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ORIGINAL ARTICLE

Efficacy of L-proline administration on the early responses during cutaneous wound healing in rats

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Abstract Proline (Pro) plays a versatile role in cell metabolism and physiology. Pro and hydroxypro are major imino acids present in collagen, an important connective tissue protein, essential for wound healing, which is a primary response to tissue injury. This study explains the role of L-pro on cutaneous wound healing in rats when administered both topically and orally. Open excision wounds were made on the back of rats, and 200 µl (200 mg) of pro was administered topically and orally once daily to the experimental rats until the wounds healed completely. The control wounds were left untreated. Granulation tissues formed were removed after day 4 and 8 of post excision wounding, and biochemical parameters such as total protein, collagen, hexosamine, and uronic acid were estimated. Levels of enzymatic and non-enzymatic antioxidants such as catalase, superoxide dismutase, glutathione peroxidase, ascorbic acid, and reduced glutathione were evaluated along with lipid peroxides in the granulation tissues. Tensile strength and period of epithelialization were also measured. It was observed that the treated wounds healed very fast as evidenced by augmented rates of epithelialization and wound contraction, which was also confirmed by histological examinations. The results strappingly authenticate the beneficial effects of the topical administration of L-proline in the acceleration of wound healing than the oral administration and control.

Keywords L-Proline · Collagen · Excision wound · Tensile strength · Epithelialization

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Introduction

Wound healing is a basic response to tissue injury, the end product of which is a dense connective tissue (scar) consisting predominantly of collagen. Wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: *hemostasis*, *inflammation*, *proliferation,* and *remodeling* (Iba et al. 2004). The process of wound healing is promoted by several natural products (Sumitra et al. 2009), plant products (Nayak et al. 2010, 2011a, 2012), Shetty et al. (2008), and biomolecules (Shi et al. 2002, 2003; Aoki et al. 2010, Simon et al. 2012).

Nutrition plays a key role in proper wound healing. Biomolecules such as vitamins, amino acids, micro and macro elements are very important for normal wound healing. Vitamin A increases macrophage influx and activation into the wounds, stimulate fibroblast to synthesize collagen, and increases cellular proliferation (Zaidi et al. 2005). Vitamin C or ascorbic acid is an essential

water-soluble nutrient that plays a role in wound healing by increasing collagen and elastin synthesis (Nicosia et al. 1991). It also augments proliferation of neutrophils (which helps in preventing infection) and increases the synthesis of blood vessels that supply the new skin tissues with nutrients (Nicosia et al. 1991). Thiamine, a B vitamin, also plays an essential role in the metabolism of carbohydrates and branched-chain amino acids (Tanphaichitr 1994), and thus is an important factor in collagen synthesis and healing process.

The role of arginine (Patel and Knight 2005), poly gamma glutamic acid (Bae et al. 2010), ornithine, and citrulline which are the precursors of pro has already been reported (Aoki et al. 2010). The elements such as zinc and selenium also play an important role in wound healing. Girodon et al. (1997) have shown that patient receiving zinc + selenium experienced less infection at 2 years.

Pro and hydroxypro are unique amino acids, rather imino acids, which constitute one-third of amino acids in collagen, a structural protein, which comprises approximately 30 % of total body proteins (Hu et al. 2008). Pro has a very important role in protein synthesis and metabolism. It plays a key role in the synthesis of arginine, polyamines, and glutamate via pyrroline-5-carboxylate. It is a main nitrogenous substrate for the synthesis of polyamines in the small intestine of neonatal pigs (Wu et al. 2000), in the placenta of gestating pigs (Wu et al. 2005), and sheep (Wu et al. 2008).

Previous reports have demonstrated that the availability of pro pool at the wound site predicts enhanced collagen synthesis (Kershenobich et al. 1970). Metabolic precursors such as ornithine, arginine, glutamate, and glutamine also alternatively enhanced the local synthesis of pro to balance the relative deficiency of preformed pro (Kershenobich et al. 1970).

Arginine, ornithine, glutamate, and glutamine have been identified as precursors for the synthesis of pro by mammalian cells (Shen and Strecker 1975). Few amino acids have been reported for their wound healing efficacy such as taurine with chitosan gel formulation (Degjim et al. 2002), ornithine (Shi et al. 2002), and arginine (Debats et al. 2009). Glutamine being the most abundant amino acid in the body, accounting for 20 % of the total circulating free amino acid pool and 60 % of the free intracellular amino acid pool. Glutamine is a critical fuel for many cells, including fibroblasts, macrophages, neutrophils, and lymphocytes, all of which participate in wound healing (Tapiero et al. 2002). Glutamine (Gln)-enriched feeding on incisional healing in rats was also investigated (Tekin et al. 2000).

Collagen plays a crucial role in wound healing. Its biosynthesis is complex and involves many successive and interrelated steps (David Stain and Keiser 1970). Fibroblasts are responsible for collagen synthesis in the wound region. Level of fibroblasts in the wound tissue can be taken as a gauge for wound healing (Savunen and Viljanto 1992). Wound strength depends on collagen reinstallation and deposition of newly synthesized collagen fibers at wound site (Paul et al. 1997). Both pro and hydroxypro are important for collagen biosynthesis, structure, and strength. Their cyclic structure restricts the rotation of the polypeptide collagen chain and creates and strengthens the helical characteristic of the molecule (Grant and Prockop 1972).

