Cardiac Fibrosis Attenuation by Chlorogenic Acid and Epigallocatechin-Gallate Mediated by Suppression of Galectin-3 Gene Expression and Collagen Deposition in Rat Metabolic Syndrome Model

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Abstract

Objective: Metabolic syndrome (MetS) is a set cluster of risk factors for metabolic abnormalities that can develop cardiovascular disease (CVD), one of that is remodeling or cardiac fibrosis. Cardiac fibrosis identified from the high levels of the profibrotic molecule; one of them is galectin-3. **Aim:** This study intended to determine the effect of combination therapy of green coffee (CGA) and green tea (EGCG) on cardiac fibrosis by raise of the galectin-3 gene expression and collagen deposition in rat cardiac tissue in the rat metabolic syndrome model. **Methods:** Twenty-four male MetS rats (Sprague-Dawley) divided into two control groups and three groups therapy (n=5) administration of the CGA 200mg/kgbw (body weight in kilograms) and EGCG 300mg/kgbw orally. After eight weeks of treatment, rats euthanized, then mRNA expression of galectin-3 was measured. Furthermore, collagen deposition of cardiac tissue carried out in histology slides. **Results:** Research reveals that the expression of galectin-3 decreased in the metabolic syndrome model group, which given combination therapy compared with metabolic syndrome model mice that did not receive any therapy (P=0.000). Collagen deposition in cardiac tissue also found less than in the therapy group compared with the group not treated with both compounds (P=0.000). The correlation between the two parameters shows a positive association with low strength. **Conclusion:** This study shows that the combination therapy of CGA and EGCG is an engaging therapeutic candidate. It expected to reduce the progression of cardiac fibrosis in metabolic syndrome.

Keywords: *metabolic syndrome, chlorogenic acid, collagen deposition, epigalocathecin-gallate, galectin-3.*

Introduction

Metabolic syndrome (MetS) is a cluster of risk factors for metabolic abnormalities that can lead to many organs' dysfunction to death. Metabolic syndrome is defined based on several pathophysiologies: insulin resistance, central obesity, hypertension, hyperglycemia, and triglyceride dyslipidemia [(1)]. The number of metabolic syndrome events continues to increase from year to year. The prevalence of the metabolic syndrome is estimated to have reached more than 20% of the adult population worldwide [(2,3)]. Indonesian Ministry of Health (2013) reported the prevalence of metabolic syndrome in Indonesia reached 23%, with the proportion of adult obesity increasing to 21.8%, hypertension to 34.1%, and

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an increase in the prevalence of type 2 diabetes up to 8.5%

Obesity and insulin resistance, along with other risk factors in metabolic syndrome, also play a role in the development of cardiovascular disease. One of the cardiovascular diseases found in metabolic syndrome sufferers is a change in the histological structure of the heart or known as cardiac remodeling. Remodeling of the heart can be a direct effect of an increase in adipocyte cells or due to the risk of hypertension that is an agonist with obesity, resulting in excess pressure on the heart. Cardiac remodeling arises from an increase in extracellular matrix deposition, referred to as fibrosis[4]. Increased oxidative stress in response to fluctuations in glucose in blood plasma, can activate an inflammatory pathway that is significantly involved in the pathogenesis of reactive fibrosis that leads to the accumulation (deposition) of type 1 collagen in cardiomyocyte tissue [5]. An increase in collagen deposition, which is not compensated by its degradation, limits contractility and relaxation of cardiac cells and inhibits electrical conductivity and regional nutritional diffusion, leads to a cause cardiac dysfunction [6].

Cardiac fibrosis can be identified early from the high levels of profibrotic molecules; one of them is galectin-3. Galectin-3 expression consistently found in fibrosis models. Galectin-3 is a molecule consisting of an N-terminal domain, 100-150 amino acids, with a C-terminal domain consisting of about 135 amino acids, containing one carbohydrate recognition domain (CRD). Galectin-3 is formed in pentamer formation using ligands that go through the N-terminal domain. On the cell surface, galectin-3 plays a role in cell-cell interaction, and between cells with extracellular matrix (ECM), activation of growth factor receptors, signal transduction, and plays a role in the formation of a glycoprotein-galectin lattice [7].

Metabolic syndrome therapy has long focused on the individual risk factor, for example with metformin therapy in patients with insulin and hypertension resistance, and some anti-hypertensive agents, such as angiotensin II converting enzyme inhibitors (ACEi), or angiotensin II receptor antagonists [8,9]. However, in the last few decades, metabolic syndrome therapy through dietary interventions provides a reasonably good prospect of improving the condition of sufferers of the MetS. Functional food studies are quite interesting because medicines extracted from plants contain many essential compounds such as polyphenols, flavonoids, and terpenoids.

