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Effects of oleogels (alpha-linolenic acid plus beeswax) extracted supplementation for approaching the therapeutic food ingredient: *in vitro* model

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Abstract

The dietary fatty acid intake for vascular disease has been highlighted for a decade. This study aimed to determine the effect of palmitic acid and fatty acid-incorporated beeswax (oleogels: OG) supplementation on anti-atherosclerotic effects through vascular smooth muscle cell (VSMC) activity. The A7r5 cells were cultured, cells were treated with three treatments, including 1) control, 2) palmitic acid (PA), and 3) alpha-linolenic acid (ALA) + beeswax, ratio 1:1 (OG) at different concentrations of 0, 25, 50 and 100 µM for 48 hrs. Cell proliferation, cell apoptosis, wound repair, and relative quantity of mRNA level of inflammatory cytokines and angiogenetic transcription factors were determined. The results showed there was a significantly reduced VSMCs proliferation with concentration-dependent ($p < 0.05$). In the presence of OG at 100 µM, the wound area had healed after 24 hrs (59.2%) compared to PA (28.0%) group treatments. OG and PA supplementation significantly up-regulated eNOS and VEGF but down-regulated the TNF-α, CRP, CD36, iNOS, and NF-κB mRNA expression levels, while PA was a contrasting pattern ($p < 0.05$). Therefore, supplementation of OG reduced the pro-inflammatory effects of inflammatory cytokines in macrophages and induced VEGF expression in VSMCs, contributing to anti-atherosclerotic effect.

1. Introduction

The chronic inflammatory effect of the endothelial cell leads to vascular disorders. It is established as a cause of death globally, such as sudden heart failure and induced atherosclerosis diseases. Previous research has demonstrated that the damage and dysfunction of the endothelial cell results in atherosclerosis development (Virdis *et al.,* 2010; Zhuang *et al.,* 2013; Wu *et al.,* 2014). The imbalance of endothelial nitric oxide synthesis (eNOS) in blood circulation also directly affects vasodilating and vasoconstricting substances (Farhangkhoee *et al.,* 2006). Moreover, it is also associated with cytokines stimulating migration and proliferation, deposition of molecules such as calcium (Chang *et al.,* 2015) as well as the production of inflammatory mediators in vascular smooth muscle cells (VSMCs) (Yang *et al.,* 2015). Once clinical studies have shown that a diet rich in PUFAs can reduce the risk factor of vascular disease (Mozaffarian and Wu, 2011). Furthermore, the supplementation of EPA and DHA omega-three fatty acids enhances the eNOS production of the endothelial cell and improves the VSMC function (Tagawa *et al.,* 1999; Sala-Vila *et al.,* 2011; Zhuang *et al.,* 2013). Meanwhile, intake of saturated fatty acids (SFA) stimulates endothelial damage and dysfunction, which contribute to inflammatory mediators and many cytokines production in macrophages such as TNF-α, NF-κB, CRP, IL-6, and MCP-1 (Wu *et al.,* 2014). Besides, Moers and Schrezenmeir (2009) also reported that NO production in endothelial cells had been suppressed by palmitic acid (PA). According to Lu *et al.* (2011), patients with vascular pathology mainly consist of the inducible NOS in their body, especially in the lipid core enclosed by macrophages. However, either the effect of fatty acids ester or fatty acid derivative on VSMCs function has not been indicated and reported. The vascular endothelial growth factor (VEGF) is a critical protein signaling that leads to the repair or remodeling the damaged blood vessels, generating an angiogenesis process and vascular disorder prevention (Kalka *et al.,* 2000; Hamdollah Zadeh *et al.,* 2008). The damaged blood vessels could be recovered medically or surgically, and vascular growth factor gene therapy (Kalka *et al.,* 2000; Zhuang *et al.*, 2013).

Interestingly, Lee *et al.* (2016) illustrated that the

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fatty acid methyl ester form has promoted a cerebral vasodilatory effect and delayed the cerebral vasospasm causing a potential therapeutic against neurodiseases. Moreover, Wong *et al.* (2013) have shown strong evidence of omega-3 FA ethyl esters supplementation in the diet by clinical studies for increasing the artery elastic properties in obese subjects. Indeed, although the medical approaches are under the examination stage for endothelial repairing, the tube formation or proliferation and the migration of VSMC in the in-vitro study should be investigated through angiogenic growth factor mechanisms (Cheng *et al.,* 2006; Hamdollah Zadeh *et al.,* 2008).

