Nanocurcumin Preparation for Reducing Vcam-1 and IL-6 in High Fat Diet-Induced Hyperlipidemic Rats

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ABSTRACT

Hyperlipidemia is a pathological condition due to lipid metabolism abnormalities. Increased and oxidized LDL in hyperlipidemia will trigger an inflammatory response and produce proinflammatory cytokines, such as vascular cell adhesion molecule-1 (VCAM-1) and Interleukin-6 (IL-6). Curcumin can be used as antioxidants, anti-inflammatory and antihyperlipidemia, but as it is practically insoluble in water, formulation of curcumin nanosuspension is made to improve the effects of curcumin therapy. This study was conducted to determine the activities of nanocurcumin preparation as preventive measures for rats induced with hyperlipidemia. The test animals used were 49 male Wistar rats divided into 7 test groups: normal control group, negative control group, 80mg/kg BW/day curcumin control group, and nanosuspension curcumin dose of 80mg/kg BW/day, nanoemulsion curcumin dose of 80mg/kg BW/day, SNEDDS curcumin dose of 80mg/kg BW/day and solid lipid nanoparticles dose of 80mg/kg BW/day. Provision of preventive measure was performed on days 1-67. On day 11-67, the induction of lard and egg yolk (1:1) was administered with a volume of 2mL/200g of rat BW. On the 68th day, blood samples were taken for the determination of VCAM-1 and IL-6 parameters using Biotin-Streptavidin-Amplified Enzyme-Linked Immuno-sorbent Assay method. Data analysis of VCAM-1 and IL-6 levels between test groups were conducted by means of normality test and One-way ANOVA ($p < 0.05$). Based on the data of VCAM-1 and IL-6 levels, nanocurcumin dose of 80mg/kg BW preparation was able to significantly improve the preventive activity of curcumin compared to curcumin suspension dose of 80mg/kg BW by reducing VCAM-1 levels in SNEDDS (53.260%), nanoemulsion (52.737%), nanosuspension (52.325%) and solid lipid nanoparticles (51.444%) and decreasing IL-6 levels in SNEDDS (33.030%), nanoemulsion (31.568%), nanosuspension (29.898%), and solid lipid nanoparticles (28.875%).

Keywords: high-fat diet, curcumin, SNEDDS, nanosuspension, nanoemulsion, solid lipid nanoparticles, VCAM-1, IL-6.

INTRODUCTION

Hyperlipidemia, a pathological condition caused by lipid metabolism disorders, is characterized by an increase in lipid fractions such as LDL-cholesterol in the blood (Nelson, 2013). One of the hyperlipidemic conditions can occur due to a high-fat diet treatment by providing high-fat ingredients to induce an increase in blood LDL (Murwani *et al.,* 2006). Increased and oxidized LDL in the blood vessel walls will trigger an inflammatory response

and produce proinflammatory cytokines, such as vascular cell adhesion molecule-1 (VCAM-1) and Interleukin-6 (IL-6) (Hansson, 2005). VCAM-1 plays a role in inflammatory cell recruitment by stimulating monocytes adhesion to the endothelial wall, which marks the initial stage of atherogenesis in both experimental animals and humans (Tanuwidjojo, 2005). IL-6 is a cytokine that can function as a predictor of coronary heart disease and has been proved to be involved in

inflammatory disorders associated with atherosclerosis (Omoigui, 2007).

Increased atherosclerosis prevalence in Southeast Asia is dominated by hyperlipidemia as the major risk factor (Ricvan, 2015). Atherosclerosis can lead to death and predictably becomes a leading cause of morbidity and mortality in developing communities in 2020 due to their unhealthy lifestyle changes (Libby, 2005). In Indonesia, the prevalence of coronary heart disease in 2013 reached 1.5% or approximately 2,650,340 people. This has placed coronary heart disease in the seventh largest prevalence rates of non- communicable diseases in Indonesia (Kemenkes, 2013).