Reactive oxygen species (ROS) are essential to remove bacteria and microorganisms as part of the defense system (Baeuerle and Henkel 1994). During the inflammation step of wound healing, ROS are generated for the repair process when molecular oxygen is reduced by NADPH oxidases in macrophages (Lambeth 2004). However, sustained excessive levels of ROS induce oxidative stress, which can inhibit cell migration and proliferation and affect the expression and function of inflammation mediators (Steiling et al. 1999). Without proper antioxidant activities, wound healing might be delayed or severe tissue damage can occur. Therefore, normal wound repair promotes the expression of many antioxidant genes such as glutathione peroxidase (GPx), catalase (CAT), and SOD (auf dem Keller et al. 2006).

Previous reports have been clearly demonstrated that the availability of pro pool at the wound site predicts enhanced collagen synthesis (Kershenobich et al. 1970). It has been reported that supplementation of metabolic precursors of pro such as ornithine, arginine, glutamate, and glutamine enhanced the local synthesis of proline (Barbul 2008).

Recently, Simon et al. (2012) have reported that supplementation of arginine plus proline favors wound healing in diabetic rats. But, they have not measured the collagen deposition at the wound site. As there is no report on the direct administration of pro, an important substrate for collagen on wound repair mechanism so far, we have carried out this study to examine the role of pro on full thickness cutaneous wound healing in rats.

Materials and methods

Chemicals

L-Hydroxyproline, D-Glucuronic acid, Chloramine-T, Glucosamine, 1,1,3,3-tetraethoxypropane and Bovine serum albumin, Epinephrine, Ascorbic acid, and reduced glutathione were purchased from Sigma Chemical Company, St. Louis, USA. L-Proline was purchased from Qualigens, and *P*-dimethyl amino benzaldehyde, diphenylamine, and Folin's Phenol reagent were from Loba Chemie, Mumbai, India. Methyl cellosolve was obtained from Merck, Darmstadt, Germany. All other reagents were of high analytical grade.

Animals grouping

Male Wistar rats, weighing between 180 and 200 g were chosen for the study. The rats were housed in wire topped cages with sterilized rice husk bedding under controlled conditions, of light/dark cycle (12:12 h), temperature at 29–31 °C, and rats were fed with commercial rat feed and water ad libitum. All procedures were carried out according to the stipulations of the Institutional Animal Care and Use Committee (IACUC). A formal approval from the Animal Ethical Committee from CLRI has also been obtained.

The rats were divided into three groups comprising six rats in each group as mentioned below:

Group I: Control rats, treated with phosphate buffered saline (PBS), once daily.
Group II: Rats treated with Pro (200 mg dissolved in 200 µl of PBS) topically once daily until complete healing.
Group III: Rats treated with Pro (200 mg dissolved in 200 µl of PBS) orally once daily until complete healing.

Wound creation

Rats were anesthetized by mild dosage of diethyl ether according to the method of Zardooz et al. (2010). In brief, small amount of cotton was drenched with diethyl ether at a concentration of 2.75 ml/L. This cotton was put inside a transparent glass desiccator under a metal mesh to avoid contact of the rat with the diethyl ether soaked cotton. The desiccator was covered with a solid lid to saturate the desiccator with ether. Each rat was monitored after being placed inside the desiccator with a tightly closed lid. A reduction in the animal's respiratory rate (nearly 50 %) and loss of the righting reflex were indicative of a state of deep anesthesia which occurred 4–5 min after diethyl ether exposure.

A 2-cm^2 full thickness open excision wound was made on the back of the rat as reported in our earlier studies (Ponrasu and Suguna 2012). The granulation tissue was removed on day 4 and 8 post wounding and used to evaluate different biochemical parameters. Separate groups of animals were maintained to find out the rate of contraction and period of epithelialization of wounds.

Biochemical parameters

To extract proteins (Porat et al. 1980), 100 mg of granulation tissue was homogenized in 5 ml of ice cold distilled water. 5 ml of 10 % trichloroacetic acid (TCA) was added and the samples were kept in an ice bath for 30 min to precipitate the proteins and nucleic acids. The contents were centrifuged, and the pellets were first washed with 1 ml of 10 % of TCA and then with 3 ml of absolute alcohol. The lipid free sediment was resuspended in 5 ml of 5 % TCA and kept at 90 °C for 15 min to separate the nucleic acids. An aliquot from this was taken and used to estimate total protein (Lowry et al. 1951).

To estimate collagen and hexosamine, the tissue samples were defatted in chloroform:methanol (2:1v/v) and dried in acetone, before use. Weighed tissues were first hydrolyzed in 6N HCl for 18 h at 110 °C, evaporated to dryness, and then made up with a known volume of water. From this, an aliquot was taken to estimate collagen and hexosamine by the method of Woessner (1961) and Elson and Morgon (1933), respectively.

Uronic acid was extracted from the granulation tissue according to the method of Schiller et al. (1961). In brief, digestion of the wound tissue was carried out with crude papain (10 mg/g wet weight of the tissue) in 0.5 M acetate buffer, pH 5.5, containing 0.005 M cysteine and 0.005 M disodium salt of EDTA at 65 °C for 24 h. An aliquot of the sample was taken to estimate uronic acid by the method of Bitter and Muir (1962). Lipid peroxide levels in granulation tissues were determined by the thiobarbituric acid reaction (Santos et al. 1980).

