The Role of Coconut Oil to Increase Expression of MMP-9, PDGF-BB, and TGF-β1 in NIH-3T3 Cell Line

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Abstract

AIM: The objective of the study was to evaluate protein expression in NIH 3T3 cells that are treated with virgin coconut oil (VCO) and hydrolysed of virgin coconut oil (HVCO) in vitro.

METHODS: Coconut oil used in this study was virgin coconut oil (VCO) and VCO hydrolysed by *Rhizomucor miehei* (HVCO). NIH 3T3 cells (5×10^5 cells/well) were seeded in nine wells and incubated for overnight, then divided into three groups. Each group consisted of three wells. Group one without treatment, group two added VCO, and group three added HVCO and then incubated for overnight. One well in each group was added MMP-9, PDGF-BB, and TGF-β1 and incubated one hour. Finally, expressions of MMP-9, PDGF-BB, and TGF-β1 were detected using immunocytochemistry method.

RESULTS: The results of the study showed that VCO and HVCO increased protein expressions of MMP-9, PDGF-BB, and TGF-β1. Percentage of MMP-9 expressions treated by VCO increased from 2.89 ± 0.07 to 28.16 ± 0.34, PDGF-BB from 28.11 ± 0.13 to 48.53 ± 0.49, and TGF-β1 from 4.19 ± 0.08 to 18.41 ± 0.54. Percentage of MMP-9 expressions treated by HVCO increased from 2.89 ± 0.07 to 55.40 ± 0.94, PDGF-BB from 28.11 ± 0.13 to 61.65 ± 0.42, and TGF-β1 from 4.19 ± 0.08 to 36.35 ± 0.67.

CONCLUSION: VCO and HVCO increase the expression of MMP-9, PDGF-BB, dan TGF-β1 in NIH3T3 cells and therefore, coconut oil active in the wound healing process. HVCO is more than active than VCO.

Introduction

Wound healing is a complicated and dynamic process including proliferation, differentiation, migration of keratocytes and their elaboration of the new extracellular matrix (ECM) [1]. Wound healing consists of four phases, namely hemostasis, inflammation, proliferation, and remodelling [2]. Hemostasis phase is the beginning of the wound healing process by involving platelets [3]. During the inflammatory phase, fibroblasts function as cytokine secretion, and growth factors to activate the body's defence system [4]. During the proliferation Wound healing is a complicated and dynamic process including proliferation, differentiation, migration of keratocytes and their elaboration of the new extracellular matrix (ECM) [1]. In the hemostasis, the phase is the beginning of the wound healing process by involving platelets [3]. During the inflammatory phase, fibroblasts function as cytokine secretions, and growth factors to activate the body's defence system [4]. During the proliferation and remodelling phases, fibroblasts are important for granulating and reorganising tissues of the extracellular matrix. So, a healing wound shows an elaborate series of interactions cytokines, growth factors, ECM constituent, receptors, proteases, cells, and dissolved mediators [5]. Matrix metalloproteinases (MMPs) take part in many physiological and pathological processes, such as morphogenesis, wound healing, tissue repair, and remodelling [6] and increasing cell

growth, migration, invasion, metastasis, and angiogenesis [5]. Lately, substantial interest has concentrated on matrix metalloproteinase 9 (MMP-9), a significant MMP family member. Transforming growth factor-beta 1 (TGF-β1), platelet-derived growth factor-BB (PDGF-BB), and fibroblast growth factor (FGF) are examples of GFs involved in cell proliferation and migration [1], [6].