Antioxidants, one of which is curcumin, can be used to inhibit molecular oxidation and free radicals in atherosclerosis. Extracts of *Curcuma longa* have proved to be capable of inhibiting nearly all expressions of VCAM- 1 by suppressed almost completely VCAM-1 protein expression. It is also reducing IL-6 production levels in rat cell tissue by blocked the mRNA expression of IL-6 in the Lipopolysaccharide (LPS) induced vascular smooth muscle cells in vitro. (Kawasaki *et al*., 2015) (Yanti *et al*., 2014).

Curcumin as a natural compound is practically insoluble in water and has low bioavailability. Nano formulation has been proved to be able to improve curcumin dissolution and solubility (Carvalho *et al*., 2015), making it possible to increase the therapeutic efficacy of curcumin with nanoparticles formulation. This study aimed to examine the activities of a variety of nanocurcumin preparations compared to curcumin suspension as a preventive measure in high-fat diet-induced hyperlipidemic rats based on the levels of VCAM-1 and IL-6. Preventive measure research is performed with the underlying principle that prevention is better than cure.

MATERIAL AND METHODS
Equipment

The equipment used was electric scales (Mettler Toledo ML-303 E), a set of laboratory glassware (Pyrex), micropipette, oral syringe, centrifugator, rotary evaporator (Heidolph), ELISA reader (Thermo Scientific), incubator (Memmert), ultra turrax (digital IKA T25), magnetic stirrer (IKA®18 BASIC), particle size analyzer (HORIBA Scientific Nano Partica SZ100), and ultrasonic homogenizer (HORIBA Scientific Nano Partica SZ100).

Materials

The test materials used in this study were pure curcumin pro analytic which was purchased from MERCK Singapore. Elisa VCAM-1 kit and Elisa Interleukin-6 kit were purchased from ExpressBio USA. Short chain chitosan, sodium tripolyphosphate and cremophor EL were purchased from Sigma-Aldrich Iceland. Sodium acetate distilled water was purchased from Ikapharmindo Putramas Jakarta Indonesia. Buffer acetate pH 4 consisting of acetic acid 96% v/v alcohol, NaCMC, tween 80, propylene glycol, miglyol 318, tween 20 and span 80 were purchased from Brataco Yogyakarta Indonesia. Glyceryl monostearate was purchased from PT. Sumber Berlian Kimia Jakarta Indonesia.

Research Design

This study employed the true experimental post-test control study. The test animals were physically healthy male Wistar rats fulfilling the weight range criteria (180-250g). Rats were fasted for 8h before induction and treatment. A total of 49 male Wistar rats were divided into 7 groups each consisting of 7 rats, namely the normal control group, the negative control group (high-fat diet induction), the curcumin suspension control group dose of 80mg/ kgBW/day, the curcumin nano-suspension treatment group dose of 80mg/ kgBW/day, the curcumin nanoemulsion treatment group dose of 80mg/ kgBW/day, the curcumin SNEDDS treatment group dose of 80 mg/kgBW/day, and the solid lipid nanoparticles curcumin treatment group dose of 80mg/ kgBW/day. Administration of preventive measure using curcumin suspension dose of 80mg/ kgBW/day and nanocurcumin dose of 80mg/ kgBW/day was carried out on days 1-67. On days 11-67, an induction of high-fat diet with lard and quail egg-yolk was given (1: 1).

Curcumin Nanosuspension Preparation

As much as 50mL of 0.1% w/v curcumin solution was mixed with 50mL of 0.02% w/v chitosan and stirred with a magnetic stirrer for 20 minutes at 60℃. After 20min, 6mL of 0.01% w/v sodium tripolyphosphate was added and stirred for 20min. The mixed preparation was evaporated to remove ethanol solvent (Chabib *et al*., 2012).

Curcumin Nanoemulsion Preparation

As much as 5mL miglyol and 15mL of propylene glycol were mixed and stirred for 5min. Then, 0.8mg curcumin was dissolved and put into the mixture of miglyol and propylene glycol then

stirred at 60ºC for 10min. Tween 80 (25mL) was added and stirred for 30min. Aqua Bidestilata was added as much as 54.2mL by means of titration then homogenized using vortex for 5min (Lagonah, 2014)

Curcumin SNEDDS Preparation

Mixture 1 of cremophor EL and tween 20 as well as Mixture 2 of PEG and miglyol 318 were made. Each was sonicated for 2min then both were mixed and re- sonicated for 1min. Curcumin was added to the mixture and then re-sonicated for 2min (Bagiana, 2015).

