ORIGINAL ARTICLE

Protective Effect of *Stichopus hermanii* Against Oxidative Stress Induced by Chronic Cigarette Smoking in Rat Salivary Glands

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ABSTRACT

Introduction: *Stichopus hermanii* (SH), which contains various antioxidant agents, tends to protect oxidative stress caused by chronic cigarette smoking (CCS). This study, therefore, aims to investigate the protective effect of SH supplementation against CCS-induced oxidative stress in rat salivary glands. **Methods:** A total of 30 male Wistar rats, which were equally divided into the control (C), cigarette smoke (CS), and treatment (T) groups, were used to carry out this research. In T group, 17 mg/kg BW of SH was administered for 90 days. Their salivary glands were removed for oxidative stress marker analyses ie malondialdehyde (MDA) level, total antioxidant status (TAS), superoxide dismutase (SOD) and catalase (CAT) activity, with the data analyzed using one-way ANOVA and Tukey's multiple comparison test to obtain a p-value of less than 0.05, which were considered statistically significant. **Results:** The results showed that in the CS group oxidative stress occurred which was characterized by significantly increased MDA levels, reduced TAS, SOD and CAT activity. While the T group significantly decreased MDA levels, enhanced TAS, SOD and CAT activity. **Conclusion:** In conclusion, SH supplementation tends to prevent oxidative stress produced by CCS in rat salivary glands.

Keywords: Chronic cigarette smoking, *Stichopus hermanii*, Salivary glands, Oxidative stress

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INTRODUCTION

Free radicals and oxidants are produced physiologically in the process of cell metabolism. When the balance between oxidants and antioxidants is disturbed it causes oxidative stress. Oxidative stress is the cause of several pathological conditions. Physiologically, free radicals are produced by immune reactions, wound healing, ATP synthesis, radiation exposure, drugs, pesticides, pollutants, smoking, and stress (1, 2).

Chronic cigarette smoking (CCS) is widely associated with an increased risk of various adverse health conditions. Many studies have shown that tobacco smoking increases the prevalence of periodontal diseases and the rate of tooth loss (3, 4). Cigarette contains nicotine, along with over 4000 bioactive and toxic compounds such as carbon monoxide and hydrogen carbide, capable of inducing oral cancer (5, 6).

The smoke also contains a number of free radicals, which increases reactive oxygen species (ROS) that cause extensive oxidative damages. In addition, the oxidants derived from smoke are a major factor in the inflammatory reaction of cigarettes. This oxidant has the ability to change the antioxidant defenses and regulate inflammation (7, 8).

Most of the pathogenesis of oral inflammation are related with reactive oxygen species (ROS) which can cause tissue damage due to oxidative stress. The human body combats oxidative stress by activating an antioxidant defense system. Antioxidants work as free radical scavengers and neutralize excess ROS, thereby protection tissue damage. ROS can cause tissue cell membrane damage known as lipid peroxidation. Lipid peroxidation can be measured by the end product such as malondialdehyde (MDA) (9,10).

Saliva is an oral cavity complex fluid that produces by

major and minor salivary glands. The saliva consists of an antioxidant capacity, which plays an important role in health (9, 10). It is the first line of defense against free radical-mediated oxidative stress, with the antioxidant capacity in patients with periodontal significantly lower compared to those that are healthy (11). Total antioxidant status (TAS) is the total salivary component which act as antioxidant properties. Salivary TAS is comprised of many elements such as superoxide dismutase, peroxidase (SOD), catalase (CAT) and non-enzyme elements including uric acid, vitamin C, reduced glutathione, and oxidized glutathione (12-17).

It is usually the first biological medium to come in contact with cigarette smoke. Therefore, smoking tends to decrease the activity of the salivary antioxidant system (10, 12). There are many studies about the role of salivary antioxidant and some of them claimed recently that an imbalance between free radicals and antioxidants in saliva may play significant roles in beginning and developing of oral disease. Furthermore, CCS also has the ability to alter the salivary antioxidant capacity that causes oxidative stress and progression of several oral inflammations (18, 19).

