TIPS

Wound Healing Activity of a New Formulation from Platelet Lysate

Akram Jamshidzadeh[1,2], Omid Koohi Hosseinabadi[3], Reza Heidari[1], Soliman Mohammadi-Samani[1,*], Sara Rajabzadeh[4], Seyed Mojtaba Seyed Raoufi[2], Alireza Ahmadi Vadeghani[3]

[1]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]Department of Pharmacology and Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
[3]Laparoscopy Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[4]International Branch, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Platelet-rich plasma (PRP) is an attractive preparation in regenerative medicine due to its potential role in the healing process in different experimental models. This study was designed to investigate the wound healing activity of a new formulation of PRP. Different gel-based formulations of PRP were prepared. Open excision wounds were made on the back of male Sprague-Dawley rats, and PRP gel was administered topically once daily until the wounds healed completely (12 days). The results revealed that the tested PRP formulation significantly accelerated the wound healing process by increasing the wound contraction, tissue granulization, vascularization, and collagen regeneration. Interestingly, this study showed that there were no significant differences between the PRP and its gel-based formulation in all the above mentioned parameters. Although this investigation showed that PRP formulation had significant wound healing effects, the PRP gel-based formulation also had significant wound healing properties. This might indicate the wound healing properties of the PRP gel ingredients in the current investigation.

Keywords: Gel-based new formulation, Growth factors, Platelet-rich plasma, Regenerative medicine, Wound.

1. Introduction

Platelets are anucleated planar cells that are derived from megakaryocytes in the bone marrow and they are functionally constructed properly for their crucial role in hemostasis and healing process (1). Platelets contain two distinguishable parts, hyalomere, and granulomere. Hyalomere is a part that plays a role in the dynamic of the cell, where granulomere contains granules including lysozyme, alpha (α) and dense granules (2). α-granules contain more than 300 kinds of proteins that some of them play important roles in hemostasis and healing processes (3, 4). Platelet-derived growth factor (PDGF), tumor growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) I and II are some of the most important growth factors (GFs) known for their role in cell proliferation, intercellular matrix synthesis, angiogenesis and chemotaxis properties (5). Dense granules contain vasoactive amines and other bioactive molecules that at least according to our knowledge, have roles in vasoconstriction, increasing the permeability of capillaries and other important functions in tissue healing and clot formation (6). The tissue repairing and healing properties, are the most appealing characteristic of platelet growth factors (PGFs) (7, 8). The wound healing process consists of four overlapping phases: a) Microvascular damage that leads to activation of coagulation cascade and attraction of inflammatory cells; b) Inflammation; in which polymorphonuclear leucocytes, monocytes, and macrophages will be activated; c)

Corresponding Author: Soliman Mohammadi-Samani, Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
Email:smsamani@sums.ac.ir.

Proliferation and remodeling; in which fibroblasts will be activated and will produce collagen and lead to the formation of intercellular matrix; d) Scar formation; which is an equilibrium between fibroblasts and extracellular matrix. At the end of the scar formation phase, a new epithelium will be formed (9). Growth factors which control this complicated process include PDGF, VEGF, and TGF β (10). Therefore, it can be guessed that PGFs play a substantial role in the wound healing process (11). According to this fact, platelet and platelet-rich plasma (PRP), can be considered as a promising new therapeutic option in wound healing and regenerative medicine. PRP is applied adventurously in almost all fields of regenerative medicine (12-14). In the field of cutaneous wound healing, several investigations have been conducted in order to evaluate the effect of PRP on chronic, diabetic, surgical and other types of wounds. Almost in all of them, PRP was accounted as an effective treatment (6, 11, 15, 16). PRP is a condensed suspension of platelets in plasma, hence, it needs to be activated in order to release its growth factors and eventually manifesting its healing properties. Several reagents including thrombin, calcium, and collagen type I or methods such as freeze and thaw cycles are applied to PRP to release its active ingredients (17). The duration of action and effects of PRP-derived GFs on its site of administration along with the physiochemical stability of GFs, is another challenge attributed to PRP administration in regenerative medicine (13, 18, 19). In this regard, we aimed to evaluate the effect of the formulation parameters on the physiochemical stability of PRP lysate and to introduce an appropriate topical formulation for PRP in a rat model with open excision wounds.

