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Green Tea and Green Coffee Therapy for Aortic Calcification Prevention in Metabolic Syndrome Model Rats: Effects on Expression of AKT, mTOR, RUNX2, and Osteopontin Levels

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Abstract

Metabolic syndrome (METS) consists of several independent risk factors for cardiovascular disease (CVD), one of which is vascular calcification (VC). Increased oxidative stress is essential in the pathogenesis of CVD in METS. One of the pathways involved in the pathogenesis of VC is the AKT pathway. Green tea and green coffee have many health benefits, including treating METS risk factors. Although several benefits of green tea and green coffee are known, there is not much information regarding the effects of these 2 extracts for treating heart disease, which is often found in METS sufferers. This research explores the benefits of green tea and green coffee extracts in preventing VC in METS through the AKT/MTOR mechanism. This research focuses on the mechanism of CVD in the AKT-mTOR, RUNX activity, and osteopontin (OPN) expression as one of the downstream pathogenesis of CVD in METS. The research was conducted on METS model rat treated with metformin and green tea with green coffee extracts. Male Sprague-Dawley rats were given a diet high in fat and sugar until they met METS for 4 months, then given treatment for 9 weeks. After 9 weeks, the rat had their aortas isolated for staining with specific antibodies to AKT1, MTOR, RUNX2, and serum OPN levels were measured using ELISA-sandwich methods. There was an increase in the expression of AKT1 and MTOR and a decrease in RUNX2 and OPN2, which was better than rat not treated with green tea and green coffee ($p < 0.05$). A significant reduction was found in the green tea and green coffee therapy group, which was better than when given metformin alone ($p < 0.05$). The results of this research showed that there is good potential for therapy using green tea and green coffee extracts to prevent VC in metabolic syndrome models.

Keywords: Cardiovascular disease, Green tea, Green coffee, Metabolic syndrome, Vascular calcification

Introduction

Metabolic syndrome (METS) is a group of risk factors that can cause organ dysfunction and even death. According to the 2005 National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III), risk factors that characterize METS include hyperglycemia/insulin resistance, hypertension, obesity, and triglyceride dyslipidemia. A diagnosis of METS is made when a person has 3 or more of these risk factors [1-3]. Other factors such as age, gender, and postmenopausal hormone also influence the development of METS [4]. The prevalence of METS increases as these risk factors increase, reaching more than 20 - 25 % in the adult population and 19.2 % in children worldwide [5]. In Indonesia, the prevalence of METS in 2019 was reported to be 21.66 %, with central obesity, hypertension and low HDL levels as the most common risk factors. Each METS component is an independent risk factor for cardiovascular diseases such as microvascular dysfunction, atherosclerosis, and aortic calcification, which can lead to heart failure and death [6-9]. Therefore, it is urgent to conduct research regarding the mechanisms of VC in METS, and pathways that may be observed as targets for preventing VC in METS.

Several molecular mechanisms mediate the occurrence of VC in METS conditions. This mechanism is known to be involved in the worsening of VC in METS, and has also been suggested as a target in the treatment of VC [10-12]. One of these mechanisms is cell metabolism via the AKT pathway. Because activation of AKT is necessary to induce other oxidative stress-induced pathways [13], and activated AKT is sufficient to cause vascular calcification [14,15]. At the mechanistic level, when mutations are made in the AKT T430/T479 amino acids in such a way, it is known that no posttranslational modification by O-GlcNAcylation occurs. This condition causes AKT phosphorylation at S473 and cellular calcium content to decrease to control levels. Additionally, binding of AKT to the mTOR complex 2 is known to result in reduced expression due to the mutation. Based on the results of these studies, it can be seen that O-GlcNAcylation can assist the binding of AKT to the mTOR complex 2, leading to the phosphorylation of AKT at S473 [16]. The role of mTOR in this pathway was further confirmed by blocking calcification driven by O-GlcNAcylation in smooth muscle cells through rapamycin treatment. These findings point to AKT T430/T479 amino acids as essential regulators of the AKT signalling pathway driven by O-GlcNAcylation leading to calcium accumulation [17]. This leads to AKT-mTOR becoming a pathway that can be used as a potential target in treating diabetes-induced CVD. Meanwhile, the runt transcription factor runt-related transcription factor 2 (RUNX2) is also known to have a role in the pathogenesis of CVD and its increase is associated with many CVD events. RUNX2 is a transcription factor that plays a role in bone and cartilage development. However, research also reveals that RUNX2 expression in cardiomyocyte cells regulates vascular calcification, leading to vascular stiffness. The study is increasingly interesting because it connects sugar metabolism and heart dysfunction [17].