Coconut oil is the most saturated oil compose of medium-chain fatty acids, including capric (7%), lauric (49%), myristic (18%), palmitic (9%), stearic (2%), and small percentages of unsaturated fatty acids that including oleic (6%) and linoleic acids (2%) [7]. VCO isolated from mature coconut fruit and processed at low temperature without chemical refining, bleaching or deodorising, and which does not lead to the conversion of the nature of the oil [8]. VCO contains more biological active constituent such as tocopherols, sterols, polyphenols, and squalene [9]. VCO contains lauric acid, which has antimicrobial, anti-viral, anti-fungal and antibacterial properties [8]. Partial hydrolysis of VCO (HVCO) using lipase from Rhizomucor miehei which is active on sn-1, 3 positions in triglyceride molecule resulted in free fatty acids and 2-monoglyceride mainly a mixture of lauric acids and more active antibacterial monolaurin [10], [11], [12]. VCO and HVCO had been tested by in vivo method and found to be active in wound healing and more active compared to the bioplacenton as a standard medicine for burn wound healing [13].

This research aims to determine the role of VCO and HVCO in wound healing by measuring the expression of MMP-9, PDGF-BB, and TGF-β1 in NIH 3T3 in vitro.

Material and Methods

Materials

Virgin coconut oil (VCO) (Palem Mustika®, Indonesia), NIH 3T3 fibroblasts were purchased from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal bovine serum and kept at 37°C with a CO_2 supply of 5%. Lipase from R. miehei 20.000 U/g (Sigma) and reagents used were buffer Tris-HCl, sodium hydroxide, concentrated hydrochloric acid, distilled water, n-hexane, sodium sulfate anhydrous, potassium hydrogen phthalate, phenolphthalein, primer antibody (MMP-9, PDGF-BB, TGF-β1) and ethanol. Reagents that used to immunocytochemical assay. All chemicals and reagents used in this work were of analytical grade.

Enzymatic hydrolysis of VCO

Thirty (30) g of oil was transferred into 250 ml Erlenmeyer flask, and then, 30 ml distilled water, 12.5 ml 0.063 M $CaCl_2$, 25 ml buffer Tris-HCl 1 M pH 8, and 3 ml lipase R. miehei were added. The mixture was stirred at 200 rpm for 10 min of every 1 h incubation time. The mixture was incubated at 50°C at 10 h. At the end of the mixture incubation time, the mixture was transferred into the separating funnel, and then, extracted with 50 ml n-hexane. The mixture was allowed to stand for some time until two layers were separated. The upper layer (n-hexane fraction) was separated as the first extract, while the bottom layer (water fraction) was extracted again as above and separated as the second extract. The first and the second extracts were mixed, and then, 250 g sodium sulfate anhydrous was added to absorb the water residue. The combined extract was allowed to stand for 15 min, filtered, and the n-hexane was evaporated using the water bath. The hydrolysed VCO (HVCO) was then used in the experiment [10], [11], [12].

Immunocytochemical Assay

NIH 3T3 cells (5 x 10^5 cells/well) were seeded into 9 wells with a coverslip in 24-well plate and incubated for 24 h. Wells were grouped, they consisted of control groups (without VCO or HVCO), VCO groups, and HVCO groups. After that, the cells were treated with 500 µL VCO (62.5 µg/mL) in medium and 500 µL HVCO (62.5 µg/mL) in medium and then incubated for 24 h. The medium was taken, washed with PBS 2 times. Then cells in coverslips fixed with cold methanol and washed PBS 2 times, then washed 2 times with distilled water. Coverslips moved in a slide then added 300 µL H_2O_2 (1: 9 in distilled water), then incubated for 10 minutes, discarded and washed with PBS 2 times. Then added 100 µL Blocking (Background Snipper), incubate for 15 minutes at room temperature, discarded and added 50 µL of primary antibody (MMP-9, PDGF-BB, TGF-β1) incubation for 60 minutes. After discarded again with PBS 2 times, then added 100 µL of secondary antibody (Trekkie Link) incubated for 20 minutes on room temperature.

