COMPARISON OF PEROXIDASE RESPONSE TO MENTAL ARITHMETIC STRESS IN SALIVA OF SMOKERS AND NON-SMOKERS

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ABSTRACT — Saliva is the first body fluid to encounter exogenous materials or gases such as cigarette smoke (CS). The aim of this study was to examine whether smoking affects oral peroxidase (OPO) reactivity to mental stress. The subjects were 39 non-smokers and 10 smokers. In the experiment, the Kraepelin psychodiagnostic test as a psychological stressor and saliva was sampled 30 min before, just before, immediately after, and 30 min after the beginning of the test. OPO reactivity to the test between smokers and non-smokers was measured in addition to uric acid concentration, flow rate, IgA, thiocyanate (SCN⁻) concentration, amylase activity as a salivary stress marker, and ultra-weak chemiluminescence (UCL) level, which is indicative of salivary antioxidative and antibacterial abilities. Moreover, we studied the effect of smoking on the response of salivary peroxidase (SPO) and myeloperoxidase (MPO) activity to mental stress, respectively. The results showed that the IgA concentration, amylase activity, SCN⁻ concentration, and UCL level are higher in the non-smoking group than smoking group and the IgA concentration and UCL level increased in the non-smokers significantly just after the Kraepelin test. The levels of SCN⁻ were higher in smokers than in non-smokers and OPO activity was greater in the non-smoking group in all sessions. Furthermore, only the non-smokers had significantly increased MPO activity just after the test. MPO may play a crucial role in the response to acute psychological stress besides inflammation, and CS suppresses this response significantly.

KEY WORDS: Cigarette smoke, Oral peroxidase, Psychological stress, Salivary peroxidase, Myeloperoxidase

INTRODUCTION

Saliva is the first biological medium confronted by external materials such as food, drink, or inhaled volatile factors. An extensive amount of research has been devoted to the immunological defense mecha-

nisms of saliva, primarily based on secretory IgA (sIgA) and protein defense based on lactoferrin, lysozyme, agglutinin, mucin etc. (Nogueira *et al.*, 2005; Amerongen *et al.*, 2004; Tenovuo, 2002). Recently, the importance of another salivary defense system has become obvious; the antioxidative defense which

involves various molecules and enzymes (Nagler, 2003). The most important antioxidant is uric acid, which contributes approximately 70% of the total salivary antioxidative capacity (Nagler *et al.*, 2002), with the role of ascorbic acid being secondary (Nagao and Terao, 1990). In the enzymatic salivary antioxidative system, peroxidase is by far the most important enzyme. Other salivary enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase, and glutathione reductase, have secondary or marginal antioxidative significance (Kranendonk *et al.*, 1997).

The oral peroxidase (OPO) found in the cavity is a very important salivary antioxidant. This OPO is composed of two enzymes, salivary peroxidase (SPO) and myeloperoxidase (MPO). SPO is secreted from the major salivary glands, mainly the parotid gland (Nagler *et al.*, 2002), while MPO is produced by leukocytes in inflammatory regions of the oral cavity (Pruitt *et al.*, 1990). SPO contributes 60-80% of peroxidase activity and MPO contributes the remaining 20-40% (Nagler *et al.*, 2002; Pruitt *et al.*, 1990).

Many studies indicate the important role of cigarette smoking in reducing the body's antioxidant content and emphasize the protective effect of antioxidant nutrients in preventing tissue damage (Stone *et al.*, 1995; Zappacosta *et al.*, 1999; Reznick *et al.*, 2003; Zappacosta *et al.*, 2002). A fairly recent study demonstrated a sharp drop in OPO activity after smoking a single cigarette in both non-smokers and smokers (Reznick *et al.*, 2003) and suggested that reactive oxygen species (ROS), such as the hydroxyl radical, might cause oxidative DNA damage to the surrounding tissues (Pryor, 1997). In this respect, the saliva antioxidative system also contributes to anticarcinogenic capability.

