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ORIGINAL ARTICLE

Role of collagen cross-linking on equine wound contraction and healing

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Abstract The present study was carried out to evaluate the effect of collagen cross-linking inhibition on equine wound contraction and healing. In five male adult donkeys, two full-thickness skin wounds (20×20 mm in diameter) were created on the lateral aspect of forelimbs, at the mid-point between the carpal and fetlock joints under general anesthesia. Two other wounds were created on the neck of each donkey symmetrically. Left-side wounds (test group) and right-side wounds (control group) were treated topically with beta-aminopropionitrile fumarate, 5 mg/ml, added to methyl cellulose gel and only methyl cellulose gel, respectively. Treatment of wounds were started at 24 h after wounding and continued every other day for ten successive days. The wounds were evaluated over a 3-week period. On days 0, 1, 3, 5, 7, 9, 12, 15, 18, and 21, digital photographs were taken of all wounds after careful shaving to visualize the wound margin. Rulers were held vertically and horizontally close to the wound as a reference. Epithelialization and granulation tissue formation were measured for each wound using Scion Image software. Percentage of the wound contraction, epithelialization, and healing were calculated for each wounds. At the end of the study, biopsy was taken from the center of each wound for hydroxyproline measurement and the same corner of each wound for histopathological examination. Macroscopic evaluation revealed significant differences in wound contraction and healing process between test and control groups in wounds located in neck ($P<0.05$), but there was no significant difference in percent of epithelialization at the same area ($P>0.05$). Significant differences were observed in the percent of wound contraction and healing between the test and control groups in wounds located in the forelimb ($P<0.05$), but no significant difference was observed in percent of epithelialization at this area ($P>0.05$). There were no significant differences between median of hydroxyproline levels of left and right wounds in forelimb and neck ($P>0.05$). Histopathological examination revealed no significant differences between median of epithelialization, inflammatory infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation scores in the specimens prepared from left and right wounds in forelimb and neck ($P>0.05$). Our data demonstrated that collagen cross-linking could play a key role in equine wound contraction and healing at the limb and neck area.

Keywords Wound · Collagen cross-linking · Cross-linking inhibitor · Contraction · Healing · Beta aminopropionitrile fumarate · Equine

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Introduction

Wound contraction and epithelialization are clinically important biological processes in healing. Closure of a full-thickness cutaneous wound occurs as a result of two

independent processes, contraction, and epithelialization. Contraction reduces the size of a wound by centripetal movement of dermis and epidermis that border the defect (Lee et al. 1986; Swaim and Lee 1987; Swaim and Henderson 1990). Epithelialization is the process by which cells from the epidermis at a wound's edge proliferate and migrate to cover the surface of the cutaneous defect (Swaim et al. 2001). Epithelial regeneration begins soon after a wound is created, continues independently, and covers the wound's granulation bed (Swaim et al. 2001). Wound contraction is a major component of second-intention wound healing, and the center for contraction is granulation tissue (Swaim et al. 2001). Although wound contraction is a clinically important biological process, it frequently results in contraction, stricture, and stenosis (Joseph et al. 1997). The mechanism of this process is not completely understood. Two theories have been described for the mechanism of wound contraction. One theory suggests that wound contraction results from migrating fibroblast that move through and rearrange connective tissue in granulating wounds. The activity of the fibroblasts on the connective tissue, composed predominately of collagen, is sufficient to cause centripetal movement of the skin margin. Simultaneous formation of collagen cross-linking maintains the dimensions of the wound as it decreases its surface area over time (Ehrlich 1988). The second theory, which carries the weight of current opinion, has been described as myofibroblasts; these highly specialized contractile fibroblasts found in granulating wounds are attached to one another by cell–cell connection and to the extracellular matrix (Gabbiani et al. 1971). Thus, they are capable of contracting synchronously to generate the centripetal force of wound contraction. Proponents of the latter theory suggest that collagen has very little to do with wound contraction (Gabbiani et al. 1971). Supportive of this idea are studies using controlled systemic lathyrism to prevent the formation of contractures (Peacock 1984; Peacock and Madden 1969). But recently, an in vitro model has been shown that the collagen cross-linking inhibition significantly reduce the wound contraction (Redden and Doolin 2003). Covalent cross-links are formed between the collagen fibrils by two different processes. Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) cross-links are the end products of the enzyme-mediated hydroxylysine aldehyde pathway and thus of one of the post-translational modifications of collagen (Eyre 1987; Last et al. 1990). The drug beta aminopropionitrile fumarate (BAPN-f), a powerful inhibitor of collagen cross-linking, has been reported to have an effect on the formation of scar tissue by inhibiting of lysyl oxidase when applied intralesionally, thus blocking lysine deamination, which is an important first step in the formation of covalent HP and LP cross-links (Nimbi 1988; Cohen 1985; Hoffman et al. 1983). In addition, it has been reported that the BAPN-f affects the collagen cross-linking by inhibiting lysyl oxidase and causing an accumulation of neutral salt soluble collagen in the skin of rats (Nimni et al 1969). Furthermore, it has been reported that BAPN-f penetrates quickly into the wound, when applying locally (Gibeault et al. 1989). Wound healing in equine distal limb is a challenge, and over the past two decades, extensive cellular and molecular details have been elucidated regarding the regulation of wound healing in equine distal limb (Carter et al. 2003). During wound healing in some cases, exuberant granulation tissue formation is a problem at this part of the equine body, even in well-treated animals (Arguelles et al. 2006). Regarding the two theories about wound contraction and the role of the collagen and collagen cross-linking in this process, a question has been arisen about the collagen cross-linking and its effect on wound contraction and healing in equine clinical cases. The aim of the present study was to determine the role of collagen cross-linking in wound contraction and healing in clinically normal donkeys, after blocking of the collagen cross-linking by BAPN-f in the full-thickness wounds on distal limb and neck area.

