

Effect of Andrographolide on Monocyte Chemoattractant Protein-1 Expression at the Initiation Stage of Atherosclerosis in Atherogenic Diet-Fed Rats

Muhammad Yulis Hamidy^{1,2*}, Fadil Oenzil³, Yanwirasti⁴ and Yufri Aldi⁵

¹Biomedical Programme, Faculty of Medicine, Andalas University, Padang, Indonesia.

²Department of Pharmacology, Faculty of Medicine, Universitas Riau, Pekanbaru, Indonesia.

³Department of Biochemistry, Faculty of Medicine, Andalas University, Padang, Indonesia.

⁴Department of Anatomy, Faculty of Medicine, Andalas University, Padang, Indonesia.

⁵Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Andalas University, Padang, Indonesia.

*Corresponding author E-mail: yulis.hamidy@gmail.com

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To evaluate the effect of andrographolide on monocyte chemoattractant protein-1 (MCP-1) expression at the initiation stage of atherosclerosis in rats induced by an atherogenic diet. The research was conducted on 27 rats divided into 3 groups (n=9). Group 1 was given a standard diet. Group 2 was given an atherogenic diet of vitamin D3 700.000 IU/kg on the first day followed by 5% goat fat, 2% cholesterol, 0.2% cholic acid and standard diet up to 100% for 2 days to induce atherosclerosis initiation stage. Group 3 was given an atherogenic diet and treated with andrographolide 40 mg/kg. An immunohistochemical examination was performed to determine the expression of MCP-1. Data analysis using one-way Anova followed by post hoc test. The results showed the expression of MCP-1 in group 1 was 6.61 ± 1.90 , in group 2 was 32.99 ± 3.74 and in group 3 was 9.61 ± 2.47 . There was a significant difference between group 3 treated with andrographolide 40 mg/kg compared with group 2 ($p < 0.001$). There was no significant difference between group 3 treated with andrographolide 40 mg/kg and group 1 ($p > 0.05$). In conclusion, andrographolide may inhibit MCP-1 expression at the initiation stage of atherosclerosis in the andrographolide treated rats. Thus, andrographolide could be a potential anti-atherosclerosis drug.

Keywords: Andrographolide, MCP-1, Atherosclerosis.0

Atherosclerosis is a chronic inflammatory disease that occurs in the walls of blood vessels. Various processes are involved in the pathogenesis of atherosclerosis, including inflammatory cells infiltration, smooth muscle cells proliferation, increasing extracellular matrix, and formation of thrombus¹. Inflammation has an important role and is the main mechanism underlying the pathogenesis of atherosclerosis². This inflammation

occurs through the interaction between monocytes and cells found in the walls of arteries such as endothelial cells and vascular smooth muscle cells³.

Monocytes play an essential role in the initiation stage of atherosclerosis⁴. In early atherosclerotic lesions, accumulation of monocytes/macrophages can be found in the intima of blood vessels. Macrophages accumulate cholesteryl esters resulting in foam cell formation¹.

Various existing studies show the important role of monocyte chemoattractant protein-1 (MCP-1) in the process of recruiting monocytes/macrophages⁵. Monocyte chemoattractant protein-1, a C-C chemokine family member, has a potent chemoattractant activity for monocytes/macrophages⁶. This chemokine is highly expressed in the macrophage-rich area of the atherosclerotic lesions in human as well as experimental animal models⁵. These findings suggest that monocyte chemoattractant protein-1 has an important role in the initiation stage of atherosclerosis.

The development of anti-atherosclerosis drugs can be done through the study on anti-inflammatory drugs that act more specifically by influencing pathogenesis of atherosclerosis. One of the ingredients that has anti-inflammatory effect and potentially as anti-atherosclerosis is andrographolide, the main active ingredient contained by *Andrographis paniculata*. Studies that have been carried out prove that andrographolide has anti-inflammatory effect^{7,8,9} and potential to be developed as anti-atherosclerosis¹⁰.

This study aimed to evaluate the andrographolide effect on MCP-1 expression at the initiation/early stage of atherosclerosis in rats induced by an atherogenic diet.

MATERIALS AND METHODS

Experimental animals

Twenty-seven male Wistar rats, 10 weeks of age (150-200 g) were obtained from School of Pharmacy, University of Riau. Rats were placed in cages in a well-ventilated room and allowed for food and water *ad libitum*. The room temperature was 20–26 °C and room lighting was regulated light and dark for 12 hours, alternately. Rat health was well monitored and rat cages were cleaned everyday. Acclimatization of rats was carried out for one week before being used in this study. The treatment of animals was subjected to the declaration of Helsinki. Ethical approval was obtained from the Ethical Review Board for Medicine & Health Research, Faculty of Medicine, University of Riau (No:457/UN.19.5.1.1.8/UEPKK/2017).

