Original Article
Angiogenic and wound healing potency of fermented virgin coconut oil: *in vitro* and *in vivo* studies

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Abstract: Objective: The process of wound healing involves activation of keratinocytes, fibroblasts, endothelial cells, etc. Angiogenesis is crucial during the process of wound healing. Virgin coconut oil is widely utilized in South Asia for various purposes including food, medicinal and industrial applications. This study aimed to evaluate the potency of fermented virgin coconut oil (FVCO) in angiogenesis and wound healing via both *in vitro* and *in vivo* assays. Methods: Human umbilical vein endothelial (HUVEC), fibroblast (CCD-18) and retinal ganglion (RGC-5) cells were cultured in medium containing different concentrations of FVCO. The proliferation, migration and morphological changes of cells were determined. The angiogenic effect of FVCO was evaluated by rat aortic assay. The therapeutic effect of FVCO on wound healing was further assessed in a wound excision model in Sprague Dawley rats. The expression of phospho-VEGFR2 (vascular endothelial growth factor receptor 2) in HUVECs was detected by Western blot. Results: FVCO (6 and 12 µg/mL) significantly improved the proliferation of HUVEC, CCD-18 and RGC-5 cells ($P < 0.05$ or 0.01). FVCO (25 µg/mL) markedly increased the migration ability of CCD-18 and RGC-5 cells ($P < 0.05$). FVCO did not affect cell morphology as indicated by fluorescein diacetate (FDA), rhodamine 123 and Hoechst staining. FVCO (25, 50 and 100 µg/mL) significantly stimulated the *ex vivo* blood vessel formation as compared with negative control ($P < 0.05$). Rats in FVCO group had significantly smaller wound size, higher wound healing percentage, and shorter wound closure time when compared with control group since day 8 ($P < 0.05$), suggesting that oral FVCO administration notably promoted the wound healing process. FVCO treatment (6 and 12 µg/mL) significantly enhanced the phospho-VEGFR2 expression in HUVECs ($P = 0.006$ and 0.000, respectively). Conclusion: Our study confirms a high angiogenic and wound healing potency of FVCO that might be mediated by the regulation of VEGF signing pathway.

Keywords: Virgin coconut oil, angiogenesis, wound healing, cell proliferation, VEGF

Introduction

Angiogenesis, the formation of new blood vessels in the body, plays a crucial role in a variety of biological processes such as initial embryonic development, growth, tissues granulation and wound healing. During the process of wound healing, angiogenic capillary sprouts invade the wound clot, forming a micro-vascular network throughout the granulation tissue within a few days [1]. Angiogenesis is also essential for several diseases including stroke, myocardial infraction, and cardiovascular diseases. The absence of blood vessels in metabolically active tissues may inhibit the repair of injury or other essential functions [2]. Therefore, identification and application of specific compounds that may induce the creation of new blood vessels may help combat such diseases.

Normal coconut oil is usually extracted from the kernel of matured coconut through dry method, and has been widely utilized in food, medicine and industry throughout the world, especially in South Asia. Virgin coconut oil (VCO) is extracted through a low-heat process from fresh coconut

without the use of chemicals, and thus contains much more biologically active components such as polyphenols, medium-chain fatty acids, tocopherols, squalene, and sterols when compared with normal coconut oil [3]. VCO has proven to have various potential medicinal values, such as antioxidant, antihypercholesterol and antithrombotic activities [4-11]. Its major component, lauric acid (60%), is known to have antimicrobial properties [12, 13]. FVO has also been commonly used in the Ayurvedic medicine for various skin disorders, especially wound healing. Intahphuak et al have also reported the therapeutic effects of VCO on wounds due to its anti-inflammatory, analgesic, and antipyretic activities [14]. Nevertheless, little has been studied regarding the angiogenesis properties of VCO. Hence, the present study has been undertaken to evaluate the angiogenic and wound healing activities of fermented-produced VCO (FVCO) through both *in vitro* and *in vivo* assays. We also detected the phosphorylation level of vascular endothelial growth factor receptor 2 (VEGFR2), a major receptor for VEGF-induced signaling in endothelial cells, in order to elucidate the molecule mechanism underlining the angiogenic effect of FVCO.

