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Original Article

Decaffeinated green tea and green coffee extracts as metformin's add-on enhance metabolic syndrome risk factors and improve the cardiac insulin-gene-related pathway

[Extractos de té verde y café verde descafeinados como complemento de la metformina mejoran los factores de riesgo del síndrome metabólico y la vía cardiaca relacionada con los genes de la insulina]

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Abstract

Context: Dysregulation of glucose metabolism in metabolic syndrome (METS) is allegedly due to the disruption of insulin as the main pathway in cellular metabolism. Green tea and green coffee are known to have potential benefit in METS therapy.

Aims: To evaluate the effect of therapy using decaffeinated green tea-green coffee extract as metformin's add-on in the risk factors of METS and its effect on the cardiac insulin-gene-related pathway, such as IRS1, PI3Kr1, and GLUT4.

Methods: METS model rats were divided into five groups. The rats' level of body weight (BW), fasting blood glucose (FBG), triglycerides (TG), high-density lipoprotein (HDL), insulin (INS), and homeostatic model assessment for insulin resistance (HOMA-IR) were measured periodically. After nine weeks of treatment, the rats were euthanized, and the heart was isolated for measurement of IRS1, PI3Kr1, and GLUT4 gene expression by reverse-transcriptase polymerase chain reaction.

Results: This study found that there was a decrease in BW, FBG, TG and an increase in HDL in METS model rats given therapy with metformin and green tea-green coffee extract (COMB) ($p<0.0001$). There is also improvement in insulin resistance by reducing HOMA-IR in the COMB group ($p = 0.0434$ for INS and $p<0.0001$ for HOMA-IR). This study found that IRS1, PI3K, and GLUT4 gene expression increased in the COMB group. The five groups differ significantly, with a $p = 0.000$.

Conclusions: Therapy using a combination of decaffeinated green tea and green coffee extract as an add-on of metformin improved METS risk factor via a significant reduction in BW, FBG, TG, increasing HDL and improving insulin resistance. It also increased the IRS1, PI3Kr1, and GLUT4 gene expression as markers of cardiac insulin-gene-related pathways.

Keywords: green tea; green coffee; insulin signalling; metabolic syndrome; metformin.

Resumen

Contexto: La desregulación del metabolismo de la glucosa en el síndrome metabólico (METS) se debe supuestamente a la alteración de la insulina como vía principal en el metabolismo celular. Se sabe que el té verde y el café verde tienen beneficios potenciales en la terapia del METS.

Objetivos: Evaluar el efecto de la terapia con extracto descafeinado de té verde y café verde como complemento de la metformina en los factores de riesgo del METS y su efecto en la vía cardiaca relacionada con los genes de la insulina, como IRS1, PI3Kr1 y GLUT4.

Métodos: Las ratas del modelo METS se dividieron en cinco grupos. Se midieron periódicamente el peso corporal (PC), la glucemia en ayunas (GSA), los triglicéridos (TG), las lipoproteínas de alta densidad (HDL), la insulina (INS) y el modelo homeostático de evaluación de la resistencia a la insulina (HOMA-IR) de las ratas. Tras nueve semanas de tratamiento, se practicó la eutanasia a las ratas y se aisló el corazón para medir la expresión de los genes IRS1, PI3Kr1 y GLUT4 mediante la reacción en cadena de la polimerasa con transcriptasa inversa.

Resultados: En este estudio se observó una disminución del BW, FBG, TG y un aumento de HDL en ratas modelo METS tratadas con metformina y extracto de té verde y café (COMB) ($p<0,0001$). También se observa una mejora de la resistencia a la insulina mediante la reducción del HOMA-IR en el grupo COMB ($p = 0,0434$ para INS y $p<0,0001$ para HOMA-IR). Este estudio descubrió que la expresión de los genes IRS1, PI3K y GLUT4 aumentaba en el grupo COMB. Los cinco grupos difieren significativamente, con una $p = 0,000$.

Conclusiones: La terapia con una combinación de té verde descafeinado y extracto de café verde como complemento de la metformina mejoró el factor de riesgo METS a través de una reducción significativa de BW, FBG, TG, aumentando HDL y mejorando la resistencia a la insulina. También aumentó la expresión de los genes IRS1, PI3Kr1 y GLUT4 como marcadores de las vías cardíacas relacionadas con los genes de la insulina.

Palabras Clave: café verde; metformina; señalización de la insulina; síndrome metabólico; té verde.

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INTRODUCTION

Metabolic syndrome (METS) is an accumulation of several disorders or risk factors for developing various organ dysfunction complications. Risk factors for METS include obesity and insulin resistance, hyperglycaemia, hypertriglyceridemia, low high-density lipoprotein (HDL) cholesterol, and hypertension (NCEP-ATP III, 2002). METS are also the main result of a sedentary lifestyle and excess nutrition. Other contributing factors are the ageing of the male gender and the effects of postmenopausal hormones (Kautzky-Willer et al., 2016; Lin et al., 2019; Magge et al., 2017). The global METS prevalence in 2022 is 12.5% to 31.4%. This number is expected to continue to increase every year as the prevalence of each risk factor increases (45.1% obesity, 24.5% high fasting blood glucose, 28.9% hypertriglyceridemia, 40.2% low HDL cholesterol, 42.6% hypertension) (Noubiap et al., 2022). These risks are interrelated factors that increase the risk of developing cardiovascular disease (CVD).