Palmitic acid (PA) is a saturated fatty acid that has an adverse effect on mammalian health especially, cardiovascular disease development. Over intake of PA can promote the dysfunction of endothelial cell activity (Wu *et al.,* 2014). On the other hand, alpha-linolenic acid (ALA) also known as the omega-3 fatty acid may help improve heart and brain function which was reported and proved by several scientific evidence (Lee and Lip, 2003; Zhuang *et al.,* 2013). However, imbalanced consumption of all types of fatty acids may result in abnormal adipose tissue and endothelial cell functions. Beeswax is an organic material which is composed of fatty acid esters and long chain alcohol groups. The EFSA Journal reported that there was no adverse effect observed for the short-term of this kind of wax application using the rats feeding model (not over 500–10,000 mg/kg) (Issara *et al.,* 2019). Moreover, this wax can be combined with fatty acid and structured for a semi-solid form as a fat replacer in the food application.

Oleogel (OG) is obtained by the organogelation process, which is an innovative technology that combines vegetable oil or fatty acid derivative and edible waxes (composed of fatty acid ester and long-chain alcohol group) (Kaushik *et al.,* 2017). It became a fat replacer in the food industry due to its physical properties and health function (Marangoni and Co, 2012). Issara *et al.* (2020) reported that supplementation of oleogel made of beeswax and canola oil in the diet could reduce the risk factor of obesity and liver dysfunction in animal studies. Also, Limpimwong *et al.* (2017) illustrated that rice bran wax oleogel replacement in a high-fat diet containing margarine improved the blood characteristics of rats and contributed to a decrease in lipid digestibility. Lately, the OG has been accepted as a food additive and applied in several food products, such as baked products, sausage, ice cream, and confectionery products. Nevertheless, there are no substantial reports about the effect of OG related to endothelial cell activity and their beneficial effects on blood vessel health. Therefore, this study aimed to investigate the effect of OG on cell remodeling and repair in VSMC, contributing to anti-atherosclerosis through inflammatory mediator cytokines immune response, TNF-α, and NF-κB, and VEGF signaling pathways.

2. Materials and methods

2.1 Palmitic acid, alpha-linolenic acid (n-3), and beeswax preparation

The palmitic acid (PA), alpha-linolenic acid (ALA: n-3), and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Seoul, Korea). The PA and ALA were dissolved in BSA with individual mixing following the manufacturer's guideline for the stock working solution at 10 mM. Before use, this solution was diluted in a culture medium (Dulbecco's modified Eagle's medium; DMEM) to the final concentration treatment at 0, 25, 50 and 100 μM (Slightly modified from Zhuang *et al.,* 2013). For beeswax (BW) preparation, it was prepared with 0.1% (w/v) in the ethanol for making a stock solution (the concentration of about 15 mM) and diluted at the concentration of 0, 25, 50 and 100 μM before cell treatment. The mixture of ALA and beeswax (OG) solution was performed at 1:1 and prepared to the concentration of 0, 25, 50 and 100 μM as the final solution before further use.

2.2 Cell culture

The rat thoracic aorta (A7r5) cells line purchased from ATCC company were cultured in DMEM mixed with FBS (10% v/v) and antibiotic (1%, v/v), and 1.5 g/L of sodium bicarbonate. Then cells were incubated at 37°C in the incubator under controlled conditions. Before cell treatment, the 0.5% FBS medium was applied for 48 hrs as pre-cultured. Afterwards, PA and OG working solutions were added to the culture medium (Slightly modified from Yang *et al.,* 2015).

2.3 Cell proliferation assay

The proliferation of A7r5 cells was determined by MTT assay following Zhuang *et al.* (2013). The rat aortic cells (1×10^4) were seeded into 96-well plates and cultured with or without different concentrations of PA and OG at 0, 25, 50 and 100 μM. After 48 hrs, the MTT solution was added and incubated for 4 hrs at 37°C. Then cells were performed by DMSO and conducted to 540 nm absorbance measurement, and the data was recorded and presented as the percentage of inhibition rate.

