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Original Article

Oxidative stress biomarkers and complexity of heart rate variability during acute single cigarette smoking

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Abstract

Smoking causes sympathovagal changes and peripheral vessel injury, which can be assessed by heart rate variability (HRV) that includes endothelial nitric oxide (NO) and sLOX-1 markers, respectively. This study aimed to investigate the HRV and NO changes in acute smoking and sLOX-1 overproduction in chronic smoking. A total of 60 males were recruited. Thirty smokers and 30 non-smokers were studied. At first the HRV parameters were obtained from all subjects. Only smokers were then consecutively recorded for 5 min and 15 min after smoking a single cigarette with simultaneous blood collection at each time point for NO and sLOX-1 measurements. We found a significant lowering of HRV and a positive correlation between sLOX-1 and mean arterial pressure in smokers. After 5 min of smoking, an immediate adverse effect of autonomic function was represented by the HRV along with an immediate lowering of NO which is a vasodilator. Acute smoking influences the autonomic neural control from NO-mediated modulation which can be assessed by the noninvasive technique of HRV analysis.

Keywords: heart rate variability, autonomic system, nitric oxide, sLOX-1, smoking

1. Introduction

Cigarette smoking commonly causes heart disease, stroke, chronic obstructive pulmonary disease, cancer, and vascular injury. The duration and number of cigarettes smoked are factors leading to atherosclerosis. In some studies, sudden cause of death from heart disease is higher in smokers (Erblich, Bovbjerg, & Sloan, 2011; Karakaya *et al*., 2006; Powell, 1998; Shah & Cole, 2010).

Recently, soluble lectin-like oxidized low-density lipoprotein receptor-1 (sLOX-1) from serum, which is related to stable atherosclerotic plaque formation, was found to increase in cigarette smokers (Takanabe-Mori *et al*., 2013). Vascular wall inflammation can be induced by oxidative stress from smoking with a great oxidative combination of low-density lipoprotein (oxLDL) aggregation through a major receptor known as lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) on the layer of endothelial cells (Moriwaki *et al*., 1998). LOX-1 is readily released into the blood circulation in the form of sLOX-1. sLOX-1 is excised from ligand specificity of LOX-1. A higher level of sLOX-1 in the serum indicates the role of atherosclerotic stenosis and plaque rupture. However, sLOX-1 decreases after gradual cessation of smoking (Takanabe-Mori *et al*., 2013). The plasma levels of sLOX-1 have been used as a predictor of peripheral artery disease (Otsuki *et al*., 2015) and as a marker for acute coronary syndrome (Hayashida *et al*., 2005; Kume *et al*., 2010).

Epidemiological studies show a correlation of smoking to venous disease of most forms of arterial disease (Powell, 1998). Inhalation of cigarette smoke also has immediate effects on the endothelial blood vessels causing potent vasodilation (Ambrose & Barua, 2004; Barnoya &

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Glantz, 2005) which results in systemic release of epinephrine and norepinephrine that cause muscle contraction and vasoconstriction (Powell, 1998). Also, there is a decrease in endothelial nitric oxide (NO), mediated endothelium-dependent vasodilator with an increased risk of vascular inflammation (Barnoya & Glantz, 2005; Huang & Vita, 2012). Smoking can cause endothelial damage from loss of NO bioactivity in vascular walls which results in enhanced platelet aggregation that leads to atherosclerotic plaque formation and consequent heart disease and stroke (Ambrose & Barua, 2004; Barnoya & Glantz, 2005; Huang & Vita, 2012).

Sympathetic nerve hyperactivity is also one reason for sudden death (Triposkiadis *et al*., 2009). Enormous amounts of increased plasma catecholamines, epinephrine, and norepinephrine from smoking affect the sympathetic outflow which results into higher blood pressure and heart rate (HR) (Karakaya *et al*., 2006). In addition, cigarette smoking causes acute constriction in the peripheral blood vessels (Akishima *et al*., 2007). Smoking influences the brain-heart axis by increasing the sympathetic activity while decreasing the parasympathetic activity. These changes can be measured by the heart rate variability (HRV) which is an electro-cardiogram (ECG) marker that reflects the activity of the sympathetic and vagal components of the autonomic system on the sinus node of the heart. An analysis of the HRV from an ECG is comprised of the time domain, which is assessed by a statistical operation on the RR intervals, and the frequency domain, which is calculated from the power spectral density (PSD) of an array of RR intervals (Samuel, Anandhalakshmi, Rekha, & Akhil, 2016). However, since the specific autonomic components may not be selective with PSD components, it has been suggested that nonlinear dynamics from the HRV should be analyzed (Pitzalis *et al.*, 1996). It is well known that the HRV index causes an increase in blood pressure, HR, vascular resistance, sympathetic discharge, the decrease in baroreflex activity (Manzano, Vanderlei, Ramos, & Ramos, 2011) and blunted vagal modulation in heavy smokers (Smith & Fischer, 2001).