Experimental design

Rats were randomly divided into 3 groups (n=9), Group 1 (normal control) was given a standard diet. Group 2 (atherogenic control)

was given an atherogenic diet of vitamin D3 (cholecalciferol) 700.000 IU/kg (Sigma, St. Louis, MO, USA) on the first day followed by 5% goat fat, 2% cholesterol, 0.2% cholic acid (Sigma, St. Louis, MO, USA) and 92.8% standard diet for 2 days to induce atherosclerosis initiation stage^{11,12}. Group 3 (treated group) was given an atherogenic diet and treated with andrographolide 40 mg/kg (Andalas Sitawa Fitolab, Padang, Indonesia). Andrographolide was given orally once a day using a gastric tube.

Evaluation of initiation stage of atherosclerosis

The initiation stage of atherosclerosis was assessed based on foam cell formation. After the treatment was completed, ether anesthesia was used to sacrifice the rats. Abdominal aortic tissues were excised rapidly and fixed in 10% neutralized formaldehyde in 0.1 M phosphate buffer. The aortic sample was embedded in paraffin. We performed a hematoxylin and eosin (H&E) staining to measure the number of foam cells in the rat abdominal aortic. Foam cells were calculated with ×400 magnification at 9 fields of view. The images were obtained using a light microscope equipped with a camera (Leica, Wetzlar, Germany) and connected to a computer monitor.

Immunohistochemical staining

MCP-1 in rat abdominal aortic were identified using MCP-1 polyclonal antibody (Bioss Antibodies Inc., Massachusetts, USA). Paraffin embedded mouse kidney with positive IHC for MCP-1 was used as positive control. Slides were observed using light microscope at ×400 magnification. Images were taken using microscope camera (Leica, Wetzlar, Germany) as many as 10 pictures each slide. MCP-1 expression in abdominal aortic was assessed by Photoshop image analysis software to calculate the percentage of area¹². The percentage of area presented the comparison area containing MCP-1 with a whole cross section of the aorta, which is assumed to be width of expression. Two independent pathologists were invited to examine the slides.

Statistical analysis

Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's posthoc test was used to analyze the results. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of atherogenic diet on foam cell formation at the initiation stage of atherosclerosis

The H&E staining showed that in Group 1 (normal control) and Group 3 (treated group) demonstrated few foam cells (Figures 1A and 1C), while in Group 2 (atherogenic control) showed abundance of foam cells in the aortic lesion (Figure 1B). These results demonstrated that the administration of an atherogenic diet induced foam cell formation in rats and andrographolide treatment reduced the accumulation of these cells in aortic lesion.

Table 1 shows the number of foam cells among different experimental groups. Atherogenic diet-fed animals exhibit increased number of foam cells in the aortic lesions compared to control rats ($P < 0.05$). Andrographolide treatment significantly reduced the accumulation of foam cells in the aortic lesions ($P < 0.05$).

Effect of andrographolide on MCP-1 expression

Immunohistochemical evaluation of MCP-1 in aorta section is shown in Figure 2 and Table 2. MCP-1 expression in atherogenic control group

Table 1. Number of foam cells among different experimental groups

Animal group	Number of foam cells
Group 1	$5,33 \pm 1,73$
Group 2	$82,33 \pm 13,10^{a,b)}$
Group 3	$7,44 \pm 1,62$

Group 1: normal control group; Group 2: atherogenic control group; Group 3: treated group.

Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test for average comparison on SPSS 17.0.

Values are presented as mean \pm SEM (N=9).

^{a)} $P < 0.05$ vs. Group 1. ^{b)} $P < 0.05$ vs. Group 3.

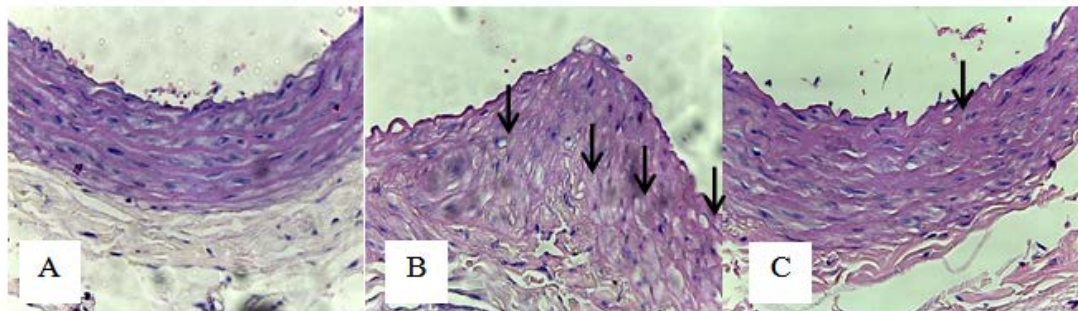


Fig. 1. Photomicrographs showing histological sections of aorta abdominal of rats. (A) Group 1 (normal control) was given a standard diet, (B) Group 2 (atherogenic control) was given an atherogenic diet, (C) Group 3 (treated group) was given an atherogenic diet and treated with andrographolide 40 mg/kg, at magnification of $\times 400$ stained with H&E. The arrow indicated foam cell

