

# Mechanism of Moxa Combustion Products Processed Under Different Conditions on Regulating Vascular Endothelial Function in Atherosclerotic Mice

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## Research

**Keywords:** moxa smoke, atherosclerosis, vascular endothelial function

**Posted Date:** January 11th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-141956/v1>

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# Mechanism of Moxa Combustion Products Processed Under Different Conditions on Regulating Vascular Endothelial Function in Atherosclerotic Mice

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## Abstract

**Background:** Atherosclerosis (AS) is a kind of chronic progressive inflammatory disease, moxibustion is an increasingly popular alternative therapy that reduces the risk of AS by regulating blood lipid levels. Although moxibustion for AS is yet to be conducted, its underlying mechanism remains unclear. In this study, we investigated the anti-atherogenic effect of moxa combustion products processed under different conditions (moxa smoke, filtered moxa smoke, volatile components of moxa floss and essential oil of *Artemisia Argyi*).

**Methods:** The mice in all groups were regularly grabbed and fixed. The mice in moxa smoke group were exposed to 2% concentration of moxa smoke, the mice in filtered moxa smoke group were exposed to filtered moxa smoke environment, the mice in volatile components of moxa floss group were exposed to 150°C moxa floss heating environment, the mice in essential oil of *Artemisia Argyi* group were exposed to *Artemisia Argyi* essential oil atomization environment. All interventions were carried out in the cabinet and were performed for 20 min per day, 6 days per week for 14 weeks. After the treatment, the mice were euthanased. The mice of plasma were measured by biochemical or ELISA method, the thoracic aorta was collected for red oil O staining.

The mRNA levels in the thoracic aorta were analyzed by RT-qPCR.

**Results:** The group moxa smoke group and filtered moxa smoke group showed a significantly lower plaque area percentage in the thoracic aorta, and higher expression of AMPK-mRNA and eNOS-mRNA in the thoracic aorta compared with the AS mice.

**Conclusion:** Moxa smoke and filtered moxa smoke equally suppressed the progression of atherosclerotic lesions in ApoE<sup>-/-</sup> mice. It is suggested that the particles in moxa smoke may not be the key component of moxibustion.

**Keywords:** moxa smoke, atherosclerosis, vascular endothelial function

## Background

Atherosclerosis (AS) is a kind of chronic progressive inflammatory disease that occurs in large and medium-sized arteries, where a large number of lipids and inflammatory cells gather and form fibrous plaques, resulting in vascular lumen stenosis gradually. It is the pathological basis of acute coronary syndrome [1-3]. Vascular endothelial cells (VEC) are the barrier between blood and vascular wall, which can synthesize and secrete a variety of vasoactive components, and participate in physiological and pathological processes such as vascular tension regulation, coagulation and fibrinolysis, inflammatory reaction, neovascularization and so on [4]. Endothelial cells dysfunction (ECD) is not only the initial link in the formation of AS [5], but also the key factor in all stages of AS development [6].

Nitric oxide (NO) and endothelin-1 (ET-1) are a pair of antagonistic active substances synthesized by VEC, which cause vasodilation or contraction. In addition, they are common indicators to reflect the function of vascular endothelial cells. The imbalance of NO and ET-1 in plasma is one of the main causes of ECD [11]. It was characterized by the decrease of plasma NO production, the decrease of NO biological activity, the dysfunction of NO pathway [7,8,9] and the increase of plasma ET-1 production [10]. eNOS is a key enzyme in the synthesis of NO, and its activation can promote the secretion of NO by endothelial cells [12]. Endothelial cell culture experiment showed that AMPK activated eNOS and promoted NO production by activating Akt-eNOS pathway or PI3K/Akt/eNOS pathway [13]. It can be seen that

AMPK and its downstream cascade PI3K-Akt-eNOS play an important role in the regulation of endothelial function.

The effect of moxa combustion products (MCP) is one of the effect mechanisms of moxibustion. MCP refers to the product generated in the process of moxa combustion, and moxa smoke is the most important part. MCP contains a certain amount of particulate matter (PM). The existing certain proportion of PM<sub>2.5</sub> in MCP particles causes concern while whether the particles in MCP are the key factors of moxibustion efficacy has not been studied. Thus, we extract MCP products in different stages of moxa floss combustion process, to explore the key effective link of moxibustion, and to study whether the particles in moxa smoke are the key material of moxibustion.

Confirmed by our team in the early stage, the apolipoprotein E-knockout (ApoE<sup>-/-</sup>) mice could be prevented and treated by the intervention of moxibustion or moxa smoke, by benign regulating lipid metabolism, reducing inflammatory response and endothelial damage [14-17]. Thus, we will observe the effectiveness of MCP processed under different conditions (moxa smoke, filtered moxa smoke, volatile components of moxa floss and essential oil of *Artemisia Argyi*) on AS. This study is helpful to further reveal the effective link of moxibustion and the mechanism when treating atherosclerosis.

## **Methods**

### **Animal preparation and grouping**

Eight-week-old male ApoE<sup>-/-</sup> mice (n=60) and control C57BL/6 mice of the same background (n=12) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (SCXK (Jing) 2018-0006).

The study commenced 7 days after arrival of the animals to the laboratory to allow for acclimatization. Sixty ApoE<sup>-/-</sup> mice were randomly divided into five groups (n=12 per group): moxa smoke group, filtered moxa smoke group, volatile components of moxa floss group, essential oil of *Artemisia Argyi* group and model group. SPSS software (SPSS Inc., Chicago, IL, USA) was used for group allocation and a completely randomized block design was adopted taking weight as the block factor. C57BL/6 mice

of the same background (n=12) were selected as the control group.

The ApoE<sup>-/-</sup> mice were fed a high-fat, cholesterol-rich/atherogenic diet (containing 15% fat, 2% cholesterol and 0.05% cholic acid) and the C57BL/6 mice were fed normal food. All animals were housed in individual cages and received ad libitum access to water and food in a temperature (22±2) °C and humidity (50–60%) controlled environment under a 12 hour light/dark schedule (lights on at 08:00).

### **Moxa combustion products treatment processed under different conditions**

The operation method of the automatic exposure device (HOPE-MED 8050) is as follows: turning on the switch of the automatic exposure device and the oxygen making machine, setting the shading rate (SR: x %) of the exposure cabinet at 2 %, and starting the smoke control system. The control group and model group: the mice were placed in the non-moxa smoke exposure cabinet. Intervention group: the mice were placed in the exposure cabinet which environment is full of moxa combustion products processed under different conditions. Moxa smoke generation: researcher ignites 1.5g of moxa floss (three years) in the burning furnace which is connected to the exposure cabinet, moxa smoke is introduced into the exposure cabinet through the pipeline, and the flow rate is automatically controlled according to the preset shading rate. When the concentration of moxa smoke reaches between 10 mg/m<sup>3</sup>~15 mg/m<sup>3</sup>, the shading rate of the exposure cabinet is about 2% [18]. Filtered moxa smoke generation: the Whatman cambridge filter is installed at the entrance of the exposure cabinet to intercept particulate matter [19]. The process of moxa smoke is the same as above. Volatile components of moxa floss generation: the moxa floss is laid in the glass tube, setting 150 °C to preheat the annular heater, then starting the annular heater to heat the glass tube at a constant temperature and uniform speed, and the weight of the moxa floss is equal to that of the moxa group. Essential oil of Artemisia Argyi generation: 3.75 µl essential oil of Artemisia Argyi (equivalent to 1.5g Artemisia Argyi leaf extraction) and 16mL of distilled water are mixed into the atomizer and connected to the glass entrance of the exposure cabinet. The atomization rate was 0.8ml/min [20]. All interventions were carried out in the cabinet and were performed for 20 min per day,

6 days per week for 14 weeks. And at the end of daily intervention, clean the exposure cabinet. Twenty-four hours after the last treatment in the experiment, all mice were weighed and then anaesthetised using an intraperitoneal injection of pentobarbital (1%) at 50 mg/kg. Blood samples were obtained from each mouse through the common ophthalmic artery. Cervical dislocation was conducted shortly afterwards to ensure painless and ethical death of the mice.

Plasma total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and nitric oxide (NO) were measured by automatic biochemical analyzer, plasma oxidized-low density lipoprotein (ox-LDL), apolipoprotein A-I (ApoA-I), ET-1 were measured by enzyme-linked immunosorbent assay (ELISA) method. The thoracic aorta was collected for red oil O staining, to analyse the atherosclerotic lesions. The mRNA levels of AMPK、PI3k、Akt and eNOS in the thoracic aorta were analyzed by real-time quantitative polymerase chain reaction (RT-qPCR). Statisticians and laboratory technicians were blind to the treatment allocation, while researchers who performed treatment on the mice were not.