Chronic smoking is one of the major coronary risk factors especially in oxidative damage and has a higher risk than in non-smokers. Little is known about the association of the oxidative stress biomarker with autonomic impairment. In this study, we hypothesized that acute effects of cigarette smoke will increase oxidative stress and is characterized by decreased endothelial NO with sLOX-1 overproduction and may affect autonomic control. Thus, we investigated the relationships among those parameters in acute smokers and used them for an index or indicator of subsequent underlying coronary heart disease which is found normally after atherosclerosis leading to hypertension, arterial stiffness, and other vascular diseases.

2. Materials and Methods

A cross-sectional study of 60 non-sick males over 20 years of age were the participants. Thirty habitual cigarette smoking males who smoke at least 1 cigarette daily consecutively for 1 year and 30 of non-smoking males served as the subjects and controls, respectively. None of the participants were receiving any medication for any ailment during the previous 1 month and gave written informed consent to participate in this study. The research was considered and approved by The Human Ethics Committee, Faculty of Medicine, Thammasat University (MTU-EC-DS-6-068/57).

2.1 Study design

Studies were conducted in a comfortable room and the procedures for performing the HRV and blood collection were explained to the subjects in detail. Anthropometric parameters such as age, height, weight, and smoking status were recorded. All participants rested for 15 min in the supine position. A lead II-ECG 5-min recording of the baseline HR was then performed. After the baseline recordings were complete, only the smokers were asked to smoke a cigarette. Then 5-min segments over 15 min of ECG were recorded after the smoking was complete. Blood serum samples were collected from the subjects simultaneously with the ECG recordings before smoking and at 5 min and 15 min after the smoking was complete. The sLOX-1 and NO were analyzed from the serum.

2.2 Analysis of serum sLOX-1 and NO concentration

Blood samples were centrifuged for 15 min at 3000 *g*. The serum samples were separated and kept in a freezer at – 20 ^{o}C until the assay. The sLOX-1 serum analysis before smoking was measured using an *in vitro* enzyme-linked immunosorbent assay (ELISA) kit for the quantitative measurement of LOX-1 (The RayBio® Human LOX-1/ OLR1). This assay employed an antibody specific for human LOX-1 immobilized on a well plate. The serum samples were pipetted into the wells and LOX-1 expressed can be bound by the immobilized antibody then recombinant with anti-human LOX-1 antibodies after that sLOX-1 was assay.

NO was measured from the same serum by an electrochemistry technique. Serum NO concentration was detected using an amino-700 NO sensor (Innovative Instrument, Inc. USA) according to the manufacturer's instructions. This technique measures the nitrite level from an electrical current generated through the surface of a carbon fiber electrode. The reaction is followed by oxidizing the NO on a microelectrode probe and converted to become nitrite.

2.3 HRV analysis

The ECG data were digitized and converted using LabChart 7, version 7.0.2. The analyses of the HRV recordings were performed by Kubios HRV 2.0 software. Abnormal beats and artifact areas were excluded. For the time domain, the mean RR interval (RRI), the standard deviation of all RR intervals (SDNN), the root mean square of successive RR interval differences (RMSSD), and the proportion of adjacent normal RRIs differing more than 50 ms in length from the preceding RR (pNN50) were measured. For the frequency domain, the PSD of the RR series was calculated by the fast Fourier transformation algorithm. The selected frequency bands were low-frequency band (LF, 0.04-0.15 Hz), high-frequency band (HF, 0.15-0.4 Hz), and LF to HF ratio (LF/HF). The nonlinear components of HRV were computed. The common nonlinear parameters were standard deviation of instantaneous variation of RR intervals (SD1), standard deviation of continuous long-term RR interval

variability (SD2), Poincaré plot, α1, α2, and Sample entropy (SampEn).

