

Peppermint oil prevented oxidative stress in experimental animal – induced acute single bout of eccentric exercise (ASBEE): study on blood catalase and hydrogen peroxide (H₂O₂) and glucose transporter-4 (GLUT-4) expression on the muscle cells

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ABSTRACT

Peppermint oil is one of the essential oils with antioxidant activity that can reduce levels of reactive oxygen species (ROS). An acute single bout of eccentric exercise (ASBEE) is an acute exercise activity that can lead to increased ROS and cause skeletal muscle injury. This study aimed to assess the effect of peppermint oil in experimental animals induced with ASBEE with the purpose to measure catalase, hydrogen peroxide (H₂O₂) blood and glucose transporter-4 (GLUT-4) expression of skeletal muscle cells. A total of 30 Wistar rats (*Rattus norvegicus*) aged 20-24 weeks, weighing 160-350 g were divided into six groups i.e. T₁ (n=5), T₂ (n=4) and T₃ (n=5) given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C₀ (n=5) not given peppermint oil and not induced with ASBEE; C_A (n=5) not given peppermint oil and induced with ASBEE and C_E (n=5) given vitamin E 400 mg/kg one h before induced with ASBEE. ASBEE induction was done by downhill running on a rat treadmill -5° with a load index of 70% VO₂ max for 30 min. Twenty four h after induction of ASBEE, blood samples and muscle tissue were taken for examination of catalase, H₂O₂ and GLUT-4 expression. The results showed increased levels of blood catalase and decreased blood H₂O₂ levels in groups T₁, T₂, T₃, and CE. The opposite occurred in the group CA. The GLUT-4 expression did not show any significant difference between groups. It was concluded that peppermint oil can improve the condition of oxidative stress caused by ASBEE.

INTISARI

Minyak pepermin merupakan salah satu minyak esensial yang memiliki aktivitas antioksidan yang dapat menurunkan kadar *reactive oxygen species* (ROS). *Acute Single bout eccentric exercise* (ASBEE) merupakan olahraga akut yang dapat menyebabkan peningkatan ROS dan berakibat cedera otot rangka. Penelitian ini bertujuan untuk mengkaji efek pemberian minyak pepermin terhadap hewan coba yang diinduksi ASBEE terutama mengkaji katalase, hidrogen peroksida (H₂O₂) darah dan ekspresi *glucose transporter* (GLUT-4) sel otot rangka. Sebanyak 30 ekor tikus wistar (*Rattus norvegicus*) usia 20-24 minggu dengan berat 160-350 g dibagi menjadi enam kelompok perlakuan. Kelompok T₁ (n=5), T₂ (n=4) dan T₃ (n=5) diberi minyak pepermin per oral dosis bertingkat 0,25; 0,5 dan 1,0 g/kg BB satu jam sebelum diinduksi ASBEE; kelompok C_A (n=5) tidak diberi apapun dan diinduksi ASBEE; kelompok C₀ (n=5) tidak diberi apapun dan tidak diinduksi ASBEE; dan kelompok

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C_E (n=6) diberi vitamin E dengan dosis 400 mg/kgBB satu jam sebelum induksi ASBEE. Induksi ASBEE dilakukan dengan *downhill running* -5⁰ pada treadmill tikus (Gamatread) dengan beban 70% indeks VO₂ *max* selama 30 menit. 24 jam setelah induksi ASBEE, sampel darah dan jaringan otot diambil untuk pemeriksaan katalase, H₂O₂ dan GLUT-4. Hasil penelitian menunjukkan terjadi peningkatan kadar katalase darah serta penurunan kadar H₂O₂ darah pada kelompok T₁, T₂, T₃, dan C_E. Terjadi sebaliknya pada kelompok C_A. Ekspresi GLUT-4 tidak menunjukkan adanya pengaruh bermakna pada setiap kelompok. Minyak peppermint dapat memperbaiki kondisi stress oksidatif yang diakibatkan oleh ASBEE akan tetapi tidak terbukti mencegah resistensi insulin sementara setelah induksi ASBEE.

