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Effect of Topical Application of Virgin Coconut Oil on Skin Components and Antioxidant Status during Dermal Wound Healing in Young Rats

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Key Words
Virgin coconut oil · Collagen · Antioxidants · Dermal wound

Abstract
Objectives: The present study was undertaken to evaluate the effect of a topical application of virgin coconut oil (VCO) on excision wounds in young rats. ***Methods:*** Three sets of experiments with 3 groups of female Sprague-Dawley rats each consisting of 6 animals were used for studying wound closure time, antioxidant status and biochemical parameters. Group 1 was the control; groups 2 and 3 were treated with 0.5 and 1.0 ml VCO, respectively, 24 h after wound creation for 10 days. After the experimental period, the healing property of VCO was evaluated by monitoring the time taken for complete epithelization as well as levels of various parameters of the wound's granulation tissue. The collagen solubility pattern, glycohydrolase activity, and histopathology of the granulation tissue were also analyzed. The antioxidant status during wound healing was monitored continuously for 14 days. ***Results:*** VCO-treated wounds healed much faster, as indicated by a decreased time of complete epithelization and higher levels of various skin components. Pepsin-soluble collagen showed a significant increase in VCO-treated wounds, indicating a higher collagen cross-linking. Glycohydrolase activities were also found to be increased due to a higher turnover of collagen. Antioxidant enzyme activities, and reduced glutathione and malondialdehyde levels were found to be increased on the 10th day after wounding, which were found to have returned to normal levels on day 14 in the treated wounds. The lipid peroxide levels were found to be lower in the treated wounds. A histopathological study showed an increase in fibroblast proliferation and neovascularization in VCO-treated wounds compared to controls. ***Conclusion:*** The beneficial effect of VCO can be attributed to the cumulative effect of various biologically active minor components present in it.

Introduction

Cutaneous wound healing is a complex and well-defined process which is initiated in response to injury [1]. The healing process occurs in five phases: inflammation, neovascularization, formation of granulation tissue, reepithelization and, finally, formation of new extracellular matrix and tissue remodeling [2–4]. New stroma begins to invade the wound space approximately 4 days after injury [5]. The structural molecules of the newly formed extracellular matrix (provisional matrix) con-

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tribute to the formation of granulation tissue by providing a scaffold or conduit for cell migration [6, 7]. Collagen is the major protein of the intracellular matrix and the predominant constituent of the final scar [8]. Therefore, the synthesis, secretion and subsequent organization of this triple-helical protein in the wound's granulation tissue become significant in the healing process since enhanced cross-linking of collagen results in higher wound tissue strength [9]. The objective in wound management is to heal the wound in the shortest time possible with minimal pain, discomfort and scarring [10] by providing adequate nutrition and by the use of topical and systemic antimicrobial agents. While some topically used antimicrobial agents can kill cells such as bacteria, they can equally interfere with cell proliferation during wound healing (i.e. immature and nonadherent keratinocytes), followed by delayed wound closure [11].

Plant products are found to be effective in promoting wound healing since they are composed of various antioxidant and anti-inflammatory principles [12, 13].

Coconut oil has long been used in the Ayurvedic system of medicine for various skin disorders including wound healing and microbial infections. It is used by some ethnic groups of Ngada on Flores, an eastern Indonesian island, to treat wounds and to preserve medicinal plants [14]. Virgin coconut oil (VCO), in contrast to the usual processing from copra, is obtained directly from coconut milk by wet process under controlled temperature. Wet processing can avoid the loss of biologically active minor components like vitamins and polyphenols. Previous studies have proved that VCO is more beneficial than copra oil in reducing the oxidation of low-density lipoproteins and plasma lipid levels, and in enhancing the antioxidant status in rats [15, 16]. Since no previous studies on the wound-healing activity of VCO are reported, we investigated the influence of a topical application of VCO on the healing of dermal wounds in young rats.

