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Exploring the role of the combination of propolis and vitamin D3 on VCAM-1 and Caspase-3 expression in preventing atherosclerosis in chronic kidney disease rats

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ABSTRACT

Background: Increased blood pressure that occurs in chronic kidney disease (CKD) is influenced by the occurrence of thickening of blood vessels. This study evaluates the utilization of propolis which acts as an anti-inflammatory and vitamin D3 which plays a role in calcium-phosphate metabolism, that when combined are expected to prevent atherosclerosis.

Methods: This experimental research used a post-test only group design with a total of 24 male rats divided into 3 groups. Subjects were induced to develop CKD with unilateral ureteral obstruction. A combination of 50mg/kgBW of propolis and 0.126mcg/kgBW of vitamin D3 was given orally every morning in the intervention group. Hemodynamics and atherosclerosis status were observed using sonography until week 4. Inflammatory markers with vascular cell adhesion molecule 1 (VCAM-1) and Caspase-3 were evaluated weekly via plasma and target organ samples were immunohistochemically examined at the study's end.

Results: Propolis and vitamin D3 significantly reduced blood pressure from 156 mmHg at the beginning of the study to 117mmHg at week 4 (p=0.000). Thickening of the aorta occurred in the control and intervention groups but the thickening in the control group (0.90mm) was statistically significantly (p=0.000) higher than the intervention group (0.30mm). There were decreased VCAM-1 and Caspase-3 levels in the intervention group compared to the control group, both in plasma and target organ levels.

Conclusion: Utilization of propolis and vitamin D3 combined has a good effect on lowering inflammation, improving hemodynamics and preventing atherosclerosis in chronic kidney disease rat models.

Keywords: Chronic kidney disease, Atherosclerosis, Propolis, Vitamin D3, Inflammation, Rat models.
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INTRODUCTION

Chronic kidney disease (CKD) is a serious problem globally with high morbidity and mortality.[1,2] CKD is often accompanied by complications of increased blood pressure so proper management is needed to maintain stable blood pressure.[3,4] Controlling blood pressure with medication interventions can prevent worsening of kidney function and also prevent cardiovascular disease.[3]

CKD can also accelerate the occurrence of calcification in atherosclerotic plaques.[2,5] Inflammation due to CKD is believed to be the main mechanism of atherosclerosis.[6-8] The high level of pro-inflammatory factors in kidney failure triggers an increase in inflammation-triggered reactive oxygen species (ROS), which will cause vascular damage and narrowing.[8] Inflammation and oxidative stress have the role of positive feedback with each other.[4] In addition, caspase-3, which is a key executioner protease in the apoptosis of macrophages and vascular smooth muscle cells (VSMC), contributes to the progression and instability of atherosclerosis.[9,10] Apoptosis is influenced by several proinflammatory, prooxidative and cytotoxic factors.[9] Besides that, the formation of atherosclerosis is also strongly influenced by the expression of vascular cell adhesion molecule-1 (VCAM-1) which has an important role since the early formation of lesions.[8,11] Expression of VCAM-1 on the cell surface is induced by proinflammatory cytokines and oxidative stress.[8,11]

Propolis and vitamin D3 are substances that are believed to suppress inflammation which is the basic cause of atherosclerosis in CKD cases.[12,13] In addition to having an anti-inflammatory effect, propolis has antioxidant, immunomodulatory and anti-proliferative effects.[12] It can reduce vascular endothelial damage and prevent stiffness and constriction of blood vessels.[12] In this study, we evaluated the combined administration of propolis and vitamin D3 for its effects on preventing constriction and stiffness of blood vessels in chronic kidney disease rat models.

RESEARCH DESIGN AND METHODS

Drugs and reagents

This study used a combination of propolis extract and vitamin D3. Propolis was obtained by maceration. A total of 500 g of dry propolis were taken from Mount Lawu, cleaned and mashed. Then 3.75 liters of 70% ethanol were added as a solvent. Next, it was stored in a closed room for 7 days and stirred vigorously twice a day.[14,15] The filtrate was separated from the pulp and then evaporated at 45°C with vacuum pressure (<1atm) for 4 hours and thick propolis was obtained (100 g). The thick propolis extract was evaporated for 24 hours to evaporate the ethanol content.[14,15] The dose of propolis given was 50mg/kgBW orally every morning. Meanwhile, vitamin D3 (cholecalciferol) used in this study used a pharmaceutical patent preparation of D-VIT syrup, with a dosage of 0.126 mcg/kgBW orally every morning.

