Protective Effects of Pterostilbene against Cardiac Oxidative Stress and Dysfunction in Nicotine-Induced Cardiac Injury Rat Model

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Prolonged nicotine exposure escalates the onset and development of cardiovascular diseases in both active and passive smokers via cardiac injury. Pterostilbene, a resveratrol derivative, has been shown to exhibit high anti-inflammatory, antioxidant and antitumor properties. Nevertheless, its role as a cardioprotective agent in a nicotine-induced rat model is still scarce. Therefore, our study was aimed to investigate the effects of co-administered pterostilbene against nicotine-induced cardiac injury rat model. Twenty-six male Sprague-Dawley rats were randomly allotted and treated with nicotine (0.6 mg/kg) or in-combination with pterostilbene (10 mg/kg) for 28 consecutive days. Non-invasive tail cuff blood pressure measurements were taken at day-0, day-14 and day-28. Rat hearts were harvested at study end point and the changes in cardiac function parameters and oxidative stress markers were evaluated. The findings have shown that pterostilbene co-administration significantly (P<0.05) reduced the blood pressure and ameliorated nicotine-induced cardiac systolic dysfunction by improving the left ventricular developed pressure (LVDP). In addition, pterostilbene also significantly (P <0.05) attenuated the thiobarbituric acid reactive substances (TBARS) level, indicative of protection against nicotine-induced cardiac oxidative stress. In summary, our findings suggest that pterostilbene has the potential to be developed as a natural alternative in protecting the cardiac injury induced by nicotine. However further studies are warranted to investigate its efficacy and the underlying mechanism in cardioprotection.

Keywords: Antioxidant; Cardiac dysfunction; Nicotine; Oxidative stress; Pterostilbene.

Smoking is one of the main risk factors contributing to chronic diseases, such as cardiovascular diseases and cancer. According to World Health Organization in 2020, tobacco use primarily cigarette smoking is one of the biggest public health threats in world history, with more than 8 million deaths reported annually worldwide. A cigarette is made using tobacco leaves, which usually contain nicotine. Prolonged nicotine exposure can escalate the onset and development of cardiovascular diseases in both active and passive smokers via cardiac injury.

Nicotine is known to induce oxidative stress which increases reactive oxygen species (ROS) and therefore lipid peroxidation. Oxidative stress causes cardiac dysfunction through the amelioration of mitochondrial respiration that attenuates ATP production and necrosis.
Besides that, nicotine triggers sympathetic stimulation which further increase the heart rate and vasoconstriction, thus elevating the peripheral resistance and blood pressure\(^5,6\). Subsequently, hypertension leads to left ventricular hypertrophy and cardiac dysfunction\(^7\). Several animal studies have shown that prolonged nicotine as long as 28 consecutive days of administration was capable of causing cardiac dysfunction in rat model\(^8,9\).

There has been an abundance of studies that investigates the potential use of natural products as therapeutic agents against the nicotine-induced cardiac dysfunction. Recently, stilbenes have attracted the interest of the public due to their various beneficial health effects such as anti-inflammatory, anticancer, antioxidant, and anti-dyslipidemia activities\(^10\). An example of stilbene is pterostilbene (Figure 1B), which is an analogue of resveratrol (Figure 1A)\(^11\). Pterostilbene is a naturally occurring polyphenol compounds that can be found in antioxidant-rich foods like grape wine and blueberries\(^12,13\). It has been suggested to have greater bioavailability due to the presence of two methoxy groups (Figure 1B)\(^14\).

Pterostilbene has previously been shown to reduce cardiac oxidative stress in several types of cardiovascular diseases, including myocardial infarction\(^15\), diabetic heart disease\(^16,17\) and acute doxorubicin-induced cardiotoxicity\(^18\). Nevertheless, its role as cardioprotective agent in a nicotine-induced rat model has not yet been explored. Hence, this study was carried out to examine the effects of co-administered pterostilbene on the blood pressure, cardiac function as well as cardiac oxidative stress in rat model of prolonged nicotine administration.

