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Efficacy of Autologous Platelet-Rich Plasma (PRP) Activated By Thromboplastin-D on the Repair and Regeneration of Wounds in Dogs

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Abstract

Objective- To evaluate the effects of autologous platelet-rich plasma (PRP) on wound healing in dogs.
Design- Experimental in-vivo study.
Animals- 5 mix-breed male adult dogs.
Procedures- Under general anesthesia, six 2 × 2-cm, full-thickness skin wounds were created on the back of dogs symmetrically. In each dog, three right side wounds were treated topically with 1.5 ml PRP jelly, whereas left side wounds received no treatment. For macroscopic evaluation, at days 0, 3, 5, 7, 10, 13, 17, 20, and 24, digital photographs were taken from wounds. At days 10, 17 and 24 after wounding, skin biopsies were taken from the center and corner of each wound for hydroxyprolin measurement and histopathologic evaluation respectively.
Results- No statistically significant differences were found in percentage of wound contraction, epithelialization and healing between test and control group during study (P>0.05). There were no significant differences between median of hydroxyprolin levels, median of inflammatory cells infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition and collagen bundle formation scores, in the specimens from left and right wounds (P>0.05).
Conclusion and Clinical Relevance- Topical application of platelet-rich plasma can not accelerate repair of the small granulating wounds of dogs.
Key Words- Dog, Wound healing, Platelet- rich plasma, Growth factors.

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Introduction

Wound healing is a complex multifactorial process that results in the contraction and closure of the defect and restoration of a functional barrier. This process occurs as a sequence of events including hemostasis, inflammatory cell infiltration, tissue regrowth, and remodelin.[1,2] Wound healing biologists aim to understand how a wound healing procedure can be induced to repair the damaged tissues faster and more efficiently. Enhancement of dermal and epidermal regeneration is an extremely important goal for the treatment of many different types of wounds.[3] Wound healing is regulated by several cell types and by a cascade of peptides such as cytokines or growth factors. Following injury, growth factors secretion by platelets and macrophages are induced and inflammatory process which is needed for healing is initiated.[2,4]

A new area of tissue engineering including bioactive molecule-based treatments has currently gained much attention. Several studies have shown that cytokine treatment might accelerate healing of tissues and especially promote the repair of impaired wounds in the variety of animals.[5-7] Unfortunately, these purified bioactive agents has fallen short of expectations in clinical studies, as it is currently clear that no single exogenous agent can effectively mediate all aspects of wound healing process.[7-9] This may be due to the fact that cytokines work in concert, both temporally and spatially.[10] So, the dynamic nature of wound repair process bears out the need for cytokine combination therapies.[7]

In an effort to provide this combination treatment, investigators have turned to Platelet-rich plasma (PRP), which is a rich source of different cytokines essential for natural healing process. It is released from platelet α-granules at sites of tissue injury.[7,11,12] These include catecholamines, serotonin, adenosine triphosphate (ATP), albumin, fibrinogen, osteonectin, osteocalcin and calcium ions. Various clotting factors and locally active growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor-α (TGF- α), transforming growth factor-β (TGF- β), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) also initiate wound healing.[11-18] Platelets also secrete fibrin, fibronectin, and vitronectin, which provide a matrix for connective tissue and act as adhesion molecules for epithelial migration.[19]

These whole ranges of bioactive factors present in PRP gel seem to result the principal therapeutic advantage of autologous platelet-rich plasma over isolated purified cytokines, accelerating the healing sequences in order to mimic the natural process as physiologically as possible.[7,10]

Although several in vitro studies have been carried out in this field, there are some studies in dogs or cats evaluating PRP effectiveness during cutaneous wound healing and/or tissue regeneration. There is only a single study illustrating the effectiveness of PRP gel in treatment of chronic wounds on the tail of a dog.[20] To the best of our knowledge, there are no previous studies assessing use of PRP in fresh wounds in dogs. Herein, we aimed to evaluate the effects of topical application of PRP, coagulated with different activators. We appraised whether increasing concentration of mediators in the wound with PRP can enhance healing rate and time required to achieve adequate tissue regeneration.

Materials and Methods

Animals
Five male, mixed-breed healthy adult dogs, 3.5 to 4 years of age and weighing between 26 and 30 kg, were used in this study. The dogs were housed in kennels, fed a maintenance ration twice daily and had free access to water.