Materials and methods

Animals and experimental set-up

Five male, native donkeys (185±17 kg) were selected. They were approximately 5 years old. The donkeys were housed in stable, fed a maintenance ration three times daily with alfalfa hay and commercial concentrate and had free access to water. To investigate the health condition of the animals, clinical examination, complete blood count, and blood serum biochemical analysis (blood urea nitrogen, creatinine, alanine aminotreasferase, asparatate aminotrasnferase, alkaline phosphatase, gamma-glutamyltransferase, cholesterol, and glucose) were carried out. Skin preparation protocol consisted of hair clipping and povidon iodine scrubbing. Under general anesthesia (using xylazin 1.1 mg/kg, i.v. and ketamine 2.2 mg/kg, i.v.), two full-thickness skin wounds (20×20 mm in diameter) were created on the lateral aspect of forelimbs at the mid-point between carpal and fetlock joints, and two other wounds were created on the neck of each donkey symmetrically. Bleeding was controlled by pressure with a sterile tampon. Left-side wounds (test group) and right-side wounds (control group) were treated topically with BAPN-f, 5 mg/ml (ICN Biomedical, USA) added to methyl cellulose gel (Kruse Company, Denmark) and only methyl cellulose gel, respectively. The wounds were covered with a sterile bandage with an absorbent layer of cotton wool and elastic tape. Wounds were started to treat at 24 h after wounding

and continued every other day for ten successive days. The experimental protocol had been approved by the ethical committee of the university.

Macroscopic evaluation of the wounds

The wounds were evaluated over a 3-week period. On days 0, 1, 3, 5, 7, 9, 12, 15, 18, and 21, digital photographs were taken of all wounds after careful shaving to visualize the wound margin. The scab of each wound was carefully removed for better visualization of epithelialization and granulation tissue area by using saline as the cleaning liquid. 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 Scion Image software. Percentage of wound contraction, epithelialization, and healing were calculated for each wound. The following formulate were used:

- Wound contraction
  1. Wound size at the day $(x)^{mm2}$/wound size at the day $(0)^{mm2} \times 100$=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 $(x)^{mm2} \times 100$=percent of the epithelialization

- Wound healing
  1. Granulation tissue at the day $(x)^{mm2}$/wound size at the day $(0)^{mm2} \times 100$=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

Hydroxyproline measurement;

At the end of the study, biopsy was taken from the center of each wound using 0.7-mm biopsy punch for hydroxyproline measurement. The tissue samples for hydroxyproline assay were washed with physiologic saline and dried in a 100°C oven for 72 h. Hydroxyproline levels were determined spectrophotometrically using the previously described method (Woessner 1961) in μg/mg of 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 the end of the study, biopsy was taken from the same corner of each wound using 0.9-mm biopsy punch for histopathological examination. The wound specimen from each donkey was fixed in 10% buffer formalin and embedded in paraffin. A microdermatome was used to cut 5-μm sections from the block, and these were mounted on slides. The slides from each wound were stained with hematoxylin and eosin and Masson's trichrome. In each sample, 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.