Keywords: peppermint oil - acute single bout of eccentric exercise - glucose transporter - 4 - catalase - hydrogen peroxide

INTRODUCTION

Acute single bout of eccentric exercise (ASBEE) is an exercise activity that is done with a duration of 30 minutes or less and maximum intensity greater than or equal to 80% ± 6 metabolic equivalents (METs). The ASBEE is included in the category of very hard that can cause physical stress due to the increase of free radicals especially reactive oxygen species (ROS).^{1,2} Imbalance production of ROS and mitochondrial antioxidant can lead to oxidative stress.^{3,4} In addition, the eccentric contractions can induce muscle fibers damage lead to inflammatory process, oxidative stress and delayed onset muscle soreness (DOMS).⁵

The DOMS are the effects of inflammation and oxidative stress in muscles that were damaged during ASBEE. The inflammation further increases ROS production through the activity of neutrophils, lymphocytes and proinflammatory cytokine activity. One of the increased ROS molecules and primary cause of DOMS is hydrogen peroxide (H₂O₂).⁶ This molecule can stimulate a number of other ROS molecules, such as the highly reactive radicals hydroxyl.⁶ Increasing H₂O₂ molecules that are not the same as antioxidants that reduce hydrogen peroxidase, there will be an increase in oxidative stress that can damage other molecules in the body such as proteins, lipids and DNA.⁷

The increase of H₂O₂ in skeletal muscle tissue causes insulin resistance by disrupting glucose uptake into muscle cells

through translocation of glucose transport-4 (GLUT-4) inhibition to the sarcoplasm.⁸ The degradation of signal proteins GLUT-4 by ROS during ASBEE increases oxidative stress, insulin resistance, and DOMS.^{9,10} These problems can reduce exercise performance and slow the recovery process. To prevent such problems many athletes use exogenous antioxidant supplements, such as vitamin C and E. However, Paulsen *et al.*¹¹ reported that the vitamins supplementation in athletes for 11 weeks before exercise can reduce proteins that are essential for the adaptation of aerobic exercise. In addition, Steinbachr and Eckl¹² reported that vitamin C and E can inhibit transcription of endogenous antioxidants.

Essential oils from medicinal plants are potential exogenous antioxidants sources. Peppermint oil is one of the essential oils that is widely available in the market and inexpensive. It is widely used for the pharmaceutical, cosmetic, and food industries. Peppermint oil contains high monoterpenes, poliphenol and flavonoids. These molecules were proven to have high antioxidant activity.^{13,14} This study aimed to investigate the effects of antioxidant activity of peppermint oil in animal model induced with ASBEE. It's effect on catalase, H₂O₂ blood and GLUT-4 of skeletal muscle cells were reported.

MATERIAL AND METHODS

Animals model and testing materials

Thirty males Wistar rats (*Rattus*

norvegicus) aged 20-24 weeks were used in the study. Rats were divided into 6 groups i.e. T₁ (n=5), T₂ (n=4) and T₃ (n=5) given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C₀ (n=5) not given peppermint oil and not induced with ASBEE; C_A (n=5) not given peppermint oil and induced with ASBEE and C_E (n=5) given vitamin E 400 mg/kg one h before induced with ASBEE. The rats were acclimatized in a 12 : 12-h light-dark cycle in individual cage for 7 days and allowed to food as well as to water *ad libitum*. The protocol of the study was approved by the Ethics Committee of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta (number: 00 098/04/LPPT/XI/2016).

Peppermint oil was purchased from PT. Bratachem, Indonesia and prepared as solution in 10% Tween 20 at 10% concentration. Vitamin E (E-300 Nature) was purchased from PT Darya-Varia, Indonesia and prepared as solution in 10% Tween 20 at dose of 400 mg/kg.

ASBEE induction

On day 7 of acclimatization, VO₂max in rats were measured. Furthermore, on day 8 the rats were placed on a rats treadmill with the lowest rate (10 m/min) with tilt -5°, followed by increasing running speed of 5 m/min every 3 min. The treadmill was discontinued when rats were experiencing fatigue as indicated by exposed to foot shock > 3 times or the rats reversed direction and not move. The maximum speed and duration of the running rats were recorded. VO₂max index was calculated by multiplying the maximum achievable speed (m/min) with body weight (kg).¹⁵ The rats were then rested without exercise for 2 days for recovery. Induction of ASBEE was performed on the 11th day by downhill running on a rats treadmill tilt -5° with a load index of 70% VO₂max for 30 min. Twenty four hours after ASBEE induction, blood samples and muscle tissue were taken for catalase, hydrogen peroxide and GLUT-4 examinations. During the induction, the other groups of rats were placed in another

room to minimize additional stress.

