

# Vagus Nerve Stimulation Ameliorates L-NAME-induced Preeclampsia-like Symptoms in Rats Through Inhibition of the Inflammatory Response

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## Research article

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# Abstract

**Background** Preeclampsia is characterized by an excessive inflammatory response. Recent studies have shown that vagus nerve stimulation (VNS) has anti-inflammatory properties in vivo. This study aims to investigate whether VNS is safe for use during pregnancy and to explore the therapeutic potential and underlying mechanisms of VNS in PE.

**Methods** Pregnant Sprague-Dawley rats were randomly chosen to receive N-nitro-L-arginine methyl ester (L-NAME)-containing water (preeclampsia-like mouse model) or saline (normal pregnancy control) daily at gestational days 14.5-20.5. VNS and the  $\alpha 7$ nAChR antagonist methyllycaconitine citrate (MLA, 1 mg/kg/d) were given daily at the same time.

**Results** VNS decreased the high systolic blood pressure and urinary protein observed in the PE rats. In addition, VNS mitigated abnormal pregnancy outcomes. Moreover, VNS alleviated the inflammatory response by decreasing the levels of inflammatory cytokines. VNS significantly increased the expression of  $\alpha 7$ nAChR and attenuated the activation of NF- $\kappa$ B p65 in the placenta.

**Discussion** Our findings indicate that maternal VNS treatment is safe during pregnancy and has a protective effect in a pregnant rat model of preeclampsia induced by L-NAME.

## 1. Background

Preeclampsia is a multifactorial and multisystemic disorder that occurs during pregnancy and that can lead to increased morbidity or mortality in both the mother and her unborn child.<sup>1</sup> A recent report shows that 2–8% of all pregnant women worldwide suffer from preeclampsia.<sup>2</sup> Preeclampsia is a disease that presents with hypertension and proteinuria after 20 weeks of gestation, and the complications include neurologic dysfunction, liver damage, renal insufficiency, fetal growth restriction, and even neonatal mortality.<sup>3</sup> The considerable variation in the onset, clinical manifestation, and severity of this pregnancy-specific disease creates major challenges in identifying risk factors for clinical and/or laboratory deterioration. Currently, pregnancy termination remains the only definitive cure for preeclampsia.<sup>1,4</sup>

Despite the severity of preeclampsia, the precise pathogenesis is not yet fully understood and is currently an area of active research.<sup>4</sup> A series of potential etiologies of preeclampsia, including endothelial dysfunction,<sup>5</sup> excessive inflammation,<sup>6</sup> immunological dysregulation<sup>7</sup> and oxidative stress<sup>8</sup>, has been identified. Among these potential etiologies, the imbalance in pro- and anti-inflammatory networks has emerged as the one phenomenon that is most strongly related to the clinical symptoms and disease severity of preeclampsia. There is growing evidence that preeclampsia is closely linked to an abnormal inflammation response that reveals both locally in the placenta and systemically in the mother. Quenching or inhibiting inflammatory regulation pathways may be a real mechanism of the pathogenesis of preeclampsia. As such, therapies that selectively suppress the excessive inflammatory response without leading to broad immunosuppression are desired.

Preventing pro-inflammatory cytokine production through endogenous “neuro-immune” interactions has emerged as one such treatment. Tracey has revealed that the vagally mediated cholinergic anti-inflammatory pathway is capable of acutely attenuating inflammation.<sup>9</sup> Electrical stimulation of the vagus nerve is known to have anti-inflammatory effects on multiple diseases, including ischemia reperfusion injury,<sup>10</sup> sepsis<sup>11</sup> and rheumatoid arthritis.<sup>12</sup> Vagus nerve stimulation (VNS) is an effective nonpharmacologic approach for the treatment of inflammatory disease, but

the use of VNS to inflammatory response following preeclampsia has not been thoroughly examined. Therefore, this research aimed to investigate whether VNS attenuate preeclampsia and to explore the underlying mechanisms involved using a preeclamptic model in rats

## **2. Methods**

### **2.1 Animals**

The sprague-Dawley rats, approximately 6–8 weeks old (weight range: 250–300 g), were obtained from the Medical Experimental Animal Center of Hainan. The animals were fed with regular chow and water ad libitum under controlled conditions of temperature (22–24 °C), humidity (50–70%), and lighting (12:12-h light–dark cycle). The protocols for animal use and the procedures for the experiments described here were approved by the Animal Ethics Committee of Hainan Medical University, China, and the experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals.

### **2.2 Experimental design**

After 7 days of acclimatization, the rats were placed with the female-male ratio of 2:1 and the presence of sperm in vaginal smears was used to define gestational day (GD) 0. The experimental rats were assigned to receive a single oral dose of 50 mg/kg L-NAME or distilled water daily from GD 14.5 to GD 20.5 during pregnancy.<sup>13</sup> Then, the rats were randomly divided into 8 groups, with 7–8 rats in each group, as follows: Group 1, the normal control group, received distilled water. Group 2, the P + Sham control group, was treated as group 1 but subjected to sham stimulation for 7 days. Group 3, the P + VNS control group, was treated as group 1 but received vagus nerve stimulation for 7 days. Group 4, the P + VNS + MLA control group, was treated as group 3 but received i.v. injection of MLA at a dose of 1 mg/kg/day for 7 days. Group 5, the PE group, received L-NAME for 7 days. Group 6, the PE + Sham group, was treated as group 1 but subjected to sham stimulation. Group 7, the PE + VNS group, was treated as group 1 but received vagus nerve stimulation for 7 days. Group 8, the P + VNS + MLA group, was treated as group 3 but received i.v. injection of MLA at a dose of 1 mg/kg/day for 7 days.

### **2.3 VNS and Sham Surgery**

Surgical protocols were previously described in detail.<sup>14</sup> In brief, the rats were anesthetized with isoflurane (5% in 100%O<sub>2</sub>) inhalation and maintained with a gas mask (2% isoflurane). The skin and muscles of the left cervical region were separated carefully, and then, the cuff was placed and fixed with

a suture around the nerve. Continuous stimulation was delivered by a stimulator (BL-420, TME Technology Co., Ltd., Chengdu, China) on the cervical vagal trunk connected to a control module. The stimulation duration of the implanted VNS device was set to 30 min at the given frequency and was followed by an off-time of 5.5 h; this procedure was repeated 4 times per day from GD 14.5 to GD 20.5. The stimulation parameters are identical to those used in our previous study,<sup>15</sup> namely, a stimulation frequency of 5 Hz, a low voltage of 3 V, a current of 1 mA and a pulse duration of 500  $\mu$ s. For the sham rats, the surgery was performed in the same way, but the cuff electrode was not implanted.