2.4 Statistical analysis

The results are reported as mean±standard error and percentages. The demographic characteristics and HRV parameters comparing the smokers and nonsmokers were obtained by independent-sample *t*-test. The NO and HRV parameters after smoking were compared with the smoking baseline parameters and analyzed by paired-sample *t*-test. A comparison of the sLOX-1 and NO levels between the normal and higher mean arterial pressure (MAP) readings among the smokers were obtained by the Mann-Whitney U Test. The scatter plots of sLOX-1 and MAP and other correlations between the NO and HRV parameters were analyzed by simple linear regression. Statistical significance was set at $P<0.05$ for all of the analyses.

3. Results and Discussion

The general characteristics of the subjects were collected using questionnaires and results are shown in Table 1. The analyzed factors were age, weight, height, body mass index (BMI), and blood pressure. There were no significant differences between the two age-matched groups in age, weight, height, and BMI. However, the systolic blood pressure (SBP) and diastolic blood pressure (DBP) were higher in the smokers than in the non-smokers. None of the subjects in this study had any major illness during the previous 1 year and did not use any drugs during the previous 1 month.

Table 1. General characteristics of the participants.

Characteristics	Nonsmokers	Smokers
Age (years)	33.8±9.11	35.4±10.21
Weight (kg)	70.57±15.35	64.27±10.40
Height (cm)	170.67±5.83	167.93±5.73
BMI (kg/m^2)	24.13±4.44	22.78±3.46
SBP (mmHg)	123.03±11.17	133.80±15.04†
DBP (mmHg)	76.27±11.47	82.33±9.73*

Significance compared with nonsmoker; *P<0.05 and † P<0.01
BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure.

3.1 Serum oxidative stress markers analysis

The results showed a significant moderate positive correlation between the baseline serum sLOX-1 and MAP among smokers in which the MAP was calculated as DBP + (SBP-DBP)/3 (Sesso *et al*., 2000) (Figure 1). This result showed that cigarette smoking may cause increased oxidative stress and induced inflammatory reaction in the blood vessels. In addition, the serum sLOX-1 levels from the smokers were also correlated with various physiologic markers, such the serum high-sensitivity C-reactive protein (hsCRP) and the expired air carbon monoxide (CO) concentrations (Takanabe-Mori *et al*., 2013). The baseline serum NO concentrations (55.58±1.42 nmol/L) among the smokers and late-recovery NO after 15 min of smoking (54.47±1.47 nmol/L) were higher than early-recovery NO concentrations after 5 min of smoking

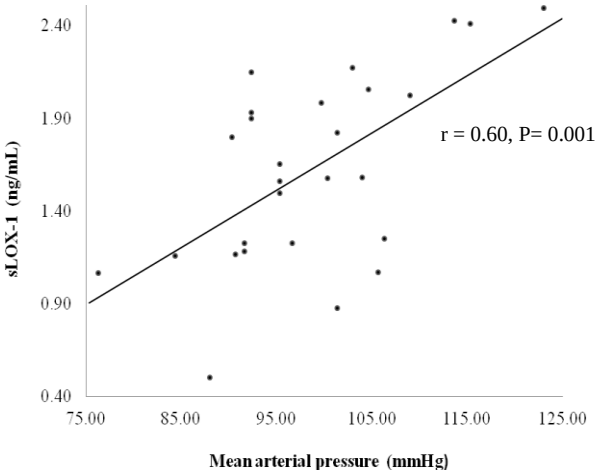

Figure 1. Serum sLOX-1 levels showed moderate positive correlation with mean arterial pressure.

(31.21±3.39 nmol/L) (Figure 2). The serum NO level in the smokers significantly decreased at 5 min after the smoking was complete compared with baseline, and significantly increased during the 6-15 min period compared with 5 min after smoking but insignificantly different with baseline. Figure 2 illustrates the effect of smoking on NO secretion from the endothelium which is the first inner layer of a blood vessel. NO controls the vascular tone and the vascular inflammatory process (Pittilo, 2000). The decrease in level of serum NO after 5 min of complete cigarette smoking may show abnormal NO mediated endothelium dependent dilation of arterial vessels leading to vasoconstriction response, increased platelet aggregation, and cell proliferation in the arterial wall. In addition, NO also reacts with superoxide free radical (O_2^-) to form peroxynitrite ($ONOO^-$) which is a NO-derived reactive nitrogen species (Patel *et al*., 1999). This dysfunction contributes to the progression of atherosclerotic lesions (Akishima *et al*., 2007; Barnoya & Glantz, 2005). A decrease of synaptic neurotranmission on the level of medullary neuron may also cause a decline of systemic NO that affects the signal processing of the autonomic system (Patel, Li, & Hirooka, 2001).