Statistical analysis

Statistical analysis was performed using the SPSS 11.5 program for Windows (SPSS, Chicago, IL, USA). Effect of time on healing was examined using analysis of variance (ANOVA). Effect of time on wound healing, epithelialization, and contraction was examined using repeated measurements and included time as fixed factor and donkeys as random factor. In addition, paired *t* test was used for the comparison of each day between groups. The median of the groups for hydroxyproline were compared using Wilcoxon

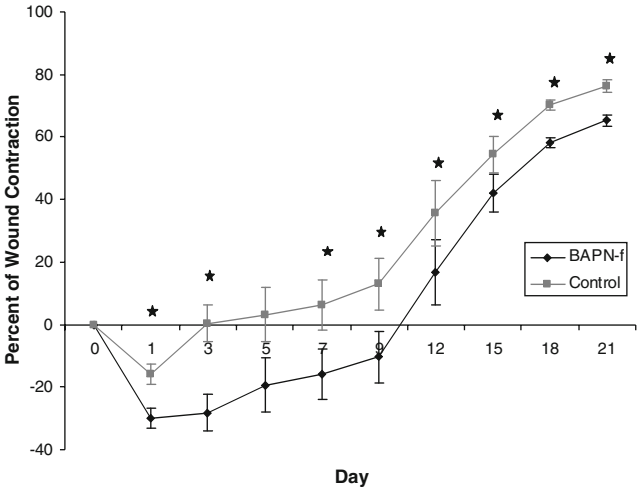

Fig. 1 Percent of wound contraction in the control and test wounds at the neck area. Significant differences were seen between groups (*$P<0.05$)

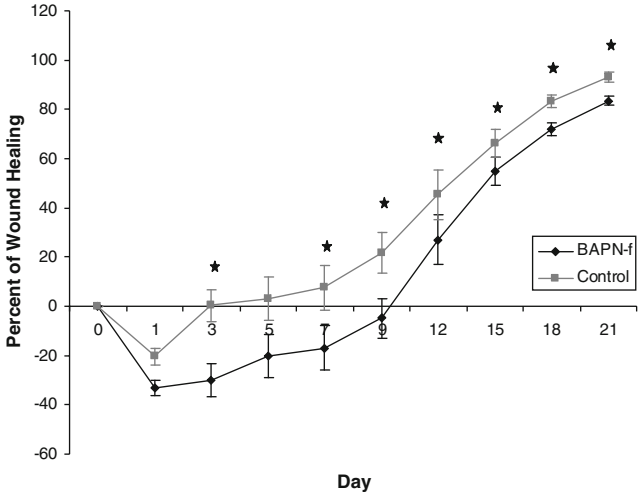

Fig. 2 Percent of wound healing in the control and test wounds at the neck area. Significant differences were seen between groups (*P<0.05)

signed ranks test. For histopathological examination, the median of the groups were compared using a non-parametric sign test. Differences were considered statistically significant when $P<0.05$.

Results

Macroscopic evaluation

Initially, all wound areas increased in size especially in neck. After the initial enlargement, forelimb wound areas decreased in size between day 3 up to 21 in the control groups and day 7 up to 21 in test groups, and neck wound areas decreased rapidly in size between day 3 up to 21 in the control groups and day 10 up to 21 in test groups. Significant differences were observed in the percent of wound contraction on days 1, 3, 7, 9, 12, 15, 18, and 21

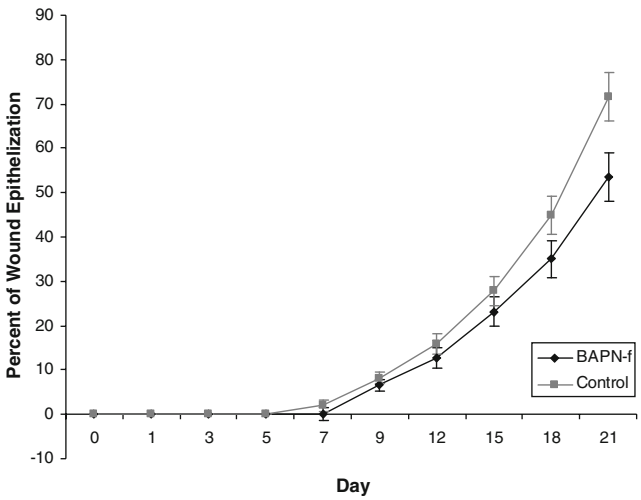

Fig. 3 Percent of wound epithelialization in the control and test wounds at the neck area. No significant differences was seen between groups ($P>0.05$)