### **Biological sample collections and measurement of Plasma**

Mice were anaesthetized with 1% pentobarbital sodium (50 mg/kg intraperitoneally) and 1–1.5 mL blood samples were then collected from the common ophthalmic artery. After centrifugation for 15 min (4°C, 1500 rpm), the serum was stored at –20°C until use. Levels of TC, TG, HDL-C, LDL-C and NO were measured using automatic biochemical analyzer. Ox-LDL, ApoA-I and ET-1 were measured using Elisa. (TC: RGB& CHN Lot: 20210207. 30027, TG: RGB& CHN Lot: 20210207. 30027, HDL-C: RGB& CHN Lot: 20210207. 30029, LDL-C: RGB& CHN Lot: 20210207. 30030, ox-LDL: RGB& CHN Lot: 20200210. 60518M, ApoA-I: RGB& CHN Lot: 20200210. 60492M, ET-1: RGB& CHN Lot: 20200210. 60265M).

### **Analysis of lesions in the aortic root and thoracic aorta**

After blood collection, the mice were perfused with phosphate buffer saline (PBS) (0.1

mol/L), the aortic root and the thoracic aorta were dissected and fixed overnight in 4% paraformaldehyde, then the aortic root was paraffin-embedded and sectioned at 5  $\mu$ m thickness and stained with hematoxylin-eosin staining (HE) to evaluate the lesions and plaque area. The thoracic aorta was dehydrated with 20% and 30% sucrose, embedded in LEICA compound and frozen immediately in liquid nitrogen, then stored in a fridge at  $-80^{\circ}\text{C}$ . Each thoracic aorta was sectioned (6  $\mu$ m) using a freezing microtome (Leica CM1850) and stained with oil red O to visualise the extent of the lipid deposition.

### Real-Time Quantitative Polymerase Chain Reaction

The mRNA levels of AMPK、PI3k、Akt and eNOS in the thoracic aorta were analyzed by RT-qPCR. Total RNA was extracted with Trizol reagent according to the manufacturer instructions from full-length aorta. All the PCR primers used are listed in Table 1. Reverse transcription kit was used for reverse transcription reaction to synthesize cDNA, RT-qPCR was performed to determine mRNA levels of AMPK、PI3k、Akt and eNOS with the SYBR PCR Mixture. Each sample was analyzed in triplicate, normalized to GAPDH. RT-qPCR conditions were  $95^{\circ}\text{C}$  for 2 min followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 sec,  $60^{\circ}\text{C}$  for 45 sec,  $60^{\circ}\text{C}$  for 60 sec, and  $95^{\circ}\text{C}$  for 15 sec.

TABLE 1 | Primers used for RT-qPCR

Gene marker	primer F	primer R
AMPK	TTGAAACCTGAAAATGTCCTGCT	GGTGAGCCACAACCTGTTCTT
PI3K	AATGTGCCCTCTTTCGTTGT	TGAATGGTGACTGGCTGACT
Akt	ACTCATTCCAGACCCACGAC	CACAATCTCCGCACCATAGA
eNOS	TGTCTGCGGCGATGTCACT	GTCTCACCCCTTAGGACCAAGA
actin	GCTCTTTTCCAGCCTTCCTT	CTTCTGCATCCTGTCAGCAA

### Statistical analysis

Data were expressed as mean $\pm$ SD. Groups were compared by one-way analysis of variance (ANOVA) followed by post-hoc test of least significant difference (LSD) using SPSS20.0 software. A probability level of  $P<0.05$  was set as the threshold of statistical significance.

## Results

### The level and comparison of TC and TG in each group of mice

The content of plasma TC in the model group was significantly higher than that in the control group ( $P=0.000$ ). The content of plasma TC in the moxa smoke group, filtered moxa smoke group, volatile components of moxa floss group and essential oil of Artemisia Argyi group were significantly lower than that in the model group ( $P=0.000$ ,  $P=0.000$ ,  $P=0.000$ ,  $P=0.035$ ). Comparison among the MCP groups, the effects of moxa smoke group, filtered moxa smoke group, volatile components of moxa floss group and the essential oil of Artemisia Argyi group in lowering plasma TC level decreased in turn, and there were statistical differences between the groups ( $P<0.05$ ).

The content of plasma TG in the model group was significantly higher than that in the control group ( $P=0.005$ ). The content of plasma TG in the moxa smoke group and filtered moxa smoke group were significantly lower than that in the model group ( $P=0.046$ ,  $P=0.016$ ). The content of plasma TG in the volatile components of moxa floss group and essential oil of Artemisia Argyi group were lower than that in the model group, but there is no significant difference ( $P=0.362$ ,  $P=0.351$ ). Comparison among the MCP groups, the content of plasma TG in the moxa smoke group and filtered moxa smoke group were significantly lower than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ), there was no statistical difference among other groups ( $P>0.05$ ). These results were shown in Figures 1A–B.



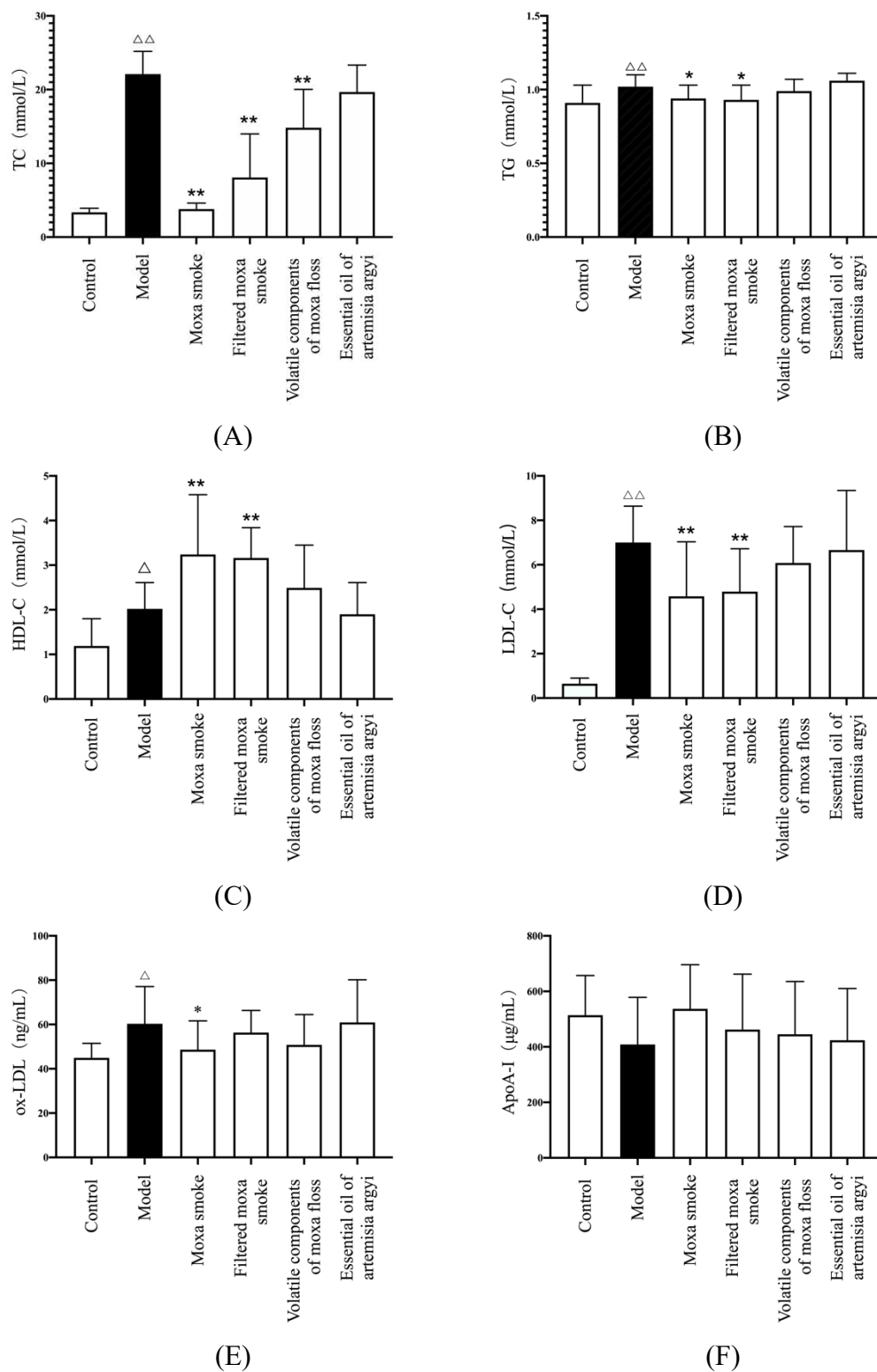


FIGURE 1: Levels of plasma TC, TG, HDL-C, LDL-C, ox-LDL and ApoA-I. (A) Plasma concentrations of TC, (B), Plasma concentrations of TG, (C) Plasma concentrations of HDL-C, (D) Plasma concentrations of LDL-C, (E) Plasma concentrations of ox-LDL, Plasma concentrations of ApoA-I. Compared with Control,  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$ ; Compared with Model group,  $*p < 0.05$ ,  $**p < 0.05$  (Mean  $\pm$  SEM, n = 12). TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; ox-LDL, oxidized-low density lipoprotein-

