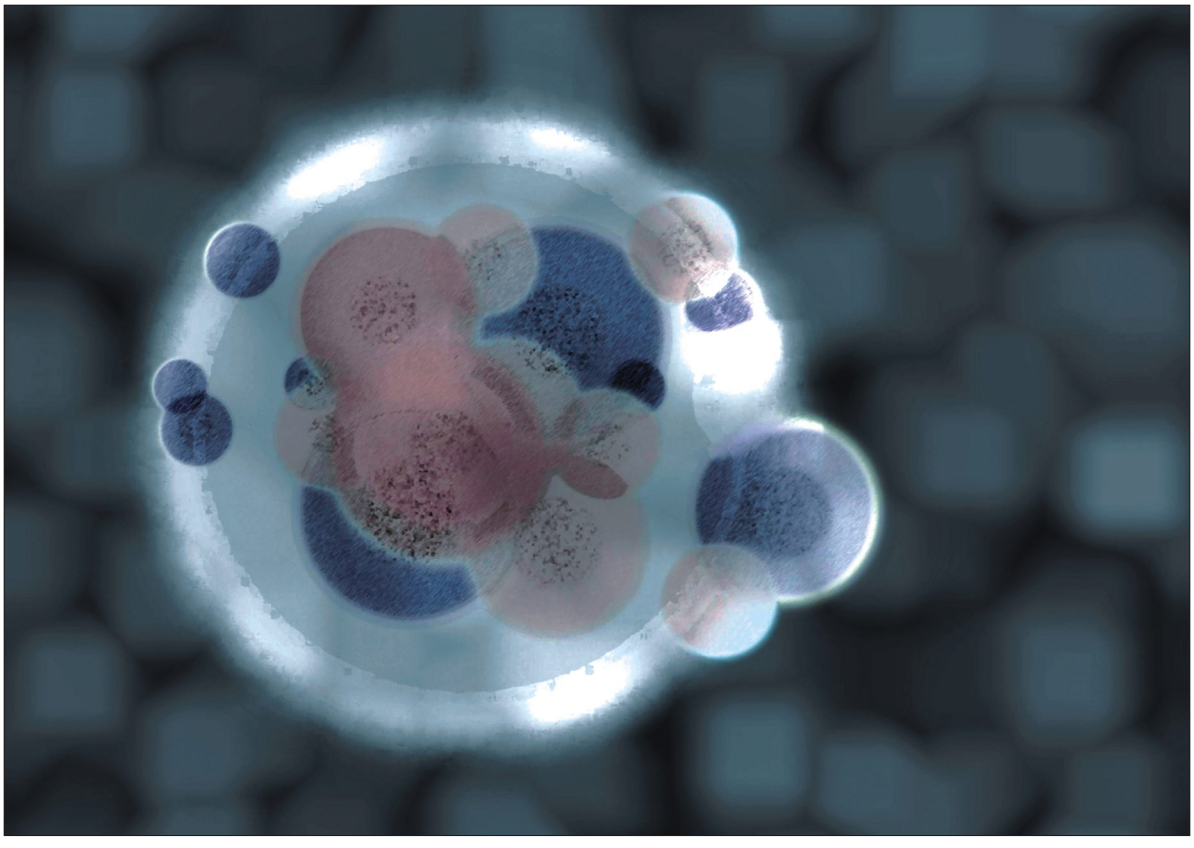

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Research Article
Analysis of Endogenous Antioxidants of Rat Blood Exposed to E-Cigarette

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Abstract

Background and Objective: E-cigarettes (e-cig) produce smoke with high levels of free oxidants, which can be eliminated by endogenous antioxidants such as glutathione peroxidase (GPx) and catalase (CAT) in the human body. The GPx catabolizes hydrogen peroxide (H_2O_2), while catalase counteracts free radicals by catalyzing H_2O_2 into H_2O and O_2. However, oxidative stress is shown in the imbalance condition between free oxidants and endogenous antioxidants. The effect of e-cigarette smoking on the human body has not been discovered. Therefore, this research aimed to analyze the levels of GPx and CAT in the rat blood exposed to e-cig. **Materials and Methods:** The exposure to smoke with nicotine 3, 6 and 9 mg was applied to 30 rats divided into five groups for 30 days. The analysis results by one-way ANOVA showed significant differences in the GPx and CAT levels between the control and treatment groups. Nicotine at 9 mg gave the GPx level at 40.25 ± 2.03 U mg^{-1} and CAT at 2.46 ± 0.50 nmol mL^{-1}. **Results:** The results indicated that e-cigarette smoke reduced the levels of endogenous antioxidants in rat blood. Moreover, the body weight of rats increased and decreased due to the unstable metabolic rate. **Conclusion:** Exposure to e-cigarette smoke has an effect on reducing glutathione peroxidase (GPx) and catalase (CAT) levels in rat blood.

