

# TaVNS reduces inflammatory responses in a L-NAME-induced rat model of pre-eclampsia

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**Key words:** Pre-eclampsia, Cholinergic anti-inflammatory pathway, TaVNS, Inflammation

**Abstract:** Pre-eclampsia is characterized by an excessive maternal inflammatory response. The cholinergic anti-inflammatory pathway (CAP) has been shown as the efferent arm of a vagal reflex with the potential to limit inflammatory responses. Therefore, in this study, the CAP regulation through the nervous vagal stimulation (VNS) reduced the severity of NG-nitro-L-arginine methyl ester (L-NAME)-induced pre-eclampsia was determined in a rat model. Rats were given 125 mg/kg/day of L-NAME via subcutaneous injection on gestational day (GD) 10–16. In addition, the rats were treated by active or sham electrical stimulation once a day during GD 13–19. Systolic blood pressure (SBP), urinary albumin, and pregnancy outcomes were documented for each rat. The average fetal weights and crown-rump length (CRL) as well as the placental weights of rats in both control and experimental groups were recorded on the 13th day, 16th day and 20th day of gestation. Subsequently, placentas were collected from the rats on GD20 to measure the level of cytokines. In addition, qRT-PCR and Western blot analysis were used to detect the mRNA and protein expression of  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) and nuclear factor- $\kappa$ B (NF- $\kappa$ B), respectively. Immunohistochemistry assays were also carried out to determine the location and level of  $\alpha 7$ nAChR and NF- $\kappa$ B in placentas. CAP regulation through the transcutaneous auricular nerve stimulation alleviated the clinical symptoms in the rats after L-NAME induction, including hypertension, proteinuria, fetal growth retardation and fetal death. In addition, TaVNS also increased  $\alpha 7$ nAChR expression, reduced NF- $\kappa$ B p65 expression, and reversed L-NAME-induced proinflammatory cytokines in the placenta tissues, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), high mobility group box 1 (HMGB-1) and interleukin-6 (IL-6). The findings of this study showed that TaVNS might be used as a promising tool to attenuate pre-eclampsia-like symptoms. In addition, the protective effect of TaVNS was associated with the improvement of  $\alpha 7$ nAChR expression and the inhibition of inflammatory reactions at the maternal-fetal interface through activating cholinergic anti-inflammation pathway.

## Introduction

Pre-eclampsia is a disease during pregnancy and is featured by hypertension, proteinuria, maternal endothelial dysfunctions and chronic immune activation. As a type of pregnancy-related disorders, pre-eclampsia is the leading cause of neonatal and maternal morbidity and mortality worldwide, and can affect up to 5–7% of pregnancies (Henderson *et al.*, 2017; Hutcheon *et al.*, 2011; Say *et al.*, 2014). Currently, there are no medical therapies available to halt the progression of this disease after its onset. Therefore, delivery

remains the main treatment for pre-eclampsia, thus leading to an increased rate of iatrogenic preterm birth.

Pre-eclampsia can be diagnosed after 20 weeks of gestation characterized by systemic inflammation, endothelial dysfunction, and oxidative stress (Chaiworapongsa *et al.*, 2014; Roberts *et al.*, 2013). Although the definite pathophysiology of pre-eclampsia is still unknown, existing evidence suggests that the endothelial dysfunction may act as a leading cause of pre-eclampsia (Ahmed, 2011). Both, observational and experimental studies have revealed a correlation between inflammation and endothelial dysfunction (Mantovani and Dejana, 1989; Zimmerman *et al.*, 1992). More evidence of excessive inflammation responses in pre-eclampsia has also been demonstrated by the uncontrolled elevation in the activation of the complement system during pre-eclampsia (Lynch *et al.*, 2010).

