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Original article

Invalid freeze-dried platelet gel promotes wound healing

Aporn Chuncharunee [a], Saranatra Waikakul [b], Adisak Wongkajornsilp [c], Viroje Chongkolwatana [d], Lancharat Chuncharunee [e], Aunchalee Sirimontaporn [a], Thanaporn Rungruang [a], Gopinathan Pillai Sreekanth [f,*]

[a] Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[b] Department of Orthopedic Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[c] Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[d] Department of Transfusion Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[e] Department of Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
[f] Siriraj Center of Research Excellence for Molecular Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

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ABSTRACT

Wound healing is the curative process of tissue injury, composed of three phases: the inflammatory phase, proliferative phase, followed by the maturation cum remodeling phase. Various treatment options were previously depicted for wound healing, however a treatment that accelerates these phases would be highly valuable. Platelet aggregation at the bleeding vessels and release of various growth factors are the most promising factors that stimulates the wound healing progress. In the present study, we hypothesized that the freeze-dried platelet which were normally discarded from the blood banks due to invalidity, might be promising to accelerate the phases of wound healing. The invalid freeze-dried platelets were prepared to a gel form called invalid freeze-dried platelet gel (IF-PG), which was tested for its efficacy in a cutaneous punch wound model in rats. Mupirocin antibiotic gel was used as a bio-equivalent formulation. The wound healing phases and changes in the wound sites were determined by assessing the wound sizes, histopathological analysis, immunohistochemical staining. The re-epithelialization at the wound sites at different time intervals till the wound closure was also determined. Our results suggest the beneficial effects of IF-PG; in reducing the wound area and accelerating wound closure in the cutaneous punch wound in rats. Histopathology and immunostaining results support the improvements in the wound when treated with IF-PG, which were similar to that of mupirocin antibiotic gel. Our preliminary findings also warrant the competency of IF-PG in modulating the different phases of wound healing process. In conclusion, IF-PG might be a resourceful alternative for the wound care management, however further studies are required to validate its impact on various growth factors before proceeding to clinical studies.

1. Introduction

Wound healing is a normal curative reaction to tissue injury by the influence of a complex cascade of cellular events that spawns resurfacing and reconstitution, which further restores the tensile strength of injured skin area. The wound healing process is composed of three sequential overlapping phases: inflammatory, proliferative, and followed by maturation and remodelling phase (Collier, 2002; Midwood et al., 2004; Nguyen et al., 2009). The initial inflammatory phase proceeds immediately after the blood vessel injury, then to a cascade of vasoconstriction and coagulation to develop blood clot via activating platelet degranulation. After homeostasis, blood vessels subsequently dilate, which allows the inflammatory cells to surround the wounded area. The macrophages phagocytize bacteria and damaged tissue to secrete growth factors and cytokines, that stimulates fibroblasts and keratinocytes to initiate the proliferation phase. This phase comprising of re-epithelialization, angiogenesis, and granulation. At the final phase,

* Corresponding author at: Siriraj Center of Research Excellence for Molecular Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Wanglang Road, Bangkok Noi, Bangkok 10700, Thailand.
E-mail address: sreekanth.gop@mahidol.ac.th (G.P. Sreekanth).

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maturation and remodelling, where collagen forms tight cross-links and further to a networking which increases the tensile strength and develop scar (Broughton et al., 2006; Gilmore, 1991).

Invalid freeze-dried platelets (IFP) were generally stored in the Blood Bank for 3–5 days and discarded due to their invalidation. Survival of living fibroblasts was observed after incubation with IFP (Ruangchainikom et al., 2009), which suggests IFP might promote wound healing. IFP set to a gel formula is termed as invalid freeze-dried platelet gel (IF-PG) which has a long shelf life. Autologous PGs were used in clinical medicine, as it shows the possibility to accelerate wound healing (Diegelmann and Evans, 2004; Jurk and Kehrel, 2005). Application of PGs were shown to improve wound healing and reduced hospitalization time of patients (Piccin et al., 2016). The efficacy of IF-PG on a naturally curing wound was not well-studied, which aids reduced hospitalization time to the patients. Therefore, we investigated the efficacy of IF-PG and how treatments with IF-PG influences the different phases of wound healing in an experimental punch wound model in rats.

2. Materials and methods

2.1. Animals

Female Wistar rats (6 weeks old, 180–200 gm.) were obtained from the National Laboratory Animal Centre, Mahidol University (Salaya campus) and in-housed at Faculty of Medicine Siriraj Hospital, Mahidol University (Bangkok Noi campus) under standard sterile conditions. The animals were maintained in stainless steel wire hanging cages and were allowed to acclimatize for 1 week before included to the experimentations. The animal room was maintained at a temperature of 25 °C ± 2 °C, relative humidity of 65% and 12-hour light/dark cycles. All animals were free access to food (C.P Mice Feed, S.W.T Co., Samutprakhan, Thailand) and sterile water *ad libitum*. The experimental protocol was approved by Siriraj Animal Care and Use Committee, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (No. SI-ACUP 007/2552).