Key words: Antioxidants, e-cigarette, glutathione peroxidase, catalase, glycerol, reactive oxygen species, carcinogens

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The e-cigarette (e-cig) is a popular electronic vaping device that produces an aerosol from liquid heating. The power source for the heat is a lithium-ion battery. Now-a-days, people use it as a part of their lifestyle as an alternative replacement to traditional smoking. People assume that e-cig smoking is healthier than tobacco-based cigarettes. They believe that e-cig composition is less harmful than the conventional one, i.e., nicotine, water, propylene glycol and flavour enhancers. Moreover, it seems safer than tobacco cigarettes and does not violate smoking-free regulations. However, the smoke generated from the device enters the respiratory tract directly into the lungs, the same as the process in tobacco cigarette smoking[1,2].

The results of the study reported that e-cigs contain toxic Tobacco-Specific Nitrosamines (TSNA) and diethylene glycol (DEG), which are known carcinogens[3]. The liquid in the e-cig contains propylene glycol, glycerol, distilled water, flavourings and nicotine solvents or nicotine plus plaster, which will turn into nitronism when exposed to heat. Nitronism is one of the causes of cancer[4]. Also, the nicotine levels in e-cigs vary from 0, 3, 6, 9 and 12 mg at each refill. After heating and evaporation occur, the nicotine in e-cigs is not wholly turned into vapour. Therefore, the nicotine concentrations in e-cigs smoke are unstable depending on the smokers' smoke[5]. Many studies on e-cigs have been carried out, but there is no agreement on the conclusion related to their impact on health due to the unavailability of sufficient objective data to serve as strong reasons.

The particles produced from the e-cig's combustion can potentially cause an excessive number of free radicals, which endogenous antioxidants cannot neutralize. Thus, oxidative stress will occur, leading to reactive oxygen species (ROS) formation and causing oxidative instability. The instability occurs when free radicals cause lipid peroxidation in cell membranes and affect cell integrity, which can eventually form several degenerative diseases such as cancer, atherosclerosis, hypertension, ischemia, Alzheimer's, Parkinson and inflammation[6].

Moreover, the smoke from e-cigs also contains pollutant gases, including carbon monoxide (CO). When it enters the bloodstream, CO binds to the haemoglobin replacing the oxygen molecules, causing the limitation of oxygen distribution to the tissues. On average, after smoking e-cigs, the CO concentration in the human body was found at 2.5-5.0%[7]. Free radicals caused by cigarette smoke are unstable and have high reactivity. This high reactivity can damage all cellular macromolecules, including carbohydrates, proteins, fats and nucleic acids. Macrophages, neutrophils and eosinophils will detoxify physiologically free radicals from e-cigarettes that enter the bronchial airways. However, an increase in the amount of excess free radicals in the airways will trigger the movement of macrophages, neutrophils and eosinophils which can cause an inflammatory reaction.