Fractionation of collagen was performed by the method of Piez (1963). In brief, for the neutral salt soluble collagen (NSSC), the granulation tissue was minced well, homogenized in 10 volumes of a neutral salt solvent (1.0 mol/L NaCl, 0.05 mol/L Tris, pH 7.5) containing 20 mmol/L EDTA and 2.0 mmol/L N-ethyl maleimide, and stirred for 24 h. The suspension was then centrifuged at 10,000 rpm for 1 h at 4 °C, and the extraction was repeated with the pellet. The supernatants were pooled and an aliquot was used for the assay of hydroxyproline by the method of Woessner (1961), and for the acid soluble collagen (ASC), the residue obtained was resuspended in 10 volumes of 0.5 mol/L acetic acid and extracted for 24 h with constant stirring, after which the contents were centrifuged at 10,000 rpm for 1 h at 4 °C. The pellet was reextracted with acetic acid, the supernatants were pooled, and an aliquot was used for the determination of hydroxyproline by the method of Woessner (1961). Finally, for insoluble collagen (IC), the residue obtained after acid extraction was hydrolyzed in 6N HCl and hydroxyproline content was estimated (Woessner 1961).

Estimation of catalase (CAT)

The levels of CAT in the granulation tissue were analyzed according to the method of Aebi et al. (1975). In short, to 1.2 ml of phosphate buffer pH 7.0, 0.5 ml of tissue homogenate was added. The enzyme reaction was started by the addition of 1.0 ml of 30 mM hydrogen peroxide, the substrate for catalase. The decrease in absorbance was

measured at 240 nm for 3 min in UV–visible spectrophotometer. An enzyme blank was run simultaneously with 1.0 ml of distilled water instead of hydrogen peroxide. The enzyme activity was expressed in moles of hydrogen peroxide decomposed per min per mg of protein.

Superoxide dismutase (SOD)

Superoxide dismutase activity was measured by the method of Misra and Fridovich (1972). To 0.5 ml of tissue homogenate, 0.75 ml of ethanol and 0.15 ml of chloroform (chilled in ice) were added and centrifuged at 2,000 rpm for 20 min. To 0.5 ml of supernatant, 0.5 ml of 0.6 mM EDTA solution and 1 ml of carbonate bicarbonate buffer (0.1 M, pH 10.2) were added. The reaction was initiated by the addition of 0.5 ml of 1.3 mM epinephrine and the increase in absorbance at 480 nm was measured in spectrophotometer. The enzyme activity was expressed as mg of protein required to give 50 % inhibition of epinephrine auto oxidation.

Glutathione peroxidase (GPx)

Glutathione peroxidase levels of the granulation tissues were evaluated by the method of Rotruck et al. (1973). To 0.2 ml tissue homogenate, 0.2 ml of 0.8 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of 4 mM reduced glutathione, 0.4 ml of phosphate buffer (0.4 M, pH 7.0) were added and incubated at 37 °C for 10 min. The reaction was arrested by adding 0.5 ml of 10 % TCA and the tubes were centrifuged at 2,000 rpm for 20 min. To the supernatant, 3 ml of disodium hydrogen phosphate (0.3 M) and 1.0 ml of 5,5′-Dithiobis-2-nitrobenzoic acid (40 %, DTNB) were added, and the color developed was read immediately at 420 nm in spectrophotometer.

Assay of ascorbic acid

The amount of ascorbic acid in the granulation tissues was measured by the method of Omaye et al. (1979). Granulation tissue was homogenized in 9 ml of 5 % ice cold TCA per g of tissue and centrifuged for 30 min at 5,000 rpm. 0.5 ml of supernatant, 0.1 ml of DTC reagent (3 g of 2,4-dinitro phenyl hydrazine DNPH, 0.4 g of thiourea, 0.05 g of copper sulphate dissolved in 100 ml of 9 N H_2SO_4) was added and incubated for 3 h at 37 °C. Then 0.75 ml of ice cold 65 % sulphuric acid was added and mixed well, and the solutions were allowed to stand at room temperature for 30 min. The color developed was read at 520 nm using spectrophotometer. Series of standard solution were also treated in similar manner. The ascorbic acid content was expressed as µg per mg of protein.

Assay of reduced glutathione

The amount of reduced glutathione in granulation tissues was assayed by the method of Moran et al. (1979). Tissue homogenate was precipitated with 10 %TCA and centrifuged at 2,000 rpm for 20 min. To 1.0 ml of aliquot, 1.0 ml of 0.6 mM 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) was added, and the final volume was made up to 5.0 ml with 0.33 M phosphate solution. The color developed was read at 420 nm in spectrophotometer. Reduced glutathione in granulation tissues was expressed as µg per mg of protein.

Biophysical analyses

Wound contraction rate was determined by tracing the wound area on to a transparent graph sheet and measuring the surface area by planimetry (Nayak et al. 2011b). The wounds were inspected for complete epithelialization as indicated by shedding of eschar without any raw wound left behind; days required for this sloughing was taken as the period of epithelialization.