PRP isolation
Canine platelet-rich-plasma was prepared freshly at each treatment day by collecting whole blood into sterile bags containing acid citrate dextrose formula as anticoagulant. Samples were centrifuged at 120g for 5 minutes while the first supernatant plasma fraction (about 50% volume) adjacent to the buffy coat was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged again at 280g for 5 minutes and 25% volume from the first fraction was obtained to yield a PRP of about 8×105 /µl platelets. To gain 1 ml of the PRP using this protocol 40 ml of whole citrated blood was needed. 1 ml of the prepared PRP was used for platelet cell count analysis.

PRP platelet count
To evaluate the enhancement of platelet concentration in the PRP, baseline platelet counts were obtained on all of the blood samples before processing and after PRP preparation. Platelet counts were performed using a hematology analyzer.

PRPs activation
To prepare PRP jelly the platelets were activated by calcium chloride (4.5 mEq/5 ml, Zist Faravar Co., Iran) 50 µl/ml and thromboplastin-D, 200 IU/ml (commercially available for PT test; Fisher Diagnostics, USA).

Surgical procedure
Under general anesthesia six 2 × 2-cm full-thickness wound (three in each side) were created on each dog (n=30 wounds). The protocol approved by the local Health Sciences Laboratory of Animal Policy and Welfare Committee of the University. Bleeding reduced significantly by pressing sterile tampon. In each dog, wounds on the right side were treated topically with 1.5 ml PRP jelly (test group), whereas left side wounds received no treatment (control group). In this manner, each dog served as its own control. Wounds were covered with sterile non adhesive bandage. Wounds were started to treat at 24 hours after wounding by 1.5 ml of PRP jelly and continues every other day for 3 successive days. Bandages were changed once daily. Biopsies were taken from each pair of the wounds at the day, 10 (pair 1), 17 (pair 2) and 24 (pair 3) after wounding for healing evaluation.

Macroscopic evaluation of the wounds
The wounds were evaluated over 24 days period. At the days 0, 3, 5, 7, 10, 13, 17, 20, and 24. Digital photographs were taken of all wounds after the area had been carefully cleaned to visualize wound margin. The scab of each wound was carefully removed using saline for better evaluation of epithelialization and granulation tissue. Rulers were held vertically and horizontally close to the wound as a reference. The area of the epithelialization and granulation tissue were measured for each wound using Sigma Scan software Version 5.

Percentage of the wound contraction, epithelialization and healing were calculated for each wounds. The following formulate were used:

-Wound contraction:

1. Wound size at the day (x) mm2 / wound size at the day (0) mm2 × 100 = percent of the wound size at the day (x)
2. 100 – percent of wound size at day (x) = percent of wound contraction

-Wound epithelialization:

Size of epithelialization area at the day (x) mm2 / size of the wound at the day (x) mm2 × 100 = percent of the epithelialization

-Wound healing:

1. Granulation tissue at the day (x) mm2 / wound size at the day (0) mm2 × 100 = percent of the non healed area to compare of the wound size at the day (0)
2. 100 - percent of the non healed area to compare of the wound size at the day (0) = percent of the healing.

Hydroxyprolin measurement

At the day 10, 17 and 24 after wounding, biopsies were taken from the center of each wound using 0.7 mm biopsy punch for hydroxyprolin measurement. Tissue samples for hydroxyprolin assay were washed with physiologic saline and dried in a 100°C oven for 72 hours. Hydroxyprolin levels were determined spectrophotometrically using the previously described method in µg/mg dry matter. 21 Initially, each specimen was weighed and hydrolyzed in 12-N HCl at 130°C for 3 hours. Then each sample was adjusted to a final volume of 1 ml and centrifuged at 3000 × g for 15 minutes. The supernatant was separated off and equal volume of isopropanol was added to each sample. Then this mixture was centrifuged at 2500 × g for 10 minutes. Serial dilutions of pure hydroxyprolin were used as standards. Concentration of hydroxyproline in each sample was calculated using the absorbance – concentration curve for the standard hydroxyproline solution.

Histopathologic examination

At the day 10, 17 and 24 after wounding, biopsies were taken from the same corner of each wound using 0.9 mm biopsy punch for histopathological examination. The wound specimens were fixed in 10% buffer formalin and embedded in paraffin. Samples subjected to hematoxylin-eosin and mason's trichrome staining. Epithelialization, inflammatory infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition and collagen bundle formation were scored as follows: absent: 0, Occasional presence:1, Slightly distributed: 2 and Abundant: 3 in each sample.

Statistical analysis

Statistical analysis was performed using the SPSS 9 program for Windows (SPSS Inc., Chicago, IL, USA). Effects of time on wound healing, epithelialization and contraction was examined using repeated measurements ANOVA, and includes time as fixed factor and dogs as random factor. In addition paired t-test was used for the comparison of mean of measured parameters in each day between groups. Median of the groups hydroxyprolin values were compared using pair t-test.