The prominent free radicals that play a role in inflammatory reactions are superoxide radicals because they have a longer half-life, so the number of target cells affected increases and can cause more cell damage and death[8]. Under conditions of oxidative stress, free radical compounds increase lipid peroxidation. In regular enzyme activity, an increase in the concentration of the substrate, the molecule hydrogen peroxide (H_2O_2), can increase the enzyme activity. However, when the active side of all enzymes binds to the substrate, the addition of the substrate cannot increase the speed of the subsequent enzyme reaction, resulting in a decrease in enzyme activity. Glutathione peroxidase (GPx) and catalase (CAT) are enzymatic antioxidants that are essential in dealing with oxidative stress. The GPx enzyme functions to catalyze hydrogen peroxide (H_2O_2). The mechanism of the GPx is one of the main ways the body uses to protect itself from oxidative damage. The GPx also functions to catalyze the reduction of hydrogen peroxide and fat peroxide (LOOH) by glutathione (γ-glutamyl stein sintetase)[9]. The CAT enzyme functions to catalyze H_2O_2 to H_2O and O_2. If the amount of free radicals is not balanced with the ability of antioxidants to reduce it, then the enzyme catalase activity will be reduced. Catalase enzymes include hydroperoxides enzymes that protect the body against harmful peroxide compounds. The buildup of peroxide compounds can produce free radicals, which will further damage the cell membrane and possibly cause cancer and atherosclerosis, which can inactivate hydrogen peroxide. The activity of oxidase enzymes produces H_2O_2 compounds. The H_2O_2 can potentially cause free radical effects because it forms OH[10].

There have been many kinds of research on free radicals and impact-based e-cigarettes. The body's defenses to overcome these effects have not been discussed, namely through endogenous antioxidants. A study is needed relating to endogenous enzymes and antioxidants to find out whether the smoke generated by suction contains nicotine which has the potential to become free radicals and affects cellular metabolism in the body. The endogenous antioxidants associated with free radical activity need to be primarily studied in the GPx and CAT.

MATERIALS AND METHODS

Study area: This research was conducted in 2021 for 6 months. The e-cigs were purchased in the local market in Semarang, Indonesia. The Wistar rats were obtained and maintained in the Biology Laboratory, Universitas Negeri Semarang, Indonesia. All chemicals used in this research were of analytical grade and procured from local suppliers and distributors.

Research design: Wistar rats were exposed to e-cigs and the GPx and CAT levels were analyzed after treatment. Thirty experimental animals were used for male Wistar rats, aged 2-3 months, weighing 200-300 g and healthy. Rats were divided into five groups into randomized posttest only with control group design with group design as presented in Table 1. The research flow and design were shown in Fig. 1.

E-cigs treatment for rats and blood plasma analysis: The control group was exposed to traditional cigarettes 2 times per day in the morning and afternoon. Then, the treatment group was exposed to 2.5 mL e-cigs in the morning and afternoon. The smoking treatment was conducted in the chamber using a nebulizer (Fig. 2). After 30 days of exposure, rat blood samples were withdrawn to examine the concentration of GPx and CAT.

Blood sampling was performed on the 31st day of the study. Blood was withdrawn from the orbital sinuses with 3 mL of hematocrit and collected in Eppendorf tubes containing EDTA. Then, the collected blood was centrifuged at 1,000 rpm for 10 min at 4°C. The blood plasma was transferred into a new tube and stored at -80°C until analysis. The plasma was measured for its GPx and CAT.

Glutathione peroxidase (GPx) assay: The GPx level was measured using a spectrophotometer at 340 nm wavelength at the PAU Food and Nutrition Laboratory at Gadjah Mada University, Indonesia. The blood plasma sample (100 µL) was diluted with 200 µL of 0.85% (w/v) NaCl solution. Subsequently, 0.1 mL of the solution was transferred into a new tube and added with 0.4 mL of 0.5% (v/v) Triton X-100. This mixture was called a hemolysate. Then, 100 mL of

Table 1: Group of treatment on Wistar rats

Groups	Treatments
K-	A negative control group, rats were fed and drinking without treatment
K+	A positive control group, rats were exposed to clove cigarette smoke for 30 days
KP_1	A treatment group, rats were exposed to 3 mg of cigarette smoke for 30 days
KP_2	A treatment group, rats were exposed to 6 mg of cigarette smoke for 30 days
KP_3	A treatment group, rats were exposed to 9 mg of cigarette smoke for 30 days