H₂O₂ and catalase measurements

Twenty four hours after ASBEE induction (on day 12), rats were anesthetized using ketamine at a dose of 60 mg/kg. Blood samples were taken and centrifuged at 10,000 g for 15 min at a temperature of 4° C. The blood plasma was taken for H₂O₂ and catalase examinations. The H₂O₂ scavenging activity was measured according to the method of Gulcin *et al.*¹⁶ A solution of 40 mM H₂O₂ as control solution (A₀) was prepared in phosphate buffer at pH 7.4. The blood plasma sample as test solution (A₁) was added to 0.6 mL of 40 mM H₂O₂ solution and leaved at room temperature for 10 min. The absorbance of H₂O₂ in control and test solution were measured spectrophotometrically at 230 nm against a blank solution containing phosphate buffer without H₂O₂. The percentage of H₂O₂ scavenging of the solutions were calculated using the following equation : % scavenged [H₂O₂] : [(A₀ - A₁)/A₀ x 100%], where A₀ was the absorbance of the control solution, and A₁ was the absorbance of the test solution.

The catalase activity was measured colorimetrically using colorimetric assay kit (Biovision-US). As much as 2-78 µL of blood samples or 1-5 µL of positive control solution was added and adjusted volume to total 78 µL with Assay Buffer. Sample High Control (HC) was prepared in separate well with the same amount of sample then bring total volume to 78 µL with Assay Buffer. Stop Solution (10 µL) was added into the sample HC into the sample HC, mix and incubated at 25 °C for 5 min to completely inhibit the catalase activity in samples as High control. Fresh 1 mM H₂O₂ (12 µL) was added into each well of samples, positive control, and sample HC to start the reaction, incubated at 25 °C for 30 min and then 10 µL Stop Solution into each sample well, except the sample HC, to stop the reaction. The absorbance was then measured colorimetrically at 570 nm. Signal change by catalase in sample was $\Delta A = A_{\text{HC}} - A_{\text{sample}}$. A_{HC} was the reading of sample High Control, A_{sample} was the reading

of sample in 30 min. ΔA was then applied to the H_2O_2 standard curve to get B nmol H_2O_2 decomposed by catalase in 30 min reaction. Catalase activity was calculated using the following equation : catalase activity = $[(B/30 \times V) \times \text{sample dilution factor mU/mL}]$, where B was the decomposed H_2O_2 amount from H_2O_2 Standard Curve (in nmol), V was the pretreated sample volume added into the reaction well (in mL) and 30 was the reaction time 30 min. One unit of catalase was amount of catalase that decomposed 1.0 μmol of H_2O_2 per min at pH 4.5 at 25 °C.

GLUT-4 expression examination

The GLUT-4 expression was immunohistochemically analysed using anti GLUT-4 antibody (IF8): sc 53566 (Santa Cruz Biotechnology, Inc., USA). At the end of the experiment, rats were euthanized through intramuscular injection of ketamin 80 mg kg^{-1} . The muscle tissue was taken from the triceps brachii of the right front foot. The tissue was fixed in formalin solution for 24 h and then processed for immunohistochemical examination by a standard method using paraffin. The tissue was incubated with H_2O_2 in methanol for 15 min and then washed with phosphate buffered saline (PBS) pH 7.4. The tissue was incubated with background sniper solution at room temperature for 15 min and washed with PBS. The tissue was then spilled with anti-GLUT-4 primary monoclonal antibody and left at room temperature for 2 h. After washing with PBS, the tissue was spilled with biotinylated IgG and left at room temperature for 20 min. Furthermore,

the tissue was spilled with avidin biotin HRP and left at room temperature for 30 min and then it was visualized by using diaminobenzidine (DAB). The tissue was counterstained with HE and washed with PBS. The observations were performed using a photomicroscopic camera and a light microscope of 10 time-magnifications in 10 visual fields per sample preparation. The results were considered as GLUT-4 positive expression if the cell indicated brown color. The color intensity was calculated by using a computer programs (Adobe Photoshop CS6 and Macbiophotonic Image J) and scored as (negative); 1 (low positive); 2 (positive); and 3 (high positive).