Animals

Subjects were white male rats (*Rattus norvegicus*) from the animal house of Universitas Gadjah Mada, 3-4 months old, weighing 150–300 g. Normal rat-feed with BR-1, and water *ad libitum* were provided.

Experimental design

This research was an experimental study with a post-test only control group design. A total of 24 male rats with relatively the same weight distribution were randomly divided into 3 groups: the healthy group, the control group and the intervention group. In the control group, mice were induced into CKD by the unilateral ureteral obstruction (UUO) method, with left ureteral ligation. Blood flow to the kidney was drastically reduced by this procedure, causing an interstitial inflammatory response to tubular atrophy and tissue fibrosis.[16-18] After day 10 until day 28, the healthy and control group were given aquadest (0.2 ml/day) with a probe, while the intervention group was given a combination of propolis extract and vitamin D3 orally every day. On day 28, rats were euthanized by the cervical dislocation method.

Measurements

Blood pressure was measured weekly. Evaluations of the diameter and thickness of the abdominal aorta and inferior vena cava were performed by vascular Doppler ultrasound. Measurements of systolic blood pressure (SBP), peak systolic velocity (PSV) and end-diastolic volume (EDV), the thickness of the tunica intima of abdominal aorta and the diameter and thickness of the inferior vena cava (ICV) were measured. Blood samples were taken every week to measure Caspase 3 and vascular cell adhesion molecule (VCAM-1) in circulation.

Immunohistochemistry

After euthanasia, histopathological preparations were taken for immunohistochemistry examination (IHC) with Masson-trichrome staining on peripheral blood vessels from the arteries and veins of the anterior extremity to measure accumulation of collagen in blood vessels as an indicator of vascular thickening. The samples of the preparations were made at the anatomical pathological laboratory of Universitas Sebelas Maret and examined by an anatomical pathologist.

Statistical analysis

All data were analyzed using version 22 SPSS software (IBM Corp., Chicago). Normality test was performed using Shapiro Wilk tests. Data with normal distribution were analyzed by parametric analysis using repeated ANOVA and one-way ANOVA, while data that were not normally distributed were analyzed by non-parametric analysis using Friedman, Wilcoxon and Kruskal Wallis tests. Parametric data were displayed in mean ± standard deviation (SD), while non-parametric data were shown in median and minimum-maximum values. The results were considered significant if $p<0.05$.

RESULTS

A total of 24 male rats were randomly divided into 3 groups with the same weight distribution. There was no significant difference in body weight of mice in the healthy group from the beginning to the end of the study. In the control group, there was a periodic weight loss from 178 g to 163 g at the end of the study ($p=0.000$). The opposite occurred in the intervention group with the combination of propolis and vitamin D3 where there was an average increase in body weight from 178 g to 200 g at week 4 of study ($p=0.000$) (Table 1).

Table 2 shows a comparison of hemodynamic parameters and the incidence of atherosclerosis measured periodically at week 0, week 2 and week 4. The SBP in the control group showed a significant increase to 181 mmHg at the end of the study, compared to the SBP in the intervention group which had a periodic decrease from the initial week to 117 mmHg at the end of the study ($p=0.000$). There was an increase in PSV and EDV values, but the controls and the controls were higher than in the intervention group. The PSV values between the two groups statistically were significantly different, namely 57.82 cm/s in the control group and 25.65 cm/s in the

Table 1. Comparison of body weight by the time of study.