In previous studies we showed that acute stress such as mental arithmetic increased OPO activity and changed several salivary biochemical parameters (Goi *et al.*, 2007). A possible anticarcinogenic role of OPO against the most prevalent and lethal cancer of the oral cavity has been mentioned (Nagler *et al.*, 2002; Zappacosta *et al.*, 1999; Reznick *et al.*, 2003), but whether the response of OPO activity to mental stress changes due to habits such as smoking has scarcely been investigated. The aim of this study was to examine the response of OPO reactivity to the Kraepelin test as a mental arithmetic stressor in smokers and non-smokers in addition to measuring uric acid and IgA concentrations, flow rate, amylase activity as a salivary stress marker, thiocyanate (SCN⁻) level and ultra-weak chemiluminescence (UCL) level, which is indicative of

salivary antioxidative and antibacterial abilities. Furthermore, we studied the effect of smoking on the response of SPO and MPO activity to mental stress.

MATERIALS AND METHODS

Experimental design and saliva collection

The subjects were 39 non-smokers (20 male and 19 female) and 10 smokers (9 male and 1 female), all students at the School of Pharmaceutical Science, University of Shizuoka. Their mean age $\pm$ standard deviation (SD) was 21.3 ± 1.1 (range 20-25) and 21.8 ± 0.7 (range 21-23) years, respectively. The aim of the experiment was explained to the subjects and consent was obtained after confirmation that they fully understood the experiment. The Kraepelin psychodiagnostic test (KN type, Employment Research, Tokyo, Japan) (Kuraishi *et al.*, 1957) was used as a psychological stressor and was conducted between 14:00 and 16:00, 1 hr or more after lunch or their last smoke. Subjects were assigned a Salivette (SARSTEDT, Nümbrecht, Germany) to collect saliva according to instructions. During collection, the Salivette was freely positioned in the oral cavity and therefore whole saliva composed of parotid, submandibular and sublingual saliva, was collected. Subjects were placed in a sitting position in a quiet environment for 5 min to ease their psychological stress, and then saliva was sampled 30 min before, just before, immediately after, and 30 min after the beginning of the experiment. Subjects could move freely for 30 min before and after the test. The samples were stored at -30°C until the measurements. This experimental design was approved by the Ethics Committee of the University of Shizuoka.

Salivary flow rate and protein assay

Saliva was defrosted on ice and centrifuged at 1000 g for 20 min. Only the liquid component volume of saliva was measured and the salivary flow rate was expressed in ml/min. The secretory immunoglobulin A (sIgA) level was measured by ELISA using peroxidase-conjugated goat antibody to human IgA as previously described (Takagi *et al.*, 2005). Amylase activity was determined using the quantitative kinetic determination kit (Wako, Osaka, Japan) (Flick *et al.*, 1970).

Detection of UCL

The detection of salivary UCL was measured as described before (Takagi *et al.*, 2005). In brief, 200 μl of saliva was placed on filter-paper (ADVANTEC, Tokyo, Japan) in a plastic dish (Nunc, Tokyo, Japan)

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and 1 ml of 3 mM gallic acid was added. After mixing for 30 sec at 100 rpm at room temperature, the dish was set in the UCL measuring counter C767 (Hamamatsu Photonics, Hamamatsu, Japan). After the addition of 1 ml of 3%(w/v) hydrogen peroxide (H_2O_2) to the saliva preparation, the reaction commenced. The UCL in the present study was defined as the total number of photons for 100 sec after addition of H_2O_2 .

Determination of SCN^- concentration

SCN^- concentration was measured spectrophotometrically as described by Aune and Thomas (Aune and Thomas, 1977). In brief, 50 μ l of the sample was added to a mixture of 400 μ l of 0.1 M HCl and 100 μ l of 0.1 M ferric chloride. After centrifugation at 1000 g for 1 min, the absorbance of the supernatant due to $FeSCN^{2+}$ was measured at 450 nm.