Statistical analysis

Data were presented as mean \pm standard error of mean (SEM) and analysed using one-way ANOVA or Kruskal Wallis test depend on normality of the data. A p value < 0.05 was considered to be significant.

RESULTS

The maximum running speed and VO_2 max index

The mean maximum running speed and VO_2 max index of each group are presented in TABLE 1. No significantly difference in maximum speed running and VO_2 max index were observed in this study.

Overview of inflammation of the muscles in general

The HE staining results showed some mononuclear cells slightly more dominant in

TABLE 1. The maximum speed running and VO₂ max index (mean ± SEM) of each group

Group	n	Maximal speed running (m/min)	VO ₂ max index (kg m/min)
T1	5	41.60±3.867	11.97±1.428
T2	4	48.50±4.907	11.93±1.232
T3	5	42.00±5.366	9.36±1.265
C0	5	-	-
CA	5	42.40±3.600	11.64±1.389
CE	6	46.83±2.508	12.29±0.985

Note: T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C0 was normal rat which not given peppermint oil and not induced with ASBEE; CA was negative control which not given peppermint oil and induced with ASBEE and CE was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

group C_A. In this group mononuclear collection of cells were found in muscle interstitial tissue. In the other groups (except group C₀) mononuclear cells were also present in the

interstitial area. Group C₀ did not appear to have mononuclear cells in muscle tissue and interstitial area (FIGURE 1).

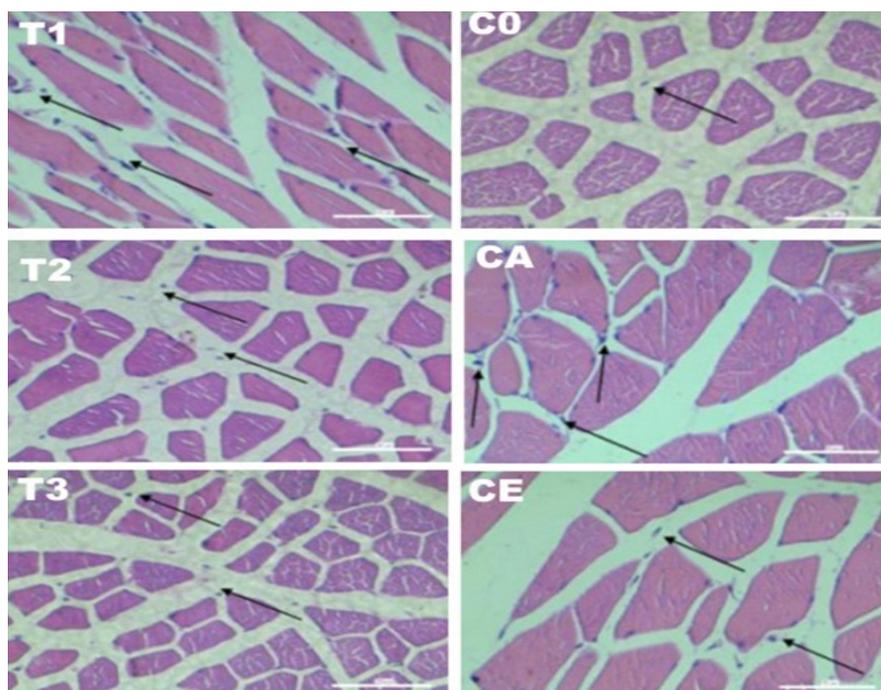


FIGURE 1. Hematoxylin-eosin staining results of skeletal muscle tissues. T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C0 was normal rat which not given peppermint oil and not induced with ASBEE; CA was negative control which not given peppermint oil and induced with ASBEE and CE was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE. The arrow (→) indicates the collection of mononuclear cells (10x magnification light microscopy) and scale 50 μm.