### **The level and comparison of HDL-C and LDL-C in each group of mice**

The content of plasma HDL-C in the model group was significantly higher than that in the control group ( $P=0.022$ ). The content of plasma HDL-C in the moxa smoke group and filtered moxa smoke group were significantly higher than that in the model group ( $P=0.006, P=0.002$ ). Compared with the model group, there was no significant difference in the content of plasma HDL-C in the volatile components of moxa floss group and essential oil of Artemisia Argyi group ( $P=0.205, P=0.870$ ). Comparison among the MCP groups, the content of plasma HDL-C in the moxa smoke group and filtered moxa smoke group were higher than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ), and there was no statistical difference among other groups ( $P>0.05$ ).

The content of plasma LDL-C in the model group was significantly higher than that in the control group ( $P=0.000$ ). The content of plasma LDL-C in the moxa smoke group and filtered moxa smoke group were significantly higher than that in the model group ( $P=0.001, P=0.002$ ). Compared with the model group, there was no significant difference in the content of plasma LDL-C in the filtered moxa smoke group and volatile components of moxa floss group ( $P=0.183, P=0.617$ ). Comparison among the MCP groups, the content of plasma LDL-C in the moxa smoke group was higher than that in the volatile components of moxa floss group and essential oil of Artemisia Argyi group ( $P<0.05$ ), and there was no statistical difference among other groups ( $P>0.05$ ). These results were shown in Figures 1C–D.

### **The level and comparison of ox-LDL and ApoA-I in each group of mice**

The content of plasma ox-LDL in the model group was significantly higher than that in the control group ( $P=0.008$ ). The content of plasma ox-LDL in the moxa smoke group was significantly lower than that in the model group ( $P=0.043$ ). Compared with the model group, there was no significant difference in the content of plasma ox-LDL in the filtered moxa smoke group, volatile components of moxa floss group and

essential oil of Artemisia Argyi group ( $P=0.912$ ,  $P=0.098$ ,  $P=0.486$ ) . Comparison among the MCP groups, the content of plasma ox-LDL in the moxa smoke group was lower than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ) , and there was no statistical difference among other groups ( $P>0.05$ ) .

Tested by ANOVA, there was no significant difference in the content of plasma ApoA-I among all groups ( $P>0.05$ ) . These results were shown in Figures 1E–F.

### **The level and comparison of NO and ET-1 in each group of mice**

The content of plasma NO in the model group was significantly lower than that in the control group ( $P=0.001$ ) . The content of plasma NO in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group were significantly higher than that in the model group ( $P=0.001$ ,  $P=0.029$ ,  $P=0.046$ ) . There was no significant difference in the content of plasma NO between the model group and essential oil of Artemisia Argyi group ( $P=0.308$ ) . Comparison among the MCP groups, the content of plasma NO in the moxa smoke group was higher than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ) , and there was no statistical difference among other groups ( $P>0.05$ ) .

The content of plasma ET-1 in the model group was significantly higher than that in the control group ( $P=0.002$ ) . The content of plasma ET-1 in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group were significantly lower than that in the model group ( $P=0.001$ ,  $P=0.000$ ,  $P=0.000$ ) . There was no significant difference in the content of plasma ET-1 between the model group and essential oil of Artemisia Argyi group ( $P=0.094$ ) . Comparison among the MCP groups, the content of plasma ET-1 in the filtered moxa smoke group and volatile components of moxa floss group were lower than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ) , and there was no statistical difference among other groups ( $P>0.05$ ) .

The content of plasma NO/ET-1 in the model group was significantly lower than that in the control group ( $P=0.030$ ) . The content of plasma NO/ET-1 in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group

were significantly higher than that in the model group ( $P=0.002, P=0.025, P=0.047$ ). There was no significant difference in the content of plasma NO/ET-1 between the model group and essential oil of *Artemisia Argyi* group ( $P=0.426$ ). Comparison among the MCP groups, the content of plasma NO/ET-1 in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group were higher than that in the essential oil of *Artemisia Argyi* group ( $P<0.05$ ), and there was no statistical difference among other groups ( $P>0.05$ ). These results were shown in Figures 2A–C.

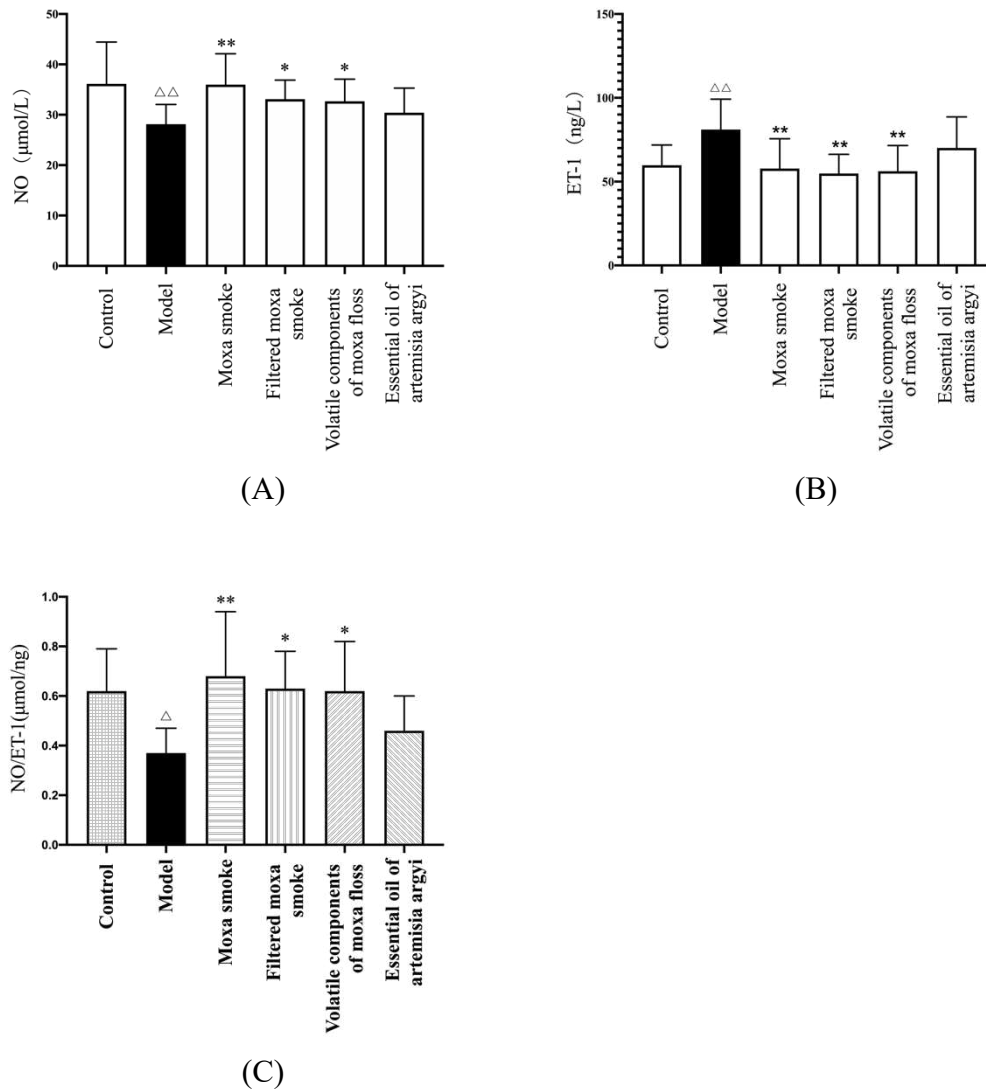


FIGURE 2: Levels of plasma NO, ET-1 and NO/ET-1. (A) Plasma concentrations of NO, (B) Plasma concentrations of ET-1, (C) Ratio of NO/ET-1. Compared with Control,  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$ ; Compared with Model group,  $*p < 0.05$ ,  $**p < 0.05$  (Mean  $\pm$  SEM,  $n = 12$ ). NO, Nitric oxide; ET-1, endothelin-1.