2.2. Preparation of IF-PG

Invalid anticoagulated blood was collected from the Blood Bank due to its expiry and was placed into centrifuge tubes. These centrifuge tubes were spun at 1200*g* for 15 min to separate plasma from the erythrocytes. The plasmatic fraction was further centrifuged at 1800*g* for 10 min to separate platelet-rich plasma (PRP) and platelet-poor plasma (PPP). The PRP was freeze-dried at −70 °C, sterilized with gamma ray of cobalt source 25 kGy and again stored at −70 °C. The prepared invalid freeze-dried platelets (IFP) was thawed and mixed with a gel base to form IF-PG. The concentration of IF-PG was maintained to 30 mg of platelets per gram of gel base. The formulation of gel base was composed of 5% sodium carboxy-methyl-cellulose solution, 14% glycerin as emollient and moisturizer, 0.1% w/w methyl-paraben and 0.02% propyl-paraben for preservation. The quality control was performed at the Department of Medical Sciences and the sterility test was conducted at the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

2.3. Wound punch and treatment

The rats were anesthetized with pentobarbital sodium (50 μg/g BW, i.p.), and then the dorsal aspect of upper part of the body was shaved and disinfected with 70% alcohol. A 6 mm skin biopsy punch was induced to create full-thickness wounds under aseptic conditions. The wound sites were labeled and treatments were applied topically once at the wound site with either a gel-base or Mupirocin 2% w/w or IF-PG. An un-treated group of rats were also maintained. The wounds were covered with Urgodrem No.1681 (hypoallergenic dressing retention sheet) and Neotape was used to prevent Urgoderm slipping and furthermore, sutured tightly to skin with silk, U.S.P 2/0. The experimental rats were sacrificed with an overdose pentobarbital sodium on 3, 7 and 12 days post-wound surgery. The wounds were visualized and photographed.

2.4. Quantification of wound area

To determine the wound healing, wound site was photographed after the punch (day 0) and at different time intervals (3/7/12 days post-wound) by a digital camera which was placed on a fixed position to eliminate technical error. Changes in the wound area was measured by Motic Image plus program of stereoscope and expressed as the percentage of wound healing using the formula:

$$\%\text{wound healing} = \frac{\text{wound area day 0} - \text{wound area days (3/7/12)} \times 100}{\text{Wound area day 0}}$$

2.5. Histopathological preparation and analysis:

The wound tissues were harvested on day 3, 7 and 12 post-wound care, fixed in 4% paraformaldehyde, dehydrated, embedded and sectioned. Hematoxylin-Eosin and Masson's trichrome aniline blue staining were performed to evaluate histological changes and collagen deposition, respectively. Histopathological changes of wound were evaluated under light microscopy equipped with a digital camera.

2.6. BrdU staining

Rats were administered with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU) 1 h before the sacrifice. The wound tissues were harvested and fixed in Bouin's buffer, embedded, and sectioned. The sections were undergone standard immunohistochemical procedures and peroxidase activity was visualized using a 3, 3′-diaminobenzidine tetrahydrochloride (DAB) reaction. The nuclei were counter-stained with hematoxylin and BrdU-positive cells showed brown nuclei.

2.7. Analysis of wound re-epithelialization

The analysis of re-epithelialization was performed according to the procedure of Lai et al. (2009). Each H&E stained slide was placed on the stage of a microscope, and the image was sent to computer-assisted morphometric analysis software. The area of the regenerated epithelium of each wound was calculated from dermal edge and epithelial edge. The degree of re-epithelialization (epithelium closure) was calculated.

2.8. Statistical analysis

The data were expressed as means ± standard error of mean, and analyzed using one-way ANOVA followed by Bonferroni's test. The $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Invalid freeze-dried platelet gel (IF-PG) treatment improved the wound healing in rats

Experimental rats were closely monitored during the treatment period for any noticeable changes in the gross appearance of the

wounds. The rats remained healthy, without any clinical evidence of infection. The wounds were observed and macroscopically recorded as photographs after surgery (day 0) and on days 3, 7 and 12 post-wound (Fig. 1A). The wound sizes of rats treated with IF-PG and Mupirocin on days 3 and 7 were rapidly reduced compared to that of control groups. However, the surface area of IF-PG-treated wounds on Day 7 was found to be more clear compared to that of Mupirocin-treated rats (Fig. 1A). Upon Day 7 post-wound, no signs of swelling, redness, exudates or inflammation were seen in rats treated with IF-PG compared to that of control groups. The wounds of all groups were found to be completely closed on day 12, and their surfaces were covered with complete epidermis with keratin (Fig. 1A).