Materials and Methods

Animals

Four-week-old female Sprague-Dawley rats weighing about 100–130 g from the animal house of the Department of Biochemistry, University of Kerala, were used for the present study. The animals were placed singly in cages with wire net floors in a room with controlled temperature (22–24°C) and humidity (70–75%) with a lighting regimen of 12/12 h and were fed a normal laboratory diet. All experiments were conducted according to the guidelines of the Institutional Animal Ethics Committee.

Extraction of VCO

The solid endosperm of mature coconut (7 months old; west coast tall variety grown at the Kerala University Campus) was crushed, made into viscous slurry and squeezed through cheesecloth to obtain coconut milk, which was refrigerated for 48 h to separate the fat and water layers. After 48 h, the fat layer was removed and subjected to mild heating (50°C) in a thermostat oven [15]. The obtained virgin oil was filtered through cheesecloth and used for the present study.

Fatty Acid Analysis of VCO

The fatty acid composition of VCO was analyzed by gas chromatography. Fats were methylated with trimethylsulfonium hydroxide [17]. Fatty acid methyl esters were separated by gas chromatography using a system (HP 5890; Hewlett Packard GmbH, Waldbronn, Germany) equipped with an automatic on-column injector, a polar capillary column (30 m FFAP, 0.53 mm i.d.; Macherey and Nagel, Düren, Germany) and a flame ionization detector [18]. Helium was used as carrier gas at a flow rate of 5.4 ml/min. Fatty acid methyl esters were identified by comparing their retention times with those of individually purified standards.

Wound Creation

Animals were anesthetized by sodium Pentothal injection and the skin on the dorsolateral flank was shaved. An excision wound of a size of 4 cm^2 was made by cutting out a 2 × 2-cm piece of skin from the shaven area. The wounds were of the full-thickness type extending down to the subcutaneous tissue [8].

Experimental Protocol

The rats were grouped (6 rats/group) as follows: group 1 – untreated controls; group 2 – treated with 0.5 ml VCO, and group 3 – treated with 1 ml VCO. Twenty-four hours after wounding, VCO was applied topically to the wounds, continuously for 10 days, using a micropipette. The wounds were left open during the entire experimental period. The animals were continuously assessed for microbial attack on a daily basis. After 10 days, the animals were killed and the granulation tissues were surgically removed and stored at –70°C for various biochemical estimations.

Estimation of Collagen and Elastin

Collagen was estimated by a method based on oxidation with peroxide of hydroxyproline to a pyrrole-2-carboxylic acid. This is then condensed with *p*-dimethylaminobenzaldehyde to form a red chromogen [19]. Elastin was extracted after removing nucleic acid from acetone-dried tissue by successive extraction with 5% TCA at 90°C for 15 min and again with 0.1 N NaOH, and the residue was washed with H_2O and autoclaved with 0.1 N acetic acid at a 15-lb pressure for 2 h. Elastin precipitated with 5% TCA and was estimated by the micro-Kjeldahl digestion method [20].

Estimation of Collagen Solubility Pattern

The collagen solubility pattern of the granulation tissue was determined as described by Miller and Rhodes [21]. Acid-soluble collagen was obtained by resuspending the residue obtained after removing neutral salt-soluble collagen in 10 vol of 0.5 M acetic acid, kept for 24 h under constant stirring and separated by centrifugation. A pellet was again extracted with acetic acid, super-

natants pooled and the hydroxyproline content estimated. The residue obtained after acid extraction was resuspended in 0.5 M acetic acid containing 100 mg pepsin per gram of wet tissue to obtain pepsin-soluble collagen. Digestion was carried out for 24 h followed by centrifugation and reextraction. Aliquots from pooled samples were used for collagen estimation. The residue remaining after pepsin digestion was referred to as insoluble collagen.

