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Syzygium Polyanthum Reduced TNF-α and ADAM17 Protein Expression in Myocardial Infarction Rat Model

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ABSTRACT

Background: In myocardial infarction (MI), inflammatory cytokine including tumor necrosis factor-α (TNF-α) plays pivotal role throughout worsening processes and recovery, whereas its cleavage is controlled by a disintegrin and metalloproteinases-17 (ADAM-17). *Syzygium polyanthum* (Wight) is widely used plant in Indonesia and Southeast Asian countries to treat various illnesses. **Objective:** This study aimed to analyze the effect of *S. polyanthum* extract towards TNF-α and ADAM17 expressions in MI rat model. **Methods:** Myocardial infarction were surgically induced in wistar rats by ligating left anterior descending coronary artery in both treatment and control group. Around 3.6 mg of *S. polyanthum* extract (SPE) was administered via nasogastric tube to treatment groups, while control group received only saline. Serum TNF-α level and expression of TNF-α and ADAM17 in blanching heart muscle was examined in both groups on day 1, day 4, day 7, and day 14 after treatment with SPE. **Results:** Reduction of serum TNF-α levels was markedly seen from day 4 in treatment group and was lower than in control group since day 4. Expression of ADAM17 was reduced and expression of TNF-α was only seen in myocardial membrane in group with SPE treatment. **Conclusion:** *Syzygium polyanthum* exerts its anti-inflammatory effect by decreasing ADAM17 expression subsequently lowering TNF-α regulation in MI rat model.

Keywords: myocardial infarction, Syzygium, TNF-α, metalloproteinases, disintegrins.

1. INTRODUCTION

The prevalence of cardiovascular disease according to National Health and Nutrition Examination Survey (NHANES) in 2013-2016 was 48% and tends to rise along with increasing age. Moreover, cardiovascular events are the leading mortality etiology in the United States (1). Based on the Indonesian basic health research (Riskesdas) in 2018, coronary heart disease (CHD) shared the highest prevalence of cardiovascular disease (2). Acute coronary syndrome (ACS) occurs suddenly due to the lack of blood flow into myocardium accompanied by changes in the ST segment on the ECG and increased level of cardiac biomarkers. It covers the spectrum of unstable angina pectoris (UAP), non-ST-segment elevation myocardial infarction (NSTEMI), and ST-segment elevation myocardial infarction (STEMI) (3).

During myocardial infarction (MI) injury, multiple factors at cell level are involved. Among them is the inflammatory immune responses occur in infarcted myocardium and neighboring tissues. These immune responses are including acute necrosis, hypertrophy, apoptosis of cardiomyocytes, and a subsequent ventricular remodelling. In recent years, studies showed that tumor necrosis factor-α (TNF-α), contributes as a key regulating factor in the inflammatory reaction. It is not only act in combination with its ligand as a mediator in the inflammatory immune response but also works independently in the setting of myocardial repair (4, 5).

As an inflammatory cytokine, TNF-α is reported to be increased in heart failure and associated with mortality. The signaling pathways induced by TNF-α play pivotal role in cellular responses to inflammation and injury. In the cardiovascular system, the activated TNF-α signal transduction pathway contributes to blood vessel dysfunction, development and progression of atherosclerosis, and adverse post-MI cardiac remodelling (6). TNF-α exerts its function via two different type I transmembrane receptors, TNFR1 and TNFR2. Both TNFRs can interact with either two forms of TNF-α in the body: membrane-associated TNF-α (mTNF-α) and soluble TNF-α (sTNF-α) (7,8). Soluble TNF-α is generally considered the active form of mTNF-α;

whereas this activation is facilitated by TNF-α converting enzyme (TACE) also known as ADAM17 (a disintegrin and metalloproteinases-17) (5).