Determination of uric acid concentration

The uric acid concentration was measured with a kit (Wako). In this assay, uric acid was transformed by uricase into allantoin and H_2O_2 , which, under the catalytic influence of peroxidase, oxidized the chromogen (4-aminophenazone/N-ethyl-methylaniline propane-sulfonate sodium) to form a red compound, the intensity of whose color was proportional to the amount of uric acid present in the sample; and it was read at a wavelength of 540 nm.

Peroxidase activity

Oral peroxidase (OPO) activity was measured according to the 2-nitro-benzoic acid-thiocyanate (NBS-SCN) assay (Pruitt *et al.*, 1990). Briefly, in this assay, 5,5'-dithiobis, 2-nitrobenzoic acid (DTNB) is reduced to NBS by the addition of β -mercaptoethanol.

The disappearance of NBS while it reacted with $OSCN^-$, the product of OPO, was monitored at 412 nm at pH 5.6. One unit of activity was defined as the level needed to cleave 1 μ M of NBS/min at 22°C using a molar extinction coefficient of 12,800.

Salivary peroxidase (SPO) and myeloperoxidase (MPO) were also determined using a kinetic spectrophotometric method. The NBS-Cl activity was used to calculate the contribution of MPO to the total (SPO + MPO) NBS-SCN activity.

Statistical analysis

GraphPad Prism was used for statistical calculations. A one-way analysis of variance (ANOVA) for repeated measures was utilized for evaluating the flow rate and UCL level; concentrations of sIgA and uric acid and activities of amylase and peroxidase, following a Dunnet multiple comparison test as described in the instruction manual of GraphPad Prism. An unpaired T-test was used to evaluate differences between groups. The level of statistical significance was set at $p < 0.05$.

RESULTS

Effect of the Kraepelin test on biochemical parameters in whole saliva

To examine the effect of the Kraepelin test on biochemical parameters in whole saliva, we measured several parameters. A significant increase of flow rate in the non-smoking group was detected at 30 min after the test, but there was no significant change in the smoking group (Table 1). The IgA concentration and salivary UCL level also increased significantly just after the test in the non-smoking group but these

Table 1. Effect of the Kraepelin test on biochemical parameters in whole saliva of smokers and non-smokers.

		Time before Test (min)		Time after Test (min)	
		30	0	0	30
Flow rate (ml/min)	non-smokers ¶	1.44 ± 0.51	1.74 ± 0.56	1.73 ± 0.60	1.94 ± 0.69**
	smokers	1.68 ± 0.71	1.84 ± 0.65	1.81 ± 0.74	1.81 ± 0.72
IgA (μ g/ml)	non-smokers ¶	9.85 ± 5.20	9.72 ± 5.78	12.71 ± 5.78*	8.37 ± 4.17
	smokers	6.80 ± 2.82	5.50 ± 1.58 [#]	7.38 ± 2.08 ^{##}	6.14 ± 1.60
α -Amylase ($\times 10^4$ U/ml)	non-smokers	17.41 ± 13.50	15.66 ± 8.49	18.45 ± 11.55	16.41 ± 9.24
	smokers	14.72 ± 9.38	15.58 ± 10.08	16.86 ± 9.24	16.41 ± 9.56
UCL ($\times 10^4$) (counts/100 sec)	non-smokers ¶	13.02 ± 5.92	13.02 ± 6.53	16.62 ± 7.72*	11.44 ± 4.28
	smokers	9.73 ± 3.46	9.40 ± 2.10	11.40 ± 3.98 [#]	10.33 ± 2.85

Date are expressed as the mean $\pm$ SD (39 non-smokers and 10 smokers). ¶one-way analysis of variance $p < 0.05$. *Dunnet and **Dunnet test vs. 30 min before the test $p < 0.05$ and $p < 0.01$. [#]Unpaired and ^{##}Unpaired T-test test vs. non-smokers $p < 0.05$ and $p < 0.01$.

parameters in the non-smoking group did not change significantly. In addition, the IgA concentration and UCL level just after the test were significantly higher in the non-smokers than smokers. Amylase activity slightly increased but was not significantly affected by the test in either group.