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Received: 28 March 2021; Accepted: 08 May 2021



The vagus nerve is a key component of the autonomic nervous system and plays a pivotal role in the modulation of inflammatory responses (Bonaz *et al.*, 2016; Koopman *et al.*, 2016). Tracey (2002) have demonstrated that vagus nerve stimulation (VNS) could inhibit immune activation and could successfully reduce the production of pro-inflammatory cytokines in a murine model of septic shock. In fact, through the activation of  $\alpha 7$ nAChR and the cholinergic anti-inflammatory pathway (CAP), the VNS inflammatory reflex is considered as a main neural control mechanism to prevent the production of proinflammatory mediators from innate immune cells (Borovikova *et al.*, 2000). In a previous study, we reported that treatment with VNS significantly improved adverse pregnancy outcomes through anti-inflammatory pathway in L-NAME -induced PE model rats (Zheng *et al.*, 2021). However, the clinical application of VNS is limited by its invasiveness. Recently, devices have been developed to noninvasively stimulate the vagus nerve. For example, functional magnetic resonance imaging of brain has demonstrated that the transcutaneous auricular VNS (TaVNS) could activate the same areas in the brain as those activated by surgically implanted VNS (Frangos *et al.*, 2015).

It was demonstrated that TaVNS could evoke parasympathetic excitation and potentially reduce vagus-mediated symptoms, thus establishing a safer and more affordable treatment modality (Hsu *et al.*, 2006; Wu *et al.*, 2009). Zhao *et al.* (2012) have exhibited that Ta-VNS could be utilized to suppress inflammatory responses via  $\alpha 7$ nAChR-mediated CAP in an experimental model of sepsis. Aberrant  $\alpha 7$ nAChR and NF- $\kappa$ B protein expression in placenta and pre-eclampsia has been reported previously (Zheng *et al.*, 2018). In the present study, it has been hypothesized that TaVNS might play a role in activating the vagus nerve-based CAP. In particular, the effect of TaVNS on the levels of NF- $\kappa$ B p65 and pro-inflammatory cytokines was explored in this study to clarify the role of TaVNS in the regulation of inflammatory diseases.

## Materials and Methods

### Animals

Female Sprague-Dawley rats at 8 weeks (220–240 g in body weight) were obtained from the SPF Animal Laboratory of Hainan, China. Rats were housed in a temperature-controlled room ( $23 \pm 1^\circ\text{C}$ ) and kept under a 12:12-h light/dark cycle with food and water ad libitum. Female rats

were mated with fertile male rats overnight. Subsequently, the female rats with timed pregnancy (the first day of positive vaginal smear results was considered as gestational day 0 (G0)) were used for the following experiments.

### Study design

We randomly divide the pregnant rats into control (VC group,  $n = 21$ ) and L-NAME groups ( $n = 63$ ). The preeclamptic model was established as previously described (Tian *et al.*, 2016; Zhu *et al.*, 2017). In brief, 125 mg/kg/day of L-NAME (Sigma, St Louis, MO, USA) was used to cause pre-eclampsia-like symptoms in the pregnant rats by injecting subcutaneously from day 10 to day 16 of gestation. The rats were provided an equivalent volume of saline solution as in the control group. The L-NAME groups contained three sub-groups ( $n = 21$  per group): (1) L-NAME group (LN), receiving no stimulation. (2) L-NAME+sham group (LN+sham), treated with sham stimulation from GD 13 to 19. (3) L-NAME+TaVNS group (LN+TaVNS), treated with TaVNS from GD 13 to 19.