### **Pathological morphology of the aortic root and thoracic aorta**

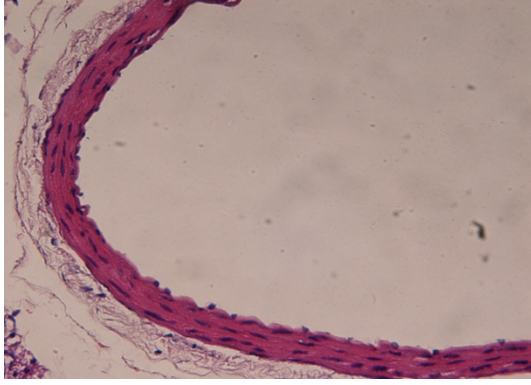
The thoracic aorta of each group was stained with HE and oil red O staining, and the histopathological changes such as gross morphology, vascular structure and integrity, plaque structure and lipid infiltration of aorta were observed from cross section and longitudinal section respectively, as shown in Figures 3A-F and Figures 4A-F.

Control group. The three-layer structures of intima, media and adventitia were clearly visible, the vascular endothelial cells were intact, the internal elastic membrane was obvious, the smooth muscle of vascular media was as usual and arranged in order. Otherwise, there was no other abnormalities and obvious lipid infiltration found.

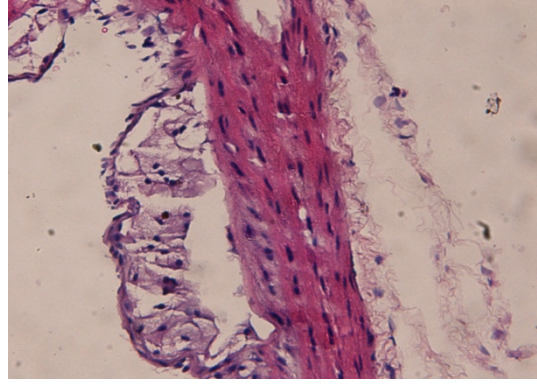
Model group. The vascular inner wall was not smooth with exfoliated endothelial cells, intima and media thickened obviously and elastic fibers were broken accompanied by the formation of atherosclerotic plaques, obvious stenosis of the whole vascular lumen, and there were more fat droplets in the plaques.

Moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group. The three-layer structure of the intima, media and adventitia of vascular wall were clearly visible, a small amount of elastic fibers was broken and fibrous plaques are formed, and the whole vascular lumen was slightly narrowed. Otherwise, a small amount of lipid infiltration was found.

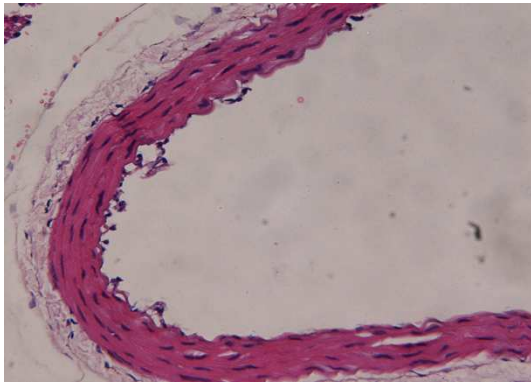
Essential oil of *Artemisia Argyi* group. The inner wall of the blood vessel was not smooth with exfoliated endothelial cells, intima and media thickened obviously and elastic fibers were broken accompanied by the formation of atherosclerotic plaques, obvious narrowing of the whole vascular lumen, and there were more fat droplets in the plaques.



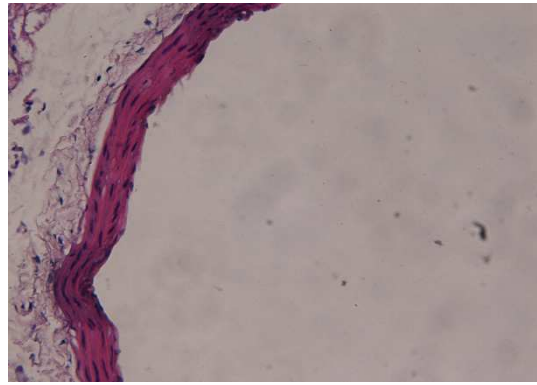
(A)



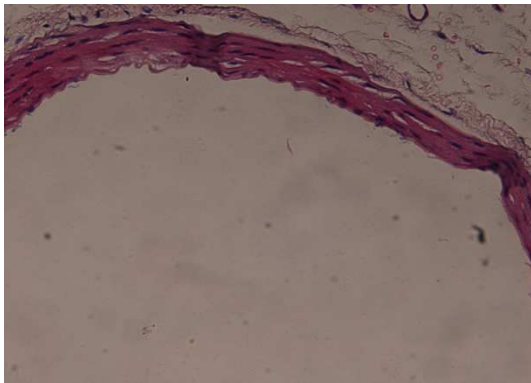
(B)



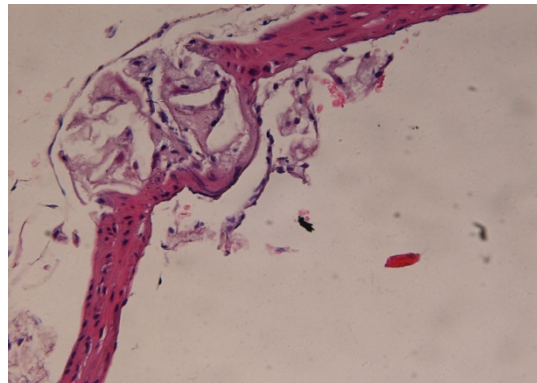
(C)



(D)



(E)



(F)

FIGURE 3: Representative images of HE the aortic root in each group (A) Control group, (B) Model group, (C) Moxa smoke group, (D) Filtered moxa smoke group, (E) Volatile components of moxa floss group, (F) Essential oil of Artemisia Argyi group.



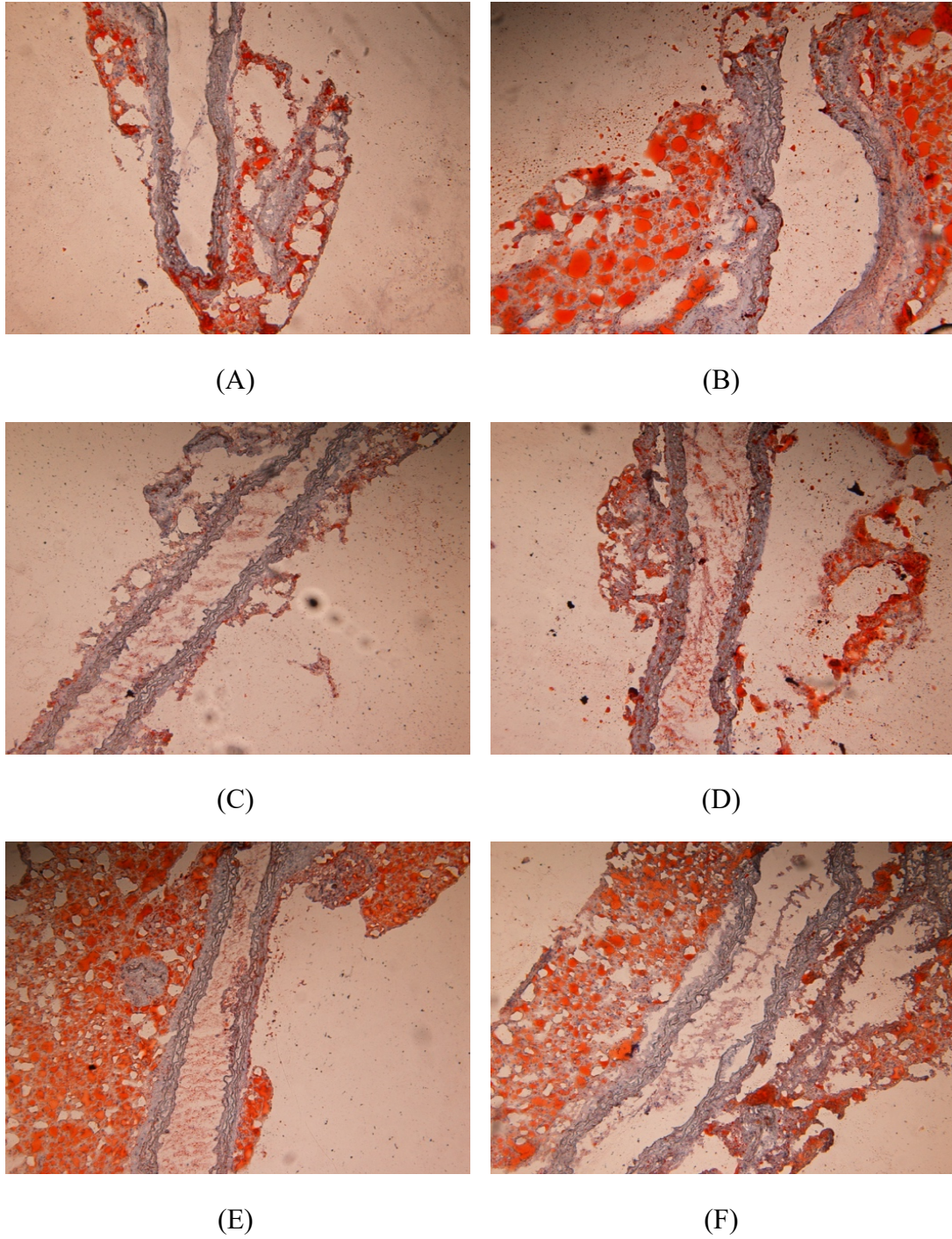


FIGURE 4: Representative images of oil red O staining aortic trunk in each group (A) Control group, (B) Model group, (C) Moxa smoke group, (D) Filtered moxa smoke group, (E) Volatile components of moxa floss group, (F) Essential oil of *Artemisia Argyi* group.