Tensile strength of incised wounds is an important parameter to be studied to find out the efficacy of any wound healing agent. Six animals for each group have been used for tensile strength analysis. The skin on the dorsal side was shaved and a 6-cm linear full thickness incision was created on either side of the midline with a sterile scalpel blade. After wound had been cleaned dry, intermittent sutures (1 cm apart) were put with sterile cotton thread. Pro treatment was given as mentioned above. The sutures were removed on 7th day of wound creation, and on 10th day, tensile strength was measured at a minimum of three sites on each wound and the average was taken (Vogel 1971).

Histology

Tissues of individual animal were removed from the wound site after sacrificing the rats. These samples were then separately fixed in 10 % formalin-saline, dehydrated through graded alcohol series, cleared in xylene, and embedded in paraffin wax (melting point 56 °C). Serial sections of 5 µm were cut and stained with Hematoxylin and Eosin (H and E) and Van Gieson's (VG). The sections were examined under light microscope and photomicrographs were taken.

Statistics

Data were expressed as mean ± SD of six animals in each group and the results were statistically evaluated using students paired *t* test and one-way ANOVA. All statistical analyses were performed using graph pad prism (version 5.0; Graph Pad software Inc. San Diego CA, California, USA).

Results

Effect of pro on biochemical analyses

Table 1 shows the total protein, collagen, hexosamine, and uronic acid content of control and pro treated wounds. In group II, topical treatment of pro significantly increased the total protein content on day 4 (36 %) which further increased to 40 % on day 8 when compared to control (group I). A similar fashion was observed in group III also, with significant increase on day 4 (24 %) and day 8 (27 %). A considerable increase in collagen content from day 4 to 8 by 37–47 % was observed in group II animals. In group III animals, about 25 and 36 % of increased collagen content was found on day 4 and 8, respectively. The synthesis of ground substances (hexosamine and uronic acid) was found to be high till day 8 of post wounding in group II rats. The amount of hexosamine and uronic acid was significantly higher on earlier days of post wounding in both group II (35 and 47 %) and group III (17 to 31 %), when compared to control.

As shown in Fig. 1, a prominent mitigation in the level of lipid peroxides was observed. There was about 63 and 84 % reduction on day 4 and day 8, respectively, in topically treated rats. Oral treatment also showed a reduction of 25 and 59 % on 4th and 8th day, respectively, when compared to control.

The activities of enzymatic antioxidants viz. CAT, SOD, and GPx were considerably increased ($p < 0.001$) in pro treated groups as compared to control. Subsequently, non-enzymatic antioxidants viz. ascorbic acid and RG showed a significant increase ($p < 0.001$) in treated animals than control (Table 2).

Table 1 Effect of L-proline on various biochemical parameters

Group	Day 4	Day 8
Total protein (mg/100 mg wet tissue)		
Control	7.72 ± 1.50	11.03 ± 1.11
Topical	12.05 ± 1.86***	18.26 ± 1.38***
Oral	10.12 ± 1.56***	15.14 ± 1.83
Collagen (mg/100 mg dry tissue)		
Control	3.78 ± 0.97	5.78 ± 1.17
Topical	5.97 ± 0.83***	11.0 ± 0.81***
Oral	5.02 ± 0.84***	8.83 ± 0.91***
Hexosamine(μg/100 mg dry tissue)		
Control	214 ± 18	258 ± 21
Topical	332 ± 24***	486 ± 34***
Oral	257 ± 32**	375 ± 32**
Uronic acid (μg/100 mg dry tissue)		
Control	28.74 ± 3,046	43.09 ± 4.54
Topical	42.76 ± 3.74***	127.10 ± 5.82***
Oral	37.42 ± 4.77**	72.06 ± 4.24**

Values are expressed as mean ± SD of six animals in each group
** $p < 0.01$, *** $p < 0.001$ as significant compared to corresponding control

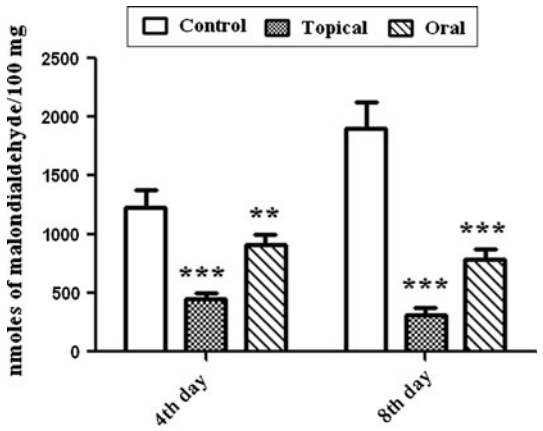

Fig. 1 Levels of lipid peroxides in granulation tissues on various days. Values are expressed as mean ± SD for six animals and level of significance is expressed as **$p \leq 0.01$ and ***$p \leq 0.001$, respectively, compared with the corresponding control

Table 3 illustrates the solubility pattern of collagen extracted from day 8 tissues of control and treated rats. The amount of ASC fraction in control was around 45 % higher than that of NSSC fraction, whereas more than threefold increase in ASC was found in group II and 2.5 fold increase in group III animals. It shows that there was abundant and earlier maturation of collagen fibers in pro treatment.