CVD in METS includes changes in the heart's structure due to overload so that it is compensated through several mechanisms (Tune et al., 2017; Ulasova et al., 2011; Xia et al., 2009). One of the heart's structure changes related to METS is the occurrence of interstitial fibrosis. Interstitial fibrosis is a prominent characteristic of diabetic cardiomyopathy. Cardiac fibrosis that occurs in METS is also an adaptive response to the stress of obesity (Cavalera et al., 2014; Hu et al., 2010). Impaired glucose metabolism and excessively increased pressure on the heart can trigger the activation and expression of profibrotic molecules, leading to cardiac fibrosis (Jia et al., 2016; Kiernan et al., 2022; Vileigas et al., 2021).

Dysregulation of glucose and lipid metabolism in METS is allegedly due to the disruption of insulin as the main pathway in cellular metabolism (Lee et al., 2022; Savage et al., 2007). Insulin signalling occurs after insulin binds to the receptor (Insulin Receptor Substrate, IRS). Binding of insulin to its receptor causes tyrosine phosphorylation and activates two parallel pathways, the phosphoinositide 3-kinase (PI3K) pathway and the mitogen-activated protein kinase (MAPK) pathway (De Meyts, 2016; Shepherd et al., 1998). Tyrosine phosphorylation of IRS activates PI3K, activating protein kinase 1 (PDK1), which is PI3K and Akt kinase dependent. The PI3K pathway is responsible for the downstream metabolic effects of insulin (De Meyts, 2016; Højlund, 2014; Huang et al., 2018).

Increased reactive oxygen species (ROS) in METS is a factor that also increases cell resistance to insulin (Castro et al., 2016; Tangvarasittichai, 2015). Insulin resistance inhibits or reduces the activation of the PI3K pathway and protein kinase B (PKB/Akt) as the insulin pathway (Huang et al., 2018; Li et al., 2017; Tong et al., 2022). Decreased activation of the PI3K-Akt pathway reduces the translocation of the glucose transporter (GLUT-4) to the cell surface. This condition has an impact on reducing glucose absorption and directly increasing hyperglycaemia conditions. The decrease in PI3K-Akt activity also improves the expression and translocation of the nuclear factor *kappa* B (NF-κB), thereby increasing levels of inflammatory cytokines (Hutti et al., 2012; Lingappan, 2017). The increase in NF-κB translocation and the high levels of ROS in cells simultaneously increase the expression of the profibrotic protein in cardiomyocytes. Conditions of insulin resistance and impaired insulin signalling may interfere with METS through other pathways, such as impaired hepatic glucose uptake and glycogen deposition (Jiang et al., 2020; Petersen et al., 2017), overactivation of gluconeogenesis and de novo lipogenesis (Warner et al., 2020; Winnick et al., 2011), and accelerated delivery of fatty acids and esterification of triglycerides (Czech et al., 2013; Girousse et al., 2013).

Drinking coffee or tea worldwide is an effort to get the better of polyphenols from green tea leaves and coffee beans. Green tea leaves (*Camellia sinensis*) are a source of epigallocatechin-gallate (EGCG), which contains 50-80% of the total catechins in green tea. Research conducted by Dulloo et al. (1999) found that the administration of green tea extract significantly increased energy expenditure and lost fat in a group of young men. In 2005, it was reported that treatment with green tea extract containing ≥94% EGCG and ≤0.1% caffeine (TEAVIGO) significantly reduced body weight (BW) and body fat in various types of rats fed a high-fat diet (Klaus et al., 2005; Wolfram et al., 2005). Since reporting these findings, clinical studies have reported the effects of tea consumption on increased energy expenditure, fat oxidation, weight loss, fat mass, and weight maintenance after weight loss (Roberts et al., 2021; Rondanelli et al., 2021).

Meanwhile, green coffee is considered the highest source of polyphenol chlorogenic acid (CGA) compared to other sources, such as fruits and vegetables (Hsu et al., 2006; Manach et al., 2004) reported that chlorogenic acid inhibited the growth of the preadipocyte cell population, which means that CGA has good potential to reduce obesity. The prospect of CGA associated with METS therapy is to reduce fasting blood glucose in 15 patients with impaired glucose tolerance who were given 400 mg CGA for 12 weeks (Zuñiga et al., 2018). CGA extracted from green coffee beans also lowered fasting blood glucose in 21

patients with metabolic diseases during eight weeks of therapy (Roshan et al., 2018). Because its role in glycaemic control is quite good, this is a promising potential provided by green tea and green coffee in METS therapy. Even though it has been widely used with drugs, the mechanism accompanying the benefits of herbal medicines is not yet known (Baker et al., 2021).

