EXECUTIVE SUMMARY

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KAJIAN EFEK KOPI DAN PERIODONTITIS PADA ATEROSKLEROSIS KORONER

SUB JUDUL: PROTECTIVE EFFECT OF COFFEE AGAINST CORONARY ATEROSCLEROSIS IN PERIODONTITIS RAT MODEL

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Abstract

Objectives. Effect of habitual coffee consumption on cardiovascular disease is controversial. Some epidemiological studies, however, reported the beneficial effect of coffee consumption on cardiovascular health. The objective of this study was to conduct an in vivo experiment to prove the effect of coffee consumption to reduce coronary atherosclerosis in periodontitis rat model.

Methods. Twenty one rats (Rattus norvegicus) were divided into three groups, i.e. 1) periodontitis, 2) periodontitis + coffee, 3) control group. Periodontitis rat model was created by means of inserting wire ligature around left molar mandibular teeth followed by injecting periodontitis bacteria Porphyromonas gingivalis in gingival sulcus thrice a week. One dose of decocta coffee (representing one cup) was fed once per day by stomach sondation. The experiment was conducted for 35 days. At the end of experiment, all rats were sacrificed. Their hearts which contained coronary arteries were removed and prepared for histopathologic examination for media-intimal thickening and ratio, the existence of atheroma, stenosis, endothelial cell disintegration and lipids deposit.

Results. Arterial wall of rats that consumed coffee demonstrated more symmetric media-intimal thickness and intimal collagen showed intact, denser and thicker. Fewer stenosis, atheroma, lipid deposit and endothelial cell disintegration were identified in coffee group.

Conclusions. One cup coffee consumption per day improved the morphology of coronary artery leading to protect against atherosclerosis. A novel perspective was the improvement of the structure of intimal collagen, it might also provide the resistance of vasculature against rupture and thrombosis, further studies are needed particularly focusing on this intimal collagen.

Keywords. Atherosclerosis; Coffee; Collagen; Periodontitis; Rat.
EFEK PROTEKTIF KOPI TERHADAP ATEROSKLEROSIS KORONER PADA MODEL TIKUS PERIODONTITIS

Abstrak


Kata kunci: Aterosklerosis; Kopi; Kolagen; Periodontitis; Tikus.
INTRODUCTION

Perception on effect of coffee beverage consumption on cardiovascular disease has changed [Lopez Garcia, 2012]. Several earlier studies had shown the adverse effect of coffee consumption on cardiovascular system, such as, related to the risk of acute coronary events [LaCroix 1986, Happonen 2004], cause extracellular accumulation of low density lipoprotein (LDL) [Rustam, 1997], increased inflammation [Zampelas, 2004], heightened acute vascular inflammatory (Hammer, 2006), induced unfavorable effects on endothelial function [PAPAMICHAE, Buscemi]. Results from more recent studies, however, reported beneficial of habitual coffee consumption on cardiovascular health, such as effects on subclinical inflammation and HDL cholesterol [Kempt, 2010], against coronary calcification [Geertruida J. van Woudenbergh 2008], inversely associated with markers of inflammation and endothelial dysfunction [Lopez-Garcia E 2006], reduce risk of death attributed to inflammatory and cardiovascular disease [Anderson, 2006], inversely associated with risk of heart failure [Mostofski, 2012] improved endothelial function [Shecher, 2011; Siosa 2013], increase the resistance of LDL to oxidative modification [Natella, 2007].

Many studies on coffee concerned with the harmful effect of caffeine used in isolation [Adebayo, 2006; Corneli 2007; Silvas 2004], in fact, coffee has a very complex chemical composition. In addition to caffeine, coffee contained many other substances that act as antioxidants [Natella 2007; Silvas 2004; Halvas 2002; Moriera 2012] and anti-inflammatory [Kempt 2010; Anderson 2006; moriera 2012], that may counteract the adverse effect of coffee. Considering these properties of antioxidant and anti-inflammatory, therefore it is plausible to assume that coffee consumption may have protective effect against atherosclerosis. Since, oxidation and inflammation represent important mechanism in atherogenesis [Fong 2002].

The currently accepted hypothesis is that atherosclerosis develops as a response to injury and that is primarily a chronic inflammatory condition [stocker]. Chronic bacterial inflammatory disease of tooth surrounding tissue (periodontitis) had been shown to play an important role in pathogenesis of atherosclerosis. Periodontitis bacteries, were identified using immunostaining and polymerase chain reaction (PCR) in some autopsy specimen of coronary atherosclerotic plaque in human who died for heart attack [Harazthy, Kozarov, stelzel]. Animal experimental studies consistently shown the positive role of periodontitis in increase risk of atherosclerosis [li, brodala].

