Effect of *Vernonia Cinerea* Extract on Heart and Coronary Artery Fibrosis of Chronic Nicotine-Treated Rat

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**Abstract**

The aim of this study was to investigate the effect of *Vernonia Cinerea* extract on the occurrence of heart and coronary artery fibrosis in rats treated with chronic nicotine. Rats were divided into control (C) group, nicotine-treated (N) group, and nicotine-treated group with Vernonia Cinerea extract (NV) (100 mg/kg [n=10]). The hearts were collected after 3, 6 months, and the histopathological changes were compared using H&E and Masson’s trichrome staining. Compared to the control group, the thickness of coronary artery walls and the collagen fibrils in the heart wall were significantly increased in the N group. The percentage of fibrosis in the heart wall was higher in the N group compared to the control group. The NV group showed a significant decrease in fibrosis compared to the N group. These results suggest that the extract of *Vernonia Cinerea* has potential to prevent or delay the occurrence of heart and coronary artery fibrosis in rats treated with chronic nicotine.

**Key words:** *Vernonia Cinerea*, nicotine, heart, fibrosis, coronary artery.
Abstract

This study was designed to investigate the effects of *Vernonia cinerea* (VC) on heart fibrosis and coronary artery inflammation in chronic nicotine treated rats. Male Wistar rat were divided into control (C), nicotine group (N) received daily dose of nicotine i.p. (1 mg/kg), and nicotine with VC orally is a dose of 100 mg/kg/day (NV) for 3 and 6 months respectively. The histopathological changes of heart tissue and coronary wall by H&E staining was examined whereas the heart fibrosis was evaluated by Masson’s trichrome staining. LM observation demonstrated that there was histopathology of coronary wall revealed thickness in N group (p<0.05). The histopathological study showed increasing of intercellular collagen bundles accumulation, representing of pro-fibrosis in heart tissue. Regarding inflammation, the nicotine treated rats at 3 and 6 months presented increasing of inflammatory leukocytes neighboring the vascular wall. Moreover, hypertrophy of cardiomyocytes with irregular sizes in diameters were critically demonstrated. The intercellular space was obviously larger when compared with the control group. In addition, the accumulation and deposition of collagen bundles and plasma cells allocating within intercellular space were conspicuously observed. Interestingly, an extract from VC revealed for protection effect in NV rats for 3 and 6 months by reducing the pro-collagen fibers accumulation, decreasing of the intercellular space, and diminishing tissue inflammation. In conclusion, the supplementation with VC provided benefit to prevent the heart fibrosis and coronary wall thickness from nicotine toxicity created in the receiving chronic nicotine replacement therapy (NRT).

Keywords: *Vernonia cinerea* Less., nicotine, heart tissue, fibrosis, coronary artery

Introduction

Cigarette can cause many diseases in organ systems such as respiratory system, cardiovascular system, central nervous system, digestive system, and reproductive system. When cigarette is burned, reaction with oxygen produces smoke and chemical compounds that harmfully affect the functions of many body organs. Absolutely, Tar can cause lung cancer (Harris et al., 2004). Carbon monoxide produces a protein marker of inflammation, C-reactive protein (CRP). Furthermore, increased plasma levels of platelet factor 4, epinephrine and norepinephrine in urine are also verified in smoking person (Zevin et al., 2001).

Nicotine replacement therapy is a medically approved procedure to help people stop smoking nevertheless nicotine itself is an addictive substance having similar effects to cocaine. It induces on neuron in the brain to secrete dopamine neurotransmitter relating with human pleasure and satisfaction (Nisell et al., 1994; Schilström et al., 1998; Sziráki et al., 2002). Generally, nicotine has a half-life of 2-3 hours, so smokers need nicotine repeatedly and gradually for much more demand. Beside, nicotine has a chemical structure similar to acetylcholine (ACh) neurotransmitter (Jie, 2009; Benowitz, 2010). Additionally, the structure of nicotine directly affects the smooth
muscle cells and endothelial cells of arterial wall, in which the tunica media and intima was further altered and degraded (Cucina et al., 2000; Yoshiyama et al., 2014). Moreover, an alkaloid cotinine which is a main metabolite of nicotine, can have a half-life in blood plasma for 16 hours (Benowitz & Jacob, 1994).