Currently, the treatment of METS uses metformin as the primary agent to improve insulin resistance. Metformin side effects have been reported, including digestive problems, diarrhoea, vomiting, and concerns that lactic acidosis may require patients to discontinue taking their medicine (Ferreira et al., 2017). Metformin can be intensified in METS patients by combining lifestyle changes with complementary therapies (Tahrani et al., 2007). Metformin therapy alone has not been proven to prevent cardiac fibrosis directly (Rena and Lang, 2018). To enhance the potency of metformin's cardioprotective effects and the mechanisms by which it is controlled, it is thus essential to pursue a complementary agent that works well with metformin with fewer side effects. Based on the positive effects that have been reported, this study used decaffeinated green tea and green coffee extracts as metformin add-ons.

So that, this study aims to evaluate the effect of therapy using decaffeinated green tea-green coffee extract as metformin's add-on in the risk factors of METS and its effect on the cardiac insulin-gene-related pathway, such as IRS1, PI3Kr1, and GLUT4.

MATERIAL AND METHODS

Plant material

The green tea leaf utilized in this study was grade 1 quality, the top new leaf, and leaf buds. All the leaves were purchased from Ciwidey, Indonesia (7°9'24.48"S-108°0'23.4"E). Dried green tea and coffee beans were sorted to remove contaminants or low-quality green tea after harvest. Meanwhile, the green coffee bean in this study was a premium coffee bean purchased from Dampit, Indonesia (8°44'16.64"S-113°41'52.26"E). After confirmation from farmers and distributors, the laboratory assistant carried out the identification and quality checking of the variants. The sample was deposited as an example in the Molecular Biology Laboratory at Universitas Brawijaya with the reference number TSN-506801 for green tea and KAD-001 for green coffee beans.

Green tea and green coffee extraction

Sorted green tea leaves and green coffee beans were roasted in an oven at 180°C for 3 min or until the first crack appeared. Demineralized drinking water was used as the solvent for the extraction. The ratio of sample and solvent was 1:15, processed at 90°C for 10, 20, and 30 min. The solution was then using coarse filter paper to filter the sample. The green coffee extract was decaffeinated using activated carbon. Then, 2.5% w/v water, 0.5% w/v water formic acid, and 25% w/v water-activated carbon were added with cane sugar (2.5% sugar w/v water). The mixture was incubated for 6 h at 80°C in a water bath shaker. Then, the activated carbon (200% v/w activated carbon) was removed from the solvent and washed.

The decaffeination of green coffee extract was accomplished using activated carbon at a ratio of activated carbon to green coffee extract of 1:75 (w/v extract). A water bath shaker was used to conduct the decaffeination process at 60, 70, and 80°C for 6, 7, and 8 h before filtering. The blanching procedure was modified to decaffeinate green tea (Liang et al., 2007). The decaffeinated process for green tea was done at 50, 75, and 100°C for 1, 3, and 5 min, respectively. The decaffeinated green tea and coffee extracts were combined with 5% maltodextrin (w/v extract) and dried at 60°C in a food dehydrator for 5 h. A size reduction was accomplished using a dry blender and an 80-mesh sieve. Using high-performance liquid chromatography (HPLC) equipment, the EGCG content of green tea extract and the CGA portion of green coffee extract were examined (Shimadzu Corporation, Japan). According to HPLC measurement, green coffee extract's CGA fraction had a concentration of 27.134 g/g, while green tea extract had a concentration of 74.126 g/g of EGCG.

Experimental animals

Using rats as experimental animals in this study was based on Replacement, Reduction, and Refinement (3R) principles. The ethical commission strictly monitored the procedure to comply with the experimental animal guidelines of the institution. This study used the minimum number of rats and reduced pain in each treatment applied to experimental rats. Acclimatization was carried out during the first seven days of arrival. Each rat was placed in a polycarbonate cage (90 × 60 × 60 cm) with hemp bedding. The environment was maintenance stable at 12-hour light/dark cycles, temperature 25°C, and humidity 50%. Food was provided and replaced daily. The drink was provided ***ad libitum***. 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.

Research design

Four-week-old male Sprague-Dawley rats (150 grams) were randomized into five groups (n = 5) after a week of acclimatization. The METS model was fed a high-fat-high-sucrose (HFS) diet. Then, the rats were administered intraperitoneally with low doses of streptozotocin (30 mg/kg body weight diluted with 10% citrate buffer pH 4.5) at 10-11 weeks of age with a body weight of 480-500 g. To become a METS model, the rat must meet three main criteria recommended by NCEP-ATP III (2002): high fasting blood glucose (>200 mg/dL), high triglycerides (TG>200 mg/dL), high-density lipoprotein (HDL<40 mg /dL). All risk factors must be stable for 4-6 weeks (Rohman et al., 2017). The rat model of METS was then divided into three treatment groups: one that received metformin (MFN, 100 mg/kg BW), one that received green tea and green coffee extracts (GTCE, 200 mg/kg BW, and 300 mg/kg BW, respectively), and group who received a combination of metformin and the green tea-green coffee extracts (COMB, 100 mg/kg, 200 mg/kg, and 300 mg/kg, respectfully). Two other groups negative control (NORM) and positive control (METS) were used. Green tea extract, green coffee, and metformin doses are the results of a preliminary study to determine the optimal dose that provides a significant outcome and is known to be safe (Lukitasari et al., 2018). The therapeutic component was dissolved in mineral water and administered orally via gavage. Therapy was given for nine weeks. Food intake and fluid intake (*ad libitum*) were measured daily.

