochemical and hematological parameters and all 13 animals were subjected to detailed gross pathology evaluation. There was slight reduction in body weight in all diabetic animals compared to their initial weight.

There was no significant difference in the hematology and clinical chemistry data of SVF+PRP treated and PI treated diabetic animals except mild to moderate increase in triglyceride and cholesterol was observed in diabetic rabbits apart from the significant elevation in blood glucose. On the basis of ear wound healing assessment, the diabetic animals treated with SVF and PRP exhibited indication of healing, including early development of granulation tissue and early signs of wound closure compared to diabetic animals with routine wound dressing animals.

The intensity and magnitude of epithelization and fibrotic changes at ulcerative wound sites were much more evident in diabetic animals treated with SVF and PRP as compared to that of PI treated animals. The epithelial

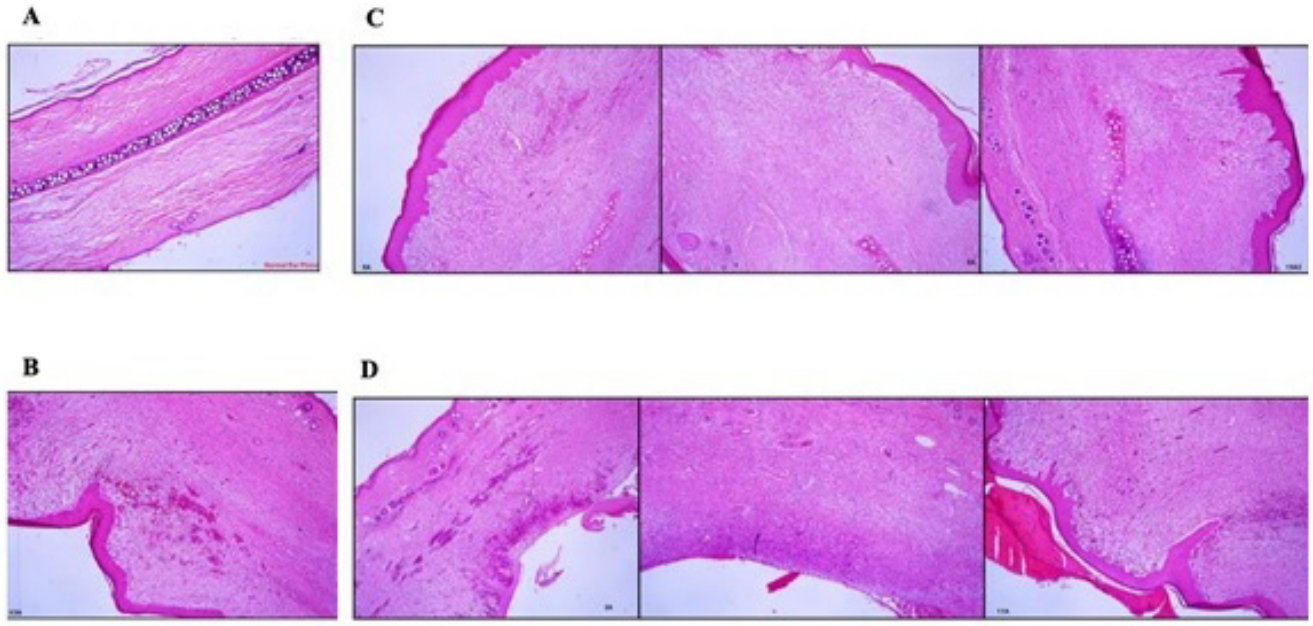

Fig. 8: a: photomicrograph of a skin section from "normal" ear pinna.
b: photomicrograph of a skin section from non-diabetic non-treated group showing unhealed ulcer.
c: photomicrograph of a skin section from the diabetic treated group showing healed ulcer. Marked fibrosis, complete epithelialization, less vascularization, thickened epithelial layer and advanced healing stage.
d: photomicrograph of a skin section from diabetic PI treated group showing unhealed ulcer. Incomplete closure of epithelial layer, marked inflammatory infiltration, more vascularization indicates early stages as compared to treated animals.

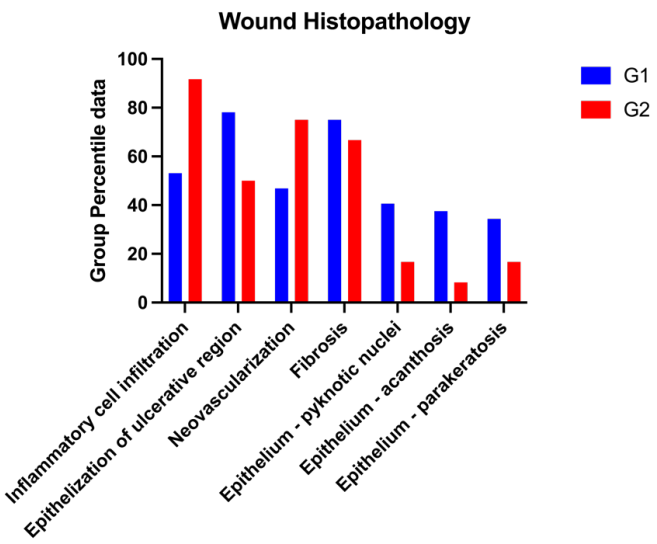

Fig. 9: Wound histopathology group percentile summary of diabetic SVF+PRP treated compared to PI treated diabetic animals.

changes such as pyknotic epithelium, acanthosis and parakeratosis were more intense in diabetic treated animals than PI treated animals. PI treated diabetic animals showed more intense inflammatory changes and neovascularization at the wound site than that of treated animals (Fig. 9).

