

The Activity of Hydrolyzed Virgin Coconut Oil to Increase Proliferation and Cyclooxygenase-2 Expression towards on NIH 3T3 Cell Line in Wound Healing Process

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Abstract

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Aim: This study aims to determine the effect of hydrolysed virgin coconut oil (HVCO) to increase cell proliferation, COX-2 expression of NIH 3T3.

METHODS: The sample used was Virgin Coconut Oil (VCO). VCO was partially hydrolysed using lipase from *Rhizomucor miehei* (active on sn-1,3 position) to produce hydrolysed VCO (HVCO) composed of free fatty acids, 2-monoglycerides. Then acid value was determined. The effect of HVCO on proliferation was evaluated using the MTT method. Wound healing assay was established by a cell migration method, and COX-2 expression was determined using RT-PCR.

RESULTS: Acid value is 135.89 ± 0.12 mg NaOH/g oil and free fatty acids (FFA) is $48.50 \pm 0.06\%$. The effect of HVCO $62.5 \mu\text{g/mL}$ on cell proliferation after 24h, 48h, and 72h incubation found as viable cells are $109.24 \pm 0.52\%$; $118.26 \pm 0.91\%$ and $106.59 \pm 0.74\%$. Percent of wound closed after 24 h and 48 h incubation are $69.94 \pm 0.54\%$ and $100.00 \pm 0.00\%$, and expression of COX-2 increased from 1 (control) to 1.83 (HVCO).

CONCLUSION: The results suggest that HVCO is effective to increase cells proliferation and hence wound healing process.

Introduction

Wound healing is a process involving many cells consisting of four stages, namely hemostasis, inflammation, proliferation, and remodelling [1]. In the hemostasis, stages are the beginning of the wound healing process by involving platelets [2]. During the inflammatory phase, fibroblasts function as cytokine secretions, and growth factors to activate the body's defence system [3]. During the proliferation and remodelling phases, fibroblasts are important for granulating and reorganising tissues of the

extracellular matrix [3].

Wound healing is associated with bacterial contamination in the wound area. The ultimate goal of wound healing is to restore the functional properties of the skin and prevent infection [4]. The COX enzyme consists of 2 isoenzymes such as COX-1 (COX-3 = COX-1 variants), and COX-2. COX-2 plays a role in the process of angiogenesis [5]. The expression of COX-2 affects the process of angiogenesis, migration, and proliferation of fibroblasts which is very important in wound healing [6]. The expression of COX-2 affects the process of angiogenesis, migration, and proliferation of fibroblasts [6].

VCO is obtained from the flesh of mature fresh coconuts fruit (*Cocos nucifera*) processed at low temperature or without heating [4]. VCO contains phytosterol that can be beneficial as anti-inflammation. Coconut oil as triglyceride does not have antimicrobial and antiviral activities, but when VCO is partially hydrolysed, it will generate free fatty acids and monoglycerides [7]. The combination of free fatty acids and monoglycerides are proved to be an antibacterial and antiviral agent, whereas diglycerides are not [8], [9]. Lauric acid and monolaurin (monoglyceride of lauric acid) are antibacterial and antiviral through several mechanisms including by liquefying and damaging the lipid layer structure in virus and cell membrane of bacteria [8], [9].

This study aims to determine the effect of HVCO in the wound healing process by measuring the activity of cell proliferation, COX-2 expression, and cell migration in NIH 3T3 cells.

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 CO₂ supply of 5%. Lipase from *R. miehei* 20.000 U/g (Sigma), and reagents used were buffer Tris-HCl, nuclease-free water, sodium hydroxide, concentrated hydrochloric acid, distilled water, n-hexane, sodium sulfate anhydrous, potassium hydrogen phthalate, phenolphthalein, ethanol, and demineralized water, DMSO, phosphate buffer saline (PBS), and fetal bovine serum (FBS). All chemicals and reagents commonly used in COX-2 expression assay.

Enzymatic hydrolysis of VCO

Thirty (30) g of oil was transferred into 250 ml Erlenmeyer, and then, 30 ml distilled water, 12.5 ml 0.063 M CaCl₂, 25 ml buffer Tris-HCl 1 M pH 8, and 3 ml lipase *R. miehei* were added. The mixture was incubated at 50°C for 10 h and stirred at 200 rpm for within every 1 h incubation time. At the end of the mixture incubation time, the mixture was transferred into the separating funnel and extracted, 50 ml n-hexane was added, and shaken at 5 minutes was done. The mixture was allowed to stand for some time until two layers were formed. The upper layer (n-hexane fraction) was separated as the first extract, while the bottom layer (water fraction) was extracted again with 50 ml n-hexanes 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 and resulted in HVCO, and then the acid value was determined. HVCO was then used to determine biomarkers in the wound healing process [10], [11], [12].

Acid value determination

Five (5) g VCO was weighed, and procedure titration carried out, then the acid value and free fatty acid (FFA) percentage of HVCO was calculated as previously described [10], [12].