Material and methods

Cells, animals and main reagents

Human umbilical vein endothelial cell line (HUVEC), human colon normal fibroblast cell line CCD-18 and the transformed RGC-5 retinal ganglion cell line were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Eight-week-old male Sprague Dawley (SD) rats were obtained from the animal house facility at the Universiti Sains Malaysia (USM). The animal study was approved by USM Animal Ethics Committee (reference number: PPSG/07(A)/044/(2010)). The FVCO samples for this study were prepared by dispersing 2 g of FVCO (commercial product from Genome Life Limited, Malaysia) in 10 mL of distilled water containing 0.5% Tween 80. FVCO was then diluted to achieve the desired concentrations. DMEM, M199 culture media and fetal bovine serum (FBS) were purchased from HyClone Company (Waltham, MA, USA). CCK-8 kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Protein extraction kit and BCA kit were purchased from Qiagen (Valencia, CA, USA). Rabbit anti-human phospho-VEGFR2 (Tyr1054) antibody, mouse anti-human β-actin antibody, and HRP-labeled goat anti-rabbit IgG were purchased from Merck Millipore (Billerica, MA, USA).

In vitro proliferation assay

The effect of FVCO on the proliferation of HUVEC, CCD-18 and RGC-5 cells was assessed by CCK-8 assay. Cells were seeded at a density of 2×10^4 cells/well into 96-well plates containing DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and FVCO in serial concentrations (3, 6, 12, 25, 50 and 100 µg/mL). The plates were incubated at 37°C in an incubator with 5% CO_2 for 48 hours. Control cells were cultured in medium containing vehicle Tween 80 (0.5%). CCK-8 solution (10 µL) was added to each well. After 4 hours, the optical density of each well was measured by a plate reader (Bio-Rad Laboratories) at 450 nm.

Would scratch assay

The migration ability of CCD18 and RGC5 cells was determined by scratch wounding assay. Briefly, cells were seeded into 6-well plates at a density of 2×10^4 cells/well, and incubated at 37°C, 5% CO_2 for 24 hours. The center of the cell monolayer was scraped with a sterile pipette tip and washed twice with PBS to create a straight, cell-free gap. Cells were incubated at 37°C, 5% CO_2 in serum-free media containing FVCO in serial concentrations (3, 6, 12, 25, 50 and 100 µg/mL). Wound closure was monitored and photographed at 12, 18 and 24 hours with a Nikon inverted microscope. Images were analyzed by Image J software (NIH, Bethesda, MD, USA). The distance between the wound was measured after capture of 5 random sites in the visual field, and the percentage of wound closure in each group was calculated.

Cells viability and morphology

CCD18 cells were seeded at a density of 2×10^4 cells/well into 6-well plates containing FVCO in serial concentrations (25, 50 and 100 µg/mL), and incubated at 37°C, 5% CO_2 for 24 hours. Control cells were cultured in medium containing vehicle Tween 80 (0.5%). The cells were respectively treated with fluorescein diacetate (FDA), rhodamine 123 (FLUKA, Mil-
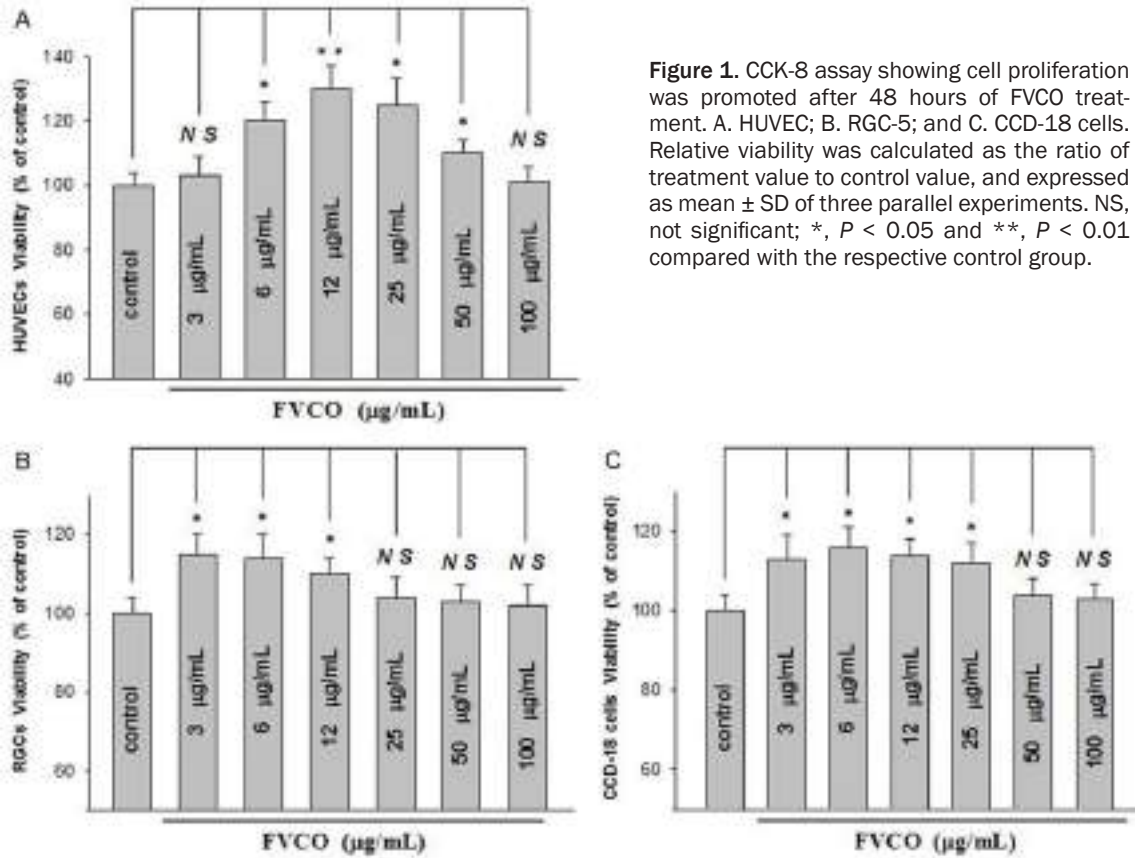
Figure 1. CCK-8 assay showing cell proliferation was promoted after 48 hours of FVCO treatment. A. HUVEC; B. RGC-5; and C. CCD-18 cells. Relative viability was calculated as the ratio of treatment value to control value, and expressed as mean ± SD of three parallel experiments. NS, not significant; *, $P < 0.05$ and **, $P < 0.01$ compared with the respective control group.