A disintegrin and metalloproteinases-17 releases soluble TNF-α, and acts by cleaving within the extracellular domain of membrane-bound pro-TNF-α, leading to increasing of proinflammatory TNF-α secretion (9, 10). ADAM17 expression has been involved in several inflammation-related diseases including rheumatoid arthritis, psoriasis, pulmonary inflammation, multiple sclerosis, breast cancer, lung cancer, renal cancer and cardiovascular diseases (9, 11). In experimental study reported by Satoh *et al.*, patients with MI also showed increased expression of ADAM17 in areas of ruptured coronary plaques (12).

Syzygium polyanthum (wight), a plant belongs to *Myrtaceae* family, is well-known among Indonesian as bay leaf or *"salam"*. This plant is widely distributed throughout Southeast Asian countries including Myanmar, Thailand, Malaysia, Singapore and Indonesia. Moreover, it is one of the ethnomedicinal plants that is seemingly gaining attention for its various pharmacological potentials treating various illnesses such as diabetes mellitus, hypertension, gastritis, ulcers, diarrhea, skin diseases as well infections (13, 14). Phytochemical screening showed that its leaves contained essential oils, tannins, flavonoids, terpenoids and fatty acids (13). In this study, we investigated *S. polyanthum* effect regarding inflammatory processes in MI rat model.

2. OBJECTIVE

This study aimed to analyze the effect of *S. polyanthum* extract towards TNF-α and ADAM17 expressions in MI rat model.

3. METHODS

3.1 Sample and Study Design

This was an experimental in vivo study using post test with control group design, conducted in December 2019. All the experiments used in the given study were approved by Institutional Ethical Committee, Faculty of Medicine, Universitas Sumatera Utara, Indonesia. A total of 32 healthy 3-month-old male wistar rats (300 g) were obtained from the Faculty of Medicine, Brawijaya University, Indonesia. All the animals were maintained under the standard condition and protocol given as per the ethical guidelines.

The method chosen for achieving the MI was direct ligation of left anterior descending (LAD) coronary artery by transthoracic surgical approach. Anesthesia was induced by an intra-peritoneal injection of phenobarbital (40 mg/kg) and ceftriaxone was given intramuscularly as for the infection prophylaxis.

The ligation was deemed successful when left ventricular anterior wall turned pale or have visible blanching. After the procedure, the chest wound was sutured and the rats were allowed to recover.

The animals were divided into two groups comprise control group which received only saline and treatment group which received 3,6 mg/rat of *S. polyanthum* extract (SPE) via nasogastric tube. On day 1, day 4, day 7 and day 14 after LAD artery ligation, four animals in both groups were scarified under anesthesia by ketamine. Transcardial blood puncture was done and the hearts were removed. Blood sample was taken directly from the heart using 3 ml syringes then centrifuged. Serum was separated and stored in -20°C until further used.

3.2 Collection of *S. polyanthum* Extract

Syzygium polyanthum (Wight) leaves were collected from Medan, Indonesia and identified at the Faculty of Medicine, Universitas Sumatera Utara, Indonesia. The dried leaves were powdered using a milling machine. The powder was sequentially extracted by maceration and prepared in 0,5% sodium methyl cellulose suspension. The extract was kept in the freezer (-20°C) until further use in the designated experiments.

3.3 ELISA

Serum TNF-α level was measured by ELISA (Mouse TNF-α Elabscience ELISA Kit) according to the manufacturer's instruction. Briefly, 100 uL of assay buffer solution was added to standard protein and serum samples, incubated, and washed afterwards.

To avoid unspecific binding, blocking was done using blocking buffer (1% BSA + 0.02 gr NaN3 in PBS, pH 7.0). After that 100uL (2ug/mL) of anti TNF-α in buffer blocking was added, incubated, and washed. Finally, 100 ml antibody labeled HRP (horse radish peroxidase) was added, followed by substrate addition. Optical density results were read by ELISA reader at 450 nm of wavelength.

