Combination Therapy of Green Tea and Green Coffee on Improving Cardiomyocyte Metabolism Through Increased Expression of AMPK and AKT Genes in Metabolic Syndrome Model Rats

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Abstract. Metabolic syndrome (MetS) is a set of risk factors for metabolic abnormalities that can lead to dysfunction of organs, one of which is the heart. Each risk factor component of MetS represents a significant economic burden for patients, health plans, and society at large. One of the cardiovascular diseases (CVD) found is decrease in heart contractility. This cardiac dysfunction begins with abnormal expression of several proteins involved in lipid and glucose metabolism pathways, such as AMP-activated protein kinase (AMPK) and Protein Kinase B (Akt). One of the efforts to increase the use of environmentally friendly and sustainable resources, the use of herbs is the main focus in various treatments, one of which is MetS. This study aims to determine the effect of combination therapy of green tea and green coffee with metformin on cell metabolism pathways through the expression of AMPK and AKT genes in the heart of MetS model rats. Rats model were treated with metformin (MFN) 100 mg/kgbw, and/or green tea and green coffee extract (GTCE and/or COMB) 300 mg/kgbw and 200 mg/kgbw respectively. Rat hearts were isolated and analyzed by *reverse-transcriptase* PCR method using AMPK and AKT primers. The results showed that there was a significant difference in AMPK gene expression (p-value = 0.000) and AKT (p-value = 0.002) between all groups. The correlation between the two shows that they have a correlation with each other. This study showed that the combination therapy of green tea and green coffee with metformin was able to improve cell metabolic pathways by increasing the expression of AMPK and AKT genes in the heart of MetS model rats.

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1 Introduction

Metabolic syndrome (MetS) is a set of risk factors for metabolic abnormalities that can lead to dysfunction of a number of organs and even death. This definition is contained in the agreement of experts who are members of the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) in 2005, which states that the risk factors established as a diagnosis of MetS sufferers include hyperglycemia/insulin resistance, hypertension, obesity, and triglyceride dyslipidemia [1]. According to the NCEP ATP III definition, a person is considered to have MetS if three or more of the five associated risk factors are met. This includes supporting risk factors, namely, age, male sex, and the influence of postmenopausal hormones. The prevalence of MetS is reported to continue to increase from year to year, and has reached more than 20–25% of the adult population [2–4] and reached 19, 2% in children worldwide [5]. The average prevalence of metabolic syndrome in Indonesia in 2019 was reported to be 21.66%, with the most common risk factors being central obesity, hypertension, and low HDL levels [6].

One of the cardiovascular diseases found in patients with metabolic syndrome is a change in histology structure (remodeling) and a decrease in heart contractility. This can be a direct effect of the increase in adipocytes, or it can be due to the risk of hypertension being agonist with obesity, resulting in excess pressure on the heart. This cardiac dysfunction begins with abnormal expression of several proteins involved in lipid and glucose metabolism. Increased oxidative stress (ROS) in response to glucose fluctuations disrupts the glucose metabolism pathway (phosphatidylinositol 3′-kinase (PI3K)-Akt/PKB). Impaired Akt activation, especially in insulin-responsive tissues, will lead to a decrease in the translation of glucose transporter 4 (GLUT4), which exacerbates hyperglycemia [7]. The inhibition of glucose uptake, and the increased cell demand for glucose along with insulin resistance, causes a decrease in intracellular cell energy (AMP/ATP) which causes a transition of cell metabolism from energy consumption to energy production (anabolic to catabolic) [8]. As an intracellular energy sensor, AMPK is phosphorylated to the Ser485/491 subunit, contributing to a sustained reduction of phosphorylation of $AMPK^{Thr172}$, so that cells obtain energy through gluconeogenesis and *de novo* lipogenesis, as well as accelerated fatty acid delivery and triglyceride esterification. Decreased AMPK and AKT activation, which indirectly activate pro-inflammatory genes will cause wound healing responses to appear through the expression of several profibrotic proteins, one of which is collagen 1 (COL1A1) which leads to cardiac fibrosis [9].

