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Effect of Platelet Rich Plasma Combined with Chitosan Biodegradable Film on Full-Thickness Wound Healing in Rat Model

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ABSTRACT

Objective: To assess the effects of platelet rich plasma (PRP) with chitosan biodegradable film on full thickness wound healing in rat.
Methods: This was an experimental study being performed in 2015 during a 4-month period. Twenty-four male white Wistar rats were divided into four groups of 12 rats each, randomly: Control group (SHAM) with creation of wounds and no treatment, PRP group with creation of wounds and application of one milliliter PRP, Chitosan group (CHIT) with dressing the wound with chitosan and CHIT/PRP group with application of one mL PRP and dressing the wound with chitosan. The wounds were created by cutting healthy skin. Wound size was measured on 6, 9, 12, 15, 18 and 21 post surgery and was compared between groups.
Results: Reduction in wound area, hydroxyproline contents and biomechanical parameters indicated there was significant difference (p=0.001) between group CHIT/PRP and other groups. Biomechanical testing was performed on day 9 post surgery in incisional model. Quantitative histological studies and mean rank of the qualitative studies demonstrated that there was significant difference (p<0.001) between group CHIT/PRP and other groups.
Conclusion: PRP with chitosan have beneficial effects on wounds repair and could be suggested for treating various types of wounds in animals and human being.

Keywords: Chitosan; Platelet rich plasma (PRP); Wound healing; Full-thickness wound.

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Introduction

Wound is defined as disruption of cellular, anatomical, andfunctional continuity of a living tissue.Wound care and maintenance involves a number of measures including dressing and administration of painkillers, use of anti-inflammatory agents, topical systemic antimicrobial

agents, and healing promoting agents [1].Wound healing is the interaction of a complex cascade of cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength of injured tissues [2].

Platelet-rich plasma (PRP) is an autologous product that concentrates a large number of platelets in a small volume of plasma [3,4]. PRP functions as a fibrin tissue adhesive with hemostatic and tissue sealing properties and,provides an immediate surgical hemostatic agentthat is biocompatible, safe, and effective. It accelerates endothelial, epithelial, and epidermal regeneration, stimulates angiogenesis, enhances collagen synthesis, promotes soft tissue healing, decreases dermal scarring, enhances the hemostatic response to injury, and reverses the inhibition of wound healing caused by glucocorticoids. The high leukocyte concentration of PRP has an added antimicrobial effect. In addition to its effectiveness for patients with chronic non-healing wounds, it has also been used as an antiangiogenic agent and as a carrier for growth factors [5]. PRP decreases the frequency of intraoperative and postoperative bleeding at donor and recipient sites, accelerates soft-tissue healing, supports the initial stability of grafted tissue at recipient sites as a result of its cohesive and adhesive nature, promotes rapid vascularization of healing tissue by delivering growth factors and, when used in combination with bone replacement materials, induces regeneration [5].

In cases of severedistortion of the tissue architecture, the healing process may not lead to morphofunctional normality but result in the formation of disoriented connective tissue with a fibrous appearance [6,7]. This abnormal tissue architecture reduces the mechanical strength and leads to scar formation. Biomaterial scan assist the proper physiological reconstruction of the skin and reduce or prevent scar tissue formation. Chitin, chitosan, and their oligomers have been found to promote wound healing, especially in the phases of proliferation and matrix formation [8]. Chitosan and its oligomers are well known for their interesting biological properties, which have led to various applications. Lysozyme slowly hydrolyzes chitosan membrane and produces chito-oligomers that stimulate correct deposition, assembly and orientation of collagen fibrils in extracellular matrix components [9]. Moreover, it has been indicated that chitosan membrane stimulates the migration of inflammatory cells and promotes cellular organization [10,11].

Various wound models have been investigated in the literature including chronic and pressure ulcers [12]. In the present study the cutting healthy skin wound model was adopted. There is no report in the literature concerning the effect of chitosan combined with PRP on full thickness wound healing created by cutting healthy skin. The aim of the present study was to evaluate the wound healing activity of chitosan and PRP combination on full thickness wounds created by cutting healthy skin in rat. Assessment of the healing process was based on excision, incision, hydroxyproline estimation and biomechanical studies.