The secondary antibody was removed, washed with PBS 2 times and added Avidin HRP Tracks, incubated for 10 minutes at temperature room, discarded and washed with PBS 2 time. Continued with the addition of 100 µL of DAB, incubated for 5 minutes, removed, and washed with distilled water. After being discarded and cleaned with tissue, added with 300 µL Mayer-hematoxylin and incubated 5 minutes. Then washed with distilled water until clean. Slide (preparation) dipped in ethanol, then dipped in xylol. After drying it was covered with a glass cover and added enter. Protein expression observed using a microscope. If protein gave brown/dark colour in the cytoplasm of all cells was positive. If protein gave blue/purple was negative Data

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analysis with Image Raster software was carried out to calculate the expression of MMP-9, PDGF BB, and TGF-β1 protein. The results of the analysis are through the image quantification of the average 5 (five) field of view [14].

Statistics

One-way analysis of ANOVA followed by Tukey's post hoc test was used to analyse data ($p < 0.05$).

Results

Observation of protein expression of MMP-9, PDGF-BB, and TGF-β1 in NIH 3T3 cells was done by comparing the protein expression of MMP-9, PDGF-BB, and TGF-β1 without treatment (as a control) with VCO and HVCO treatment. The data were obtained in the form of percentage expressions of MMP-9, PDGF BB, and TGF-β1 in 5 fields of view (Figure 1, Table 1, and Figure 2).
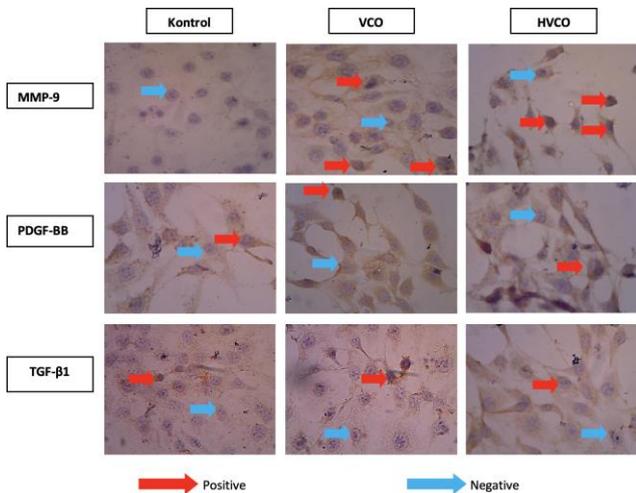
Figure 1: Effect of VCO 62.5 μg/mL and HVCO 62.5 μg/mL treatment on NIH 3T3 cells. Cells were observed with a microscope (40 x magnification)

As can be seen, VCO and HVCO increased protein expression tested. Percentage of MMP-9 expressions were increased from 2.89 ± 0.07 (control) to 28.16 ± 0.34 (VCO), and 55.40 ± 0.94 (HVCO).

Table 1: Effect of VCO and HVCO on NIH 3T3 cells towards MMP-9, PDGF-BB, and TGF-β1

Group (n = 3)	Protein Expression (%) (Mean ± SEM)		
	MMP-9	PDGF-BB	TGF-β1
Control	2.89 ± 0.07	28.11 ± 0.13	4.19±0.08
VCO	28.16 ± 0.34*	48.53 ± 0.49*	18.41±0.54*
HVCO	55.40 ± 0.94*	61.65 ± 0.42*	36.35±0.67*

Values are mean ± SEM; Statistics-one-way ANOVA followed by Tukey's post hoc test; *p < 0.05 significantly difference; n = number of observers in each group; SEM: Standard error of the mean.