In addition, the inflammatory processes can lead to the pathogenesis by irritating/worsening the smooth muscle cells of the heart vessels and cardiomyocytes. Nicotine can additionally induce the thickness of tunica adventitia of blood vessels, in which collagens type I and type III are abnormally accumulated in connective tissue layer (Sekhon et al., 2004). Simultaneously, nicotine administration is also correlated with vascular endothelial injury (Pittilo, 2000). In pregnant women, chronic nicotine damages and weakens the tunica intima, causing vascular lesions, and inflammation in newborn (Lim & Sobey, 2011). It has also been reported that nicotine induces cardiac apoptotic cell death by inducing oxidative stress (Zhou et al., 2010; Lan et al., 2016). The high levels of proinflammatory cytokines are associated with many cellular mechanisms, leading to aortic atherosclerosis (Lau et al., 2006). Nicotine affects the increase of LDL-C cholesterol levels in the blood (Gepner et al., 2011) and raises total cholesterol and glucose levels in mice and worsens coronary artery contractions, leading to cardiac arrhythmias, ischemic heart disease, hypoxemia, and acute heart failure (Benowitz et al., 2003; Benowitz & Burbank, 2016). Simply, a mechanism of wound healing and repair, is formation of cardiac fibrosis in response to inflammation. It is characterized by the imbalance of extracellular matrix (ECM), which results in the anomalous accumulation of proteins and nonproteins production and degradation, particularly collagen. Then, cardiomyocytes are replaced by connective tissue fibers of ECM. A step of fibrogenesis has been as follows: epithelial cells are injury; transforming growth factor-beta1 (TGF-beta1) is released by epithelium cells; inflammatory cells are infiltrated; reactive oxygen species (ROS) are produced; myofibroblasts are induced and produce of collagen fibers (Herum et al., 2017). A chronic nicotine administration can cause angiogenesis and fibrogenesis of mouse corneal tissue (Kim et al., 2012) and cholangiocyte proliferation and profibrotic gene expression of rat biliary fibrosis (Jasen et al., 2013).

VC is classified as Thai herbal medicine (NLEM, 2013), can be found in all regions of the country. VC used to quit smoking is the knowledge that was passed down from their predecessor. It is reported that this herb can reduce the symptoms of smoking addiction (Wongwiwatthanukit et al., 2009; Chaikoolvatana et al., 2018; Puttarak et al 2018). However, some research data on this herb in the laboratories present that it can influence antioxidant and antimicrobial activities in cell cultured of bacterial and fungi (Sonibare et al., 2016). Additionally, the VC at a dose of 20 mg/kg b.w. i.p. can help to suppressing the reactive oxygenase species (ROS), protection of liver tissue and gastrointestinal tract, reducing proinflammatory cytokines and preventing radiation induced free radical mediated DNA injury in BALB/c mice (Pratheeshkumar & Kuttan, 2010). VC also improves physical strength of the body, demonstrating by the increased antioxidant property in blood and the decreased carbon monoxide in lung. Nowadays, there are further studies on the major active
ingredients of VC in chewing form by research grants from the Tobacco Control Research and Knowledge Management Center (TRC) and Thai health promotion foundation during 2009-2010. It has been found that each part of VC plant has different chemical substances and properties. Leaves have the highest total antioxidant capacity, with total phenolics and catechin, flavonoid, and isoflavone compared to flowers and stalks (Ketsuwan et al., 2017). The properties of antioxidant and anti-inflammation of this herb and cigarette smoking have not been comprehensively studied. Moreover, no studies have been conducted on the use of VC herbal extracts in combination with chronic nicotine exposure. Moreover, Promputta et al. (2012) have reported that VC extract had anti-inflammatory activity in lung tissue. For these reason, it can be seen that the exposure of nicotine to the body can adversely affect the structures and functions of various systems. Therefore, this study was interested in studying the changes in cardiomyocytes and heart tissue together with coronary artery in chronic nicotine exposure. Moreover, the effect of herbal extracts of VC on cardioprotective tissue in chronic nicotine condition was also investigated.