Materials and Methods

Approvals

Our study protocol was reviewed and approved by Urmia University ethical committee and institutional review board (IRB).All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85–23, revised 1985).

Preparation of Chitosan

Chitosan solution was prepared by dissolving medium molecular weight, crab shell chitosan (~400kDa, 85% deacetylated) (Fluka, Sigma-Aldrich St. Louis, MO, USA) in an aqueous solution (1% v/v) of glacial acetic acid (Merck, Darmstadt, Germany) to a concentration of 2% (w/v) while stirring on a magnetic stirrer-hot plate. The solution was stirred with low heat (at 50^{oC}) for 3hour. The resultant chitosan solution was filtered through a what man No. 3 filter paper then vacuum filtration to remove any un-dissolved particles. To overcome the fragility of chitosan, glycerol (Sigma Chemical Co., St. Louis, MO, USA) was added as 30% (w/w) of the total solid weight in solution [13]. Chitosan (2%, w/v) in acetic acid was freeze-dried,and cross-linked with 5% (w/v) tripolyphosphate and freeze-dried again to produce a sponge-like matrix. Chitosan sheets were prepared and dressed on created wounds.

Preparation of Platelet Rich Protein

The platelet-rich plasma was prepared based on a method described by others [14]. In brief, the fresh blood sample was obtained by heart puncture with a tube containing sodium citrate. The collected blood was firstly centrifuged at 160 g, for 20 minutes, at environmental temperature (22 ºC). Then, a red lower fraction (red cell component) and an upper straw-yellow turbid fraction (serum component) were observed. A point was marked at 1.4 mm below the line dividing the two fractions. All the content above this point was pipetted and transferred to other 5 ml vacuum tube in which a line corresponding to 0.35 ml was drawn from the tube's bottom. The sample was then submitted to a new centrifugation at 400 g, for 15 minutes, resulting in two components: one above was platelet-poor plasma and other below the line drawn on the tube was PRP. Two rats were serves as PRP donors.

Excision Wound Model and Planimetric Studies

For excisional wound healing model 24 healthy male Wistar rats weighing 160-180 g approximately

seven weeks of age were divided into four groups of 6 rats each, randomly: Sham surgery group (SHAM) with creation of wounds and no treatment, PRP- treated group (PRP) with creation of wounds and application of one milliliter adipose derived nucleated cell fractions, Chitosan group (CHIT) with dressing the wound with chitosan and CHIT/PRP group with application of one milliliter platelet rich plasma and dressing the wound with chitosan. All the wounds in all experimental animals were rinsed daily by 10-ml sterile normal saline for 10 days. After induction of anesthesia with Xylazine HCL 2% (5 mg/kg/IP, Alfasan International, Woerden, Holland) and ketamine HCL 10% (60 mg/kg/IP, Alfasan International, Woerden, Holland) rats were fixed in a ventral posture on a surgery table. A circular excision wound was made by cutting away approximately 300 mm^2 full thickness of predetermined area on the anterior-dorsal side of each rat. All the test formulations were applied for 10 days starting from the day of wounding.

Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18 and 21 post-operation by a digital camera while a ruler was placed near the wounds (Figure 1). The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc., San Jose, CA, USA) and wound contraction percentage was calculated using the following formula: Percentage of wound contraction = $(A_0 - A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging [15]. The animals were left in separate cages for four days at room conditions for acclimatization. Animal houses were in standard environmental conditions of temperature (22±3 °C), humidity (60±5%), and a 12h light/dark cycle. The animals were maintained on standard pellet diet and tap water. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced.

Determination of Hydroxyproline Levels

On the day 21 of the post-surgery of excision, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content, which is a basic constituent of collagen. The hydroxyproline contents were estimated using a method described by others [16]. Briefly, tissues were dried in a hot air oven at 60–70 ◦C to constant weight and were hydrolyzed in 6N 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 terminated by addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60 ºC and measured at 557 nm using UV-visible spectrophotometer (CamSpec M330, Cambridge CB2 4BG, UK).