Prevention of VC in METS has so far focused on controlling risk factors and managing complications that may arise. Several preventive options using herbs are known to be an attractive solution because of the convenience and reported health benefits related to controlling risk factors in METS. Research conducted by a research team at the Center for Cardiovascular Studies, Universitas Brawijaya, shows that herbs based on green tea and green coffee are promising in improving metabolism and preventing gene expression from METS-related complications. Preliminary study also found that green coffee extract improved the metabolic profile, adiponectin, and insulin resistance of the MetS model [18]. Other research also shows that green tea extract has an important role in improving lipid metabolism in MetS model animals by increasing the expression of PPARα and PPARγ and the expression of adiponectin receptors after 9 weeks [19,20]. In addition, the combination of green coffee and green tea extracts reduces fasting blood sugar and TG levels in plasma systolic blood pressure and on a molecular scale, is more effective in lowering angiotensin-2, inflammation-related genes (NF-κB, TNF-α, IL-6) and fibrosis gene expression (Tgf-β1, Rac-1, α-SMA, and Galectin-3) [21-23].

From the previous study and findings, all this suggests that it is appropriate to carry out studies on the use of green tea and green coffee extracts by targeting related pathways, namely AKT1 and mTOR, and their effects on RUNX2 and osteopontin levels. Based on the potential findings, this research aims to explore the benefits of decaffeinated green tea and green coffee extracts in preventing heart disease through the AKT-mTOR complex pathway, RUNX activity, and osteopontin expression as one of the downstream patogenesis of VC in METS.

Materials and methods

Animals treatment

The Sprague-Dawley rats used in the research were 5 weeks old with an average weight of 250 - 300 g, obtained from the National Agency of Drug and Food Control, Central Jakarta, Indonesia. Before the research process, acclimatization will be carried out for 7 days. Acclimatization occurred in the Animal House, Biology Department, Universitas Brawijaya. During acclimatization, rat will be given standard food and drinks that comply with laboratory standards. Drinking water is provided ad libitum using the drip method to avoid contamination with faeces.

The high-fat and sugar diet was prepared independently in the laboratory using a composition consisting of standard pellets, sucrose, methionine, hydrogenated vegetable fat, salt, monosodium glutamate and duck egg yolk. Standard rat pellet diets were converted into powder by grinding and mixing with 20 % sucrose, 0.5 % methionine, 2.5 % salt, 2 % monosodium glutamate, 15 % egg yolk and 20 % white fat. After adapting for 1 week, the rat will receive treatment in the form of a diet high in fat and 5 % fructose until they reach a body weight of 500 g. After that, rat was given an injection of streptozotocin at a low dose of 30 mg/kgBW to obtain an experimental animal model for hyperlipidemia and hyperglycemia. Body weight and biochemical parameters (blood glucose, triglycerides, HDL cholesterol) were measured 7 days after vehicle or STZ injection. High fat and sugar diet diet was continued for 6 weeks. Rat with blood glucose (> 200 mg/dL), triglycerides (> 200 mg/dL), high systolic blood pressure (≥ 140 mmHg), and reduced HDL levels (< 40 mg/dL) confirmed the presence of metabolic syndrome criteria according to NCEP-ATP III in 2005. Metformin, green tea and green coffee extracts were given in liquid form, orally using oral gavage in the morning. The dosage of green tea and green coffee extracts is determined based on preliminary studies. The optimal dose is 100 mg/kgBW for metformin, 300 mg/kgBW for green tea extract and 200 mg/kgBW for green coffee. This dose is known to have the most significant effect on the markers used in previous studies: lipid profile, fasting blood sugar, PPARα and PPARɣ, adiponectin, and inflammation markers [18-21].