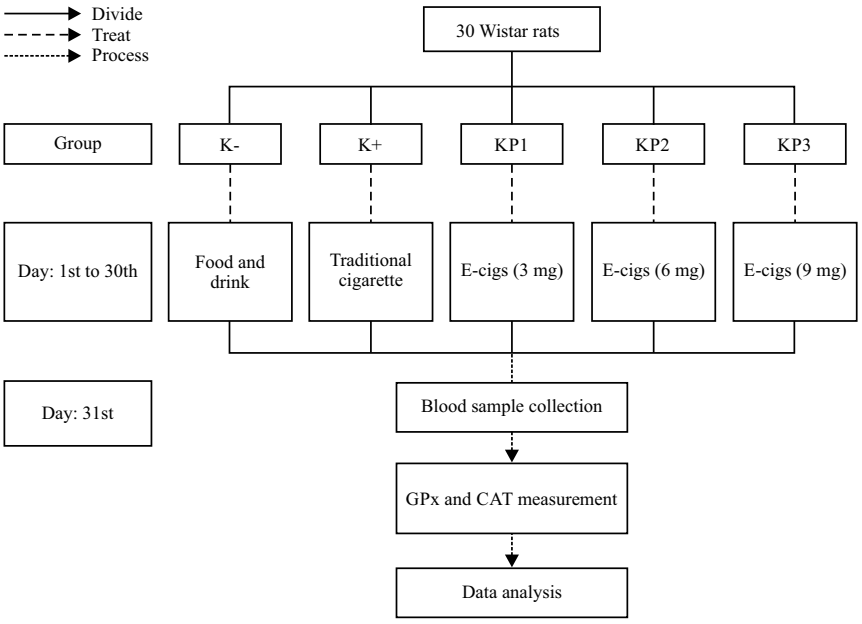

Fig. 1: Research flow and design used in this study, 30 Wistar rats were subjected to the treatment for GPx and CAT measurement

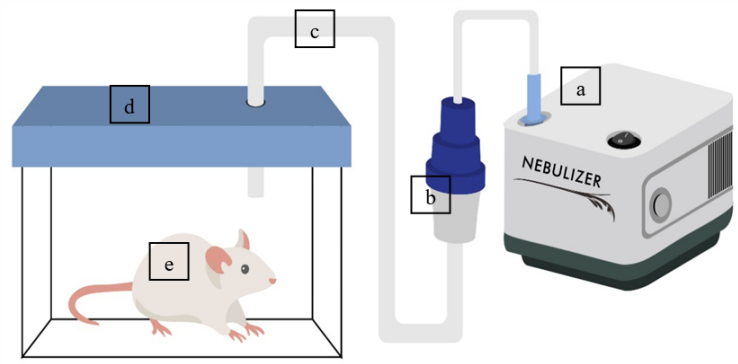

Fig. 2: Tools for smoking treatment process which consists of (a) Nebulizer, (b) E-cigs, (c) Hose, (d) Lid and (e) Smoking chamber

hemolysate was taken into the test tube and 100 mL of Drabkin solution was added. The solution was mixed using a shaker, added with 2.6 mL of 0.1 M phosphate buffer pH 7.0 and slowly shaken at 50 rpm. Next, 0.1 mL NADPH, 0.01 mL GSSG-R, 0.01 mL NaN_3 and 0.1 mL GSH were added and shaken. Finally, 1 mL H_2O_2 was added to the cuvette and mixed with the 100 µL hemolysate. The reaction was performed between 1-2 min intervals and the OD_{340} was measured. Distilled water (100 uL) was used as the blank instead of hemolysate. The following Eq. 1 performed the calculation of the GPx level[11,12]:

$$\text{GPx (U mg}^{-1}) = 8412 \times \Delta\ OD_{340}/\text{time (min)} \quad (1)$$

Catalase (CAT) assay: The CAT level assay was carried out by adding 800 µL 0.5% (v/v) Triton X-100 to 200 µL blood plasma. Then, the stock solution was prepared by dissolving 10 µL of catalase in 50 mL 0.1 M phosphate buffer pH 7.0. The standard solution was prepared by dissolving 0.5 mL of stock solution in 9.5 mL phosphate buffer (ratio 1:20) and 0.5 mL of stock solution in 19.5 mL phosphate buffer (ratio 1:40). Next, 10 µL lysate was mixed with 12.5 mL phosphate buffer. The reaction started after 1 mL of H_2O_2 was added. The whole solution was slowly vortexed and then the decrease in absorbance was measured by a spectrophotometer at a wavelength of 240 nm[11,12]. The catalase calculation was performed following Eq. 2:

$$\text{CAT (nmol mL}^{-1}) = \frac{\text{Absorbance test-}\Delta\text{ blank absorbance/time (min)}}{(\text{Molarity } H_2O_2)\times(\text{Measured sample volume})} \times \text{Dilution factor} \quad (2)$$

Data analysis: Data analysis was carried out statistically in this study using parametric statistics. If the data were not normally distributed and homogeneous, then Mann-Whitney non-parametric statistics were used. The data normality was tested using the Kolmogorov-Smirnov Test and then the data homogeneity was measured with the Levene Test[12]. Data analysis was performed with one-way ANOVA and continued with the Least Significant Differences (LSD) Test. Statistical analysis was assisted by the SPSS program for windows version 2.0.