Today, many people consume antioxidant supplements to improve their health, in the form of pills or tablets, which have no preventive effects and are harmful to our health (20). In many developing countries, natural ingredient is an essential alternative antioxidant. According to a number of studies, *Stichopus hermanii* (SH) comprises antioxidant activities, which is due to the riboflavin, flavonoid, saponin, tannin, and omega-three compounds contained therein (21-23). SH is safe for treatment, as there is no adverse effect on its daily oral extraction for six months (24).

However, there have not been any study conducted on the evaluation of the effect of SH on oxidative stress due to incessant cigarette smoking. Therefore, this study aims to analyze the protective effect of SH supplementation against CCS-induced oxidative stress in rat salivary glands.

MATERIALS AND METHODS

SH Extraction

SH was obtained from Raas Island, Madura, East Java, Indonesia. It was cleaned, cut into pieces and dried for 3-4 days. The body wall dried samples were then pulverized in a blender, followed by the extraction process using the maceration method. Dried body wall of SH powder extracted in a row using three types of solvent by maceration method to produce different fractions namely n-hexane, ethyl acetate and ethanol. Maceration with n-hexane is continued with semipolar (ethyl acetate) and solvents ethanol as a polar solvent. Each stage is repeated until the solvent clear. The solvent is evaporated with rotary vacuum evaporator at 50^{o}C, until nonpolar fractions are formed, semi-polar and polar. Furthermore, the crude extract, which is stored at -200C in the vial (21, 22). Then a qualitative identification of secondary metabolite as bioactive compounds was tested, ie alkaloids, flavonoids, terpenoids, tannins and steroids.

Animals

A total of 30 healthy 12 weeks old male Wistar (Rattus norvegicus) rats, weighing between 200 to 250 g were used for this study. They were housed in a cage under standard conditions with the administration of commercial standard diet (triglycerides, free fatty acids, free amino acids, sugars, vitamins, and minerals) and distilled water ad libitum. The rats were also acclimatized for seven days before the experiment was conducted (25).

CCS Exposure

Exposure to cigarette smoke is carried out using an electronic machine consisting of an electronic valve, vacuum pump, timer, and inhalation chamber. A timer is used to adjust the fresh air and cigarettes smoke that enter and exit to the inhalation chamber, which are used to prevent oxygen deficiency. Rats are passively exposed to smoke once a day in the morning. Exposure was immediately removed from the burning of 5 kretek cigarettes per rat, which was carried out for 10 minutes and carried out for 90 days (26, 27).

SH Administration

SH extract was dissolved in Na-CMC 2% to form a supency and administrated using gavage at a dose of 17 mg / kg BB before CCS exposure every morning for 12 weeks.

Experimental Design

This study was carried out in line with the approved animal research and the ethical clearance committee guidelines of the Universitas Hang Tuah (No.112/KEPK/VIII/2017). The randomized post-test control group design was used to carry out the experimental laboratory research on thirty male Wister rats, which were equally divided into the control (C), cigarette smoke (CS), and treatment (T) groups. The C group (as control put into the room air), CS group (cigarette smoke exposure), and T group (CS and 17 mg/kg BW SH supplementation for 90 days). After 90 days, rats were fasted overnight and anesthetized with intraperitoneal ketamine/xylazine (60 mg/kg and 6 mg/kg) respectively. Rats were euthanized by decapitation and salivary glands were quickly biopzied (28).

Tissue Preaparation

Both of the parotid and submandibular salivary glands were taken to obtain sufficient sample volumes for a number of biochemical examination parameters. Both salivary glands are taken immediately washing out the blood with ice-cold 0.9% saline solution and

placed in microtube individually used for preparation of tissue homogenates. Specimens from each organ were weighted and stored at -80°C. Homogenates of the tissues were prepared as 1.0 g/10 mL in 250 mM sucrose, 1 mM EDTA, 1 mM DL-dithiothreitol and 15 mM Tris HCl (pH 7.4), using tissue homogenizer. Each homogenate was centrifuged for 30 min at 800 g. The resulting supernatant fraction was used to determine tissue MDA levels, TAS, SOD and CAT activities (29).

Oxidative Stress Measurement in Rat Salivary Glands

The MDA level assay method depends on the formation of MDA as an end product of lipid peroxidation, which reacts with thiobarbituric acid producing thiobarbituric acid reactive substance (TBARS), a pink chromogen, which can be measured spectrophotometrically at 532 nm (30).