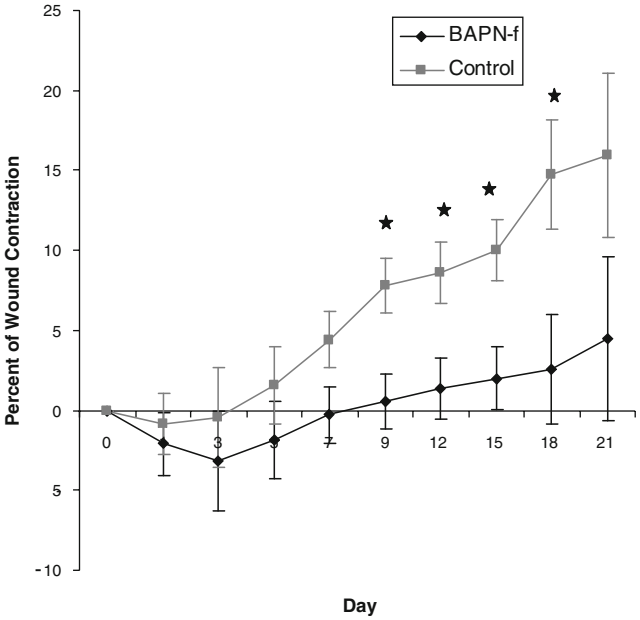

Fig. 4 Percent of wound contraction in the control and test wounds at the forelimb area. Significant differences were seen between groups (*$P<0.05$)

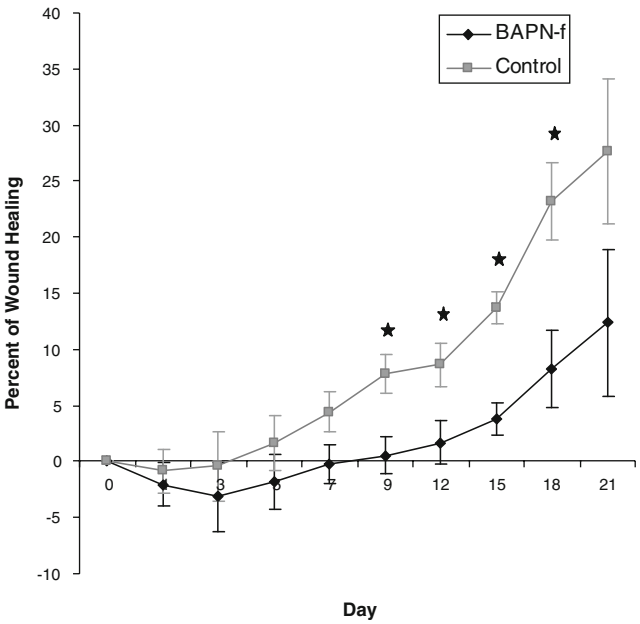

Fig. 5 Percent of wound healing the control and test wounds at the forelimb area. Significant differences were seen between groups (*$P<0.05$)

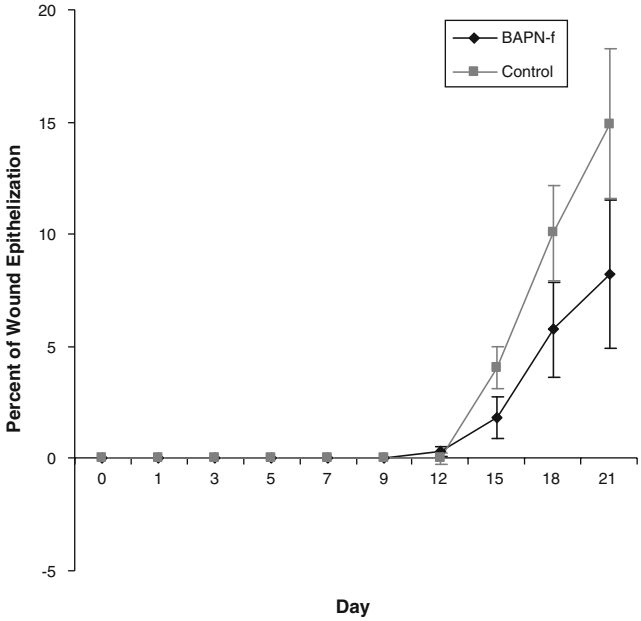

Fig. 6 Percent of the epithelialization in the control and test wounds at the forelimb area. No significant differences was seen between groups ($P>0.05$)

and healing on days 3, 7, 9, 12, 15, 18, and 21 between the test and control groups in neck wound area ($P<0.05$; Figs. 1 and 2), but no significant difference was seen in percent of epithelialization at this site ($P>0.05$; Fig. 3). Significant differences were observed in the percent of wound contraction on days 9, 12, 15, and 18 and healing on days 9, 12, 15, and 18 between the test and control groups in forelimb wound area ($P<0.05$; Figs. 4 and 5), but no significant difference was seen in percent of epithelialization at this site ($P>0.05$; Figs. 6, 7, and 8).