Incision Wound Model and Biomechanical Testing

Twenty-four healthy male Wistar rats weighing 160-180 g approximately seven weeks of age were

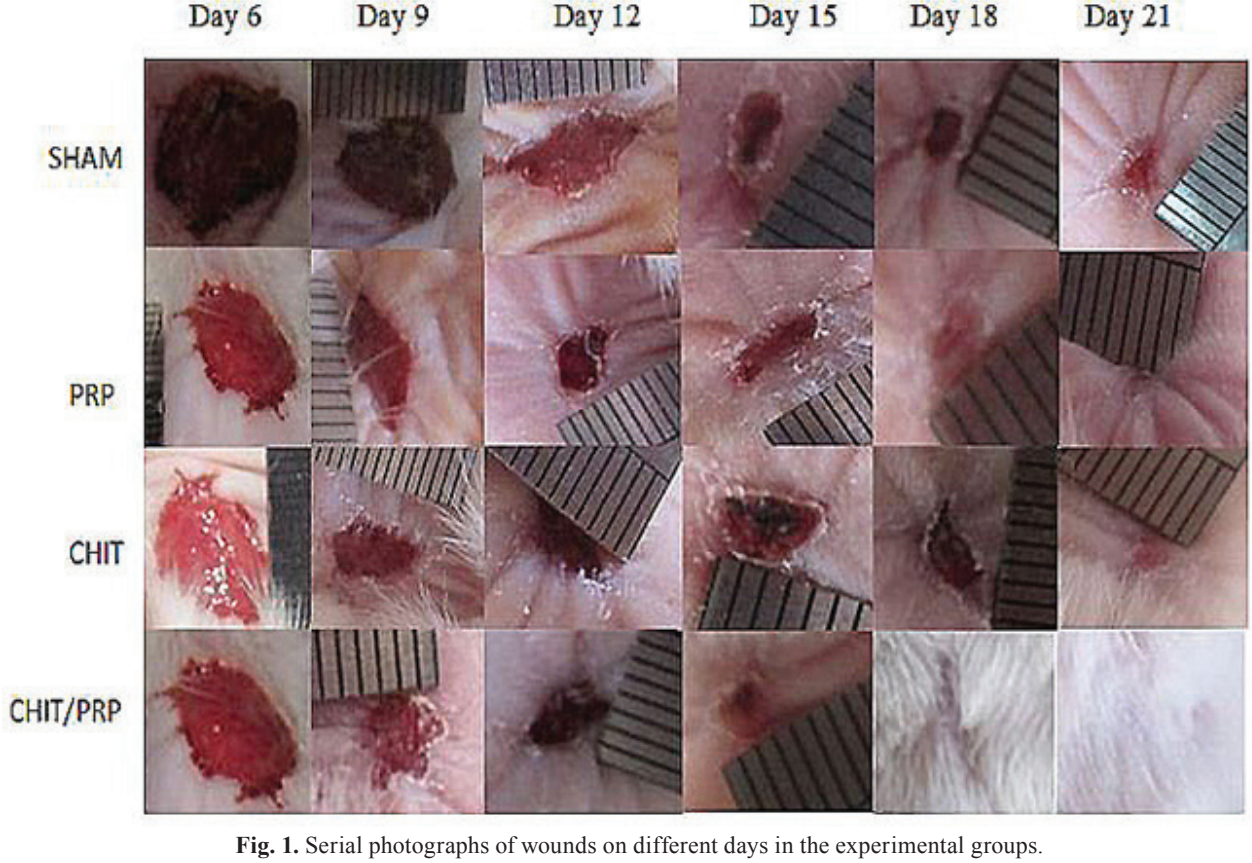

Fig. 1. Serial photographs of wounds on different days in the experimental groups.

divided into four groups of 6 rats each, randomly (see above). All animals of four groups were anesthetized as mentioned above and a paravertebral long incision of 4 cm length was made through the skin and cutaneous muscle at a distance about 1.5 cm from the middle on right side of the depilated back. After the incision was made, the two ends of the wound were sutured at 0.5 cm intervals with 3/0 nylon. All the groups were treated as the same in the excision model. Ointments, standard were applied once daily for 9 days. On day 9, sutures were removed and a strip of skin, 7 cm long, with the same widths of wound diameter, in the manner that the wound was located at the middle of the strip, was removed by a double-blade scalpel. The skin was then wrapped in Ringer's soaked gauze, aluminum foils, and plastic bags and kept in −20°C freezer until mechanical testing. The TA. XT Plus Texture Analyzer mechanical test device was used for the assessment (Stable Micro Systems, Surrey GU7 1YL, UK). The samples were fitted with appropriate clamps, the distance between the clamps at the start of testing being 4 cm. The strips were loaded with 0–30 kg load cell, with strain rate of 1 cm/min and the load elongation curves were obtained. Yield strength (yield point) (kg), ultimate strength (kg), maximum stored energy (kg/cm), and stiffness (kg/cm) were measured from the load elongation curves.