Levels of catalase and H₂O₂ in Blood

The blood catalase level of rats without peppermint oil administration and induced with ASBEE (C_A group) was significantly lower and the blood H₂O₂ level was significantly higher than that without induced with ASBEE (C₀ group) (p<0.05). In addition, the blood catalase level of rats after peppermint oil administration and induced

with ASBEE (T₁, T₂ and T₃ groups) was significantly higher than that C_A group and it was significantly lower than that C₀ group. In contrast, the blood H₂O₂ level of T₁, T₂ and T₃ groups were significantly lower than that C_A group and they were significantly higher than that C₀ group (p<0.05). No significantly different in blood catalase and H₂O₂ levels between C₀ group and C_E group were observed (p>0.05).

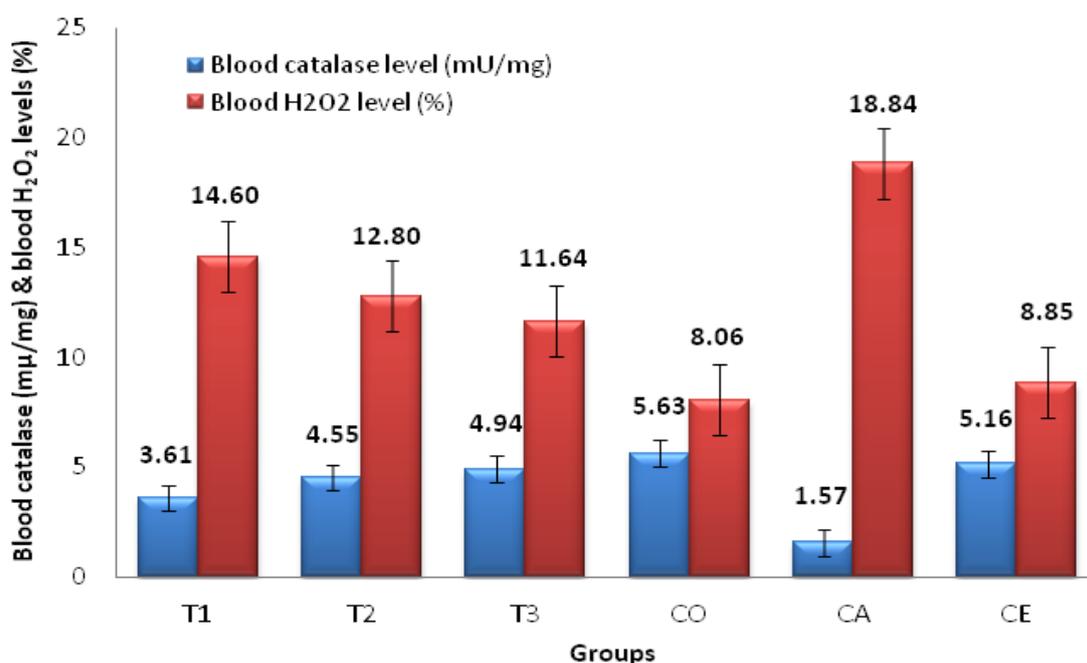


FIGURE 2. Blood catalase enzyme and H₂O₂ levels. T1, T2, and T3 were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C₀ was normal rat which not given peppermint oil and not induced with ASBEE; C_A was negative control which not given peppermint oil and induced with ASBEE and C_E was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

GLUT-4 expression

Low positive in the GLUT-4 expression (score <1) in the muscle tissue was observed in all groups (FIGURE 3 and 4). Moreover, no significantly different in the GLUT-4 expression was reported (p>0.05). In general,

the GLUT-4 expression was observed in closed sarcoplasm. However, in the C_A group the GLUT-4 expression appeared less strong and was observed cytoplasm. The GLUT-4 expression in group C_E, C₀, T₁, T₂ and T₃ was similar in color and location of the expression.