Measurement of body weight, fasting blood glucose, triglyceride, and high-density lipoprotein cholesterol level

The rat's body weight (BW) was measured using a scale periodically. Venous blood was taken from rats aged 25, 30, 35, and 40 weeks in a fasted state for 8 h. Blood serum was separated from red blood cells by centrifugation at 4500 rpm for 15 min at 4°C. Blood serum was then separated to measure the fasting blood sugar (FBG), triglyceride (TG), and high-density lipoprotein cholesterol (HDL) levels using the GPO method.

Blood glucose levels were measured by mixing 1000 µL of glucose reagent (Biolabo France, Ref. 87409) with 10 µL of blood serum. The mixture was then incubated at room temperature for 1 min and read on a 500 nm spectrophotometer. This method also measured TG levels (Biolabo France, Ref. LP80519). HDL levels were measured by mixing 100 µL blood serum with 10 µL HDL precipitate reagent (Biolabo France, Ref. 86516), incubated for 10 min, then centrifuged at 4500 rpm, 15 min at 4°C. Next, 25 µL of the supernatant was mixed with 1000 µL total cholesterol reagent (Biolabo France, Ref. 80106). The mixture was incubated at room temperature for 10 min and then read by a spectrophotometer with a wavelength of 500 nm. The measurement was repeated three times for each sample, and the average was taken.

Measurement of insulin level using sandwich ELISA method

Blood serum samples and reagents were prepared at room temperature. A volume of 100 µL of the sample, standard solution, or blank was added to each well. The plate was covered with a sealer and incubated at 37°C for 90 min. The liquid in each well was decanted and then added with 100 µL Biotinylated Detection Antibody in each well. The plate was covered with a sealer and incubated at 37°C for 60 min. The liquid in each well was decanted, and 350 µL wash buffer was added and incubated for 1 min. Washing was carried out 3 times. 90 µL of substrate reagent was added to each well. The plate was then covered with a new sealer and incubated for 15 min at 37°C. 50 µL of stop solution was added to each well. The plate was then read on a Microplate Reader (Zenix-320) at a wavelength of 450 nm, which had been pre-heated for about 15 min before the optical density (OD value) measurement. The measurement was repeated three times for each sample, and the average was taken.

Measurement of HOMA-IR

The Homeostasis Model Assessment-estimated Insulin Resistance (HOMA-IR) is an index used to determine the occurrence of insulin resistance in metabolic syndrome rat models or the diagnosis of type 2 diabetes mellitus (T2DM). This index was measured using two parameters, FBG and insulin levels. HOMA-IR was determined using the formula: fasting insulin level (µU/mL) × fasting blood glucose (mg/dL):405. The HOMA-IR value of >2.00-2.20 was assigned as the cut-off for diabetic rats (Antunes et al., 2016). HOMA-IR was monitored periodically at 25, 30, 35, and 40 weeks to determine disease progression in the model used. The measurement was repeated three times for each sample.

Measurement of IRS1, PI3K, and GLUT4 gene expression

According to the manufacturer's instructions, RNA was extracted from cardiac tissue using PrimeZol. Approximately 3 g of tissue was collected and mashed with a sterile mortar and pestle, and 500 µL of PrimeZol was added gradually until tender. RT reactions were done using a ReverTra Ace kit (Toyobo, Japan). The RNA expression level was determined

using the LightCycler 96 PCR system (Takara, Japan) and the GoTaq Green Master PCR kit (Promega, Madison, United States) according to the manufacturer's instructions. The sequence of the primers was 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'; IRS1 forward 5′-AAG CAC CTG GTG GCT CTG TA-3′, reverse 5′-TCA GGA TAA CCT GCT AGA CC-3; PI3K-r1, forward: 5′-CCT CTC CTT ATA AAG CTC CTG GAA-3'; reverse: 5′-GAT CAC AAT CAA GAA GCT GTC GTA A-3'; and GLUT4, forward: 5′-CTT CCT TCT ATT TGC CGT CCT C-3'; and reverse: 5′-GCT GCT GTT TCC TTC ATC CTG-3'. The cycle of PCR was as follows: 5 min at 95°C for predenaturation; 29 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C; and a 10 min extension at 72°C. The mRNA expression level of the target gene was normalized to the expression level of β-actin. The results were analyzed with ImageJ software. Each sample was replicated three times.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 and GraphPad Prism 9.0 software. Data were presented as mean ± standard deviation. The mean area under the curve (AUC) was calculated. Comparison of BW, FBG, TG, HDL, INS, and HOMA-IR results are shown as t-test results for the AUC of the curve. Test for data normality and homogeneity using the Kolmogorov-Smirnov/Shapiro-Wilk test and Levene's test ($p > 0.05$). Statistical tests were carried out using Duncan's ANOVA and post-hoc Tukey test. P-values less than 0.05 were considered significant.