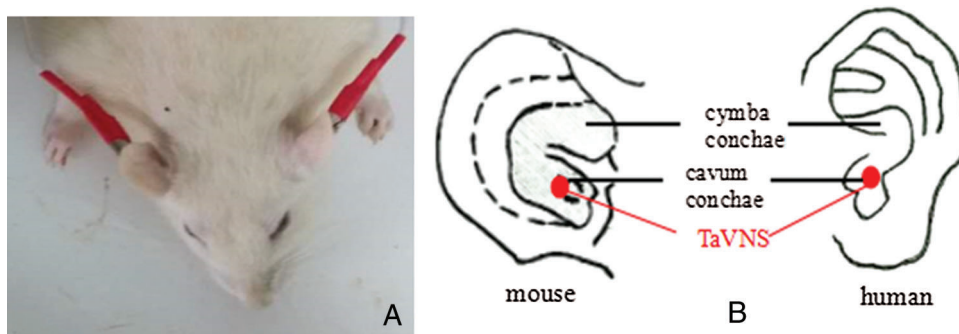
### Noninvasive TaVNS

After a low level of anesthesia achieved by inhalation of 3% isoflurane, electrodes were placed on the auricular conchae bilaterally. A daily transcutaneous electrical stimulation was applied via a stimulator (SEN-7203, Nihon Kohden) for 7 days at 8:00–10:00 h with the frequencies of 2/15 Hz switched every second and an intensity of 2 mA (Wang *et al.*, 2015). In the sham group, similar procedures were adopted except that the power of the stimulator was turned off, thus no electrical stimulation was generated (Fig. 1).

### Measurement of systolic blood pressure (SBP) and 24h-urinary protein

The SBP of the rats was measured using a noninvasive automatic blood pressure analyzer (Zhenghua Biological Instrument Inc., China) on the 10th, 13th, 16th, 18th and 20th day of GD as described previously (Agarwal *et al.*, 2009). The measurement was repeated at least three times to record the mean value. As a key criterion in evaluating the successful establishment of the pre-eclampsia model, the SBP should be increased by at least 20 mmHg after L-NAME treatment.

The rats were placed in separate metabolic cages for 24 h to obtain their urine samples on the 10th, 13th, 16th, 18th, and 20th day of GD. Urinary levels of albumin were measured by an automatic analyzer (HIACHI 7600-020, Japan).



**FIGURE 1.** Transcutaneous auricular vagus nerve stimulation posture (A) and position (B) of the procedure.

### Measurement of biochemical parameters

The experimental animals were observed daily for mortality, morbidity, general appearance, and behavior. On the 13th, 16th, and 20th day of GD, 7 rats were collected from each of the four groups respectively and euthanized by cardiac puncture after being anesthetized by 3% isoflurane inhalation. All fetuses were assessed for appearance, body weight and size (crown-rump length). In addition, placenta weights were recorded in each group. On the 20th day of GD, the number of alive, dead, and resorbed fetuses was recorded. Some of placenta samples were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. The remaining placenta samples were stored in a  $-80^{\circ}\text{C}$  freezer for subsequent real time PCR and Western blot analysis.

### Western blot analysis

In brief, protein extracts of the samples were prepared using a RIPA lysis buffer (Beyotime, Shanghai, China), resolved on 10% SDS-PAGE, electro-transferred onto polyvinylidene fluoride (PVDF) membranes, and blocked with PBST containing 5% nonfat-dried milk for 2 h. The membranes were then incubated with primary antibodies against  $\alpha 7\text{nAChR}$  (Cat. No. ab10096; Abcam, MA, USA) and NF- $\kappa\text{B}$  p65 (Cat. No. ab207297; Abcam, MA, USA) overnight at  $4^{\circ}\text{C}$ , followed by the incubation with HRP-labeled secondary antibodies (Cell Signaling Technology). Finally, the protein bands on the membranes were detected by an enhanced chemiluminescence (ECL) kit (Sevensapharmtech Co., Ltd., Shanghai, China).  $\beta$ -actin served as endogenous control for the Western blot analysis.