RESULTS

The results of the glutathione peroxidase (GPx) level and catalase (CAT) level measurement of all groups were presented in Table 2. One-way ANOVA test results showed that exposure to e-cigs significantly affected GPx levels in rat blood. The lowest level of GPx (40.25 U mg^{-1}) was found in the experimental animal group exposed to e-cigs smoke at 9 mg concentration. The GPx levels in this group were significantly different from other groups. The Kruskal-Wallis non-parametric test results of exposure to electric cigarettes significantly affected catalase (CAT) levels in rat blood. At the same time, the Mann-Whitney test results showed differences between treatment groups. The highest decrease in CAT level (2.46 nmol mL^{-1}) was found in the experimental animal group with exposure to smoke with a concentration of 9 mg.

The body weight of rats was measured using a balance, the results were shown in Fig. 3. The weight of rats in both the control and treatment groups experienced an increase and decrease in body weight during the treatment process. There was a significant increase in body weight at week 2 for K-, KP_1 and KP_2 rats (weight difference increased at >30 g), while the rats in the K+ and KP_3 groups gained not too significant weight at week 2 (weight difference decreased at <10 g). The highest weight gain at week 2 was obtained in KP_2 rats (weight difference increased at 86 g). KP_2 rats that experienced the most significant weight gain at week 2 experienced weight loss at week 3 and the peak continued to decrease at

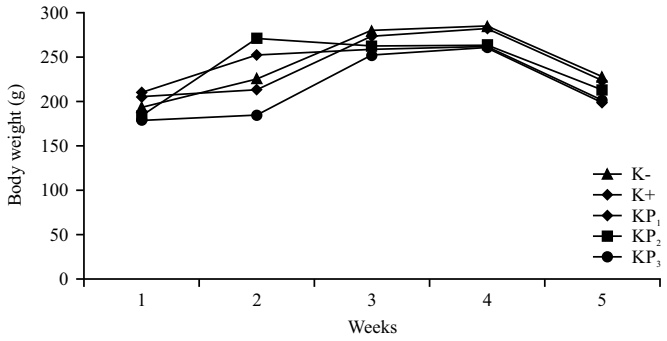

Fig. 3: Average body weight of rats (g) in all groups measured every week for 5 weeks

Table 2: Glutathione peroxidase (GPx) levels and catalase (CAT) levels in rat blood of all groups

Groups	Treatments	GPx level (U mg^{-1})	CAT level (nmol mL^{-1})
K-	Negative control/no treatment	78.33±1.90[a]	6.59±2.70[a]
K+	Positive control/traditional cigarette	19.80±2.42[b]	1.86±1.50[b]
KP_1	Electric cigarette (3 mg)	72.02±1.66[c]	5.06±1.50[c]
KP_2	Electric cigarette (6 mg)	58.52±2.82[d]	3.93±1.50[d]
KP_3	Electric cigarette (9 mg)	40.25±2.03[e]	2.46±0.50[e]

Number followed by the letters in the same column shows the significant difference in each treatment group with a level of accuracy of $p<0.05$

week 5. The pattern of significant weight gain occurred at week 2 for K+ and KP_3, but they experienced weight loss at week 3. In groups, K-, KP_1 and KP_2, the peak of weight gain was at week 3 and quite stable at week 4, but there was a pattern of decline at week 5. Rats in group K- experienced a weight gain (weight difference increased at 34 g) until week 5, while K+ increased only 18 g. The KP_1 rats experienced a drastic weight loss (weight difference decreased at 11 g) at week 5 compared to the initial weight. Meanwhile, for rats in the KP_2 and KP_3 groups, the weight gain at week 5 was significantly different with an increase at >20 g.