## **2.4 Measurement of blood pressure and heart rate**

The systolic blood pressure and heart rate were tested in all the groups of rats on the GDs 12, 14, 16, 18 and 20 by the tail-cuff technique with a BP-2000 Blood Pressure Analysis System (Visitech Systems Inc., North Carolina, USA). According to the internal programmer, the cuff was placed around the tail, inflated to block blood flow, and then slowly deflated. Each rat was prewarmed at 37 °C 30 min before each measurement was taken, and each measurement was taken 3 times to obtain the average.

## **2.5 Urine protein concentration**

On the GDs 13, 15, 17 and 19, the rats were housed separately for 24 h under the cages to collect urine samples. Urine samples were centrifuged at 2000 rpm for 15 min at 22 °C, and the supernatant was used for the analysis of protein levels by using a BCA protein assay kit (Thermo Fisher Scientific Inc, CN, Shanghai)

## **2.6 Sample preparation**

The rats underwent cesarean section after being anesthetized by an injection of 10% chloral hydrate (30 mg/kg) on GD 21. The pups and placentas were dissected, counted, inspected, and weighed. Blood was collected into plain bottles from the inferior cava vena. Two pieces of tissues were obtained from the maternal placenta immediately after delivery. One sample was washed with 0.9% NaCl, fixed in 4% paraformaldehyde and embedded in paraffin for histopathological analysis. The other samples were stored at -80 °C for the following experiments.

## **2.7 Histology and Immunohistochemistry**

The placental specimens in 4–5  $\mu$ m thick paraffin sections were stained with hematoxylin and eosin according to the standard H&E protocol and observed and photographed under an optical microscope (Olympus BX51, Tokyo, Japan). The sections were detected by a eligible and blinded pathologist to assess the extent of pathological changes.

Immunohistochemical analyses were performed to assess  $\alpha$ 7 nicotinic acetylcholine receptor ( $\alpha$ 7nAChR) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) p65 in the placentas. The placental sections selected for immunostaining were deparaffinized and hydrated in an alcohol gradient. The sections were then incubated in a 1:1,000 dilution of primary antibodies against  $\alpha$ 7nAChR (cat. no. ab10096; Abcam, MA, USA) and NF- $\kappa$ B p65 (cat. no. ab207297; Abcam, MA, USA) overnight at 4 °C. On the following day, the goat anti-mouse IgG

secondary antibodies were added to the sections at 37 °C and incubated for 30 min. The sections were dyed with 3,3'-diaminobenzidine solution (Leica Microsystems, Shanghai, China), counterstained with hematoxylin, dehydrated with ethanol and mounted in synthetic resin. The expression of  $\alpha 7$ nAChR and NF- $\kappa$ B p65 was examined by comparing positively labeled areas and total areas using Image-Pro Plus v6.0 software (Media Cybernetics, Inc., Bethesda, Maryland).

## 2.8 Enzyme-linked immunosorbent assay (ELISA) method

The concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and IFN- $\gamma$  in the maternal serum and placental homogenate were determined according to the manufacturer's instructions by ELISA kits (R&D Systems, Minneapolis, MN). The analysis was performed in duplicate using a Bio-Plex™ system (Luminex Bio-Plex™ 200 System, Bio-Rad).

## 2.9 Measurement of gene expression by quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the placental tissues by using TRIzol (Invitrogen, Carlsbad, CA, USA). The RNA concentration was determined by NanoDrop 2000 analysis (NanoDrop Products, DE, USA). The SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen, USA) was used for reverse transcription, and the TaqMan Gene Expression Master Mix (Applied Biosystems) was used for qPCR according to the manufacturer's protocol. The qPCR parameters and all the primers for the amplification of the studied genes ( $\alpha 7$ nAChR, NF- $\kappa$ B p65 and  $\beta$ -Actin) were used according to Kong et al. <sup>16</sup> Thermal cycler settings were 95 °C for 5 min, then 40 cycles of 95 °C for 20 s and 60 °C for 40 s, and 72 °C for 8 min. The primer sequences (5' to 3') for  $\alpha 7$ nAChR, NF- $\kappa$ B p65 and  $\beta$ -Actin were as follows (forward and reverse, respectively):  $\alpha 7$ nAChR (ACCTCGTGTGATCCAAAGCC and GGTTTCCTCTTGCTCAGGGT); NF- $\kappa$ B p65 (CGACGTATTGCTGTGCCTTC and TGAGATCTGCCAGGTGGTAA) and  $\beta$ -Actin (CCTCTATGCCAACACAGTGC and GTACTCCTGCTTGCTGATCC).  $\beta$ -Actin was used as the housekeeping gene. Data analysis was carried out using the  $2^{-\Delta\Delta CT}$  method.

## 2.10 Statistical analysis

All experimental data are reported as the mean  $\pm$  standard error of the mean. Comparisons between multiple groups were performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test or Dunnett's test. Data were analyzed by SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). P-values of 0.05 were considered to indicate a statistically significant difference.

## 3. Results

### 3.1 Effect of VNS on the blood pressure and heart rate of rats with L-NAME-induced preeclampsia

The baseline SBP values were not significantly different among the groups on GD 11 and GD 13 ( $P > 0.05$ ). On GD 15, the SBP was increased in the L-NAME-treated group, but to a lesser extent than the

increase observed in the non-L-NAME-treated group ( $P > 0.05$ ). On GD 17 and GD 20, we observed a significantly lower SBP in the L-NAME-treated rats that were treated with VNS than that in the untreated rats. Moreover, the SBP was not significantly different between the non-L-NAME-treated groups ( $P > 0.05$ , data not shown) at that time (Fig. 1A).

In accordance with the SBP, the average baseline HR was similar among the groups (Fig. 1B). L-NAME administration induced a rapid and progressive increase in the HR beginning on GD 17. The rats that received L-NAME and underwent VNS showed an increased HR compared to the control rats but a reduced HR compared to the rats that received L-NAME alone. We also observed that concurrent treatment with VNS and MLA significantly attenuated the elevated HR in the L-NAME-treated rats. Interestingly, among the non-L-NAME-treated groups, the P+VNS or P+VNS+MLA groups exhibited a significantly lower HR than the P and P+sham groups on GD 18 and GD 20 ( $P < 0.01$ ).

### 3.2 Effect of VNS on urinary protein in rats with L-NAME-induced preeclampsia

At the beginning of the experiment, the degree of proteinuria was not significantly different ( $P > 0.05$ ) between the groups. The increase in proteinuria after L-NAME treatment was significantly enhanced on GD 17 and 20. VNS administration showed a strong ability to reduce proteinuria in the L-NAME-treated rats. Moreover, the 24-h urinary protein level was significantly higher in the PE+VNS+MLA rats than in the PE+VNS rats ( $P < 0.05$ ). The 24-h urinary protein level did not significantly vary throughout pregnancy in the non-L-NAME-treated groups ( $P > 0.05$ ) (Fig. 2). The data for the P+sham, P+VNS and P+VNS+MLA groups are not shown.