**MATERIALS AND METHODS**

**Ethics of Animal Experimentation**

All experiments reported were in adherence with the UKM Animal Ethics Committee (UKMAEC) guidelines (Approval number: FSK/2019/SATIRAH/25-SEPT./1041-OCT.-2019-OCT.-2020). Adult male Sprague-Dawley rats (200-250 g) were acquired from UKM Laboratory Animal Resource Unit (LARU), Faculty of Medicine. All rats underwent acclimatization and housed in the standard laboratory conditions (ambient 25 ± 3°C, 12 h day/night cycle). The rats were fed with standard rodent pellet and tap water ad libitum.

**Study design**

After one week of acclimatization, 26 rats were randomly assigned into three groups (n=8/9): control, nicotine (NIC) and pterostilbene + nicotine (PTS+NIC). Rats from NIC group and PTS+NIC group received 0.6 mg/kg of nicotine (i.p.) (Tokyo Chemical Industry, Japan) dissolved in normal saline as previously described\(^9\). Rats from PTS+NIC group received 10 mg/kg of pterostilbene (J&K Scientific Ltd., China) dissolved in 10% DMSO (i.p.)\(^19\), and after 5 minutes, nicotine was administered. As pterostilbene was administered in 10 % DMSO, NIC group rats were also given 10 % DMSO (i.p.). Vehicle control rats were given normal saline and 10 % DMSO vehicle (i.p.). All animals were treated for 28 consecutive days prior to sacrifice and tissues collection. Body weight, food and water consumption were also recorded daily during the experimental period.

**Blood pressure measurement**

Blood pressure measurements were taken at day-0, day-14 and day-28 on conscious rats by using CODA\(^20\) non-invasive blood pressure system (Kent Scientific, USA). All rats were accustomed to the CODA\(^20\) blood pressure system for three consecutive days prior to blood pressure baselines for the purpose of animal acclimatization to the procedure. The variables measured on non-invasive tail cuff apparatus were systolic blood pressure (SBP), diastolic blood pressure (DBP) as well as mean arterial pressure (MAP).

**Langendorff heart perfusion ex vivo**

On the 29th day, rat hearts were isolated for the use of Langendorff heart perfusion to study the cardiac function. Before that, heparin (500 unit/kg, i.p.) were injected into the rats to prevent blood agglutination, and followed by KTX (1 ml/kg, i.p.) for anaesthesia\(^21\). After which the rats have become unconscious and lost their pedal reflex activity, the rats’ hearts were rapidly excised by performing thoracic surgery and taken out to be immersed in ice-cold Krebs-Henseleit buffer solution before immediately cannulating them to Langendorff isolated heart system (ADInstruments, Australia) via aorta\(^22\). The rat hearts underwent retrograde perfusion at constant pressure mode of ~40–60 mmHg, with Krebs-Henseleit buffer solution (in mM: NaCl 118.0;
KCl 3.2; MgSO₄ 1.2; NaHCO₃ 25.0; KH₂PO₄ 1.18; CaCl₂ 2.5; glucose 11.1, pH 7.4), which was constantly supplied with 95% O₂ and 5% CO₂ and maintained at 37 °C. Perfusion pressure and coronary flow (CF) changes were continuously monitored using flow and pressure transducers. In order to measure pressure changes inside the left ventricle, a small and thin latex balloon filled with water was attached to pressure transducer (MLT844, ADInstrument, Australia) was placed into left ventricle (LV) by inserting it via the bicuspid valve, allowing isovolumic contraction. Rat hearts were left to stabilize for 20 minutes under continuous flow. Hearts that showed poor function (e.g., CF < 10 ml/min and heart rate < 70 beats/min) during equilibration were excluded from the study.

After the stabilization period, data of left ventricular developed pressure (LVDP), left ventricular maximum contraction rate (LV +dP/dt max) and relaxation rate (LV –dP/dt max) as well as the isovolumic relaxation time constant (Tau) were collected using PowerLab data acquisition system and evaluated with LabChart 8.0 (ADInstrument, Australia). The amount of perfusate that flow out from coronary collected in one minute was recorded as the rate of coronary flow.

Tissues collection
Heart tissue was collected and weighed after Langendorff analysis. The heart was then excised, and a portion of the left ventricle (LV) was cut for analysis of oxidative stress markers. Hind leg was removed to measure tibia length to normalize heart and other organ weights.