Among the smokers, the serum sLOX-1 levels were higher in the higher MAP (excess 110 mmHg) group than in the normal MAP (70 to 110 mmHg) group (Xu *et al*., 2015). The sLOX-1 results of the normal MAP and higher MAP groups were 1.48±0.10 ng/mL and 1.98±0.21 ng/mL, respectively, which were significantly higher in the higher MAP group ($P<0.05$). The serum NO results from the higher MAP group were lower than the normal MAP group (50.80±1.94 nmol/L) vs. 57.32±1.67 nmol/L) ($P<0.05$) (Figure 3). The results in Figure 3 showed that smoking induced inflammatory reaction in the blood vessels and increased the risk of cardiovascular incidence. Reactive oxygen species from cigarette combustion is a major cause of antioxidant depletion (Lykkesfeldt *et al*., 2000) and consequent oxidative stress leading to blood vessel injury by increasing the level of inflammatory marker sLOX-1 which supports the relationship between smoking and inflammation and atherosclerosis

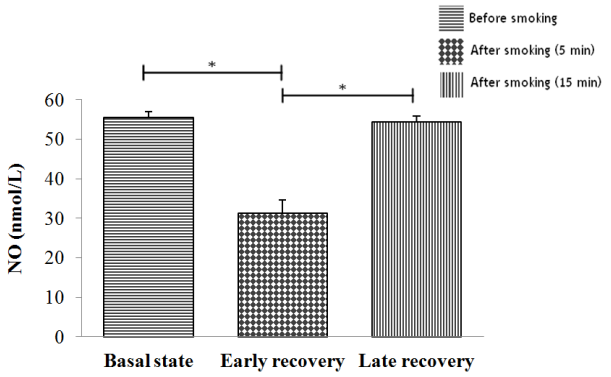

Figure 2. The change in serum nitric oxide (NO) of smokers after smoking a cigarette * P<0.001.

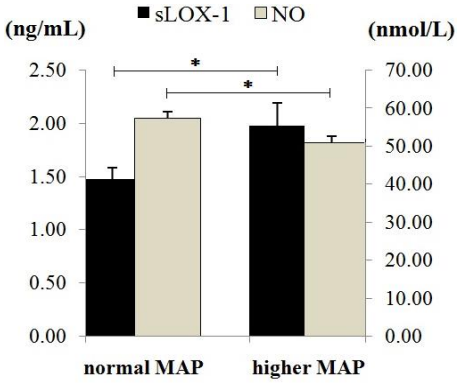

Figure 3. Levels of serum sLOX-1 and NO compared to normal and higher MAP groups from smokers, * P<0.05

(Takanabe-Mori *et al*., 2013). According to Jaimes (2004), the oxidants in second-hand smoke can depress the production of NO. This vasodilator marker by the endothelium independently has an effect on mitochondrial respiration (Jaimes, DeMaster, Tian, & Raij, 2004). In addition, serum sLOX-1 from this study tended to be inversely related with baseline serum NO concentrations (Figure 3). According to these results smoking is one of the major routes of oxidative stress causing systemic impairment from the tendency to have a negative correlation between serum sLOX-1 and NO concentrations.

3.2 Analysis of heart rate variability

HR was found to be higher in smokers than in non-smokers, while RRI, SDNN, RMSSD, pNN50, SD1, and SD2 were significantly decreased in smokers. There were no significant differences between the groups in terms of LF, HF, LF/HF, α1, and α2 (Table 2). From this study, we found that the power activity of HRV from smokers was significantly lower than non-smokers because of oxidative stress from long-term smoking. The decreased RRI, SDNN, and other HRV parameters except HR among smokers suggested that smoking affected the sympathovagal modulation that resulted in increased sympathetic activity and decreased parasympathetic activity. It is known that nicotine, free radicals, and CO in cigarette smoke may cause increased activation of the sympathetic nervous system resulting in the systemic release of norepinephrine and epinephrine (Papathanasiou1, Mamali, Papafloratos, & Zerva, 2014), thereby increasing heart rate and blood vessel contraction that contributes to increased blood pressure.