Methods

Animals

In this experimental study, 30 male Wistar rat about 8 weeks old and weighing 180-200 grams were obtained from the National Laboratory Animal Office, Mahidol University Salaya Campus. The rats were allowed to acclimatize for a period of 1 week. Animals received adequate food and water throughout the three-month and six-month trials. They were fed in a temperature-controlled room at 20 ± 2°C, relative humidity at 40-75% with light-dark 12/12 hours alternatively. Animal husbandry was processed at the faculty of medicine, Srinakharinwirot University. Three months and six months of experimental animals were desired and the animals were divided into 3 groups, each group consisted of 5 rats in a plastic cage. All administrations were injected intraperitoneally (i.p.) at concentrations measured as mg/kg in volumes of 0.5 ml per animal. Control group (C) received 0.5 ml of 0.9% normal saline per day whereas nicotine group (N) received 1 mg / kg of nicotine / day were dissolved in normal saline. Nicotine supplemented with VC group (Nicotine+VC) received 1 mg / kg of nicotine / day were dissolved in normal saline after each received the VC extract orally. The VC extract were dissolved in water 100 mg / kg/ day. Notes were recorded daily about rat’s weight throughout the trial.

Fixation and Staining Procedure

At the end of 3 months and 6 months, the rats were anesthetized by anesthetic overdose with intraperitoneal pentobarbital. Heart tissues were collected in order to investigate fibrosis by staining with Masson’s trichrome (Jong et al. 2012) and H&E techniques (Anderson, 2018). The chest and abdomen were opened, the heart was perfused via the aorta and rapidly removed. Then, the proximal part of left and right coronary artery was
fixed with 4% formaldehyde and Bouin's fluid, the left and right ventricle was cut, stored at −80°C and kept into liquid nitrogen and fixed with 4% formaldehyde and Bouin's fluid.

Tissue processing

After fixation, the left ventricles were cut into 6 pieces vertical to the long axis. Tissues were dehydrated, embedded in paraffin, cut into slices 5 μm thick, and mounted on glass slides. Sections of formaldehyde-fixed tissues were stained with H&E and sections of Bouin’s fluid-fixed tissues were stained with Masson’s trichrome staining solutions for histopathological morphometry.

Morphometry of coronary artery

The whole area of all histopathological slides was scanned at ×400 magnification using a high-resolution camera (Panoramic digital slide scanner, 3HISTECH Co). Coronary artery fibrosis was determined by quantitative morphometry as encircle images of the large coronary arteries (diameters ≥ 200 mm) were studied. The inner border of the lumen and the outer border of the tunica adventitia were traced in each arterial image with Masson’s trichrome staining at magnifications of 100. Perivascular fibrosis was determined as the ratio of the area of fibrosis surrounding the vessel wall to the total vessel area. In each heart, ~80 small arteries examined (right coronary artery, 40 and left coronary artery, 40). Average values for each size of vessel were used for analysis.

VC extract

Whole plant was collected from Ongkharak by washing, sunshine drying, and incubating in hot air oven. The crude extract was prepared at Pharmacology Department, Faculty of medicine, SWU, Bangkok, TH. Primarily, mixed and squeezed dried herbal powdered plant material with distill water in dilution 1:4, then cell sonication was performed by ultrasonic bath at 40°C for 30 minutes. Consequent, filtered the solvent by Watman grade filter paper, passing through Buchner funnel which was connected with vacuum pump. The clear solvent was separated for four flasks inside the deep freezer at -20°C, collected in refrigerator at -80°C, and dehydrated by freeze-dryer. The crude extract was completely removed under reduced pressure and a semisolid mass was obtained (yield 10.09% w/w with respect to dried powder). The ready extract was reserved at 4°C previous to use.

Percent yield of herbal extract from dry weight

\[
Yaa \text{ Dok Kao} = \frac{54.03 \text{ g}}{535 \text{ g}} \times 100 = 10.09\% 
\]

Statistical analysis

Data are conveyed as mean±SE. Consecutive time- correlated changes in parameters of a group were compared by 1-way ANOVA and Bonferroni’s multiple comparison test. Differences between groups were
determined using 2-way ANOVA and a multiple comparison test. A P-value <0.05 was measured statistically significant.