Histological Preparation and Quantitative Morphometric Studies

The tissue samples were taken on 7, 14, 21 days after surgery and fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) and Masson's trichrome stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, poly morphonuclear cells and fibroblastic aggregation were quantitatively evaluated. Acute hemorrhage, congestion, vascularization, epithelialization, collagen production and density were also evaluated qualitatively. Morphological findings were scored using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). The histological parameters were classified according to the intensity of occurrence in five levels (- absence; + discrete; ++ moderate; +++ intense; ++++ very intense) [17].

Statistical Analysis

Differences among groups for wound area, hydroxyproline level were evaluated by Kruskal–Wallis variance analysis. When the p-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. Student's t-test was used for evaluation of mechanical test results. Comparison among days was assessed by Mann–Whitney U-test. The Bonferroni correction was applied for all possible multiple comparisons. Statistical package for social sciences (SPSS Inc., Chicago, IL, USA) version 11.0 was used for statistical analysis. A two-sided p-value of less than 0.05 was considered statistically significant.

Results

Reduction in Wound Area

Wound contraction percentage in different groups over time series is shown in Table 1. The healing rate of ointment treated groups was significantly different compared to the control group (p<0.001). However, time had significant effect on wound contraction of all wounds (p=0.032) (Figure 1). In SHAM group, where no PRP and chitosan were treated, wounds were healed after 21 days, however, in PRP/CHIT group the wounds were healed by day 18.

Hydroxyproline Content of Wound

Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content [18] Hydroxyproline contents in the groups SHAM, CHIT, PRP and CHIT/PRP were found to be 52.33±3.50, 65.42±3.37, 73.12±4.20 and 85.46±3.52 mg g^{-1}, respectively. Hydroxyproline contents were increased significantly in the CHIT/PRP group which implies more collagen deposition compared to other experimental groups (p=0.001).

Table 1. Effect of adipose derived nucleated cell fractions combined with chitosan biodegradable film on circular excision wound contraction area (mm^2). Values are given as mean±SEM

Wound area on days (mm^2)						
Groups	**Day 6**	**Day 9**	**Day 12**	**Day 15**	**Day 18**	**Day 21**
SHAM	252.75±0.94	103.33±1.41	86.51±0.34	42.12±1.71	22.12±1.11	7.38±0.15
PRP	148.14±1.62	65.03±1.12	56.60±1.12	25.60±0.22	3.15±1.10	1.70±0.33
CHIT	181.25±1.80	71.40±1.20	62.52±0.37	30.64±0.27	3.66±1.18	1.38±0.46
CHIT/PRP	84.23±2.79[a]	48.17±0.49[a]	23.68±0.42[a]	2.12±0.11[a]	0.00±0.00[a]	00.00±0.00[a]

[a]The treated groups are compared by Student t test with other groups; The mean difference is significant at the .05 level *vs* SHAM, ADNC and PRP

Biomechanical Findings

The biomechanical indices, maximum stored energy, stiffness, ultimate strength and yield strength obtained for CHIT/PRP group were significantly higher than those obtained for other groups (p=0.002) (Table 2). This indicated better biomechanical properties of the PRP and chitosan combination on treated tissues.

Histological and Morphometric Findings

There were significant differences in comparisons of CHIT/PRP and other groups, particularlyin terms of cellular infiltration, acute hemorrhage, congestion, edema, collagen production and density, re-epithelialisation and neovascularization. During the study period, scores for re-epithelialisation and neovascularisation were significantly higher in CHIT/PRP rats than other groups (p=0·001) were observed. Polymorphonuclear and mononuclear cell count, fibroblast cell proliferation and also Mean Rank of the qualitative study of acute hemorrhage, edema and collagen production score in CHIT/PRP group were significantly higher than those of other experimental groups (p=0.001) (Table 3) (Figures 2,3,4 and 5).