### 3.3 Effects of VNS on the morphological changes in the placenta in rats with L-NAME-induced preeclampsia

As shown in Fig. 3, optical microscopic observation (H&E staining) revealed slight calcification and a few syncytiotrophoblast nodules of the placenta in the non-L-NAME-treated groups. The placentas were seriously impaired in the PE and PE+Sham groups, as indicated by substantial inflammatory cell infiltration, large-scale villous infarction and fibrin-like substance deposition. Chronic VNS reversed the detrimental changes in the placenta in the PE+VNS rats. However, the morphological characteristics of placentas in the PE+VNS+MLA group did not differ from those in the PE and PE+Sham groups.

### 3.4 Effects of VNS on pregnancy outcomes in rats with L-NAME-induced preeclampsia

We chose various indexes to assess pregnancy outcomes, mainly maternal and placental weight, pup weight and live pup number (Tab. 1). There were no differences in maternal and placental weight among the various groups. L-NAME administration significantly lowered the weight of the pups and the number of living pups compared to those of the pregnant control groups. In addition, VNS treatment during pregnancy significantly alleviated the LPS-induced reduction in placental weights, but the effects of VNS were abolished by MLA. Notably, one of the pregnant rats in the PE group was born prematurely on GD 16, and one fetus was malformed (Fig. 4).

### 3.5 Effects of VNS on the levels of proinflammatory cytokines in both the serum and placenta in rats with L-NAME-induced preeclampsia

We measured the serum and placental protein concentrations of TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10 and IFN- $\gamma$  in the 8 groups. In the PE and PE+Sham rats, TNF- $\alpha$  was significantly upregulated in both the serum and placenta compared with the levels in the non-L-NAME-treated rats, and the upregulation was significantly inhibited by VNS; this trend was also observed for IL-1 $\beta$  and IL-6. However, in the PE+VNS+MLA group, the apparent suppression of the TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 concentrations did not reach statistical significance (Fig. 5). The levels of IL-10 and IFN- $\gamma$  in the serum and placenta did not vary significantly across different groups ( $P > 0.05$ , data not shown).

### 3.6 VNS increased placental $\alpha 7nAChR$ mRNA and protein expression in rats with L-NAME-induced preeclampsia

The real-time PCR results showed that the  $\alpha 7nAChR$  mRNA expression was significantly higher in the P+VNS group than in the other groups. The downregulated  $\alpha 7nAChR$  mRNA levels following L-NAME treatment were elevated by VNS treatment but not by concurrent treatment with VNS and MLA. No statistically significant differences were observed among the P, P+sham, P+VNS+MLA, PE, PE+sham and PE+VNS+MLA groups ( $p > 0.05$ ) (Fig. 6A). The immunohistochemical assay results also revealed that the  $\alpha 7nAChR$  protein expression was significantly higher in the P+VNS group than in the P and P+sham groups ( $p < 0.05$ ), and its levels were even higher in the PE+VNS group than that in the PE and PE+sham groups. Moreover, the inhibitory effects of nicotine were significantly blocked by MLA ( $p < 0.05$ ) (Fig. 7).

### 3.7 VNS increased placental NF- $\kappa B$ 65 mRNA and protein expression in rats with L-NAME-induced preeclampsia

The placental NF- $\kappa B$  65 mRNA and protein expression levels showed no significant difference ( $P > 0.05$ ) among the non-L-NAME-treated groups. Maternal exposure to L-NAME significantly increased ( $P < 0.05$ ) the level of NF- $\kappa B$  65 mRNA in the placenta. L-NAME-induced placental NF- $\kappa B$  65 mRNA expression was attenuated ( $P < 0.05$ ) by VNS treatment during gestation (Fig. 6B). Immunohistochemical assay results also indicated that the level of placental nuclear NF- $\kappa B$  p65 was significantly increased ( $P < 0.05$ ) in rats treated with L-NAME (Fig. 7), and this level was decreased ( $P < 0.05$ ) by VNS administration. However, MLA supplementation dramatically inhibited the VNS-induced decreases in NF- $\kappa B$  p65 mRNA and protein expression ( $P < 0.05$ ).

**Table 1:** The effects of VNS treatment on pregnancy outcomes in L-NAME induced PE rats on GD 21.

Group (n=8)	Live fetuses	Maternal weight (g)	Fetal weight (g)	Placental weight (g)
P	15.22 ± 0.35	401.21± 11.37	4.77 ± 0.12	0.56 ± 0.021
P+VNS	14.19 ± 0.54	398.20± 15.64	4.69 ± 0.06	0.52 ± 0.023
P+Sham	15.70 ± 0.61	400.41± 13.12	4.70 ± 0.09	0.54 ± 0.035
P+VNS+MLA	15.42 ± 0.39	397.03 ± 14.31	4.72 ± 0.11	0.51 ± 0.041
PE	10.18 ± 0.84 <sup>*/#</sup>	396.21± 13.72	3.58 ± 0.08 <sup>*/#</sup>	0.50 ± 0.027 <sup>*/#</sup>
PE+VNS	13.16 ± 0.68 <sup>*</sup>	399.21. ± 12.03	4.41 ± 0.13 <sup>*</sup>	0.53 ± 0.033 <sup>*</sup>
PE+Sham	10.41 ± 0.75 <sup>*/#</sup>	400.48± 14.90	3.67 ± 0.10 <sup>*/#</sup>	0.52 ± 0.042 <sup>*/#</sup>
PE+VNS+MLA	11.17 ± 0.42 <sup>*/#</sup>	398.92. ± 13.82	3.89 ± 0.07 <sup>*/#</sup>	0.55 ± 0.019 <sup>*/#</sup>

Data are analyzed by One-way ANOVA, and presented as Mean±SEM. \**P*<0.05 vs P, P+VNS, P+Sham and P+VNS+MLA; #*P*<0.05 vs PE+VNS group.

## 4. Discussion

In this study, we found that VNS treatment improved adverse pregnancy outcomes, such as decreasing the high SBP and HR, reducing the urinary protein excretion, promoting recovery from placental injury, and suppressing inflammation in rats with L-NAME-induced preeclampsia. Moreover, VNS also increased placental  $\alpha 7$ nAChR expression and effectively inhibited placental NF- $\kappa$ B p65 activation. Meanwhile, the protective effects of VNS could be blocked by systemic pretreatment with a specific  $\alpha 7$ nAChR antagonist.

Inflammatory pathways are implicated in several adverse obstetrical outcomes, specifically preeclampsia.<sup>17</sup> Human and animal data have shown that the main presentation of preeclampsia is the release of a large quantity of inflammatory cytokines, which indicates an aberrant inflammatory process in preeclampsia.<sup>18-19</sup> The increased levels of inflammatory cytokines in the amniotic fluid and/or cord blood during preeclampsia are involved in adverse neonatal outcomes.<sup>20</sup> Taken together, these studies suggest that inflammatory pathways are not only associated with preeclampsia but also may be responsible for adverse neonatal outcomes.