Green tea is one of the most frequently consumed drinks globally, occupying the highest production, about 20% of total global tea production. Epigallocatechin-3-gallate (EGCG) in green tea considered a significant contributor to various health benefits [10]. Previous studies revealed an increase in adiponectin and its receptor after administering green tea extract at a dose of 300-400 mg/kg bb for nine weeks[11]. Also, they increased PPARα and PPARγ expression in rat metabolic syndrome models[12]. Another popular beverage that is consumed by most people in the world is coffee. Green coffee beans are rich in chlorogenic acid, compared to other sources such as fruit and vegetables [13,14]. The administration of green coffee extract for seven weeks is known to reduce fasting blood glucose, lipid profile, blood pressure, increase adiponectin and HOMA-IR index in rat metabolic syndrome models[15].

However, the involvement of green coffee and green tea as a therapeutic agent for cardiac fibrosis in the metabolic syndrome remains unclear and needs further investigation. Therefore, this study will examine the effect of combination therapy of green coffee and green tea on cardiac fibrosis. It is characterized by increased expression of the gene galectin-3 and collagen deposition in rat cardiac organs in the metabolic syndrome model.

Methods

Research Design

The study was a true experimental design with a post-test only control group design with a simple random sampling technique. Sprague-Dawley rats aged 8-12 weeks, weighing 250-300gram acclimatized for seven days, which further divided into five groups, the negative control group was healthy mice (n=5) fed with commercial pellet feed. The positive control group (n = 5) were mice with metabolic syndrome through the high-fat diet (HFD) consist of powdered mouse pellets, 20% sucrose, 0.5% methionine, 2.5 salt, 2% MSG, 15% egg yolk, and 20% white fat) treatment for 14 days. Streptozotocin (STZ) injection 30 mg/kg body weight

(BW) given on the 15th day. Then, HFD continued until the end of the study [16]. Group mice must meet the criteria for the rat metabolic syndrome model, which is an increase in blood glucose levels >126 mg/dL, HDL levels <40 mg/dL, and triglyceride levels >150 mg/dL. Treatment group 1 was mouse metabolic syndrome model (n=5) who were given 200 mg/kg body weight green coffee extract (CGA) therapy for seven weeks via oral gavage. Treatment group 2 was metabolic syndrome model mice (n=5) which received green tea extract (EGCG) therapy with a dose of 200 mg/kgbw via oral gavage. Treatment group 3 (Combination) was a metabolic syndrome mouse model which (n=4) given therapy using a CGA combination of 200 mg/kgbw and EGCG 300 mg/kgbw by oral gavage.

Green Tea and Green Coffee Extraction Process

Coffea canephora robusta beans are roasted with an automatic coffee roasting machine at 180-200° C for 6-8 hours. Furthermore, the coffee beans were macerated with 95% ethanol to produce a crude extract. The crude extract is filtered using a filter cloth to separate the liquid phase from the solid phase. Besides, the liquid phase concentrated using a rotary evaporator at ±40°C. Green tea extracted from light green tea leaves. Green tea leaves 500 grams are dried using a drying cabinet (50ºC) for 8 hours to get green tea with a moisture 8-10%. Green tea blended and boiled at 80°C for 30 minutes. Then, the crude extract is filtered to separate the liquid phase from the solid phase—the concentrated liquid phase prepared using a rotary evaporator at ± 40 °C.

Dosage Determination

The dosage of administration of green coffee extract and green tea is determined based on preliminary studies. The optimal dose is 200 mg/kgbw for green coffee extract and 300 mg/kg BW for green tea extract.[12,15] Daily food intake and fluid intake are measured every day, and body weight is measured every week—fluid intake given *ad libitum*.

Blood Pressure Measurement and Biochemical Examination of Blood

Blood pressure was measured using the tail-cuff method with the sphygmomanometer at the beginning and the end of the experiment as systolic blood pressure (SBP). Serum concentrations of fasting glucose, triglycerides (TG), and HDL were measured enzymatically (Biolabo, France). The sample measured using a spectrophotometer.