3.4 Immunohistochemical Analysis

The blanched area of myocardial infarcted heart tissue was separated and fixed using 10% formalin buffer for 24 hours. The following actions were dehydration, clearing, impregnation, and blocking. Tissue was made of 6 μm thickness and immunohistochemistry was performed to see the expression of ADAM17 (anti-ADAM17, Santa Cruz Biotechnology, United States) and TNF-α (anti-TNF-α, Santa Cruz Biotechnology, United States). Samples were washed with PBS pH 7.4 and immunohistochemical staining was carried out according to the kit's instruction (novoLink novocastra, Leica paint # RE7150-CE).

Resulted slides were observed using a Nikon E100 microscope. The measurements of each parameter (expression of ADAM17 and TNF-α) used a calculation technique of 20 fields with a magnification of 1000×, each containing approximately 1500 cells. The images were documented with a 400× magnification by using Panasonic GX-8 camera.

3.5 Statistical Analysis

All data were expressed as mean ± standard deviation (SD). The difference of serum TNF-α level each group were analyzed by one-way ANOVA and Tukey post hoc study. Independent t-test was used to determine difference of TNF-α between groups on the same day. In this study, values $p < 0.05$ was considered as significant.
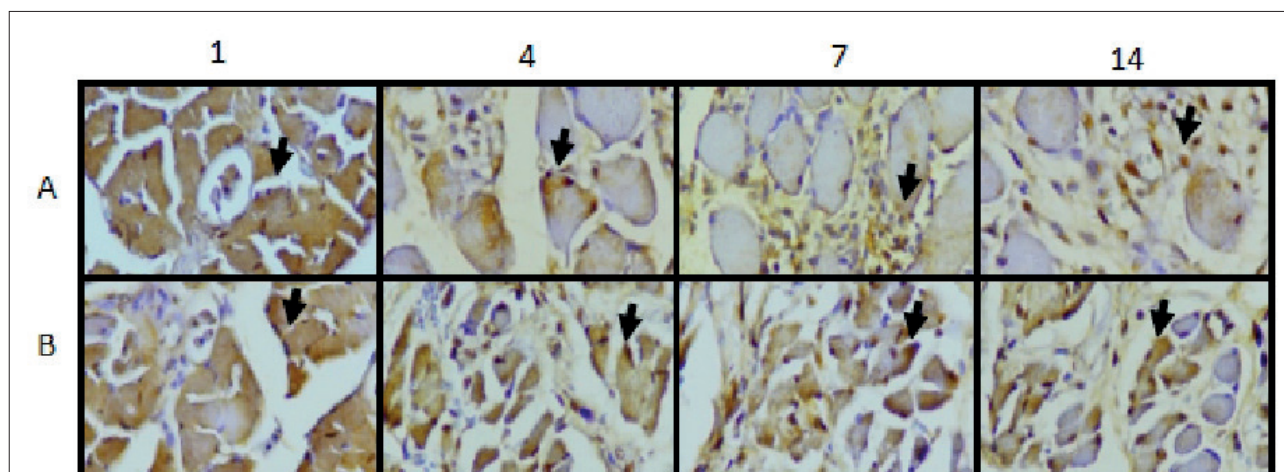
Figure 1. Representative photographs of immunohistochemical analysis using anti-TNF- antibody in MI rat model (400×). Incubation time of days 1 (1), 4 (4), 7 (7) and 14 (14) were showed for control groups (A) and S. polyanthum extract treatment groups (B). TNF- tends to be highly expressed in myocardial membrane in SPE treatment groups compared to control groups. Arrow sign indicates TNF- expression (brown spot). (TNF-tumor necrosis factor)
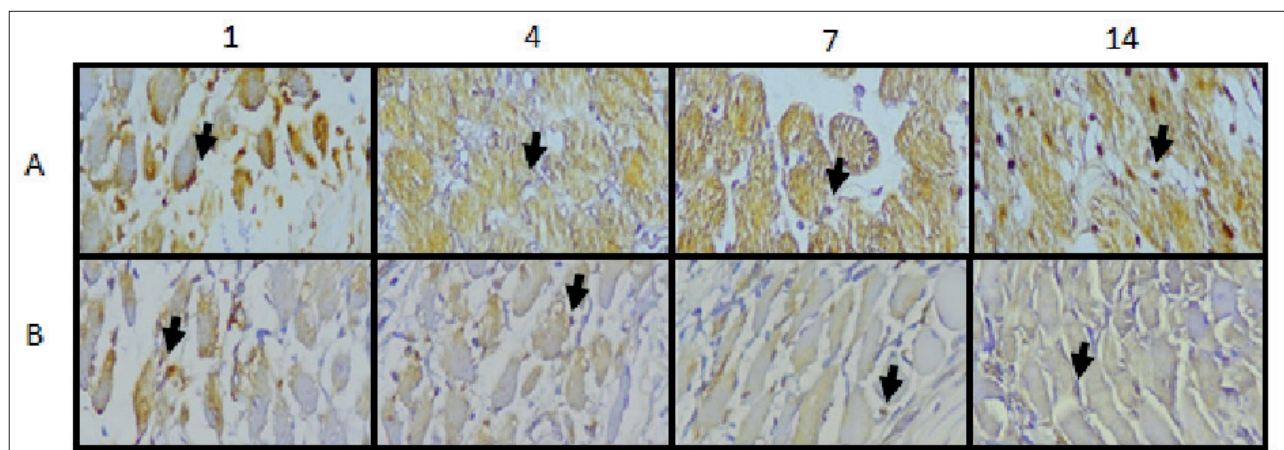