Solid Lipid Nanocurcumin Preparation

A lipid phase consisting of Glyceryl monostearate (GMS) Span 80 and curcumin was dispersed into the aqueous phase consisting of aquadest and tween 80 while being heated at 75℃ and stirred. The emulsion produced was then homogenized with ultra turrax at a speed of 30,000rpm for 5min and ultrasonicated with 30% amplitude for 10min. The results were cooled at room temperature to produce SLN curcumin in the form of emulsion (Mujib, 2011).

Measurement of nanoparticle size distribution and stability was conducted using Particle Size Analyzer (PSA) at the Pharmacy Technology Laboratory of Islamic University of Indonesia. Nanoparticle is confirmed when the particle size is 10-1000nm and Polydispersity index <0.3.

Curcumin Suspension Preparation

As much as 8mg of curcumin was weighed thoroughly, and then 0.5% w/v Na-CMC solution was added until the volume reached 50mL. The concentration of curcumin suspension obtained was 0.016% w/v.

High-Fat Diet Induction

Induction of a high-fat diet was carried out according to the method employed in a previous study (Sa'adah and Pratiwi, 2016) with a modification in the use of lard and quail egg-yolk (a ratio of 1:1) orally each day for 57 days. The volume of lipid mixture administered to the rats was 2mL/200g/BW.

Determination of VCAM-1and IL6 Levels

Determination of VCAM-1 and Interleukin-6 levels was carried out by taking blood plasma samples of each group on 68th day. The blood plasma sample was then analyzed by means of Biotin-Streptavidin- Amplified Enzyme-Linked Immunosorbent Assay. Tests were carried out using an ELISA kit produced by ExpressBio with duplo samples.

Blood sample collection

Blood samples were taken as much as 500 mL from orbital vein of Rats on day 68th. Then the blood is processed into plasma by adding heparin as an anticoagulant and centrifuging for about 15min at 4000rpm.

ELISA kit protocol for VCAM and IL-6 examination

Wells and 37℃ incubator were prepared, and wells were washed two times with 350µL of wash buffer (left for ±1min prior to disposal). Then, 100µL of standard solution, 100µL of sample, and 100µL of standard dilution buffer (blank) were added into each well. Wells were covered with plate sealers and incubated at a temperature of 37℃ for 90min. Plate sealers were removed and the well content was discarded. The wells were rinsed twice with 350µL wash buffer and left for 1min, then the buffer was disposed into the sink and the content was slammed onto tissue. A total of 100µL biotin-labeled antibody solution was added to each well. The wells were covered with plate sealers and incubated at 37℃ for 60 min. The plate sealers were opened and content of the wells was discarded. Wells were rinsed three times using 350µL wash buffer. Before being disposed into the sink and slammed onto tissue, the content was let rest for 1min. Into each well was added 100µL SABC solution followed by sealing with plate sealers and aluminum foil and incubation at 37℃ for 30min. Plate sealers were removed and well content was discarded. Wells were washed five times using 350µL wash buffer and left for 1min prior to disposal into the sink and a smash onto tissue. Each well was filled with 90µL TMB substrate solution, closed with aluminum foil, and incubated at 37℃ for 30min in the dark. Next, 50µL stop solution was put into each well and mixed to obtain a homogeneous solution. The color would instantly turn into yellow, and result was read for the optical density at 450nm absorbance in a microplate reader immediately after the addition of stop solution (XpressBio, 2018a) (XpressBio, 2018b).