2.4 Apoptotic cell determination by PureBlu DAPI nuclear staining

Rat aortic VSMCs were cultured in 6-well plates until reaching subconfluence. Cells were supplemented

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with PA and OG at a concentration of 100 μM for 48 h and were kept in an incubator (37°C, in a 5% CO_2). The DAPI powder was dissolved in distilled water (500 μL) and mixed well until homogeneous. Then 49.5 mL of PBS was added as referred to stock working solution. Before the staining procedure, the culture medium was removed from each well, and cells were fixed with 10% formaldehyde-PBS for 15 mins. Then the DAPI solution was added directly into the live cells for 20-30 mins without light conditions at room temperature. After incubation, cells were washed with DPBS 2 times, and the growth medium was added to each well. The stained cells were observed by using a fluorescence microscope (EVOS™ FL Imaging Systems for Fluorescence and Transmitted Light Application, ThermoFisher, Life Technologies Corporation, Bothell, WA 98021), five pictures (center and surrounding zone) were collected in each well. Cells with uncondensed nuclei were interpreted as living cells. Each image was counted with at least 100 cells, and the average number of cells from five images was performed for further analysis.

2.5 Cell migration assay by scratch-wound repair

The VSMCs determined the in-vitro migratory activity using a scratch-wound migration assay, which was adapted from Zhuang *et al.* (2013) and Yang *et al.* (2015). In brief, cells were seeded (1×10^6) in a 6-well plate for culture until reaching the monolayer. Then, an injury line (cell-free zone) was created with a sterilized pipette at the center, and cells were continuously cultured. The severed cells were washed with the medium and followed by a PA or OG solution treatment with concentrations at 100 μM. The fresh medium was used as the vehicle control in this trial. About three images of cells-free zone per well were observed and captured using an inverted microscope with a camera (OLYMPUS model CXX53SF Tokyo 163-0914, Japan) under a 40× lens magnification. After treatment, wound repair was observed by measuring the injury areas in each well at the initial state (0 hrs) and after 24 hrs. The healing area of each treatment was presented as % of closure zone areas related to fresh wounds by counting the migrated cells and averaging. The experiments were performed with three replications.

2.6 RT-PCR determination

The total RNA was isolated from the A7r5 by MagListoTM 5M Tissue Total RNA Extraction Kit (Bioneer Corporation, Seoul, Korea). After that, it was conducted to cDNA kit (AccuPower® CycleScript RT PreMix, Bioneer Corporation, Seoul, Korea) for making a cDNA synthesis according to the manufacturer's instructions. The RT-qPCR was performed to determine the mRNA expression level of each gene and calculated via the comparative ΔΔCT method using StepOne Software v2.3. The quantification was performed in triplicate, and the results were expressed as relative mRNA expression (fold change). Primers used for RT-PCR analysis were included TNF-α: Forward 5'-GCTGGTAGGTTCCTGTTGTTTC-3'; Reverse 5'-CACCACGCTCTTCTGTCTACTG-3', CRP: Forward 5'-CATCTGTGCCACCTGGGAGTC-3'; Reverse 5'-AAGCCACCGCCATACGAGTC-3', iNOS: Forward 5'-GAGATTTTTCACGACACCCTTC-3'; Reverse 5'-GAGATTTTTCACGACACCCTTC-3', CD36: Forward 5'- AGGAAGTGGCAAAGAATAGCAG-3'; Reverse 5'-ACAGACAGTGAAGGCTCAAAGA-3', eNOS: Forward 5'- TTCCGGCTGCCACCTGATCCTAA-3'; Reverse 5'- AACATGTGTCCTTGCTCGAGGCA-3', NF-κB: Forward 5'-ACAACCCCTTCCAAGTTCCCT-3'; Reverse 5'-TGGTCCCGTGAAATACACCT-3', VEGF: Forward 5'-CGTCTACCAGCGCAGCTATTG-3'; Reverse 5'-CACACAGGACGGCTTGAAGAT-3' and GAPDH (Housekeeping gene): Forward 5'-GACATGCCGCCTGGAGAAAC-3'; Reverse 5'-AGCCCAGGATGCCCTTTAGT-3' based on the previous study and synthesized by Bioneer Corporation, Seoul, Korea. (Slightly modified from Zhuang *et al.,* 2013; Wu *et al.,* 2014).