Amount of hydroxyproline

There were no significant differences between median of hydroxyproline levels (μg/mg dry matter) in the specimens from left and right wounds in forelimb and neck ($P>0.05$; Table 1).

Histopathological evaluation

There were no significant differences between median of epithelialization, inflammatory infiltration, presence of

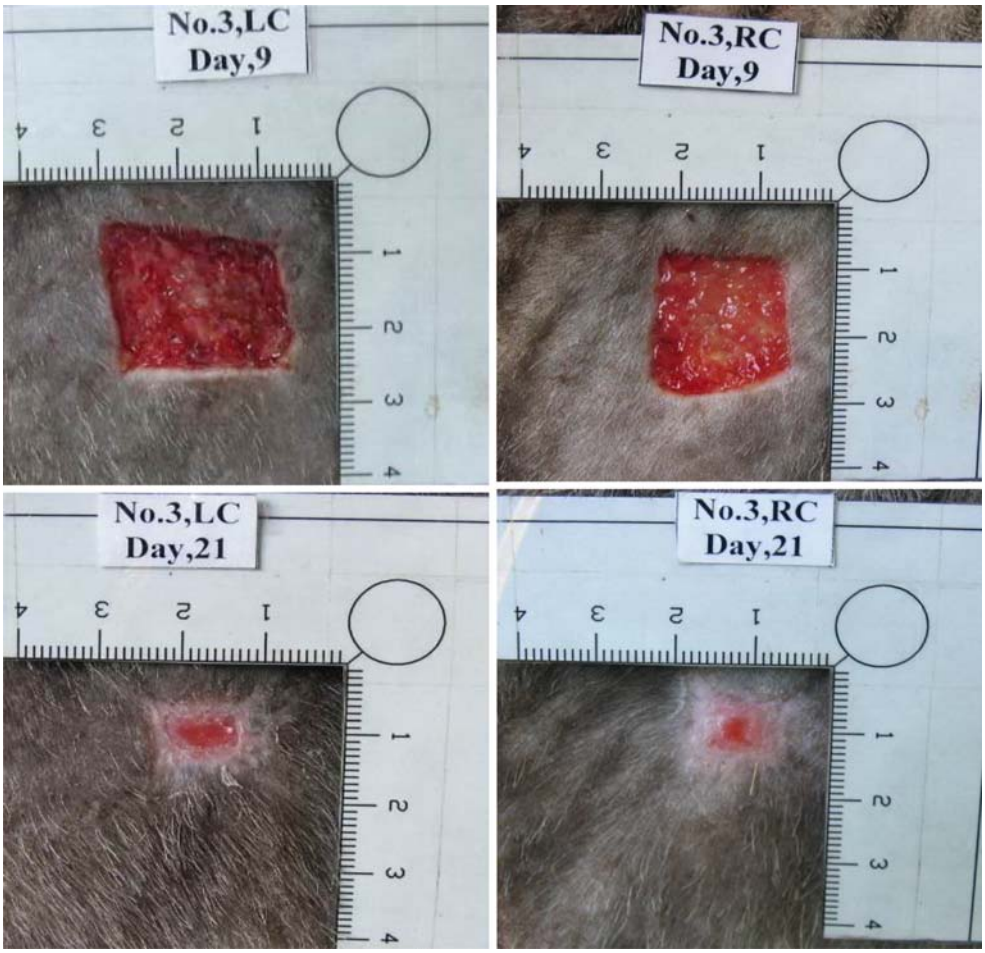

Fig. 7 Wounds at days 9 and 21 in test (*left*) and control (*right*) group in neck area. *RC* right cervical, *LC* left cervical