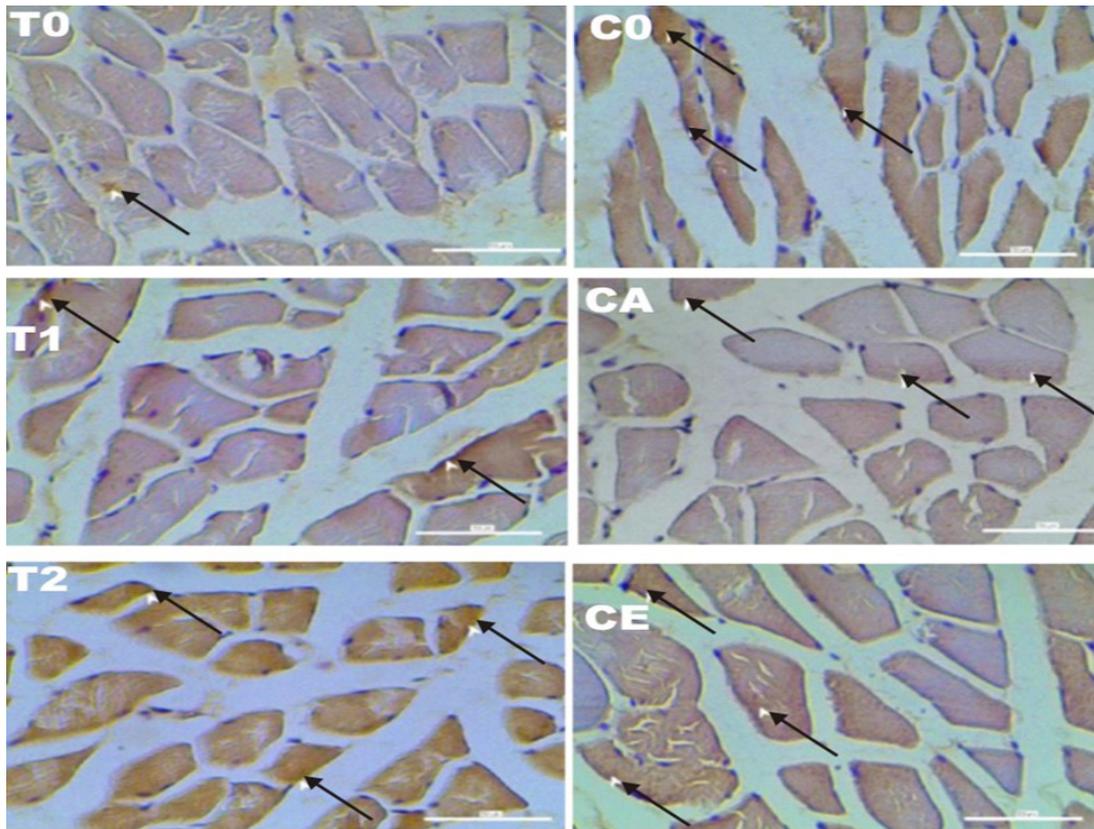


FIGURE 3. The expression of GLUT-4 in skeletal muscle tissue in each group. T₁, T₂, and T₃ were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C₀ was normal rat which not given peppermint oil and not induced with ASBEE; C_A was negative control which not given peppermint oil and induced with ASBEE and C_E was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE.