The slides were scanned with a high-end whole slide by panoramic digital slide scanners (Panoramic scan, SWU, Bangkok, TH). Evolution of total aortic wall thickness was determined by panoramic viewer program. The values are expressed as mean ± standard error of mean (SEM). They were calculated by using one-way ANOVA. T-test (Graphpad prism 5.1) was used at 95% confidence intervals (p <0.05).

Results

This study showed that nicotine no had effects on the body weight of all three groups. The left ventricle sections were stained with H&E at 3- and 6-months experiments (fig. 1). Regarding H&E staining, our results showed that the normal histology of the 3 and 6-month (fig. 1A&D) heart tissue of both control rats. The histology of heart tissue rats of 3-month groups was not shown. Typically, normal myocardium is composed of cardiomyocytes (Fig. 1A&D). Each cardiomyocyte was enclosed by a sarcolemma which then was surrounded and maintained by endomysium (Fig. 1A&D). Endomysium generally showed normal connective tissue characteristic, composed of fine collagen fibers, network of reticular fibers, and some fibroblasts. Consequently, the clear spaces of endomysium and perimysium were typically regular (Fig. 1A&D). The normal cardiomyocyte was generally presented dark blue concentric nuclei (Fig 1A&D). The cross striations pattern was arranged in the eosinophilic cytoplasm (Fig. 1A&D). The ventricular cardiomyocyte diameter was regular shaped (Fig. 1A&D).

Nicotine and cardiac tissue inflammation

Based on our data collected in this study, we proposed the following analyzed histological images (Fig. 1A-F) representing the characteristic of how nicotine enhanced inflammation and how VC protected heart inflammation. Our result showed that N rats at 6-month period revealed the abnormal intercellular spaces which were wider than normal ones (Fig. 1B). Moreover, many cardiomyocytes showed hypertrophy in which the diameters were irregular in sizes (Fig. 1B&E). Additionally, proinflammatory cells showed an increasing of proinflammatory leukocytes. (Fig. 1E and 2E)

Nicotine and cardiac tissue pro-fibrosis

The present study offers confirmation for long-term histopathological injury and compromised heart function caused by nicotine induction in heart tissue. Intense red normal muscle cells and blue myocardial interstitial collagen fibers were examined as a result of a microscopic examination of Masson trichrome stained shown in Figure 2. To confirm the toxicity of chronic nicotine exposure, our result demonstrated that nicotine had an enhancing effect on pro-fibrosis in the N rats, as confirmed by deep blue color of the endomysium, and pericellular space (Fig. 2B). In addition, the N group showed that differences in the 3- and 6-months periods, but 3-months nicotine administration is not report.
**Figure 1** Left ventricle sections were stained with H&E at 6-month experiment; control (A&D), Nicotine (B&E), and Nicotine+VC (C&F); Cardiomyocytes (c) and nucleus (arrow heads), cardiofibroblasts (asterisk), pericellular spaces (black arrows), proinflammatory cells (yellow arrows). The scale bar: 100 µm.

At 3-month not showed.

**Effect of Vernonia cinerea in anti-inflammatory and anti-profibrotic of heart tissue**

Results of our study supported the hypothesis that VC extract could play role on cardioprotective tissue confirmed by H&E staining (Fig. 1C&F) and Masson’s trichrome staining of nicotine+VC rats at 6 months (Fig. 2C&F). The VC extract has been shown anti-inflammation ability in cardiac tissue during chronic nicotine exposure, as supported by the 3-month nicotine+VC rats which did not present proinflammatory cells and cardiofibroblasts were a small amount in heart tissue. Moreover, VC extracts has been shown anti-fibrosis activity in 3-month nicotine+VC rats, because there was no collagen bundles accumulation in the pericellular space of heart tissues when staining with Masson’s trichrome. The histology of heart tissue rats of 3-month groups was not shown in this report. However, nicotine+VC rats at 6 months remained a small number of proinflammatory leukocytes in heart tissue (Fig. 1C&F), when compared with N group (Fig. 1B&E). Regarding Masson's trichrome method, collagen fibers can be specifically identified in dark blue staining and is used to evaluate the pathology of fibrosis. According 6-month heart tissue, collagen bundles of nicotine+VC group was reduced in numbers (Fig. 2C) compared with N rats (Fig.2B).
Figure 2  Left ventricle sections were stained with Masson’s trichrome (Fig. 2A-F) at 6-month experiment; Control (A&D), Nicotine (B&E), and Nicotine+VC (C&F); Collagen bundles (yellow arrows) dominated in pericellular space, perimysium (P), vessels (Vv), and proinflammatory cells (black arrows).