Biochemical Parameters

Wet granulation tissue was homogenized to obtain a 5% homogenate in saline, centrifuged at 6,300 *g* for 10 min. Supernatant was removed and used for estimating DNA using diphenylamine [22], protein was estimated by the method of Lowry et al. [23], total glycosaminoglycans (GAG) by the procedure of Bitter and Muir [24], sialic acid was estimated by the procedure as described by Warren [25], and total hexose by the method of Spiro [26]. Glucuronidase and hexosaminidase activities were estimated by the method described previously by Kawai and Anno [27] using *p*-nitrophenyl-β-D-glucuronide and *p*-nitrophenyl-β-acetyl glucosaminide, respectively, as substrates. Malondialdehyde (MDA) in the granulation tissues was estimated by the procedure of Ohkawa et al. [28]. Reduced glutathione (GSH) content in the granulation tissue of treated and control wounds were estimated by the method described by Benke et al. [29].

Antioxidant Enzyme Activities

Separation and Purification of Antioxidant Enzymes

The granulation tissue was homogenized in 0.25 M sucrose and differentially centrifuged at 10,000 rpm under cold conditions to get the cytosolic fraction. Before estimating the activity, superoxide dismutase (SOD) was purified by precipitating, using 90% ammonium sulfate, dialyzed against 0.0025 M Tris-HCl buffer (pH 7.4) and used as the enzyme source.

Assay for SOD

SOD was determined by the method described by Kakkar et al. [30]. The assay mixture contained 1.2 ml pyrophosphate buffer (0.052 M; pH 8.3), 0.01 ml phenazonium methosulfate (186 μM), 0.3 ml nitroblue tetrazolium (300 μM), 0.2 ml NADH (780 μM) and 0.1 ml of enzyme solution and H_2O_2. The reaction was started by adding NADH and stopped after 90 s by adding 1 ml glacial acetic acid. The reaction mixture was stirred and 4 ml butanol added; after 10 min, it was centrifuged. The color intensity of the chromogen in the butanol layer was measured at 560 nm.

Assay for Glutathione Peroxidase

Glutathione peroxidase (GPx) was determined by the method of Lawrence and Burk [31]. The reaction mixture contained 2 ml phosphate buffer (50 mM; pH 7.0), 0.2 ml EDTA, 0.3 ml sodium azide (1 mM), 0.1 ml GSH (1 mM) and 0.1 ml NADPH (0.2 mM). To this mixture, 0.2 ml of enzyme solution was added, incubated for 5 min at room temperature, and the reaction started by adding 0.2 ml of 0.25 mM H_2O. Optical density was measured at 340 nm at 20-min intervals. Enzyme activity was measured as micromolar of NADPH oxidized per minute per milligram protein.

Assay for Glutathione Reductase

Glutathione reductase (GR) activity was measured by the method of David and Richard [32]. The reaction mixture contained 2.6 ml of 0.12 M phosphate buffer (pH 7.2), 0.1 ml of 15 mM EDTA, 0.1 ml of 65.3 mM oxidized glutathione and 0.1 ml of the enzyme solution. To the reaction mixture, 0.05 ml of 9.6 mM NADPH was added, and the absorbance measured at 340 nm at 30-second intervals. Enzyme activity was measured as micromolar of NADPH oxidized per minute per milligram protein. Protein was measured by the method of Lowry et al. [23].

Histopathology

For the histopathological observations, fresh pieces of the granulation tissue were immersion fixed in formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in benzene and embedded in paraffin wax. Blocks were made and 5- to 7-μm-thick sections were double stained with hematoxylin and eosin and observed under the light microscope [33].

Statistical Analysis

Statistical differences were determined using one-way ANOVA followed by Duncan's post hoc test to identify the differences, using SPSS version 10. Differences of $p < 0.05$ were considered to be significant. Data are reported as means ± SEM unless otherwise stated.

Results

Table 1 indicates that the granulation tissue weight of the treated animals (132.7 mg for 0.5-ml-treated, and 157.7 mg for 1.0-ml-treated wounds) were significantly changed compared to the control wounds (59.0 mg). The time of complete epithelization was found to be lower in treated wounds (18.67 days) compared to the control wounds (22.37 days). Total GAG levels were also elevated in treated wounds on the 10th day. Total protein, hexose, sialic acid and elastin levels in VCO-treated wounds were found to be increased significantly compared to the control wounds (table 1).