2. Materials and methods
2.1. Materials
Glycerol, glycine, dextrose, sodium hydroxide, sodium chloride, sodium metabisulphite and EDTA were purchased from Merck (Germany). Carbopol 934 was used as a gelling agent and methylparaben was used as a preservative which is proper for protein formulations (Sigma-Aldrich, USA). PRP was obtained from Fars blood transfusion organization (Shiraz, Iran). Platelet growth factors were obtained by conducting the freeze and thaw method (frozen at -80 °C for 5 hours and defrosted at 30 °C in a water bath) on PRP (17, 20).

2.2. Formulation preparation
In order to evaluate the effects of excipient on physical stability of PGFs, different formulations were prepared (Table 1). Each of the formulations was obtained by dissolving pre-determined percent of EDTA, methylparaben, sodium metabisulphite, sodium chloride, glycerol, glycine and dextrose in 10ml of deionized water. Carbopol 934 was added at the moment that other excipients had been dissolved entirely. Finally, pH of the solution was adjusted to 7 by the use of sodium hydroxide (21, 22).

2.3. Turbidity study
To indirectly assess the physical stability of GFs in different formulations, turbidity study was conducted for all formulations by the use of UV-Vis spectrophotometer (Shimadzu, Japan) at 350 nm. Heat-induced aggregation study was performed at 53 °C by a water bath equipped with a thermometer. For each of the formulations, samples were prepared by adding 2.5 ml of platelet lysate to 2.5 ml of the vehicle. The blanks were

Table 1. Constituents of three formulations of platelet lysate topical gel evaluated in the current investigation.

Formulations	Constituents								
	NaCl	SMBS	MPB	EDTA	DX7	GE	GL	CP	Water
F1	0.9	0.2	0.1	0.1	-	2	2	0.5	q.s.
F2	0.9	0.2	0.1	0.1	2	2	2	0.5	q.s.
F3	0.9	0.2	0.1	0.1	1.5	2	4	0.5	q.s.

NaCl, Sodium chloride; SMBS, Sodium metabisulphite; MPB, Methylparaben; EDTA, Ethylenediaminetetraacetic acid ; DX, Dextrose; GE, Glycine; GL, Glycerol; CP, Carbopol. All values are in w/v% except dextrose, glycine, and glycerol which are in molar scale (M).

prepared by adding 2.5 ml of deionized water instead of platelet lysate. The change in absorption at 350 nm was recorded every 30 seconds until the rate of increase in optical density reached a plateau (23, 24).

2.4. Kinetics of proteins aggregation

In order to normalize heat-induced aggregation profile of the formulations, the kinetics of protein aggregation was calculated and the order of reaction was determined by conducting a graphical method (24, 25).

2.5. Animal study

Forty-two male Sprague-Dawley rats (180–250 g) were selected and housed in separate cages having a temperature-regulated conventional animal room at 22 ± 3 °C, 40-60% relative humidity with an artificial light cycle of 12-12 hours light/dark. The research protocol complied with the guidelines for animal care of Shiraz University of Medical Sciences, Shiraz, Iran. Rats were randomly allocated into three independent equal groups:**A)** Wound-induced without treatment, **B)** Wound-induced and PRP gel-treated, and **C)** Wound-induced and gel base-treated. PRP gel and the gel base formulation were applied to the excision wound once daily using a sterile cotton swab throughout the period of study (12 days).