The LV tissue was homogenized in cold 0.01 M Tris-HCl buffer. Then, supernatant was collected from centrifugation (12,000 rpm, 4°C, 30 minutes) and used for oxidative stress markers analysis.

Assessment of oxidative stress indicators
The indicator of oxidative stress such as TBARS (thiobarbituric acid reactive substances) and GSH were measured using colorimetric assay method. According to Stock and Dormandy (1971), malondialdehyde, an indication of lipid peroxidation was estimated by concentration of TBARS in LV tissue. The TBARS concentration in the sample was measured spectrophotometrically at 532nm based on standard curve produced using 1,1,3,4-tetraethoxypropane. Meanwhile, reduced glutathione (GSH) was measured using Ellman assay as previously described.

Statistical analysis
The data are portrayed as mean ± standard error of mean (SEM). Graph Pad Prism 8.3 was used as a tool for statistical analysis. Comparisons between groups were performed using one-way or two-way analysis of variance (ANOVA) and subsequently a Tukey’s post-hoc test unless stated otherwise, where P < 0.05 was considered as statistically significant.

RESULTS
Pterostilbene significantly reduced body weight gain and total water intake in 28 days when compared to control group (both P < 0.05; Table 1). In contrary, nicotine administration had no significant effects (P > 0.05) on the body weight gain, total food and water intake.

Table 2 shows the postmortem systemic analysis of rat’s organ weight. After 28 days of treatment, neither administration nicotine nor pterostilbene significantly changed the organ weight of rats. Relative organ weight was also unaltered in each experimental group at end point.

Table 1. Body weight gain, total food and water intake in all experimental groups

| Parameters          | Control      | NIC          | PTS+NIC      |
|---------------------|--------------|--------------|--------------|
| Body weight gain (g) | 83.7 ± 9.87  | 70.4 ± 8.21  | 53.7 ± 5.98* |
| Total food intake (g)| 634.3 ± 40.45| 691.4 ± 66   | 543.4 ± 8.42 |
| Total water intake (ml)| 1668 ± 77.02 | 1526 ± 46.81 | 1362 ± 44.78* |

Note: All values are portrayed as mean ± SEM (n=8-9 per group).
*significant (P < 0.05) relative to control group, using one-way ANOVA with Tukey post-hoc test
At day-28, nicotine group exhibited no significant differences \((P > 0.05)\) in all the blood pressure parameter when compared to the control rats (Figure 2A-C). However, the SBP, DBP and MAP were significant decreased \((P < 0.05)\) in PTS+NIC group as compared to the nicotine group (Figure 2A-C).

Cardiac function was determined \textit{ex vivo} using Langendorff apparatus. Nicotine group tended to increase the heart rate compared to the vehicle control groups \((P =0.3817; \) Figure 3A). However, the heart rate in PTS+NIC vs. nicotine group was significantly reduced \((P<0.01)\) (Figure 3A). As shown in Figure 3B, coronary flow for all experimental groups was not altered in the perfused hearts. On Langendorff analysis, nicotine rats showed a tendency of deterioration in cardiac systolic function parameters which are left ventricular developed pressure (LVDP) \((P =0.0987)\) and maximal contraction rate \((LV +dP/\text{dt}_{\text{max}}})(P=0.2701)\) as well as impairment of diastolic function such as maximal relaxation rate of the left ventricle \((LV–dP/dt_{\text{max}})(P=0.0788)\) and isovolumic relaxation time constant (Tau) \((P=0.2674)\) (Figure 3C-F). Co-administration pterostilbene attenuated the nicotine-induced cardiac systolic dysfunction significantly \((P <0.05)\) in LVDP but LV +dP/dt\text{max} only exhibited increasing trend compared to the nicotine group (Figure 3C-D). Meanwhile, cardiac diastolic function parameters such as \(LV–dP/dt_{\text{max}}\) and Tau in PTS+NIC group tended to improve