MetS treatment has focused on individual components by suppressing insulin resistance using metformin [10]. Metformin (1,1-dimethylbiguanide) is a biguanide derivative, which acts by leading to the activation of AMP-activated protein kinase (AMPK) with an increase in phosphorylated AMPK levels [11, 12]. Physiologically, metformin works to decrease glucose production, by increasing glucose utilization, increasing GLP-1, decreasing ATP thereby activating AMPK, increasing insulin sensitivity (through

effects on fat metabolism) and decreasing cAMP, thereby reducing the expression of gluconeogenic enzymes [13]. Reports related to side effects arising from regular consumption of metformin, such as digestive tract disorders such as diarrhea and vomiting, as well as concerns that lactic acidosis may lead to discontinuation of treatment [14]. In addition, the cost-effectiveness of long-term pharmacological treatment from the use of metformin needs special attention. Recent increases in prevalence, also increased resource utilization by affected patients, and increased morbidity and mortality place a big economic burden on society.

Therefore, it is necessary to conduct research to improve the efficiency of the glucose regulation mechanism and the cardioprotective properties of metformin by utilizing herbal agents such as green tea and green coffee extracts. This study aims to determine the effect of combination therapy of green tea and green coffee with metformin on cell metabolism pathways through the expression of AMPK and AKT genes in the heart of MetS model rats.

2 Results and Discussion

2.1 Research Design

The study was a true experimental design with a post-test-only control group design with a simple random sampling technique, from March to October 2022 in Molecular Biology Laboratory, Faculty of Mathematics and Sciences, University of Brawijaya, Malang. Rat model of metabolic syndrome is made by following methods that have been done by Rohman et al. [15]. Sprague-Dawley rats aged 8–12 weeks, weighing 250–300 gram acclimatized for seven days. Rats were divided into five groups: the negative control group (NORM) was healthy mice ($n = 5$) fed commercial pellet feed. The positive control group ($n = 5$) were with metabolic syndrome rats (METS) through the high-fat high sucrose diet (HFHS) consist of powdered mouse up to 500 gram weight. Streptozotocin (STZ) injection 30 mg/kg body weight (BW) after body weight reaches 500 grams. Then, HFHS continued until the end of the study. The MetS rats must meet the criteria for the metabolic syndrome model, i.e. FBG >200 mg/dL; TG >200 200 mg/dL; HDL <40 mg/dL; and SBP >120/80 mmHg. Therapy was given to rats after rats met these criteria constant for 4 weeks. The therapeutic dose given to rats was Metformin (MFN) 100 mg/kgbw; green tea and green coffee extract (GTCE) 300 mg/kgbw and 200 mg/kgbw respectively. Meanwhile for the combination therapy group (COMB) is a combination of Metformin (MFN) 100 mg/kgbw; green tea and green coffee extract (GTCE) 300 mg/kgbw and 200 mg/kgbw. Both herbs and drugs will be dissolved using the same mineral water used to drink mice via oral gavage. Daily food intake and fluid intake are measured every day, body weight is measured every week, and fluid intake is given ad-libitum. 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 was measured using a spectrophotometer.

2.2 Green Coffee and Green Tea Extraction Process

Green coffee was extracted from roasted *Coffea robusta* beans at 180–200 °C for 6–8 h. Then, the coffee beans are macerated with 95% ethanol to produce a crude extract. The crude extract is filtered to separate the liquid phase to be concentrated using a rotary evaporator at ± 40 °C. Green tea extracted from 500 g of green tea leaves, dried using a drying cabinet (50 °C) for 8 h to get 8–10% moisture. Dried green tea leaves blended and boiled at 80 °C for 30 min. Then, the crude extract is filtered to get a concentrated liquid phase using a rotary evaporator at ±40 °C.