There is growing evidence indicating that administering anti-inflammatory agents to the mother may ameliorate the adverse perinatal outcomes of preeclampsia. The treatment of rats in preeclampsia with anti TNF-alpha antibodies attenuated hypertension and decreased IL-6 and sVCAM-1 levels.<sup>21</sup> As an anti-inflammatory constituent of the herb, *Uncaria rhynchophylla* can suppress inflammation and mitigating preeclampsia-like symptoms in a rat model.<sup>22</sup> There is some evidence suggesting that in addition to

anticoagulation, the potential effects of low molecular weight heparin in preventing preeclampsia progress are mediated by suppressing inflammation.<sup>23</sup>

The cholinergic anti-inflammatory pathway (CAP) bridges the immune and nervous systems<sup>9</sup> and plays multi-effect roles in modulating inflammation. These pathways affect the afferent sensory nerves of the solitary nucleus, which in turn activates the efferent vagus nerve and promotes the release of acetylcholine (ACh). Then, ACh stimulates its receptor,  $\alpha 7$ nAChR, which results in the inhibition of a key molecular mediator of inflammation.<sup>9</sup> The CAP can also be activated by VNS or  $\alpha 7$ nAChR stimulation. Previous research has shown that treatment with  $\alpha 7$ nAChR agonists, such as nicotine<sup>24</sup> and choline,<sup>25</sup> relieved preeclampsia symptoms and improved adverse fetal outcomes, including fetal loss and intrauterine growth restriction, in pregnant rats administered LPS. Therefore, we aimed to explore whether the stimulation of efferent vagus nerve directly regulate the inflammatory response to L-NAME during SD mouse pregnancies..

L-NAME is a nonspecific nitric oxide synthase inhibitor that has been confirmed to increase the vascular response, block the relaxation of the vascular endothelium and form a narrow spiral artery. Many studies have shown that injection of pregnant rats with L-NAME exhibit preeclampsia-like symptoms.<sup>26</sup> In the present study, a rat model of preeclampsia was successfully established and suitable for further analysis. The results indicated that L-NAME led to an increase in the systolic blood pressure and heart rate during pregnancy. Furthermore, substantial proteinuria, decreased fetal and placental weight, increased embryonic resorption, and high proinflammatory cytokine production in the maternal and placenta of rats were induced.

VNS was a common treatment for epilepsy in more than 100,000 patients, and it is generally well tolerated.<sup>27</sup> Recently, VNS has been found to provide protection against systemic inflammation injury in rats.<sup>10</sup> VNS regulates the CAP and inhibits cytokine synthesis, and subsequent prevention of organs or tissues injury. In addition, VNS is associated with decreased heart rate variability, which is a diagnostic tool in the detection of autonomic impairment in a rat model of inflammatory disease.<sup>28</sup> This study shows that chronic VNS also causes an attenuation of SBP elevation during the development of hypertension in preeclampsia. The effectiveness of VNS's blood pressure-lowering effects depends on several factors, including the stimulus parameters, position, administration route, and experimental model.<sup>29</sup> VNS also increases the levels of nitric oxide, the major vasodilator in the circulation, thereby improving endothelial function and reducing vascular resistance,<sup>30</sup> which suggests that lower peripheral resistance may lead to reduced blood pressure in response to VNS treatment. In addition, our data further demonstrated that MLA failed to block the effect of VNS on the lower heart rate in VNS-treated animals. A possible explanation is that cardiac electrophysiological regulation by VNS may be mediated through muscarinic acetylcholine receptors (mAChRs).<sup>31</sup>

Proteinuria is not essential to diagnosis but is related to disease severity and fetal outcomes in preeclampsia. In the study, we found proteinuria and fetal malformation in L-NAME-treated rats, which is

consistent with previous studies.<sup>32</sup> However, mild proteinuria and no fetal malformations were observed in the VNS-treated rats. We also identified lower placenta and fetus weights in the L-NAME-treated groups, which concur with published research papers.<sup>33</sup> Partial reversal of growth retardation was observed in the VNS group, where the weight of the fetuses was similar to that of fetuses from the control group.

The placenta of the rats that received only L-NAME showed histopathological changes including inflammatory cell infiltration, villous infarction and fibrin-like substance deposition. The effect of VNS seems to be reconstructive because these changes were maintained but at mild intensity. Overall, VNS improved the placental pathology, suggesting that VNS may provide protection for the fetus during preeclampsia.

Previous research has shown that CAP is involved in the limitation of inflammatory responses, which depends on  $\alpha 7$ nAChR in animal models.  $\alpha 7$ nAChR was initially found in the nervous system; however, its expression in nonneuronal cells, such as macrophages, endothelial cells, smooth muscle cells and the placenta,<sup>34</sup> was only recently discovered.  $\alpha 7$ nAChR expressed in the placenta may have an anti-inflammatory role and inhibit the activation of endothelial cells, which are involved in the pathophysiology of preeclampsia.<sup>25</sup> In this study, the lower mRNA and protein levels of  $\alpha 7$ nAChR were observed in the placentas of L-NAME groups. Studies indicate that higher levels of ACh are found in the placenta<sup>35</sup> but that its actual synthesis<sup>36</sup> and transport<sup>37</sup> are greatly reduced in pre-eclampsia. Hence, compensatory changes in the expression of its receptors may contribute to the change in ACh levels in preeclampsia. Interestingly, we also found that VNS significantly increased the level of  $\alpha 7$ nAChR expression, which reconfirmed its anti-inflammatory effects.

It is well known that NF- $\kappa$ B plays a central role in the expression of genes involved in immune and inflammatory responses.<sup>38</sup> Human study have demonstrated increased NF- $\kappa$ B activation and increased NF- $\kappa$ B activation in vessels and placenta in women with preeclampsia.<sup>39</sup> NF- $\kappa$ B inhibition leads to the suppression of TNF- $\alpha$ , TGF- $\beta$ 1, IL-10 and NO production.<sup>40</sup> Thus, the pathways involved in NF- $\kappa$ B activation are likely targets for reducing inflammation. The anti-inflammatory properties of  $\alpha 7$ nAChR-mediated CAP have been attributed to the inhibition of NF- $\kappa$ B activation. NF- $\kappa$ B activation was represented by the ratio of the cytoplasmic to nuclear localization of the NF- $\kappa$ B p65 subunit. Research has shown that VNS exerts anti-inflammatory effects on monocytes challenged with the release of the proinflammatory cytokine TNF- $\alpha$  by partially inhibiting NF- $\kappa$ B p65 activation.<sup>72</sup> VNS treatment attenuated the L-NAME-induced increase in placental nuclear NF- $\kappa$ B p65, while MLA significantly antagonized these effects. Therefore, we conclude that chronic VNS blocks the inflammatory response in rats with preeclampsia partly through the release of ACh from vagus nerve endings, which binds to  $\alpha 7$ -nAChR and inhibits the activation of the NF- $\kappa$ B pathway.