### RNA extraction and real-time qPCR

According to the manufacturer's instructions, TRIzol<sup>®</sup> reagent (Invitrogen, CA, USA) was used to extract total RNA from placenta. Following the manufacturer's protocols, reverse transcription was performed using 5  $\mu\text{g}$  of total RNA in conjunction with a reverse transcription primer and RevertAid<sup>TM</sup> M-MuLV Reverse Transcriptase (Thermo, USA). Subsequently, qPCR was performed using a 20  $\mu\text{L}$  system containing 10  $\mu\text{L}$  of TaqMan<sup>®</sup> Universal PCR Master Mix (TliRNaseH Plus; Takara Bio, Shiga, Japan), 1  $\mu\text{L}$  each of forward and reverse primers, 2  $\mu\text{L}$  of cDNA template, and 7  $\mu\text{L}$  of ultrapure water. The reaction conditions are as follows:  $50^{\circ}\text{C}$  for 2 min and  $94^{\circ}\text{C}$  for 10 min, followed by PCR which consisted of 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $60^{\circ}\text{C}$  for 1 min. Primer sequences designed by Shanghai Biotechnology Co., Ltd., Shanghai, China. The sequences of the primers for  $\alpha 7\text{nAChR}$ , NF- $\kappa\text{B}$  p65 and  $\beta$ -Actin were as follows (forward and reverse, respectively):  $\alpha 7\text{nAChR}$  (ACCTCGTGTGATCCAAAGCC and GGTTTCTCTGCTCAGGGT); NF- $\kappa\text{B}$  p65 (CGACGTATTGCTGTGCCTTC and TGAGATCTGCCAGGTGGTAA) and  $\beta$ -Actin (CCTCTATGCCAACACAGTGC and GTACTCCTGCTTGCTGATCC).  $\beta$ -Actin was used as internal control. Relative mRNA expression was adjusted by using  $2^{-\Delta\Delta\text{Ct}}$ .

### Immunohistochemical assay

Placental tissues were paraffin-embedded and subsequently cut into 3–4  $\mu\text{m}$ -thick serial sections. The sections were pretreated

with 0.3% hydrogen peroxide, followed by incubation in a humidified chamber with a purified rabbit polyclonal antibody against  $\alpha 7\text{nAChR}$  and NF- $\kappa\text{B}$  p65 (Zhongshan Jinqiao Co., Ltd., Beijing, China) at  $4^{\circ}\text{C}$  overnight. After being washing with PBS, the slides were incubated with a solution containing a suitable secondary antibody (Biogototechnology Co., Ltd., Nanjing, China). Finally, the sections were incubated in streptavidin-peroxidase, stained with diaminobenzidine, washed with hematoxylin, dehydrated, and determined under an optical microscope (OLYMPUS, BX60).

### ELISA assay

According to the manufacturer's instructions, the expression of TNF- $\alpha$ , HGMB-1 and IL-6 in placenta was tested utilizing an ELISA kit (R&D Systems, Minneapolis, USA).

### Statistical Analysis

The data were analyzed by version 13.0 (SPSS Inc, Chicago, IL) using two-tailed Student's *t*-test (for comparison between two groups), one-way ANOVA (for comparison among three or more groups), or two-way ANOVA (when two-variables were involved). All data were expressed as mean  $\pm$  SEM.  $P < 0.05$  indicated significant difference.

### Results

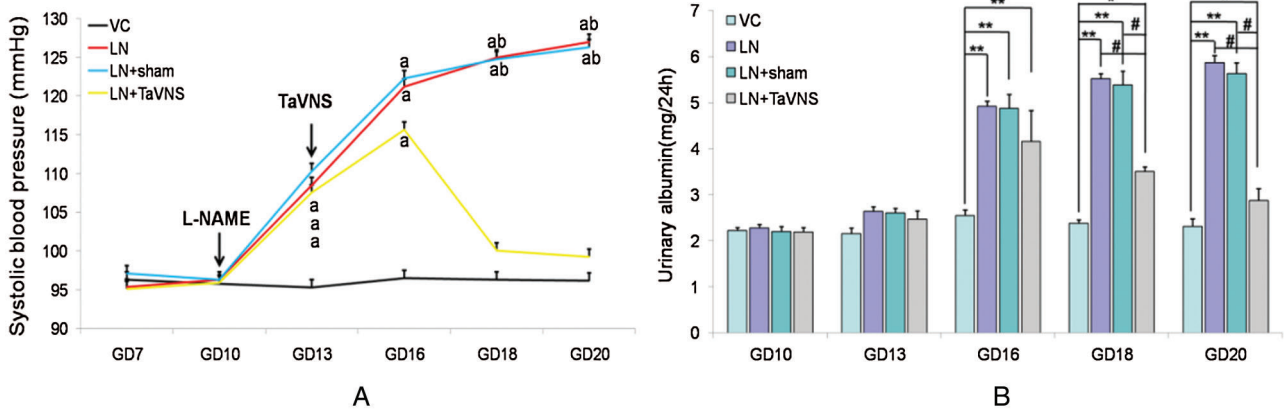
#### *TaVNS ameliorated L-NAME-induced pre-eclampsia-like symptoms in rats*

