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ORIGINAL ARTICLE

Effects of platelet-rich plasma (PRP) on cutaneous regeneration and wound healing in dogs treated with dexamethasone

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Abstract To evaluate the effects of platelet-rich plasma (PRP) on cutaneous regeneration and wound healing in dogs treated with dexamethasone, the present study was undertaken. Under general anesthesia, six full-thickness skin wounds were created on the back of five male adult dogs symmetrically. Left side wounds were left without any treatment, and right side wounds were treated topically with PRP jelly. Six days before creating the wounds, dogs received dexamethasone, 0.5 mg/kg IM, and every other day up to day 8 after wounding. For macroscopic evaluation, digital photographs were taken from wounds. In days 10, 17, and 24 after wounding, skin biopsies were taken from the center and corner of each wounds for hydroxyproline measurement and histopathological evaluation. No significant difference was seen in the percentage of wound contraction, epithelialization, and healing between test and control groups during the study ($P>0.05$). There were no significant differences between median of hydroxyproline levels between left and right wounds in dogs treated with dexamethasone ($P>0.0.5$). There were no significant differences between median of epithelialization, inflammatory cell infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation scores, in the specimens of left and right wounds ($P>0.05$). The results of the present study demonstrated that PRP did not have significant effects to promote cutaneous regeneration and wound healing in dogs treated with dexamethasone at least 16 days after last injection.

Keywords Dog · Cutaneous wound healing · Platelet-rich plasma · Growth factor

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Introduction

The major goal of wound healing biology is to determine how a wound 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 (Ferguson et al. 2005). The healing of wounds is regulated by several cell types and by a cascade of peptides known as cytokines or growth factors. After an injury, growth factor secretion by platelets and macrophages is induced, and inflammation-healing process is initiated (Theoret 2005). Although numerous growth factors are involved, transforming growth factors-β (TGF-β1) and platelet-derived growth factor (PDGF) play key roles in wound healing (Carter 2003; Anitua et al. 2004). TGF-β1 favors the chemoattraction of monocytes and macrophages, and a combined attraction and proliferation of fibroblasts (Carter 2003; Theoret 2005). These peptides also regulate the transcription of extracellular matrix proteins, including fibronectin, collagen, and

glycosaminoglycans (Braun et al. 2002; Theoret 2005). The ability to repair cutaneous wounds following injury is critical for survival. Advanced age and diseases such as diabetes or some drug such as corticosteroids alter the effectiveness of the wound healing process (Ashcroft et al. 1995; Singer and Clark 1999). Local application of growth factors has important therapeutic potential in the treatment of chronic wounds, although the exact mechanisms of synergistic action are not completely understood (Debus et al. 2000). Growth factors and their receptors regulate key aspects of soft and hard tissue repair (Benndt and Schultz 1993). Several clinical studies demonstrate that growth factor treatment accelerates healing of normal tissues and promotes healing of impaired wounds (Benndt and Schultz 1993). A previous study using topical application of diluted platelet-derived locally acting growth factors resulted in repair of previously nonhealing cutaneous wounds in human (Knighton et al. 1990). Growth factors are essential for regulating the cellular events involved in wound healing. Growth factors attract cells into the wound, stimulate their proliferation, and significantly influence on extracellular matrix deposition (Declair 1999; Nishimoto et al. 2007). TGF-β is particularly important because once it is activated, it affects most aspects of tissue repair, including its initiation and termination. TGF-β was predicted to be of therapeutic value in the treatment of chronic, nonhealing, or slow healing wounds over a decade ago (Ksander et al. 1990). PDGF improves dermal regeneration, acts locally to promote protein and collagen synthesis, causes endothelial migration or angiogenesis (Ross 1987), and induces the expression of TGF-β (Pierce et al. 1989a, b). TGF-β and PDGF have been reported to be released at sites of tissue damage during degranulation of platelet α-granules (Assoian et al. 1983). In addition to its therapeutic potency, a series of studies suggest an important role of endogenous PDGF in the repair process. Upon injury, PDGF is released in large amounts from degranulating platelets (Reuterdahl et al. 1993; Carmona et al. 2007), and it is presented in a wound's fluid, particularly early after injury (Breuing et al. 1997; Harris et al. 1995; Marikovsky et al. 1993; Soma et al. 1992). Furthermore, expression of PDGF and its receptor has been demonstrated in various cells of murine, pig, and human wounds using in situ hybridization and immunohistochemistry (Ansel et al. 1993; Antoniades et al. 1991; Whitby and Ferguson 1991; Arguelles et al. 2006). The patterns of PDGF and PDGF receptor expression suggest a paracrine mechanism of action, since the ligands are predominantly expressed in the epidermis, whereas the receptors are found in the dermis and granulation tissue. Interestingly, expression of PDGFs and their receptors was reduced in wounds of genetically healing-impaired diabetic *db*/*db* mice and glucocorticoid-treated mice (Beer et al. 1997, 2000), indicating that a certain expression level of PDGFs and their receptors is essential for normal repair. This hypothesis was supported by the finding that impaired wound healing in aged mice is associated with a delay in appearance of PDGF A and B isoforms and α- and β-receptors (Ashcroft et al. 1997). Finally, the levels of PDGF in nonhealing human dermal ulcers were strongly reduced compared with surgically created acute wounds (Pierce et al. 1995) and provided further supporting of the important role of PDGF for normal healing. On the other hand, augmented PDGF production might be involved in the pathogenesis of hypertrophic scars and keloids as suggested by the potent effect of PDGF on fibroblast proliferation and extracellular matrix production, the presence of enhanced levels of this growth factor in hypertrophic scar tissue (Niessen et al. 2001), and the increased responsiveness of keloid fibroblasts to PDGF (Haisa et al. 1994). In the present study, the potential roles of PDGF in preventing the negative effects of glucocorticoids in cutaneous wound healing of dogs have been investigated.