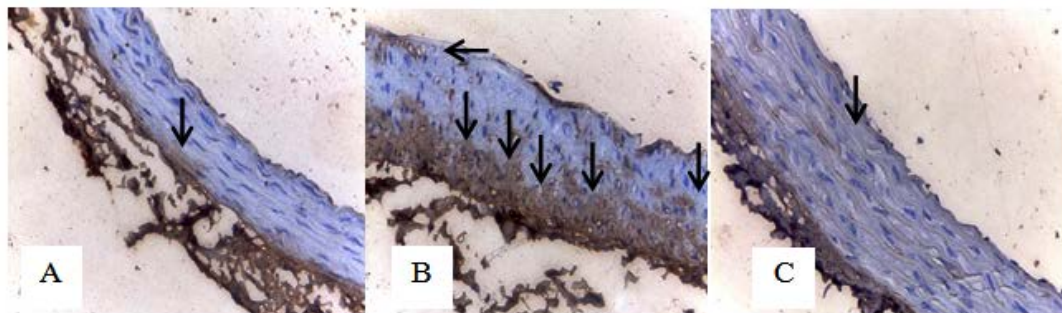


Fig. 2. MCP-1 immunoreactivity in rats abdominal aorta. (A) Group 1 (normal control) was given a standard diet, (B) Group 2 (atherogenic control) was given an atherogenic diet, (C) Group 3 (treated group) was given an atherogenic diet and treated with andrographolide 40 mg/kg, at magnification of $\times 400$. Diaminobenzidine stains brown to MCP-1. Slides were counterstained with H&E staining. The arrow indicated MCP-1 expression area

Table 2. MCP-1 expression among different experimental groups (by Photoshop)

Animal group	Percentage area (%)
Group 1	6,61 ± 1,90
Group 2	32,99 ± 3,74 ^{a),b)}
Group 3	9,61 ± 2,47

Group 1: normal control group; Group 2: atherogenic control group; Group 3: treated group.

Statistical analysis of the data was carried out using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test for average comparison on SPSS 17.0.

Values are presented as mean ± SEM (N=9).

^{a)}P<0.05 vs. Group 1. ^{b)}P<0.05 vs. Group 3.

(Group 2) was markedly higher than normal control group (Group 1). However MCP-1 expression was reduced by andrographolide treatment (Group 3).

DISCUSSION

The results of this study showed that andrographolide inhibits MCP-1 expression at the initiation stage of atherosclerosis in rats induced by an atherogenic diet. Andrographolide may reduce foam cell formation in the early stages of atherosclerosis.

Andrographolide was one of the main active ingredients contained by *Andrographis paniculata*. Recent studies were reported that andrographolide was effective in treating inflammatory diseases because it was a powerful NF-κB inhibitor^{13,14}. Andrographolide has been shown to use anti-inflammatory effects in experimental models of respiratory inflammatory diseases including asthma¹⁵, acute lung injury (ALI)¹⁶, and idiopathic pulmonary fibrosis (IPF)¹⁷. Andrographolide has been shown to protect against stroke by inhibition of NADPH oxidase 2 (NOX2) and inducible-nitric oxide synthase (iNOS) production via inhibition of NF-κB¹⁸.

In order to develop andrographolide as an effective anti-inflammatory drug, various clinical trials have been carried out, including for the treatment of sinusitis, where the drug was reported to be effective in relieving inflammatory symptoms and well tolerated by patients¹⁹. Beneficial effects of *A. paniculata* have been shown in a phase 2 trial of rheumatoid arthritis²⁰ and

significantly reduced fatigue in multiple sclerosis patients²¹.

Toxicological study on andrographolide has been carried out both pre-clinical and clinical studies. In an acute toxicity study it was found that andrographolide up to 5 g/kg given orally daily for up to 14 days in rats had no observable adverse effect. The same results were also found in a sub-acute toxicity study which showed administration of oral dose of andrographolide up to 500 mg/kg given daily for up to 21 days in rats also had no observable adverse effect²². In a clinical trial, administration of 170 mg of *A. paniculata* purified extracts (about 85 mg andrographolide) given orally every 12 h for 12 months was found to be well tolerated in patients with relapsing-remitting multiple sclerosis. Only one patient received *A. paniculata* presented with a mild skin rash, which was attenuated with anti-histamine treatment²¹.