Measurement of Galectin-3 Gene Expression

Total RNA of heart tissue isolated using Easy Blue (Intron Biotechnology) following the manufacture's protocol. Reverse transcription reactions carried out using the ReverTra Ace-α kit (Toyobo, Japan). Then the level of RNA expression was performed using the LightCycler 96 PCR system (Takara, Japan) using the GoTaq Green Master PCR Kit (Promega, Madison, USA) following the manufacture's protocol. The primary sequence is as follows: B-actin, forward: 5'- TGA GAG AAT CGT GCG TGA CAT-3′ and reverse: 5'-ACC GCT CAT TGC CGA TGA TGA TGA-3′; galectin-3 (LGALS3) forward:5′-GGCCACTGATTGTGCCTTAT-3′; and reverse: 5′-TCTTTCTTCCCTTCCCCAGT-3′. The PCR cycle is as follows: 5 minutes at 95°C pre-denaturation; 35 cycles of 30 seconds at denaturation of 95°C, 30 seconds of annealing at 57.1°C, followed by extensions for 30 seconds at 72 °C; and final extension for 10 minutes at 72 ° C. The mRNA level of the target gene normalized to the level of b-actin expression.

Histology Examination of Cardiac Tissue

The heart organ is put into a cassette, soaked in a level of ethanol solution that is 70% to 100% each for 60 minutes at room temperature. Cassette was removed, clearing using xylol for 15 minutes at room temperature three times. Infiltration with liquid paraffin for three times each transfer 60 minutes in an incubator temperature of 60 ºC, then removed to get a paraffin block. The paraffin block sliced 12 mm thick horizontally using a rotary microtome.

Analysis Data

Data obtained tabulated descriptively in mean (± standard deviation) for galectin-3 gene expression from each group. Data obtained from histological observations of cardiac collagen deposition by field of view, taken on average from five fields of view for each mouse model in each group treatment. The normality and homogeneity of the data tested using the Shapiro-Wilk test and the Levene test, followed by the Kruskal Walis test. The

confidence interval set to 95% (P = 0.05). Correlation of galectin-3 and the average area of collagen deposition from each group using Spearman's-rho correlation test.

Ethical Clearance: This experimental design has fulfilled and approved by the Ethics Committee of Faculty of Medicine, Brawijaya University, Malang, Indonesia, by registered number: 405/EC/KEPK/2020.

Result

Galectin-3 gene expression

The galectin-3 gene expression is relative to β-actin presented in Figure 1. It is indicating that there are differences in galectin-3 gene expression in each group. The negative control group (Normal) had the lowest gene expression, with an average of 0.2 ± 0.047. The positive control group (MetS) had the highest expression of the galectin-3 gene, among other models, which was 1.02 ± 0.228. The MetS + CGA group had an average of galectin-3 expression 0.94 ± 0.208, which was the highest value among the groups given the intervention. The MetS + EGCG treatment group, was known to affect the expression of galectin-3, which had the lowest average of all groups given therapy, 0.67 ± 0.124. The third treatment group (MetS+ CGA+ EGCG) produced an average expression of 0.70 ± 0.2901, which was the lowest mean after the second treatment group in the five groups used in this study. Based on Kruskall-Wallis analysis, showed the value of 19.251, obtained a statistical table value of 9.488 (df = 4; 5%). So that, there is an effect of giving a combination of CGA and EGCG to galectin-3 expression. Based on the independent T-test analysis compared with MetS group, all treatment groups produced significantly different levels of galectin-3 with a P-value <0.05 (Table 1).

Collagen deposition in heart tissue

Collagen deposition examination is a further histological procedure after collecting rat heart organs of each group (Figure 2). Data is converted into semi-quantitative data (%) in the percentage of collagen deposition compared to normal cardiomyocyte cells. The normal group had the lowest percentage of collagen deposition compared to other groups (1.8% ± 0.447). The highest collagen deposition was found in the MetS group, 8.52% ± 2.261. Meanwhile, the MetS + CGA group have a percentage of collagen deposition of 3.41% ±0.67, the MetS + EGCG treatment group with a percentage of 2.33% ± 0.095, and the combination therapy group with a percentage of 2.86 % ±0.58. Based on the Kruskal-Wallis analysis, obta11,175. This value, when compared to obtained a statistical table 9,488 (df=4; 5%), there is an effect of giving a combination of CGA and EGCG to collagen deposition. Compared with the negative control group, only MetS, MetS + EGCG, and combination treatment groups produced significantly different collagen deposition with a P-value <0.05 (Table 1).

Correlation between Galectin-3 gene expression and collagen deposition in cardiac tissue

The correlation test on the two dependent variables determines the closeness of the relationship between the two variables (Table 2). The results of correlation analysis between galectin-3 gene expression and collagen deposition for five treatment groups, obtained positive correlation (Sig.2-tailed; P-value> 0.05). The correlation coefficient is positive, indicating that the relationship between the two variables is unidirectional. It suggests that the higher the expression of the galectin-3 gene, the more collagen deposition in cardiomyocyte tissue.