Materials and methods

Dogs and experimental setup

Five male, mixed breed dogs (31±4 kg) were used in the study. They were approximately 4 years old. The dogs were kept in kennels, fed a maintenance ration twice daily, and had free access to water. They were given rabies vaccine and antiparasitic drugs (praziquantel 5 mg/kg and piperazine 100 mg/kg, orally). Clinical examination, complete blood count and blood serum biochemical analysis (blood urea nitrogen, creatinine, alanine aminotreasferase, asparatate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, cholesterol, and glucose) were carried out to confirm the health status of the animals. Skin preparation protocol consisted of hair clipping and povidon iodine scrubbing. Under general anesthesia (using acepromazine 0.05 mg/kg IM and ketamine 20 mg/kg IM, followed by halotan), six full-thickness skin wounds (20× 20 mm) were created on the back of each dog symmetrically (three wounds in each side). Bleeding of the wound bed significantly reduced by pressing sterile tampon. Left side wounds (control group) were left without treatment, and right side wounds (test group) were treated topically with platelet-rich plasma (PRP) jelly. Six days before creating of the wounds, dogs received dexamethasone 0.5 mg/kg IM every other day up to day 8 after wounding. They received eight injections of dexamethasone totally. The wounds were covered with a sterile nonadhesive bandage, and the back of each dog was bandaged with an absorbent layer of cotton wool and elastic tape. Treatment of wounds was started 24 h after wounding by 1 ml of PRP jelly and continued every other day for three successive

days. The bandages were changed once daily. Biopsy were taken from the each pair of the wounds at the days 10 (pair 1), 17 (pair 2), and 24 (pair 3) after wounding to evaluate the healing process. The experimental protocol was approved by the University Ethical Committee.

Preparation of platelet-rich plasma

Dog platelet-rich plasma was prepared by collecting whole blood into a sterile bag containing acid citrate dextrose formula A anticoagulant, each morning before treating the wounds by PRP at the days 1, 3, and 5 after wounding. Afterward, they were centrifuged at 120×*g* for 5 min. The first supernatant plasma fraction (about 50%), adjacent to the buffy coat, was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged at 280×*g* for 5 min, and 25% from the first fraction was obtained. A present harvesting program for single donor platelet with concurrent plasma collection was used to collect a target yield of about 8×10^5/μl platelet (PRP). Normally, 40 ml whole citrated blood was used to prepare 1.5 ml of the PRP evaluated in the present study. Calcium chloride (4.5 mEq/5 ml, Zist Faravar Co., Iran) 50 μl per milliliter PRP and thromboplastin-D (commercially available for PT test) 200 IU/ml (Fisher Diagnostics, USA) were used to activate the platelets.