Discussion

Inflammation, proliferation and tissue remodeling is three phases of healing process which occurs following tissue damages as closely as possible to its natural state. The healing process is activated when platelets come into contact with exposed collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing. Inflammatory cells also arrive along with the platelets at the injury site providing key signals known as growth factors. The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength [18].

Attempts to deliver growth factors to wounds utilizing platelets have been developed and have shown beneficial efficacy on chronic wound healing [19,20]. PRP therapy is a concentration of platelets with at least five growth factors important in wound healing. In addition, PRP may help fight infections by recruiting white blood cells and through platelet release of bactericidal factors. Much of the literature supports the use of PRP to enhance healing, but a few studies have failed to determine a benefit. Variations in devices and study designs may be responsible for negative results [21]. Marx *et al*., [22] provided a good discussion of issues leading to false-negative results and stressed that PRP should be autologous and must contain viably active platelets in sufficient

Table 2. Biomechanical parameters assessed for each of the experimental groups. Values are given as mean±SEM.

Biomechanical Parameters				
Groups	**MES [a] (Kg/cm)**	**Stiffness (Kg/cm)**	**Ultimate Strength (Kg)**	**Yield Point (Kg)**
SHAM	0.67±0.14	0.46±0.13	0.76±0.13	0.65±0.14
PRP	1.30±0.11	1.28±0.18	1.12±0.14	1.09±0.19
CHIT	1.10±0.21	0.97±0.13	0.93±0.12	0.74±0.11
CHIT/PRP	1.72±0.35 [b]	1.61±0.12 [b]	1.49±0.10 [b]	1.43±0.13[b]

[a]MES: Maximum Stored Energy; [b]The mean difference is significant at the 0.05 level vs SHAM, PRP and CHIT

Table 3. Intensity of histological parameters assessed in experimental animals.

Histological parameters						
Groups	**Days**	**Acute Hemorrhage**	**Congestion**	**Vascularization**	**Epithelialization**	**Collagen**
SHAM	7	++++	++++	+[b]	-	+
	14	+++	++	++	++	++
	21	-[a]	-	+++	++	++
PRP	7	+++	+++	++	+	++
	14	++	-	+++	++	+++
	21	-	-	+++	+++	+++
CHIT	7	+++	++	++	-	+
	14	++	-	+++	++	++
	21	-	-	+++	++	+++
CHIT/PRP[f]	7	++[c]	++[c]	+++[d]	++[c]	++[c]
	14	-	-	++++[e]	++++[e]	++++[e]
	21	-	-	++++[e]	++++[e]	++++[e]

Classification of histological parameters according to the intensity of occurrence: [a]- absence; [b]+ discrete; [c]++ moderate; [d]+++ intense; [e]++++ very intense. Histopatological damages were assessed as explained under material and methods on days, 7, 14 and 21 of lesion. [f]p<0.05 vs other experimental groups.