TAS was measuresing the oxidation of ABTS (2,2-Azino-di-[3-ethybenzthiazoline sulphonate]) by metmyoglobin, which is inhibited by non-enzymatic antioxidants contained in the sample. Oxidized ABTS can then be detected via spectrophotometry at a wavelength of 740 nm. The capacity of antioxidants in the sample to inhibit oxidation of ABTS is compared with the capacity of known concentrations of Trolox, and results are expressed as nmol of Trolox equivalents per g tissue or ml plasma (31).

SOD is an enzymatic antioxidant that catalyses the dismutation of superoxide anions to oxygen and hydrogen peroxide. SOD activity assay by measures the percentage of superoxide radicals that undergo dismutation in a given sample. Absorbances were read at 440 nm using a spectrophotometer. Results are expressed as units of SOD activity per mg tissue (32).

CAT activity assayed based on formation of chromic acetate from dichromate and glacial acetic acid in presence hydrogen peroxide, chromic acetate that produced was measured colorimetrically at 570 nm, one enzyme unit was defined as the amount of enzyme which catalyzed the oxidation of 1 µmole H_2O_2 per minute under assay conditions. The values of catalase activity are expressed as U mg-1 protein units. One unit catalase is responsible for consumption of 1 mol of H_2O_2 per minute (33).

Total protein content was measured to determine the amount of protein contained in each gram of tissue. It was measured using the bicinchoninic acid (BCA) method. Bovine serum albumin (BSA) was used as a standard (31).

Statistical Analyses

The data are presented as mean ± standard deviation (SD). All data were tested for normality with the Kolmogorov-Smirnov test and for homogenity with the Levene test. After confirming the normality and homogeneity of data distribution, the parameters of the groups studied were compared by analysis of variance (ANOVA) and Tukey's multiple-comparison test, and a p value of <0.05 was considered significant using SPSS.

RESULTS

Secondary metabolites are non-essential metabolites for the growth of organisms and found in a unique form or vary between species and one the other. Secondary metabolism in marine organisms has produced a number of very important compounds because of their diversity and habitats. Bioactive components extracted from SH body wall samples are presented in Table I. The presence of secondary metabolite compounds in crude extracts of the SH's body wall sampled was confrmed, positive based on the test.

Table I: Qualitative analysis of crude extracts of body wall samples of SH

Secondary metabolite compounds	Present
Alkaloids	+
Flavonoids	+
Terpenes	+
Tannins	+
Steroids	+

Based on the ANOVA test, it was discovered that there was a significant difference among groups in MDA levels (p=0.000) as shown in Table II. It was discovered that there was a significant difference among groups in TAS, SOD, and CAT activities (p=0.000) as shown in Table III.

Table II: Assessment of lipid peroxidation markers (MDA level) in rat salivary gland (nmol Eq/mg protein)

Group	MDA level (Mean ±SD)	ANOVA (p-value)
C	0.29 ± 0.05	0.000*
CS	0.68 ± 0.07	
T	0.31 ± 0.06	

Note: Data are presented as mean ±standard deviation (n = 10 per group). * Significant different

Table III: Assessment of antioxidant markers ie TAS (µmol uric acid Eq/mg protein), SOD and CAT activity (unit/mg protein) in rat salivary gland

Group	Marker			ANOVA (p-value)
	TAS	SOD	CAT	
C	0.24 ± 0.05	1.24 ± 0.07	31.42 ±6.18	0.000*
CS	0.12 ± 0.03	0.58 ± 0.07	17.23 ±2.22	0.000*
T	0.23 ± 0.07	1.15 ± 0.09	30.15 ±6.32	0.000*

Note: Data are presented as mean ±standard deviation (n = 10 per group). * Significant different

Based on the results of Tukey's multiple-comparison test discovered that the lipid peroxidation (MDA) average levels was significantly higher in group CS compared with group C and group T (p=0.000). TAS, SOD and CAT average activities was significantly lower in group

CS compared with group C and group T (p=0.000) as shown in Table IV. The oxidative stress markers were significantly different (p<0.005) in group C compared to group CS and group T. Furthermore, based on the results of Tukey's multiple-comparison test discovered that the lipid peroxidation (MDA), TAS, SOD and CAT average was found no significant difference in group C when compared to the group T with a p-value above 0.05, as shown in Table IV. In summary, SH supplementation was significant less than 0,05, which proved to be able to prevent an increase in MDA levels and also the amount of reduction in TAS, SOD, and CAT activities at rat salivary glands.