Effect of Pro on biophysical analyses

Biophysical changes were observed by monitoring the reduction in wound size, tensile strength, and time, in days, required for complete epithelialization of the wounds. Figure 2a, compares the tensile strength of control and pro treated incision wounds (day 8). A significant increase in tensile strength was observed in group II (60 %) and in group III rats (48 %). The epithelialization period (time taken for complete healing) of treated wounds showed a decrease of 51 % (10 days) in group II and 24 % in group III, respectively (Fig. 2b).

Wound contraction

The photographs of control and treated wounds taken on various days are shown in Fig. 3a. Percentage wound contraction observed at regular intervals depicts the efficacy of pro on healing pattern (Fig. 3b). Significant reduction in wound size in all the days explores the efficacy of pro on wound healing. Entire healing took place in 10 days in group II animals and 16 days in group III animals, when compared to control wounds which took 21 days to heal.

Table 2 Changes in the levels of enzymatic and non-enzymatic antioxidants in granulation tissues of control and L-proline treated animals

Group	Day 4	Day 8
Catalase (moles of hydrogen peroxide decomposed per minute per milligram of protein)		
Control	1.07 ± 0.04	1.19 ± 0.08
Topical	1.46 ± 0.07***	2.05 ± 0.09***
Oral	1.24 ± 0.06***	1.67 ± 0.06***
Superoxide dismutase (mg of protein required to give 50 % inhibition of epinephrine auto oxidation)		
Control	22 ± 0.7	36 ± 0.8
Topical	47 ± 0.9***	94 ± 1.10***
Oral	32 ± 0.6***	65 ± 0.10***
Glutathione peroxidase (nmol of glutathione utilized per minute per milligram of protein)		
Control	38 ± 0.6	48 ± 0.6
Topical	63 ± 0.4***	89 ± 0.6***
Oral	54 ± 0.7***	73 ± 0.5***
Ascorbic acid (μg per milligram of protein)		
Control	4.53 ± 0.85	6.02 ± 0.79
Topical	6.80 ± 2.88***	9.78 ± 0.61***
Oral	5.69 ± 0.75**	7.81 ± 0.70***
Reduced glutathione (μg per milligram of protein)		
Control	1.04 ± 0.09	1.12 ± 0.09
Topical	1.61 ± 0.11***	1.87 ± 0.10***
Oral	1.50 ± 0.09***	1.68 ± 0.07***

Values are expressed as mean ± SD of six animals in each group

** $p < 0.01$, *** $p < 0.001$ as significant compared to corresponding control

Table 3 Solubility pattern of collagen from 8th day granulation tissue (μg/100 mg wet tissue)

Group	Neutral salt soluble	Acid soluble	Insoluble
Control	710 ± 8.89	1,032 ± 69.60	2,007 ± 69.08
Topical	1,107 ± 57.38***	3,534 ± 158.0	3,872 ± 142.30***
Oral	852 ± 81.41**	2,010 ± 91.68	2,958 ± 209.80***

Values are expressed as mean ± SD of six animals in each group

** $p < 0.01$, *** $p < 0.001$ as significant compared to corresponding control

Microscopic analyses of granulation tissues

Figure 4 shows the histological sections for the 4th and 8th day of control as well as treated. Untreated wound shows minimal cellular infiltration, less number of fibroblasts, and inflammatory cells (Fig. 4a). In topically treated wound, increased fibroblasts and macrophages with numerous blood vessels were seen on day 4 (Fig. 4b). Proliferating blood capillaries and inflammatory cells such as neutrophils and fibroblasts could be seen on day 4 oral treatment (Fig. 4c).

On day 8, control tissue showed loosely laid collagen fibers with irregular pattern with few proliferating blood capillaries. Incomplete epithelialization with lesser fibrous tissue at the wound site was also observed (Fig. 4d). 8th day topically treated wound showed cluster of collagen fibers parallel to the wound region with complete epithelialization, prominent thick bundles of collagen fibers deposited with proliferating fibroblasts were also seen (Fig. 4e).

Figure 4f shows the histological observation of 8th day oral group. Mild collagen formation, large number of proliferating capillaries, complete fibrous tissues, and epithelialization with less collagen deposition were observed at the wound site.

Discussion

Wound healing mechanism requires a well orchestrated integration of a series of dynamic biological events such as cell migration, proliferation, and extracellular matrix deposition at the wound surface (Martin and Parkhurst 2004). Impaired macrophage activity during inflammation leads to a proliferation phase in which angiogenesis, fibroblast proliferation, and matrix formation are defective (Beer et al. 1997). Angiogenesis during wound repair serves a dual function of providing the nutrients required by the healing tissue and serving for structural repair through the formation of granulation tissue (Martin et al. 2003).

Pro is a non-essential imino acid, synthesized from glutamic acid. It is an essential component of collagen and