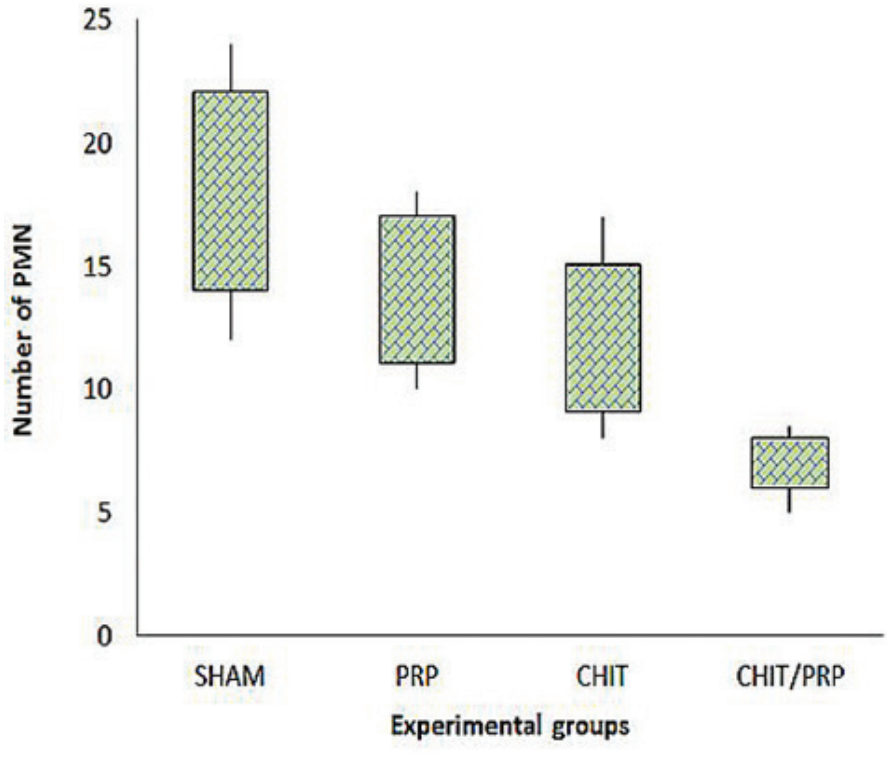

Fig. 2. Box-and-whisker plots of number of polymorph nuclear cells in excisional model of the rat's skin in experimental groups. Results were expressed as mean±SEM.

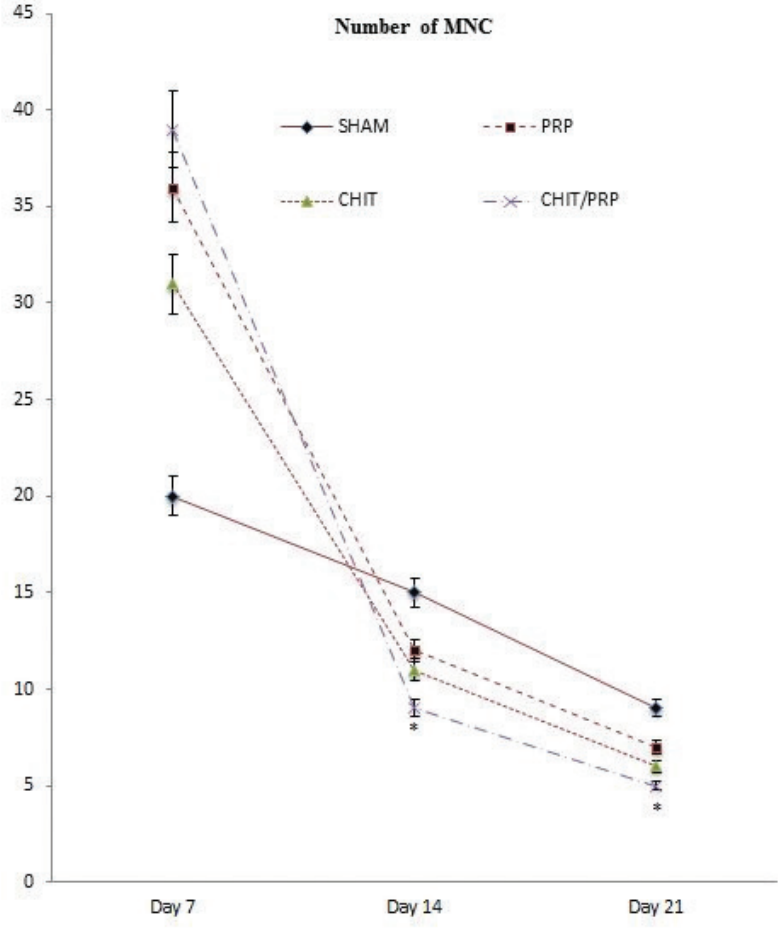

Fig. 3. Line graph indicating number of momonuclar cellsin excisional model of the rat's skin in experimental groups. Results were expressed as mean±SEM. * $P< 0.001$ vs other experimental groups.