DISCUSSION

Many people think that e-cigs or electronic nicotine delivery systems (ENDS) are healthier when compared to conventional cigarettes made from tobacco due to the chemicals contained in e-cigs being less than conventional cigarettes[13]. However, the Indonesian Ministry of Health reports that e-cigs composition will turn into nitronism when heat exposure occurs. Nitronism is one of the causes of cancer[14] and the added flavour content of e-cigarettes is harmful to humans, including nitrosamines, toxic chemicals such as diethylene glycol and tobacco-specific components of anabasine, myosmine and betanicotyrine[15].

Table 2 showed the antioxidant enzymes GPx and CAT in both negative control and treatment groups. The GPx and CAT were lowest in the e-cigs treatment at 9 mg and comparable to the positive control results (smoking with traditional cigs). The significant differences in GPx and CAT levels may be due to the increased generation of superoxide anion and hydrogen peroxide, resulting in the generation of hydroxyl free radicals. It has been demonstrated that the formation of these free radicals participates in a variety of hazardous processes and elevated levels of superoxide and hydrogen peroxide may harm alveolar macrophages by releasing proteolytic enzymes[16]. Exposure to traditional cigs in rats reduced levels of GPx significantly compared to normal conditions. The GPx is one of the main compounds for endogenous protection from oxidative damage. This enzyme catalyzes the reduction of hydrogen peroxide and fatty peroxide (LOOH) by glutathione. The sulfhydryl group on glutathione (GSH) functions as an electron donor and is oxidized to the disulfide form (GSSG), which will be reduced by glutathione reductase to the sulfhydryl form. The GPx is an enzymatic antioxidant, one of which requires Selenium (Se) for its activity so that GPx levels can describe Se levels as well. The lower the GPx level, the lower the Se level in the blood. This is due to the effects of cigarette smoke which contains potent free radicals and can cause oxidative stress. Oxidative stress can cause disturbances in the oxidation process, resulting in increased free radicals production. This increase in free radicals is due to the antioxidants available in the body no longer able to convert reactive oxygen (O*) into neutral compounds (O_2). These antioxidants provide a very effective defense, but severe oxidative stress can deplete available antioxidants.

The K- group (negative control) had the highest CAT level, significantly different from all other groups. The K+ group (positive control) had lower catalase levels than the K- group (negative control) and was the group with the lowest catalase levels compared to the other groups. Exposure to traditional cigs given to the K+ group (positive control) decreased the level of the catalase enzyme in the blood. Traditional cigs contain harmful substances such as nicotine, tar and cadmium, which can cause liver cell damage and inhibit/reduce catalase biosynthesis. The CAT enzyme levels in the blood in the K- group (negative control) had normal levels and this was due to the absence of harmful substances in the form of free radicals that entered the blood, where CAT in the liver would continue to biosynthesis effectively.

Meanwhile, the group exposed to traditional cigs experienced a decrease in CAT levels because cell damage occurred and there was a significant inhibition of catalase biosynthesis in the liver, which caused CAT to diffuse into the blood. The lowering in CAT levels in the blood of the e-cigs treatment group was significantly comparable to the negative control group. Enzymes may take the form of free radicals scavengers such as GPx and CAT in smokers. The obligatory use of reserve antioxidants to detoxify the excess free radicals results in alternating the levels of different antioxidant enzymes[17]. At the same time, the increased GPx level occurred due to increased lipid peroxidation. The GPx and CAT are among the most essential antioxidant enzymes as free radical scavengers in normal conditions. In this research, the damage caused by ROS of smoking occurs as a consequence of the imbalance between the generation and detoxification of these species, defense against oxidative stress is provided by oxidative enzymes such as GPx and CAT, which are the first line of cellular defense against oxidative damage[18].

The significant increase and decrease in body weight of rats were due to smoking chemicals within smoke on the appetite regulation centre of the hypothalamus[19]. Returning to behavioural aspects, there appears to be a significant correlation between smoking behaviours, lifestyle, body weight, BMI and the number of cigarettes smoked each day. This could be another explanation. Additionally, a higher metabolic rate could be the cause of decreased body weight and BMI[20]. Another way of this result may be to generate free radicals in tissues because of increasing lipid peroxidation by smoking[21].