For histopathology examination, the median of the groups were compared using a non-parametric Sign test. A value of $P<0.05$ was considered significant.

Results

PRP Preparation

Platelet counts in PRP showed substantial increase above the baseline number of platelets in the whole blood. The PRP centrifugation technique used in this experiment resulted in an average platelet enrichment of 3.56 ± 0.36X (range = 3.1X to 4X).

Macroscopic evaluation

The repeated-measures linear model with treatment and time as within-subject factors revealed no significant difference in percentage of contraction, epithelialization and healing among wounds. Initially, all wound areas increased in size. After the initial enlargement, wound areas decreased in size between days 10 up to 24 in control and test group ($P>0.05$) (Fig. 1, 2, 3).

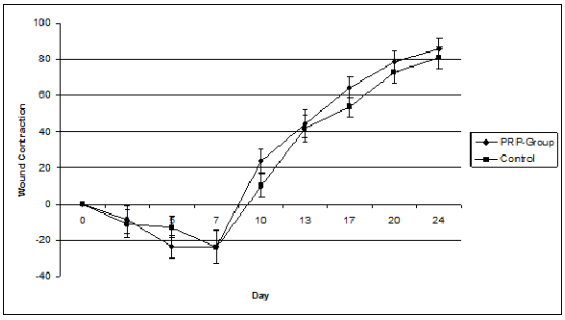

Figure 1. Percent of wound contraction in the control and test wounds. There were no significant differences between left (control) and right (PRP treated) wounds ($P>0.05$).

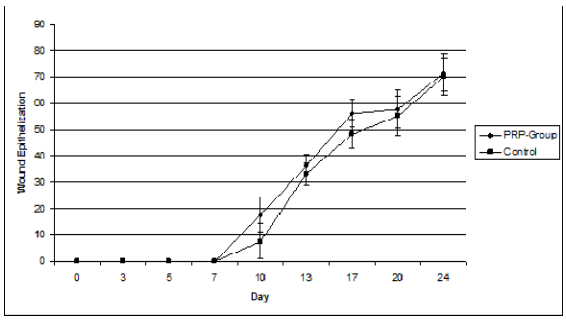

Figure 2. Percent of wound epithelialization in the control and test wounds. There were no significant differences between left (control) and right (PRP treated) wounds ($P>0.05$).

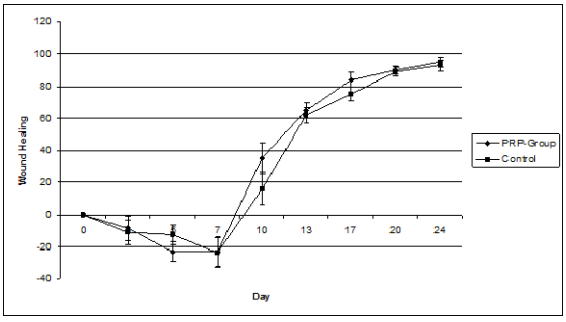

Figure 3. Percent of wound healing in the control and test wounds. There were no significant differences between left (control) and right (PRP treated) wounds ($P>0.05$).

Amount of hydroxyprolin

The differences in mean ± SD concentration of hydroxyprolin levels (µg/mg dry matter) between left and right wounds were not significant ($P>0.0.5$). Although, at the day 17 and 24 level of hydroxyprolin was higher in wounds treated with PRP (Fig. 4).

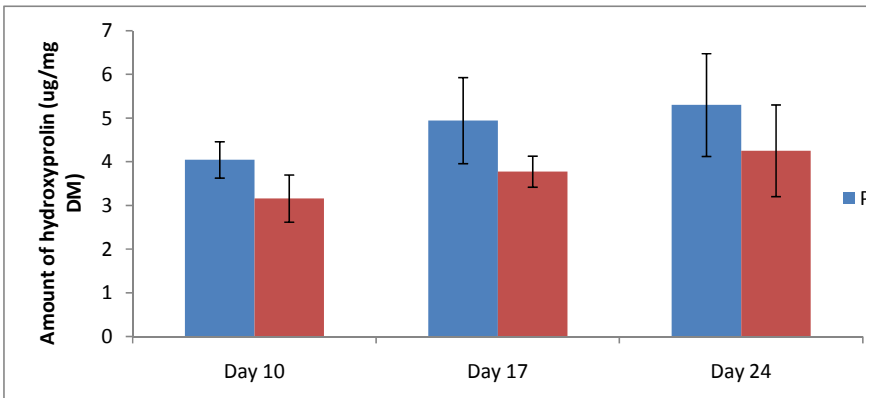

Figure 4. Amount of hydroxyprolin (µg/mg dry matter). There were no significant differences between left (control) and right (PRP treated) wounds (P>0.05)

Histopathologic evaluation

There were no significant differences between median of inflammatory cells infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition and collagen bundle formation scores, in the specimens from left and right wounds at the 10, 17 and 24 (P>0.05). The PRP-treated wounds, except at the day 24, showed a tendency toward increased epithelialization rate in both periods, but no statistical significance was found (Fig. 5). Descriptive studies have shown an increase in epithelialization, fibroblast proliferation and collagen bundle formation at the day 24 in PRP treated wounds.