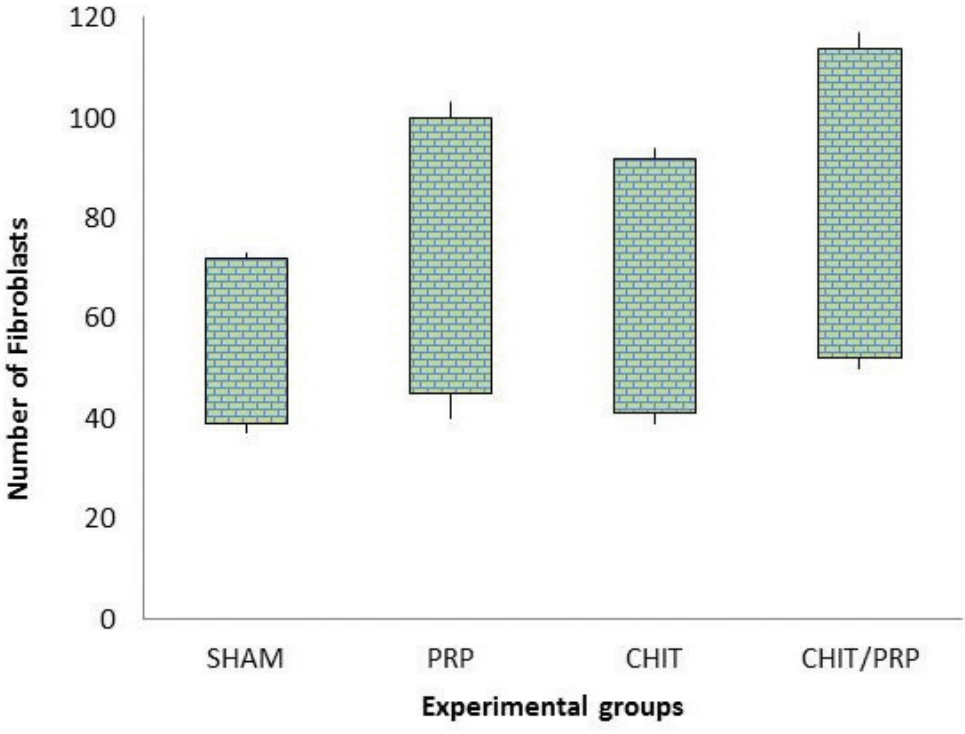

Fig. 4. Box-and-whisker plots of number of fibroblasts in excisional model of the rat's skin in experimental groups. Results were expressed as mean±SEM.