Staining uses Immunohistochemistry techniques

Cardiac tissue paraffinized on a glass slide is deparaffinized using xylol and graded alcohol to dissolve the wax on the tissue. The deparaffinized slides were soaked in a chamber containing pH 6 citrate buffer, then heated in a microwave at 95 ºC, 300 V, for 5 min. The slides were removed from the microwave and left at room temperature for 20 min. The slides were then rinsed with PBS 3 times, waiting for 2 min each time. After antigen retrieval, the tissue specimens on the slides were blocked by endogenous peroxidase using 3 % hydrogen peroxide dissolved in methanol. Nonspecific protein blocking is carried out with a blocking buffer.

Primary antibody staining uses primary antibodies dissolved in a blocking buffer on the specimen. The antibodies used in this study were AKT (Cell signalling, Cat. Number 4060), MTOR (Cell signalling, Cat. Number 2983), and RUNX2 (Cell signalling, Cat. Number 9647). Secondary antibody staining was carried out by dropping Biotin-Conjugated Anti-globulin. The specimen slide was followed by administering the Strepavidin-Horse Radish Peroxidase (SA-HRP) substrate. Chromogen administration is carried out by administering DAB chromogen dissolved in DAB buffer (1:40). Specimens that have been incubated from the previous process are continued with background staining (apart from target cells) by dripping Mayer's Hematoxillen which has been diluted with distilled water (1:3). Incubation was carried out for 10 min at room temperature. The slides were then rinsed with distilled water. The specimen was dried until it was completely free of water, then dripped with Entellan^{TM} (Sigma, Cat No. 1.07961) and covered with a covered glass. The immunohistochemical slides were then observed with a microscope.

Serum osteopontin level measurement

Serum OPN measurements were performed using the ELISA-sandwich method (Elabscience, E-EL-R0702). Serum samples were prepared at ambient temperature, and subsequently, each well was allotted 100 µL of either sample, standard solution, or blank. The plate was sealed and incubated at 37 °C for 90 min. After decanting the liquid in each well, 100 µL of biotinylated detection antibody was added to each well, and the plate was resealed for an additional 60 min of Incubation at 37 °C. After decanting the liquid from each well, 350 µL of wash buffer was added, and the mixture was allowed to stand for 1 min before being washed thrice. Next, 90 µL of substrate reagent was added to each well. Subsequently, the plate was resealed and further incubated at 37 °C for 15 min. Finally, 50 µL of stop solution was introduced to each well. The plate was then measured using a Microplate Reader (Zenix-320) at a wavelength of 450 nm, with the instrument being pre-warmed for approximately 15 min before obtaining the optical density (OD value).

Data analysis

The data obtained was tabulated to receive an average figure (mean). Research data is presented in quantitative and qualitative form. Descriptive statistics in the form of frequency tables, mean and standard deviation and percentages are given for each parameter. Proving the research hypothesis is preceded by determining the normality and homogeneity of the research data. Data normality and homogeneity tests used the Kolmogorov-Smirnov/Shapiro-Wilk and Levene's tests. Data can be concluded to be normal and homogeneous if the Levene and Shapiro-Wilk test results have a p-value of > 0.05. The degree of error for this study was determined to be 5 % with a confidence interval of 95 %. If the data is normal and homogeneous, then parameter statistics are used.

Ethical clearance

This research has been approved by the Health Research Ethics Committee of Saiful Anwar General Hospital, Malang, Indonesia, by registered number: 400/211/K.3/302/2021.