## 5. Conclusions

Our research showed that VNS improved PE symptoms and inhibited pro-inflammatory cytokines in a rat model of PE. These findings revealed a novel therapeutic use of VNS for preventing excessive pro-inflammatory cytokines during pregnancy complications. VNS is a non-pharmacological treatment. VNS has emerged as a potential non-pharmaceutical treatment strategy with anti-inflammatory and anti-hypertensive properties. However, different stimulation parameters and sites have been shown to exert distinct therapeutic effects. In this study, we only verified the current stimulation parameters of left-side VNS and found that the heart rate is regulated in normal pregnant rats. Further studies will aim to explore the best stimulation parameters and the underlying mechanisms. Furthermore, the beneficial effects of HRV biofeedback on preeclampsia symptoms and cardiovascular system by increasing vagal tone need to be elucidated in further research.

## Abbreviations

VNS: Vagus nerve stimulation; L-NAME: NG-nitro-L arginine methyl ester; GD: gestational day; NF-kB: Nuclear factor-kB;

ELISA: Enzyme-linked immunosorbent assay;  $\alpha 7nAChR$ : Nicotinic acetylcholine receptor type 7. MLA: Methyllycaconitine citrate. CAP : Cholinergic anti-inflammatory pathway.

## Declarations

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### Availability of data and materials

The datasets used and/or analysed during the current study are available upon reasonable request from the corresponding author

### Authors' contributions

The study was conceived by Lei Shi and Mei Zhong. Linmei Zheng and Rong Tang carried out the experiment and analysed the data. Linmei Zheng wrote the first version of the paper. All authors contributed to revisions of the paper and approved of the final manuscript.

### Competing interests

The authors declare that they have no conflicts of interest.

### Consent for publication

Not applicable.

## Ethics approval and consent to participate

Study did not involve human subjects. It's not applicable.

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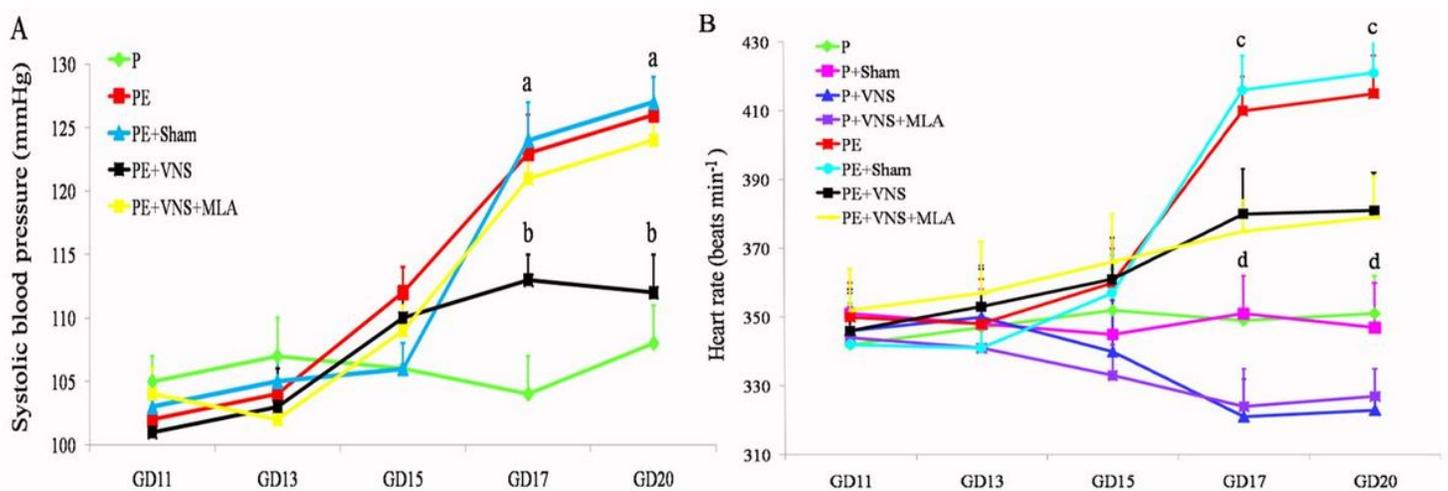
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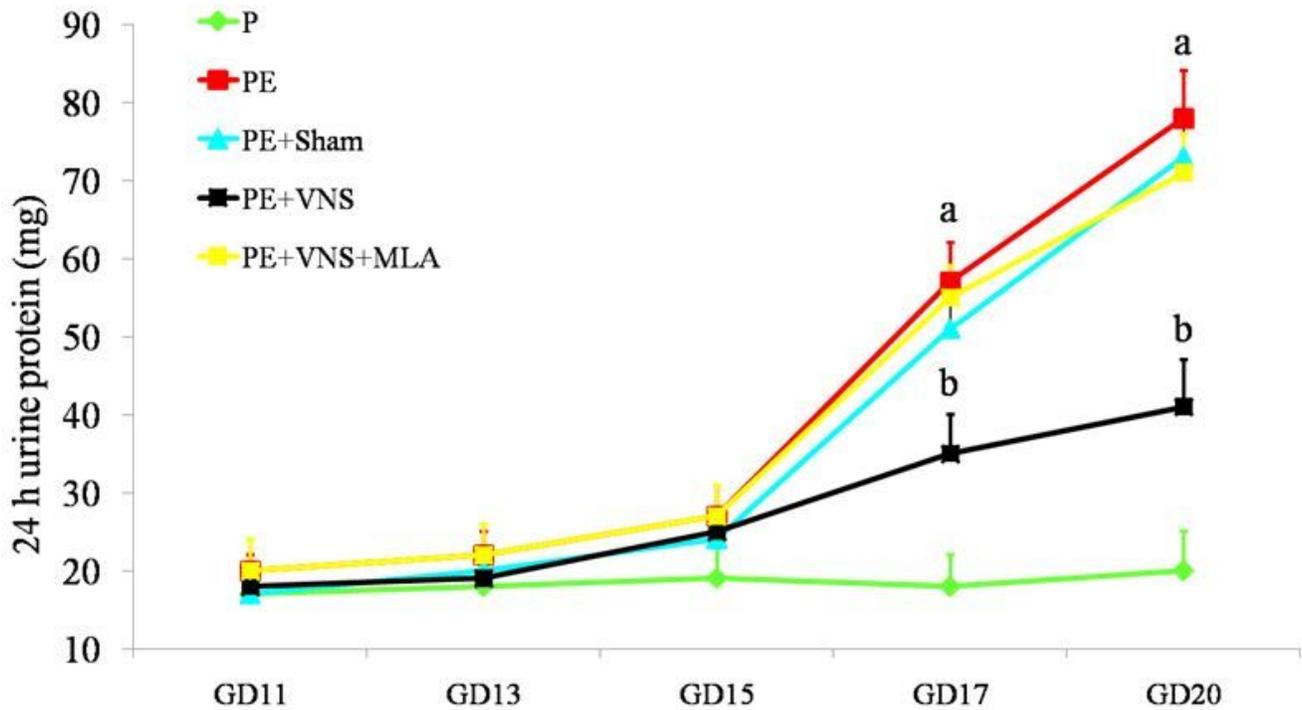
## Figures