2.7 Statistical analysis

All experiment was performed in three replications, and the results were expressed as mean ± standard deviation (SD). The analysis of variance (ANOVA) and Duncan's multiple range tests at a 95% significant level ($p < 0.05$) was performed by using SPSS software (SPSS 16.0 for Windows, SPSS Inc., Chicago, USA).

3. Results and discussion

3.1 Effect of palmitic acid and oleogel on vascular endothelial cell proliferation and apoptosis

The proliferation, as well as the apoptosis process, play a vital role in cell function. Anti-proliferative and anti-migratory effects in the cell may cause endothelial cell dysfunction via the inflammation pathway, generating several ischemic diseases. The representative anti-proliferative and anti-apoptotic VSMCs are shown in Figure 1. Here, the results showed that endothelial anti-proliferation had increased when the concentration of fatty acid and OG treatment increased. At 50 μM treated cells, a significant increase in the percentage of proliferative inhibition was observed in the PA group compared to OG and control ($p < 0.05$). However, in a comparative effect of PA and OG, the high proliferation rate of VSMCs was observed to significantly increase with OG treatment when compared to PA treatment ($p < 0.05$) even the concentration increased up to 100 μM

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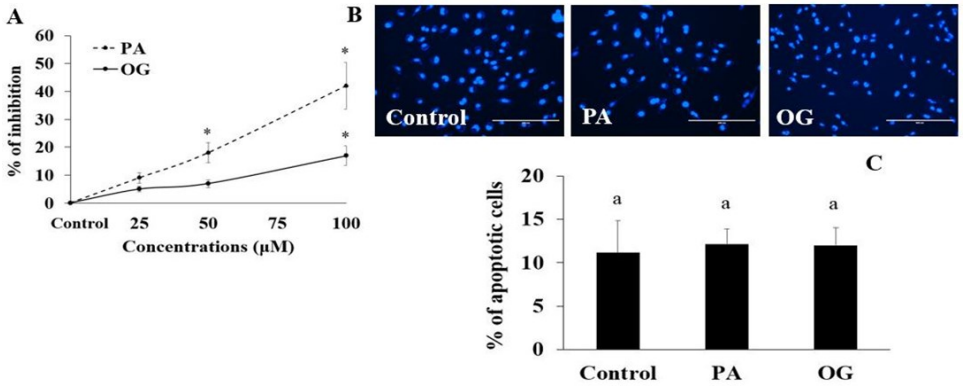

Figure 1. The effect of oleogels on endothelial proliferation (A) and cell death (B and C). The A7r5 cells were seeded in 96-well plates and cultured with different concentrations of PA or OG. MTT assay was performed to investigate cell proliferation. DAPI staining was applied for cell apoptosis observation. The % of inhibition was calculated from each group. The data was expressed as mean±SD (n = 3). Bars with different notations are statistically significantly different at $p<0.05$. *significant difference compared to the control group. PA: Palmitic acid, OG: Oleogels.

(Figure 1A). Based on this result, the 100 μM of both treatments was conducted to examine the apoptotic cells by DAPI nuclear staining technique, indicating the activated endothelial cells. According to Figures 1B and 1C, no significance was observed in vascular endothelial cells' apoptosis rate ($p > 0.05$) with the presence of PA and OG or without their supplementation. Our results imply that PA and OG did not influence endothelial apoptosis but affected the VSMCs proliferation. St-Denis *et al.* (2012) elucidated that a combination of saturated (SFA) and monounsaturated (MUFAs) fatty acids with different ratios and dose-dependent-manner has suppressed the VSMCs proliferation due to their pro-apoptotic ability. Besides, they also noted that a higher proportion of SFA is a major mediating generating the apoptosis of endothelial cells. On the other hand, Yang *et al.* (2015) have been found to stimulate endothelial proliferation and migration induced by oleic acid (MUFA) at 100 and 200 μM. Similarly, Wu *et al.* (2014) reported that rat aortic cells treated with PA and its combination with BSA declined the numbers of VSMCs through the whole inflammatory process associated with CRP, TNF-α, and iNOS synthesis pathways in the vascular wall. In 2019, the study by Issara *et al.* (2019) demonstrated that the effects of edible fatty acids and beeswax extracted solution on 3T3-L1 cells were no toxicity observed with low concentration, contributing to a normal state of cell proliferation activity.