2.3 Measurement of AMPK and AKT Gene Expression

Total RNA of heart tissue was isolated using PrimeZol according to the manufacturer's protocol. Tissue samples were taken ± 3 g, crushed using a sterile mortar and pestle, and 500 μL PrimeZol was added gradually until smooth. Reverse transcription reactions were performed using a ReverTra Ace-α kit (Toyobo, Japan). Then the RNA expression level was performed using the LightCycler 96 PCR system (Takara, Japan) using the GoTaq Green Master PCR Kit (Promega, Madison, USA) according to the manufacturer's protocol. The primer sequences are as follows: β-actin, forward: 5′- TGA GAG GGA AAT CGT GCG TGA CAT-3′ and reverse: 5′-ACC GCT CAT TGC CGA TAG TGA TGA-3′; AMPK forward: 5′-GGCCACTGATTGTCCGCTAT-3′; and reverse: 5′-TCTTTGCCTCCCTTCCCCAGT-3′; and AKT forward: 5′-CTG GGC TAA CT GAT GAT CT -3'; and reverse: 5′-TGC TTT GGA GG CTT CGG TGC TCT C-3′. The PCR cycle was as follows: 5 min at predenaturation of 95 °C; 29 cycles of 30 s at denaturation at 95 °C, 30 s of annealing at 55 °C, followed by extension for 30 s at 72 °C; and a final extension of 10 min at 72 °C. The mRNA level of the target gene was normalized to the level of β-actin expression.

2.4 Analysis Data

The data obtained were tabulated to obtain the average (mean) for AMPK and AKT expressions from each group. Research data is presented in quantitative and qualitative forms. Descriptive statistics in the form of tables of frequency, mean and standard deviation, percentages are presented for each parameter. The proof of the research hypothesis is preceded to determine the normality and homogeneity of the research data. Test for normality and homogeneity of data using the Kolmogorov-Smirnov/Shapiro-Wilk test and Levene's test ($p > 0.05$). The degree of error in this study was set at 5% with a confidence interval of 95%. Different tests were carried out by statistical tests using Duncan's ANOVA-Post Hoc variation test. Correlation test is done by Pearson test.

2.5 Ethical Clearance

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

3 Results and Discussion

3.1 Gene Expression of AMPK and AKT

AMPK gene expression is a relative expression of -actin presented in Fig. 1A. AMPK expression in this study showed different results between groups. In the group of normal rats, the relative expression of AMPK was 0.800 ± 0.144. AMPK expression was known to decrease in the METS group up to 0.205 ± 0.026. Meanwhile, in MetS rats with therapy, it was found that AMPK expression increased, namely MFN 0.781 ± 0.304, GTCE 0.566 ± 0.215, and COMB 0.685 ± 0.075. This expression was found to be