The histopathological evaluation of the ear wound tissue indicates that the healing process of PI-treated animals is slower than that of SVF and PRP-treated animals, thus exhibiting the beneficiary effect of SVF and PRP treatment in diabetic rabbits.

Based on ear wound healing assessment, the diabetic animals treated with SVF and PRP exhibited indication of healing, including early development of granulation tissue and early signs of wound closure compared to diabetic animals with routine wound dressing animals.

The histopathological evaluation of the ear tissue indicates that the healing process of PI-treated animals is slower than that of SVF and PRP-treated animals, thus exhibiting the beneficiary effect of SVF and PRP treatment in diabetic rabbits.

Discussion

Adipose stem cells are extensively used in wound healing, and adipose has gained much attention as a source of stem cells because of their ease to isolation. However, to obtain clinical grade and large amounts the isolation is time-consuming and labor extensive. In this study, we evaluated the potential of non-expanded adipose stem cells i.e., SVF, as an alternative to expanded and cultured adipose stem cells. Our understanding that growth factors play an important role in wound healing, combined with the knowledge that SVF has approximately 10% of multipotent stem cells, we combined 2 autologous treatments reported and used independently in treating diabetic wounds.

Wound healing involves several overlapping processes, with the first stage of healing being the inflammatory phase in which edema occurs. Prolonged edema should

be prevented as this can cause tissue ischemia and infection, ultimately delaying the healing process. We noted that the PI-treated animals showed more intense inflammatory changes indicating a slower healing process than the SVF+PRP treated animals on histopathological evaluation. An anti-inflammatory effect of the SVF+PRP has been observed, as evidenced by a lower inflammatory cell infiltration in the SVF+PRP treated group than the PI group. A common marker to assess inflammatory response is nitric oxide (NO). NO stimulation increases vascular permeability. In an in vitro osteoarthritis model, a PRP treatment group produced less NO than the PI treated group.[56] Another study used TNF-alpha as an inflammatory marker and IL-10 as an anti-inflammatory marker, TNF-alpha levels were significantly lower and IL-10 levels significantly higher in the PRP group compared to the control.[57] These results support our findings that the combination of SVF+PRP has an anti-inflammatory effect.

The intensity and magnitude of epithelization and fibrotic changes at ulcerative wound sites were much more evident in diabetic animals treated with SVF and PRP than that of PI-treated animals. The epithelial changes such as pyknotic epithelium, acanthosis and parakeratosis were more intense in diabetic treated animals than PI treated animals. PI-treated diabetic animals showed more intense inflammatory changes and neovascularization at the wound site than treated animals. This variation indicates that the healing process of PI-treated animals is slower than that of SVF + PRP treated animals, thus exhibiting the beneficiary effect of SVF+PRP.

The histopathological evaluation of the ear wound tissue indicates that the healing process of PI treated animals is slower than that of SVF and PRP-treated animals, thus exhibiting the beneficiary effect of SVF and PRP treatment in diabetic rabbits.

Based on ear wound healing assessment, the diabetic animals treated with SVF and PRP exhibited indications of healing, including early development of granulation tissue and early signs of wound closure compared to diabetic animals with routine wound dressing animals. Inflammation regulation, the proliferation of cells, remodeling, deposition of extracellular matrix, angiogenesis and epithelization play important roles in skin wound healing. All these events were noted in the macroscopic and microscopic evaluation of the wounds. Based on wound healing assessment and histopathological examination, the diabetic rabbits treated with stromal vascular fraction and platelet-rich plasma application exhibited early development of granulation tissue and early signs of wound closure as compared to diabetic animals with normal dressing. Based on this study, further research is suggested in understanding the roles of major growth factors in wound healing such as vascular endothelial growth factor. Understanding the synergistic effects of SVF and PRP on wound healing combined with a dose-dependent study would help establish a platform for developing a treatment for diabetic ulcers in humans. The fact that there are limited studies on the combination of SVF and PRP, highlights the need for a well-structured clinical trial. We hope this research would serve as a fundamental platform that demonstrated that combining the therapeutic advantages of both autologous treatments: SVF and PRP enhanced wound healing in diabetic wounds.

In conclusion, we have demonstrated that topical application of SVF + PRP enhanced wound closure compared to PI, which is used almost universally as a standard of care dressing. We also demonstrated histological differences, allowing us to infer that SVF+PRP has a definitive advantage in wound management.

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