Proliferative Activity

HVCO (1000 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; 62.5 µg/mL; 31.25 µg/mL; and 15.625 µg/mL in *co-solvent* DMSO (Sigma) was submitted for proliferative test. In that way, NIH 3T3 cell line (1 x 10⁴ cells/mL) was grown in DMEM complete medium. After 24; 48 and 72 h treatment, MTT assay was performed and cell viability was counted to determine the proliferative activity [13], [14], [15].

Wound Healing Migration Assay

The migration assay was carried out with NIH 3T3 cells seeded at 5 x 10⁴ cells/well in 24-well plates and incubated for 24 h at 37°C. Cultured cells were washed with PBS and added culture media which containing 0.5% FBS and incubated for 24 h. Scratch was done in the bottom centre of the well within the cell layer using a yellow tip. Cell residues in the plate were washed with PBS and treated with HVCO and incubated for 48 h at 37°C and documented under the inverted microscope against cell migration rapidity after 0, 24, and 48 h. The space from scratch treatment between control and treatment culture cell was quantified using Image J software and defined as cell migration area [16].

Expression of COX-2

NIH 3T3 cells (5 x 10⁴ cells/well) were seeded into 6-well plate and incubated for 24 h. After that, the cells were treated with HVCO and then incubated for 24 h. Both floating and adherent cells were collected in a conical tube using trypsin 0.025%. The cells were washed thrice with cold PBS and centrifuged at 2500 rpm for 5 min. The supernatant was separated and used for RNA extraction (Genaid, USA) and RNA concentration was determined by spectrophotometric method (Nanodrop) and stored at -80°C until used. Complementary DNA (cDNA) was synthesised from 3.0 µg total RNA using RT-PCR kit (Toyobo, Japan) in

a final volume of 20 μL using random primers based on the manufacturer's instructions. RT-PCR was carried out in Apply Biosystem Proflex. The reaction mixture consisted of GoTaq Green (12.5 μL) (Promega), 1.0 μL of cDNA 1 μL forward primers, 1 μL reverse primers, and 9.5 μL nuclease-free water to make a total volume of 25 μL . β -actin was used as internal reference control. The PCR primers were used for β -actin (F: 5'-gtc gta cca ctg gca ttg t-3'; R: 5'-cag ctg tgg tga agc t-3'), Cox-2 (F: 5'-cca gca ctt cac gca tca gt-3'; R: 5'-acg ctg tct agc cag agt ttc ag-3'). The PCR condition was comprised of first incubation at 95°C for 2 minutes, 95°C for 30 sec, annealing at 55°C 30 sec, extension at 72° for 1 minute, and 35 cycles. The PCR products were detected by electrophoresis in 2% agarose gels and added gel red 10 μL . Then, they were visualized with gel doc [13], [17], [18].

Statistic Analysis

The results were presented as means \pm SD. The statistical analysis was carried out by using SPSS edition 21.

Results

Acid value and %FFA of HVCO

The acid value is 96.89 ± 0.12 mg NaOH/g oil and free fatty acids (FFA) is $48.50 \pm 0.06\%$.

Wound Healing Migration Assay

A little wound repair was observed in wells with HVCO at 62.5 $\mu\text{g}/\text{mL}$ after 24 and 48h incubation with $61.94 \pm 0.54\%$ and $100.00 \pm 0.00\%$ respectively closure area. The wound healing migration of HVCO is given in Figure 1.

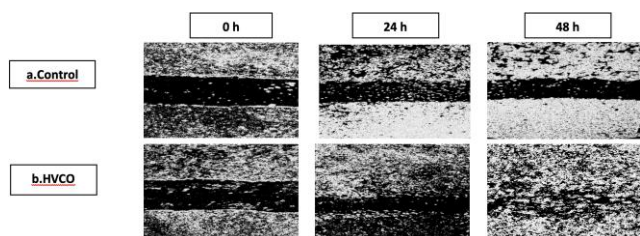


Figure 1: Wound Healing Migration Assay. NIH 3T3 cells were treated by HVCO 62.5 $\mu\text{g}/\text{mL}$ for 0; 24 and 48 h and measured the closure area; A) control cells; B) HVCO 62.5 $\mu\text{g}/\text{mL}$. Proliferative Activity

The percentage of viable cells after treatment and incubation for 24 h, 48 h, and 72 h (109.98 ± 0.52 ; 118.26 ± 0.53 ; 106.59 ± 0.43) showed the stimulation effect of HVCO towards the proliferation of

NIH 3T3 cells. The effect of HVCO is given in Figure 2.