The scale bar: 20 µm.

Table 1  Effect of Vernonia cinerea extract on the wall-to-lumen ratio (µm) at 3- and 6-months periods.

The results were expressed as Mean ± S.E.M (n = 10)

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Wall-to-lumen ratio of left and right coronary artery (µm)</th>
<th>p-value compared with control group</th>
<th>p-value compared with nicotine group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± S.E.M.)</td>
<td>3 months</td>
<td>6 months</td>
</tr>
<tr>
<td>Control</td>
<td>116.10 ± 1.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nicotine</td>
<td>163.40 ± 1.59*A</td>
<td>0.0019</td>
<td>0.0009</td>
</tr>
<tr>
<td>Nicotine + VC</td>
<td>131.50 ± 1.25</td>
<td>0.1485</td>
<td>0.9011</td>
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</table>

*A statistically significant difference compared to the control (p < 0.01). **B was statistically significantly different from nicotine (p < 0.01).
Effects of VC in nicotine exposure with the wall of coronary artery thickness

LM observation demonstrated that there was histopathology of the wall of coronary artery revealed thickness (Fig. 2-3). Figure 3A-D represents the total thickness of coronary arteries from different groups. Control group with normal wall thickness as shown in figure 3A, while total layers of coronary in nicotine treated rats at 6 month showed abnormalities, characterized by tunica intima, media, and adventitia thickening (Fig. 3B and Table 1). These histopathological changes were not seen in N/coine+VC groups at 3 and 6 months. Cross sections of coronary artery from N group showed thickness and vascular fibrosis characterized by high density of blue color of collagen fiber appearance (Fig. 3B). Our findings indicate that the effects of nicotine, may promote coronary tissue remodeling pathways, specifically in the regulating of systemic hypertension and coronary fibrosis. However, histological appearance of coronary is very similar to control groups (Fig. 3C and Table 1). In 100 mg/kg^-1 NV group, vascular fibrosis and blue density were reduced (Fig. 3C and Table 1). Considering the VC consumption, the protection of coronaries walls was clearly demonstrated. Interestingly, VC consumption followed in marked significant reductions of pro-fibrosis. In addition, the protections of pro-fibrosis and anti-inflammation have been recognized (Table 1).

Figure 3 A-C Cross sections of coronary artery were stained with Masson’s trichrome at 6-month. Control (C), nicotine (N), nicotine +VC, coronary artery (A), collagen fibers (yellow arrows), and coronary thickness (yellow lines). A comparative study of coronary wall thickness at 3 and 6 months (P<0.01) (Fig. D).
Discussion