Figure 2. Histological myocardial infarcted heart sections stained with immunohistochemical using anti-ADAM17 antibody (400×). Incubation time of days 1 (1), 4 (4), 7 (7) and 14 (14) were showed for control groups (A) and S. polyanthum extract treatment groups (B). Notable high expression of ADAM17 were shown in control groups but not in SPE groups. Arrow sign indicates ADAM17 expression (brown spot). (ADAM17- a disintegrin and metalloproteinases-17)

4. RESULT

4.1 Serum TNF-α Level in MI Rat Model

Table 1 depicts the effect of SPE on serum TNF-α level. In control group, TNF-α level was decreasing gradually since day one, reaching significant reduction after day 7 after myocardial infarction (day 7 vs day 1, p<0.05). Treatment of SPE swiftly decreased TNF-α level since day 4 (day 4 vs day 1, p<0.001). When compared between groups, TNF-α level was lower in SPE group than control group since day 4 after SPE administration.

4.2 Immunohistochemistry of TNF-α and ADAM17 in Infarcted Myocardial Heart Tissue

Expression of TNF-α and ADAM17 had different pattern in our study. TNF-α seemed to predominantly expressed at immunocompetent cells at day 7 and 14. On the contrary, different results was found in SPE treatment groups, which showed that TNF-α was predominantly expressed at myocardial membrane (Figure 2). Immunohistochemical analysis of ADAM17 showed that ADAM17 was upregulated in control groups in accordance with incubation period (day 1 to day 14). On the other hand, ADAM17 was downregulated in SPE treatment groups (Figure 3).

Day	control group (pg/ml)	SPE group (pg/ml)	p
1	980.1 ± 133.36	958.2 ± 90.21	0.795
4	788.24 ± 123.16	574.32 ± 81.4ᵃ*	0.027
7	688.67 ± 61.62 ᵇ†	498.07 ± 67.04ᵇ*	0.006
14	539.21 ± 22.42 ᶜ*,ᵈ†	404.41 ± 33.25e ᶜ*	0.001

Table 1. TNF-α level in control and treatment group. ᵃday 4 vs day 1, ᵇ day 7 vs day 1, ᶜday 14 vs day 1, ᵈday 14 vs day 4, *p < 0.001, †p < 0.05. SPE – Syzygium polyanthum