The HR significantly increased within the first 5 min after smoking was complete compared with baseline and then decreased during the 6-15 min period but the RRI was exactly contrary to the HR. Meanwhile SDNN significantly decreased during the 6-15 min period after smoking. In addition, there was no difference in values between baseline and after smoking in RMSSD and pNN50 (Table 3). LF and LF/HF significantly increased within the first 5 min after smoking was complete, and then decreased during the 6-15 min period while HF significantly decreased during 5 min after smoking compared with baseline, and then increased during the 6-15 min period (Table 3). SD2 and α1 significantly increased within the first 5 min after smoking a cigarette compared with baseline, and then decreased during the 6-15 min period but SD1 decreased 5 min after smoking was complete and continued to decrease during the 15 min after smoking. In addition, SD1/SD2 and SampEn significantly decreased within the first 5 min after smoking, and then increased during the 6-15 min period (Table 3).

We found an immediate autonomic modulation in cardiac regulation from acute smokers by instantaneous alteration in increased HR and decreased HRV, particularly within the first 5 min after smoking was complete as Karakaya *et al*. found (Karakaya *et al*., 2007). We observed a decrease in pNN50 5 min after smoking was complete and continued to decrease during the 15 min. However, an increase in LF and LF/HF was found 5 min after smoking which returned to baseline 15 min later (Table 3). This occurred because LF and LF/HF represent the sympathovagal balance of autonomic function while HF and pNN50 represent the parasympathetic nervous system since smoking causes blunting of vagal control (Cagirci *et al*., 2009).

An increase in SD2 after 5 min of smoking completely recovered 15 min later because SD2 represents the sympathetic autonomic function, while SD1 continued to decrease because smoking affected the sympathovagal balance of autonomic function by parasympathetic withdrawal and predominant sympathetic activation (Manzano *et al*., 2011). The α1 significantly increased immediately after 5 min of smoking and showed a change in sympathetic activation or vagal deactivation, in which α1 was due to a change in the same direction of peripheral resistance, whereas with α2 there was no significant change because this index was affected by the decrease in peripheral resistant (Rojo-Alvarez *et al*., 2007). These α1 and α2 parameters characterize the intrinsic and extrinsic factors, respectively, in vasomotor tone of cerebral autoregulation (Toth, Rozsa, Springo, Doczi, & Koller, 2011). We believe there is an intrinsic factor from impairment in endothelial factor such as decreased NO which influences the effect on vasomotor tone regulation. In addition, SampEn relates to a quantity of dynamical systems that can be used to examine physiological systems. Greater regulation systems expressed as a reduction of entropy reflects the sympathovagal balance toward sympathetic predominance (Richman & Moorman, 2000; Rojo-Alvarez *et al*., 2007). This result shows irregularity in systemic signal alteration from disorder to order immediately after smoking a cigarette may be related to the risk of cardiovascular events (Table 3).

Table 2. Heart rate variability data of the participants.

Variable	Nonsmokers	Smokers	P-value
HR (beats/min)	70.93±1.82	85.54±2.74	< 0.001
RRI (ms)	864.07±21.69	725.81±25.36	0.0001
SDNN (ms)	33.85±2.64	22.57±2.45	< 0.01
RMSSD (ms)	35.57±3.31	22.56±3.06	< 0.01
pNN50 (%)	15.88±3.11	6.34±1.80	< 0.05
LF (n.u.)	54.00±92.92	57.14±3.04	NS
HF (n.u.)	45.99±89.45	42.86±3.04	NS
LF/HF ratio	1.41±0.16	1.99±0.39	NS
Total power (ms^2)	1227.13±181.41	614.23±120.71	< 0.01
SD1 (ms)	25.63±2.44	16.08±2.18	< 0.01
SD2 (ms)	61.07±4.83	40.71±3.49	< 0.01
α1	1.04±0.04	1.12±0.05	NS
α2	0.90±0.03	0.91±0.04	NS

NS = not significant; HR = heart rate; RRI = RR interval; SDNN = standard deviation of all RR intervals; RMSSD = root mean square of successive RR interval differences; pNN50 = proportion of adjacent normal RRIs differeing more than 50 ms in length from the preceding RR; LF = low frequency; HF = high frequency; SD1 = standard deviation of instantaneous variation of RR intervals; SD2 = standard deviation of continuous long-term RR interval variability.

Table 3. Alteration in the time domain, frequency domain, and nonlinear parameters of heart rate variability after smoking a cigarette.