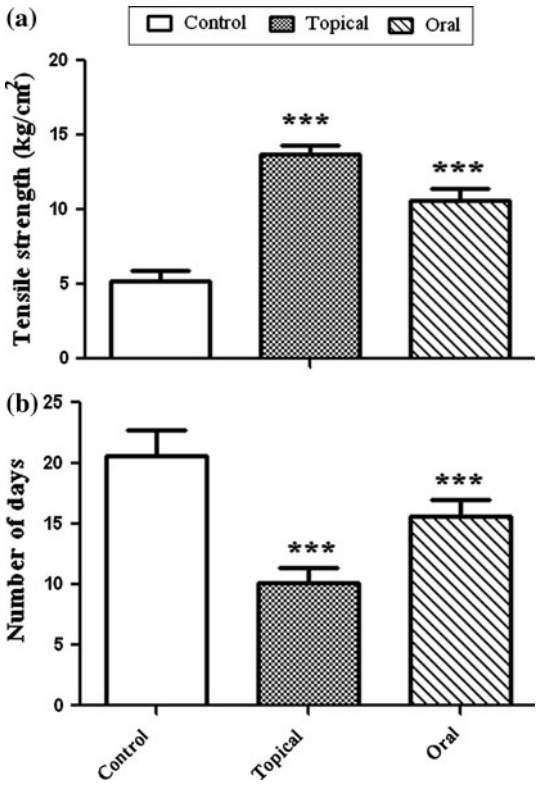

Fig. 2 **a** Tensile strength of incision wounds from control and treated rats. Values are expressed as mean ± SD for six animals, ***$p \leq 0.001$. **b** Period of epithelialization (measured as the number of days required for complete healing) in control and treated rats. Values are expressed as mean ± SD for six animals, ***$p \leq 0.001$

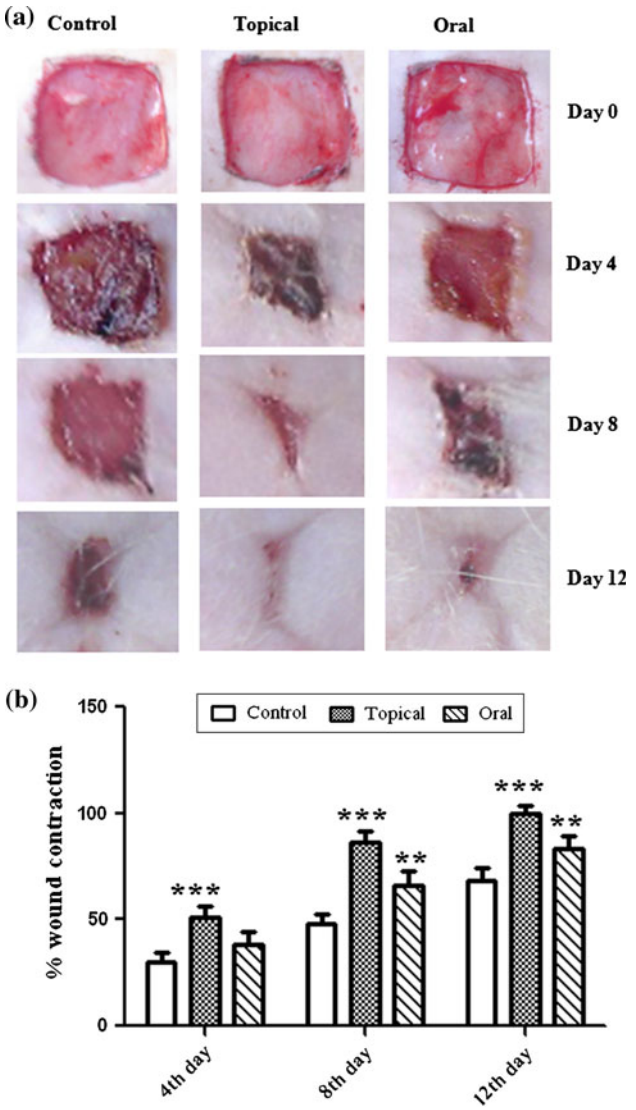

Fig. 3 **a** Photographical images of wound contraction rate on different days of control and treated animals. **b** Graphical representation of percentage wound contraction on various days of control and treated wounds. Values are expressed as mean ± SD for six animals **$p \leq 0.01$ and ***$p \leq 0.001$, as significant compared with the control. *Scale bar* 1 cm

is important for proper functioning of joints and tendons (Krane 2008). Pro plays a major role in protein synthesis and structure, metabolism, nutrition as well as wound healing, antioxidative reactions, and immune responses (Guoyao et al. 2011). Pro, acts in accordance with arginine, glutamine, and leucine to enhance protein synthesis in cells and tissues. It serves as a major amino acid for the synthesis of polyamines, key regulators of DNA, and protein synthesis as well as cell proliferation and differentiation (Wu et al. 2010). Pro, being a non-essential amino acid, its insufficiency occurs only in prolidase deficiency, an autosomal inborn metabolic error, where lack of the enzyme does not allow for the degradation of the imino-dipeptides (Pro-containing) generated during collagen lysis, with large excretion of these dipeptides in the urine. Patients with prolidase deficiency have low pro levels and a variety of wound healing deficits (Trent and Kirsner 2004).

While the role of proline precursors such as arginine, glutamic acid, ornithine, and citrulline on wound healing has been extensively studied, the efficacy of supplementation of pro on wound healing has not been reported so far. Hence, in this manuscript, a preliminary study has been carried out to investigate the potential of pro as a wound healer.