Table IV: Intergroup comparison of the lipid peroxidation and antioxidant activities markers in rat salivary gland

Marker	p-value		
	Group C versus CS	Group C versus T	Group CS versus T
MDA level	0.000*	0.502	0.000*
TAS	0.000*	0.823	0.000*
SOD activity	0.000*	0.832	0.000*
CAT activity	0.000*	0.520	0.000*

Note: Data are presented p-value (n = 10 per group). * Significant different

DISCUSSION

Smoking is a bad habit that interferes with oral health and has the most important role as the initiation and development of several oral inflammatory conditions. Cigarette smoke contains a large number of toxic agents and free radicals, which can induce cytotoxic destroy in cellular components, and cellular function. Cigarette smoke changes the antioxidant capacity of saliva. Cigarette smoke exposure increases the production of ROS, which can result in impaired redox homeostasis in human bodies (6, 34). The results of the preliminary study prove that smoking can interfere with the oxidative stress mechanism of periodontal tissue, inhibit defense mechanism against plaque bacteria, blood vessel obstruction and delay wound healing (35).

Saliva is the first fluid that is exposed to cigarette smoke. Its antioxidant system plays an important role in oral health (3, 4). Therefore, this study was designed to investigate the balance between oxidant and antioxidant activity that cause an oxidative stress in saliva induced by CCS. CCS cause oxidative stress which is characterized by an imbalance between ROS and antioxidant activity in saliva. The increase in ROS was marked by a significant increase in MDA levels as one of the end products of lipid peroxidation in the CS group compared to C group. In this study, CCS significantly increased the lipid peroxidation levels of salivary glands, with MDA mostly studied. It is typically quantified with TBARS assay (30). Generally, a rise in oxygen-free radicals tend to increase the oxidative stress damaged through lipid peroxidation (14). Thousands of ROS and RNS (Reactive Nitrogen Species) in cigarette smoke can damage the structure of the cell membrane known as lipid peroxidation and produce various toxic compounds including various types of aldehydes, such as MDA. High MDA levels are an indication of high cell membrane damage and also as a marker of free radical activity (9). Previous study proves that cigarette smoke exposure indeed can also increase MDA levels and reduce antioxidant activity in blood plasma and saliva (36).

On the other hand, there was a significant decrease in the salivary antioxidant activity which was marked by a decrease in the TAS, SOD and CAT activity in the CS group compared to the C group. The prior study showed that the parameters of TAS were significantly lower in smokers (37). Lowered TAS, SOD, and CAT in this study are evidence that cigarette smoke-induced free radical generation, which significantly reduced the antioxidant (38).

The toxic agents contained in cigarette smoke, such as ROS and RNS stimulating lipid peroxidation and oxidation of proteins and DNA bases; aldehydes can modify; hydroquinone complexes diffuse across cell membranes, give rise to semiquinones and lead to the formation of superoxide radicals and hydrogen peroxide (H_2O_2). It is proved that smoking causes increased oxidative stress damage. The damage caused by ROS in cigarette smoking occurs as an imbalance between oxidant and antioxidant of these species. Defence against oxidative stress is provided by a system of enzymes and antioxidants capable of preventing excess production of ROS and neutralizing free radicals (9,38). The antioxidants have the ability to protect the body from damage due to the induction of oxidative stress. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) are an important line of defense against oxidative cell damage preventing lipid peroxidation, and protein and DNA oxidation. It also exists in submandibular and parotid salivary glands, however, its amount in the saliva of cigarette smokers is lower than that of healthy people (38).

The most promising strategy to prevent oral cavity from the oxidative damage caused by these reactive species is the use of antioxidant molecules. These compounds can act as direct antioxidants through free radical scavenging mechanisms and/or as indirect antioxidants by enhancing the antioxidant status (enzymatic and non-enzymatic). An antioxidant agent is needed that is able to prevent oxidative stress in the oral cavity due to exposure to cigarette smoke which has a key role in the initiation and development of several inflammatory conditions of the oral cavity. One natural ingredient that has antioxidant potential is SH.