As expected, L-NAME-induced pre-eclampsia-like symptoms in rats. In addition, the rats in LN and LN+sham groups showed a continuously increased level of SBP from GD14 to delivery. In contrast, the TaVNS treatment significantly reduced the magnitude of SBP elevation in pre-eclampsia rats. After the TaVNS treatment (Fig. 2A), the SBP in pre-eclampsia rats on GD 16 was significantly lower compared to the SBP in non-treated pre-eclampsia rats, although the SBP in TaVNS treated rats was still higher than that in the normal control group. However, the SBP in TaVNS treated rats was similar to that in normal rats on GD18 and GD20. Interestingly, the SBP between sham-treated and non-treated L-NAME groups showed no significant difference from GD10 to GD 20.

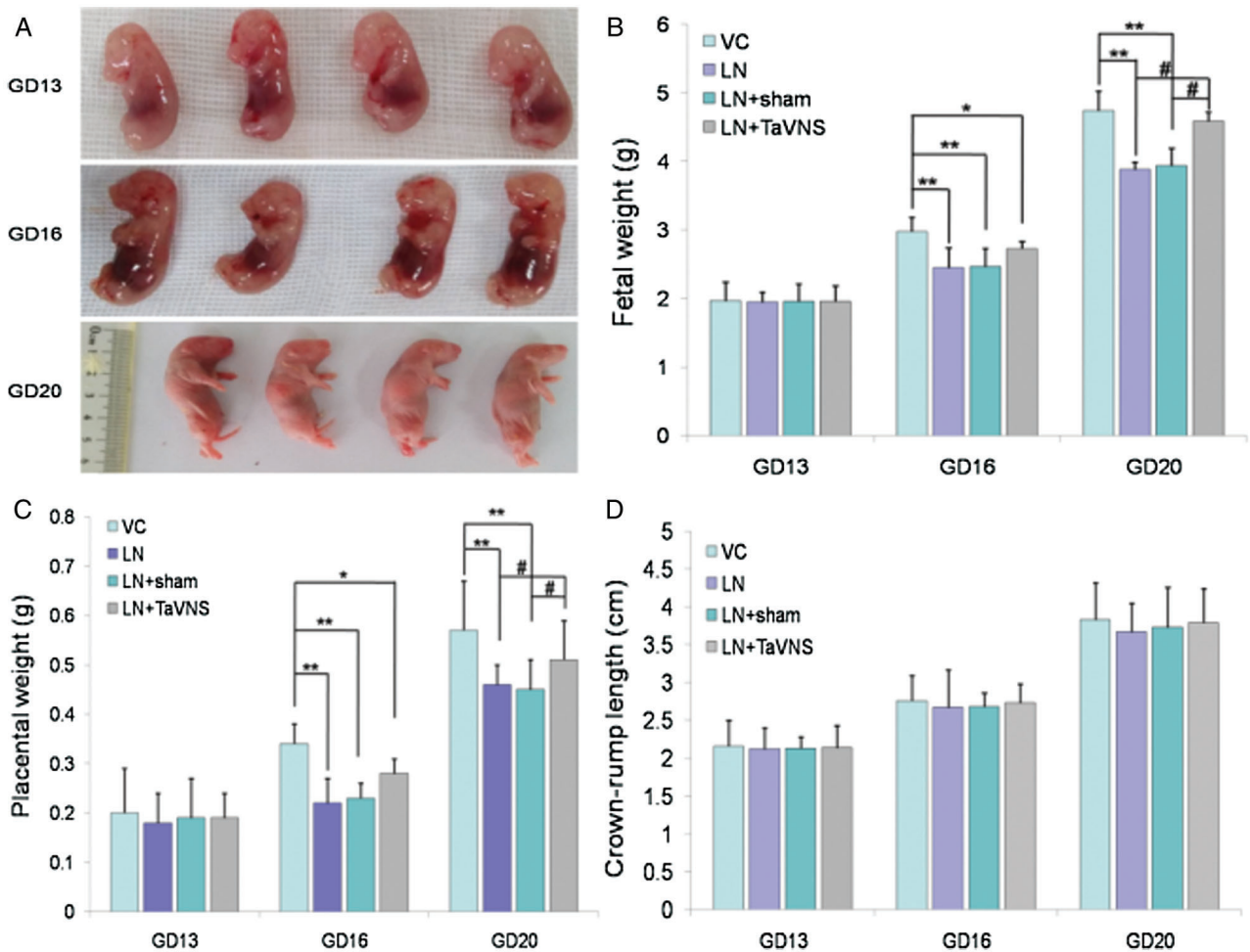
Proteinuria is closely related with pre-eclampsia and is used as a marker of renal malfunction. In this study, the level of proteinuria was also evaluated in the rat model. Although the levels of urine protein were similar in all groups before pregnancy and on GD 10, the L-NAME group showed a significantly increased level of proteinuria in pre-eclamptic rats ( $P < 0.01$ ) (Fig. 2B), indicating the presence of renal dysfunction in this group. The treatment with sham stimulation failed to alleviate this condition of renal dysfunction. However, after the TaVNS treatment, the level of urinary protein significantly decreased by 11% on GD 18 and 20 ( $P < 0.01$ ), suggesting that the TaVNS treatment partly reduced kidney injuries.