**Figure 1**

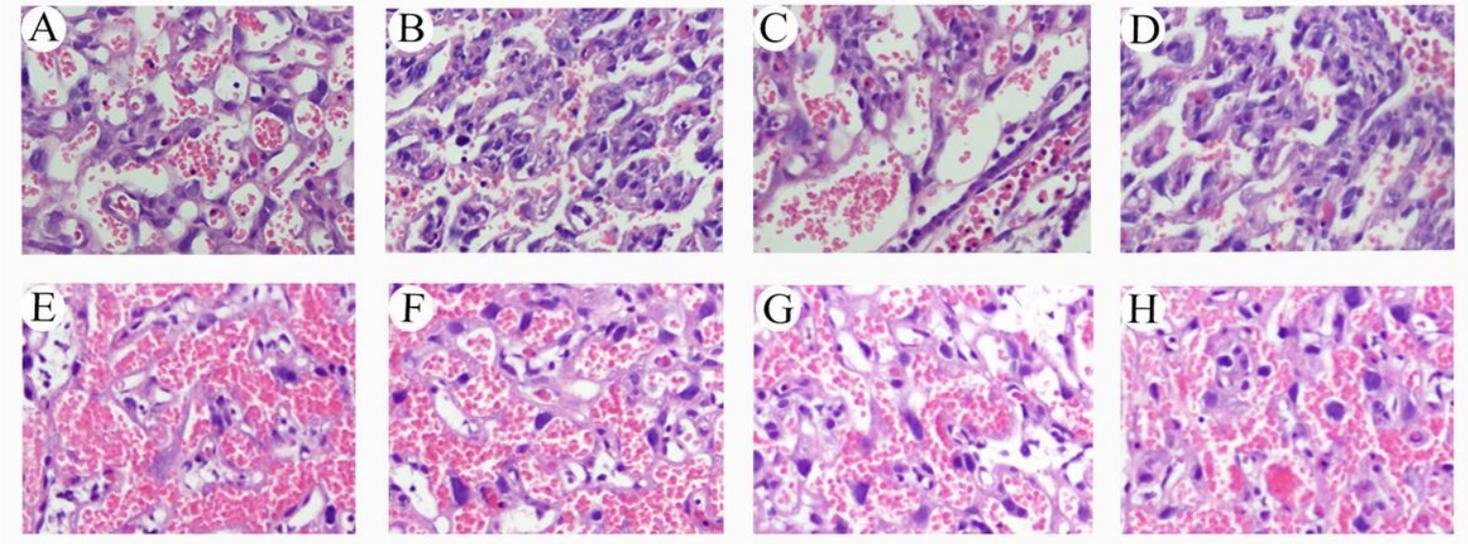
Effect of VNS on the blood pressure and heart rate of rats with L-NAME-induced PE Systolic blood pressure (A) and heart rate (B) of pregnant rats treated or not (control) with L-NAME or L-NAME plus VNS

in different periods of pregnancy. Data are presented as Mean+ SEM. (a)  $p < 0.05$ , the PE, PE+ sham and PE+ TaVNS and PE+VNS+MLA groups compared to the P group on GD 17 and GD 20; (b)  $p < 0.05$ , the TaVNS group compared to the P, PE, PE+ sham and PE+VNS+MLA on GD 17 and GD 20; (c)  $p < 0.05$ , the PE and PE+ sham groups compared to and PE+ TaVNS and PE+VNS+MLA groups on GD 17 and GD 20; (d)  $p < 0.05$ , the P and P+ sham groups compared to and P+ TaVNS and P+VNS+MLA groups on GD 17 and GD 20.