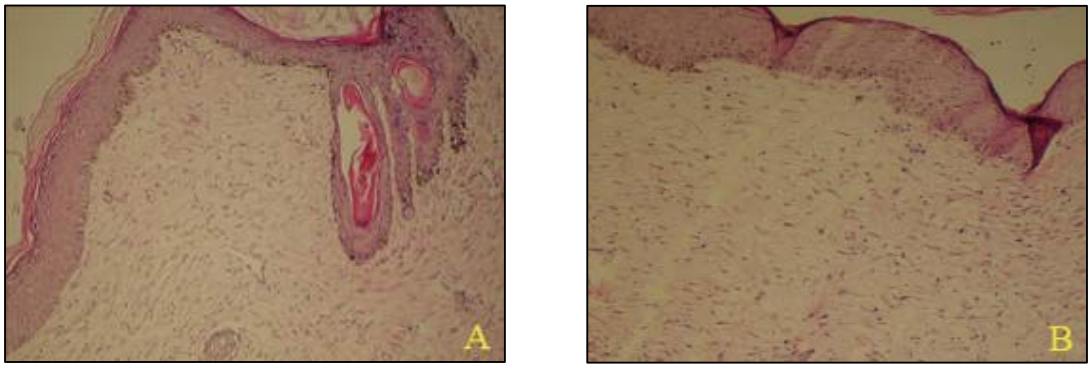

Figure 5. Thicker epithelium with acanthosis and prominent Rete pegs in PRP-treated skin (A) in comparison to the control skin (B), Day 17. Proliferation of fibroblasts is also more prominent in the PRP-treated skin. Hematoxylin and eosin stained sectioned through the middle of the wounds. X64.

Discussion

In the recent years, application of platelet-rich plasma to enhance bone regeneration and soft tissue maturation has been widely extended in the fields of orthopedic, periodontic, maxillofacial, plastic, thoracic and vascular surgeries, as well as ophthalmological procedures.[2,22-31] However, some controversies exist about the efficacy of PRP application.[2] While some authors reported the effectiveness of PRP gel in the treatment of nonhealing chronic wounds, others did not report any improvement.[7,12,32-36] This might be due to differences in experiment (animal, human), wound defect model, differences in PRP biology

among species, differences in PRP preparation techniques, differences in PRP activity and differences in investigated time points.[37]

Herein, we have developed a new wound sealant composed of concentrated PRP, thromboplastin, and chloride calcium that is delivered as a topical gel to cutaneous wounds. Findings in this study do not support the hypothesis that application of PRP (coagulated with thromboplastin and chloride calcium) as wounds treatment can accelerate or improve quality of repair. Macroscopic, biochemical and histological studies showed that wound healing was not enhanced when PRP is used in this particular model of wound in dogs.

In this study, the rate of healing was assessed in a relative manner (ie, reducing the percentage of wound surface area over time), which took into account the exact dimensions of the original wounds. Indeed, despite all the efforts made to standardize wound size at surgery, it was almost impossible to ensure that each wound possessed identical dimensions at the beginning of the study. Data presented here has revealed no significant difference in macroscopic evaluation of the wounds during the time period assessed.

The therapeutic impact of platelet concentration and hence growth factor release in animal PRPs has still to be defined. Some in vitro studies showed concentration-dependent effects of PRP on the proliferation of human mesenchymal stem cells, fibroblast proliferation and type I collagen production.[37,38] It is suggested that PRP should contain at least 3- to 5-fold increase in platelet concentration over baseline, in order to ensure a therapeutic effect.[7]