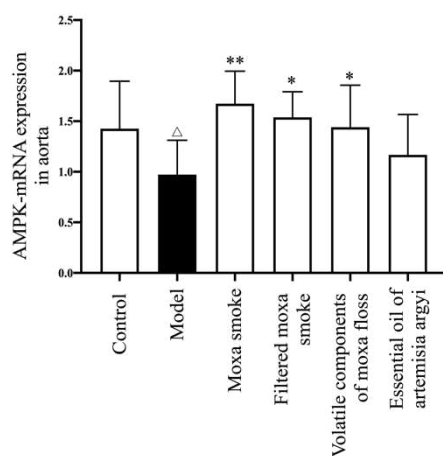
### The mRNA expression of AMPK, PI3K, Akt and eNOS in the thoracic aorta of mice

AMPK-mRNA of aorta in the model group was significantly lower than those in

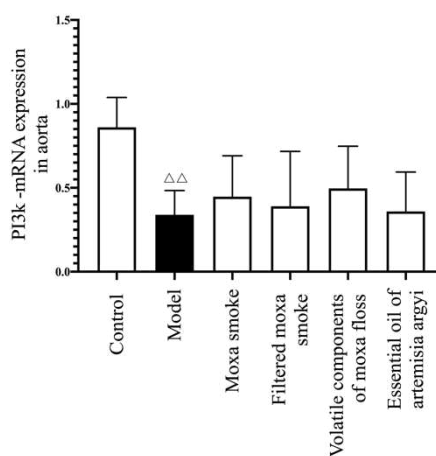
control group( $P=0.011$ ). AMPK-mRNA of aorta in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group were significantly higher than that in the model group( $P=0.017$ ,  $P=0.032$ ,  $P=0.045$ ) . There was no significant difference in AMPK-mRNA of aorta between the model group and essential oil of Artemisia Argyi group( $P=0.653$ ) . Comparison among the MCP groups, AMPK-mRNA of aorta in the moxa smoke group were significantly higher than that in the essential oil of Artemisia Argyi group ( $P<0.05$ ), and there was no statistical difference among other groups ( $P>0.05$ ) .

Tested by ANOVA, there was no significant difference in PI3K-mRNA, Akt-mRNA of aorta among all groups ( $P>0.05$ ) .

The eNOS-mRNA of aorta in the model group was significantly lower than those in control group( $P=0.002$ ). The eNOS-mRNA of aorta in the moxa smoke group, filtered moxa smoke group and volatile components of moxa floss group were significantly higher than that in the model group( $P=0.010$ ,  $P=0.021$ ,  $P=0.049$ ) . There was no significant difference in eNOS-mRNA of aorta between the model group and essential oil of Artemisia Argyi group( $P=0.705$ ) . Comparison among the MCP groups, There was no significant difference in eNOS-mRNA of aorta between the moxa smoke group and filtered moxa smoke group ( $P<0.05$ ), and there was significant difference among other groups ( $P>0.05$ ). These results were shown in Figures 5A–D.



(A)



(B)



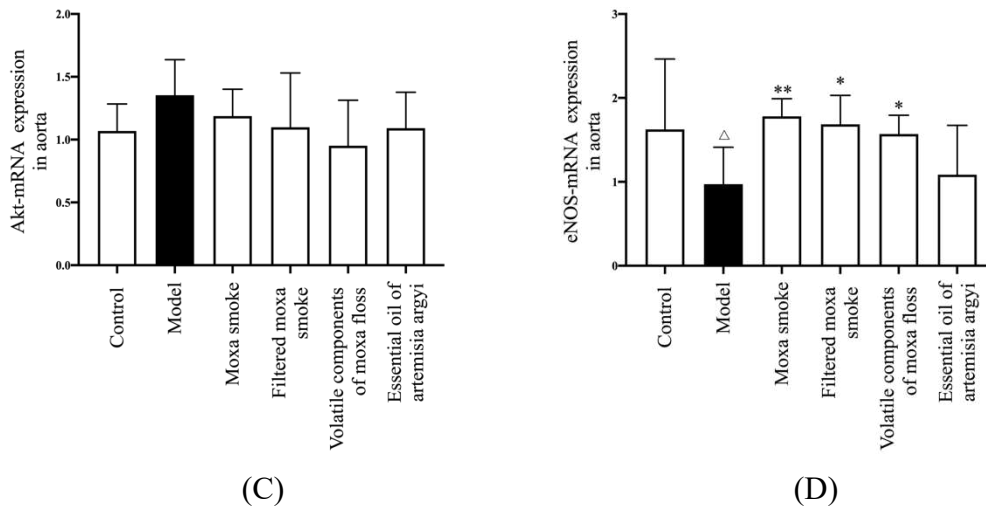


FIGURE 5: (A) mRNA expression levels of AMPK, (B) mRNA expression levels of PI3K, (C) mRNA expression levels of Akt, (D) mRNA expression levels of eNOS. Compared with Control,  $\Delta p < 0.05$ ,  $\Delta\Delta p < 0.01$ ; Compared with Model group,  $*p < 0.05$ ,  $**p < 0.05$  (Mean  $\pm$  SEM, n = 12).

## Discussion

Severe lipid metabolic disorders are the basis of the pathogenesis of AS, which is usually characterized by a significant increase in the levels of TG, TC, LDL and VLDL, a significant decrease in the levels of HDL and other related lipoproteins (such as ox-LDL, ApoA-I) with metabolic abnormalities. In this experiment, it was found that the content of plasma TC, TG, LDL-C and ox-LDL in the model group increased significantly, while the content of plasma ApoA-I decreased significantly. It is worth noting that the content of plasma HDL-C increased significantly, which may be related to the genetic background of ApoE<sup>-/-</sup> mice [21].

ApoE<sup>-/-</sup> mice are prone to form AS plaques at the root of the aorta, the smaller curvature of the aortic arch, and the branches of the aorta, pulmonary artery and carotid artery [22-24]. When 8-week-old ApoE<sup>-/-</sup> mice were fed a high-fat diet for 14 weeks, we found obvious atherosclerotic plaques in the thoracic aorta. After the intervention of moxa smoke, filtered moxa smoke and volatile components of moxa floss, the structure of aortic plaque and endothelial cells were significantly improved, but there was no significant improvement in the essential oil of Artemisia Argyi group.

AMPK-related pathways prevent and treat AS by protecting vascular function, promoting cholesterol outflow, accelerating fatty acid oxidation and inhibiting inflammation [25]. AMPK regulates the PI3K signal pathway by a complex way and stimulates the activation of the signal pathway. Similarly, Akt can also feedback and regulate the phosphorylation process of AMPK [26]. There is a wide range of adjustment between PI3K/Akt and AMPK, the relationship is mainly characterized by mutual cooperation [27]. PI3K/Akt signal pathway plays an important role in the proliferation and differentiation of vascular endothelial cells mainly through the activation of eNOS [28-30]. Studies have shown that a high-fat diet can reduce the phosphorylation of endothelial AMPK, leading to the down-regulation of the PI3K-Akt-eNOS pathway which is associated with endothelial dysfunction, and resulting in the formation of AS finally [31]. In addition, anthocyanins can increase the expression of eNOS and the production of NO by activating AMPK [32].

The results of experiment showed that the expression of AMPK-mRNA and eNOS-mRNA was enhanced by the intervention of moxa smoke and filtered moxa smoke, which further resulted in the increase of the content of NO in plasma, so as to coordinate the imbalance of NO and ET-1, and improve the function of vascular endothelial cells. It is speculated that AMPK may be related to the activation of NO signal transduction by directly affecting the activity of eNOS [33], which is worthy of further study.