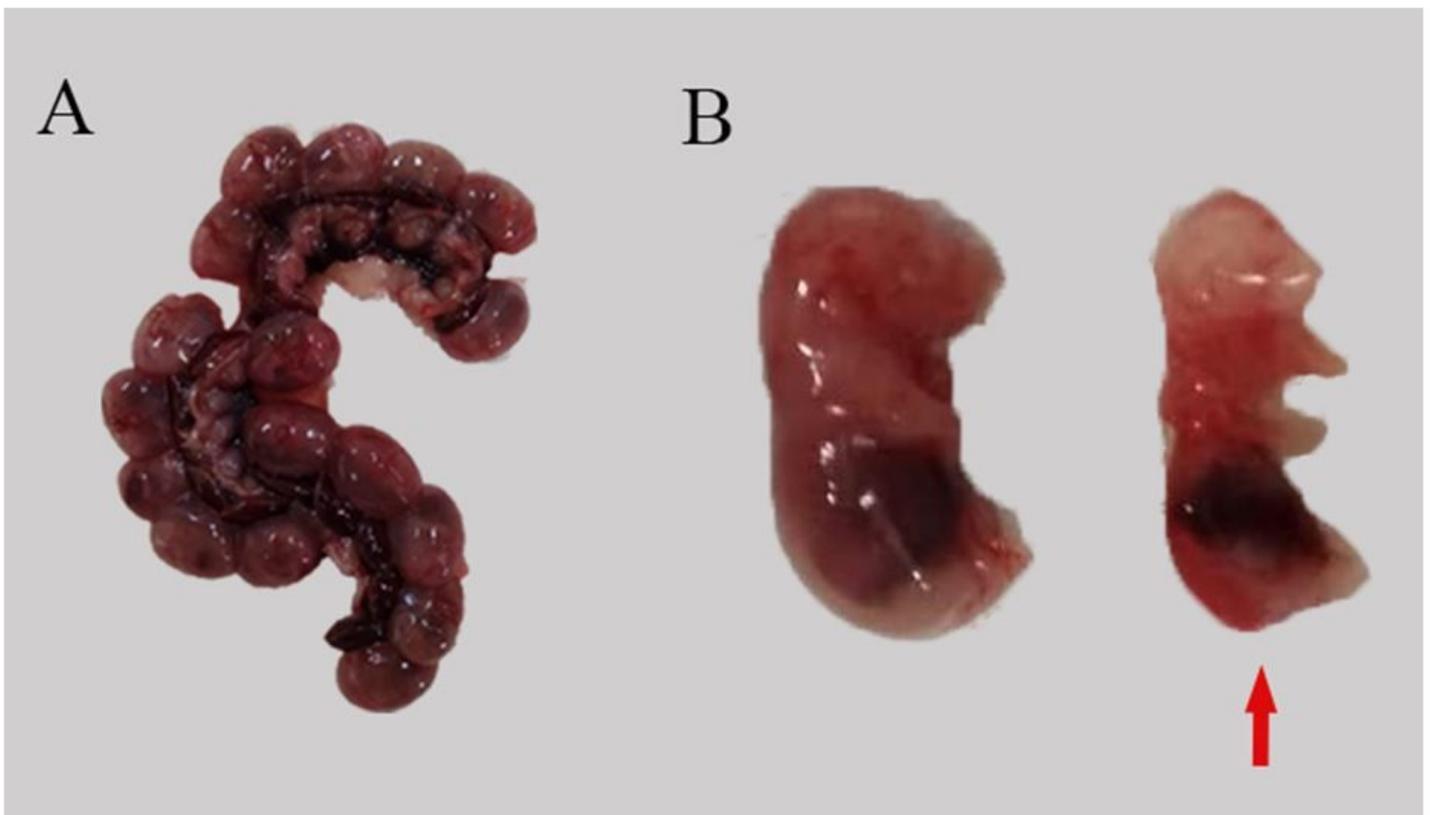
**Figure 2**

Effect of VNS on urinary protein in rats with L-NAME-induced PE VNS ameliorated urinary protein in the L-NAME induced PE like rats. The 24-h urinary protein was measured on GDs 11, 13, 15, 17, and 20. Data are presented as Mean+ SEM. (a)  $p < 0.05$ , the PE, PE+ sham and PE+ TaVNS and PE+VNS+MLA groups compared to the P group on GD 17 and GD 20; (b)  $p < 0.05$ , the TaVNS group compared to the P, PE, PE+ sham and PE+VNS+MLA on GD 17 and GD 20.



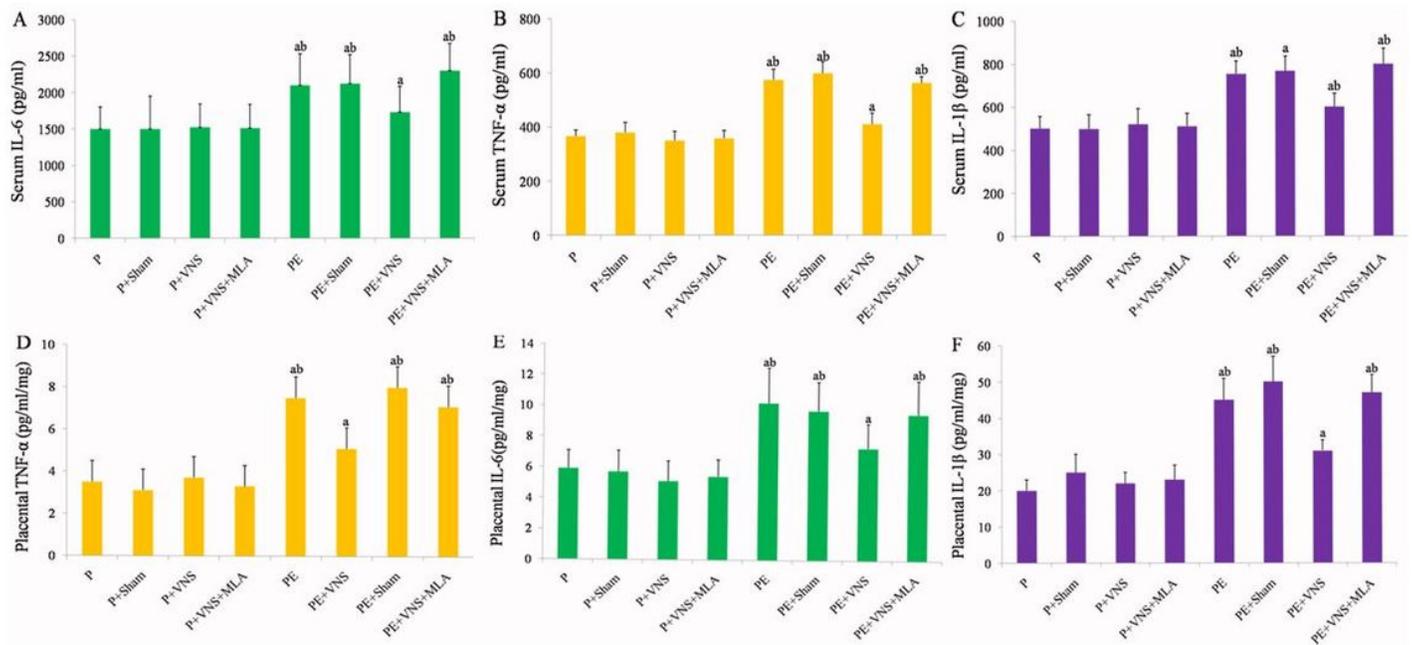
**Figure 3**

Effects of VNS on the morphological changes in the placenta in rats with L-NAME-induced PE. Changes of placenta structure in pregnant rats (GD 21). (A-H) Histopathological images of placenta in pregnant rats of (A) P, (B) P+Sham, (C) P+VNS, (D) P+VNS+ MLA, (E) PE, (F) PE+Sham, (G) PE+VNS and (H) PE+VNS+ MLA groups. (H&E; magnification,  $\times 200$ ; scale bar,  $25 \mu\text{m}$ ).