Groups	Mean body weight by time(g)					*p*
	Week 0	Week 1	Week 2	Week 3	Week 4	
Healthy (g)	185.33 ± 1.28	185.41 ± 1.35	185.54 ± 1.29	185.52 ± 1.21	185.57 ± 1.25	0.196
Control (g)	178.87 ± 3.64	175.50 ± 3.58	171.37 ± 3.42	167.25 ± 3.77	163.75 ± 3.61	0.000*
Intervention (g)	178.87 ± 3.68	182.00 ± 3.92	188.37 ± 3.62	193.75 ± 3.99	200.50 ± 3.66	0.000*

*Significant (p<0.05)

Table 2. Comparison of hemodynamic indicators and vascular condition by time.

Indicator	Group	Time			*p*
		Week 0	Week 2	Week 4	
SBP (mmHg)	Control	148.50±4.62	172.00±4.66	181.37±3.66	0.000*
	Intervention	156.50±3.33	141.62±4.13	117.50±3.77	
PSV (cm/s)	Control	18.49±1.10	38.35±3.16	57.82±4.38	0.000*
	Intervention	18.02±1.03	22.30±0.65	25.65±1.10	
EDV (ml)	Control	2.69 (2.32 – 3.43)	5.27 (5.16 – 6.74)	7.08 (6.92 – 8.69)	0.000*
	Intervention	2.63 (2.29 – 3.04)	3.70 (3.28 -4.31)	5.03 (4.72 – 5.65)	
Tunica intima of AA (mm)	Control	0.25 (0.20 -0.40)	0.60 (0.40 – 0.90)	0.90 (0.70 - 1.10)	0.000*
	Intervention	0.20 (0.20 – 0.40)	0.25 (0.20-0.40)	0.30 (0.20-0.50)	
Diameter of ICV (mm)	Control	2.04 (2.00 – 2.10)	2.96 (2.8 – 3.4)	3.35 (3.00 – 3.50)	0.000*
	Intervention	2.04 (1.80 – 2.10)	2.20 (1.90 – 2.40)	2.97 (2.90 – 3.20)	
Thickness of ICV (mm)	Control	0.55 (0.50 – 0.60)	0.80 (0.70 – 0.90)	1.00 (0.90 -1.10)	0.000*
	Intervention	0.60 (0.50 – 0.60)	0.70 (0.60 – 0.70)	0.80 (0.70-0.80)	

*Significant (p<0.05); SBP: Systolic blood pressure; EDV: end-diastolic volume; PSV: peak systolic velocity; AA: abdominal aorta; ICV: Inferior vena cava.

Table 3. Comparison of inflammatory marker.

Indicator	Group	Time				*p*
		Week 1	Week 2	Week 3	Week 4	
VCAM 1 Plasma	Control	651.06±21.78	660.10±9.69	666.16±5.38	662.011±23.48	0.000*
	Intervention	642.64±16.48	518.20±5.36	437.00±6.68	478.88±13.61	
Caspase 3 Plasma	Control	7.29±0.31	7.48±0.20	7.51±0.20	7.35±0.34	0.000*
	Intervention	7.11±0.09	5.78±0.02	4.41±0.40	1.86±0.10	
Thickness of artery	Control				73.96±19.88	0.031*
	Intervention				55.98±7.14	
Veins	Control				15.21 (3.70-19.90)	0.000*
	Intervention				4.94 (2.60-7.60)	

*Significant (p<0.05); VCAM-1 : Vascular cell adhesion molecule-1

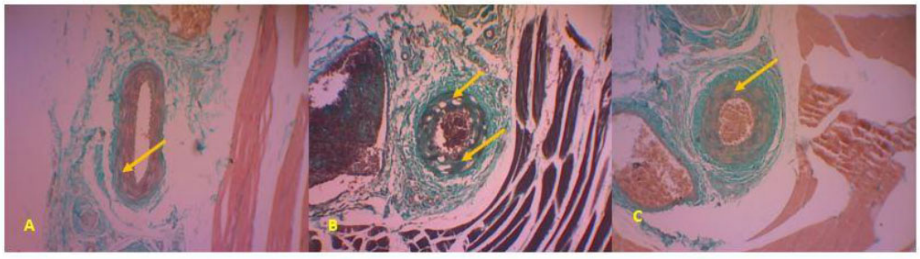

Figure 1. Collagen deposition in femoral artery wall with Masson's trichrome staining in healthy group score 0 (A), CKD group score 2 (B), intervention group score 1 (C). Microscope magnification 100x.

intervention group with almost the same initial values of about 18 cm/s (p=0.000).