waukee, WI, USA) for 1 hour to stain the mitochondria, and hoechst (Invitrogen, Carlsbad, CA, USA) to stain the DNA. The samples were washed with PBS and photographed with a Nikon inverted microscope.

Ex vivo rat aorta ring assay

The rat aorta ring assay was performed as previously described with minor modifications [15] to evaluate the angiogenic potential of FVCO. Briefly, 8-week-old male SD rats were anaesthetized with an intraperitoneal injection of 10% chloral hydrate. Thoracic aorta was excised and the fibro-adipose tissue was removed. The aorta was cut into 1-mm rings, and placed in 48-well plates containing M199 medium supplemented with finbrinogen, L-glutamin and FVCO in serial concentrations (3, 6, 12, 25, 50, 100 μg/mL), and covered with 10 μL of thrombin. The control group was cultured in medium containing vehicle Tween 80 (0.5%). After 5 days, explants were monitored for microvessel outgrowths that occurred spontaneously at a basal rate from the cut

surfaces of the aortic rings using a Nikon inverted microscope (4 ×). The vessels were measured using the image analysis software Leica Qwin and the mean vessel length was calculated.

In vivo wound healing assay

The wound healing properties of FVCO was assessed in an excision wound model as previously described [16]. 8-week-old male rats weighing 200-250 g were randomly divided into 2 groups each containing 6 animals. The treated group was given orally a single dose of 500 mg/kg FVCO per day for 20 days. The control group was given normal diet containing vehicle Tween 80 (500 mg/kg/day). Rats anaesthetized by an intraperitoneal injection of 10% chloral hydrate. An excision wound (2 cm × 2 cm) was made on the dorsolateral flank and left open. The wound area was measured on day 4, 8, 12 and 16 after wounding. The epithelialization period was noted as the number of days after wounding required for the scar to fall off leaving no raw wound behind. The per-
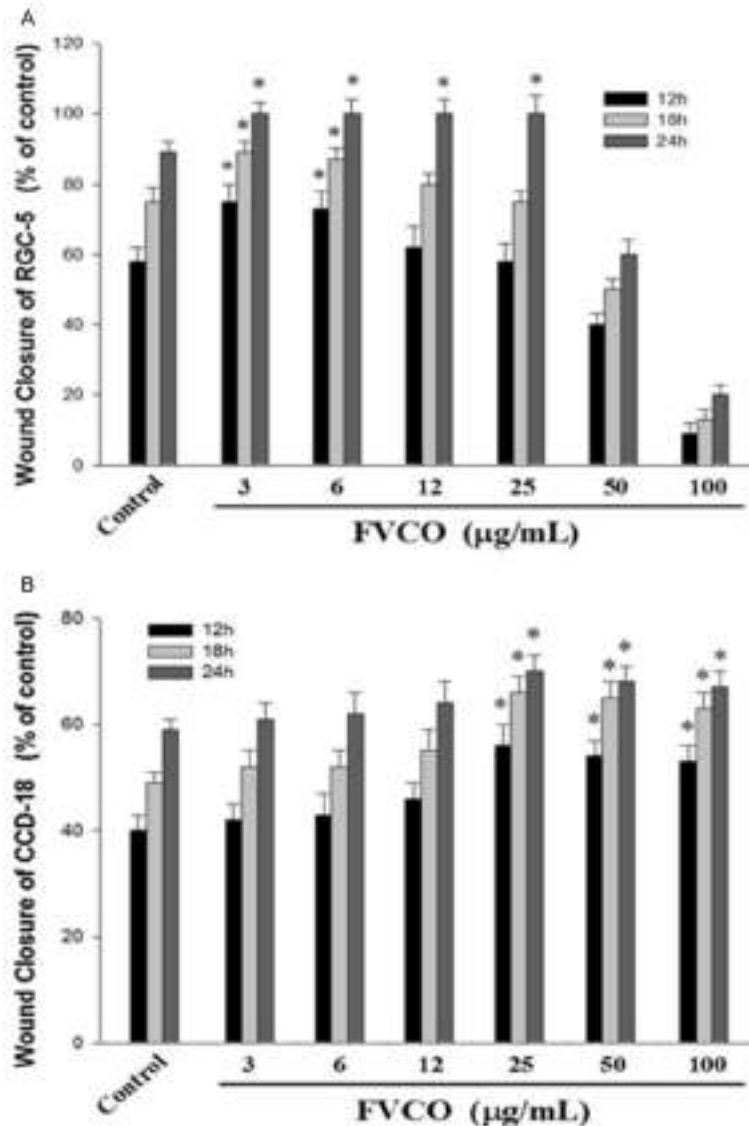
Figure 2. Cell migration was increased after 12, 18 and 24 hours of FVCO treatment by scratch wounding assay. A. RGC-5; and B. CCD-18 cells. Data were expressed as mean ± SD of three parallel experiments. *, P < 0.05 compared with the respective control group.