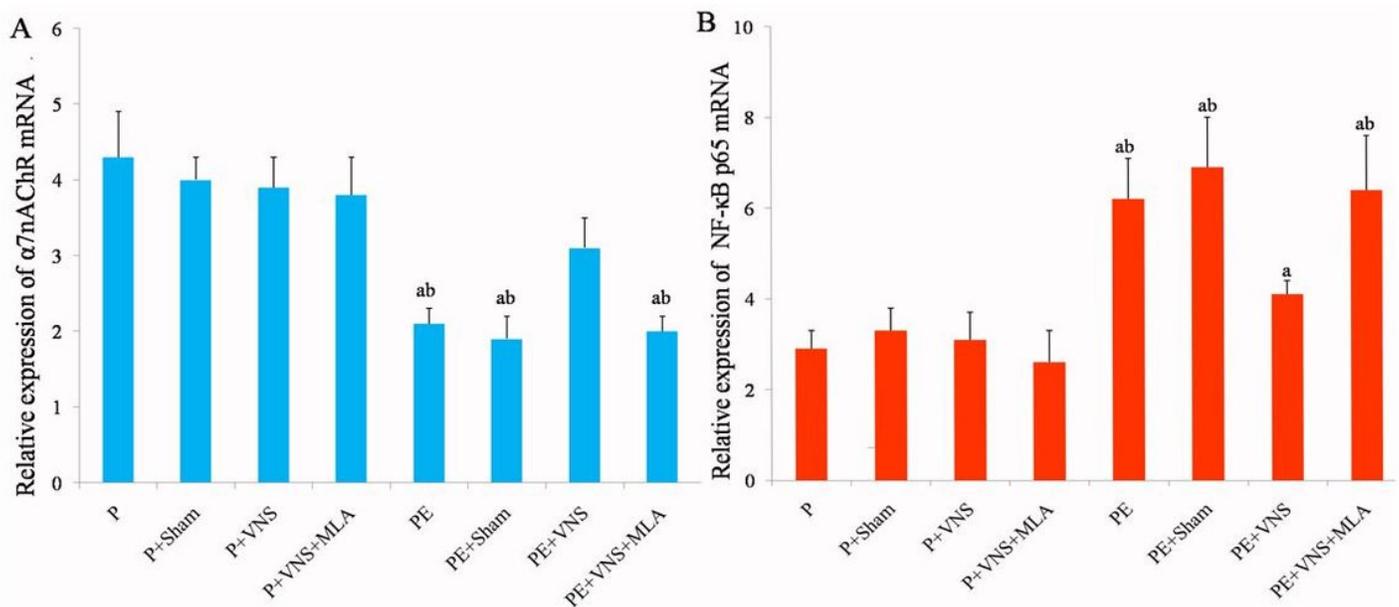
**Figure 4**

Placenta and fetus. (A) The placental tissues obtained from pregnant rats. (B) Representative pictures of normal fetal (B) and fetal congenital malformation was observed in preeclamptic rat (red arrow).



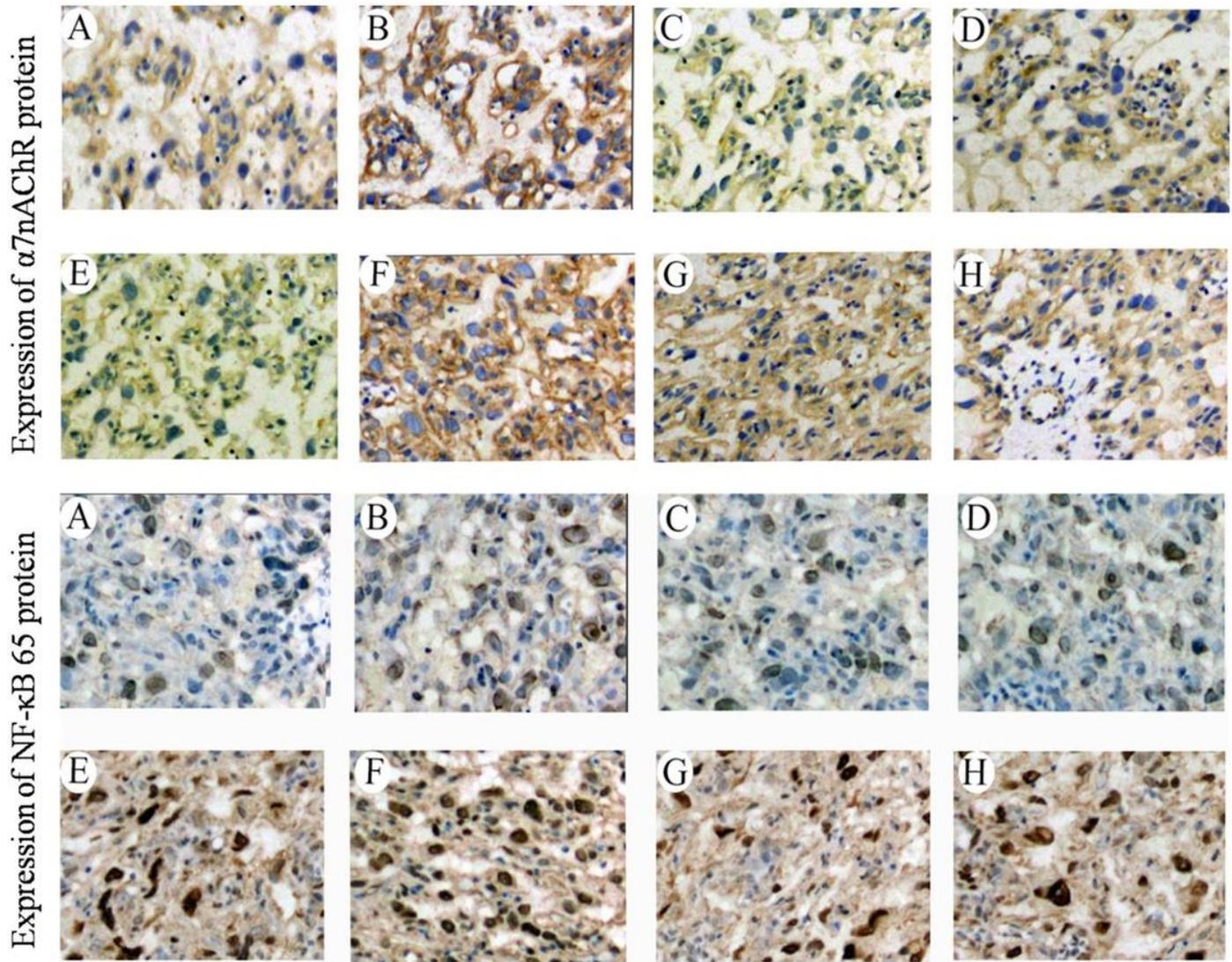
**Figure 5**

Effects of VNS on the levels of proinflammatory cytokines in both the serum and placenta in rats with L-NAME-induced PE. VNS treatment attenuated inflammatory cytokines in both plasma and placenta in L-NAME induced PE rats. Serum TNF- $\alpha$  (A), IL-1 $\beta$  (B) and IL-6 (C), placental TNF- $\alpha$  (D), IL-1 $\beta$  (E) and IL-6 (F) were measured in different group on GD 20. Data were presented as Mean+SEM. aP<0.05 vs P, P+VNS, P+Sham and P+VNS+MLA; bP<0.05 vs PE+VNS group.



**Figure 6**

The expression of  $\alpha 7nAChR$  (A) and NF- $\kappa B$  65 (B) mRNA in the placenta as determined using qPCR. Data were presented as Mean+SEM. aP<0.05 vs P, P+VNS, P+Sham and P+VNS+MLA; bP<0.05 vs PE+VNS group.



**Figure 7**

The expression of  $\alpha 7nAChR$  and NF- $\kappa B$  65 protein in the placenta as determined using immunohistochemistry. Data were presented as Mean+SEM. A: P group; B: P+Sham group; C: P+VNS group; D: P+VNS+ MLA group; E: PE group; F: PE+Sham group; G: PE+VNS group; H: PE+VNS+ MLA group. Original magnification:  $\times 400$ .

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [HumaneEndpointsChecklist.docx](#)