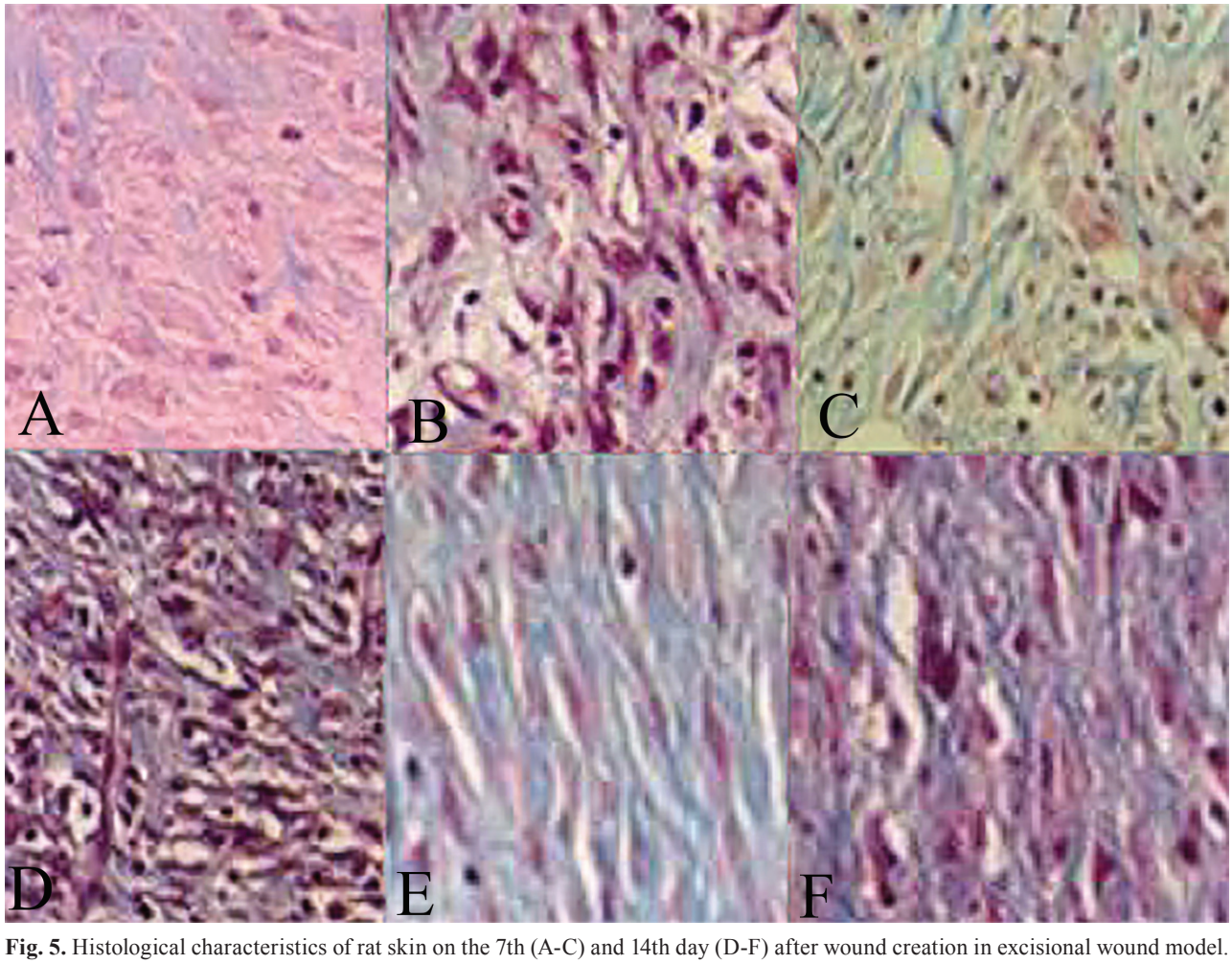

Fig. 5. Histological characteristics of rat skin on the 7th (A-C) and 14th day (D-F) after wound creation in excisional wound model. A and D: CHIT, B and E: PRP, C and F: CHIT/PRP. Wounds with surrounding skin were prepared for histological microscopic evaluation by Masson trichrome staining. Scale bar: 50 µm

concentration to aid healing. When these conditions are met, the vast majority of publications report a significant enhancement of healing when PRP is used.

Several reports have demonstrated a beneficial effect of chitosan as a biologically active dressing in wound management. It has been reported that the application of chitosan to the open wounds in dogs induced exudate, which has a high growth factor activity, and induced infiltration by inflammatory cells and granulation tissue formation accompanied by angiogenesis [23-25].

Chitosan-membrane-based wound products have been investigated both in laboratory animals and humans, but are still at the early stage of development. Since 1980, chitosan and its derivatives have been used in skin and wound management products in Japan. Beschitin W, an artificial skin prepared from chitin threads, has been developed for human use and is on the market [26,27].In excisional wound model there was a significant decrease in wound area. This indicated improved collagen maturation by increased cross linking. Whilean increase in hydroxyproline content in treatment groups indicated increased collagen content, since hydroxylproline is the direct estimate of collagen synthesis it supports the wound healing activity of the extract [28]. PRP therapy is the preferable treatment option in patients with vatoius kinds of wounds of different aetiology and localisation, particularly when other more conventional therapies lack evidence of effectiveness or when radical surgical treatment is not possible or contraindicated [29]. The efficacy of topical platelet gel application has been evaluated in clinical studies in human, observing a significant improvement in wound healing [30]. PRP is indicated for use in free connective tissue graft procedures, manipulations with mucoperiosteal flaps and soft tissue augmentation for cosmetic purposes in medicine and dentistry [29]. Chitosan has an accelerating effect on wound healing by activating immune cells through its cytokine production, giant cell migration, and stimulating type IV collagen synthesis [31]. The chitosan mesh membrane has been demonstrated to have a positive effect on the re -epithelialization and the regeneration of the granular layer and it is confirmed that chitosan mesh membrane is a potential substitute for human wound dressing [32].

Although the present study showed the promisin effect of local transplantation of PRP and chitosan combination on wound healing in rats, data regarding the molecular mechanisms leading to its action remain to be investigated in depth. The authors have not provided the molecular evidence for the action of PRP, which may be considered a limitation of this study.

In conclusion, the result of the present study demonstrated that chitosan and PRP combination were showing wound-healing property partly by increasing thecollagen synthesis.

Conflict of Interest: None declared.

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