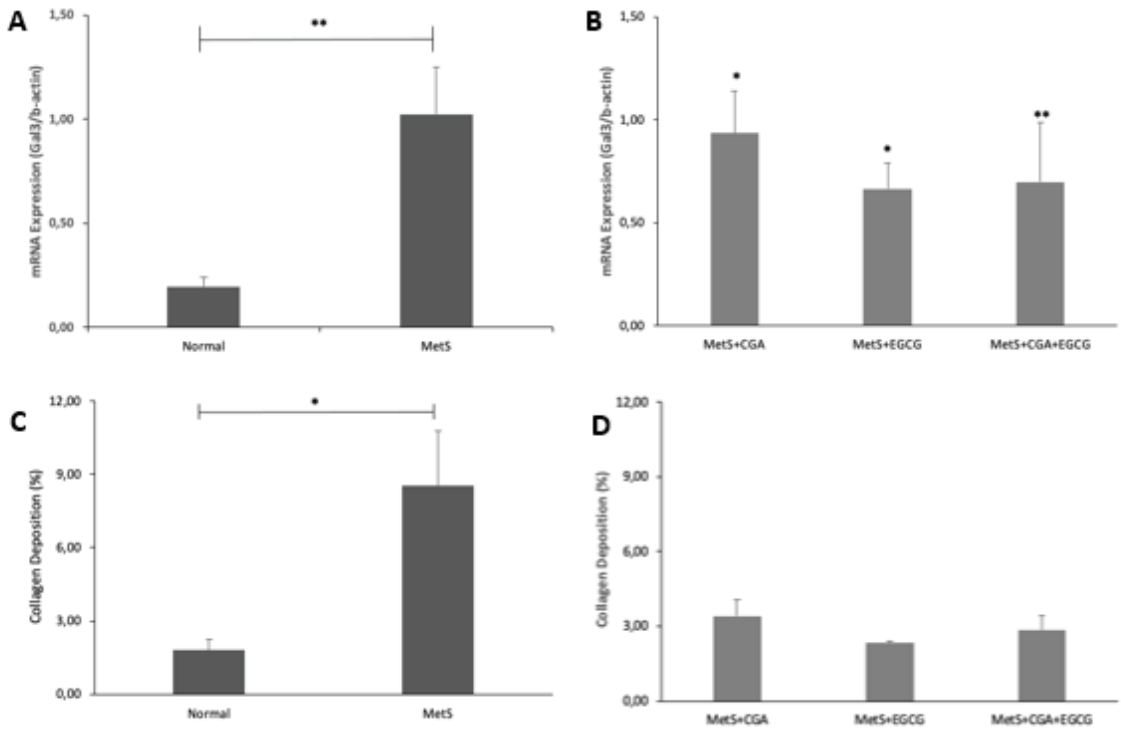

Figure 1. (A) Galectin-3 gene expression in (A) control groups and (B) treatment groups. Collagen Deposition percentage in cardiac tissue of in (C) control groups and (D) treatment groups.

Independent T-test		Sig. (2-tailed)	
		Galectin-3	Collagen Deposition
MetS	Normal	0.000**	0.045*
	MetS+CGA	0.003*	0,137
	MetS+EGCG	0.013*	0.088
	MetS+CGA+EGCG	0.000**	0,442

Table 1. p-value of Independent T-test analysis

*Significant for P-value <0.005; ** P-value=0.000

Discussion

Fibrosis is a tissue disorder that can be found in several vital human organs and is the most significant risk factor as a cause of organ dysfunction. Fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) components that disrupt the function of a cardiac tissue, that composed by cardiomyocytes [17,18]. Besides, fibrosis can also be known based on protein levels or increased gene expression in the pathogenesis of cardiac fibrosis. One of the essential proteins that are pro-fibrotic mediator is galectin-3. Cardiomyocyte almost does not express galectin-3 under normal physiological conditions [19]. But in certain pathological conditions, cardiomyocytes can also act as alternative sources of galectin-3 which induces fibroblasts to synthesize collagen and other pro-fibrotic components

from the extracellular matrix [20,21].