RESULTS

Decrease in BW, FBG, TG, and increase in HDL in METS model rats given combination therapy with green tea-green coffee extract and metformin

Based on this study, the curves and AUC values of each parameter can be seen in Fig. 1A-D and Table 1. In BW measurements, the NORM group rats had the lowest values among all groups. Meanwhile, METS has the highest score. Among the three groups given therapy, the lowest score was on MFN, followed by COMB and GTCE. Body weight after 30 weeks in the MFN, GTCE, and COMB groups decreased significantly compared to the NORM ($p<0.001$ *vs*. NORM, $p<0.05$ *vs.* METS). Levels of FBG are also reduced with the therapy. The lowest FBG value was obtained from the NORM control, and the lowest was in the COMB group. All groups had significantly different FBG values than NORM ($p<0.05$ *vs*. NORM). TG levels decreased with therapy, with the lowest values in the group given GTCE and COMB therapy. The METS, MFN, and GTCE groups had significantly different TG values than NORM ($p<0.05$ *vs*. NORM).

Meanwhile, HDL levels also experienced improvement, as indicated by an increase in AUC. The highest score was in the NORM group, followed by COMB, MFN, and GTCE. The difference in HDL levels was significant in the MFN group ($p = 0.0007$ *vs.* NORM). All treatment groups differed significantly in each parameter with a $p<0.0001$.

Improvement of insulin resistance by reducing HOMA-IR in the green tea-green coffee extract and metformin therapy group

As measured by insulin levels and FBG, insulin resistance showed promising results. Insulin levels were found to be the lowest in the MFN and COMB groups and differed significantly ($p<0.05$ *vs*. METS). Meanwhile, for METS and NORM, the results were not entirely different. Therefore, the measurement of insulin levels alone cannot show its effect on glycaemic control, so an index measuring insulin resistance through the HOMA-IR is used. The HOMA-IR curve in this study had the highest AUC in the METS group, while the COMB group had the lowest score in the treatment group ($p<0.05$ *vs*. METS) (Fig. 1E-F, Table 1). All treatment groups differ significantly in each parameter, with $p = 0.0434$ for INS and $p<0.0001$ for HOMA-IR.

Increased IRS1, PI3K, and GLUT4 gene expression in the green tea-green coffee and metformin treatment group

Gene expression on three parameters in the cardiac insulin signalling pathway showed promising results. The IRS1 gene expression observed in this study showed an increase after therapy. IRS1 gene expression in the positive control group (METS) showed low results (0.214 ± 0.038). Meanwhile, the NORM group had the highest IRS1 gene expression (1.452 ± 0.193). In the therapy group, the effect of metformin therapy alone resulted in the lowest gene expression (0.236 ± 0.029). However, gene expression in the GTCE therapy group was higher (0.459 ± 0.131). IRS1 gene expression was highest in the COMB group, 0.789 ± 0.058. The five groups differ significantly, with a $p = 0.000$ (Fig. 2A).

Measurements of PI3Kr1 gene expression yielded different values between groups, with the highest gene expression values in the NORM group (0.673 ± 0.054) and the lowest gene expression in the METS group with values of 0.129 ± 0.032. The MFN therapy group found that PI3Kr1 expression was still relatively low, with a relative expression of 0.158 ± 0.045. The administration of single green tea and green coffee

extract (GTCE) therapy also did not significantly increase the expression of PI3Kr1, 0.160 ± 0.027. However, an increase in PI3Kr1 gene expression occurred in the COMB group by 0.283 ± 0.028. Although unable to restore PI3Kr1 gene expression as in the NORM group, increased PI3Kr1 gene expression was indicated as a positive effect of METS given green tea-green coffee combination therapy as a metformin add-on. Based on the one-way ANOVA analysis, it was found that there were significant differences between the treatment groups with p = 0.000 (Fig. 2B).

Downstream of insulin signalling are proteins for glucose uptake. GLUT4 carries out this process as a glucose molecule transport protein. Although GLUT4 works via translocation from the cytoplasm to the plasma membrane, measurement of GLUT4 gene expression is also known to start from the transcription level. GLUT4 gene expression in the NORM group was the highest in all groups. In the NORM group, GLUT4 gene expression was 1.198 ± 0.030 and decreased in the METS group to 0.151 ± 0.010. GLUT4 gene expression increased with metformin (MFN) therapy at 0.167 ± 0.060. In this study, GLUT4 gene expression did not improve much by green tea-green coffee extract therapy only in the GTCE group 0.158 ± 0.051. In combination therapy, there was a significant increase in GLUT4 expression to 0.437 ± 0.114. GLUT4 gene expression in this study differed significantly between groups with p = 0.000 (Fig. 2C).

Table 1. Total area under curve (AUC) analysis results.