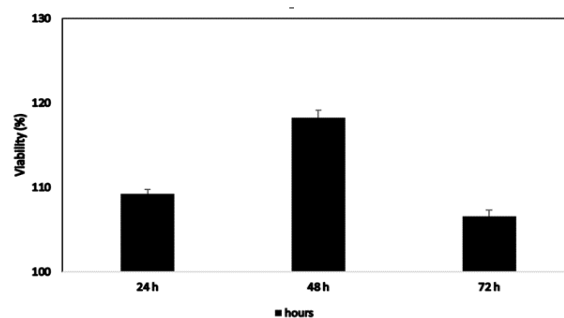


Figure 2: Percentage of viable cells of NIH 3T3 cells were treated by HVCO 62.5 $\mu\text{g}/\text{mL}$ for 24; 48 and 72 h and measured viable cells

COX-2 Expression

HVCO were showed a significant up-regulatory effect on the expression of COX-2 (1.93 ± 0.49). COX-2 expression is given figure 3.

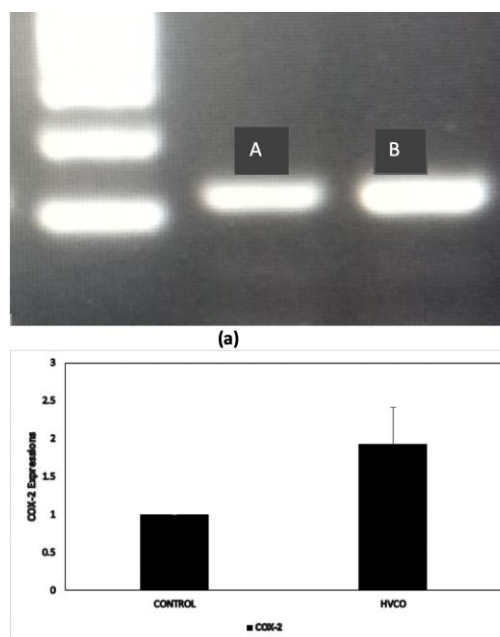


Figure 3: Representative figures showing COX-2 expression after treatment with HVCO 62.5 $\mu\text{g}/\text{mL}$ (a) bands of COX-2 expressions (A = Control Cell; B = HVCO = 62.5 $\mu\text{g}/\text{mL}$), and (b) chart of COX-2 expressions

Discussion

Enzymatic hydrolysis of VCO was done using lipase from *R. miehei*, which is specific for acyl groups at sn-1 and sn-3 position in triglyceride molecule [10], [11]. At the temperature of 50°C, this enzyme hydrolyses fatty acids on sn-1 and sn-3 positions in triglyceride molecule which generates two free fatty acids (FFAs) and 2-monoglyceride in hydrolyzed VCO (HVCO) [10], [11]. Acid value is defined as mg NaOH

used to neutralise FFA contained in 1 g of fats or oils to indicate the amount of FFA in one-gram fats or oils. HVCO composed of FFAs mainly as lauric acids and 2-monoglycerides mainly as 2-monolaurin [12].

Lauric acid and monolaurin are the antibacterial and anti-inflammatory agent that able to overcome skin problems [19]. Lauric acid and monolaurin decrease the time for complete epithelialization because lauric acid and monolaurin can increase proliferation cells and migration cells [4]. During the wound healing process, cells at the wound edges proliferate and migrate, leading to re-epithelialization of the wound surface [20].

Migration of NIH 3T3 fibroblasts was assessed using the wound healing scratch assay. Lauric acid and monolaurin increase proliferation cells and migration cells [4]. Cell migration activity in the HVCO group is faster than the control group. Lauric acid and monolaurin increase proliferation cells and migration cells. Lauric acid and monolaurin are found in HVCO that stimulate cells to migrate. So, the percentage of HVCO group cell migration is faster than the control group.

Cyclooxygenase-2 (COX-2) is an inducible enzyme which plays a critical role in multiple pathophysiological processes including inflammation, atherosclerosis, tissue injury, angiogenesis and tumorigenesis [6]. According to Futagami et al., study, COX-2 mRNA in the normal rat skin with a wider allocation than the COX-2 protein but with less intensity. After the injury, this COX-2 protein and mRNA were expressed primarily in the head and basal layers of the epidermal wound edges, which are structured of migratory and proliferative cells [6].

In the Ebeling et al., study, COX-2 is one of the wound healing parameters. Pentacyclic triterpene and botulin are an active compound of birch bark extract. They influence the inflammatory phase of wound healing by upregulating proinflammatory cytokines, chemokines and cyclooxygenase-2 (COX-2) in human primary keratinocytes. Exemplarily, Ebeling et al., confirm upregulation in the ex-vivo pig wound healing model for IL-6 and COX-2. They provide evidence for COX-2 and IL-6 that their mRNA increase is due to an mRNA stabilising effect, a process in which p38 MAPK and HuR (human antigen R) are essentially involved [21]. In this study, Lauric acid and monolaurin increase of COX-2 expression which mediated angiogenesis and migration NIH 3T3 cell.

In conclusion, HVCO increase cell proliferation after 24 hours incubation, constant at 48 and decreased after 72 hours incubation, cell proliferation decreases. Percent of the wound closed at 48 hours incubation, closing up to 100%. HVCO is also able to increase COX-2 expression. HVCO increase cell proliferation, percent of the wound closed, and COX-2 expression, so HVCO is useful as wound healing.

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