In this study, researchers used a metabolic syndrome model that was given CGA therapy of 200 mg/kgbw and/or EGCG 300 mg/kgbw to investigate the potential of the two compounds in inhibiting galectin-3 overexpression. The treatment group with CGA therapy 200 mg/kgbb was known give effect on decreasing galectin-3 expression in cardiomyocyte tissue (P=0.000), compared with the group given EGCG therapy of 300 mg/kgbw alone. Meanwhile, the combination group of the two therapies had a good effect, not much different from the group given EGCG therapy of 300 mg/kgbw only. Although the lowest expression was obtained in the EGCG 300 mg/kgbw therapy group only, the combination of CGA 200 mg/kgbw and EGCG 300 mg/kgbw also suppressed galectin-3 expression (P=0.000). So, CGA and EGCG expected to reduce the progression of cardiac fibrosis. Raised galectin-3 expression in the metabolic syndrome model group, has the potential to make it possible that even in models that do not have cardiac fibrosis, the galectin-3 expression is a mediator that plays a role in the mechanism of diabetic cardiomyopathy. The correlation analysis between galectin-3 gene expression and collagen deposition for five treatment groups obtained a positive correlation coefficient, i.e. the higher the expression of the galectin-3 gene, it is possible to increase collagen deposition in cardiomyocyte tissue.

Other studies suggest that the use of EGCG as a supplement can significantly reduce systemic galectin-3 levels in wildtype mice and overexpress dual-specificity tyrosine phosphorylation-related kinase 1A (Dyrk1A) mice. It also followed by a decrease in the expression of collagen-1 and collagen-3, which are the main biomarkers for fibrosis heart[22]. Based on other studies, the administration of EGCG therapy to aged rats was able to provide a significant effect on increasing cardiomyocyte diameter and volume with a decrease in numerical density [23]. Oral EGCG therapy for one month after diabetes induction proved significantly inhibits over-expression of serum levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) as well as ICAM-1 and VCAM-1[24]. So overall, EGCG is showing anti-inflammatory potential to inhibit cardiac hypertrophy, fibrosis, and apoptosis that are triggered by ageing.

Research conducted by Tian et al. showed that CGA could repair the cellular injury caused by TNF-α[25]. Later, it has implications for increased cell viability, increased mitochondrial membrane potential, and inhibited cardiomyocyte apoptosis in mouse models of heart failure induced by transverse aortic constriction (TAC). Research on myocardial infarct (MI) mice reports that in the group given CGA therapy 30 mg/kg BW there was a significant increase in contractions compared to the MI vehicle group. Day-14 of the study showed an increase in macrophage infiltration in the MI vehicle group. This result shows the role of CGA in inhibiting fibrotic pathogenesis which is characterized by macrophage infiltration, without giving systemic side effects [26]. The fibrosis signalling pathway is a complicated mechanism. However, TGF-β1/α-smooth muscle actin (SMA)/ Collagen-1 pro-fibrotic pathway has widely used to explain the induction of cardiac hypertrophy and fibrosis in models or patients with heart failure [27]. TGF-β1 is known to bind to receptors in fibroblasts and myocytes, thereby activating the Smad 2/3/4 complex to facilitate nuclear translocation, and initiate the synthesis and secretion of pro-fibrotic cytokines, such as pro-collagen and α-SMA, which can induce fibroblasts to differentiate into myofibroblasts [28]. Galectin-3 is known to act by maintaining or increasing TGF-β receptor binding on the myofibroblast cell membrane, thereby promoting pro-fibrotic signalling through the Smad and PKB / Akt pathways and stimulating the production of the extracellular matrix (ECM) [29].

Based on this research, EGCG and CGA are synergistic in inhibiting cardiac fibrosis which is one of the complications due to diabetes, as seen from suppression of cardiomyocyte galectin-3 levels. This synergy can be caused by the two molecules being antioxidants that work to suppress inflammation. EGCG and CGA, which are a group of polyphenols, are known to be able to inhibit oxidative stress which is its central role as an antioxidant. Although the whole mechanism not widely understood, the anti-inflammatory effect given by the two components is quite good.

The difference between the two pathways in inhibiting fibrosis; EGCG that tends to be an anti-inflammatory agent, inhibiting the overexpression of cytokines and infiltration of inflammatory cells. Meanwhile, CGA is known to be quite useful in inhibiting

the pathogenesis of fibrosis through the TGFb pathway, so that it can reduce the expression of collagen 1 and 3, which expression has not measured in this study.

Conclusion

Therapy using a combination of chlorogenic acid (CGA) green coffee extract and epigallocatechin-gallate (EGCG) from green tea extract orally on animal models of metabolic syndrome is known to reduce the expression of galectin-3 gene percentage in collagen deposition in heart tissue. This study shows that the administration of combination therapy CGA and EGCG is an excellent therapeutic candidate, so that is supposed to reduce the progression of cardiac fibrosis in metabolic syndrome.

Declaration of Conflict: Author declares there is no conflict interest

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