#### *TaVNS attenuated L-NAME-induced restriction on fetal growth*

The effects of L-NAME on fetal growth in the utero are shown in Fig. 3. Daily L-NAME administration on GD 10–16



**FIGURE 2.** (A) The SBP was measured on gestational days (GDs) 7, 10, 13, 16, 18, and 20 in pregnant groups. (a)  $P < 0.05$ , the LN, LN+sham and LN+TaVNS groups compared to the VC group on GD13 and GD16; (b)  $P < 0.05$ , the TaVNS group compared to the VC, LN, and LN-sham. (B) The 24 h urinary protein excretions were measured on days 10, 13, 16, 18 and 20 of pregnancy. \* $P < 0.05$  vs. VC, \*\* $P < 0.01$  vs. VC, # $P < 0.05$  vs. LN+TaVNS, ## $P < 0.01$  vs. LN+TaVNS.



**FIGURE 3.** Fetal weights, placental weights and CRL (crown-rump length) are shown on GDs 13, 16 and 20. Representative examples of embryos (3A). Graphical representation of average fetal weight (3B), placental weight (3C), and crown-rump length (3D) of pups in four groups on GDs 13, 16 and 20. \*  $P < 0.05$  vs. VC, \*\*  $P < 0.01$  vs. VC, #  $P < 0.01$  vs. LN+TaVNS, ##  $P < 0.05$  vs. LN+TaVNS.

significantly decreased fetal weight and placental weight. However, after the TaVNS treatment starting from GD 13, both fetal weight and placental weight were significantly increased ( $P < 0.01$ ) compared to those in the LN and LN+sham groups ( $P < 0.05$ ) on GD 20. However, there was no significant difference in terms of CRL among these groups.