The total collagen content of VCO-treated wounds showed a significant increase compared to the control. Collagen levels were slightly lower in the wounds treated with 1.0 ml VCO (table 1). The solubility pattern of collagen from the granulation tissue indicates higher levels of pepsin-soluble collagen in the VCO-treated group than in the control group (fig. 1). Acid-soluble and insoluble collagen also increased in VCO-treated wounds. DNA levels in wounds treated with 1 and 0.5 ml VCO showed a significant increase compared to the control wounds. Glycohydrolase, β-glucuronidase and N-acetyl-β-glucosaminidase were also found to be significantly increased in wounds treated with 1 ml VCO. The levels of β-glucuronidase were found to be lower in wounds treated with 0.5 ml VCO (fig. 2).

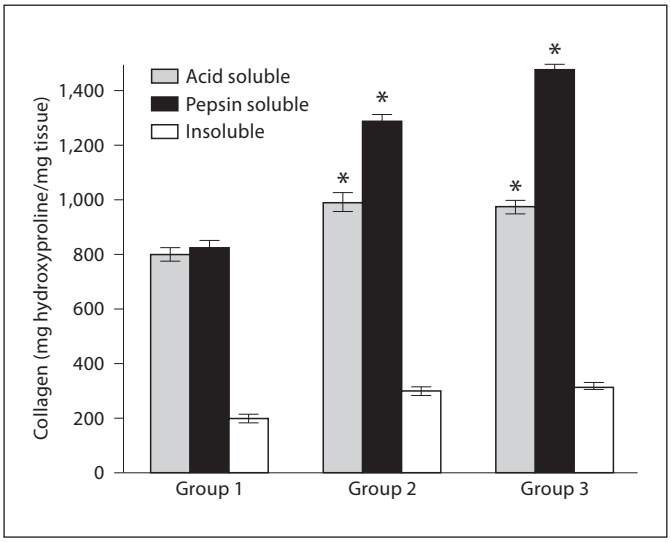

Fig. 1. Collagen solubility pattern. Values are means ± SEM of 6 rats. Group 1: controls. Group 2: VCO topically applied (0.5 ml). Group III: VCO topically applied (1.0 ml). * Statistically significant compared to controls ($p < 0.05$).

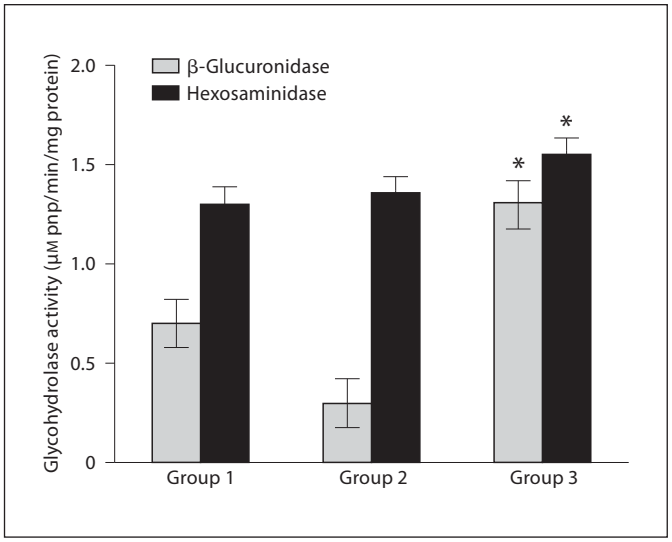

Fig. 2. Glycohydrolase activities in the granulation tissues of control and test wounds. Values are means ± SEM (n = 6). Group 1: controls. Group 2: VCO topically applied (0.5 ml). Group 3: VCO topically applied (1.0 ml). * Statistically significant compared to controls ($p < 0.05$).