Influence of smoking on thiocyanate concentration and antioxidant factors

The mean level of SCN^- was significantly higher in the smoking group (0.99 ± 0.71 mM) than non-smoking group (0.49 ± 0.36 mM) at 30 min before the Kraepelin test and increased significantly just after (0.77 ± 0.38 mM) and decreased 30 min after (0.57 ± 0.29 mM) the test in the non-smoking group (Table 2). No significant change in the SCN^- level was observed in the smoking group.

The uric acid concentration was slightly higher in the non-smoking group than smoking group but not significantly so, and was not affected by the test in either group. The OPO activity was greater in the non-smoking group within each session and a significant increase was observed just after the test in the non-smoking group.

Response of SPO and MPO activity to Kraepelin test

OPO activity is mainly composed of SPO and MPO activity. We next studied whether the response of SPO and MPO activity to mental stress was different between non-smokers and smokers. SPO activity was not changed by the Kraepelin test in either group. On the other hand, in non-smokers, MPO activity increased significantly just after the test (227.8 ± 229.4 mU/ml), compared with that 30 min before (102.3 ± 118.3 mU/ml) (Fig.1). A meaningful change in MPO activity was not detected in the smoking group.

DISCUSSION

Saliva is the first body fluid to encounter exogenous materials or gases such as cigarette smoke (CS) that penetrate the human body. Free radicals, reactive oxygen species (ROS), and reactive nitrogen species in inhaled CS have been suggested to mount a constant and direct attack on the oral epithelial cells, gradually accumulating and causing a step-wise malignant transformation. Therefore, precise information on the anti-oxidative defense system in saliva is needed. The purpose of this study was to examine whether smoking effects OPO reactivity to mental stress.

The results of the present study showed that defensive components are stronger in non-smokers than smokers. The IgA concentration, Amylase activity, and UCL level were higher in the non-smoking group than smoking group regardless of the Kraepelin test. In our system, OPO catalyzes the oxidation of salivary SCN^- by H_2O_2 to the antimicrobial component hypothiocyanate (OSCN^-) with the emission of UCL as follows (Goi *et al.*, 2007).

Therefore, the amount of UCL reflects the ability to consume H_2O_2 as an antioxidant and produces OSCN^- . The function of OSCN^- in the oral cavity has been discussed in relation to antimicrobial activity (Spiegel *et al.*, 2005; Sermon *et al.*, 2005) and the UCL level also reflects the strength of the innate immune system such as the antibacterial system. The IgA concentration and UCL level in the non-smokers increased significantly just after the Kraepelin test. Flow rate also increased 30 min after the test in the non-smokers. Some studies reported that cigarette con-

Table 2. Mean levels of SCN^- and peroxidase activity in saliva from smokers and non-smokers.

		Time before Test (min)		Time after Test (min)	
		30	0	0	30
SCN^- (mM)	non-smokers ¶	0.49 ± 0.36	0.61 ± 0.32	$0.77 \pm 0.38^{**}$	0.57 ± 0.29
	smokers	$0.99 \pm 0.71^{##}$	0.82 ± 0.64	0.85 ± 0.54	0.63 ± 0.49
Uric acid (mg/dl)	non-smokers	2.27 ± 0.75	2.39 ± 0.95	2.54 ± 1.17	2.34 ± 0.99
	smokers	2.21 ± 0.86	2.14 ± 1.05	2.31 ± 1.18	2.12 ± 0.87
OPO (mU/ml)	non-smokers ¶	711.3 ± 422.2	776.8 ± 420.9	$932.1 \pm 529.0^*$	733.2 ± 408.9
	smokers	516.2 ± 292.3	582.1 ± 387.8	661.7 ± 332.8	602.4 ± 303.2

Data are expressed as the mean $\pm$ SD (39 non-smokers and 9 smokers). ¶ one-way analysis of variance $p < 0.05$. *Dunnet and **Dunnet test vs. 30 min before the test $p < 0.05$ and $p < 0.01$. ##Unpaired T-test test vs. non-smokers $p < 0.01$.