Table I. Particle size, Polydispersity index, and Zeta potential of nanocurcumin preparation

Preparation	Particle size (nm)	Polydispersity index	Zeta Potential (mV)
Nanosuspension	154.6±2.10	0.23±0.10	+22.5±0.2
Nanoemulsion	74.9±0.80	0.36±0.02	-37.4±1.2
SNEDDS	69.0±1.15	0.32±1.15	-39.5±0.2
SLN	150.4±2.20	0.45±0.20	-62.3±0.1

Criteria of Nano Preparations: Particle size: 10-1000nm (Rawat *et al.*, 2006) Polydispersity index < 0.5 (Abdassah, 2017) Zeta potential > +/- 30 mV (Abdassah, 2017)

Calculating VCAM-1 and IL-6 Concentrations

OD of Sample = OD of sample – OD of blank
OD=Optical Density

The sample concentration was calculated based on the linear regression of absorbance (optical density) obtained from the ELISA plate reader relative to the standard concentration series. Formula for the percentage of reduced concentration:

Percentage of Reduced Concentration (%) =

$$\frac{conc.\ of\ negative\ control - conc.\ of\ curcumin}{conc.\ of\ negative\ control} \times 100\%$$

Data analysis

Data of VCAM-1 and IL-6 levels from each test group was analyzed in Normality test and compared using One-way ANOVA test with Post-hoc Tukey ($p < 0.05$).

RESULTS AND DISCUSSION

This research was approved to be carried out and can be carried out treatment on male Wistar rat test animals in accordance with the approval of Ethical Clearance by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Islamic University of Indonesia, Yogyakarta with number 76 / Ka.Kom.Et / 70 / KE / IV / 2018.

Characterization of nanocurcumin

From the characterization of four nanocurcumin preparations, the SNEDDS preparation has the smallest particle size, which is below 100nm. The particle size of SNEDDS is smaller than that of the other preparations due to the high-energy method used in the preparation process. Meanwhile, the best zeta potential is found in the solid lipid nanocurcumin preparation because it consists of solid lipid that can form capsules and absorb active substances within, enabling them to prevent environmental degradation and so the preparation becomes more stable.

Table I give information that all nanocurcumin preparation show the particle size, polydispersity index and zeta potential measurements are in accordance with the nanoparticle criteria. With the smaller particle size, drug delivery will more optimally reach the target of drug action. In addition, the drug will also be protected by the carrier when passing through several barriers such as acidic and basic conditions (Nasr *et al.*, 2016)

Polidispersity index is a value that indicates the normal distribution of particle size or distribution. Standard value of polidispersity index is <0.50 (Abdassah ,2017).

Zeta potential describes the charge difference between a dispersion medium and the liquid layer attached to the dispersed particle of substance. Zeta potential also indicates the magnitude of charge repulsion among dispersed particles in a liquid medium. Such repulsion occurs because each of the surfaces of dispersed particle has the same charge. The higher the zeta potential of a particle is, the more significant the repulsion among particles is. Repulsion prevents aggregation or flocculation between globules, leading to a more stable nanoemulsion preparation. A nanoemulsion preparation is required to have ±30 mV zeta potential to maintain good stability.

Zeta potential value shows the magnitude of repulsion between droplets. The higher the value is, the stronger the repulsion between droplets is, thus avoiding aggregation. A nanoemulsion system stabilized by a surfactant is naturally ionic since the charge of droplet surface comes from the ion of surfactant hydrophilic groups. Meanwhile, in a nanoemulsion system stabilized by a nonionic surfactant, the surface charge comes from the adsorption of ions in the water phase or due to a friction between droplets and their dispersion medium (Rosen, 2004).

Table II. Mean levels of VCAM-1 ± SE

Sample (n=5)	Kadar VCAM-1 (ng/mL)
Normal	0.985±0.336 [b,c]
Negative control	6.627±0.758 [a,d,e,f,g]
Curcumin suspension control	5.546±0.441 [a,d,e,f,g]
Curcumin Nanosuspension	3.159±0.481 [b,c]
Curcumin SNEDDS	3.097±0.282 [b,c]
Solid Lipid Nanocurcumin	3.218±0.348 [b,c]
Curcumin Nanoemulsion	3.132±0.475 [b,c]

Statistical test results from One-way ANOVA and Post-hoc Tukey (p <0.05): (a) significantly different from the normal control group, (b) significantly different from the negative control group, (c) significantly different from the curcumin suspension control group, (d) significantly different from the curcumin nanosuspension group, (e) significantly different from the curcumin SNEDDS group, (f) significantly different from the SLN curcumin group, (g) significantly different from the curcumin nanoemulsion group.