Variable		Basal state	Early recovery (5 min after smoking)		Late recovery (15 min after smoking)	
Time Domain	HR (beats/min)	85.54±2.73	92.47±2.55	*	90.46±2.54	**
	RRI (ms)	725.81±25.36	666.43±19.79	*	680.65±20.03	**
	SDNN (ms)	22.57±2.45	22.34±2.47		20.09±2.29	**
	RMSSD (ms)	22.65±3.06	18.37±3.12		18.87±3.32	
	pNN50 (%)	6.34±1.80	4.84±2.06		4.31±2.20	
Frequency Domain	LF (n.u.)	57.14±3.04	71.81±2.27	*	62.85±3.13	**
	HF (n.u.)	42.86±3.04	28.19±2.27	*	37.15±3.13	**
	LF/HF	1.99±0.39	3.58±0.53	*	2.73±0.50	**
Nonlinear Parameter	SD1 (ms)	16.08±2.18	14.26±2.29		13.46±2.36	
	SD2 (ms)	40.71±3.49	49.84±4.38	*	39.24±3.68	†
	SD1/SD2	0.37±0.03	0.27±0.02	*	0.32±0.02	†
	α1	1.12±0.05	1.34±0.04	*	1.20±0.05	†
	α2	0.91±0.04	0.90±0.04		0.94±0.03	
	SampEn	1.47±0.07	1.21±0.07	*	1.38±0.06	†

*; significance compared with basal state, †; significance compared with early recovery.
**; significance compared with both basal state and early recovery.
HR = heart rate; RRI = RR interval; SDNN = standard deviation of all RR intervals; RMSSD = root mean square of successive RR interval differences; pNN50 = proportion of adjacent normal RRIs differeing more than 50 ms in length from the preceding RR; LF = low frequency; HF = high frequency; SD1 = standard deviation of instantaneous variation of RR intervals; SD2 = standard deviation of continuous long-term RR interval variability.

The decreasing ratio of SD1/SD2 during the early recovery period was due to the reduction in SD1 compared with the marked increase in SD2 obtained through the Poincaré plot. The characteristic of a Poincaré plot is a coordinated plot between current plotted of RRI (RR_n) against the previous RRI (RR_{n+1}). Points below the line at 45° to the normal axis are called the line of identity. A common route to explain the geometry of the Poincaré plot should be a fit to an ellipse graph (Karmakar, Khandoker, Gubbi, & Palaniswami, 2009). Ellipse geometry is an alignment of the position consistent with the identity line (Figure 4). The qualitative analysis of Poincaré plot before and after smoking during the 5-15 min of smoking is shown in Figure 4. During the phase of acute single cigarette smoking, there is a decrease in the dispersion of points in the early recovery phase compared with the basal state and late recovery phase moments. This chart pattern, according to Manzano *et al.* found (Manzano *et al*., 2011). Our findings show that the qualitative Poincaré plot shape may provide additional insight of smoking on autonomic function.

3.3 Correlation between NO concentration and HRV

The serum NO level in early recovery state, i.e. 5 min after smoking was complete, from the smokers showed moderate positive correlation with HF as shown in Figure 5(a) and a significantly irreversible correlation with LF/HF ratio and SD2 shown in Figure 5(b) and 5(c), respectively. These results showed that endogenously reduced NO in the blood circulation increased the overall sympathetic excitability

within the first 5 min after finishing cigarette smoking as illustrated in Figure 5(b) and 5(c). The reason for this finding is that smoking affects systemic NOS-inhibition which influences a baroreflex mediated inhibition of sympathetic nerve activity that causes increased blood pressure (Middlekauff, Park, & Moheimani, 2014; Narkiewicz *et al*., 1998). The vascular endothelium and medullary neuron released NO effected in reduction of overall sympathetic or the baroreflex mediated activation of vagal tone elevation (Koch, Hasser, & Schadt, 1995). The overall effect of NO results in a reduction of the efferent outflow (Zanzinger, 1999). In addition, the result of circulating NO was also shown to be significantly correlated with SampEn as shown in Figure 5(d). The algorithm of SampEn is the signal complexity measuring the time sequence which can be applied to clinical data of noisy time series (Yuanyuan, Chengyu, Binhua, & Mengsun, 2014). Smoking might induce sympathetic vasomotor activity and heart rate resulting in increased sympathetic dominance on the cardiac autonomic nerve activity. The change in SampEn was displayed in these modulations. The greater complexity of SampEn indicated a physiological mechanism of systemic disorder that ranged from an orderly to a disorderly manner (Richman & Moorman, 2000). Thus the result of SampEn from this study unsurprisingly showed a significant positive correlation with NO.