## **Conclusions**

moxa smoke and filtered moxa smoke can inhibit the progression of atherosclerosis in ApoE<sup>-/-</sup> mice. The anti-atherosclerotic effect of moxa combustion products can be achieved in the following ways: (1) Regulate lipid metabolism so as to prevent early lipid accumulation. (2) The effect of moxa smoke is similar to that of filtered moxa smoke to some extent, it is suggested that the composition of particles in moxa smoke may not be the key factor in the anti-AS effect of moxibustion. This has guiding value for the smoke shield is used to filter moxa smoke during moxibustion in clinical practice, which can not only ensure the clinical effect, but also reduce patients' doubts about the

safety of moxa particles.

### **Abbreviations**

AS: atherosclerosis; VEC: vascular endothelial cell; ECD: endothelial cell dysfunction; NO: nitric oxide; ET-1: endothelin-1; eNOS: endothelial nitric oxide synthase; AMPK: adenosine monophosphate-activated protein kinase; Akt/PKC: protein kinase C; PI3K: phosphatidylinositol 3-kinase; MCP: moxa combustion products; PM: particulate matter; ApoE<sup>-/-</sup>: apolipoprotein E-knockout; TC: total cholesterol; TG: triglyceride; HDL-C: high-density lipoprotein cholesterol; LDL-C: low density lipoprotein-cholesterol; Ox-LDL: oxidized-low density lipoprotein; ApoA-I: apolipoprotein A-I; ELISA: enzyme-linked immunosorbent assay; RT-qPCR: real-time quantitative polymerase chain reaction; PBS: phosphate buffer saline; HE: hematoxylin-eosin staining; ANOVA: one-way analysis of variance;

### **Acknowledgements**

Not applicable.

### **Authors' contributions**

YPH and QH designed the project and wrote this manuscript; XLOY and JJJ conducted the NMR performed the animal experiments; XH, HW and RH analyzed the data and prepared the figures; BXZ revised the manuscript. All authors read and approved the final manuscript.

### **Competing interests**

The authors declare that they have no competing interests.

### **Availability of data and materials**

The datasets used during the current study are available from the corresponding author on reasonable request.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Ethics approval and consent to participate**

Animal care and experimental procedures used in the current study were approved by the Medicine and Animal Ethics Committee at Beijing University of Chinese Medicine (approval number: BUCM-4-2019022702-1022). All experiments were performed according to the National Guideline for the Care and Use of Laboratory Animals, Amendment 2 (State Council of China, 2013) and all efforts were made to minimise suffering.

Ethical approval of this study was obtained from the Medicine and Animal Ethics Committee at Beijing University of Chinese Medicine (approval number: BUCM-4-2019022702-1022).

**Funding**

This study was supported by National Natural Science Foundation of China (Nos. 81874503).

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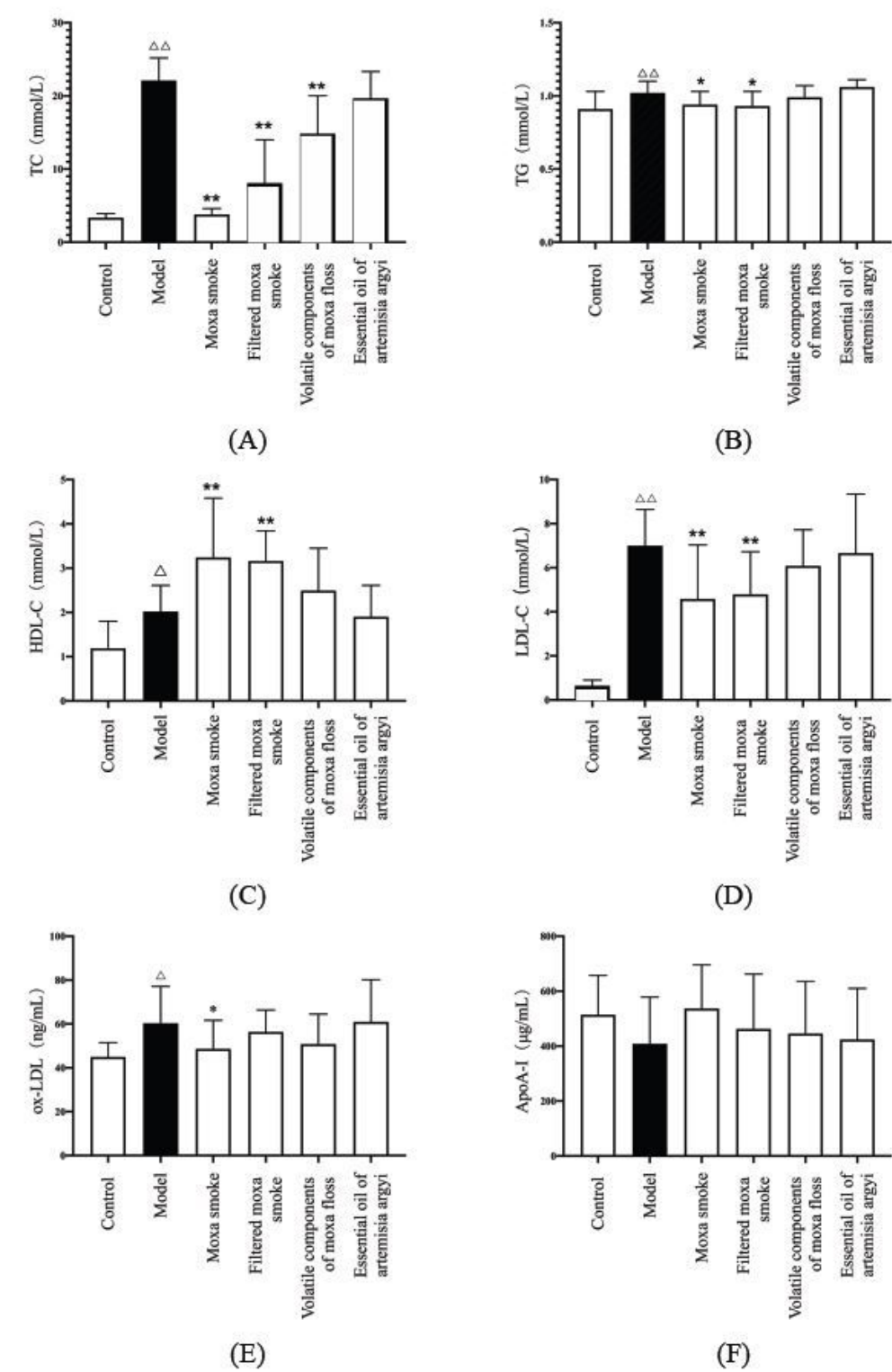
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# Figures



**Figure 1**

Levels of plasma TC, TG, HDL-C, LDL-C, ox-LDL and ApoA-I. (A) Plasma concentrations of TC, (B), Plasma concentrations of TG, (C) Plasma concentrations of HDL-C, (D) Plasma concentrations of LDL-C, (E) Plasma concentrations of ox-LDL, Plasma concentrations of ApoA-I. Compared with Control,  $p <$

0.05,  $\square\square p < 0.01$ ; Compared with Model group,  $*p < 0.05$ ,  $**p < 0.05$  (Mean  $\pm$  SEM, n = 12). TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein; LDL-C, low-density lipoprotein; ox-LDL, oxidized-low density lipoprotein cholesterol ApoA-I, apolipoprotein A-I.