Changes in wound area was quantified as the percentage of wound healing on different days of post-wound care. On day 3, the improvements of wound area were found to be 52.17 ± 1.79% in the IF-PG-treated, and 50.06 ± 4.15%, 42.08 ± 2.79%, 41.61 ± 3.10% in Mupirocin-treated, gel base-treated and untreated sites respectively (Fig. 1B). Subsequently, all the wound areas were found to be lessened on day 7 which was 88.08 ± 1.48% in the IF-PG-treated and 84.63 ± 2.63, 79.45 ± 1.63, 78.89 ± 2.40 in Mupirocin, gel base-treated and untreated groups respectively (Fig. 1B) suggesting the efficiency of IF-PG in the wound area management. The percentage wound areas on day 12 were 92.54 ± 1.54% for the IF-PG-treated, and 91.33 ± 1.98%, 90.81 + 0.92%, 91.45 ± 1.02% for Mupirocin-treated, gel base-treated and untreated sites respectively. However, we did not observe any statistical significance between the groups on day 12 (Fig. 1B).

3.2. IF-PG treatment improved the histopathology of the wound

Histopathology of the wound was studied to correlate the macroscopic changes and described individually for day 3, 7 and 12 post-wound (Fig. 2A and 2B).

On day 3 (Fig. 2**A and 2B)**, the microscopic findings of all wounds shown retirement of inflammatory phase overlapping with the beginning of proliferative phase and were characterized by deposition of hemorrhagic fibrin clots (scabs) in the wound gaps. The scabs covered the wounds were in touch with provisional matrix and the wound margins were easily recognizable which markedly presented migratory tongue of the epidermal cells (epithelial cells) towards the wounds below the scabs. The forward part of the tongue (tongue tip) was comprised of a monolayer, progressively with a bi-layer and disorganized, a multi-layered epithelium towards the wound margins (at the tongue tail). Re-epithelialization had begun in all wounds, but the epidermal development of IF-PG-treated and Mupirocin-treated wounds were found to be more prominent. In the dermis, less inflammatory cells (i.e. macrophages, mast cells, and neutrophils) and more granulation tissues (i.e. fibroblasts, collagen) were observed in the IF-PG-treated and Mupirocin-treated wound tissues compared to that of controls. The collagen fibers from the edges of each wound were migrated into the central area; particularly, they were recognized significantly intense in the IF-PG and Mupirocin-treated wounds.

On day 7 (Fig. 2**A and 2B),** the wounds were in the proliferative phases overlapping with the beginning of maturation and remodeling. Untreated and gel base-treated wounds exhibited the areas of epithelial loss surrounded by a focally extensive layer of neutrophils with loose crust, fibrin on the top. In contrast, the IF-PG and Mupirocin-treated wounds presented improvements including complete re-epithelialization of the wounds with multiple layers of thick epidermis. The cells of the stratum basale was elongated perpendicular to the basement membrane, and the stratum spinosum and stratum granulosum became thick. The dermis of the IF-PG-treated group revealed the most intense granulation tissue among all groups. An increased number of collagen fibers from the edges of each wound were migrated into the central area which was best seen in both IF-PG-treated and Mupirocin-treated wounds. The collagen fibers were placed in a disorganized arrangement and were randomly distributed. The signs of angiogenesis were extant with a considerable number of new vessels in all the groups.

On day 12 (Fig. 2**A and 2B)**, the wounds were at the maturation and remodeling phase. The wound surfaces among the groups were covered with new epidermis (neoepithelium) comprised of stratum basale, stratum spinosum, stratum granulosum and stratum corneum with keratin. The new epidermis of IF-PG-treated and Mupirocin-treated wounds developed superior than those of control wounds; they were presented with thicker epidermal layers and additional columnar appearance with dark blue spot nuclei of epithelial cells in stratum basale compared to that of the control groups. In the dermis, the IF-PG-treated wounds consisted of larger numbers of well-developed dermal papillae, hair follicles, and new vessel formation (neovascularization) relative to control groups. Moreover, the density and intensity of the collagen bundles were better, whereas the number of fibroblasts reduced in the IF-PG-treated groups compared to that of control groups.

3.3. IF-PG accelerated the cell proliferation phase of wound healing

The proliferative phase of the wound healing was monitored on Day 3 post-wound by staining with bromodeoxyuridine (BrdU) into the experimental wounds. Both IF-PG-treated and Mupirocin-treated groups shows large numbers of proliferating cells (Fig. 3A, illustrated in brown nuclei) in the epidermis and dermis, whereas untreated or gel base-treated wounds characterized less BrdU-positive cells, suggesting delays in the wound closure. Keratinocytes, fibroblasts, and endothelial cells of blood vessels incorporated BrdU into their nuclei. The BrdU-labeled keratinocytes were integrated into the newly formed epithelium near the wound margin. The up-regulation of BrdU was prominent in keratinocytes at the stratum basale and suprabasal layer of stratum spinosum of developing epidermal tongue near the wound margin on day 3 and less extensive on day 7 post-wound. Our results suggest IF-PG treatment in the punch wound accelerated wound repair via proliferative activity (Fig. 3A). The BrdU positive cells were quantified for each group of rats and is shown in Fig. 3B.