The purpose of this study was to conduct an in vivo experiment to prove the effect of coffee consumption to reduce coronary atherosclerosis, in periodontitis rat model. In particular, the present study analyzed the effect of one dose of coffee decocta consumption (representing one cup per day). Indicators of coronary atherosclerosis included diffuse intimal thickening (DIT), morphological change of arterial wall, lipids deposit, foam cell, and scavenger receptor (Sc-R) expression.
METHODS

Animals and groups
Male rats (*Rattus norvegicus*), 12 weeks old (± 200 gr body weight) were purchased from the Department of Physiology Faculty of Medicine Brawijaya University, Malang, Indonesia. The animal were maintained under standard laboratory conditions and according to the guidelines established by institutional Animal Care and Ethics Committee. Animal treatment procedure was approved by Ethical committee at Faculty of Dentistry Gadjah Mada University, Yogyakarta, Indonesia. Dry pellet normocholesterol standard diet and water were given *ad libitum*. All rats were housed in pens in identical condition to minimize environment factor. Total of twenty one rats were randomly divided into three groups, namely 1) periodontitis, 2) periodontitis + coffee (coffee group), and 3) control (healthy, non-periodontitis, no coffee).

Preparation of coffee decocta
This study used commercial pure Robusta ground coffee produced by State Plantation Company PTPN XII Jember, East Java, Indonesia. To prepare coffee decocta, 3 gr ground coffee was poured into 200 ml boiled aquades, for 3 minutes, while shaking. One dose of coffee decocta representing one cup (0,6 ml) was given per day to rats by means of stomach sondation.

Periodontitis rat model.
Periodontitis model was created in rat by means injecting periodontitis bacteria *Porphyromonas gingivalis* (ATTC, 33277) in buccal gingival sulcus of left mandibular teeth, as previously described (Susilawati). Before injection, these teeth were tided using a loop wire ligature (θ: 0,5 mm, Fried Krupp) in order to make better retention for bacterial plaque to accumulate on periodontal tissue leading to accelerate periodontitis. This procedure was performed under intramuscular anesthesia using ketamin 0,02ml/rat. *P. gingivalis* was prepared in cultured medium Brain Heart Infusion Broth (BHI-B) enriched by vitamin K<sub>1</sub> and hemin (Sigma) under anaerobic atmosphere for 2 x 24 hours. To confirm bacterial purity, *P. gingivalis* was stained using Gram dye, and identification under light microscope shown that *P. gingivalis* was Gram negative with uniform rod morphology. *P. gingivalis* concentration was adjusted to Mack Farland 0,5 (1,5 x 10<sup>8</sup>) CFU per ml. Chronic periodontitis was conditioned by regular injection of 50 μl *P. gingivalis* thrice a week for five weeks (35 days). At the end of study, all of the rats in periodontitis group demonstrated alveolar bone resorption clinically and radiographically (suffered from periodontitis).

Histologic samples preparation
At the end of experiment, all of rats were fasted overnight and afterword were sacrificed using a heavily chloroform inhalation. The heart which contained coronary arteries were removed and fixed in 10% formalin (in PBS), and then trimmed crossectionally (perpendicular to the direction of blood flow) in about coronal one half area of the heart, and prepared for frozen section. Serial cryosections (10 μm thickness) were made and then mounted on to microscope slides

Coronary arterial wall thickness and morphology
To analyze coronary arterial wall thickness and morphology, samples were stained using collagen staining kit (Picro’Sirius Red). According to the instruction of the manufacturer, this stain demonstrated red color for collagen and yellow for elastin. The images were demonstrated
by light microscope magnification 400 and 1000 times and visualized using optilab viewer. Coronary arterial wall thickness was measured as media-intima thickness (μm), in the thickest area. We also observed focusing on intimal collagen integrity and thickness. In addition, symmetric and asymmetric thickness and media-intima ratio were analyzed as well. For morphologic parameters, we identified the existence of irregularity, atheroma, stenosis, luminal narrowing and occlusion.

**Endothelial cell layer integration and lipid deposit**
Morphology of endothelial layer were analyzed in histologic samples after being stained by Hematoxylin-Eosin dye. Lipid deposit, foam cell and fatty emboli were identified in frozen section slide that were stained by Oil RedO dye (Sigma) and hematoxyline (Merck) for counter stain. 5% Oil RedO was prepared by dilution in propilene glikol. Lipid deposit was demonstrated as red color droplet, while nuclei pale blue.

**Analysis of intimal collagen integrity**
Integrity of intimal collagen is important to maintain the resistance of vasculature and atherosclerotic plaque against rupture and thrombosis. Disintegration of intimal collagen could cause thinning of fibrous cap so that increase the risk of rupture. The present study analyzed quality intimal collagen such as the intact, dense and disintegration. This was analyzed in samples that were stained by collagen staining kit.