5. DISCUSSION

In the steady state, tissue resident macrophages exert homeostatic functions, including defending against infection and removing senescent or damaged cells. Moreover, macrophages exhibit distinct organ and tissue-specific physiological functions. In the heart, macrophages have an indispensable role in response to injury, including MI (15). Several studies showed that macrophages were upregulated in blanched area of myocardial infarcted heart (16, 17). Macrophage shows a pro-inflammatory M1 phenotype (classically activated) in early MI, while an anti-inflammatory M2 phenotype (alternative-

ly activated) shows up later. These phenotypes are distinct and have opposite roles (15, 18). Pro-inflammatory agent produced by macrophage is mainly TNF-α (19). Thus, measurement of TNF-α could be used to detect inflammation after MI (12).

In this study, we investigated TNF-α to determine the ability of *S. polyanthum* to ameliorate heart inflammation after MI. Treatment with SPE after MI strongly associated with reduction of serum TNF-α level since day 1 and reaching statistically significant on day 4. Systemic TNF-α was known to be elevated in the first few days after MI. Kempf *et al.,* found elevated serum TNF-α on day 1 post myocardial infarction, even after intervention (20). Elevation of TNF-α in the first few days post MI indicates inflammation, and its level must decrease thereafter (21). Prolonged elevation of serum TNF-α was associated with several complication such as severe MI, congestive heart failure, cardiogenic shock, or reperfusion injury after recanalization (20, 22, 23). Result in our study shows the potential of *S. polyanthum* in reducing heart inflammation after MI.

As outlined before, ADAM17 impacts the biology of TNF-α (24). TACE/ADAM17 has been identified by its ability to cleave (major shedding) TNF-α (10,25). Given the importance of function, our study also observed the expressions of ADAM17 along with TNF-α in infarcted myocardial tissue. There was marked reduction of ADAM17 expression after treatment with SPE. As for TNF-α, expression was likely to be more predominant in myocardial membrane in SPE treatment group rather than immunocompetent cells in control groups. Previous study suggested that ADAM17 expression was increased in infarcted myocardium (26). Its high expression promotes pro-inflammatory events in infarcted myocardium and subsequently followed by an increased TNF-α expression (27). In concordance with previous study, expression of TNF-α in control group was shown in immunocompetent cells of infarcted area indicating inflammation in myocardial tissue. Additionally, in this study, treatment with SPE reduced inflammation as characterized by decreased ADAM17 expression in cardiomyocytes and increased TNF-α expression only in myocardial membrane. In murine experimental study, it was showed that membrane-bound TNF-α has anti-inflammatory properties, which led to the hypothesis that membrane-bound TNF-α is anti-inflammatory and that cleavage of TNF-α by ADAM17 activity was a prerequisite for pro-inflammatory TNF-α activity (24, 28, 29). Taken together, this finding strongly suggests *S. polyanthum* could be used for future anti-inflammation drug.

Leaves, fruits, and barks of *S. polyanthum* are traditionally used for various medicinal and nonmedicinal purposes carried out by people in Southeast Asia, including Indonesia. The roots and the fruits are consumed to reverse the hangover effect with alcohol, whereas the leaves are traditionally consumed for treating various illnesses (13, 14). Phytochemical studies revealed that the leaves and fruits of *S. polyanthum* contain vitamin C and flavonoids, which may have anti-inflammatory activity (14). Although we did not examine the exact composi-

tion of *S. polyanthum* extract, our study suggests that *S. polyanthum* could be used in terms of anti-inflammatory effect following MI. Isolation of active agent compound, especially flavonoid, in *S. polyanthum* is needed to obtain better understanding into its role in inflammation and heart disease.

6. CONCLUSION

In conclusion, *S. polyanthum* extract might have anti-inflammatory effect in myocardial infarction condition. The effect is probably exerted via downregulation of ADAM17 subsequently affecting TNF-α regulation.

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