3.4. IF-PG treatment prompted re-epithelialization of the wound

Morphometric analysis was conducted to estimate the re-epithelialization of wound healing. On day 3 and 7, IF-PG-treated wounds were characterized with an increased epithelial migration from the wound edge along with a decreased wound gap and width, suggesting its ability to promote wound closure. The wounds in all group exhibited enhanced migration of the epithelium, however a higher degree of the epithelial migration was observed in the IF-PG-treated wounds and Mupirocin-treated wounds compared to untreated or gel-treated wounds on day 3 and 7. Moreover, a significantly higher number of cells and thicker epidermis was observed in the IF-PG treated group, which indicates a better state of wound healing. The rate of re-epithelialization was better on the day 7 post-wound treated with Mupirocin or IF-PG (Table 1).

4. Discussion

Platelets are used for a wide range of wound healing application. Recently, the therapeutic potential of PGs in different aspects of regenerative medicine was reviewed (Piccin et al., 2017a). Cord blood platelet gel (CBPG) with higher growth factor content was prepared for topical application and were reported to improve

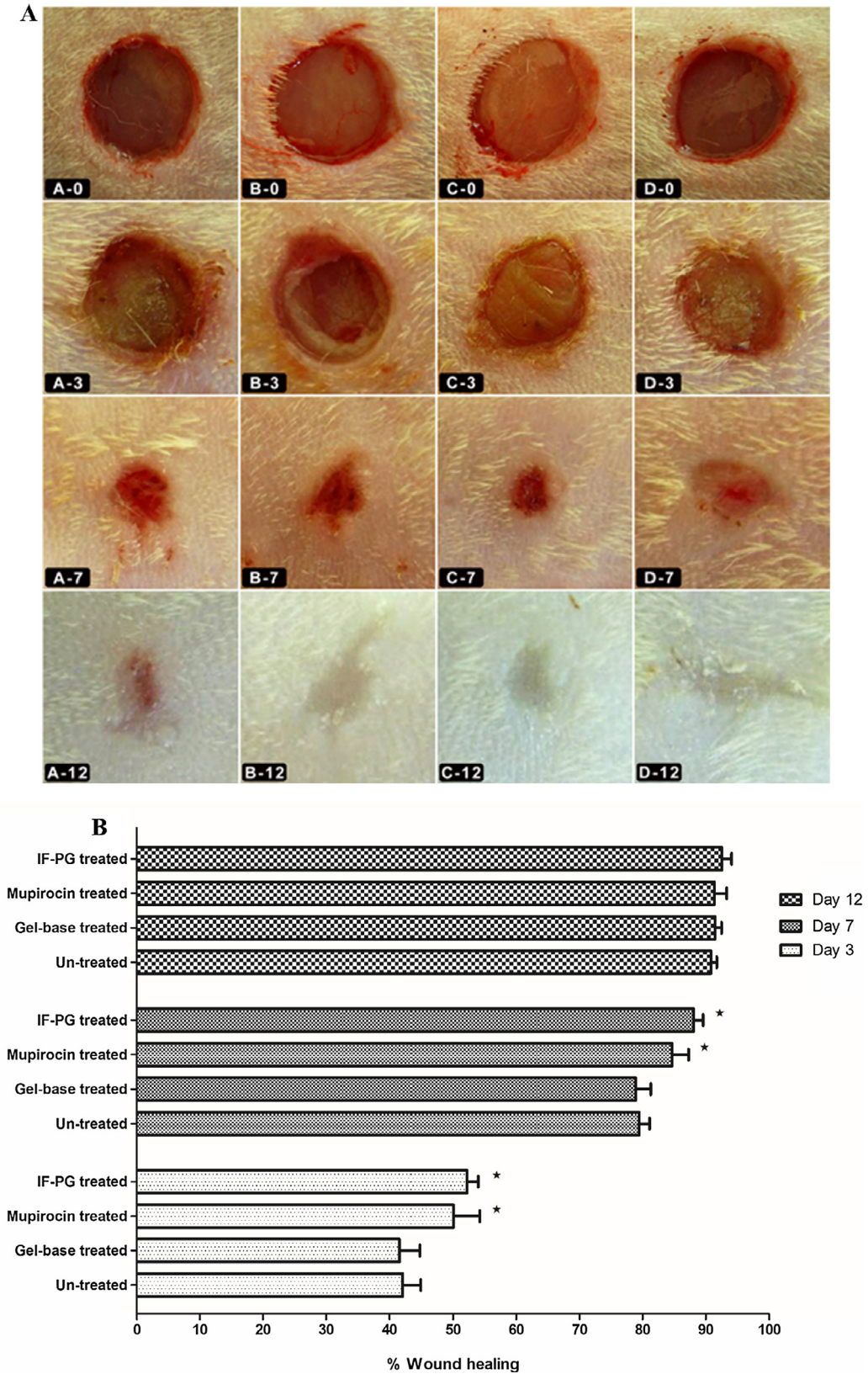

Fig. 1. A. Gross appearance of the wounds at different time intervals. Gross appearance of the wounds was photographed and represented (A) untreated control (B) treated with gel base (C) Mupirocin treated and (D) IF-PG treated at different time intervals including day 0, 3, 7 and 12 post wound. **B. Percentage of wound healing at different time intervals.** Wound healing percentage at different time intervals was represented (A) untreated control (B) treated with gel base (C) Mupirocin treated and (D) IF-PG treated at different time intervals including day 3, 7 and 12 post wound.