Macroscopic evaluation of the wounds

The wounds were evaluated over a 4 weeks' period. Digital wound photographs were taken on days 0, 3, 5, 7, 10, 13, 17, 20, and 24 after careful shaving of the area to visualize the wound margin. The scab of each wound was carefully removed for better visualization of epithelialization and granulation tissue formation by using the saline as the cleaning liquid. Rulers were held vertically and horizontally close to the wounds as a reference. The area of the epithelialization and granulation tissue were measured for each wound using Scion Image software. Percent of the wound contraction, epithelialization, and healing was calculated for each wounds based on the following formula:

- Wound contraction:
  1. Wound size at the day$(x)^{mm^2}$/wound size at the day$(0)^{mm^2}\times100$ = 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$(x)^{mm^2}$/size of the wound at the day$(x)^{mm^2}\times100$ = percent of the epithelialization

- Wound healing:
  1. Granulation tissue at theday$(x)^{mm^2}$/wound size at the day$(0)^{mm^2}\times100$ = percent of the nonhealed *area compared to the woundsize at the* day(0)
  2. 100−percent of the nonhealed area compared to the wound size at the day (0)=percent of the healing

Hydroxyproline measurement

At days 10, 17, and 24 after wounding, biopsies were taken from the center of each wound using 0.7-mm biopsy punch for hydroxyproline measurement. Tissue samples for hydroxyproline assay were washed with physiological saline and dried in a 100°C oven for 72 h. Hydroxyproline levels were determined by a spectrophotometer using the previously described method (Woessner 1961) in micrograms per gram dry matter. Initially, each specimen was weighed and hydrolyzed in 12-N HCl at 130°C for 3 h. Then each sample was adjusted to a final volume of 1 ml and centrifuged at 3,000×*g* for 15 min. The supernatant was separated off, and an equal volume of isopropanol was added. Then this mixture was centrifuged at 2,500×*g* for 10 min. Serial dilutions of pure hydroxyproline were used as standard, and the concentration of hydroxyproline in each sample was calculated using the absorbance–concentration curve for the standard hydroxyproline solutions.

Histopathological examination

At days 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 specimen from each dog was fixed in 10% buffered formalin, embedded in paraffin, cut at 5-μm sections, and stained with hematoxylin and eosin and Masson's trichrome. Different histological findings of each sample, including epithelialization, infiltration of inflammatory cells, presence of 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.

Statistical analysis

Statistical analysis was performed using the SPSS 9 program for Windows (SPSS Inc., Chicago, IL, USA). Effects of time on healing were examined using ANOVA. Effects of time on wound healing, epithelialization, and contraction were examined using repeated measurements and included time as a fixed factor and dogs as random factor. In addition, paired t test was used to compare each

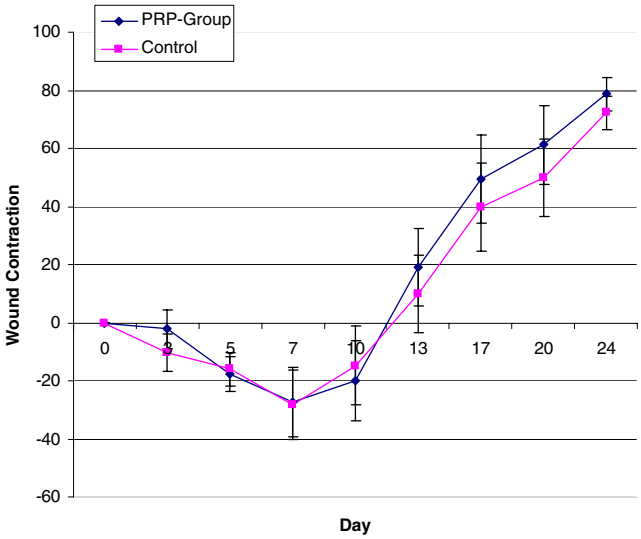

Fig. 1 Percent of wound contraction in the control and test wounds. There were no significant differences between left (*control*) and right (*PRP-treated*) wounds ($P>0.05$)

day between the groups. The median of the groups for hydroxyproline was compared using pair *t* test.