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**Table 2. Postmortem systemic analysis of rat’s organ weight**

| Parameters        | Control         | NIC            | PTS+NIC        |
|-------------------|-----------------|----------------|----------------|
| Heart weight (mg) | 947.1 ± 35.15   | 899.3 ± 50.96  | 941.9 ± 21.65  |
| Atria (mg)        | 41.4 ± 4.35     | 49.4 ± 3.63    | 46.1 ± 3.55    |
| Right ventricle (mg) | 170.2 ± 10.99  | 153.4 ± 13.70  | 175.2 ± 6.20   |
| Left ventricle (mg) | 647.8 ± 19.73  | 611.3 ± 34.58  | 638.1 ± 15.78  |
| Lung weight (mg)  | 1546 ± 84.58    | 1349 ± 113.90  | 1339 ± 68.86   |
| Liver weight (mg) | 10196 ± 466.20  | 8928 ± 471.20  | 8760 ± 340.50  |
| Right kidney (mg) | 924.0 ± 32.94   | 873.8 ± 45.07  | 824.6 ± 30.38  |
| Left kidney (mg)  | 930.0 ± 39.44   | 895.8 ± 38.26  | 847.7 ± 32.42  |
| Tibia Length (mm)| 40.4 ± 0.71     | 38.5 ± 1.00    | 39.6 ± 0.41    |
| HW: TL (mg/mm)    | 23.4 ± 0.71     | 23.3 ± 0.95    | 23.8 ± 0.40    |
| Atria: TL (mg/mm) | 1.0 ± 0.11      | 1.3 ± 0.11     | 1.2 ± 0.10     |
| RV: TL (mg/mm)    | 4.2 ± 0.25      | 4.0 ± 0.30     | 4.4 ± 0.13     |
| LV: TL (mg/mm)    | 16.0 ± 0.37     | 15.8 ± 0.65    | 16.1 ± 0.27    |
| LW: TL (mg/mm)    | 38.4 ± 2.22     | 34.9 ± 2.59    | 33.8 ± 1.51    |
| Liver: TL (mg/mm) | 252.2 ± 11.10   | 231.8 ± 10.22  | 221.1 ± 7.04   |
| LK: TL (mg/mm)    | 22.8 ± 0.59     | 22.6 ± 0.83    | 20.8 ± 0.63    |
| RK: TL (mg/mm)    | 23.0 ± 0.76     | 23.3 ± 0.75    | 21.4 ± 0.73    |

\textit{Note}: All values are portrayed as mean ± SEM \((n=8-9\) per group).

\textit{Abbreviations}: LV, left ventricle; RV, right ventricle; HW, heart weight; LW, lung weight; LK, left kidney; RK, right kidney; TL, tibia length
Fig. 2. Blood pressure parameters such as (A) systolic blood pressure, (B) diastolic blood pressure, (C) mean arterial pressure of experimental rats by group (n=8-9/group). All values are portrayed as mean ± SEM, two-way ANOVA with Tukey post-hoc test was used. *P<0.05, **P<0.01 for NIC vs. PTS+NIC group; *P<0.1 for control vs. PTS+NIC group.
Figure 4 shows the analysis of oxidative stress markers in LV tissues of experimental rats by group. After 28 days, TBARS level was significantly increased in nicotine-administered rats ($P < 0.05$; Figure 4A). Pterostilbene supplementation significantly attenuated the increment in TBARS level ($P < 0.05$; Figure 4A). GSH level was shown statistically non-significant reduction in nicotine group ($P = 0.1845$; Figure 4B). Whereas PTS+NIC group was shown no significant differences in GSH level compared to nicotine group ($P > 0.05$; Figure 4B).

Fig. 3. Cardiac function parameters such as (A) heart rate, (B) coronary flow, (C) LVDP, (D) LV $+\frac{dP}{dt}$, (E) LV $-\frac{dP}{dt}$, and (F) Tau of experimental rats by group (n=4-7/group). All values are portrayed as mean ± SEM, one-way ANOVA with Tukey post-hoc test was used. *$P < 0.05$, **$P < 0.01$ for NIC vs. PTS+NIC group

Abbreviations: LVDP, left ventricular developed pressure; LV $+\frac{dP}{dt}$, maximal rate of left ventricular contraction; LV $-\frac{dP}{dt}$, maximal rate of left ventricular relaxation; Tau, isovolumic relaxation time constant.
DISCUSSION

In this study, we have shown preliminary findings that pterostilbene supplementation attenuates nicotine-induced cardiac dysfunction and oxidative stress. Based on our previous pilot study, we have shown that nicotine administration at the dose of 0.6 mg/kg intraperitoneally for consecutive 28 days was capable of causing cardiac dysfunction in rats. Pterostilbene dosage was chosen based on previous studies where the dose chosen can reduce the cardiac oxidative stress in animal model of acute doxorubicin-induced cardiotoxicity.