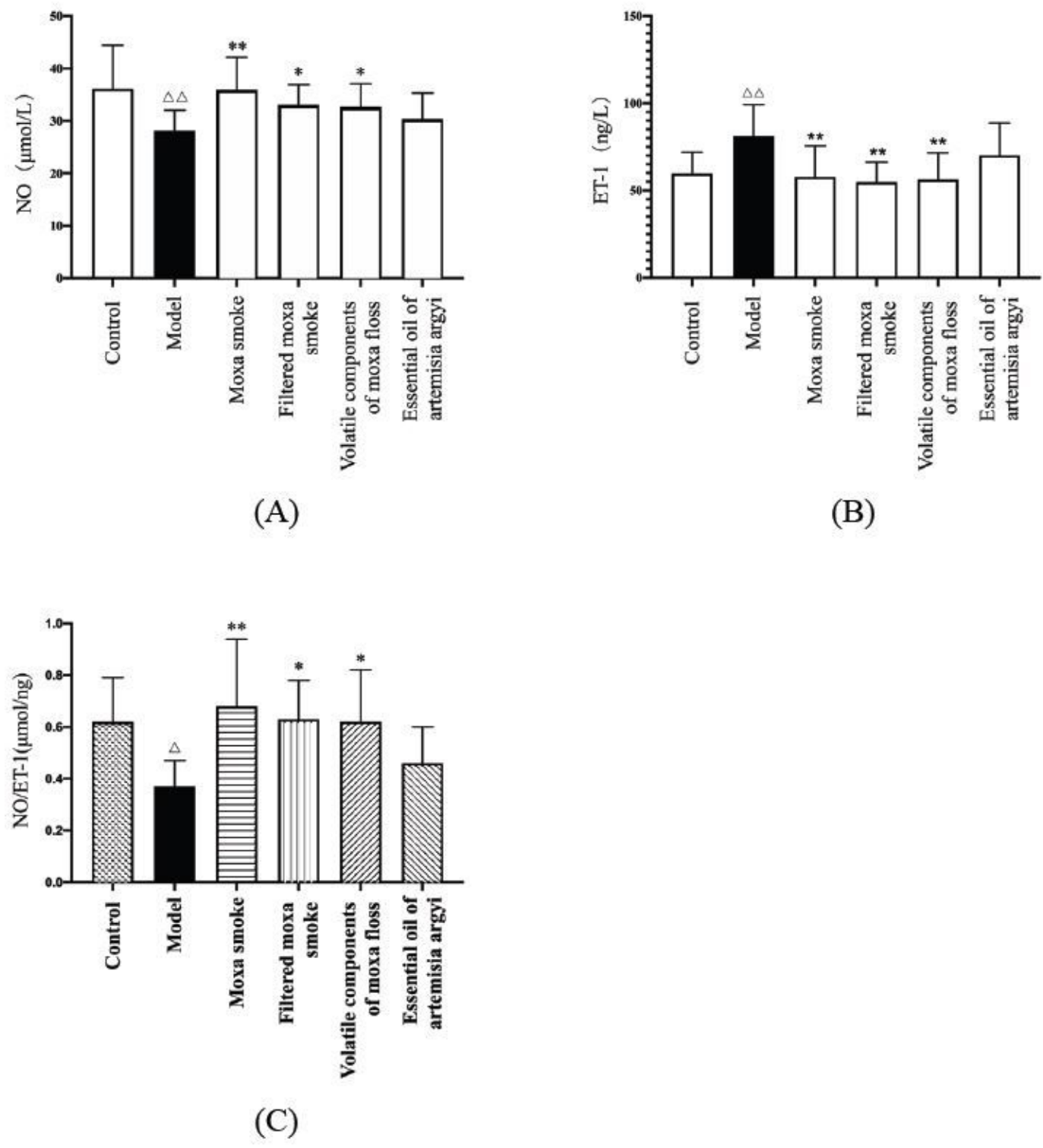
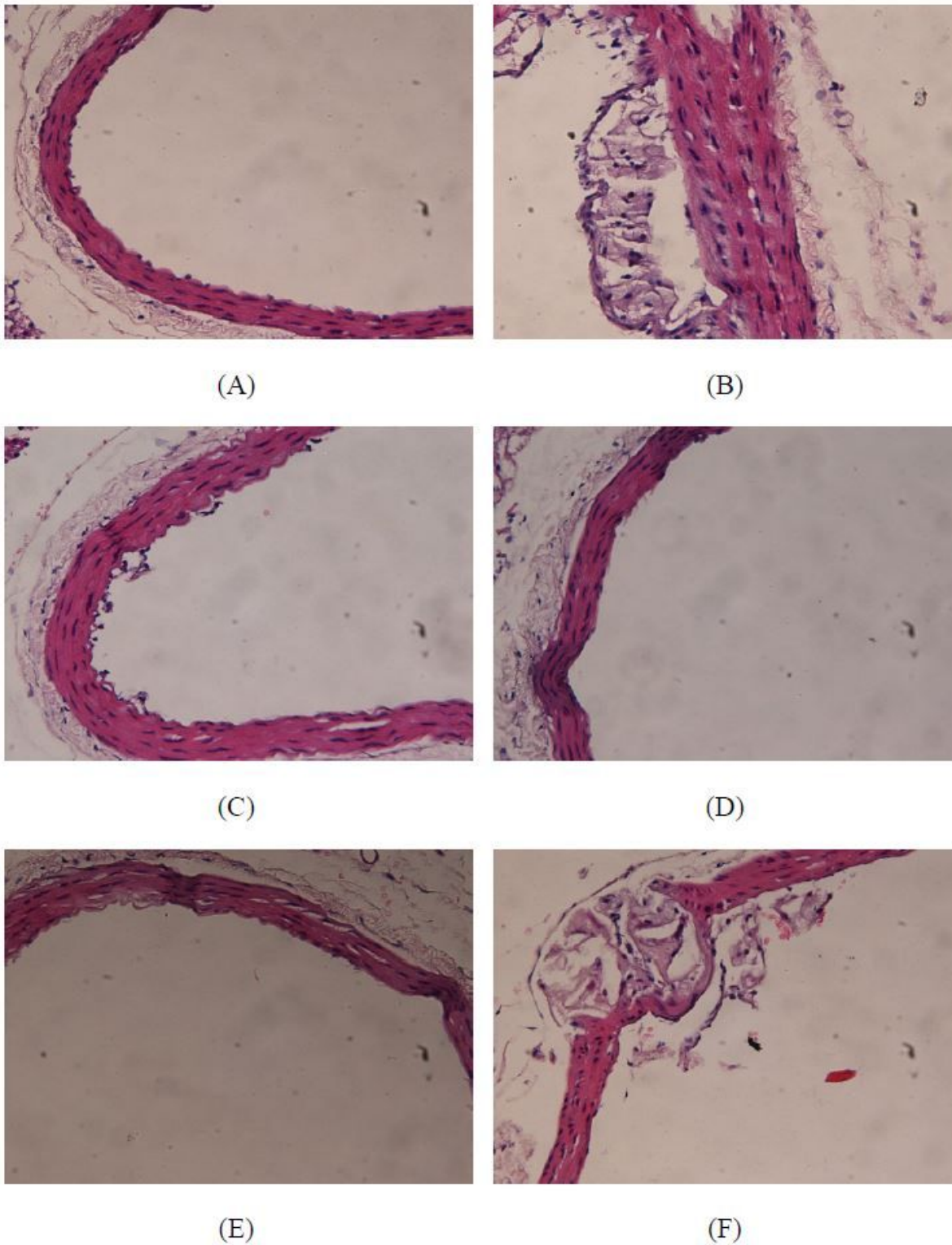


Figure 2

Levels of plasma NO, ET-1 and NO/ET-1. (A) Plasma concentrations of NO, (B) Plasma concentrations of ET-1, (C) Ratio of NO/ET-1. Compared with Control,  $\square p < 0.05$ ,  $\square\square p < 0.01$ ; Compared with Model group,  $*p < 0.05$ ,  $**p < 0.05$ .

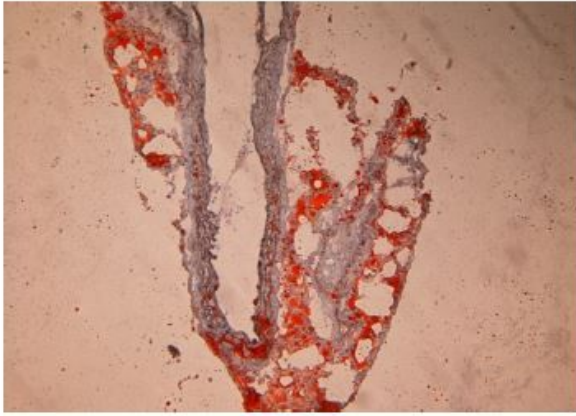
0.05, \*\*p < 0.05 (Mean  $\pm$  SEM, n = 12). NO, Nitric oxide; ET-1, endothelin-1.



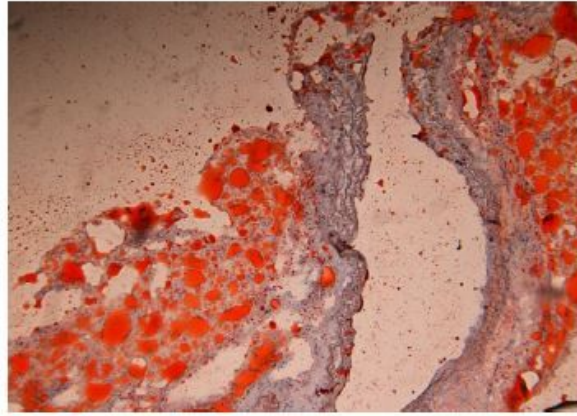
**Figure 3**

Representative images of HE the aortic root in each group (A) Control group, (B) Model group, (C) Moxa smoke group, (D) Filtered moxa smoke group, (E) Volatile components of moxa floss group, (F) Essential oil of Artemisia Argyi group.