Results and discussion

Based on the results obtained in this study, it is known that there are differences in the expression of the proteins targeted in this study. Under microscope observation, AKT1 expression was observed as brown staining localized in the cytoplasm. The intensity and distribution of staining are assessed and visualized on a chart as in **Figure 1(A)**. This study found an increase in AKT1 expression in METS model along with therapy. The average AKT1 expression in the NORM and METS control groups was the highest in the

NORM group, 28.83 ± 1.23, and the lowest AKT expression in the METS group was 3.18 ± 0.37. Based on the one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are significantly different with a *p*-value < 0.05. The mean AKT was found to be higher in the therapy group compared to the METS control. The highest mean AKT expression in the group given MFN therapy was 17.32 ± 0.93, while in the GTCE group, it was 16.99 ± 0.51. Based on one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are not significantly different compared to METS controls with a *p*-value > 0.05. However, compared with the METS group, these 2 groups differed with a *p*-value < 0.05 (**Table 1**, **Figure 1(A)**). The results of this study found that there was an increase in AKT1 expression in METS model rat along with therapy. AKT1 expression was found to decrease in the METS group and an increase in the mean AKT was found in the MFN and GTCE therapy groups (*p*-value > 0.05). These results showed that in METS conditions, the AKT1 protein experiences disturbances in the AKT1 signalling pathway or as confirmation that insulin resistance has occurred, which causes an imbalance in glucose metabolism. As is known, AKT1 plays an essential role in several mechanisms related to glucose metabolism [24]. In glucose transport, activation of AKT1 helps increase glucose transport into cells, especially in muscle and adipose tissue. This mechanism helps lower blood glucose levels. In the same pathway, AKT plays a role in glycogenolysis and gluconeogenesis. AKT1 also influences the formation and breakdown of glycogen in the liver and muscles [25]. In the context of this research, its role was also found in heart muscle. In addition, AKT1 is known to play a role in protein synthesis and cell growth. AKT1 can promote protein synthesis and cell growth, which is essential for maintaining energy balance and metabolism. Metformin administration is known to increase AKT1 but is not better than GTCE, which produced a higher mean AKT1 in this study. This study's results align with research conducted by Zhang et al. that activation of AKT1 must go through the insulin pathway, which is not facilitated by metformin [26], which, in this study, polyphenols from GTCE were superior in increasing AKT1.

The second observation was made on the MTOR protein, where it was found that there were significant differences between groups. The mean MTOR expression in the NORM and METS control groups was the highest in the METS group, 32.12 ± 1.39, and the lowest MTOR expression in the NEG group was 4.98 ± 0.61. Based on the one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are significantly different with a *p*-value < 0.05. The mean MTOR was found to be lower in the therapy group compared to the METS control. The highest mean MTOR expression in the group given GTCE therapy was 15.49 ± 0.69, while in the MFN group, it was 14.14 ± 0.80. Based on one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are not significantly different compared to METS controls with a *p*-value > 0.05. However, compared with the METS group, these 2 groups were especially other with a *p*-value < 0.05 (**Table 1**, **Figure 1(B)**). The increase in AKT1 in the group given GTCE therapy may also be related to suppressing the activation of the mTOR pathway, which regulates glucose metabolism. It is known that MTOR is a protein kinase that regulates insulin secretion by pancreatic beta cells [27]. Thus, disruption of the MTOR pathway, which also occurred in the METS group, may affect the ability of the pancreas to respond to changes in glucose levels and produce insulin. The decrease in MTOR upon administration of GTCE is in line with research conducted by Jia *et al.* [28], which stated that green tea EGCG could attenuate cardiac fibrosis in type 2 diabetic rat28, and the underlying mechanism was related to the activation of autophagy through modulation of the AMPK/mTOR pathway and then repression of the TGF-β/MMP's pathway. Meanwhile, CGA, a component of green tea, has been proven to reduce MTOR and CPT1a, which causes a decrease in the gene expression of Glucose-6-phosphatase in the liver [29].

Table 1 Results of measurement and analysis on AKT1, MTOR, RUNX2, and OPN.