2.6. Excision wound induction

Rats were anesthetized using a mixture of 100 mg/kg of ketamine and 10 mg/kg of xylazine, i.p, and the dorsum was clipped free of hair. As a model of wound induction in rats, full thickness skin samples (1×1 cm) were taken with fine scissors under sterile conditions (26).

2.7. Wound closure analysis

To determine the wound closure rate, the wounds were observed every day. At each visit, the wounds were debrided and a digital photograph was taken of each wound with a single lens, 12.1-megapixel digital Camera (Canon, Tokyo, Japan). To calibrate the magnification of photographs, the camera was held at the distance of 20 cm from the wound base and a ruler was laid against the wound. The wound area (mm^2) was estimated histomorphometrically. Wound closure rates were calculated by a previously reported method (Eq. 1):

$$\text{Wound closure rate (\%)} = \frac{\text{area at 1st visit} - \text{area at each visit}}{\text{area at 1st visit}} \times 100 \quad \text{Eq 1.}$$

2.8. Hydroxyproline estimation

After 12 days of wound induction, a part of the wounded tissues was analyzed for hydroxyproline content. The wounded tissues were dried in a hot air oven at 60-70 °C to reach a constant weight and were hydrolyzed in 6 N HCl at 130 °C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was ended by the addition of 0.4 M perchloric acid. After adding the Ehrlich reagent at 60 °C, the absorbance of the developed color was measured at 557 nm using a Pye Unicam® spectrophotometer (27).

### 2.9. Tissue preparation and processing

The rats were euthanized by ether inhalation at days 6 and 12 after wound induction. Full-thickness skin samples (1×1 cm) were taken and the wound tissues were fixed in buffered formaldehyde (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, and 10% formaldehyde in distilled water) (28). In a random sampling manner, nine pieces were chosen for histological analysis. The pieces were embedded in a cylindrical paraffin block, sectioned and then stained with hematoxylin and eosin (H&E) inorder to be viewd by the use of a light microscope.

2.10. Statistical analysis

Data were expressed as Mean±SD. The non-parametric K independent sample test (Kruskal-Wallis) followed by Mann-Whitney U test, were employed for data comparison. $P<0.05$ was considered significant between groups.

3. Results

As shown in Table 1, three different formulations containing PRP lysates, were prepared and physical stability of these formulations were assessed based on the aggregation rate and change in turbidity of the formulation at 350 nm (23).
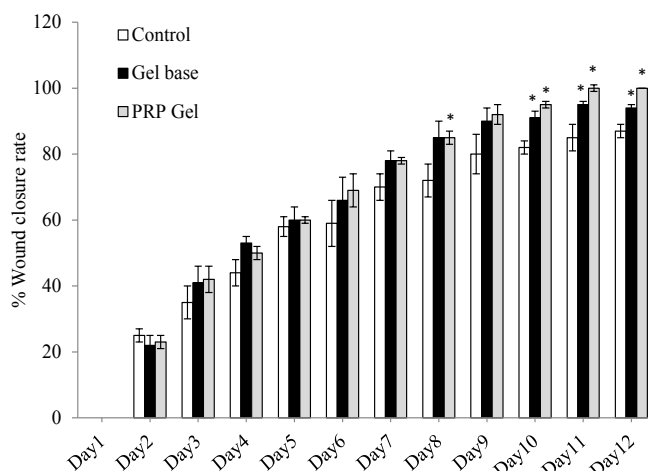
Figure 1. Effect of platelet lysate administration on wound closure rate.
Data were expressed as mean±SD (n=7).
The significant difference with the control group in each day is indicated by asterisks (*P*<0.05).

Among these formulations, the F3 formulation (Table 1) showed the best stability profile. Hence, this formulation was used in the study's animal experiments.