The results of this study demonstrate that VC water extract reduced inflammation and pro-fibrosis of heart tissue and coronary artery of chronic nicotine treated rats. It has been reported that the nicotine causes the inflammation of the heart tissue. It is indicated by cardiomyocytes hypertrophy, endomysium fibrosis, perimysium fibrosis, fatty accumulation, and inflammatory leukocytes infiltration. The migration of inflammatory leukocytes was also appeared into the inflamed area. The results of nicotine toxicity, our research showed that the heart tissue of 6-month N rats presented increasing of proinflammatory leukocytes around the vascular walls within heart tissue. Additionally, proinflammatory leukocytes and cardiofibroblasts were also presented increasing in connective tissue of the intercellular spaces, specifically in 6-month N rats, as confirmed by H&E staining and Masson’s trichrome staining. Thus, the authors finding is in agreement with the previous works of Promputta et al. (2012), they reported that VC decrease lung inflammation and improve pro-profibrotic of rat lung during chronic nicotine exposure. The authors investigate of VC supplementation can reduce the inflammation caused by chronic nicotine as shown by having less numbers of inflammatory leukocytes present in the heart tissue and the perivascular wall of coronary artery when compared with nicotine treatment only. Our finding of anti-inflammation of VC is confirmed by the previous works of Saraphanchotithaya & Sripalakit (2015), Pratheeshkumar & Guttan (2010). Concerning to the report of chemical compounds in Thai herb, the leaf of VC has the highest substance of radical scavenging and antioxidant, treated in RBCs culture (Ketsuwan et al., 2017). Appadath Beeran et al. 2014, has been reported that hirsutinolide type sesquiterpene separated from the herb influenced cytotoxic effects in cancer epithelial cells. Thus, VC might involve the reduction or suppression of certain types of inflammatory cytokines (example IL-1beta, IL-1alpha, TGFbeta, IL-6 and IL-8) and suppress pro-inflammatory cells in slow down functions. This result indicates that VC may have a supposed function in anti-inflammation process, since recently there is an evidence of anti-inflammatory activity reported in acute and chronic models in paw edema of Wistar’s rat. Moreover, VC extract can reduce the generation of free radicals NO and ROS and reduce the synthesis of iNOS enzymes is supported by Kumpunya & Praputbut (2014). In addition, there are some indication reported about the VC methanolic extract also showed an enhancement in the phagocytic activity of macrophages. These results indicate the VC extract downregulated the inducible NO synthase and cyclooxygenase-2 (COX-2) mRNA expression in LPS-promoted macrophages Pratheeshkumar & Guttan (2010). These results indicate the immunomodulatory activity of VC. Therefore, VC is probably one of the medicinal plants that have the potential to develop as an antioxidant and anti-inflammatory.

The authors finding in the 6-month N treated group showed the pro-fibrosis of heart tissue and promote perivascular pro-fibrosis of coronary artery in rats. It is indicated by increasing of proinflammatory leukocytes and cardiofibroblasts in heart tissue. Moreover, pericellular cardiocytes and perivascular of coronary artery showed that the huge of collagen bundle accumulation. This result may support the study of Lau et al. 2006, they suggested
that the chronic nicotine exposure could induce the inflammatory processes of macrophages, which were important to speed up of atherosclerosis in low density lipoprotein (LDL)-receptor of mouse. However, these results have supported that VC may slow-down the formation of pro-collagen or collagen fibers in early stages of fibrosis. Our result demonstrated that VC had a reducing effect on pro-fibrosis in the NV rats at 6 months, as confirmed by weak blue color within the endomysium, pericellular space, perimysium and perivascular wall of coronary artery stained with Masson's trichrome. This condition is in agreement with the previous works of Li et al., (2004) have reported that nicotine promoting effects on the fibroblasts and smooth muscle cells of vascular wall and enhances the angiotensin II induced vasoconstriction effects in cell cultured. Thus, VC may protect the vascular wall injury by blocked the function of cardiofibroblasts in heart tissue and smooth muscle cells in the tunica media of coronary wall condition is in agreement with the previous works of Lucas et al., (2010) have reported that disturbance of tumor growth factor beta (TGF-beta) signaling attenuated pressure-overload-induced interstitial cardiofibroblast proliferation, collagen accumulation, and encourages left ventricle dilation, as a result creating a model of enlarged cardiomyopathy. Therefore, it is predictable that some biochemical gradients of VC might disrupt the effect strengthening of nicotine by stimulating through the pro-inflammatory pathway and pro-fibrogenesis pathway.

Conclusions

The results of this study showed the protective activity of VC extracts in chronic nicotine exposure in rat models. The activities of VC might slow-down the toxicity of nicotine to cardiac tissue and coronary fibrosis by suppressing the inflammatory process and pro-fibrotic process pathways. However, the protective effect of VC extracts on the prevention of cardiac injury due to nicotine withdrawal from cigarettes or nicotine replacement therapy also is required further study. Administration of VC resulted in a beneficial effect in chronic nicotine treated condition, regarding to the protection of vascular wall. Future studies are needed to possibly test the conception of varying the type of animal models or dose of pharmacotherapy treating genotypes or phenotypes of the rate of nicotine and VC metabolism in the molecular levels.

Acknowledgements

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