Variable	Group	Min	Max	Mean square	F	*p*-value
AKT1	NEG	20.06	27.69	377.86	107.61	0.00*
	METS	2.29	4.46			
	MFN	14.6	19.48			
	GTCE	15.93	18.89			
MTOR	NEG	3.23	6.93	638.51	148.06	0.00*
	METS	28.32	35.15			
	MFN	11.43	15.49			
	GTCE	13.3	17.3			
RUNX2	NEG	3.15	4.44	965.78	458.55	0.00*
	METS	34.12	38.21			
	MFN	25.47	30.43			
	GTCE	23.3	26.3			
OPN	NEG	10.28	13.3	337.29	111.42	0.00*
	METS	28.72	33.38			
	MFN	19.07	21.6			
	GTCE	13.92	20.13			

^ Mean square in between group
* Significant different (*p*-value < 0.05)

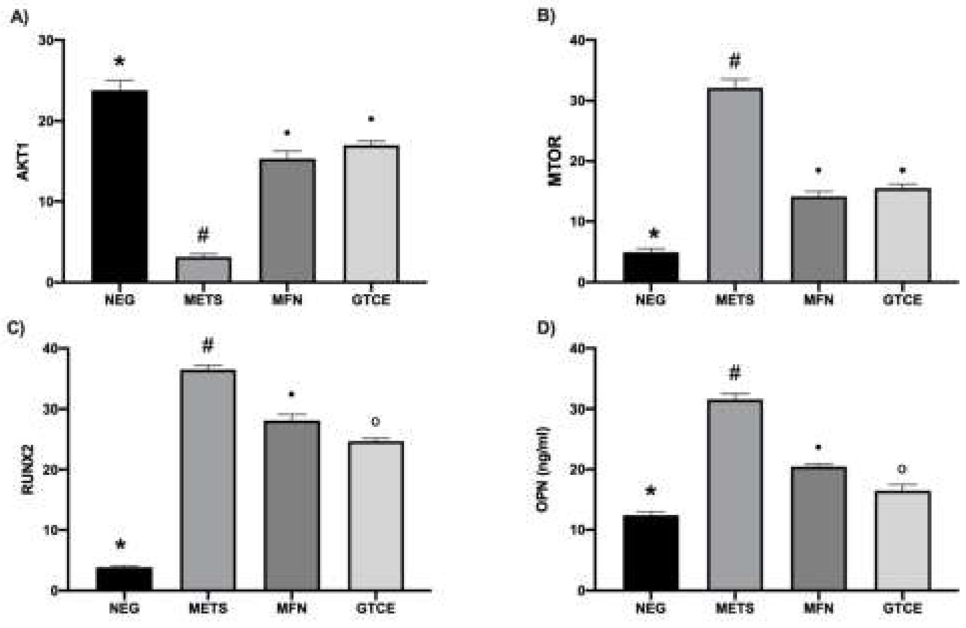

Figure 1 Results of analysis of (A) AKT1; (B) MTOR; (C) RUNX2; (D) OPN. Each group's subset signs (*#•) indicate a significance *p*-value < 0.05. Information: NORM = Negative control group of rat; METS = Positive control group of rat (METS model, no therapy); MFN = METS model rat group + 100 mg/kgBW metformin therapy; GTCE = METS model rat group + 300 mg/kgBW green tea extract therapy and 200 mg/kgBW decaffeinated green coffee.