4. Conclusions

The effects of cigarette smoking were examined in this study from the elevated vascular injury marker, sLOX-1 and autonomic modulation presented by HRV. In conclusion, lower NO, which represents a vascular oxidative stress marker, caused an acute effect from smoking a single cigarette. Moreover, the overproduction of sLOX-1 and decreased bioavailability of NO may affect the autonomic control and induce vascular lesions. Most importantly, the results of the data analysis from HRV measurements indicated that autonomic impairment occurred in chronic smokers and a top-up occurred in the 5 minutes after completely smoking a single cigarette, which immediately elevated sympathetic activity and blunted vagal tone. Furthermore, distribution in the Poincaré plot also provided a sensible and simple tool to detect an immediate change in HRV from acute smoking.

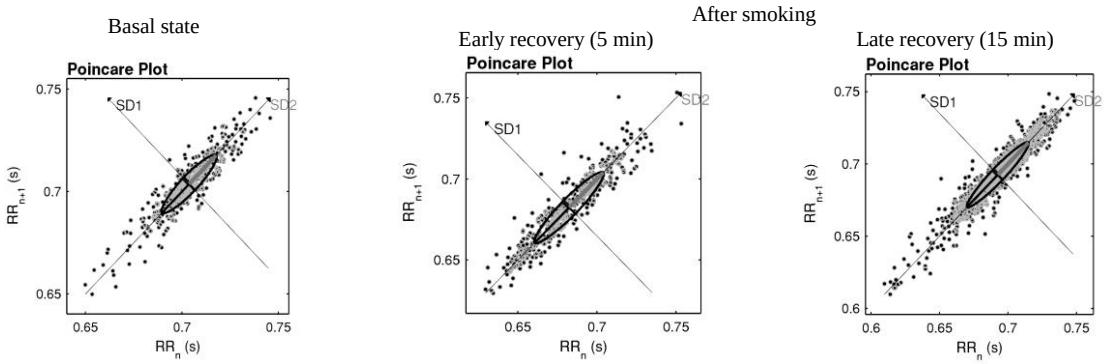

Figure 4. Distribution of Poincaré plots of smokers through the phase of smoking a cigarette.

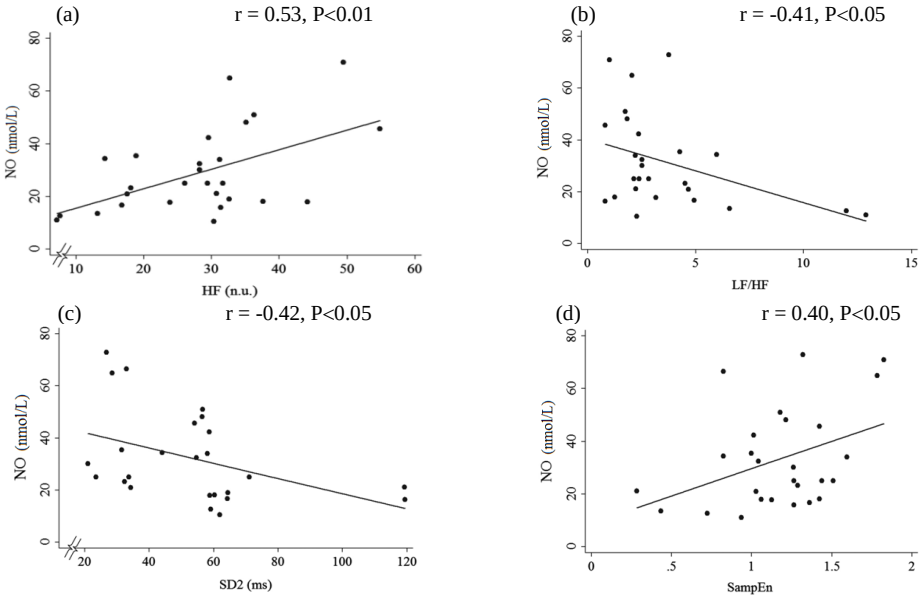

Figure 5. Correlation between circulating NO concentration of smokers and HF (a), LF/HF ratio (b), SD2 (c), and SampEn (d) in early recovery state.

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