Table 1. Effect of different VCO parameters during healing

Group No.	GT wet weight mg	TCE days	GAG mg[a]	Elastin mg[b]	Protein	Hexose	Sialic acid	DNA mg[b]	Collagen mg[c]
1	59.00 ± 3.2	22.37 ± 0.37	221.00 ± 1.5	0.02 ± 0.001	15.67 ± 0.19	1.69 ± 0.09	0.024 ± 0.0003	2.1 ± 0.02	1.2 ± 0.01
2	132.67 ± 1.8*	20.67 ± 0.33*	236.67 ± 4.4	0.23 ± 0.001*	21.58 ± 0.46*	2.06 ± 0.05*	0.039 ± 0.002*	2.8 ± 0.12*	3.2 ± 0.30*
3	157.66 ± 4.3*, #	18.67 ± 0.33*	245.33 ± 2.6	0.32 ± 0.001*	25.76 ± 0.47*	2.46 ± 0.08*	0.041 ± 0.0008*	3.9 ± 0.17*, #	3.0 ± 0.35*

Values are means ± SEM of 6 rats. GT = Granulation tissue; TCE = time for complete epithelization. Group 1: control; group 2: VCO topically applied (0.5 ml); group 3: VCO topically applied (1.0 ml). * Statistically significant compared to group 1 ($p < 0.05$); # statistically significant compared to group 2 ($p < 0.05$).

[a] Per 100 mg. [b] Per 100 mg dry tissue. [c] In milligram hydroxyproline per 100 mg dry tissue.

Table 2 shows the antioxidant enzyme activities of VCO-treated wounds as compared to the controls. All the antioxidant enzymes measured, i.e. SOD, GPx and GR, were found to be significantly expressed in VCO-treated wounds on the 10th day compared to the control wounds. After 10 days, these enzymes were found to be lowered to basal levels on day 14 in treated wounds. The GPx levels in the controls showed no significant change on the 6th, 10th and 14th days. On the 14th day, the levels of GPx in the controls remained higher than in the treated wounds.

There was no significant change in GSH level on the 6th day for VCO-treated and control wounds. The GSH content was found to be lowered in all 3 groups on day 10. But on day 14, the GSH levels of the treated wounds were found to be higher than those of the control wounds. MDA levels were found to be decreased in all groups on day 10. On the 14th day, the MDA levels were elevated in the control wounds, but the levels in the treated wounds were normal (table 2).

Table 2. Activities of antioxidant enzymes, levels of glutathione and MDA in granulation tissues of control and test wounds

	Group No.	Days after wounding		
		6	10	14
SOD[1]	1	0.10 ± 0.01	1.2 ± 0.02	0.2 ± 0.02
	2	0.50 ± 0.02	3.7 ± 0.03	0.5 ± 0.03
	3	1.20 ± 0.02	3.8 ± 0.03*	0.5 ± 0.02
GPx[1]	1	0.18 ± 0.03	0.19 ± 0.02	0.17 ± 0.03
	2	0.32 ± 0.34	0.33 ± 0.04*	0.02 ± 0.04
	3	0.37 ± 0.35	0.57 ± 0.03*	0.02 ± 0.04
GR[2]	1	0.07 ± 0.01	0.12 ± 0.01	0.02 ± 0.01
	2	0.07 ± 0.02	0.30 ± 0.02*	0.06 ± 0.02
	3	0.06 ± 0.02	0.39 ± 0.02*	0.05 ± 0.02
GSH[2]	1	0.7 ± 0.001	0.2 ± 0.001	1.2 ± 0.05
	2	0.8 ± 0.002	0.5 ± 0.001*	1.3 ± 0.02*
	3	0.8 ± 0.002	0.5 ± 0.003*	1.4 ± 0.01*
MDA[3]	1	0.5 ± 0.001	0.3 ± 0.003	0.9 ± 0.005
	2	0.4 ± 0.001	0.4 ± 0.002	0.5 ± 0.002*
	3	0.4 ± 0.001	0.3 ± 0.001	0.5 ± 0.001*

Values are means ± SEM of 6 rats. Group 1: controls; group 2: VCO topically applied (0.5 ml); group 3: VCO topically applied (1.0 ml).). * Statistically significant compared to group 1 ($p < 0.05$).