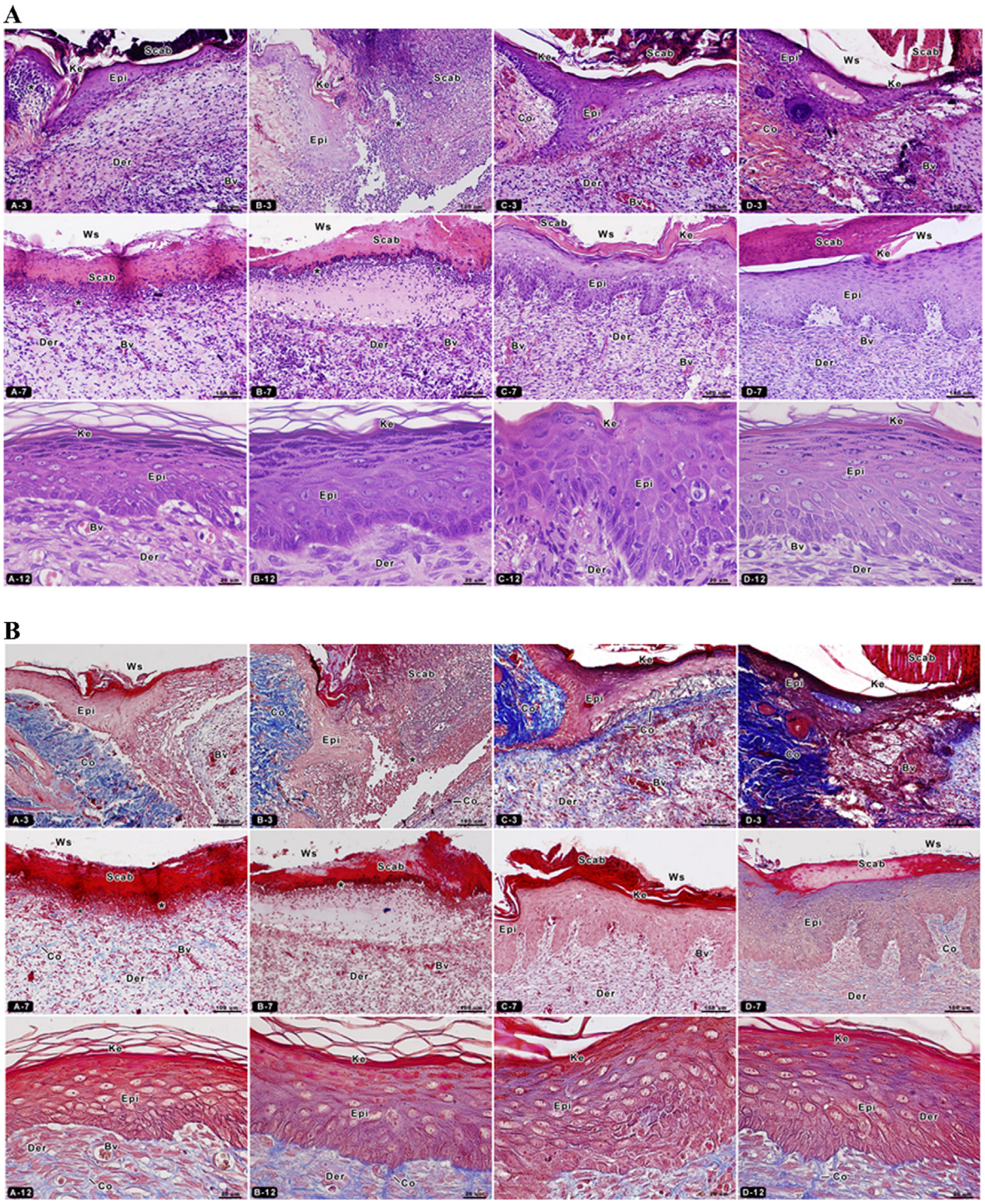

Fig. 2. A. Histopathological analysis of the skin wound tissues on day 3, 7 and 12 post wounds. The images were represented as (A) untreated control (B) treated with gel base (C) Mupirocin treated and (D) IF-PG treated **B. Hematoxylin and Eosin staining followed by Masson's Trichrome staining of the skin wound tissues on day 3, 7 and 12 post wounds.** The images were represented as (A) untreated control (B) treated with gel base (C) Mupirocin treated and (D) IF-PG treated. (Represented as arrows: wound margin, Bv: blood vessel, Co: collagen fibers, Der: dermis, Epi: epidermis, Ws: wound surface, *: inflammatory cells).

tissue regeneration and pain in a severe oral mucositis patient upon post stem cell transplantation (Piccin et al., 2017b). Platelet gel formulations were successful in the wound healing of surgical patients with neck and jaw osteoradionecrosis (Piccin et al., 2016; Scala et al., 2010). Platelet gel with chamber therapy was reported to heal the dermatitis associated with the radiation therapy in a malignant patient (Piccin et al., 2015). Autologous PGs are being used in cosmetic surgeries (Man et al., 2001). In a diabetic animal model exhibiting chronic wound, freeze-dried platelet-rich plasma fastened the healing (Pietramaggiori et al., 2006). A short literature summary on the wide applications of different platelet formulations is shown in the Table 2. In the current study, we prepared the IF-PG and tested in a punch wound model in rats. Mupirocin was used as a bio-equivalent formulation, which was previously used in several animal models of wound healing (Farahpour et al., 2017; Mahboubi et al., 2016; Nayak et al.,

2017; Theunissen et al., 2016). Controls were also maintained including punch wound untreated and treated with a gel-base. On day 12 post-wound, wound closure was observed and selected as the study endpoint. Sampling was conducted on different time points including day 3, 7 and 12 post-wound to differentiate different phases of wound when treated with IF-PG.

Our result shows IF-PG-treated wounds on days 3 and 7 show marked changes in the wound area and size compared to that of control groups; whereas, IF-PG-treated wounds did not show any significant change to that of Mupirocin-treated wounds suggesting as an alternative option to treat wounds. Interestingly, the wound healing velocities were distinctly noticed in days 3 and 7 of both IF-PG-treated and Mupirocin-treated wounds, which were consistent with the wounds of the lower equine limb treated with platelets isolated from the horse blood containing growth factor release (Carter et al., 2003). On day 12 post-wound, all the group of rats