The comparatively individual supplementation of DHA and EPA in HMEC-1 cells has been reported to significantly increase the rate of cell cytotoxicity when EPA reached 100 μM than the DHA treatment group, but not significantly was observed in cell apoptosis (Zhuang *et al.,* 2013). Consistent results have been found via Shiina *et al.* (1993) report. Villacorta *et al.* (2007) explored that nitro-linoleic acid prohibits VSMCs cell proliferation by the Nrf2 protein signaling pathway. Nevertheless, our results showed the consistency of VSMCs proliferative-pattern rate with previous studies of fatty acids or fatty acids ester from treating cells in a dose-dependent manner. Therefore, it can be suggested that the complexation of PUFAs or MUFAs with altered fatty acids ester or long-chain fatty alcohol such as beeswax may be vital for reducing the possibility of VSMCs toxicity and apoptosis rate, contributing to anti-proinflammatory effects and anti-atherosclerosis development by controlling endothelial proliferative and apoptotic cells pathways.

3.2 Effect of palmitic acid and oleogel on vascular endothelial cell wound healing

The endothelial cell proliferation and migration are critical for repairing the VSMCs injury. The representation of wound healing of endothelial cells is shown in Figure 2. After 24 hrs of treatments at 100 μM of PA and OG treatment, about 31.2% of the injury areas had healed by OG (59.2%) treatment when compared to the PA (28%) treatment group ($p < 0.05$). The control group (only ethanol) did not affect endothelial migration and wound-repairing areas. Hence, it can be suggested that endothelial cells treated with OG induce the proliferation (Figure 1A) and migration (Figure 2A), at least one part, of VSMCs, leading to the recovery of the injury zone, and promoting an anti-vascular disorder disease. Yang *et al.* (2015) reported that oleic acid exerts connective tissue recovery via a mechanism involving the vascular endothelial growth factor since 50-200 μM of oleic acid treated with A7r5 significantly increased the endothelial numbers in a dose-dependent manner. Nevertheless, up to 200 μM of oleic acid is toxic to cell activity leading to anti-proliferative and anti-migratory effects of VSMCs. Our study showed a consistent dose-dependent trend with PA and OG treatment. A study by Iwata *et al.* (2011) demonstrated that saturated and trans-fatty acids reduce vascular nitric oxide production in endothelial cells, which causes oxidative stress of vascular cells and inflammation process development.

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Not only nitric oxide levels in endothelial but also other biomarkers related to inflammatory cytokines signaling or its needed mechanisms generating the atherosclerosis stages should be investigated. A study by Wu *et al.* (2014) found that PA suppressed the vasculogenic growth factor and stimulated pro-inflammation of the same VSMCs model. However, this study determined the effect of PA and OG that promote or retard inflammation on endothelial vascular smooth muscle cells by regulating cytokine response downstream.

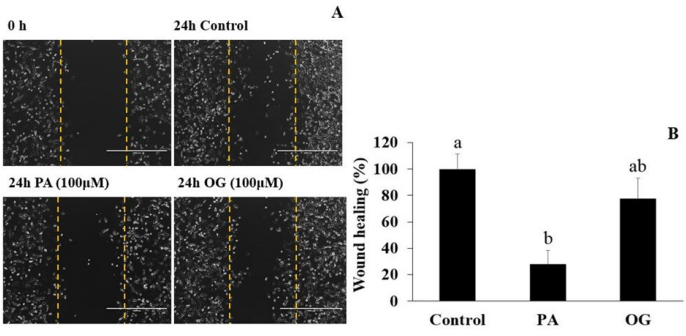

Figure 2. The representative images of wound healing of vascular endothelial cell after treated with palmitic acid and oleogels at 100 μM for 24 hrs incubation time (A). Cells were tested by scratch-wound repair assay. The wound healing area in each treatment was presented as % of closure areas related to fresh wounds (B). The data was expressed as mean±SD (n = 3). Bars with different notations are statistically significantly different at $p<0.05$. *significant difference compared to the control group. PA: Palmitic acid, OG: Oleogels.