total protein (20 µg) were separated by electrophoresis using 10% polyacrylamide gels containing 0.1% SDS, and transferred to polyvinylidene difluoride membranes. The membrane was blocked in TBS buffer containing 5% skim milk at room temperature for 2 hours, and incubated overnight at 4°C with rabbit anti-human phospho-VEGFR2 (1:1000) or mouse anti-human β-actin. The membrane was incubated with HRP-labeled secondary antibodies (1:500) at 37°C for 1 hour, washed 3 times with TBST, and subjected to ECL detection. The bands were scanned at 800 dpi using an Epson Perfection 1200 u scanner, and analyzed using Labworks software (UVP Products, CA).

Statistical analyses

All data were expressed as mean ± standard deviation and analyzed by SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Differences between any two groups were compared by student's t-tests. *p* values less than 0.05 are considered statistically significant.

Results

FVCO promoted cell proliferation

centage of wound closure was calculated using the following formula: Wound healing% = (1-(Wound area on day X/Wound area on day 1))*100%.

Western blot analysis

HUVECs were incubated in 6-well plates containing FVCO (0, 6 and 12 µg/mL) at 37°C, and harvested after 24 hours. Total protein was extracted using protein extraction kits and quantified using a BCA kit according to the manufacture's instruction. Equal amounts of