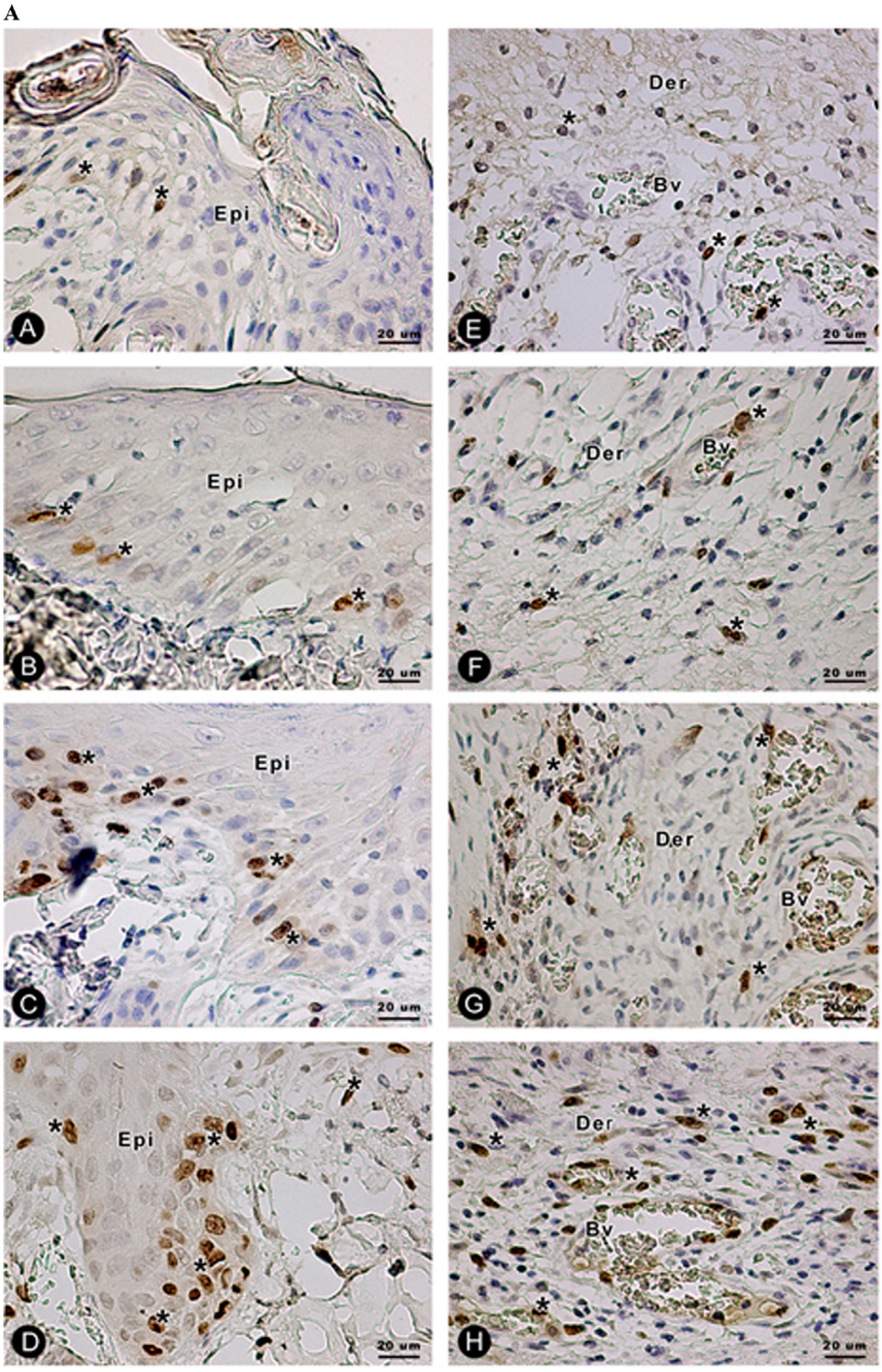

Fig. 3. A. BrdU expression in the epidermis (A-D) and dermis (E-H) of wounds. For the epidermal staining, (A) untreated control (B) treated with gel base (C) Mupirocin treated and (D) IF-PG treated. For the dermal staining (E) untreated control (F) treated with gel base (G) Mupirocin treated and (H) IF-PG treated. (Represented (Der: dermis, Epi: epidermis, Scale bar: 20 μm). **B. Quantification of BrdU stained positive cells between groups.**

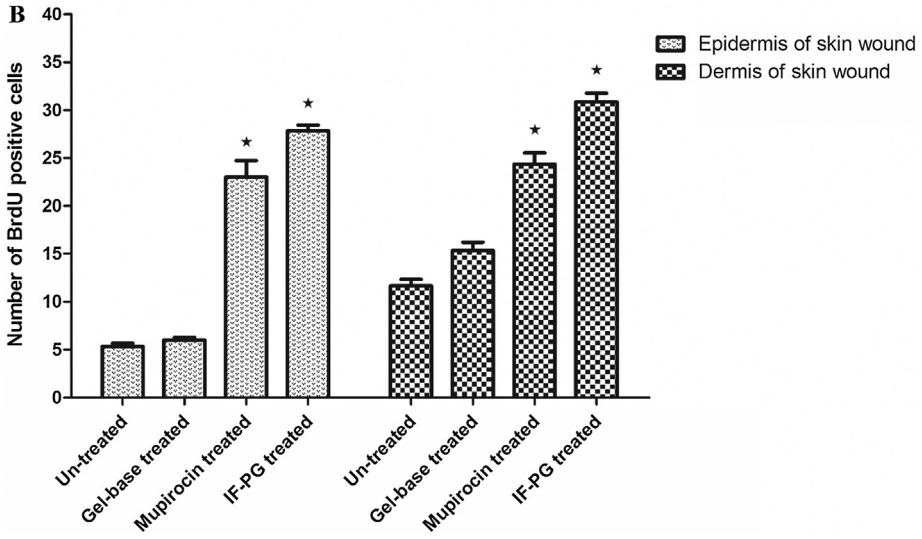

Fig. 3 (*continued*)

Table 1
Percentages of the re-epithelialization at the indicated time intervals. Punch wounds in rats were treated with IF-PG or Mupirocin or a gel base formulation control. An untreated group of rat was also maintained. Re-epithelialization was assessed and expressed in percentage. Data were represented as mean ± SEM for 6 animals. The $p < 0.05$ was considered to be statistically significant.

Groups	Re-epithelialization (%)		
	Day 3	Day 7	Day 12
Untreated wound	24.81 ± 4.46	86.70 ± 7.24	100
Gel-base treated wound	25.03 ± 1.03	89.40 ± 3.62	100
Mupirocin treated wound	43.70 ± 3.95*	100*	100
IF-PG treated wound	41.58 ± 1.90*	100*	100

achieved wound closure, however their appearance and the pattern was notably different. Platelet treatment did not show any improvements in the post-operative wound drainage after bilateral reduced mammoplasty (Anzarut et al., 2007). Though our aim was in healthy rats crafting a punch wound, so it is very important to understand the disease condition and molecular mechanism of the disease when hypothesizing IF-PG treatment.