Smoking can cause weight loss as nicotine can stimulate leptin that can suppress appetite. However, in our study, nicotine treatment had no effects on body weight gain and food intake. Our findings are in consistence with our previous research which have demonstrated that nicotine (0.6 mg/kg/day) did not affect the body weight increment. On the other hand, a study employing a higher dose of nicotine (2 mg/kg) in the same duration of 28 days was found to significantly reduce the body weight gain. The discrepancy is probably due to the low dose of nicotine used in our study that did not alter the rat’s food intake as well as body weight gain. Rats treated with pterostilbene showed a remarkable reduction in body weight gain in comparison of the control group. Our result is consistent with the past study which reported that pterostilbene was able to decrease the body weight in fructose-diet diabetes rats. Our results suggest that reduction in body weight gain may be due to the anti-obesity effect of pterostilbene.

Nicotine is known to activate the sympathetic nervous system through nicotinic acetylcholine (nAChR) receptors and stimulates catecholamines production which could increase the blood pressure and heart rate. In our present study, analysis of blood pressure revealed that SBP, DBP and MAP in nicotine rats tended to increase through the 28-day induction. It could be due to the activation of compensatory mechanism involving baroreflex to counteract the increase in blood pressure due to the induction of nicotine and this mechanism was supported by Oakes et al. On the other hand, the SBP, DBP and MAP in PTS+NIC group decreased as compared to the NIC group. The mechanisms underlying was unexplored in our study. The potential mechanisms involved maybe due to the activation of endothelium nitric oxide synthase (eNOS) phosphorylation by pterostilbene through P13K/Akt pathway, which then stimulating nitric oxide production in vascular endothelial cells, thereby lowering the blood pressure. Future work should be warranted to determine the precise mechanism to further elucidate the role of pterostilbene in blood pressure lowering effect.

Next, pterostilbene (given at high dose of 125 mg/kg twice a day) was reported in a clinical study to cause a low blood pressure and weight loss effect.

Fig. 4. Oxidative stress parameters such as (A) TBARS level, (B) GSH level in LV tissues of experimental rats by group (n=7-9/group). All values are portrayed as mean ± SEM, one-way ANOVA with Tukey post-hoc test was used. *P <0.05 for control vs. NIC group; #P <0.05 for NIC vs. PTS+NIC group.
in hypercholesterolemia patients\textsuperscript{34}. A clinical study demonstrated that weight loss can decrease blood pressure in a cohort of overweight patients\textsuperscript{35}. Apart from the vasodilatation effect of pterostilbene, the significant reduction in blood pressure as shown in PTS+NIC group could also be partially attributable to the decreased in body weight gain observed in PTS+NIC group. Next, the heart rate of nicotine-induced rats has shown an insignificant elevation. Pterostilbene has been manifested to reduce the heart rate in rats subjected to nicotine. Our finding was consistent with previous studies\textsuperscript{16,36} suggesting negative chronotropic effects of pterostilbene but its mechanisms involved are not yet fully understood.