The effect of FVCO on HUVEC, CCD-18 and RGC-5 cell viability was evaluated by *in vitro* CCK8 assay. When compared with control group, the cell viability of HUVECs in 6, 12, 25 and 50 µg/mL FVCO groups was increased by 20%, 30%, 25% and 10%, respectively (*P* = 0.042, 0.000, 0.031 and 0.020, respectively, **Figure 1A**). The cell viability of RGC-5 treated with 3, 6 and 12 µg/mL FVCO was increased by 15%, 14% and 10%, respectively (*P* = 0.017, 0.012 and 0.047, respectively, **Figure 1B**). The cell viability of CCD-18 in 3, 6, 12 and 25 µg/ mL was increased by 13%, 16%, 14% and 12%,
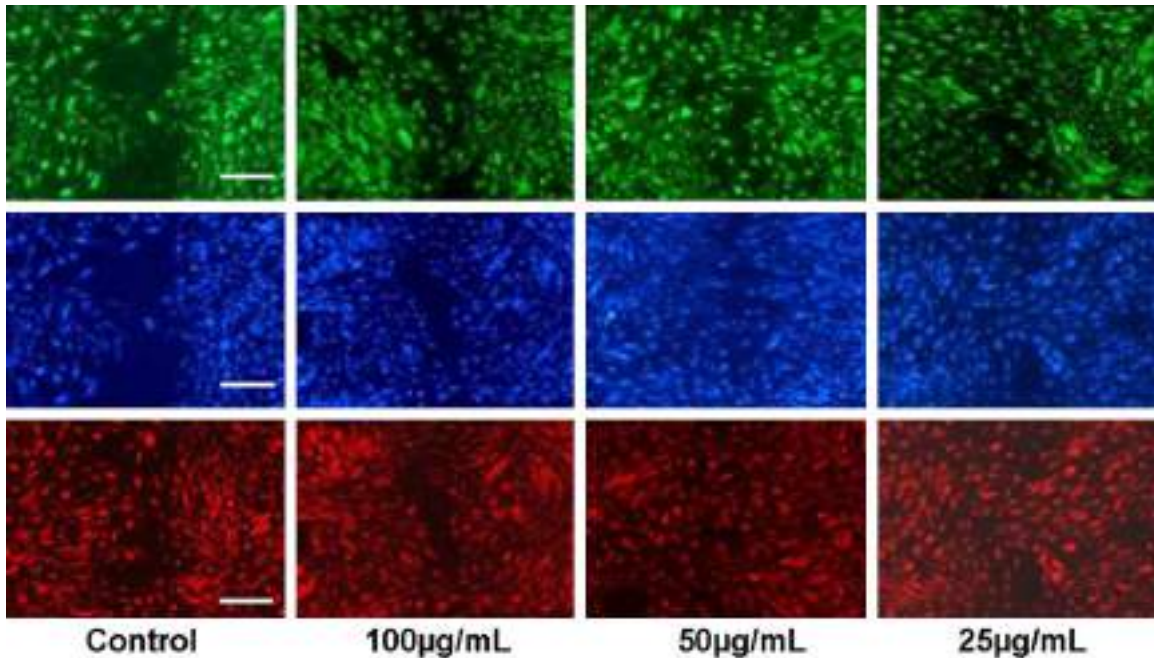
Figure 3. FDA (green color), Hoechst (blue color) and rhodamine 123 staining (red color) showing that FVCO-treated CCD18 cells exhibited similar morphology and density as control group after 24 hours of treatment. Scale bar represents 20 μm.

respectively (P = 0.042, 0.040, 0.019, 0.021, respectively, **Figure 1C**). These results suggested that FVCO markedly improved the proliferation of these cells.

FVCO stimulated cell migration

The effect of FVCO on the migration ability of RGC-5 and CCD-18 cells was determined by scratch wound assay. After 12 hours, the wound closure percentage of RGC-5 cells in 3 and 6 μg/mL FVCO group was 75 ± 1% and 73 ± 2.4%, respectively, which was significantly higher compared with control group (58 ± 1.2%, P = 0.034 and 0.018, respectively). After 18 hours, the wound closure percentage in 3 and 6 μg/mL FVCO group (89 ± 2% and 87 ± 2.7%, respectively) was significantly higher than that in control group (75 ± 2.5%, P = 0.012 and 0.036, respectively). After 24 hours, the gaps in FVCO treated groups (3, 6, 12 and 25 μg/mL) were completely closed, whereas the wound closure percentage in control group was only 89% (**Figure 2A**), suggesting that FVCO significantly enhanced the migration ability of RGC-5 cells. Similarly, wound closure percentage of CCD-18 cells in 25, 50 and 100 μg/mL FVCO group was notably higher compared with the control group at 12, 18 and 24 hours of incubation (all P < 0.05, **Figure 2B**).

FVCO did not affect cell morphology

To evaluate the effect of FVCO on the morphology of cells, CCD18 cells were stained with FDA, rhodamine and Hoechst, respectively. As shown in **Figure 3**, FVCO treated and control (non-treated) groups exhibited similar cell morphology at 24 h after incubation. DNA fragmentation was observed in none of the groups, suggesting that FVCO did not affect cell morphology.

FVCO increased in vitro blood vessel formation

The effect of FVCO on angiogenesis was evaluated by rat aorta ring assay. The aorta ring was obtained from SD rats and treated with FVCO at serial concentrations for 5 days. It was found that the vessel length and density in 25, 50, and 100 μg/mL FVCO groups were notably increased compared with control (P = 0.031, 0.037 and 0.019, respectively, **Figure 4**). FVCO (100 μg/mL) significantly increased the formation of new blood vessels by 14% (**Figure 4**).