To understand how IF-PG treatment fasten the wound healing, histopathology was conducted from day 3, 7 and 12 post-wound tissues. The findings differentiated different phases of wound healing, where IF-PG-treated wound was prominent at all the phases of wound healing compared to that of control rats. The IF-PG-treated rats show less inflammatory signs in the lesions; but more intense granulation tissues including collagen and fibroblasts; and re-epithelialization compared to that of control rats. However, the wound pathology of IF-PG-treated rats was similar to Mupirocin-treated wounds. In both IF-PG and Mupirocin-treated rats, the collagen fibers and epithelial tongues from the edges of the wounds migrating into the central area were also similar, suggesting their efficiency to promote healing. But, because of the emergence of multi-resistant organisms in antibiotic, ancient approaches of traditional and alternative medicine are still in demand for wound care (Dorai, 2012). Interestingly, IF-PG treatment improved the wound healing by exerting anti-inflammatory effects, fibroblast synthesis (fibroplasia), collagen synthesis, angiogenesis (revascularization) and re-epithelialization. This might be achieved by releasing a higher amount of growth factors, including transforming growth factor-beta (TGF-β), which stimulates proliferation of keratinocytes, cytokine release; and platelet-derived growth factor (PDGF), interleukin-1, fibroblast growth factor (FGF) which triggers fibroblast to migrate, secrete extracellular matrix and synthesize collagen (Broughton et al., 2006; Rozman and Bolta, 2007). Further investigation on the involvement of various growth factors on IF-PG-treated wound is vital to characterize the mechanism.

Cell proliferation ensued in both epidermal and dermal compartments of the wounds in all experimental groups. The

Table 2
Short literature review on Cord Blood Platelet Gel (CBPG) and Platelet concentrates in different disease conditions.

Type	Disease indication	Major outputs	Reference
Cord Blood Platelet Gel (CBPG)	Severe oral mucositis Epidermolysis bullosa (EB)	Complete cure of disease symptoms without any side effects Safe for the management of newborns with dystrophic recessive EB, prevent fluid loss and superinfection, no significant relapses in the lesions	Piccin et al. (2017a, 2017b) Tadini et al. (2015b), Tadini et al. (2015a)
Platelet Rich Plasma (PRP)	Knee Osteoarthritis (OA) Tendon and muscle injuries	Aids pain relief in knee OA, no adverse events noticed Care of muscle and tendon injuries in both elite and recreational athletes	Smith (2016) Mishra et al. (2009)
Platelet Rich Fibrin (PRF)	Regenerative dentistry	High biocompatibility, higher fibroblast migration and proliferation	Miron et al. (2017) Soffer et al. (2003) Ahila et al. (2018)
Platelet Gel Formulation	Osteoradionecrosis of jaw Radio-dermatitis Plastic and reconstructive surgical procedures	Regeneration of bone and soft tissues, shortened patient's hospital stay Successful restoration of disease condition Benefits in speeding up soft tissue healing	Piccin et al. (2016) Piccin et al. (2015) Chandra et al. (2007)

proliferative activity was monitored by evaluating the DNA-synthesizing cells using bromodeoxyuridine (BrdU) incorporation. The wound tissues of IF-PG and Mupirocin-treated on day 3 characterized a large number of proliferating cells (brown nuclei) in the keratinocytes, on stratum basale of the epidermis, and fibroblasts along with endothelial cells of new blood vessels suggesting a faster recovery. In contrast, BrdU incorporation in the wound was diminished and wound closure was delayed in both untreated and gel base-treated wounds suggesting IF-PG accelerated wound repair via proliferative activity. Our findings were consistent with earlier reports suggesting platelet-rich plasma gel augmented differentiation of the suprabasal epidermis via the expression of keratohyalin granules and cytokeratin 10 in suprabasal keratinocytes following injury (Fuchs, 1995; Kane et al., 1991). Additionally, BrdU staining of the vascular endothelial cells in the IF-PG or Mupirocin-treated wounds shows signs of angiogenesis on day 3, and reached a peak on day 7 post-wound, which leads to higher vascular density in the granulation tissue. The endothelial cells were proliferated and migrated during the new blood vessel formation; additionally, these new vessels might provide nutrients and oxygen for tissue regeneration. These events were known to be initiated by various growth factors and the most prominently, the vascular endothelial growth factor (VEGF) (Barrientos et al., 2008; Behm et al., 2012).

In conclusion, the wound in our experiments were naturally curable; however, the invalid platelets that were discarded from the blood bank was purposed for the wound care management. Studies on the application of IF-PG in intractable wounds might strengthen its effectiveness; however, toxicity, dosage and disease conditions causing the wounds need to be thoroughly studied before the therapeutic approach.

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