Variables	NORM	METS	MFN	GTCE	COMB	p-value
BW (gram)	4466.00 ± 176.0^{a}	7732.00 ± 321.0^{b}	6419.00 ± 247.0^{c}	6638.00 ± 257.0^{c}	6619.00 ± 282.0^{c}	0.000*
FBG (mg/dL)	1559.00 ± 134.5^{a}	4941.00 ± 379.9^{b}	4738.00 ± 326.4^{b}	4298.00 ± 478.3^{b}	4252.00 ± 478.3^{b}	0.000*
TG (mg/dL)	1660.00 ± 145.2^{a}	3892.00 ± 208.8^{b}	2921.00 ± 178.3^{c}	2706.00 ± 226.0^{c}	2708.00 ± 188.1^{c}	0.000*
HDL (mg/dL)	779.10 ± 35.1^{a}	445.70 ± 33.6^{c}	576.40 ± 57.3^{bc}	545.50 ± 26.2^{bc}	658.70 ± 37.4^{ab}	0.000*
INS (µU/mL)	2345.00 ± 254.4^{ab}	2427.00 ± 195.1^{a}	1830.00 ± 162.8^{b}	2244.00 ± 299.4^{ab}	1911.00 ± 309.1^{b}	0.002*
HOMA-IR	22.11 ± 1.7^{a}	71.41 ± 2.1^{b}	53.33 ± 2.3^{b}	59.05 ± 2.2^{b}	51.79 ± 4.6^{c}	0.000*

Data are shown as mean ± standard error for week 25th to 40th (n = 4 weeks). The AUC unit was represented as the units of the Y-axis (variables) times units of the X-axis (weeks). Treatments marked with an asterisk (*) or different letters (^{a,b,c}) were considered significantly different by ANOVA followed by a Tukey's test (p<0.05). BW: body weight (gram x weeks); FBG: fasting blood glucose (gram x weeks); TG: triglycerides; HDL: high-density lipoprotein cholesterol INS: insulin; HOMA-IR: homeostatic model assessment for insulin resistance; NORM: negative control; METS: positive control; MFN: metformin therapy; GTCE: green tea and green coffee extract therapy; COMB: metformin with green tea and green coffee extract therapy.

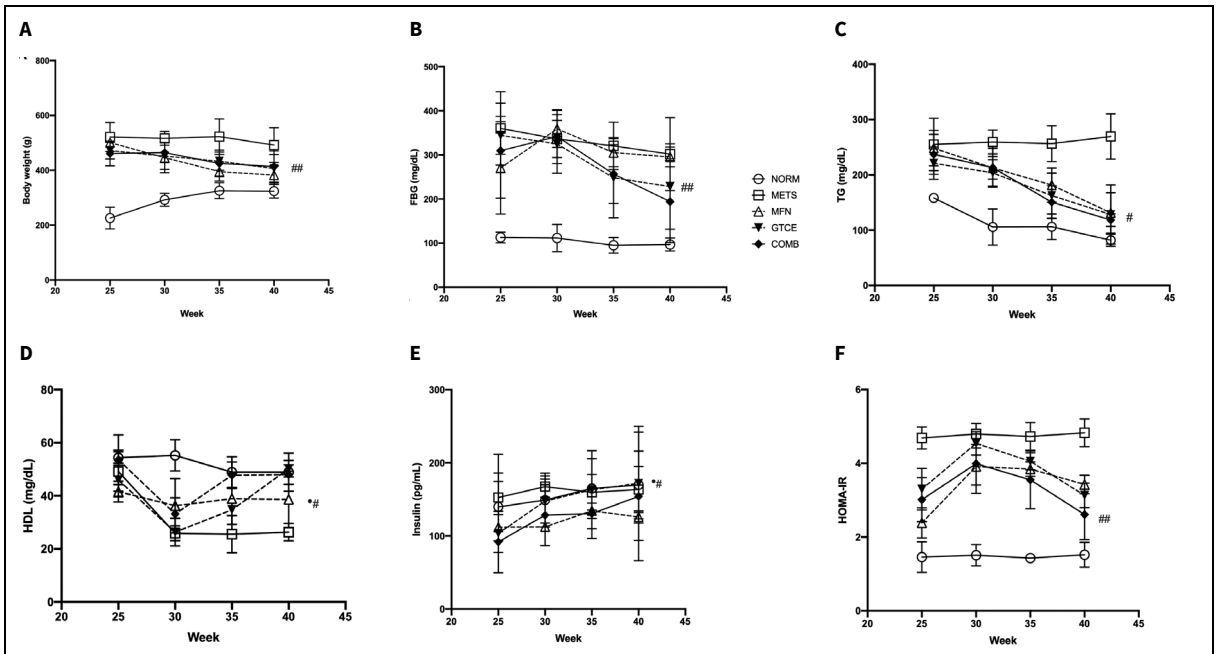

Figure 1. Measurement of METS risk factors and findings in this study. **(A-F)**: BW, FBG, TG, HDL, INS and HOMA-IR results in all groups at each time point. Data are shown as t-test results for the AUC for five rats in each group.

Data are expressed as mean ± SEM or SD (n = 5). Treatments not sharing the same letters in the same row were significantly different by ANOVA followed by a Tukey's test (p<0.05). BW: body weight; FBG: fasting blood glucose; TG: triglycerides; HDL: high-density lipoprotein cholesterol INS: insulin; HOMA-IR: homeostatic model assessment for insulin resistance; NORM: negative control; METS: positive control; MFN: metformin therapy; GTCE: green tea and green coffee extract therapy; COMB: metformin with green tea and green coffee extract therapy.