Percentage of PDGF-BB expressions were increased from 28.11 ± 0.13 (control), to 48.53 ± 0.49 (VCO) and 61.65 ± 0.42 (HVCO). Percentage TGF-β1 expressions were increased from 4.19 ± 0.08 (control) to 18.41 ± 0.54 (VCO) and 36.35 ± 0.67 (HVCO).
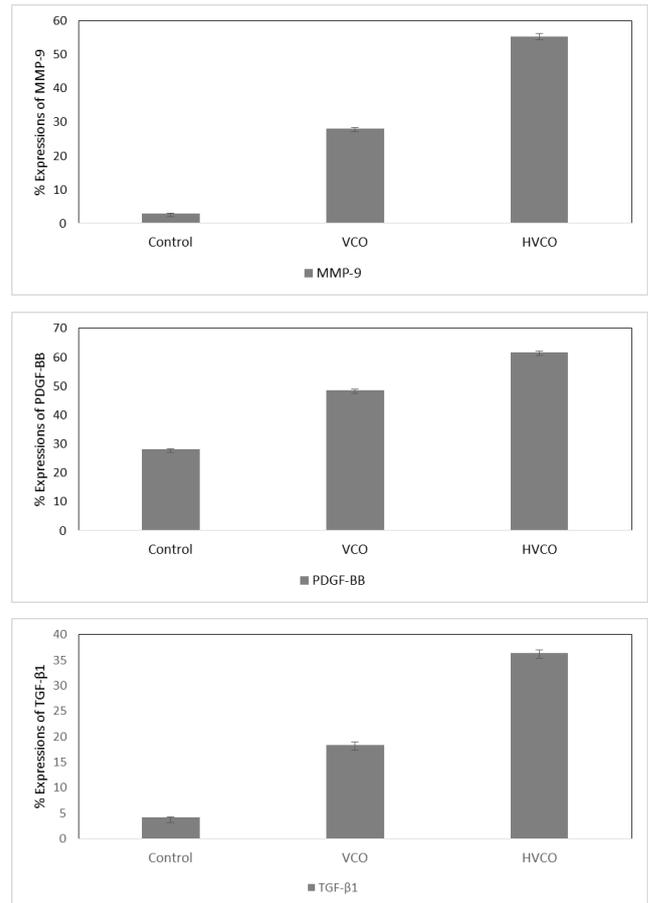
Figure 2: The effect of treatment with VCO and HVCO on the expression of MMP-9, PDGF-BB, and TGF-β1(*p > 0.05 = different significant compared control)

Discussion

According to on the above Figure 1, Table 1, and Figure 2 can be seen that VCO and HVCO can increase the expression of the protein of MMP-9, PDGF-BB, and TGF-β1 increased proliferation, migration, angiogenesis, which play a role in the wound healing process. Then VCO and HVCO can be active in the wound healing process. According to Silalahi and Surbakti (2015) reported that HVCO (12 days) found to be more effective than VCO (17 days) in the wound healing process, compared with bioplacenton (18 days) as standard medication [13]. The individual effect of TGFβ1, PDGF- BB, and bFGF on cell proliferation and differentiation during the repair process were studied at different time points until wound closure by Munoza, et al., 2017 [1]. TGF-β1 excited proliferation in the wound site and the surrounding area, persuade myofibroblast

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differentiation and inhibited cellular migration. PDGF-BB persuade rapid wound closure proliferation, high motility. Each of the GFs induced increased in responses promoting stromal repair differently. Matrix metalloproteinases (MMPs) belong to the family of zinc-dependent endopeptidases, whose primary function is to degrade the extracellular matrix (ECM) [5]. MMPs participate in many physiological and pathological processes, such as morphogenesis, wound healing, tissue repair, and remodelling [5]. MMP-9 is an important MMP family [5]. MMP-9, a type IV collagenase, may show a major role during cell growth, inflammation, and angiogenesis [15]. VCO contains lauric acid, which has antimicrobial, anti-viral, anti-fungal and antibacterial properties [8]. VCO and HVCO contain bioactive components which play a role in wound healing [15]. A few studies have reported the benefits of lauric acid as an antimicrobial and anti-inflammatory agent that can help to overcome skin problems [8]. Fatty acids are important components of cell membranes, high promotes the inflammatory process on wound healing [15]. So that, VCO and HVCO by increasing expression MMP-9, PDGF-BB, and TGF-β1may play an important role in the wound healing process.

In conclusion, treatment with VCO and HVCO on NIH 3T3 cells increase MMP-9, PDGF-BB, and TGF-β1expressions and hence play a role in the wound healing process.

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