A significant increase in total protein and collagen has been observed in pro treated rats. It has been reported that pro acts in harmony with arginine, glutamine, and leucine to enhance protein synthesis in cells and tissue (Wu et al. 2010). Hexosamine and uronic acid are the matrix molecules that act as the ground substratum for the synthesis of a new extracellular matrix. The early increase in hexosamine and uronic acid showed that the fibroblasts actively synthesize the ground substratum on which collagen is laid down. It is reported that there is an increase in the levels of these components during the early stages of wound healing, following which normal levels are restored (Nithya et al. 2003). A similar trend was observed in pro treated wounds

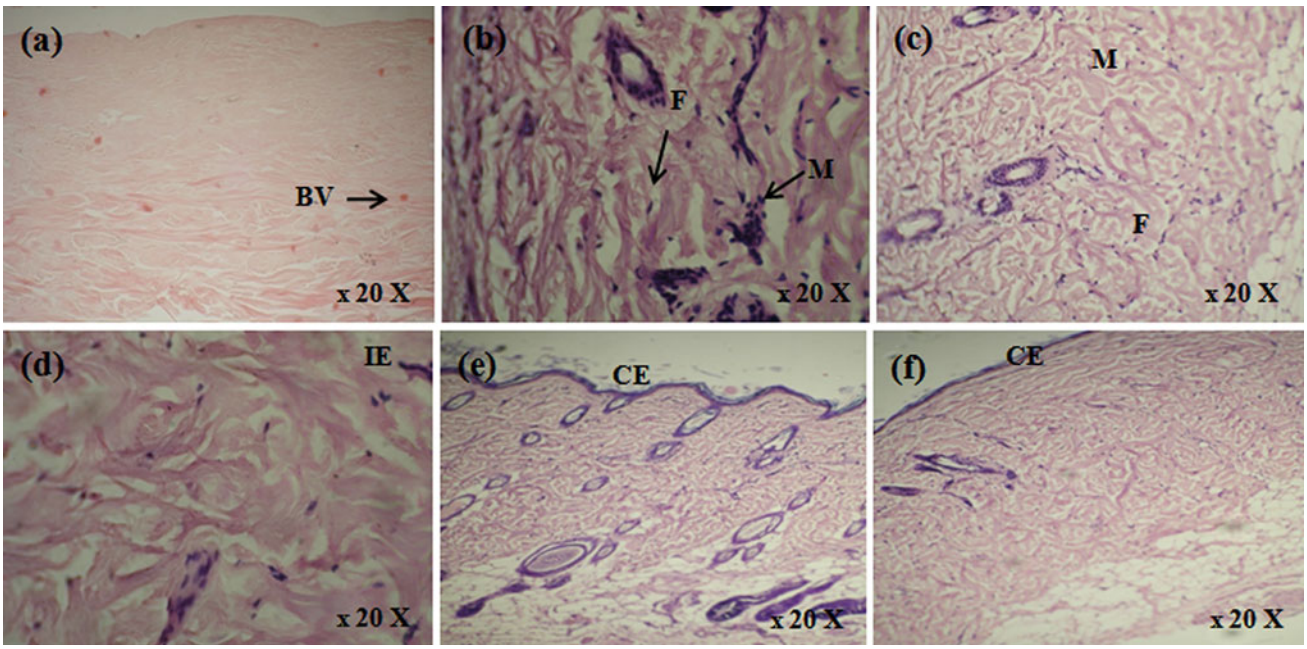

Fig. 4 Histological evaluation representing the control and pro treated granulation tissues on day 4 and 8 of post wounding, respectively, (Stained with Hematoxylin-Eosin and Van Gieson's (H&E and VG); magnification 20×). **a** During 4th day, control showing minimal cellular infiltration with less fibroblasts and blood vessels, whereas **b** topically treated tissue showing more new blood vessel formation and plenty of fibroblasts with macrophages. **c** Oral treatment showing abundant cellular infiltration mainly neutrophils and fibroblasts. On 8th day, **d** control depicts thin incomplete epithelial layer with less collagen and **e** topically treated shows complete epithelialization with regularly arranged dense collagen deposition. Whereas in group III, **f** oral treatment illustrates complete epithelialization with moderate collagen deposition. *Scale bar* 50 µm. *F* fibroblast, *M* macrophages, *IE* incomplete epithelialization, *E* epithelialization, *BC* blood vessels

wherein the levels of hexosamine (the ground substratum for collagen synthesis) and uronic acid increased significantly. Uronic acid in the wound draws fibroblasts and stimulates collagen synthesis by providing more fluid, which helps faster and greater cell mobility, early remodeling, and supports the wounds to heal faster without scar formation.

Assessment of collagen content in wound tissues of the control and pro treated wounds clearly suggests that pro augments collagen synthesis and deposition. The healing process depends, to a large extent, on the regulated biosynthesis and deposition of new collagens and their subsequent maturation. Siegel (1976) observed that any increase in collagen synthesis leading to an increase in newly formed collagen is associated with an increase in cross-linking of collagen.

Increased protein content of granulation tissues indicates the level of cellular proliferation and protein synthesis. Increased cellular proliferation may be due to the mitogenic activity of the pro, which might have significantly contributed to healing process (Dioguardi 2008). Early dermal and epidermal regeneration in treated rats also confirmed that pro has a positive effect toward cellular proliferation, granular tissue formation, and epithelialization. Also, it has been reported that the definite increase in collagen and hexosamine showed a positive correlation with that of DNA in cultures of swine aortic media (Daoud et al. 1977). Higher protein and collagen contents of treated wounds suggest that pro stimulates the proliferation of cells which actively synthesize the ECM (Ma et al. 2011).