Based on the composition of OG (combination of omega-three fatty acid and beeswax) treatment in our study, n-3 PUFAs may stimulate angiogenesis in pathophysiology study. Moreover, it is significant for reducing the risk factor of mortality associated with vascular and cardiac diseases (Surachmanto and Datau, 2011). The cardioprotective effects of omega-three were recognized almost 50 years ago. Dyerberg *et al.* (1975) published, with strengthened their hypothesis with high-fat consumption but containing a high amount of omega 3 in the patient group of Eskimos, the mortality rate of death from coronary heart disease has significantly decreased in the population. Similarly, Lee and Lip (2003) also illustrated that the positive of EPA and DHA intake could be secondary prevention of cardiovascular disease. Nguemeni *et al.* (2010) studied and demonstrated that stroke prevention by rapeseed oil (RSO) diet contained a high amount of alpha-linolenic acid (ALA) supplementation. Their experiment showed that animals fed with a 10% and 20% RSO-enriched diet had a reduced mortality rate. Besides, ALA supplemented diets; lipid peroxidation was sensitively promoted, which mediates numerous biological functions involved in vascular pathophysiology and the inflammatory effect of several cytokines in immune responses more than regular diet (Schaeffler *et al.,* 2009; Nguemeni *et al.* 2010). Likewise, the European Food Safety Authority (EFSA, 2007) reported that no adverse effect level (NOAEL) of chronic toxicity was observed from beeswax supplementation in animal diet-feeding rats for a short time. Therefore, this finding could suggest that endothelial cells treated with OG help recover the wound areas through intra-cellular cell proliferation mechanisms in VSMCs and prevent endothelial cell dysfunction.

3.3 Effect of palmitic acid and oleogel supplementation on transcription factors regulating inflammatory cytokines, vasculogenesis, and vasodilatory related gene expression pattern in endothelial cells

The intracellular proteins synthesized by mediator cytokines regarding the immune response pathway play an important function in chronic cellular inflammation. In this study, the nuclear NF-κB, C-reactive protein (CRP), and TNF-α, one of an inducible transcription factors family, control numerous gene-related immune and inflammatory responses, which are in agreement with previous studies (Popa *et al.,* 2007; Liu *et al.,* 2017). As well as vasodilatory maintenance and angiogenic effects including inducible nitric oxide synthase (iNOS), cluster of differentiation 36 (CD36), endothelial NOS (eNOS) and VEGF, respectively, were determined and presented in Figure 3 (A-G). Our results showed that vascular cell-supplemented PA had up-regulated the TNF-α, NF-κB, CRP, iNOS, and CD36 mRNA expression. In contrast, OG treatment had moderate-regulating of these transcriptional factors when compared to the control group ($p < 0.05$). On the other hand, PA has significantly suppressed the expression of endothelial NOS and vascular endothelial growth factor when compared to the OG group treatment ($p < 0.05$). Generally, TNF-α might establish several effects on the body by actions on the vascular part (Popa *et al.,* 2007). Barrera *et al.* (2002) noted that even though lipid modification is helpful in a host, TNF-α increases in a part of acute blood circulation, stimulating the chronic inflammatory effects of the blood vessel and endothelial cells with long-term activities leading to cardiovascular disease development. eNOS, NF-kB, and CD36 play a vital function in vascular nitric oxide production, inflammatory process, and lipid uptake pathway in the VSMCs. These findings were correlated with the exploration of Wu *et al.* (2014), who elevated the effect of PA inducing TNF-α protein expression in VSMCs. Moreover, Wu *et al.* (2014) reported the increase of mRNA and protein expression patterns of iNOS and CRP by PA supplementation in A7r5 cells, causing pro-inflammation. Cytokine signaling that is transcriptionally induced by immune response has potent mediators of some other cytokines and chemokines production such as