In an o/w nanoemulsion system containing a nonionic surfactant, the surfactant will form a film layer on the droplet surface. Such layer prevents droplet aggregation in the dispersion medium (Lazaridis *et al.*, 1999)(Rosen, 2004).

Determination of VCAM-1 Levels

In this study, VCAM-1 levels were determined using the Biotin-Streptavidin-Amplified Enzyme-Linked Immunosorbent Assay (BSA-ELISA) method. The BSA-ELISA system is a modification of the standard ELISA to increase the sensitivity of ELISA testing. Level determination was carried out on day 68 for each test group. Data of VCAM-1 levels in each group (Table II).

Based on statistical analysis, the mean VCAM-1 level in the normal group is significantly different (p<0.05) from that in the negative control group. This data shows that the induction of high-fat diet with lard and quail egg-yolk can significantly increase the levels of VCAM-1 in negative control rats, marking the success of the method to induce high-fat diet in rats. Quail eggs and lard are examples of animal food-source containing high saturated fat and cholesterol. The composition of lard includes saturated fatty acids (1% myristate, 25% palmitate, and 15% stearic acid) and unsaturated fatty acids (oleic 50%, 6% linoleic, and the remaining 3%) which can increase cholesterol concentration in the blood by 15-25%(Sa'adah, and Pratiwi, 2016).

VCAM-1 level in rats with curcumin suspension at a dose of 80mg/kgBW does not differ significantly (p> 0.05) from that in negative control rats, showing the disability of curcumin suspension to reduce VCAM-1 levels in high-fat diet-induced rats. Meanwhile, the levels of VCAM-1 in rats treated with nanocurcumin at a dose of 80mg/ kgBW significantly decrease (p<0.05) indicating the ability of nanocurcumin to reduce VCAM-1 levels in high-fat diet- induced rats. The antioxidant activity in curcumin is considered to relate to the reduction of VCAM-1 levels by capturing free radicals to reduce cell damage in atherosclerosis formation; therefore, VCAM-1 levels in the endothelial blood-vessel tissue can decrease. The high antioxidant content of curcumin can be observed in the structure known as functional groups, such as β- diketone groups, carbon double bonds, and phenyl rings, containing a number of hydroxyl and methoxy substituents that play a role in donating their hydrogen groups to capture oxygen free-radicals which also play an important role in the initial formation lipid peroxidation (Shin *et al.*, 2011)(Nurtamin, 2014).

The percentage of decrease in VCAM-1 levels of nanocurcumin is higher than that of curcumin suspension. The results of variance analysis (ANOVA) and Tukey test show significant differences (p<0.05) between VCAM-1 levels in the curcumin suspension group and those in the nanocurcumin group. This indicates that curcumin SNEDDS preparation has the best results among other nano preparations. It is because curcumin SNEDDS is able to increase drug surface area, thus improving curcumin bioavailability. The small particle size of curcumin SNEDDS also has an impact on the delivery system of drug particles in vitro by improving drug solubility which therefore increases the permeability of drug molecules across the intestinal membrane (Shakeel *et al.*, 2013).

Table III. Mean IL-6 levels ± SE

Sample (n=5)	IL-6 Level (pg/mL)
Normal	62.933 ± 3.148 [b,c]
Negative control	108.916 ± 5.968 [a,d,e,f,g]
Curcumin suspension control	96.067 ± 10.362 [a,d,e,f,g]
Curcumin Nanosuspension	76.351 ± 3.994 [b,c]
Curcumin SNEDDS	72.933 ± 7.238 [b,c]
Solid Lipid Nanocurcumin	77.466 ± 6.065 [b,c]
Curcumin Nanoemulsion	74.533 ± 6.762 [b,c]

Statistical test results from One-way ANOVA and Post-hoc Tukey ($p < 0.05$): (a) significantly different from the normal control group, (b) significantly different from the negative control group, (c) significantly different from the curcumin suspension control group, (d) significantly different from the curcumin nanosuspension group, (e) significantly different from the curcumin SNEDDS group, (f) significantly different from the SLN curcumin group, (g) significantly different from the curcumin nanoemulsion group.