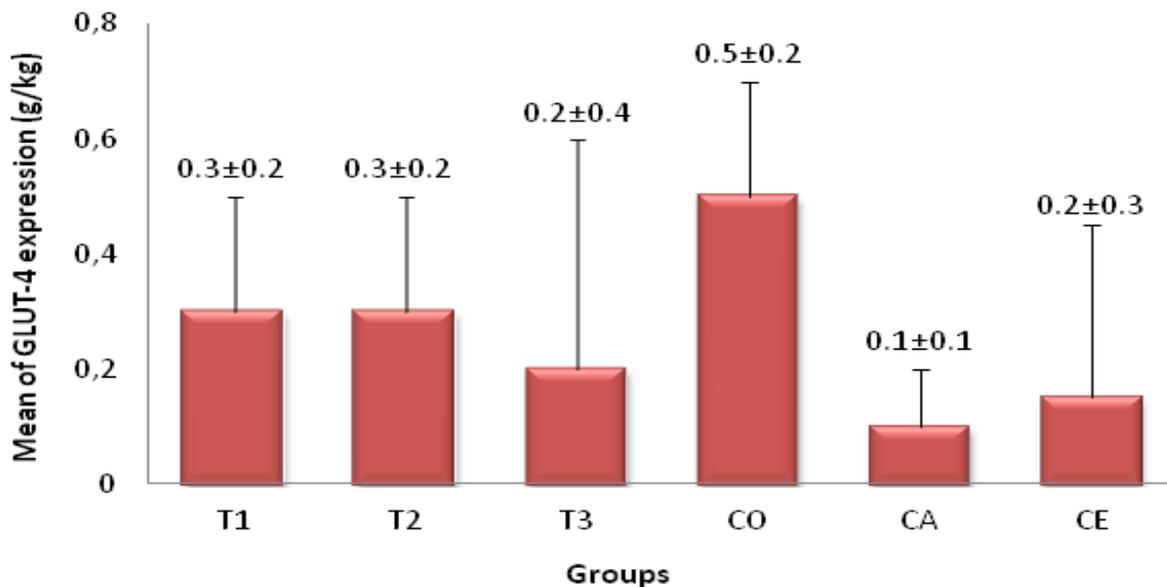


FIGURE 4. The color intensity of GLUT-4 expression in skeletal muscle tissue in each group. T₁, T₂, and T₃ were the treatment group which given peppermint oil orally at different dose of 0.25, 0.5 and 1.0 g/kg, respectively, one hour before inducing with ASBEE; C₀ was normal rat which not given peppermint oil and not induced with ASBEE; C_A was negative control which not given peppermint oil and induced with ASBEE and C_E was positive control which was given vitamin E 400 mg/kg one hour before induced with ASBEE

DISCUSSION

This study showed that the peppermint oil influenced oxidative stress and insulin resistance in dose dependent manner (FIGURE 2). The catalase and H_2O_2 of rats given peppermint oil (T_1 , T_2 and T_3) were higher than those of rats not given peppermint oil (C_A). Moreover, the GLUT-4 expression in skeletal muscle tissue of the rats given peppermint oil (T_1 , T_2 and T_3) was not significantly different compared to that of the rats not given peppermint (C_A). This effect demonstrates the ability of peppermint oil to donate a proton or hydrogen to a molecular oxidant and turn it into a molecule that reduces oxidative stress.^{13,17} These results are consistent with a study reported by Sroka et al.,¹⁴ that peppermint oil contain seriocitrin and rosmarinic acid, which have a considerable effect on the action of H_2O_2 scavenging.

The blood H_2O_2 levels of C_E group was not significantly different with group C_0 . It was indicated that vitamin E could decrease the blood H_2O_2 levels similar to the group that not induced with ASBEE. Vitamin E can prevent lipid peroxidation in cell membranes by means of interaction with unsaturated fatty acids and protection of the polypeptide chain protein. In addition, vitamin E acts as a peroxide scavenger.¹⁸ The blood H_2O_2 levels of T_2 and T_3 groups were not significantly different. However, they were significantly lower than that of C_A group, but significantly higher than that C_E groups (FIGURE 2). It was indicated that the peppermint oil at dose of 1.0 g/kg (T_3 group) had an antioxidant activity although its effect was lower than vitamin E at dose of 400 mg/kg (C_E group).

The antioxidant activity of peppermint oil was associated with its ability as a modulator to increase the internal antioxidants, such as catalase. This activity showed in dose dependent manner. In addition, post-hoc statistical analysis showed no significantly different in blood catalase levels between the T_3 and C_E groups. It was indicated that the antioxidant activity of peppermint oil was similar with vitamin

E. Song and Park¹⁹ reported that flavonoids (luteolin) in peppermint oil can increase endogenous antioxidant enzyme activities.

Blood catalase enzymes act as a catalyst for changing of H_2O_2 back into water. Catalase can reduce the levels of ROS molecules of H_2O_2 and prevent damage to biological molecules such as lipids, proteins and DNA in the body.³ ASBEE induced muscle tissue damage can cause additional oxidative stress which influences the metabolism of glucose in damaged muscle tissue.⁹ Glucose metabolism in muscle proteins involves GLUT-4 as the transporter of glucose into muscle cells.

The blood H_2O_2 levels of the C_A group was highest but the blood catalase levels was lowest compared to those of other groups. In addition, the blood H_2O_2 and catalase levels were likely associated with the GLUT-4 expression (FIGURE 2 and 4). These findings are consistent with a study reported by Aoi et al.²⁰ that many oxidative damage in lipids, proteins and DNA found in the skeletal muscle due to high levels of ROS. Wei et al.²¹ also reported that the interference in the GLUT-4 translocation due to high levels of ROS found in skeletal muscle oxidative damage. Furthermore, Maddux et al.²² showed that one of the ROS molecules that interferes with the molecule GLUT-4 translocation to the cell membrane is the H_2O_2 .