SH containing secondary metabolites as a bioactive compounds with experimentally confirmed pharmacological activity. Secondary metabolites are

non-essential metabolites for the growth of organisms and found in a unique form or vary between species and one the other. The function of secondary metabolites is to defend yourself from conditions unfavorable environment, for example to overcome pests and disease, attracts pollinators, and as signaling molecules (39). Secondary metabolism in marine organisms has produced a number of very important compounds because of their diversity and habitats. Identification of secondary metabolite content is the important initial step in natural ingredients research which can be a precursor for synthesis new drugs or prototypes of active drugs certain (40). Based on the test results of the identification of secondary metabolites as bioactive components above, the SH body wall extracts used in this study proved that SH contains alkaloids, flavonoids, terpenoids, tannins, and steroids.

The effect of antioxidant supplementation on salivary gland redox balance is relatively little known. Redox state homeostasis in living systems is very complex and lifestyle factors undeniably concur in determining the impact of changes in oxidative stress response in both unhealthy and healthy subjects. A large part of studies investigating the effectiveness of antioxidant supplementation therapy in humans raised contrasting results (41). The prior study prove that Vitamin E and C supplementation reduces oxidative stress and improves antioxidant enzymes (42). The supplementation of N-acetylcysteine during a short period was effective in reducing oxidant action and increasing antioxidant action (43). But there are no research that investigates the protective effect of SH supplementation against CCS-induced oxidative stress in saliva. Our study is the first to investigate the protective effect of SH supplementation against CCS-induced oxidative stress in rat salivary glands.

In this study, SH extract supplementation eliminates the adverse effect of CCS on oxidative stress markers. It contains bioactive compounds, especially phenolic, alkaloids, flavonoids, terpenoids, tannins, and steroids. Phenolic is the main antioxidant compounds of SH (21). It protects oxidative stress by directly neutralizing reactive oxidant capacity (44). Phenolic compounds can function as antioxidants because they can reduce ROS activity, including superoxide anion radicals and hydroxyl radicals in mitochondria and the endoplasmic reticulum through redox reactions and hydrogen peroxide in saliva. Phenolic compounds have radical elimination capabilities due to their abilities to donate hydrogen and form stable radical intermediates (44). Flavonoids have efficiencies in scavenging peroxyl radicals. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions. The chelation of metals could be crucial in the prevention of radical generation which damage targets biomolecules (46). Alkaloid scavenges free-radical such as hydroxyl radical (*OH) and chelating ferrous ion. The ferrous ion chelating effects of the alkaloids have a similar pattern with their *OH scavenging effects. The mechanism of *OH scavenging effects of the alkaloids was closely related to their ferrous ion chelating activities (47). Terpenes, one of the most extensive and varied structural compounds occurring in nature, display a wide range of biological and pharmacological activities. Their effect on the chelating properties of Fe(2+). Terpenes have been shown to provide relevant protection under oxidative stress conditions in different diseases including liver, renal, neurodegenerative and cardiovascular diseases, cancer, diabetes as well as in ageing process (48). Tannins can act as antioxidant because it has free radical scavenging activity, chelation of transition metals, inhibition of prooxidative enzymes and lipid peroxidation (49). Previous studies have also shown that golden sea cucumber extract (*stichopus hermanii*) can reduce MDA level of male rats induced with streptozotocin (50).

Therefore, it is suggested that phenolic has the ability to directly neutralize ROS, reduce lipid peroxidation, and increase the activities of antioxidant enzymes such as SOD and CAT. In addition, the antioxidant and radical activity of SH is due to the oxidative stress caused by the flavonoids and phenolic compounds, which increases the activity (45, 46). It is also possible that phenolic in SH interact synergistically to protect rat salivary glands against oxidative stress caused by CCS.

The limitation of this research is that the secondary metabolite compound has not been identified quantitatively so the antioxidant compound which have the primary role as protective agent have not been determined.

CONCLUSION

In conclusion, the toxic effects of CCS on the rat salivary glands was significantly reduced by SH through the reduction of lipid peroxidation and by restoring its antioxidant. The study showed that SH supplementation protects the rat salivary glands from the oxidative stress induced by CSS.

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