FVCO promoted in vivo wound healing

As shown in **Figure 5** and **Table 1**, the FVCO group had significantly smaller wound size, and
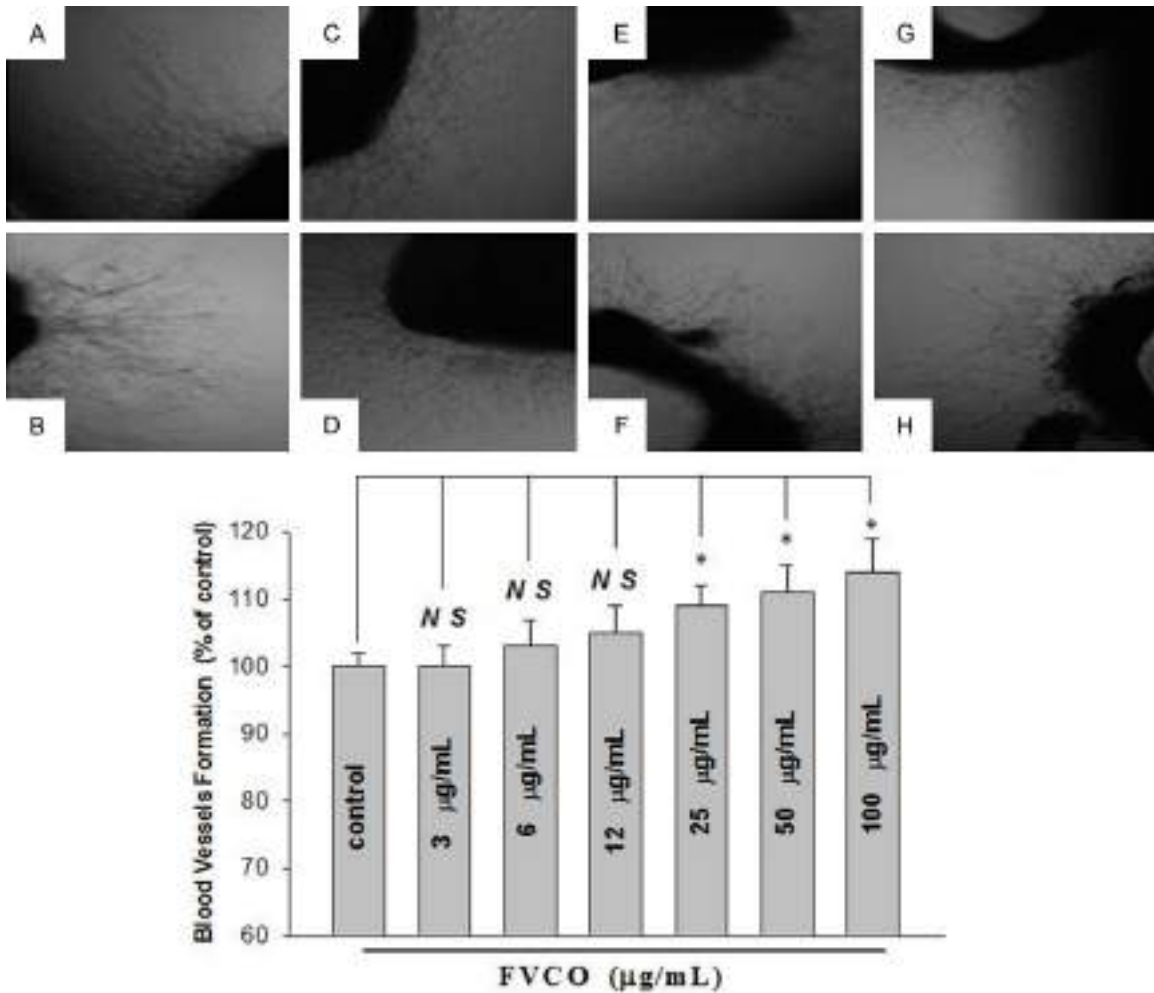
Figure 4. Angiogenesis was stimulated after FVCO treatment by *ex vivo* rat aorta assay. The thoracic aorta excised from male SD rats was cut into 1-mm rings and placed in M199 media with finbrinogen, L-glutamin and FVCO in serial concentrations (0, 3, 6, 12, 25, 50, and 100 μg/mL). Microvessel outgrowths was observed using an inverted microscope (4 ×) after 5 days, and measured using the image analysis software Leica Qwin. (Top) Representative images of the rat aorta ring in different groups. A, B: 100 μg/mL; C: 50 μg/mL; D: 25 μg/mL; E: 12 μg/mL; F: 6 μg/mL; G: 3 μg/mL; and H: Control. (Bottom) Relative blood vessel formation was calculated as the ratio of treatment value to control value. Data was calculated as mean ± SD from 3 parallel experiments. NS, not significant; *, $P < 0.05$ compared with control group.

higher wound healing percentage when compared with control group since day 8 ($P = 0.028$, 0.041 and 0.011, respectively). The wound closure time in FVCO group (16 days) was also markedly shorter than that in control group (20 days), suggesting that oral FVCO administration notably promoted the wound healing process.