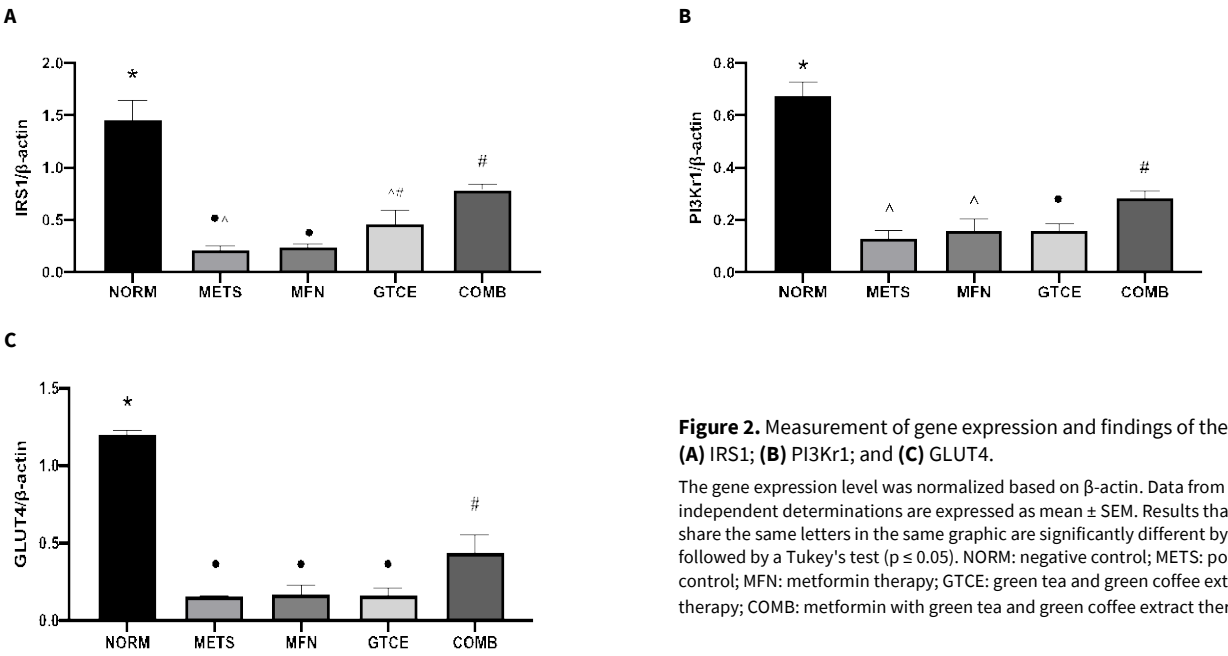

Figure 2. Measurement of gene expression and findings of the study. **(A)** IRS1; **(B)** PI3Kr1; and **(C)** GLUT4.

The gene expression level was normalized based on β-actin. Data from three independent determinations are expressed as mean ± SEM. Results that do not share the same letters in the same graphic are significantly different by ANOVA followed by a Tukey's test ($p \leq 0.05$). NORM: negative control; METS: positive control; MFN: metformin therapy; GTCE: green tea and green coffee extract therapy; COMB: metformin with green tea and green coffee extract therapy.

DISCUSSION

The risk factors for METS are related, which places a person at high risk of developing cardiovascular disease and type 2 diabetes mellitus. The NCEP-ATP III (2002) uses three components of risk factors to define a METS diagnosis, which is used in this study as the main parameter to test therapy success. The main components of METS examined in this study show promising results. Significant reductions in body weight, fasting blood sugar, and triglyceride levels occurred after being given combination therapy with green tea, green coffee extracts, and metformin. In contrast, HDL levels increased until the end of the therapy period. This condition is due to the effect of the polyphenol EGCG from green tea and CGA from green coffee, which supports metformin performance. Several other studies show that EGCG and CGA significantly reduce obesity in experimental animals and human subjects. EGCG and CGA are known to have anti-obesity properties associated with reduced epididymal white adipose mass and affect the characteristics of serum lipids (Cho et al., 2010; Legeay et al., 2015; Li et al., 2018; Rochlani et al., 2017; Wang et al., 2019), as well as increasing the excretion of fatty acids through the faeces (Janssens et al., 2016; Li et al., 2018). EGCG and CGA are also known to be able to inhibit the expression of genes involved in the synthesis of fatty acids such as ACC1 (Lin et al., 2021; Murase et al., 2011), FAS (Zhang et al., 2006; 2009; Zheng et al., 2014), C/EBPβ (Marino et al., 2022; Tipoe et al., 2010), PPARγ (Lukitasari et al., 2018; Vasileva et al., 2020), and lipolysis-related genes (HSL) in mice treated with HFD (Li et al., 2018; Wang et al., 2022; Xu et al., 2019). Meanwhile, according to a meta-analysis by Gillani et al. (2021), metformin significantly affects weight loss and lipid profile improvement. Still, several studies disagree because of differences in dosage and side effects on the digestive tract caused by metformin (Kooy et al., 2009). However, metformin influences obesity through appetite suppression with the gut-brain axis (Yerevanian and Soukas, 2019). Metformin has been shown to increase the secretion of the incretin glucagon, which promotes weight loss, peptide 1 (GLP-1) (Mulherin et al., 2011; Napolitano et al., 2014) and the anorectic hormone peptide YY (PYY) (DeFronzo et al., 2016). This condition is thought to be a secondary effect due to changes in bile acid changes through interaction with the farnesoid X receptor, which is caused by metformin in the GI tract (Lien et al., 2014). Changes in genes related to lipogenesis and lipolysis can also be one of the underlying mechanisms. This reason causes the combination of green tea, green coffee extracts, and metformin to have a good effect on weight loss and fasting blood sugar, along with improving the lipid profile in METS.