Meanwhile, RUNX2, which was observed in this study, also showed changes in expression when given therapy using MFN or GTCE. In the control group, it is known that the NEG group has the lowest mean with a value of 3.79 ± 0.25, while in the METS group, it is known to be 36.47 ± 0.66. Based on the one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are significantly different with a *p*-value < 0.05. A decrease in RUNX2 expression was found in the group given MFN and GTCE therapy. The mean RUNX2 expression in the MFN group was higher than GTCE, 28.15 ± 0.97, while in the GTCE group, it was 24.70 ± 0.48. Based on the one-way ANOVA and post-hoc Tukey test, it is known that these 2 groups are significantly different with a *p*-value < 0.05 (**Table 1**, **Figure 1(C)**). Therapy using GTCE for METS observed in this study was also known to reduce RUNX2 better than the drug control, MFN. This result can be attributed to the activity of polyphenols in inhibiting the overactivation of RUNX2, in line with research conducted by Vali *et al.* [30], which revealed that EGCG therapy could reduce RUNX2, indicating a decrease in early-stage osteogenic differentiation activity in human osteoblast-like cells [30]. Meanwhile, it is known that the OPN levels in the group given therapy also experienced a decline in mean. In line with research on a significant reduction in coronary calcification in women with a coffee intake of 3 - 4 cups and more than 4 cups, compared with a daily intake of 3 cups or less [31]. This study concluded an increase in AKT1 expression and a decrease in MTOR, RUNX2, and OPN in METS rat given GTCE therapy.

Measurements of osteopontin (OPN) levels also showed different results between treatments. In the NEG group, the level was 12.41 ± 0.55, while the highest level was found in the METS group, 31.50 ± 0.95. Based on the one-way ANOVA and post-hoc Tukey tests, it is known that these 2 groups are significantly different with a *p*-value < 0.05. A decrease in OPN levels was also found in the group given MFN and GTCE therapy. The mean OPN expression in the MFN group was higher than the GTCE, 20.48 ± 0.41, while in the GTCE group, it was 16.47 ± 1.01. Based on the one-way ANOVA and post-hoc Tukey test, it is known that these 2 groups are significantly different with a *p*-value < 0.05 (**Table 1**, **Figure 1(D)**). This study showed that OPN levels increased in the METS group and were shown to decrease when therapy was given. Osteopontin is a matricellular protein that plays an important role in the pathogenesis of VC [32]. Research indicates that OPN expression is increased in STZ-induced diabetic cardiomyopathy model rat. OPN was also increased in the development of hypertrophy in rats with pressure overload33. OPN is known to inhibit mineral deposition in vascular walls and cardiac valves [34]. Overexpression of OPN in cardiac tissue led to loss of cardiomyocytes, fibrosis, and Th1 cell infiltration [35,36]. The therapy given to METS model rat in this study caused a significant reduction in OPN expression in serum. This condition then indicates that there is a potential effect in inhibiting CV through the consumption of green tea and green coffee. This effect may originate from inhibiting chronic inflammation and reducing serum OPN levels [37]. As is known, research shows that green tea and green coffee can reduce levels of inflammatory markers that lead to CVD, such as TGF-β, and transcription factors related to CVD. However, further research needs to be done to determine whether the effect of this therapy works directly with specific targets on the markers used in this study or whether other mechanisms support this mechanism so that it inhibits the progression of CVD in METS. Additional research is needed to determine if this therapy directly affects the specific targets identified in this study and whether other mechanisms are involved in inhibiting the progression of cardiovascular disease in metabolic syndrome. Further studies are warranted to clarify these mechanisms and identify new therapeutic targets for managing cardiovascular complications associated with metabolic syndrome.

Conclusions

This study showed the potential for therapeutic use of green tea and green coffee extracts to prevent vascular calcification in rat models of metabolic syndrome by increasing AKT1 expression, decreasing MTOR and RUNX2, with the main implication of reducing the circular protein product, osteopontin. Moreover, the results emphasize the complex molecular mechanisms behind the protective properties of green tea and green coffee extracts against vascular calcification. Through enhancing AKT1 expression and suppressing MTOR and RUNX2, these extracts demonstrate significant therapeutic potential in impeding the advancement of vascular calcification in individuals with metabolic syndrome. Noteworthy is the decrease in osteopontin levels, a crucial circular protein linked to vascular calcification, which underscores the diverse effects of these extracts on essential molecular pathways related to pathological calcification. This clarification of the involved molecular pathways offers valuable insights into crafting targeted strategies to combat vascular calcification and its related complications in metabolic syndrome.

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