FVCO upregulated intracellular phospho-VEGFR2 level in HUVECs

In order to investigate the mechanism behind the angiogenic effect of FVCO, the expression of phospho-VEGFR2 level in HUVECs incubated with or without FVCO was compared by Western blot. It was found that the relative expression of phospho-VEGFR2 in 6 and 12 μg/mL was significantly increased compared with control group ($P = 0.006$ and 0.000, respectively, **Figure 6**).

Discussion

Blood vessels constitute the largest network in our body. Therefore, researches on the identification of therapeutic agents that can promote angiogenesis have received a large amount of attention. It has been known that the angiogen-
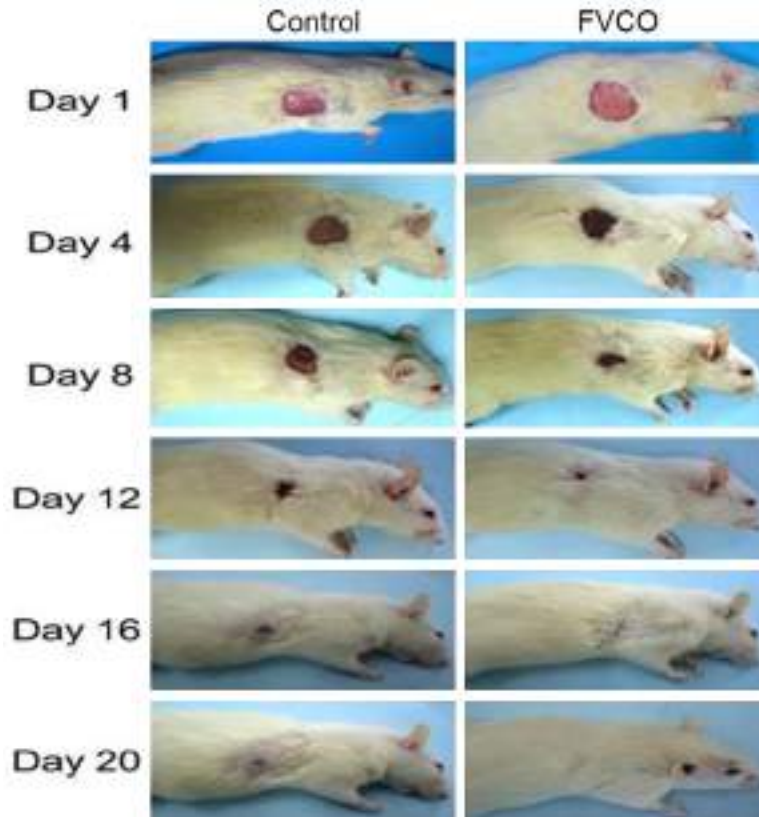
Figure 5. FVCO treatment promoted the wound healing potency in SD rats. Rats with an open excision wound (2 cm × 2 cm) on the dorsolateral flank were randomly assigned into FVCO group (n = 6) and non-treated control group (n = 6). The treated group was given orally a single dose of 500 mg/kg FVCO per day for 20 days. The control group was given normal diet containing vehicle Tween 80 (500 mg/kg/day). FVCO group had obviously smaller wound area since day 8 after wounding.

Table 1. Comparison of wound area and healing percentage in FVCO and control groups

Time	Wound area (mm²)		Wound healing%	
	Control (n = 6)	FVCO (n = 6)	Control (n = 6)	FVCO (n = 6)
Day 1	372 ± 6.1	374 ± 8.2	-	-
Day 4	245 ± 7	207 ± 3.5	35	45
Day 8	160 ± 6	88 ± 3.6	57	77*
Day 12	84 ± 9	15 ± 1.6	78	96*
Day 16	53 ± 5	0	86	100*
Day 20	0	0	-	-

*, $P < 0.05$ compared with control group.

esis process relies on the proliferation, migration, and remodeling of fully differentiated endothelial cells, such as HUVECs. Although previous studies have reported that VCO promotes wound healing due to its anti-inflammatory,

analgesic, antipyretic and antioxidant activities [11, 17], the angiogenesis properties of VCO have seldom been explored. The role of cells in angiogenesis has been well recognized. Our study showed that FVCO (6 and 12 µg/ mL) significantly promoted the proliferation of HUVEC, RGC5 and CCD18 cells as indicated by CKK8 assay. Moreover, wound scratching assay demonstrated that FVCO (25 µg/mL) also significantly enhanced the migration ability of RGC5 and CCD18 cells. To our best knowledge, this is the first study showing the beneficial effects of FVCO on the growth of HUVEC, RGC-5 and CCD-18 cells. The stimulation of FVCO on cell proliferation and migration might be attributed to its high polyphenol content, including protocatechuic, vanillic, caffeic, syringic, ferulic and P-coumaric acids and catechin [18, 19]. In this study, we also found that FVCO treatment did not affect cell morphology of HUVEC, CCD-18 and RGC-5 cells, suggesting the safety of FVCO. Consistently, we have previously reported the high safety of orally administrated FVCO in an acute, sub-chronic and chronic toxicity study using a rat model [20].