[1] U/mg proteins. [2] mM NADPH oxidized/min/mg proteins. [3] nM/100 g tissue.

Discussion

In the present study, we examined the influence of VCO, applied topically for the healing of dermal wounds in rats. The results indicate a significant beneficial effect of VCO on intracellular and extracellular matrix components and the antioxidant profile during cutaneous wound healing in animals treated.

The total collagen content of the granulation tissue from both VCO-treated animal groups were found to be significantly higher compared to the untreated controls (table 1). Collagen is the major component of the extracellular matrix and also the predominant protein of the granulation tissue. The role of collagen in the healing process starts immediately during an injury and continues for many weeks to months, even after the wound is closed [34].

The solubility pattern of collagen from the granulation tissue of VCO-treated animals showed an increase in pepsin-soluble collagen (fig. 1). This is evidence for an increased cross-linking of collagen molecules. Fibroblasts synthesize and secrete collagen into the matrix, where it undergoes cross-linking to form fibers. Increased cross-linking results in poor solubility of collagen in neutral buffer and dilute acid solution, and can only be released by limited pepsin digestion indicating a greater wound strength. VCO application increased the total DNA of the granulation tissue. This increase in DNA indicates cellular hyperplasia. VCO application also increased the amount of elastin in the granulation tissue compared to the untreated controls (table 1). Elastin is also a highly cross-linked protein similar to collagen and is found in the connective tissues like skin and large blood vessels [3].

GAG levels were found to be higher in all treated wounds than in the controls (table 1), indicating the active synthesis of extracellular matrix. GAG is the key component of the ground substance that helps the formation of connective tissue [35]. The increase in wet weight of the granulation tissue in the treated groups may be due to the greater compressive strength of newly produced GAG and its water retention capacity (table 1). The total protein content of VCO-treated wounds was significantly increased compared to that of the control wounds. This increase may be due to the expression of proteolytic enzymes which in turn affect the host tissue remodeling processes [36] and the active synthesis and deposition of matrix proteins in the granulation tissues [37]. This is evident from the higher turnover of collagen in wounds treated with VCO.

Glycohydrolase, β-glucuronidase and hexosaminidase activities were found to be increased in the granulation tissues of group 3 animals compared to the untreated control animals (fig. 2). The activity of glucuronidase was found to be lower in group 2 animals compared to control and group 3 animals. Activity of the enzyme β-glucuronidase, a marker for macrophages [38], is correlated with a high degree of cellular proliferation. The role of the enzyme in the catabolism of mucopolysaccharides is also implicated during the wound healing phase [39]. The increase in total hexose and sialic acid levels in the VCO-treated wounds may be due to this higher catabolism of mucopolysaccharides during wound remodeling. During the wound healing process, removal of the injured tissue takes place by the action of hydrolytic enzymes in lysosomes. This may also result in the removal of GAG in the extracellular matrix by highly degrading enzymes.

The skin is frequently and directly exposed to numerous environmental assaults and prooxidants, which are known to promote the generation of reactive oxygen species (ROS). During wound healing, the epidermal cells from the adjacent area cover the wound surface, and