Hydroxyproline, the main constituent of collagen, serves as a marker of collagen biosynthesis at the wound site. Collagen not only provides strength and integrity to the tissue matrix, but also plays a vital role in homeostasis and in epithelialization at the later phase of healing. Augmented levels of hexosamine and uronic acid support the stabilization of collagen molecules by accelerating electrostatic and ionic interactions (Siegel 1976). Collagen is a major protein in the extracellular matrix and is the essential component that ultimately contributes to wound tensile strength (Singer and Clark 1999). The collagen synthesized is laid down at the wound site and cross-linked to form fibers. Wound strength is acquired from both remodeling of collagen and the formation of stable intra and intermolecular cross-links (Chithra et al. 1998).

Solubility of collagen in different medium is an important parameter investigated to find out the maturity of collagen fibers present in the wound. Increase in the NSSC fraction is an index of newly formed collagen. A remarkable increase in NSSC fraction of treated wounds substantiates

that the fibroblasts actively synthesize collagen in the pro treated rats. More than twofold increase in acid soluble and insoluble collagen fractions confirms that pro accelerates not only the synthesis, but also the cross-linking of collagen which was also confirmed by augmented tensile strength of the wounds.

Pro administration remarkably accelerated the healing of both excision and incision wounds. During granulation tissue formation, as contraction proceeds and resistance increases, fibroblasts differentiate into myofibroblasts. The presence of myofibroblasts is considered to be the characteristic of tissue undergoing contraction. The faster wound contraction in excision wounds in pro treated rats might be due to the earlier differentiation of fibroblasts to myofibroblasts. (Dunphy and Udupa 1955).

Enhanced tensile strength in incised wounds could infer that pro not only increases collagen content, but also aids in cross-linking of this protein. Proline gets converted into hydroxyproline, an important amino acid, present in collagen, which plays a major role in the stabilization of collagen molecules through intra and inter crosslink formation.

The present investigation shows that the administration of pro leads to increased levels of enzymatic and non-enzymatic antioxidants in the granulation tissues, which might be due to the free radical scavenging capacity of pro (Kaul et al. 2008), and this antioxidant property of pro may explain why its concentrations increase markedly in response to cellular oxidative stress (Verbruggen and Hermans 2008).

SOD and GPx serve as free radical scavengers. SOD converts superoxide radical into hydrogen peroxide (Vervaart and Knight 1996), and GPx neutralizes the hydrogen peroxide into water (Paglia and Valentine 1984). The free radical damage reduction can be assessed by MDA levels as an indicator of lipid peroxidation. Increased CAT activity could either be due to increased expression and synthesis of collagen (Gupta et al. 2006).

Ascorbic acid has a vital role in collagen metabolism, as it is required for prolyl and lysyl hydroxylase enzyme activities (Kivirikko and Prockop 1967). Excess of ascorbic acid enhances the elevation of collagen transcription, collagen mRNA levels, and collagen synthesis in cultures of human fibroblasts (Chojkier et al. 1989).

Deposition of newly synthesized collagens at the wound site increases the collagen concentration per unit area and, hence, the tissue tensile strength. Assessment of collagen content in granulation tissues of control and treated wounds clearly suggests that pro enhances collagen synthesis and deposition. It appears to be greater and earlier maturation of collagen fibers in pro treated wounds. The significant increase in the tensile strength of wound tissues substantiates this observation (Savunen and Viljanto 1992).

Wound contraction is a process that occurs throughout the healing process, begins in the fibroplasia stage. The increased rate of wound contraction in pro treated groups might be attributed to increased proliferation and transformation of fibroblasts into myofibroblasts.

Histological evaluation also showed enhanced proliferation of fibroblasts and reepithelialization in pro treated wounds. The early reepithelialization and faster wound contraction in treated wounds could be associated with the increased keratinocytes proliferation and their transformation to the wound site (Blakytny and Jude 2006).

The healing effect of orally administered L-citrulline or ornithine was compared with L-arginine in rat pressure-ulcer wound model by Aoki et al. (2010). They have found out that L-ornithine had better healing effect. Ornithine and arginine share many biologic and pharmacologic activities as they are metabolically closely related amino acids. Levenson et al. (1980) have reported that citrulline has no effect on wound healing, while ornithine enhances wound healing remarkably.

Shi et al. (2002) reported that ornithine supplementation improves collagen deposition and breaking strength of wounds. They have concluded that the effect of ornithine is not dependent on NO metabolism and generation unlike arginine.

All these amino acids which are precursors of proline have been reported to accelerate wound healing process. Hence, direct administration of pro would definitely improve wound healing.

Topically administered drugs are effective in faster wound contraction due to the larger availability at the wound site (Ponrasu and Suguna 2012). From this investigation, we confirm that the rate of wound contraction in pro treated rats was significantly higher and period of epithelialization was shorter. These results further support the efficacy of pro on wound repair mechanism. Deposition of collagen content at the wound site increases collagen concentration per unit area and, hence, the tissue tensile strength, which is also a measure of the cross-linking of collagen. The faster contraction and healing of wounds in topical application might be due to the direct and immediate availability of pro at the wound site.

In conclusion, this preliminary study confirms that topical administration of pro promotes complex cascade of wound healing process such as fibroplasia, collagen synthesis, wound contraction, and epithelialization.

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Conflict of interest The authors declare that they have no conflict of interest.

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