In this study, it was found that there was a significant decrease in the insulin resistance index in the green tea-green coffee and metformin combination therapy group ($p<0.0001$). Tissue sensitivity to insulin greatly influences glucose metabolism. This state is because insulin has the function of increasing glucose uptake, reducing circulating glucose levels in the blood plasma, and increasing the conversion of glucose into molecules that can be stored as energy reserves, such as glycogen or fat (Forlenza et al., 2018; Nakrani et al., 2021). EGCG is known to improve in-

sulin resistance by scavenging ROS, thereby blocking IRS-1 transduction and preventing IRS-1 from binding to insulin receptors by reducing tumour necrosis factor (TNF), which is induced by c-jun NH2-terminal kinase (JNK) phosphorylation (Yan et al., 2012). On the other hand, CGA has been shown to stimulate and increase insulin-mediated glucose transport, thereby increasing the use of glucose in the muscles. The effect of CGA in insulin-mediated glucose transport suggests that CGA may act via a pathway that differs significantly from insulin signalling, which was also reported by Ong et al. (2012). The study found that the most probable is the effect of CGA on glucose transport mediated by Adenosine Mono-Phosphate-Activated Protein Kinase (AMPK).

Meanwhile, metformin activates the AMPK, inhibiting enzymes that play a role in gluconeogenesis and glycogen synthesis in the liver, along with stimulating insulin signalling and glucose transport in the muscles. The AMPK signalling pathway is the main pathway in the mechanism of action of metformin (Seo-Mayer et al., 2011; Sung and Choi, 2012). Metformin will then increase peripheral non-oxidative glucose through increased glucose disposal into skeletal muscle (Malin and Stewart, 2020; Rösen and Wiernsperger, 2006). This condition could be the underlying mechanism and increased effect of improving insulin resistance by combining green tea, green coffee and metformin.

Several conditions play an essential role in the progression of METS and its manifestations in the heart. One condition that plays an essential role in METS is glucose metabolism in the heart muscle. To investigate the underlying molecular mechanism of the improvement effect of the therapy on glucose metabolism in the heart, we analysed the genes related to insulin signalling in the heart. This study showed that by administering a combination therapy of green tea, green coffee and metformin, there was a significant increase in IRS1, PI3Kr1, and GLUT4 gene expression ($p<0.001$). This finding is supported by research by Ueda-Wakagi et al. (2019), which proved that EGCG increases GLUT4 expression and membrane translocation through PI3K/Akt activation in obesity and T2DM genetic models. Green tea also increases glucose-stimulated insulin secretion via the cyclic adenosine monophosphate (cAMP)/Akt pathway. In addition, EGCG can boost the closure of insulin stress signalling pathways caused by serine phosphorylation of insulin receptor-1 (IRS-1) substrates (Meng et al., 2019). CGA stimulates glucose transport through AMPK activation (Ong et al., 2012). Meanwhile, metformin was reported to increase insulin receptor (IRS2) activity and glucose uptake through increased translocation of the glucose transporter in the plasma membrane (Gunton et al., 2003). As a result, metformin will increase insulin-mediated suppression of the gluconeogenesis process. This result suggests that the three therapeutic components work synergistically in reducing METS parameter risk factors.

The heart is known to have the ability to utilize all classes of substrates to meet high energy demands, and its primary preference is fatty acids (Kolwicz et al., 2013; Shao and Tian, 2015). This regulation can be changed according to conditions and responds to environmental changes (Shao and Tian, 2015). However, a shift in substrate preference from fatty acids to glucose is associated with a loss of metabolic flexibility due to dependence on glucose utilization and high glucose levels (Karwi et al., 2018; 2021). This mechanism then contributes to the development of cardiac dysfunction. In this study, improvements in physiological parameters and gene expression related to glucose metabolism in the heart are expected to provide an overview of effective and efficient METS therapy development. Further research is needed to review the beneficial effects of green tea and green coffee and mechanisms in other pathways related to glucose and lipid metabolism in the heart organ due to METS.

CONCLUSION

Therapy using a combination of decaffeinated green tea and green coffee extract as metformin's add-on improved METS risk factors by reducing body weight, fasting blood glucose, and triglycerides, increased high-density lipoprotein cholesterol, and improved insulin resistance significantly. The combination of green tea, green coffee and metformin also enhanced the expression of genes related to cardiac insulin signalling, such as IRS1, PI3Kr1, and GLUT4.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Chomsy IN	Rohman MS	Khotimah H	Widodo N	Nugrahini NIP
Concepts or ideas	x	x	x	x	x
Design	x	x	x	x	x
Definition of intellectual content	x	x	x	x	x
Literature search	x		x		x
Experimental studies	x				x
Data acquisition	x				
Data analysis	x				x
Statistical analysis	x				x
Manuscript preparation	x				
Manuscript editing	x				
Manuscript review	x	x	x	x	x

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