The mean initial area of the ulcers was 91.64±8.01 mm^2 (ranging between 86.43-94.98 mm^2) at the 1st visit. There were no significant differences in primary wound surface area between the three groups. The wound area of negative control group contracted almost to 15% of the original size by day 12 post-wounding (Figure 1). There was a significant difference in wound closure rate between the three groups within 10, 11 and 12

postoperative days (Figure 1). There was no difference between the PRP group and other groups in hydroxyproline content at the 12th-day post-wound induction (Figure 2).

Rats in the PRP group tended to demonstrate enhanced fibroblast and macrophage migration, increased vascularization, collagen regeneration, and epithelialization in comparison with the control group (Table 2). A significant increase in the number of infiltrated fibroblasts in the subcutaneous tissue in the PRP treatment group was seen at 12 days post-wounding (Table 2). Furthermore, significant abundant collagen regeneration was

Table 2. Histological scoring indices of wound healing activity of platelet lysate in different days in rats.

	Control		Gel base		PRP gel	
	Day 6	Day 12	Day 6	Day 12	Day 6	Day 12
Fibrotic tissue	0.0±0.0	1.0±0.0	0.0±0.0	3.0±0.5	0.0±0.0	3.0±0.5
Cellular mitosis	1.3±0.5	1.0±0.0	1.6±0.5	0.6±0.6	1.3±0.4	0.7±0.6
Tissue granulization	1.0±0.0	1.0±0.0	2.0±0.0*	0.7±0.6	2.8±0.0*a	0.3±0.6*a
Epithelialization	1.3±0.7	2.0±0.9	1.5±0.6	2.3±0.3	2.0±0.0	2.0±0.0
Vascularization	2.0±0.0	1.0±0.0	3.0±0.0*	1.0±0.5*	3.0±0.0*	2.0±0.0*a
Neutrophils	3.0±0.0	3.0±0.0	3.0±0.0	2.0±0.0*	3.0±0.0	1.3±0.8*
Macrophages	1.0±0.0	1.0±0.5	2.4±0.0*	0.6±0.5	2.6±0.8*	0.6±0.5
Fibroblasts	1.3±0.5	0.9±0.5	1.6±0.5	2.3±0.5*	1.7±0.5	2.7±0.6*
Edema	2.0±0.0	2.0±0.0	1.7±0.5	0.8±0.0*	1.3±0.5	0.8±0.0*
Collagen regeneration	0.9±0.0	2.0±0.0	1.9±0.0*	3.0±0.0*	1.9±0.0*	3.0±0.0*

0: absent; 1: mild; 2: moderate; 3: severe. Data are presented as Mean±SD (n=7).

*Significantly different as compared with control group (*P*<0.05).

aSignificantly different as compared with gel base group (*P*<0.05).
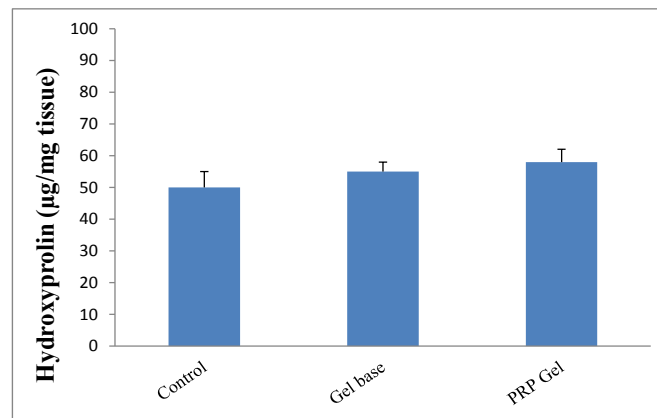
Figure 2. Hydroxyproline levels in wound tissue during the healing process. Hydroxyproline was measured in all experimental groups, 12 days after wound induction. Data were expressed as Mean±SD (n=7 for each group).
Although it tended to rise in PRP and gel base-treated groups, there was no significant difference between these groups and the control group in their hydroxyproline content.

observed in the PRP group at 6 and 12 days post-wounding (Table 2). Moreover, epithelialization was significantly greater in the PRP group than in the control group at 12 days post wounding. There was no significant difference in the cellular count (neutrophils and macrophages) between experimental groups at any time point. Interestingly, there was no significant difference between PRP treated and gel base-treated group in many parameters assessed as wound healing indicators (Table 1, Figure 1).