Atherosclerosis initiation stage was carried out in rats induced by atherogenic diet. We used rats in this study as experimental animal because these animals have several advantages including being practical, easy to obtain and are omnivorous diet animals, more like humans. Atherosclerosis can be induced in the rats by adding vitamin D3 (cholecalciferol) into high-cholesterol diet²³. Rat model of atherosclerosis created by vitamin D3 and feeding with an atherogenic diet is similar to the human. The composition of the atherogenic diet given in this study consisted of 5% goat fat, 2% cholesterol, 0.2% colic acid and a standard diet up to 100%¹². Atherogenic diet was given to produce hypercholesterolemia in experimental animals. Hypercholesterolemia causes LDL to infiltrate arteries, especially at the site of hemodynamic disturbance. Oxidative and enzymatic modifications will convert LDL to oxLDL²⁴. oxLDL causes endothelial dysfunction so that it will cause recruitment of monocytes into the arterial wall²⁵.

Monocyte infiltration into the arterial wall plays an important role in the initiation stage and development of atherosclerotic lesion. One factor that has an important role in the recruitment of monocytes is MCP-1²⁶. Various types of cells presented in the walls of blood vessels synthesize and secrete MCP-1. This chemokine has a strong chemotactic effect on monocytes/macrophages²⁷.

The chemotactic effect of MCP-1 on monocytes in circulating blood is mediated by a specific high-affinity receptor CCR2^{4,5,26}. Aiello et al demonstrated that MCP-1 can initiate atherosclerosis in mice with apolipoprotein-E deficiency²⁸. Meanwhile, studies in mice with deletion of MCP-1 or CCR2 showed a decrease in monocyte accumulation in the arterial wall and prevent the formation of atherosclerotic lesions^{29,30}. These evidences prove that MCP-1 plays an important role in the atherosclerosis initiation stage and could be a potential target for atherosclerotic therapy.

The atherosclerosis initiation stage is characterized by the foam cells formation³¹. Foam cells found at the early atherosclerotic lesion originate from monocytes in the circulation³². Monocytes that have attached to vascular endothelial cells then migrate into the arterial intima layer mediated by MCP-1²⁴. These monocytes will then differentiate into macrophages in response to Macrophage-Colony Stimulating Factor (M-CSF) and other stimuli³³. Macrophages express the scavenger receptor (SR) consisting of CD36, SR-A and lectin-like oxLDL receptors-1 (LOX-1) which facilitate oxLDL particle uptake by macrophages resulting in lipid-laden foam cells³⁴.

We suggest that the mechanism of inhibition of MCP-1 by andrographolide occurs through inhibition of NF- κ B activation. Previous studies have shown that andrographolide has anti-inflammatory effects through the inactivation of the NF- κ B¹³. NF- κ B is a transcription factor that has an important role in regulating the genes of various cytokines and chemokines involved in the inflammatory process³⁵. NF- κ B consists of: p105/p50 (NF- κ B1), p100/p52 (NF- κ B2), p65 (RelA), RelB dan c-Rel. The NF- κ B pathway is activated by the IKK (I κ B kinase) complex, which consists of the regulator subunit, IKK γ which is also known as the NF- κ B essential modifier (NEMO), and the catalytic subunit, IKK α and IKK β . As the downstream substrate is I κ B. In normal circumstances the NF- κ B p50/NF- κ B p65 dimer is bound to I κ B. In response to stimuli, IKK α and IKK β will be phosphorylated and cause I κ B phosphorylation. The phosphorylated I κ B will degrade through the ubiquitin proteasome pathway. I κ B degradation causes NF- κ B p50/NF- κ B p65 to undergo phosphorylation and translocation which eventually results in gene transcription³⁶.

Inhibition of NF- κ B activity by andrographolide occurs through dephosphorylation of p65 by activating PP2A^{9,37}. Dephosphorylation of p65 by andrographolide results in decreased translocation of NF- κ B into the nucleus and reduces binding of NF- κ B with DNA³⁷. Through this mechanism, andrographolide inhibits MCP-1 expression so that it will inhibit monocyte recruitment into the arterial wall.

CONCLUSION

The results of the present study suggest andrographolide has atheroprotective effects by inhibiting MCP-1 expression and eventually suppressing macrophage foam cell formation. Clinically, the results presented here provide insights into the potential use of andrographolide as an anti-atherosclerotic drug.

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