Sonography examination showed thickening of the tunica intima of the abdominal aorta in the CKD rat model. Thickening in the control group was significantly higher than in the intervention group, which was 0.90 mm compared to 0.30 mm (p=0.000) at the end of the study. On examination of the ICV, the diameter and thickness of the vein wall increased by the end of the study. There was a significant difference in the increase in diameter and thickness of the ICV by sonography. The diameter of the two groups was almost the same at the beginning, while at the end of the study the control group had a diameter of 3.35mm, and the intervention group was 2.97 mm (p=0.000) (Table 2).

There was a significant decrease in the levels of VCAM-1 and Caspase 3 in the intervention group with propolis and vitamin D3 as shown in the Table 3, which also shows that at the end of the study, the intervention group had a smaller mean arterial thickness (p=0.031) and venous thickness (p=0.000) compared to the control group without intervention. Similar results were found in the immunohistochemical examination where the deposition of collagen fibers that affected atherosclerosis was less in the group with combination intervention of propolis and vitamin D3 compared to the control group as shown in Figure 1.

DISCUSSION

Patients with CKD are more at risk for cardiovascular complications,

even premature mortality.[19-22] CKD is also characterized by decreased anti-atherogenic properties and has a role in the process of atherosclerosis in increased cardiovascular morbidity.[19,22-24] CKD can cause calcium deposition leading to vascular calcification. In large arteries, vascular calcification is associated with the appearance of atheromatous plaques, whereas in small arteries rich in vascular smooth muscle cells, medial calcification without plaque is common.[5,22] This may be due to instability of circulating calcium and phosphate ions and abnormal differentiation of vascular smooth muscles.[22]

Inflammation is one of the main mechanisms of atherosclerosis and CKD is closely related to systemic inflammation. Proinflammatory changes in CKD patients increase circulating concentrations of C-reactive protein (CRP) and cytokines.[24-27] The inflammation causes arterial narrowing, vascular stiffness and endothelial dysfunction.[13,21,25,27] In fact, the occurrence of endothelial dysfunction begins early in renal impairment, where the filtration rate decreases and blood pressure rises.[21] This is due to the accumulation of fatty plaques on inner walls of blood vessels that contain low-density lipoprotein (LDL) and cellular waste.[13] Oxidation of LDL in the vasculature can activate endothelial cells to produce adhesion molecules that will eventually lead to proliferation of smooth muscle and connective tissue, and bulging into arterial lumen, which cause decreased tissue perfusion.[13,25] Oxidative stress is a hallmark feature of cardiovascular disease caused by CKD.[24,28]

Propolis is a safe natural resinous product made by bee formed material extracted from plants.[29-31] Propolis has several biological modalities such as antioxidants, anti-inflammation, immunomodulatory, antihypertensive and anti-atherosclerotic.[26,30-33] Administration of propolis can cause significant decrease in mean systolic and diastolic pressure.[29] Propolis has the ability to modulate both serum lipoproteins and their handling by vascular tissues, which can slow the progression of atherosclerosis by preventing endothelial vascular dysfunction.[30-32] One study reported that propolis has antioxidant activity by means of free radical scavenging and reduces malondialdehyde (MDA) levels.[32,33] Administration of antioxidants can neutralize excess free radicals that can damage cells and tissues, thus preventing various inflammatory disease conditions such as atherosclerosis.[29,33] The anti-inflammatory activity of propolis has been tested both in vitro and in vivo by modulating inflammatory mediators, inhibiting the production of proinflammatory cytokines, increasing anti-inflammatory cytokines and blocking the activity of nuclear factor kappa B (NFκB).[32,33] Results showed that it can protect the endothelium and become an important therapy in atherosclerosis.31 Inhibition of the NFκB pathway has been demonstrated to show beneficial effects to reduce blood pressure and reduce cardiovascular complications.[28]