Cardiac function was measured using the Langendorff apparatus where the perfusion pressure was calibrated to be similar among the experimental groups. Coronary flow was almost similar in all groups, suggesting that there was no vasodilatation and ischemia\textsuperscript{22,37}. Prior to the start of systolic dysfunction, one of the initial indications of cardiac dysfunction is the left ventricle (LV) diastolic dysfunction\textsuperscript{38}. According to our results, LV diastolic dysfunction induced by nicotine was tended as indicated by increased relaxation time (Tau) together with decreased ventricular relaxation rate (LV –dP/dt\textsubscript{max}) in Langendorff-perfused rat hearts. These alterations indicate that the impaired LV relaxation was probably due to stiffness of the ventricular wall\textsuperscript{39}. On the other hand, the tendency of reduction in LV-developed pressure (LVPDP) and the LV +dP/dt\textsubscript{max} further indicated the failing of LV contraction by nicotine reduced the function of the heart potassium (K\textsuperscript{+}) channel in vitro study\textsuperscript{40}. However, co-administration of pterostilbene suggested that the cardiac dysfunction progression was attenuated, similar to the previous studies in which pterostilbene was able to improve heart function by modulation of calcium (Ca\textsuperscript{2+}) handling proteins\textsuperscript{35,41}. Among the mechanisms of myocardium protection of pterostilbene against nicotine was the role of pterostilbene as an antioxidant. At the same time, a decrease in blood pressure in pterostilbene co-administered treatment with nicotine was also believed to reduce the cardiac dysfunction. The cardioprotective mechanism of pterostilbene may also be due to the blood pressure lowering effect of pterostilbene as depicted in our study which was believed to reduce the nicotine-induced cardiac dysfunction, since hypertension itself was a major key factor in cardiac dysfunction\textsuperscript{42}. The reduction of cardiac dysfunction may not be significant due to low dose of pterostilbene as shown in an animal study using pterostilbene dose of 5 mg/kg per day for 60 days\textsuperscript{16}.

Oxidative stress, which occurs as a consequence of an imbalanced ROS production and antioxidant status, is a major mechanism resulting in nicotine-induced cardiac dysfunction rat model\textsuperscript{15,43}. In the heart, mitochondria and NADPH oxidase (NOX) are the primary sources of ROS production\textsuperscript{43}. Previously, we had shown that prolonged nicotine administration was able to cause myocardial oxidative stress evidenced by the increase of NOX2 gene expression and mitochondria ROS production after 28 days\textsuperscript{9}. Increased mitochondria and NADPH-driven ROS generation can initiate lipid peroxidation in the heart; hence augmented TBARS level in the nicotine rats. Our observation is similar to several previous studies demonstrated that nicotine administration for duration of 21–28 days could cause the increase level of lipid peroxidation in the heart\textsuperscript{8,27,45,46}. Interestingly, co-administration with pterostilbene significantly attenuated the increase in TBARS level, suggesting the protective action against the cardiac oxidative damage caused by nicotine. The potential mechanisms involved might due to the ability of pterostilbene to activate the AMPK/Nrf2/HO-1 signaling pathway which can also increase the expression of antioxidant enzymes and subsequently inhibit oxidative stress\textsuperscript{16}. Moreover, the ability of pterostilbene to activate another signaling pathways which is AMPK/SIRT1/PGC1\textalpha may also be among the other mechanisms contributing to the decrease in TBARS level\textsuperscript{18}.

GSH is one of the endogenous antioxidants which plays an important role in scavenging H\textsubscript{2}O\textsubscript{2}\textsuperscript{47}. Excessive ROS production can deplete the endogenous antioxidant levels. In the present study, nicotine administration for 28 days consecutively showed a decreasing trend of the GSH level in the heart\textsuperscript{27,28}. It was likely that sub-chronic administration of nicotine has not yet cause depletion of GSH level as GSH is the second line of defense in antioxidant defense system\textsuperscript{28}. Other endogenous antioxidant such as superoxide...
dismutase (SOD) could quickly interact with the superoxide generation by nicotine. Future work therefore is warranted to measure the activity of SOD enzymes to verify whether nicotine could inhibit SOD activity. For the PTS+NIC group, no significant changes were shown in the GSH level as nicotine itself did not affect the GSH level in the rat model. The evidence of cardiac oxidative stress would be more accurate through immunohistochemistry studies that showing an increase in 3-nitrotyrosine content in left ventricle.

CONCLUSION

Our study demonstrated pterostilbene supplementation could reduce the deterioration of heart function by possibly acting as an antioxidant in the nicotine-induced cardiac injury rat model. Future studies are warranted to further investigate the protective mechanism of pterostilbene from cardiac injury caused by the oxidative stress.

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Conflict of Interest

We declare no conflict of interest in our paper.

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Statement of Informed Consent

Not applicable

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