For histopathological examination, the median of the groups was compared using a nonparametric sign test. Differences were considered statistically significant when $P<0.05$.

Results

Macroscopic evaluation

Initially, all wound areas increased in size. After the initial enlargement, wound areas decreased in size between days

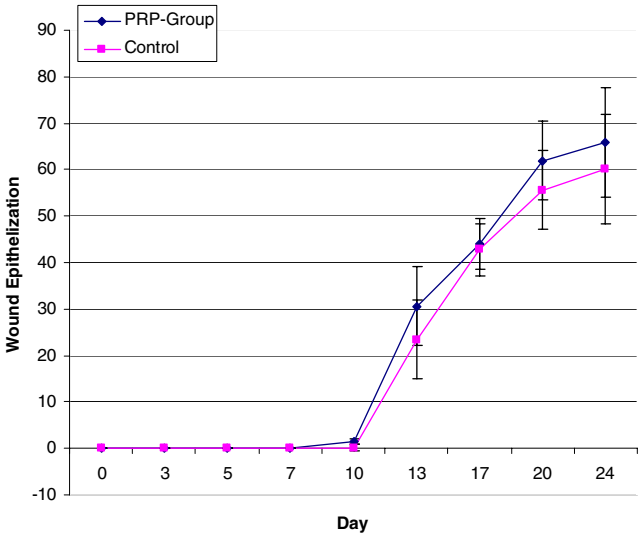

Fig. 2 Percent of wound epithelialization in the control and test wounds. There were no significant differences between left (*control*) and right (*PRP-treated*) wounds ($P>0.05$)

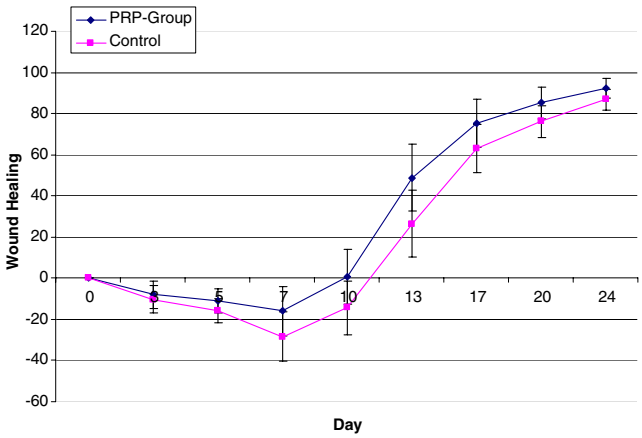

Fig. 3 Percent of wound healing in the control and test wounds. There were no significant differences between left (*control*) and right (*PRP-treated*) wounds ($P>0.05$)

10 and 24 in control and test group. No significant differences were seen in percent of wound contraction, epithelialization, and healing between test and control group during the study ($P>0.05$) (Figs. 1, 2, and 3).

Hydroxyproline levels

There were no significant differences between median of hydroxyproline levels (μg/mg dry matter) between left and right wounds in dogs treated with dexamethasone ($P>0.0.5$). Although at days 17 and 24, hydroxyproline level was greater in PRP-treated wounds (Fig. 4).

Histopathological evaluation

There were no significant differences between median of epithelialization, infiltration of inflammatory cells, presence of granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation scores in the specimens from left and right wounds at days 10, 17, and 24 of sampling ($P>0.05$).

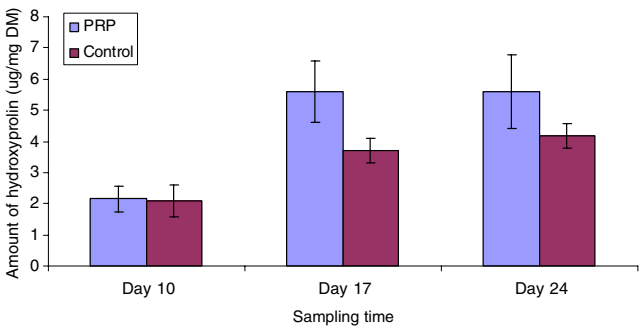

Fig. 4 Amount of hydroxyproline (μg/mg dry matter) in dogs treated with dexamethasone. There were no significant differences between left (*control*) and right (*PRP-treated*) wounds ($P>0.05$)