Another study also showed there was a decrease in the level of hydrogen peroxide (H_2O_2) and nitric oxide (NO) in the administration of propolis.[32] In addition, propolis can also down-regulate CD36 expression in animal models, which is responsible for the recognition and internalization of oxidatively LDL which leads to the formation of foam cells in the atherosclerosis process.[32] In one study, propolis supplementation was found to reduce vascular cell adhesion protein 1 (VCAM-1) expression.[31,32] VCAM-1 is a protein expressed in endothelial cells that can be activated by proinflammatory cytokines.[34] In inflammatory conditions, many pro-inflammatory cytokines induce the expression of intercellular adhesion molecules such as VCAM-1. Accordingly, anti-inflammatory administration that suppresses this process can reduce VCAM-1 expression thereby inhibiting the migration of monocytes from the blood to the vascular intima, reducing the aggregation of foam cells and lipids in the arterial wall and inhibiting monocyte chemotaxis to the lesion sites so as to reduce the accumulation of lipids in the intima.[31]

CKD causes a decrease in plasma vitamin D concentrations.[21,23] In one study, vitamin D deficiency was associated with endothelial dysfunction and atherosclerosis, as well as upregulation of renin-angiotensin-aldosterone (RAAs) and induction of hypertension.[23,35,36] Administration vitamin D3 can suppress renin and angiotensin gene expression by inhibiting the NFκB pathway.[23,35] Vitamin D3 also exhibits vasoprotective effects by improving endothelial function, inhibiting VSMC proliferation and down-regulating inflammatory processes.[35,36] Vitamin D3 is believed to affect the function of macrophages which are very crucial in the formation of foam cells.[36] This finding is in line with the results of this study where the combined administration of propolis and vitamin D3 as an anti-inflammatory agent resulted in a decrease in the concentration of VCAM-1 and Caspase-3 as well as a decrease in the diameter and thickness of arteries.

This study had several limitations such as not testing the content of the propolis used. This study also only used male rats with 3 to 4 months of age. In addition, histopathological sampling was not performed using a stereotypic method and was only performed once. Further research needs to be done by testing the content of propolis used and using a more varied sample to evaluate the effects of propolis administration on hemodynamic improvement and prevention of atherosclerosis in CKD.

CONCLUSIONS

The combination of vitamin D3 and propolis extract as an anti-inflammatory agent can reduce the process of wall thickening and narrowing of arterial diameter, and can also reduce the expression of proinflammatory agents such as VCAM-1 and Caspase-3.

ABBREVIATIONS

CKD: Chronic Kidney Disease; MDA: Malondialdehyde; NFκB: nuclear factor-κB; AA: Abdominal aorta; ICV: Inferior Vena Cava; SBP: Systolic blood pressure; PSV: Peak systolic velocity; EDV: End diastolic volume; ROS: Reactive oxygen species; UUO: unilateral ureteral obstruction; IHC: Immunohistochemistry; RAAs: Renin Angiotensin Aldosterone system; LDL: Low-Density Lipoprotein; CRP: C-reactive protein; VCAM-1: Vascular Cell Adhesion Molecule 1.

ETHICS APPROVAL AND CONSENTS TO PARTICIPATE

This study was approved by Health Research Ethics Committee of the Faculty of Medicine, Universitas Sebelas Maret (No. reg 734/X/HREC/2018).

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DECLARATIONS OF INTEREST

The authors declare that they have no competing interests.

AUTHOR'S CONTRIBUTION

Data gathering and idea owner of this study: Darmawan Ismail, Bambang Purwanto, Brian Wasita, Supomo, Ketut Putu Yasa and Soetrisno
Study design: Darmawan Ismail, Bambang Purwanto, Brian Wasita, Supomo, Ketut Putu Yasa and Soetrisno
Data gathering: Darmawan Ismail, Bambang Purwanto, Brian Wasita, and Supomo
Writing and submitting manuscript: Darmawan Ismail, Bambang Purwanto, Brian Wasita, and Supomo
Editing and approval of final draft: Darmawan Ismail, Bambang Purwanto, Brian Wasita, Supomo, Ketut Putu Yasa and Soetrisno.

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