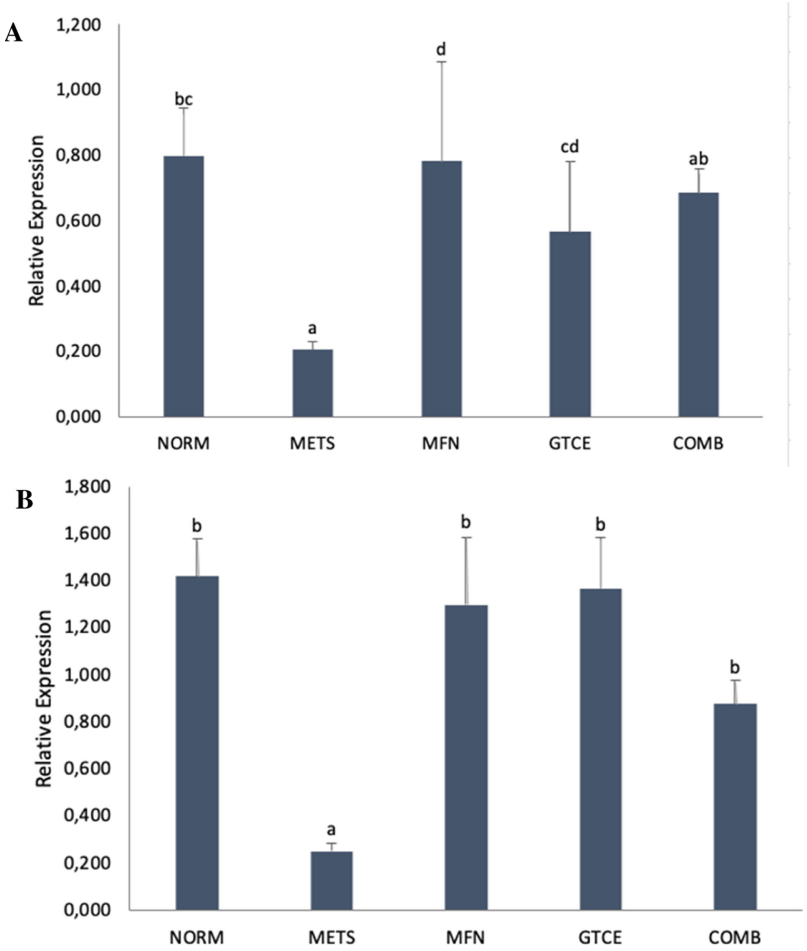

Fig. 1. The AMPK (A) and AKT (B) gene expressions in heart tissue of rat MetS model. Reverse-transcriptase PCR measures the mRNA expression that was normalized based on β-actin level—one-way ANOVA and Duncan post-hoc test was used. $P < 0.05$ was considered to indicate a statistically significant difference. Groups that appear in the same homogeneous subset (a,b,c,d) are not significantly different.

significantly different between groups ($p = 0.000$) with the most significant differences found in the METS group ($\alpha = 0.205$) and MFN therapy ($\alpha = 1.551$) (Tables 3 and 4). The decreased AMPK expression due to MetS seems to be able to be increased either by using metformin alone, or green tea and green coffee extracts. The use of a combination of metformin with green tea and green coffee extracts has shown promising results in MetS therapy. This is evidenced by the AMPK expression which almost returns to its normal expression (NORM). However, metformin as the first line of T2DM treatment still led as the highest expression among the treatment groups. However, the use of green tea extract and green coffee alone is known to have a positive effect on increasing AMPK expression in MetS therapy (Tables 1 and 2).

The expression of the AKT gene in this study is also a relative expression of -actin, presented in Fig. 1B. AKT expression in this study showed different results between groups, with a p-value of 0.002 (Tables 3 and 4). Sequentially, the highest AKT expression was produced by the normal group with a value of 1,419 ± 0.162, followed by the GTCE treatment group 1.366 ± 0.215 and MFN 1,298 ± 0.288, and finally by COMB 0.876. ± 0.100. Meanwhile, the group with the lowest AKT expression was the METS group 0.250 ± 0.033. Significant differences were known to occur between the METS groups ($\alpha = 0.251$) (Tables 3 and 4). As the main drug in the treatment of MetS, MFN

Table 1. Data Normality Test Results

Group		Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	dF	Sig.	Statistic	dF	Sig.
AMPK	NORM	0.226	5	0.200*	0.940	5	0.665
	METS	0.222	5	0.200*	0.920	5	0.531
	MFN	0.262	5	0.200*	0.901	5	0.414
	GTCE	0.194	5	0.200*	0.933	5	0.617
	COMB	0.161	5	0.200*	0.966	5	0..849
AKT	NORM	0.210	5	0.200*	0.973	5	0.893
	METS	0.348	5	0.047	0.806	5	0.091
	MFN	0.219	5	0.200*	0.930	5	0.600
	GTCE	0.247	5	0.200*	0.847	5	0.185
	COMB	0.224	5	0.200*	0.871	5	0.270

Table 2. Data Homogeneity test results

	Test of Homogenity of Variances			
	Levene Statistic	df1	df2	Sig.
AMPK	14.472	4	20	0.564
AKT	1.524	4	20	0.233

Table 3. One-way ANOVA Test Results

	ANOVA					
		Sum of Square	dF	Mean Square	F	
AMPK	Between groups	6.041	4	1.510	9.109	Sig.
	Within Groups	3.316	20	0.166		0.000
	Total	9.357	24			
AKT	Between groups	4.055	4	1.014	6.082	0.002
	Within Groups	3.333	20	0.167		
	Total	7.388	24			

Table 4. Duncan's test results

	Duncan		Subset for alpha = 0.05			
	Group	N	1	2	3	4
AMPK	METS	5	0.205	–	–	–
	COMB	5	0.485	0.484	–	–
	NORM	5	–	0.799	0.799	–
	GTCE	5	–	–	1.255	1.255
	MFN	5	–	–	–	1.551
	Sig.		0.290	0.236	0.092	0.265
AKT	METS	5	0.251	–	–	–
	COMB	5	–	0.876	–	–
	NORM	5	–	1.111	–	–
	MFN	5	–	1.298	–	–
	GTCE	5	–	1.367	–	–
	Sig.		1.000	0.096	–	–

is known to have a good effect in increasing the expression of the AKT gene, as was observed in the GTCE-treated group. The combination of the two is also thought to be able to increase the expression of the AKT gene, but in this study, it was found that its expression was still lower than the single therapy group.