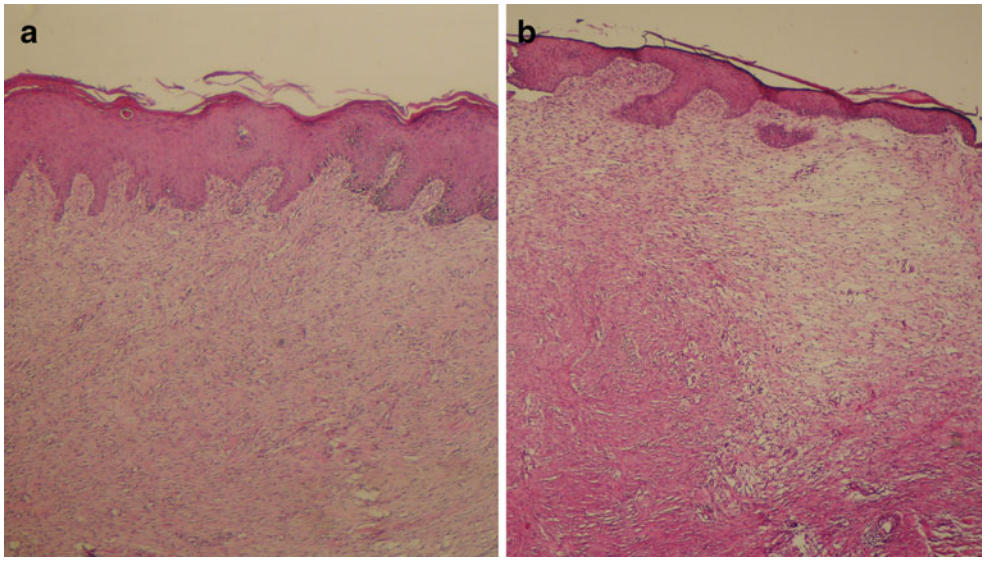

Fig. 5 Epidermal thickness is thicker in PRP-treated skin (**a**) in comparison to the control skin (**b**), day 17. Proliferation of fibroblasts is also more prominent in the PRP-treated skin. Hematoxylin and eosin-stained, sectioned through the middle of the wounds, ×64

Although at day 24, epithelialization, fibroblast proliferation, and collagen bundle formation were greater in PRP-treated wounds according to the descriptive study (Figs. 5 and 6; Table 1).

Discussion

For the activation of the platelets in the concentrated PRP, calcium chloride and thromboplastin-D were used at the present study (Mendelsohn et al. 2007). This inexpensive method is used for PT test and can be available in most veterinary clinics. A well-defined sequence of events follows dermal injury and normal healing. There is a controlled progression to re-epithelialization, scarring, and restoration of an intact epidermis. Hemostasis is promoted rapidly after injury when platelets released from blood at the wound site bind to freshly exposed tissue components (Carter et al. 2003). These cells contain many chemicals that act as messenger molecules responsible for initiating blood coagulation, inflammation, and wound healing. One of the most important molecules for healing is PDGF.