The score of GLUT-4 expression in all groups was between 0 to 1 (FIGURE 4). It was indicated that the expression of each sample is low positive quality. However, the control group (C_0) had the highest expression. Possible causes include induction of ASBEE that caused increased oxidative stress. Higaki et al.²³ reported that in low concentrations, H_2O_2 will stimulate GLUT-4 translocation and entry of glucose in the skeletal muscles and inhibit the entry of glucose if the concentration of H_2O_2 is high.

No significantly difference in the GLUT-4 expression between groups was observed in this study (FIGURE 4). The possible causes are inflammatory conditions of muscle

tissue mostly happened in muscle interstitial areas. Panza et al.⁵ reported that the peak of inflammation in muscle contraction due to improper eccentric activity is 24-48 h. Variations in peak inflammation may occur in the sample population. A study conducted by Armstrong et al.²⁴ reported the similar results. A total of 140 male rats Sprague-Dewley were subjected to acute exercise running downhill -16 ° for 90 min and the speed of 16 m/min. Twenty four h after exercise they found that the inflammation was in the interstitial area of the muscles. These conditions allow the levels of ROS in muscle tissue derived from the inflammation to remain low. In addition, due to the eccentric contractions, ASBEE causes damage to the collagen matrix of the muscle tissue. Damage to the collagen can invite mononuclear cells to the interstitial area.²⁴ One h after ASBEE, ROS is generated as a result of the ischemia-reperfusion process of muscle tissue and activates NFκB xanthine oxidase. Active NFκB causes the appearance of the inflammatory cascade to proceed slowly (inflammation after 24 h). Inflammation after 24 h of injury may increase the production of ROS. ROS are actively produced from the aging process of cell neutrophils and mononuclear phagocytes. This increase in ROS can enhance the increase damage in muscle tissue and cause widespread inflammation. As a result, DOMS can occur for an extended time.²⁰

One limitation of this study is in addition to the time variation of inflammation, the blood H₂O₂ levels were measured from venous blood and not derived from muscle. This complicates the direct identification of oxidative stress that occurs in the muscles since H₂O₂ content of the blood is an accumulation of all body tissues involved in the sport. The GLUT-4 expression in cell membranes and oxidative stress can not be separated from the other intracellular signaling proteins. One of the proteins that affect the activities above is peroxisome proliferator activated receptor γ coactivator 1-α (PGC1-α). This protein is a central regulator of energy metabolism and numerous

in cells that have a high metabolism.²⁵ Chun and Arany²⁶ concluded that PGC1-α may decrease the activation of phosphorylation of Akt protein on insulin-dependent pathways that prevents insulin resistance. Pagel-Langenickel et al.²⁷ reported that PGC1-α prevents insulin resistance by inducing mitochondrial metabolism so diacylglycerol (DAGs) molecules are decreased. The DAG molecules inactivate phosphorylation of Akt protein. If the DAG molecules are reduced, then the phosphorylation can be increased.

Related to the importance of antioxidants, proteins PGC1-α activity increases cellular defense against oxidative stress. PGC1-α is one of the molecular regulators of the energy metabolism and is associated with the expression of antioxidants. Valle et al.²⁵ said that down regulation of PGC1-α protein can decrease the production of Mn-SOD (superoxide dismutase manganese-) on endothelial cells. This result may occur in skeletal muscles. Barbosa et al.⁷ added explanation that increased expression of the enzyme catalase appears to increase transcription PGC1-α.

This study has some limitations in linking the findings with molecular PGC1-α. In addition, this study is limited to groups of adult male Wistar rats. Further research is recommended to study the difference in subjects samples if studies are conducted in groups of older male Wistar rats and the results are associated with the process of degeneration (ageing). Furthermore, exploratory study of the relationship between the regulator molecule PGC1-α with the effects of peppermint oil as an exogenous antioxidant can confirm our results as an effective antioxidant. This study contributes a review related to the benefits of peppermint oil in preventing the negative effects of exercise attributed to oxidative stress from ASBEE.

CONCLUSION

Peppermint oil can increase blood catalase levels and decrease blood H₂O₂ levels in adult male Wistar rats with oxidative stress

induced by ASBEE. However, peppermint oil has not been shown to increase the GLUT-4 expression in this study.

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