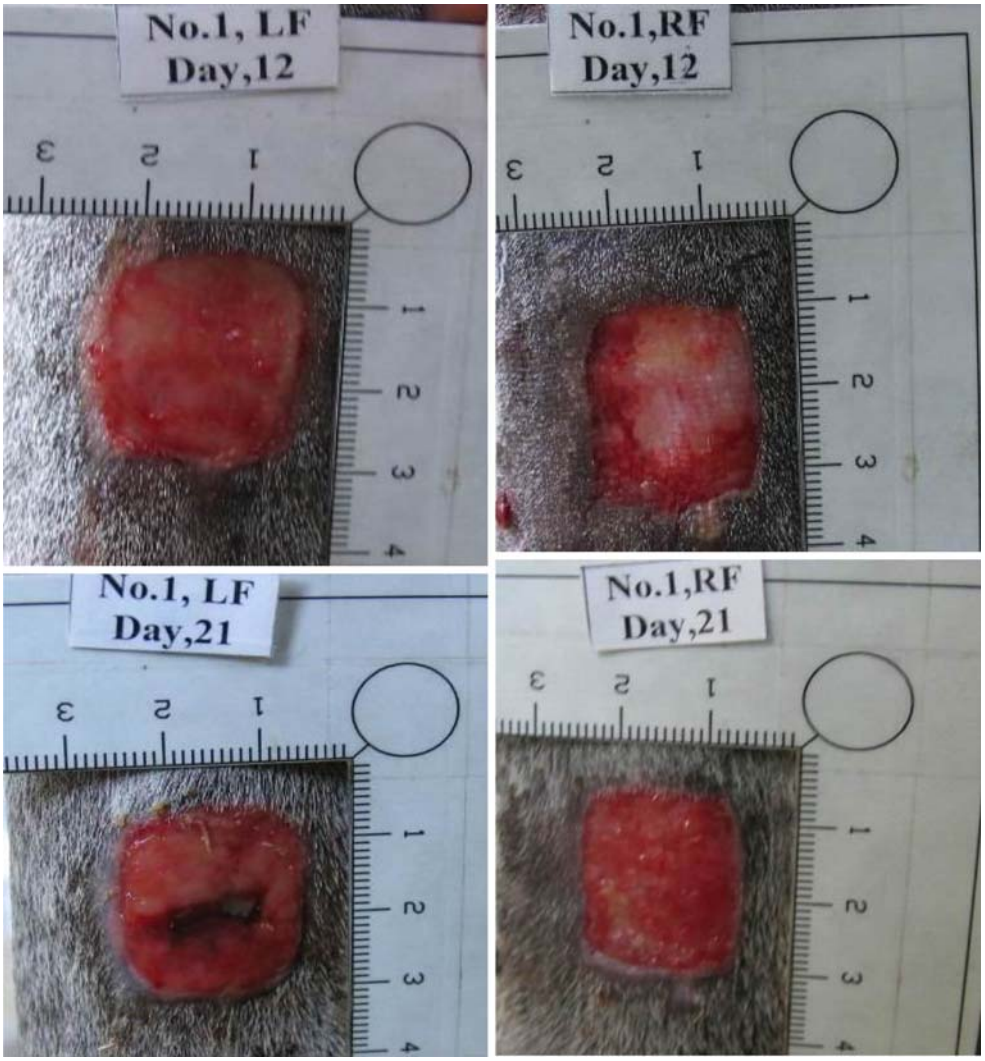

Fig. 8 Wounds at days 12 and 21 in test (*left*) and control (*right*) group in forelimb area. *RF* right forelimb, *LF* left forelimb

dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation scores in the specimens from left and right wounds in forelimb and neck ($P>0.05$; Tables 2 and 3; Figs. 9 and 10).

Discussion

In second-intention wound healing, repair of a defect is achieved by contraction and epithelialization. Wound contraction is defined as the centripetal movement of the original wound margins (Peacock 1984). Wound contraction is greater in areas of the body with loose skin than in areas where skin is stretched tightly (Swaim et al. 2001).

Successful wound healing relies on reconstitution of tissue bulk, remodeling of matrix collagen, and restoration of tissue tensile strength (McGrath and Simon 1983). Wound healing is essential and regeneration coordinated by intercellular signaling mechanisms. Multiple and specific peptidergic and chemical mediators influence cell reproduction and growth through autacoids and paracrine pathways (Johnston 1977; Steenfos 1994). In macroscopic

Table 1 Amounts of hydroxyproline (μg/mg dry matter) in tissue samples

	Percentiles		
	25th	50th (Median)[a]	75th
Left or right forelimb wounds			
Left forelimb	6.2598	7.5930	11.2664
Right forelimb	6.8677	8.8745	13.0908
Left or right neck wounds			
Left neck	7.4700	9.1200	11.8875
Right neck	8.1125	10.7750	12.4475

[a] There were no significant differences between median of hydroxyproline levels in left and right forelimb and neck wounds ($P>0.05$)

Table 2 Histological characteristics of wound healing in donkeys treated with BAPN compared with controls treated with methyl cellulose gel at the forelimb wounds

Left or right forelimb wounds	Percentiles					
	25th		50th (Median)[a]		75th	
	Left	Right	Left	Right	Left	Right
Epithelialization	0.00	0.00	0.00	0.5	0.75	1.75
Inflammatory infiltration	2	1	2.5	1.5	2.75	2
Presence of dermal granulation tissue	1	1	1.5	1	2	1
Fibroblast proliferation	2	2.25	2	3	2.75	3
Arrangement of fibroblasts	1	1.25	1.5	2	2	2.75
Collagen deposition	1.25	1.25	2	2	2	2.75
Collagen bundle formation	1	1.25	1	2	1	2