4. Discussion

Chronic wounds are imposing huge expenses on public health and the patients' health quality can be reduced in this situation. Conventional clinical wound treatments include pressure relief, treatment with antibiotics, surgery, and nutritional support. New methods of wound healing are composed of different strategies which can enhance the healing process and reduce the problems associated with the wound. These new strategies include the use of growth factors, high-pressure oxygen and electrical stimulation (29, 30). PRP is an attractive agent in regenerative medicine (29). The advantages of products such as PRP, include being cost-benefit, availability to all patients, providing comfort and safety (31). PRP is rich in growth factors. TGF-β is the major growth factor involved in collagen synthesis (32). This growth factor and PDGF which are released following the activation of platelet alpha granules, have been identified to be vital and important factors in the

wound healing process. TGF β is the most effective growth factor in the wound healing process. Because of the presence of these two growth factors in platelet concentrates, platelets are an inexpensive source of these compounds. PRP as a compound that induces regeneration of mesenchymal tissues such as connective tissue, tendons, bones and blood vessel, increases the rate of wound healing and promotes angiogenesis, stimulates mitosis and migration of fibroblasts and increases collagen synthesis by fibroblasts (6). Hence, PRP has been used in many clinical disorders in humans (6, 11, 16). Topical growth factor products are typically used as an adjuvant therapy along with the standard care for treatment of diabetic foot ulceration (5).

A major limitation in the clinical use of GFs in wound healing and in the case of chronic wounds, is finding a practical and effective method to transmit GFs to the wound. Different preparations of PRP have partially overcome some of these limitations (33). Despite the efficacy of PRP in the wound healing (34), no effect was seen in the wound healing process in some studies which assessed platelet products. In one study conducted by Eppley and coworkers, the effects of PGFs were examined on chronic leg ulcers of 18 patients and no significant differences were observed between control and treatment groups (16). In another study, platelet effect was studied on venous ulcers of 86 patients. No significant difference was observed between different groups after 9 months of treatment (35). In order to suggest appropriate topical gel formulation of PRP lysate, three

different formulations were designed and tested for physical stability in the current investigation. Glycerol could significantly reduce the aggregation of the lysate proteins (Table 1). Dextrose might play a role in stabilizing protein lysate and can be applied individually or in combination with other appropriate excipients as a proper stabilizing additive (Table 1). The F3 formulation (Table 1) seemed to be the most appropriate one for the PRP gel-based product.

Interestingly, in this study, no significant difference was detected between PRP topical gel and gelbase formulation, in fibroblast infiltration, angiogenesis, necrosis, collagen aggregation, cell degeneration, giant cell, polymorphonuclear cells, the rate of epithelialization and granulation parameters (Table 2). The wound healing effects of the gel base in the current investigation, might be due to its dressing and nutritional properties of our gel base that can promote healing activity (Table 2 and Figure 1). On the other hand, the PRP gel formulation was significantly different in some parameters such as vascularization and tissue granulation (Table 2), which indicates its more significant role in the wound healing process in comparison with our

gel base formulation(Table 2).

Based on the results of this study, there was significant differences between the wound healing rates in PRP receiving group compaired to the negative control group, but there were no significant differences between the base gel receiving group and PRP receiving ones conserning this matter. The profound healing effect of the gel base formulation can probably be due to the presence of different moisturizing factors such as glycerol, sugars and a hydrophilic polymer in the formulation.a. Furthermore, PRP itself has a healing effect. Hence, it can be concluded that application of PRP in the suitable base form, could have a synergistic effect on the wound healing process.

Acknowledgments

Conflict of Interest

None declared.

5. References

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