Hom et al., have reported the accelerating effect of autologous platelet gel on epithelialization of human full-thickness wounds. Particularly, when the platelet count in the gel is more than 6 times the baseline intravascular platelet count.[7,39] This histologic finding may be due to a higher concentration of growth factors available to influence healing from the higher number of platelets delivered. Herein, no effects of canine PRP on wound reepithelialization was found statistically. This corroborates the previous equine PRP studies by Monterio et al. These investigators evaluated the effect of topical application of autologous PRP to full-thickness skin wounds located on the distal aspect of the limb in horses. Their results have revealed that topical application of autologous PRP does not enhance wound epithelialization. Although platelet counts in the PRP were < 6 times baseline values.[7] On the contrary, in the study of Carter et al., equine PRP was reported to enhance epithelial differentiation and wound healing significantly in distal limb of horses.40 In our investigation, the epithelialization rate in the PRP treated groups increased 5- and 1.4-fold more than controls at the day 10 and 17 of study, respectively. Although, the final results of the present study did not indicate any significant difference regarding the epithelialization. This could be due to diminishing the PRP gel effects after the last application at the day 6 after wounding. These findings are consistent with earlier reports suggesting that PRP gel had its greatest effect on healing on 14 days after wounding.[39] This finding also correlates with a recent study on a rodent model showing that the effect of platelet gel on healing appears to occur in a transient fashion within 14 days of administration.[39,41]

The regenerative potency of PRP certainly depends on its growth factors content. Weibrich et al. demonstrated that neither whole blood nor PRP platelet counts can reliably predict the resultant growth factor levels in PRP.[2,42,43] Since the levels of growth factors in the PRP samples of the present study were not measured due to the experimental model used, further studies seem necessary to confirm this hypothesis, even though there were several boundaries in this study. Moreover, the platelet counts in the PRP concentrate were less than 6 times baseline values. It seems that more beneficial results in wound healing could be achieved if platelet numbers were more than 6 times the baseline platelet count levels during the PRP processing. It should also be noted that the levels of growth factors in the PRP samples need

to be measured, so further works are necessary to determine if thromboplastin has effect on the release of growth factors.
In conclusion, 3-time topical application of autologous platelet-rich plasma coagulated with thromboplastin and calcium chloride on full-thickness skin wounds can not accelerate or improve the quality of repair.

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چکیده

فعال شده با ترومبوپلاستین–د (PRP) تاثیر پلاسمای غنی از پلاکت خودی در التیام و بازسازی زخم در سگ ها

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هدف– ارزیابی تاثیر پلاسمای غنی از پلاکت فعال شده با ترومبوپلاستین در روند التیام پوست در سگ.

طرح مطالعه– مطالعه تجربی در حیوان زنده.

حیوانات– ۵ قلاده سگ نر نژاد مخلوط.

روش کار– تحت بیهوشی عمومی شش عدد زخم تمام ضخامت به اندازه ی ۲۰×۲۰ میلی متر در ناحیه ی پشت به صورت قرینه ایجاد شد. در هر حیوان زخم های طرف راست به عنوان گروه درمان انتخاب شدند که به وسیله ی ۱/۵ سی سی ژل پلاسمای غنی از پلاکت درمان شدند و بر روی زخم های طرف چپ ستون مهره ها به همین میزان متیل سلولز قرار داده شد (گروه کنترل). بیست و چهار ساعت پس از ایجاد زخم ها درمان شروع شد و به صورت یک روز در میان تا سه بار ادامه پیدا کرد. در روزهای صفر، ۳، ۵، ۷، ۱۰، ۱۳، ۱۷، ۲۰ و ۲۴ از تمامی زخم ها عکس های دیجیتالی تهیه شد. در روزهای ۱۰، ۱۷ و ۲۴ بعد از ایجاد زخم ها از مرکز و گوشه ی تمامی زخم ها نمونه ی بافتی به ترتیب برای بررسی میزان هیدروکسی پرولین و هیستوپاتولوژی گرفته شد.

نتایج– از نظر ماکروسکوپیک هیچ اختلاف معنی داری بین دو گروه درمان و کنترل از نظر درصد تشکیل بافت اپی تلیال، درصد انقباض زخم و درصد التیام دیده نشد ($p<۰/۰۵$). همچنین هیچ اختلاف معنی داری بین دو گروه درمان و کنترل از نظر میزان هیدروکسی پرولین، خونریزی، بافت نکروزه، توده فیبرینی، سلول های آماسی، بافت جوانه ای، فیبروپلازی وجود نداشت ($p<۰/۰۵$). در زخم های گروه درمان میزان اپی تلیزاسیون در روزهای ۱۰ و ۱۷ بیشتر از زخم های گروه کنترل بود اما از لحاظ آماری معنی دار نبود.

نتیجه گیری و کاربرد بالینی– درمان زخم های تمام ضخامت با اندازه ی کوچک در ناحیه ی پشتی سگ ها با پلاسمای غنی از پلاکت در سه نوبت باعث بهبود در تسریع روند التیام نمی شود.

کلید واژگان– سگ، التیام زخم، پلاسمای غنی از پلاکت، فاکتورهای رشد.