**RESULTS**

**Coronary Arterial Wall Thickness**
Analysis using collagen staining method clearly demonstrated the comparation of coronary arterial thickness between groups. Coffee group showed thinner arterial wall compared to non coffee groups. This was confirmed by figure 2, that demonstrated an obvious arterial wall thickening in periodontitis group (B) compared to coffee (C) and non-periodontitis group (A). In addition, We also found that coffee group have more symmetric intimal thickness and relatif wider lumen and minimum blood component adhesion in the luminal surface.
Coronary Arterial Wall Morphology

Further analysis of coronary arterial wall morphology using collagen staining method was focused on the morphology of intimal layer. A significant difference on intimal layer morphology depicted on Figure 3. Collagen of endothelial basal membrane in intimal layer of coffee group showed obviously thickest and intact (Figure 3C) compared to other groups. In periodontitis group, collagen basal membrane were thinnest and disintegrated (Figure 3B), while in non periodontitis group were shown as a thin layer (Figure 3A).

Figure 2. Morphology of coronary artery using collagen staining (Picro Sirius Red). A. Non-periodontitis group. B. Periodontitis group. C. Periodontitis + Coffee group. Coronary artery of coffee group demonstrated intact and dense intimal collagen (†)
Expression of Scavenger Receptor
Immunohistochemical analysis of coronary artery revealed that the expression of scavenger receptor (CD 163) were fewer in coffee and control groups compared to periodontitis group. Figure 4, coronary artery of periodontitis rat model expressed more scavenger receptors.

Figure 3. Magnification of intimal collagen of coronary artery (→)
A. Non periodontitis group demonstrated a thin collagen intimal
B. Collagen intimal of periodontitis group showed disintegrated
C. Collagen intimal of coffee group showed thickest and intact

Figure 4. Immunohistochemistry of coronary artery: expression of scavenger receptor (CD 163), 100 x magnification. Coffee group (C1, C2) demonstrated lesser expression of Sc-R compared to periodontitis group (dark brown, B1, B2). Control group (A1, A2) demonstrated similar density to coffee group.
Lipid Deposition
Histochemistry analysis of coronary artery using Oil RedO demonstrated that endothelial lipid deposition, fatty emboli and foam cell were more common found in periodontitis group compared to control and coffee groups. Rat that fed with coffee showed minimum endothelial lipid deposition and fatty emboli was absent.

![Figure 5](image.png)

**Figure 5.** Lipid deposition (demonstrated by Oil RedO staining) on endothelial surface and fatty emboli were found in coronary artery of periodontitis rat model (B) compared to control group (A). Fewest lipid deposition and fatty emboli were identified in coffee group (C).

DISCUSSION

The present study revealed that chronic periodontitis induced worsening morphology of coronary arterial wall leading to accelerate atherosclerosis. Several atherosclerosis parameters identified were arterial wall thickening, progression atheroma morphology, endothelial lipid deposition and disintegration of intimal collagen. This result is consistent with our previous study that atherosclerotic lesion were identified more frequently in periodontitis rat model (Susilawati, fdi yogya). Other authors reported that experimental bacteremia (Bordala, Lie) using periodontitis bacteria *P. gingivalis* accelerated atherosclerosis. In the present study, instead of direct bacteremia, systemic inflammation was assumed to be originated from chronic periodontal focal infection (chronic infection was maintained by regular injecting *P. gingivalis* di periodontal tissue). The level of bacteremia and systemic inflammation, however, in this study was not measured.

The deleterious effect of chronic periodontitis in coronary artery could be encountered by means of habitual one cup coffee consumption per day, in which protected against coronary atherosclerosis. Rats that consumed coffee demonstrated more symmetric arterial wall thickness, no atheroma progression, fewer endothelial lipid deposition and fatty emboli. Most importance result was the effect on the morphology of intimal collagen, in coffee group, coronary intimal collagen was intact and significantly denser and thicker compared to control and periodontitis groups. This might explain the resistance of the vasculature against injurious agents from blood stream, so that reduced the response to injury leading to reduce atherosclerosis. This is a new perspective. This is the first report of the beneficial effect of coffee on improving vasculature intimal collagen.
The integrity of vasculature intimal collagen is modulated by the activation of matrix degrading enzymes, matrix metalloproteinases (MMPs). These enzymes are mainly produced by inflammatory cells as inactive zymogen (proMMPs). Some activators such as bacterial proteinases and oxidants/free radicals can be activated proMMPs to become active MMPs by mechanism of proteolysis and cysteine switch, respectively. Considering condition of systemic infection, We speculated that entering of injurious agents into blood circulation could stimulate inflammatory response in vasculature such as production of large amount of proMMPs, oxidants and free radicals, and interaction of these substances reveal active MMPs that degrade vasculature collagen.