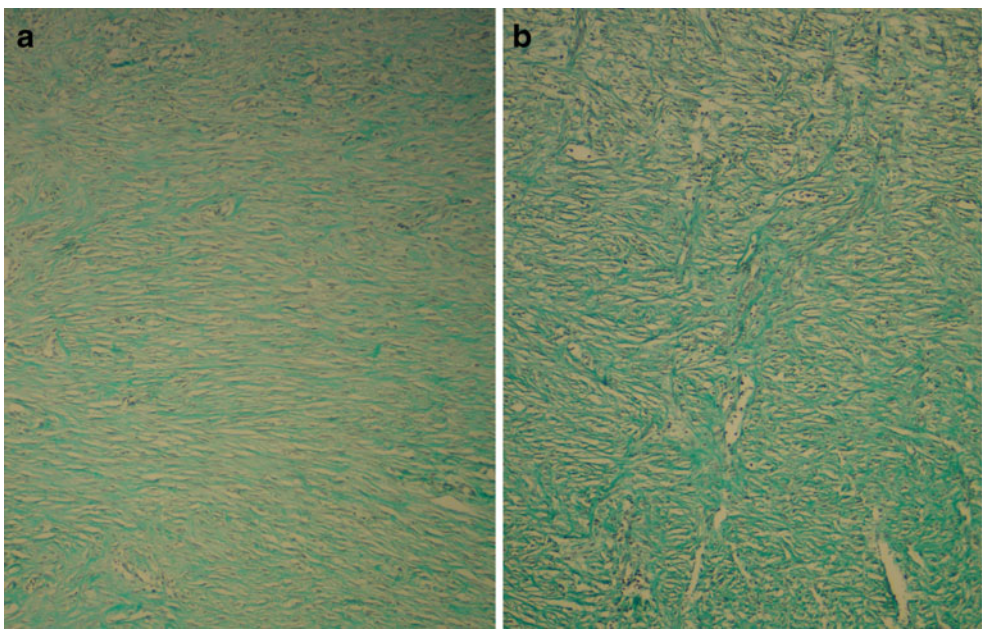

Fig. 6 Dermal collagen reorganization is more improved in PRP-treated skin (**a**) in comparison to the control skin (**b**), day 24. Masson's trichrome-stained, sectioned through the middle of the wounds, ×160

Table 1 Histological characteristics of wound healing in dogs treated with PRP

Left or right wounds	Percentiles																	
	25th						50th (median)[a]						75th					
	Left			Right			Left			Right			Left			Right		
Day	10	17	24	10	17	24	10	17	24	10	17	24	10	17	24	10	17	24
Epithelialization	0.00	0.00	0.75	0.00	0.5	0.75	0.00	0.00	0.75	0.00	0.5	0.5	0.5	0.5	0.75	0.5	1.75	1.75
Inflammatory infiltration	1	1	0.75	0.75	1	0.75	1.75	1.75	0.75	0.5	1.75	0.5	2.75	2	1.75	1.75	2	0.75
Presence of dermal granulation tissue	0.25	0.75	1	0.25	1.75	1.25	1.5	0.75	1.5	1.5	1.5	1.75	2	2.25	1.75	1	1.75	2
Fibroblast proliferation	1	1	1.75	1	1	2	1	1	1	1	1.25	1	2	2.25	2	2	2.75	3
Arrangement of fibroblasts	0.00	0.5	1.25	0.00	1.25	1.25	1	1.75	1.75	1	1.75	2.25	2	2	2	2.25	1.75	2.75
Collagen deposition	0.25	0.25	0.5	0.5	1.75	2	0.5	2	2	0.25	2	2	2	2	2	2.25	2.75	3
Collagen bundle formation	0.00	0.00	0.75	0.00	0.75	1.25	0.00	0.5	1.75	0.00	0.75	2	0.00	1	2	0.00	1.25	3

[a] There were no significant differences between median of each finding in left and right wounds ($P>0.05$)