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

evaluation, closure of a skin defect occurs as a result of two independent process, contraction, and epithelialization (Lee et al. 1986; Swaim and Handerson 1990). The effect of wound contraction is the centripetal movement of a full-thickness wound to facilitate closure of a surface defect (Swaim and Handerson 1990). Regarding wound contraction, two theories has been described for this process, including myofibroblasts- and collagen-dependent wound contraction. Scar contracture has been a problem in many aspects of healing. In general, airways, urethra, lung, and kidney all can have long-term dysfunction due to scarring. Skin wounds are especially prone to contracture if there is an abnormal or excessive population of fibroblasts or an upregulation of collagen III content especially in equine distal limb. Proponents of the myofibroblasts-dependent wound contraction suggest that collagen and collagen cross-linking has very little to do with wound contraction (Joseph et al. 1997; Ehrlich 1988). But the older theory (collagen dependent wound contraction) suggests that wound contraction results from migrating fibroblasts that move through and rearrange connective tissue in granulating wounds. The activity of the fibroblasts on the connective tissue, composed predominately of collagen, is sufficient to cause centripetal movement of the skin margin. Simultaneous formation of collagen cross-linking maintains the dimensions of the wound as it decreased its surface area over time (Gabbiani et al. 1971). At the present study, the amount of hydroxyproline did not show significant difference between groups at the forelimb and neck wounds. This finding demonstrates that the BAPN-f has no effects on the amount of collagen production when applied locally. This finding was also supported by histopathological examination at the present study. Our data demonstrated that there were no significant differences for epithelialization, inflammatory infiltration, presence of dermal granulation tissue, fibroblast proliferation, arrangement of fibroblasts, collagen deposition, and collagen bundle formation. BAPN-f is a potent inhibitor of collagen cross-linking (Peacock and

Table 3 Histological characteristics of wound healing in donkeys treated with BAPN compared with controls treated with methyl cellulose gel at the neck wounds

Left or right neck wounds	Percentiles					
	25th		50th (Median)[a]		75th	
	Left	Right	Left	Right	Left	Right
Epithelialization	2	2	2.5	2.5	3	3
Inflammatory infiltration	1	1	1	1	1.75	1
Presence of dermal granulation tissue	1.25	1	2	1	2	1.75
Fibroblast proliferation	2.25	2.25	3	3	3	3
Arrangement of fibroblasts	2	2.25	2	3	2.75	3
Collagen deposition	1.25	2	2	2	2	2
Collagen bundle formation	1	1	1	1.5	1	2

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

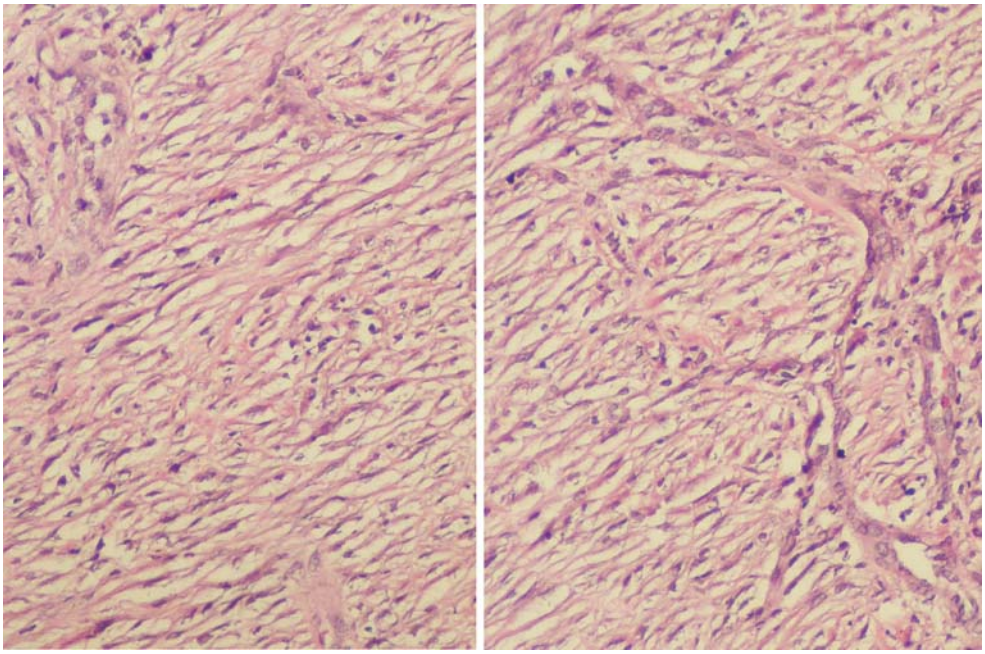

Fig. 9 Arrangement of proliferated fibroblasts in control (*left*) and test (*right*) group. Hematoxylin and eosin (×160)