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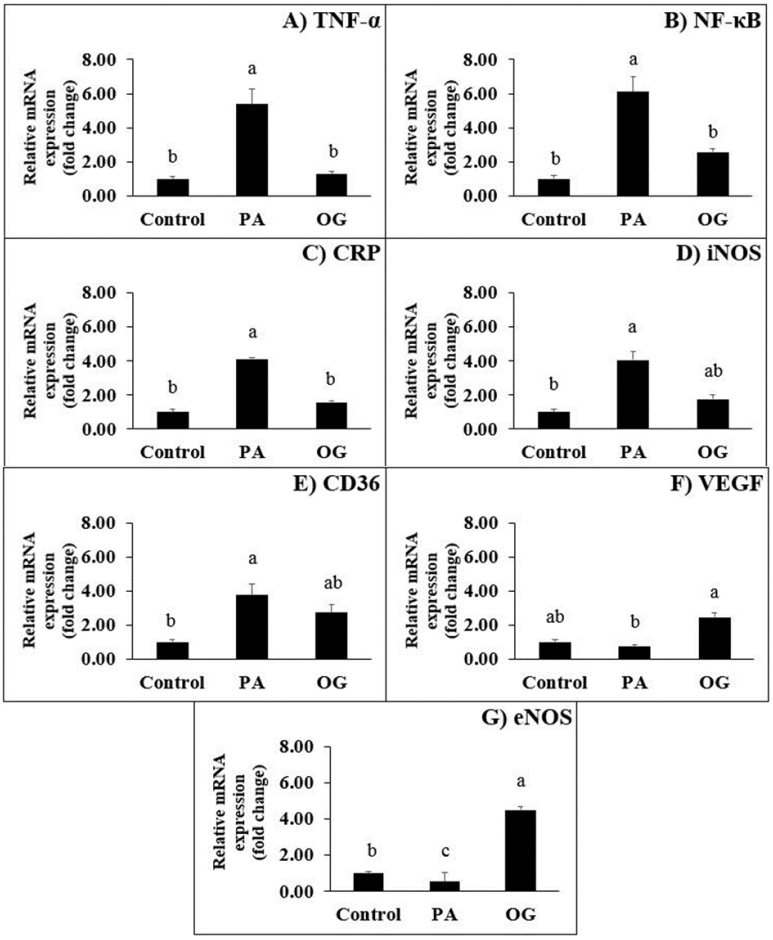

Figure 3. The relative mRNA expression of TNF-α, NF-κB, CRP, iNOS, CD36, eNOS and VEGF in A7r5 endothelial cells was determined by RT-PCR after treated cells at 100 μM for 48 hrs. The data was expressed as mean±SD (n = 3). Bars with different notations are statistically significantly different at $p<0.05$. *significant difference compared to the control group. PA: Palmitic acid, OG: Oleogels.

RANKL, NF-κB, MCP-1, matrix metalloproteinase (MMP)-2, chemokine ligand (CCL)11/eotaxin or CD36, which is associated with lipid uptake pathway in cells (Arpita *et al.,* 2006; Sprague and Khalil, 2009; Baker *et al.,* 2011). The NF-κB has mainly been realized as a pro-inflammatory pathway in physiological and pathological processes (Lawrence, 2009). Munkong *et al.* (2016) reported that the rats fed a high-fat diet (HFD) riches SFA, animals fed HFD had a risk of vascular disorder development after finding an increase of the NF-κB and CD36 mRNA as well as protein expression in rat aortic tissue when compared to regular treatment. Still, there is no injury observed in aortic morphology. Previous studies demonstrated that significant signaling of eNOS, CD36, and NF-κB p65 subunit that the up-regulation of eNOS and prohibition of CD36 and NF-κB p65 expression have been associated with anti-cardiovascular disease in rodents' model (Gareus *et al.,* 2008; Zhao *et al.,* 2009; Manning-Tobin *et al.,* 2009; Liu *et al.,* 2015). The oxidized form of omega-3 (EPA and DHA) at 100 μmol/L can inhibit the NF-kB via the regulation of PPARα dependent pathway in human umbilical vein endothelial cells (Archana *et al.,* 2004). In addition, the effect of both non-esterified and esterified PUFAs on the response of macrophages, lymphocytes, and endothelial cells has been mentioned by Calder (2010) and Fritsche (2006). The study about *in-vitro* vascular calcification, a one significant risk factor of inflammatory effect in endothelial, induced Type I-diabetes by streptozotocin on pro-inflammation via the regulating of receptor activator of nuclear factor-kappa-B ligand (RANKL) expression pattern in VSMCs (Chang *et al.,* 2015), a higher concentration of glucose treatment (hyperglycemia) had inhibited the mRNA expression of RANKL within this cultured cell leading to vasoprotective effects. However, our study results imply that lipid metabolisms and glucose pathway metabolisms in intracellular affecting on vascular disorder by regulating the cytokines associated with pro-inflammatory effects in endothelial cells, which promotes atherosclerosis.