Pathologic degradation of vascular collagen is the basis molecular process for atherosclerotic plaque rupture leading to coronary thrombosis and acute coronary syndrome (Shah 2005; Gough, 2006) It was constantinides (1994) who firstly noticed that arterial thrombosis is practically always initiated by local breaks of vascular collagen. When there is vascular collagen breakdown, platelet will encounter and readily to aggregate. Platelet aggregation will be followed by activation of coagulation cascade leading to subsequent thrombus formation. When thrombosis is occlusive and happened on atherosclerotic plaque near by coronary artery it could stop up the blood supply into the heart leading to ischemia and heart attack. Hence, pathologic vascular collagen degradation is a critical event determining the clinical symptom of ACS.

There was speculated that the benefit effect of coffee was due to antioxidant and anti-inflammatory compounds, such as polyphenol, that is rich in coffee [Moreira]. The effect of polyphenol-rich food or polyphenol extracts on development of atherosclerotic lesion had been studied in animal model [mura, Vinson, dufi, manach, bernatova]. Polyphenol improved function of endothelial cell and inhibit platelet aggregation (Vita 2005), decreased oxidation of LDL [de whalley, Natella], improved cardiovascular remodeling and vascular function [bertanova], increased plasma antioxidant carotenoid [svilaas]. In the present study, we speculated that antioxidant and anti-inflammatory compounds in coffee might modulate inflammatory response and enzymes that responsible for collagen degradation, it is matrix metalloproteinases (MMPs).

Matrix metalloproteinases is produced as inactive enzymes (zymogen) proMMPs, their activation depends on any activators such as proteinases and or reactive oxygen species (ROS). Inflammatory response increased production of inflammatory cytokines such as TNF-α, IL-1β that can induce expression of proMMPs. In addition, bacterial infection induced large amount of ROS that could activate proMMP by mechanism of cysteine switch. Therefore interaction components in inflammation could result in MMP activation leading to degrade collagen.

The most significant result of this study was the influence of coffee consumption on the morphology of collagen intimal. It may open a novel perspective study. There is only a few study or literatures that mention the role of coffee in collagen metabolism, particularly vascular collagen. Further studies are needed to elucidate this mechanism effect of coffee in collagen metabolism, in addition, the influence of its structure to cardiovascular function as well. Like as mention above, in addition to caffeine, coffee is a mixture of thousand substances including antioxidants. There is still unclear, whether the effect of coffee on collagen metabolism was mediated by its antioxidant, many works however are still needed to elucidate the role of antioxidants in collagen metabolism.
The clinical significant of coronary artery morphology that composed by intact and dense intimal collagen might provide protective effect against atherosclerosis and rupture. Since atherosclerotic plaque rupture mostly occurred in area of thin collagen fibrous cap [Gouge, 2006]. Atherosclerotic plaque rupture is the proximate event of clinical manifestation of acute coronary syndrome. Therefore it can be stated that coffee might prevent the incidence of acute coronary syndrome by improving the morphology of arterial wall particularly intimal collagen.

Cardiovascular effect of coffee is necessary to continue to be explored. Although some studies revealed the positive cardiovascular effect, many studies however suggested the adverse effect. There is still common perception that caffeine in coffee can cause blood pressure to increase. This is due to the effect of coffee to increase adrenal glands to release more adrenaline, unfavorable effect on endothelial function (Papamitchel 2006, buschemy 2010) In addition to chemical compositions, variety factors could influence the effect of coffee, such as frequency of consumption, type of coffee procescising, brewing method, host factors (e. g. age, gender), etc.

The present study use pure one dose of coffee decocta per day that represent one cup of coffee. Further study are needed to analyze effect of the dosis, since some study indicated that high intake of coffee have negative effect on cardiovascular health. In addition to dosis, coffee serving usually was mixed with other ingredients such as milk and sugar. There still not known whether coffee-nutrient interaction would influence the effect of coffee. Other factors like high lipid diet, alcohol consumption, smoking are also commonly follow the habitual coffee consumption are needed to study as well.

In conclusion, coffee consumption improved morphology of arterial wall leading to protection against coronary atherosclerosis. This protective effect might also explain the resistance mechanism of vasculature against rupture, thrombosis, and it could be meant that coffee consumption could reduce the incidence of acute coronary syndrome. Most interestingly, this study provide evidence that coffee consumption influence the structure of intimal collagen of coronary artery. Further studies will be focused on elucidation of factors that affect its collagen structure and composition.

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