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sumption had a negative correlation with salivary flow and an altered flow rate would also affect the washout, dilution, and clearance of OPO released from bleaching systems (Hannig *et al.*, 2006). These results indicate that smoking suppresses the quick response of the innate immune system to psychological stress such as the mental arithmetic test.

In an epidemiological study, measurement of the SCN^- level might be a valuable tool for studying the actual effect of smoking on the human body (Densen *et al.*, 1967). CS contains hydrogen cyanide, which is metabolized by the liver to SCN^- . SCN^- is specifically sequestered from the plasma by the parotid gland and is secreted by this gland into the oral cavity (Reznick *et al.*, 2003). The levels of SCN^- were higher in smokers than in non-smokers, a result supported by other studies (Zappacosta *et al.*, 1999; Kanehira *et al.*, 2006). However, SCN^- levels increased significantly just after the Kraepelin test only in the non-smoking group. The uric acid concentration did not differ between the groups significantly and OPO activity was higher in the non-smoking group in all sessions. These results were consistent with reports that CS attacked antioxidative enzymes, reducing their activity severely, rather than molecules such as uric acid (Zappacosta *et al.*, 1999; Nagler *et al.*, 2001). The marked 65-70% loss of OPO activity caused by cyanate in CS was prevented by pre-incubation of the saliva with hydroxocobal-

amine, a known chelator of cyanate (Klein *et al.*, 2003). This indicated that cyanide was most probably the agent responsible for the CS-associated loss of salivary OPO activity. Moreover, a significant increase was observed only in the non-smoking group. OPO, SCN^- , and H_2O_2 constitute an antimicrobial system (Tenovuo *et al.*, 1985; Prudy *et al.*, 1983; Thomas *et al.*, 1994) and a hypothesis that salivary SCN^- can protect the stomach from OH radicals formed by ascorbic acid/ $\text{H}_2\text{O}_2/\text{Fe(II)}$ systems under acidic conditions was proposed (Takahama and Oniki, 2004). Therefore, the change in OPO activity and SCN^- concentration may have a significant effect not only on the oral cavity, but also on other sites in the body, and CS could suppress the quick response of the innate immune system.

We calculated the response of SPO and MPO activity to mental arithmetic stress. SPO activity was always higher in the non-smoking than smoking group. Furthermore, MPO activity increased significantly just after the Kraepelin test only among the non-smokers. Both SPO and MPO can catalyze the oxidation of SCN^- (Thomas and Fishman, 1986), but MPO is also able to oxidize Cl^- and OCl^- is a more potent oxidizing agent than OSCN^- (Mansson-Rahemtulla *et al.*, 1986). Usually, an increased output of MPO is thought to reflect a certain grade of infection of parotid glands, resulting in leukocyte infiltration and the production of MPO or inflammatory markers. However, our results

Fig. 1. Effect of the Kraepelin test on peroxidase activity of SPO and MPO. Data are expressed as the mean $\pm$ SEM (○:39 non-smokers and ● smokers). The overall test of differences among the times was statistically significant ($p < 0.05$ by one-way analysis of variance). *Dunnet test vs. 30 min before the test $p < 0.05$.

indicated that MPO activity increased very quickly due to mental stress and that the contribution of OPO activity changed from 30 min before ($10.8 \pm 8.6\%$) to just after the stress ($23.9 \pm 14.2\%$) only in the non-smoking group. From these results, MPO may be crucial to respond to acute stress besides inflammation and CS may suppress this response significantly. The mechanism by which CS inhibits the response of MPO to acute stress is not clear, but this finding may contribute to a more profound understanding of the effect of smoking on the mind and body.

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