Determination of Interleukin-6 Levels

In this study, IL-6 levels are determined using the Biotin-Streptavidin-Amplified Enzyme-Linked Immunosorbent Assay (BSA- ELISA) method. Data of IL-6 levels in each group (Table III).

Based on the statistical analysis, the mean IL-6 level in the normal group shows a significant difference ($p < 0.05$) compared to that in the negative control group. Such data shows that the induction of high-fat diet with lard and quail egg yolk can significantly increase IL-6 levels in negative control rats, indicating the success of the method used to induce a high-fat diet condition in rats.

IL-6 levels in rats given curcumin suspension at a dose of 80mg/ kgBW do not differ significantly ($p > 0.05$) from those in negative control rats, indicating that the administration of curcumin suspension is not able to reduce IL-6 levels in high-fat diet- induced rats. On the other hand, IL-6 levels in rats with nanocurcumin at a dose of 80mg/ kgBW show a significant decrease ($p < 0.05$), marking the ability of nanocurcumin to reduce IL-6 levels in high-fat diet-induced rats.

Levels of IL-6 in rats with nanocurcumin treatment are not significantly different ($p > 0.05$) from those in normal rats. This indicates that nanocurcumin preparation is effective in reducing IL-6 levels to nearly normal. Decreased IL-6 levels in the nanocurcumin treatment group are closely related to the activity of curcumin as an antioxidant and anti-inflammatory. The antioxidant activity of curcumin reduces IL-6 levels because it can capture free radicals that cause oxidative stress. When free radicals are captured, oxidation of low density lipoprotein (LDL) into oxidized low density lipoprotein (Ox-LDL) can be prevented, thus averting endothelial dysfunction as well as reducing IL-6 levels and adhesion molecules, such as VCAM-1 in the endothelium of blood vessel tissue (Sumardika, 2012). Curcumin is rich in antioxidant because its structure consists of phenolic hydroxyl groups and β- diketone groups, which function as free radical scavengers in an antioxidative mechanism (Rosidi *et al*., 2014). Not only antioxidant activities, curcumin also has anti-inflammatory activities. Curcumin can inhibit the formation of prostaglandin and suppress the activity of cyclooxygenase enzyme, thus reducing inflammation (Basnet and Skalko-Basnet, 2011). Curcumin is also able to downregulate transcription factors, such as nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) receptor factor and activator protein 1 (AP-1). The AP-1 transcription factor acts to regulate the expression of various cell types that are important for cell proliferation, differentiation, and apoptosis. Therefore, curcumin can suppress inflammatory response in the blood vessels, which then reduces the expression of adhesion molecules and cytokines that can facilitate the formation of plaques on the walls of blood vessels (Nurtamin, 2014).

The percentage of reduction in IL-6 levels from curcumin SNEDDS is higher than that from groups of nanosuspension, nanoemulsion, solid lipid nano, and curcumin suspension, leading to a conclusion that the design of curcumin SNEDDS formulation can increase the preventive activities of curcumin and is better than the other three designs of nanocurcumin formulation as well as curcumin suspension in reducing IL-6 levels in rats. This is because the formulation of curcumin SNEDDS preparation can increase the solubility of

curcumin, and the very small particle size affects the distribution of drug molecules to go easily through the intestinal wall by binding to lipoproteins and carrying drug molecules to the lymphatic system, thereby preventing drug molecules from passingthe liver or first pass effect which results in increased bioavailability of curcumin (Makadia *et al.*, 2013).

CONCLUSION

Nanocurcumin preparation is able to increase the preventive activities of curcumin significantly better than curcumin suspension by reducing VCAM-1 levels in SNEDDS preparation (53.260%), nanoemulsion (52.737%), nanosuspension (52.325%), and solid lipid nano (51.444 %) as well as decreasing IL-6 levels in SNEDDS (33.030%), nanoemulsion (31.568%), nanosuspension (29.889%), and solid lipid nanoparticles (28.875%) in male Wistar rats induced with lard and quail egg yolk.

ACKNOWLEDGEMENT

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