Fibroblasts at the wound site are stimulated by PDGF and other growth factors to proliferate and produce extracellular matrix (ECM) as a component of granulation tissue. The presence of functional ECM is required to allow keration-cyte migration from the wound edge and re-epithelializes the wound surface with eventual scar formation. Cutaneous wound healing involves repair and regeneration. It is controlled by growth factors that regulate expression, enzyme production, cellular differentiation, proliferation, metabolism, and migration, as well as the synthesis and remodeling of ECM proteins (Steed 1998; Komarcevic 2000; Carter et al. 2003). The ECM then coordinates cellular events and modulates cellular responsiveness to growth factors and cytokines. Glucocorticoids, in contrast, inhibit wound healing in humans and in animal models of tissue repair (Kane et al. 1991; Roberts et al. 1988). The inhibition of procollagen synthesis in fibroblasts and the decrease in circulation monocyte levels associated with systemic administration of glucocorticoids sharply reduce the potential host responses for wound repair in vivo (Kane et al. 1991; Tsunawaki et al. 1988). Because of therapeutic potency of PDGF, a series of studies suggest an important role of endogenous PDGF in the repair process. Upon injury, PDGF is released in large amounts from degranulat-ing platelets (Roberts and Sporn 1990), and it is present in wound fluid, particularly early after injury (Matsuoka and Grotendorst 1989). At the present experiment, there was no significant difference in macroscopic evaluation of the wounds during the study. Dogs at the present study received dexamethasone IM and 6 days before up to 8 days after wounding, so this result could be due to the effects of dexamethasone on wound healing. Expression of PDGF and its receptor has been demonstrated in various cells of murine, pig, and human wounds using in situ hybridization and immunohistochemistry (Ono et al. 1995; Rengove et al. 2000; Vogt et al. 1998). The patterns of PDGF and PDGF receptor expression suggest a paracrine mechanism of action, since the ligands are predominantly expressed in the epidermis, whereas the receptors are found in the dermis and the granulation tissue. Interestingly, expression of PDGF and its receptor was reduced in wounds of healing-impaired genetically diabetic *db*/*db* mice and glucocorticoid-treated mice (Werner and Grose, 2003; Carter et al. 2003), indicating that a certain expression level of PDGF and its receptor is essential for normal repair. At the present study, no significant differences were seen in the amount of hydroxyproline in test and control groups. As previously mentioned, certain expression level of PDGF and its receptor is essential for normal repair. Dexamethasone can reduce the expression level of PDGF and other cytokines in wounds. Statistically there was no significant difference in the amount of hydroxyproline, but the amount of hydroxyproline increased at days 17, and 24

in PRP-treated group. This finding could be attributed to the reducing effects of dexamethasone after the last injection at day 8 after wounding.

TGF-β1 is produced by a variety of cells normally recruited to an injury site. TGF-β1 regulates cellular differentiation, proliferation, chemotaxis, and synthesis of many ECM components (Roberts et al. 1988; Kane et al. 1991; Carter et al. 2003). The release of H_2O_2 by macrophages is suppressed by TGF-β (Tsunawaki et al. 1988; Carter et al. 2003), so that growth factors and cytokines secreted by macrophages exert their effects, but the ability to induce cell death by oxidative stress is alleviated (Roberts and Sporn 1990; Carter et al. 2003). Topical application of TGF-β1 in cutaneous wounds in pig and rat promotes healing (Mustoe et al. 1987; Pierce et al. 1989a, b; Carter et al. 2003). Differentiation can be induced by natural compounds (Carter and Parham 1997; Carter and Madden 2000; Carter et al. 2003), including growth factors. TGF-β decreases basal keratinocyte proliferation and induces suprabasal cell differentiation to stimulate epidermal regeneration associated with cutaneous wound healing (Choi and Fuchs 1990; Kane et al. 1990, 1991; Carter et al. 2003). At the present study, no significant differences were seen in histopatological evaluation of healing between test and control groups. As previously mentioned results for macroscopic and hydroxyproline evaluation between test and control groups, this could be attributed to the effects of dexamethasone during the study. Although at the day 24, descriptive study of PRP-treated wounds showed mature granulation tissue characterized by dense parallel dermal collagen bundles.

Dense, tightly packed, organized collagen fiber bundles are characteristic of mature granulation tissue, whereas thin, randomly organized collagen bundles containing many fibroblasts characterize the early stages of granulation tissue deposition (Moyer et al. 2002). Treatment of fibroblasts with TGF-β significantly increases cellular synthesis of collagen, fibronectin, and glycosaminoglycans and promotes matrix formation (Hsuan 1989; Ignotz and Massagué 1986; Carter et al. 2003). TGF-β triggered synthesis and rapid maturation of collagen in early wounds caused by cutaneous incisions in rabbits (Carter et al. 2003). When PDGF was used in diabetic rats, collagen deposition in experimental wounds increased to the level of control, nondiabetic animals (Grotendorst et al. 1985). Combined PDGF and TGF-β resulted in higher collagen deposition than rats treated just by TGF-β (Lawrence et al. 1986; Carter et al. 2003). The results of the present study demonstrated that PRP cannot promote wound healing significantly in dogs treated with dexamethasone at least 16 days after last injection. But beneficial effects of PRP as a source of important growth factors needed for wound healing should be kept in mind.

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