Madden 1969). This suggests that wound contraction is not independent of collagen cross-linking formation. Macroscopic evaluation of the wounds at the forelimb and neck area has demonstrated significant differences for wound contraction and healing between test and control groups, but no significant differences in epithelialization between all groups. Redden and Doolin (2003) have reported that the collagen cross-linking and cell density have distinct effects on fibroblast-mediated contraction of collagen gels. Second intention wound healing is strongly dependent to wound contraction, especially at the equine distal limb. At the present study, wound contraction was about 20% in limb and 80% in neck wound area in control and 10% in limb and 60% in neck wound area in test group, respectively. Percentage of wound healing was also influenced by decreasing of wound contraction in test group. Distal limb wounds were affected more than the neck wound area. Local application of drugs such as BAPN-f can inhibit the enzyme lysyl oxidase. This inhibition prevents the production of HP and LP cross-linking between collagen and can influence the wound contraction. In a theory, after wounding, pre-existing

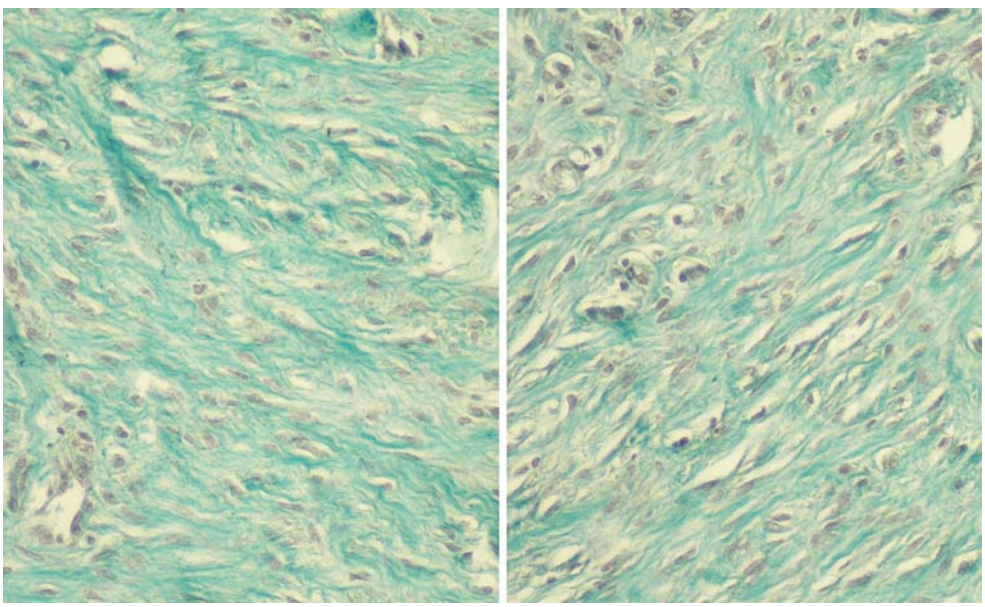

Fig. 10 New collagen bundle formation in control (*left*) and test (*right*) group. Masson's trichrome (×320)

fibroblasts and perivascular mesenchymal cells differentiate into phenotypically distinct myfibroblasts (Bouisson 1988). Myofibroblasts contain a cytoplasmic network of actin-like microfilaments oriented parallel to the long axis of the cell, providing it with contractile and migratory capabilities (Majno 1979). Myofibroblasts also possess specialized interconnections, the fibronexi, that consist of an association of intracellular, actin-like microfilaments and extracellular fibronectin fibers across the cytoplasmic membrane (Singer et al. 1984). These extracellular fibronectin fibers, in turn, form connections with collagen fibers and other myofibroblasts (Goslen 1988; McGrath and Simon 1983). This action can cause wound contraction (Doillon et al. 1985). But our data and data reported by Redden and Doolin demonstrated that wound contraction is not independent to HP and LP cross-linking between collagens. Macroscopic and histopathological findings associated with hydroxyproline assessment of the present study demonstrated that collagen cross-linking plays a key role in equine wound contraction and healing. In addition, our data have demonstrated that the other aspects of wound healing (epithelialization, fibroblast proliferation, and collagen deposition) cannot be influenced by collagen cross-linking inhibitor, beta-BAPN-f.

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