In this study, rat aortic assay was employed to study the effect of FVCO on blood vessels formation. Rat aorta ring assay is one of the most common models to mimic human tissue [21]. Our study showed that FVCO enhanced the length of blood vessels and promoted the blood vessel formation in rat aorta ring assay, indicating its angiogenesis effects. Possible reasons are the bioactive compound in coconut oil such as phenolic compound and medium chain fatty acids (MCFAs) might promote the proliferation of HUVEC through stimulating the production of VEGF, a signal protein secreted by cells that stimulates vasculogenesis and angiogenesis. VEGF is an angiogenic protein and
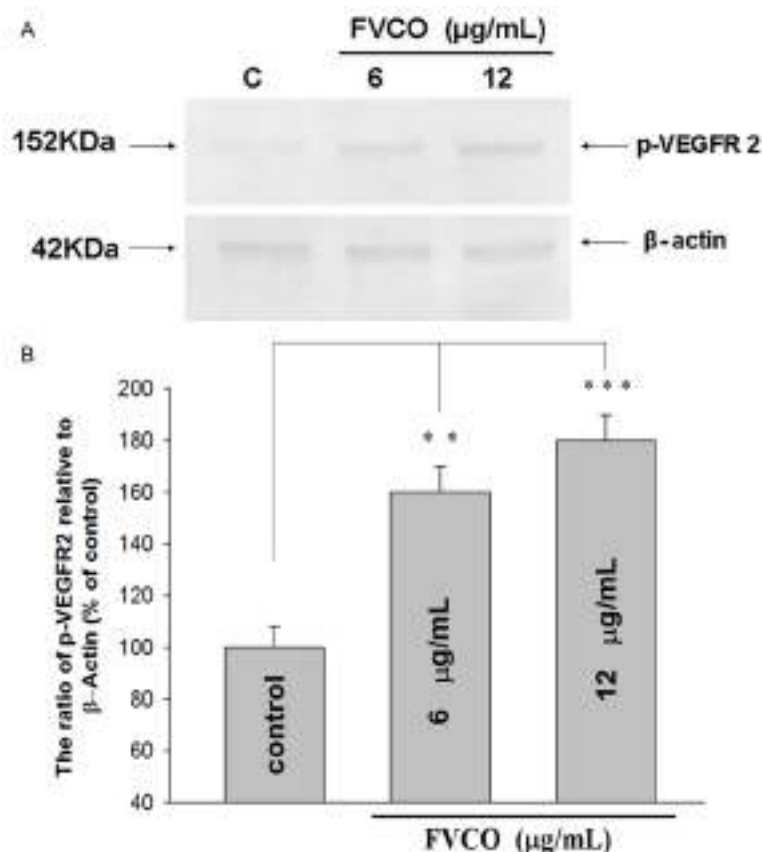
Figure 6. Phospho-VEGFR2 (p-VEGFR2) expression in HUVECs was detected by Western blot after 24 hours of FVCO treatment. A. Representative image of Western blot. B. Relative p-VEGFR2 expression was increased with increasing FVCO concentration. **, $P < 0.01$, and ***, $P < 0.001$ compared with controls.

matrix components during the process of wound healing. The therapeutic effects of FVCO might be attributed to its high content of lauric acid and phenolic compounds [7, 25].

In the this study, we demonstrated for the first time that FVCO promoted the proliferation of HUVEC, RGC-5 and CCD-18 cells and enhanced the blood vessel formation. Our study confirms a high angiogenic and wound healing potency of FVCO that might be mediated by the regulation of VEGF signing pathway.

Acknowledgements

Disclosure of conflict of interest

None.

is produced in large quantities by the epidermis during wound healing [22-24]. We therefore further detected the expression of VEGFR2, a major receptor for VEGF-induced signaling in endothelial cells, in order to elucidate the molecule mechanism behind the angiogenic effect of FVCO. It was found that FVCO treatment markedly enhanced VEGFR2 expression in HUVECs, indicating that FVCO-induced angiogenisis was associated with the activation of VEGF signaling pathway in HUVECs.

Several diseases are the result of failure or insufficient blood vessel formation, such as chronic wounds which could be treated by a local expansion of blood vessels. Transporting new nutrients to the site is very crucial for the repair of a wound [1]. In the present study, we demonstrated the promotive activities of FVCO, applied superficially for the healing of dermal wounds in rats, indicating the beneficial effects of FVCO on intra- and extra-cellular

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