It is known that cigarettes contain various kinds of chemicals contained in them. Lighting the cigarettes will produce about 4,800 chemical compounds, including nicotine, carbon monoxide gas, nitrogen oxides, hydrogen cyanide, tar, ammonia, acrolein, benzene and ethanol. Some of the content of cigarettes can harm health. The smoke from the burning has high levels of free oxidants and each inhaled cigarette smoke contains oxidant molecules. Free radicals from cigarette smoke are toxic substances for the body that has the potential to damage cells. The nicotine receptor located in the brain consists of five subunits arranged around a central space. These subunits vary in how they respond to nicotine and affect the transfer of electrical impulses, so each can produce different responses to nicotine at different levels and concentrations. Animal studies have shown that the 4β2 receptor subtype is the main receptor influencing nicotine dependence because removing the 2 subunit stops the behavioural response to nicotine. Mutations in the 4 subunits alter sensitivity to nicotine. At low doses, nicotine can stimulate the central and peripheral systems causing, among other effects, such as an increase in heart rate or blood pressure. However, at high doses, nicotine can block nAChrs, producing low blood pressure and changes in the body's capacity to release adrenaline. Nicotine can also cause the release of catecholamines and stimulate the autonomic system. There is an increase in glycogen synthesis due to adrenoceptor stimulation. Therefore, this causes a decrease in fasting blood glucose levels. It also causes lipolysis resulting in weight loss.

In a study conducted by Salam *et al.*[22] on rats exposed to e-cigarette smoke for 3 hrs a day for 3 months, with the amount of nicotine in e-cigarette smoke at 10 mg m^{-1}, they found evidence that nicotine inhaled from e-cigs can turn into chemicals that damage DNA in the hearts, lungs and bladders. Common liquid solvents, propylene glycol, glycerin and flavourings play an important role in stimulating the formation of free radicals[23]. Romagna *et al.*[24] used e-cigs containing 18 mg of cappucci-no-flavoured nicotine, experiments were carried out with four female rats exposed to e-cigs acutely (short exposure to cigarette smoke for approximately 5 min) and six female rats exposed to chronic e-cigarette smoke (4 hrs in a day for 5 days a week and 8 months of experiment). They examined the size of the diameter of the arteries and vasodilation. They found that 1 hrs after exposure to smoke from vaping for 5 min, the arteries narrowed by about 31% of their previous size. Chronic exposure to smoke will cause aortic stiffness because of cell damage and acute exposure also causes a 9% decrease in vasodilation. Toxic chemicals, such as nicotine and carbon monoxide inhaled through cigarettes that enter the bloodstream, can damage the endothelial lining of blood vessels and cause platelet interactions with blood vessels resulting in thrombosis, which causes the endothelium not to function properly. Direct exposure to cigarette smoke molecules on blood vessel walls

causes blood vessel walls to release inflammatory mediators and cytokines that indirectly cause damage to blood vessel walls. Chemicals in cigarettes also contain reactive oxygen species (ROS), which cause necrosis of vascular endothelial cells[24,25]. The serum antioxidants including GPx and CAT could be used as the basic information on the negative effects of e-cigs smoking. The chemicals in e-cigs led to many problems caused by impaired metabolisms. Also, it damages some organs such as the respiratory tract, lungs and blood vessels. Therefore, the information on the effect of e-cigs must be disseminated as fast as possible among people for better understanding.

CONCLUSION

The current study assessed the impact of e-cigarette use on serum antioxidants (GPx and CAT). To assess the negative effects of smoking on the GPx and CAT levels, the rat group was divided according to how many e-cigarettes were consumed daily. At the maximum e-cigarette intake, the results revealed a considerable drop in GPx and CAT levels, as well as a disruption in the metabolic rate that caused the increase and reduction in rat body weight. In summary, e-cigarettes impair metabolism by depleting significant amounts of serum antioxidants needed to neutralize excess free radicals.

SIGNIFICANCE STATEMENT

The study identified the endogenous antioxidants of rat blood exposed to e-cigarettes. There is a scarcity of data in Indonesia, especially on this e-cigs research. The effect of e-cigs was discussed, and this result is urgent to give the Indonesian government a point of view that they must regulate the e-cigs use among society as fast as possible, especially among teenagers.

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