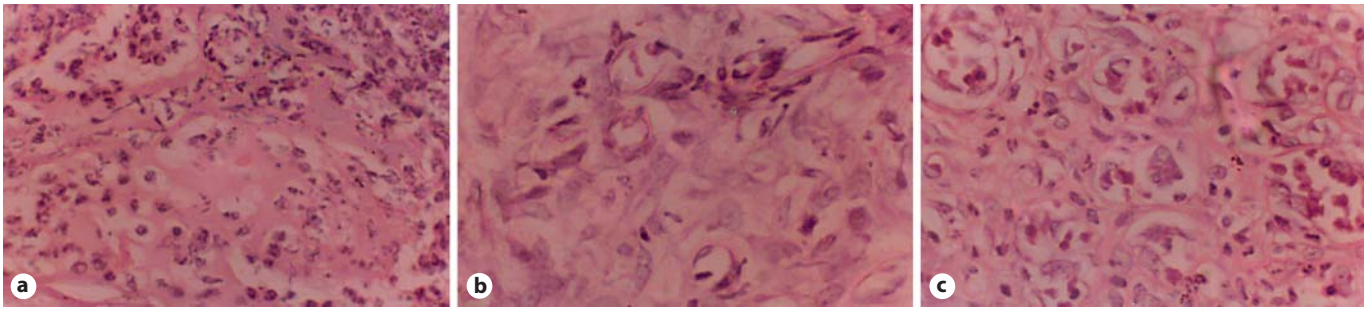

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Fig. 3. Histopathology of the granulation tissue of treated and control wounds. **a** Control. **b** Treated with 0.5 ml VCO. **c** Treated with 1 ml VCO. Granulation tissues were processed, embedded in paraffin wax. HE. ×40.

some of them become phagocytic and accumulate under the wound scab, resulting in a respiratory burst with an increased consumption of oxygen-forming SOD ions [40]. ROS function as specific secondary messengers in signaling cascades involved in cell proliferation and differentiation. The primary immediate defense against this environmental skin damage is the antioxidant capacity of the skin [41–43]. The increase in the activities of SOD, GPx and GR in both treated and untreated wounds may be due to the formation of ROS during active wound healing.

The increase in the activities of antioxidant enzymes resulted in a decrease in lipid peroxides (MDA) in treated rats. The levels remained lower even when the antioxidant enzymes reversed back to basal levels on the 14th day. There are reports that mild oxidative stress can modulate cellular growth and differentiation [44]. The wound site itself is rich in oxidants such as H_2O_2, mostly contributed by neutrophils and macrophages [45]. Studies showed that wounding results in the loss of different free radical scavengers, both enzymatic and nonenzymatic, which both partially recovered following healing [46].

VCO treatment resulted in a decrease in reduced GSH content on the 10th day, but it was found to increase thereafter compared to controls. GSH is a nonprotein thiol in living organisms, which plays a significant role in coordinating the body's antioxidant defense process [47]. Depletion of GSH can inhibit lymphocyte activation and modulates the immune function [48, 49]. Decreased GSH levels are also associated with attenuated signal transduction [50].

The histopathological examination of the granulation tissue of the control animals showed florid fibroblast proliferation (fig. 3). Granulation tissues of group 2 animals (0.5 ml VCO) showed a greater degree of fibroblast proliferation towards the periphery than the control wounds. The same result was observed in the wounds treated with 1.0 ml of VCO.

The wound healing property of VCO may be due to its minor biologically active components and antimicrobial fatty acids. Coconut oil is rich in fatty acids of medium chain length (6–12 C) of which the major fatty acid is lauric acid (12 C) [51]. Previous studies had shown that coconut oil was able to eliminate bacterial infection and can also stimulate the immune response [52]. Fatty acids are bioactive molecules [53], which have been proved to modulate cellular proliferation [54], cell signaling [55] and growth factor activities [56]. Our previous studies have shown that VCO contains higher levels of polyphenols, vitamin E and provitamin A with significant radical scavenging activities, compared to conventionally extracted coconut oil from copra [15, 57]. VCO contains higher amounts of antioxidant polyphones, (+/–)-catechins, ferulic acid, *p*-coumaric acid, caffeic acid, unidentified phenolic acid and unidentified flavonoids [58]. The cumulative effect of these compounds may be beneficial to the wound healing process. In conclusion, these results clearly indicate that VCO beneficially affects the wound environment and fastens the healing process.

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