The correlation between AMPK and AKT gene expression in this study shows the results in Table 5.

Based on the results of the analysis, it is known that there is a relationship with a positive value between AMPK and AKT. It can be concluded that both have a correlation with each other, linear and synergistic in supporting each other's performance. AMPK maintains energy homeostasis by activating catabolic pathways (producing ATP) and

Table 5. Correlation of relative genes expression of AMPK and AKT

		AMPK	AKT
AMPK	Pearson	1	0.693**
	Correlation		
	Sig. (2-tailed)		0.000
	N	25	25
AKT	Pearson	0.693**	1
	Correlation		
	Sig. (2-tailed)	0.000	
	N	25	25

inhibiting anabolic pathways (consuming ATP). Downstream effects of AMPK activation can be tissue dependent and impact, directly or indirectly, a variety of cellular processes, including lipid and glucose metabolism, energy consumption, immune responses, and growth, and cell polarity. AMPK activation was initially described by mediating AMP binding; however, studies show that binding of AMP and ADP results in a conformational change that activates AMPK in two ways. First, activation that promotes phosphorylation of Thr-172 by kinases in the upstream, and second, antagonists dephosphorylation by protein phosphatase [15, 16]. In contrast, only AMP was shown to directly increase the activity of phosphorylated AMPK (Thr-172) via an allosteric mechanism. In combination, these two activation mechanisms result in the most rapid and multiple-fold increase in activity than the single mechanism [17, 18]. The increase in AMPK gene expression as a result of GTCE therapy in this study showed the potential and benefits of green tea and green coffee in overcoming the decreased AMPK gene expression in an effort to re-increase lipid and glucose metabolism molecularly. Although it is known to have a lower value than control drugs, the ability of GTCE, either alone or with MFN, can help increase AMPK gene expression so that it is expected to improve cell metabolism.

In other metabolic pathways, the AKT signaling pathway is a regulatory pathway regulating glucose and lipid metabolism. Activation of Akt2, especially in insulin-responsive tissues, will cause the translation of glucose transporter 4 (GLUT4). AKT converts glucose to glucose 6-phosphate by stimulating hexokinase. Akt regulates two processes by glycolysis. Glycolysis is carried out through Glucose 6-phosphate and glycogen synthase kinase 3 (GSK3) to produce cellular energy. In addition, glycolysis is needed to trigger cells to produce glycogen through Forkhead Box O (FOXO) protein [19]. The FOXO protein consists of several isomers, with the main target isomer of Akt being FOXO1 for energy homeostasis throughout the body [20]. FOXO1 and peroxisome proliferator-activated receptor-coactivator 1α (PGC1α) together regulate gene expression to enhance gluconeogenesis and fatty acid oxidation [21]. In addition, FOXO1 can induce the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6PC) genes, which further enhance gluconeogenesis [22]. AKT expression was higher than in the METS group, indicating that both GTCE were

able to improve the decrease in AKT under MetS conditions. This was also supported by the combination of both herbs and drugs, with different significance to the MetS group. In this case, efforts to improve metabolism, GTCE group was able to increase AKT gene expression better than MFN. This could be due to the mechanism of action of herbal groups that are insulin responsive or known as insulin-dependent, while MFN tends to respond to energy-related signals sent by mitochondria [23, 24]. Therefore, it is necessary to carry out further research for these two proteins at the enzymatic level related to phosphorylation and to compare the phosphorylated and non-phosphorylated conditions.

4 Conclusion

This study showed that the combination therapy of green tea and green coffee with metformin was able to improve cell metabolic pathways by increasing the expression of AMPK and AKT genes in the heart of MetS model rats.

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