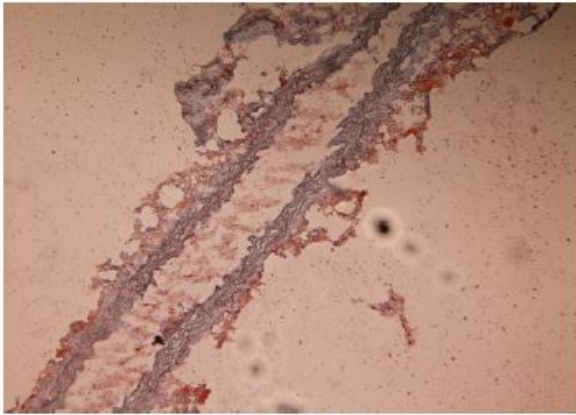




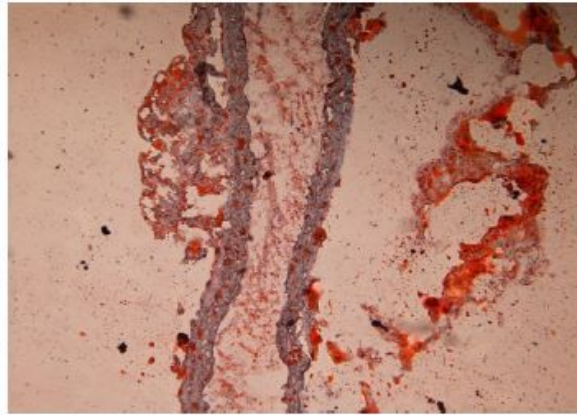
(A)



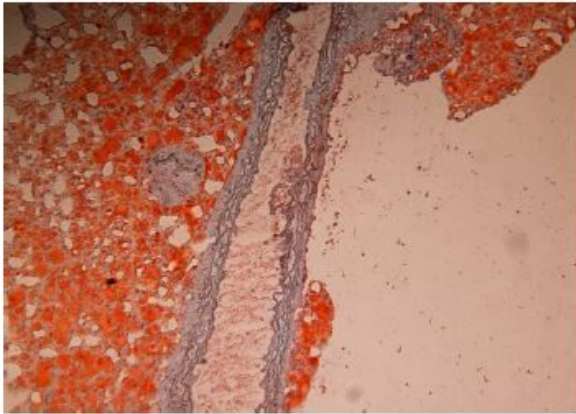
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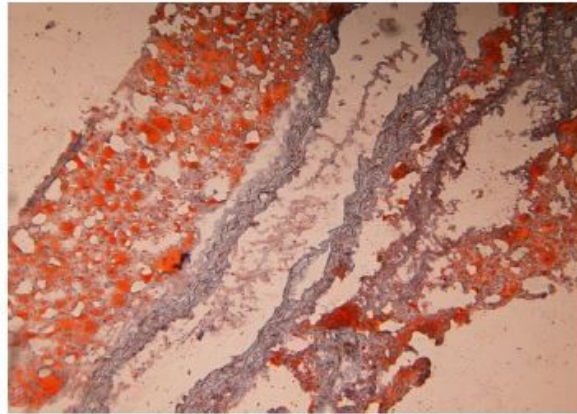
(C)



(D)



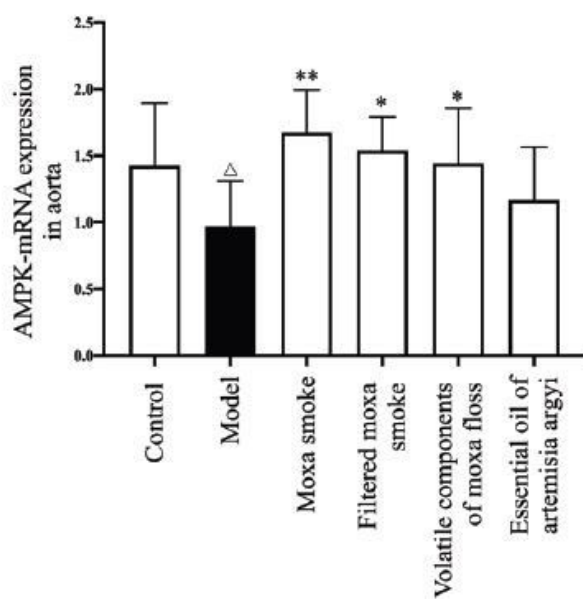
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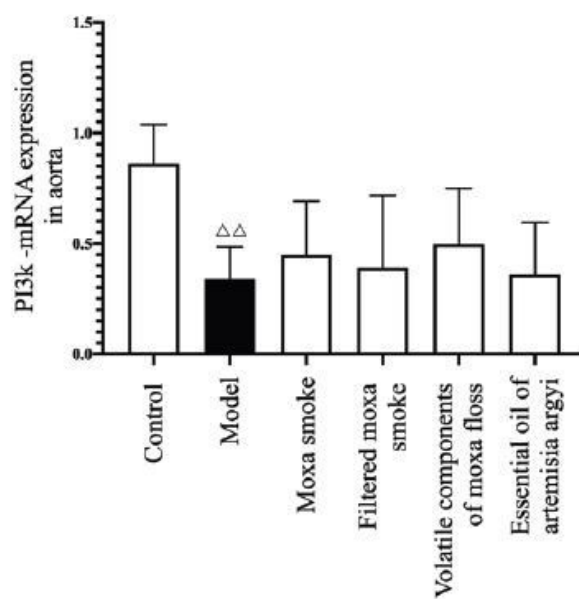
(F)

**Figure 4**

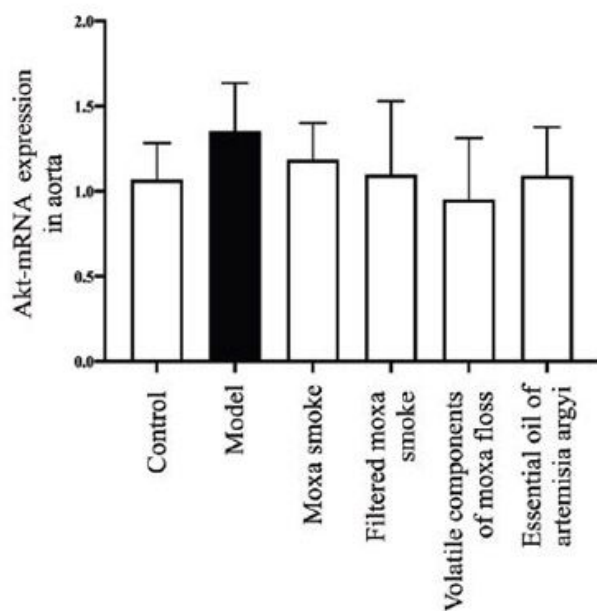
Representative images of oil red O staining aortic trunk in each group (A) Control group, (B) Model group, (C) Moxa smoke group, (D) Filtered moxa smoke group, (E) Volatile components of moxa floss group, (F) Essential oil of *Artemisia Argyi* group.



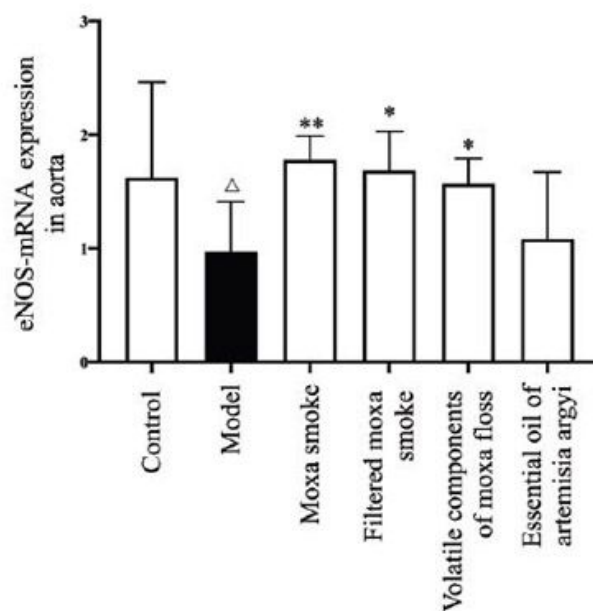
(A)



(B)



(C)



(D)

Figure 5

(A) mRNA expression levels of AMPK, (B) mRNA expression levels of PI3K, (C) mRNA expression levels of Akt, (D) mRNA expression levels of eNOS. Compared with Control,  $\square p < 0.05$ ,  $\square\square p < 0.01$ ; Compared with Model group, \* $p < 0.05$ , \*\* $p < 0.05$  (Mean  $\pm$  SEM,  $n = 12$ ).