The VEGF family has been classified as a vital key for vasculogenesis and angiogenesis process. The homeostasis regulation of VEGF produced by VSMCs leads to cell re-programming and repairing in patients with vascular disorders disease (Kim and Byzoya, 2014). Otherwise, it may negatively affect the cancer state through the vascular synthesis in tumor growth, inducing cancer cell distribution (Munkong *et al.,* 2016). According to Figure 3, the OG supplement in A7r5 cells

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at 100 μM had the highest mRNA expression of VEGF. The PA supplement showed the inhibitory effects of VEGF compared to the OG group ($p < 0.05$). Increasing VEGF production in endothelial cells treated with OG of our study supported the endothelial re-programing resulting in wound repair (Figure 2). Previous evidence has been strongly confirmed that PA actions to the pathogenesis of cardiovascular diseases via plasma metabolomics analysis (Chen *et al.,* 2010). Moreover, Ghosh *et al.* (2017) noted that PA is a biomarker promoting an inflammatory response and cellular senescence in cardiac fibroblasts, which it is interacts with cytokines stimulation through the activation of toll-like receptor 4 and 2 (TLR-4 and TLR-2) pathway, releasing the mitochondrial reactive oxygen species load and mitochondrial dysfunction in fibroblasts. Lately, the oleic acid (100 μM) treats rat aortic cells, and it shows a significant increase in the VEGF-A mRNA transcription as well as protein synthesis in VSMCs of Zucker fatty rat (genetical rat) in a time-dependent manner (Doronzo *et al.,* 2013). The application of n-3 PUFAs has been explored to reduce VEGF production in human colon cancer cells leading to anti-angiogenesis of growth tumors correlated in mice (Piccioni *et al.,* 2004). Also, Piccioni *et al.* (2004) suggested that n-3 PUFAs action is a chief target to the VEGF expression level by modulating the angiogenic pathway and decreasing the COX2 and HIF-1 together with prohibiting the phosphorylation of ERK intracellular signaling. From all the reasons, it can be suggested in our finding that endothelial cells supplemented with 100 μM of OG exert beneficial effects on anti-vascular disorders by expressing the VEGF, which is deeply involved in collateral protection of vascular therapy. However, more mechanisms need to confirm the molecular signaling related to anti- or angiogenesis, particularly ERK-1/2, Akt or mTOR pathway induced individual fatty acids ester, fatty alcohol, natural waxes, or their interaction with VSMCs. The summarized diagram of healthy VSMCs after fatty acids and natural waxes extracted solution and its combination treatment is shown in Figure 4.

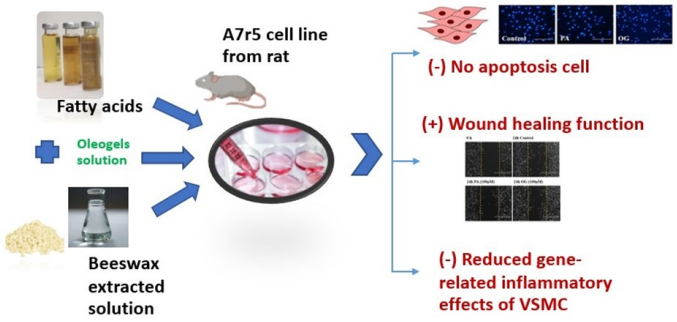

Figure 4. The summarized diagram of healthy VSMCs after fatty acids and natural waxes extracted solution and its combination treatment.

4. Conclusion

According to the results, endothelial cells supplemented OG (at 100 μM) was promoted a wound healing area by proliferation or migration processes in VSMCs while the PA (since 50 μM) was prohibiting the cell activity. Moreover, the OG can reduce the risk factor of atherosclerosis by modulating the TNF-α, CRP, NF-kB, CD36, and iNOS, causing a pro-inflammatory effect and exerting the eNOS and VEGF mRNA expression in the VSMCs, contributing to the vasculoprotective effect. Consequently, oleogel combined with beeswax and omega-3 fatty acids can be structured as the fat replacer (solid fat form) and applied in food product such as bakery or meat products etc.

Conflicts of interest

The authors declare no conflict of interest.

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