*TaVNS improved pregnancy outcomes in L-NAME-treated rats*  
No pregnant rats were dead, and no preterm delivery were observed among pregnant rats intraperitoneally injected with L-NAME. As shown in [Tab. 1](#), there was no significant difference in weight gain of pregnant rats among the groups. The injection of L-NAME resulted in an 4.6% increase in



TABLE 1

## TaVNS improved pregnancy outcomes in the L-NAME-treated rat

Group (S)	VC	LN	LN-sham	LN-TaVNS
Dams (Pregnant mice) (n)	7	7	7	7
Maternal weight gain (g)	360.04 ± 1.52	353 ± 1.07	354 ± 2.06	359.03 ± 0.94
Fetuses examined, No	104	107	102	110
Live fetuses, No (%)	100(96.2)	93(90.7)	90(91.2)	104(94.6)
Resorbed fetuses, No (%)	2(1.9)	3(2.8)	2(1.9)	3(2.7)
Death fetuses, No (%)	2(1.9)	11(6.5) <sup>ab</sup>	10(6.9) <sup>ab</sup>	3(2.7)
FGR per litter (%)	11.3 ± 2.1	30.2 ± 3.2 <sup>ab</sup>	33.6 ± 2.6 <sup>ab</sup>	16.5 ± 4.3

Note: Maternal weight gain, the number of total fetuses, live fetuses, fetal resorptions, death fetuses and FGR per litter are shown. TaVNS significantly improved the number of live fetuses and attenuated elevation of death fetuses and FGR per litter in the L-NAME -treated group ( $P < 0.05$ ). However, there was no significant difference on the number of resorbed fetuses per litter and maternal weight gain among different groups. <sup>a</sup> $P < 0.05$  vs. VC, <sup>b</sup> $P < 0.05$  vs. LN+TaVNS.

dead fetuses and led to a 5.5% decrease in live fetuses ( $P < 0.05$ ), further analysis showed that TaVNS treatment significantly blocked the effect of L-NAME. Fetal growth restriction (FGR) per litter was approximately 20% increased significantly in the LN+sham and LN groups compared to the VC group ( $P < 0.05$ ). While the administration of TaVNS reduced the rate of FGR per litter. In addition, there was no significant difference among different groups in resorbed fetuses.

#### TaVNS reduced the expression of inflammatory factors in the placenta of L-NAME-treated rats

To explore the mechanism for the protective action of TaVNS on pre-eclampsia rats, we measured IL-6, TNF- $\alpha$  and HMGB1 levels on GD20 by ELISA. As shown in Fig. 4, we found that control rats expressed low levels of IL-6 ( $6.18 \pm 0.37$  pg/mg), TNF- $\alpha$  ( $3.25 \pm 0.1$  pg/mg) and HMGB1 ( $13.2 \pm 0.49$  pg/mg). Levels of IL-6 ( $10.11 \pm 0.14$  pg/mg), TNF- $\alpha$  ( $6.9 \pm 0.09$  pg/mg) and HMGB1 ( $24.23 \pm 0.61$  pg/mg) were enhanced markedly after L-NAME compared with the VC group ( $P < 0.05$ ). Treatment with TaVNS prevented the increases in IL-6 ( $7.51 \pm 0.12$  pg/mg), TNF- $\alpha$  ( $5.56 \pm 0.33$  pg/mg) and HMGB1 ( $17.5 \pm 0.75$  pg/mg) in the placenta following L-NAME administration ( $P < 0.05$ ).

#### TaVNS up-regulated $\alpha 7nAChR$ expression in the placenta of L-NAME-treated rats

As shown in Fig. 4, the expression of  $\alpha 7nAChR$  in experimental groups was detected by qRT-PCR and Western blot. Analysis showed that the protein level of  $\alpha 7nAChR$  decreased in L-NAME-treated rats and increased significantly after TaVNS treatment (Fig. 4D). To investigate the cause, we conducted an mRNA-level verification and found that the mRNA level of  $\alpha 7nAChR$  was significantly increased (Fig. 4E). No significant difference was found between the VC group and LN+TaVNS group in the protein and mRNA levels (both  $P > 0.05$ ).

#### TaVNS down-regulated NF- $\kappa B$ p65 expression in the placenta of L-NAME-treated rats

The effects of TaVNS on the protein and mRNA expression of NF- $\kappa B$  p65 in the placenta are shown in Figs. 4G and 4F. The

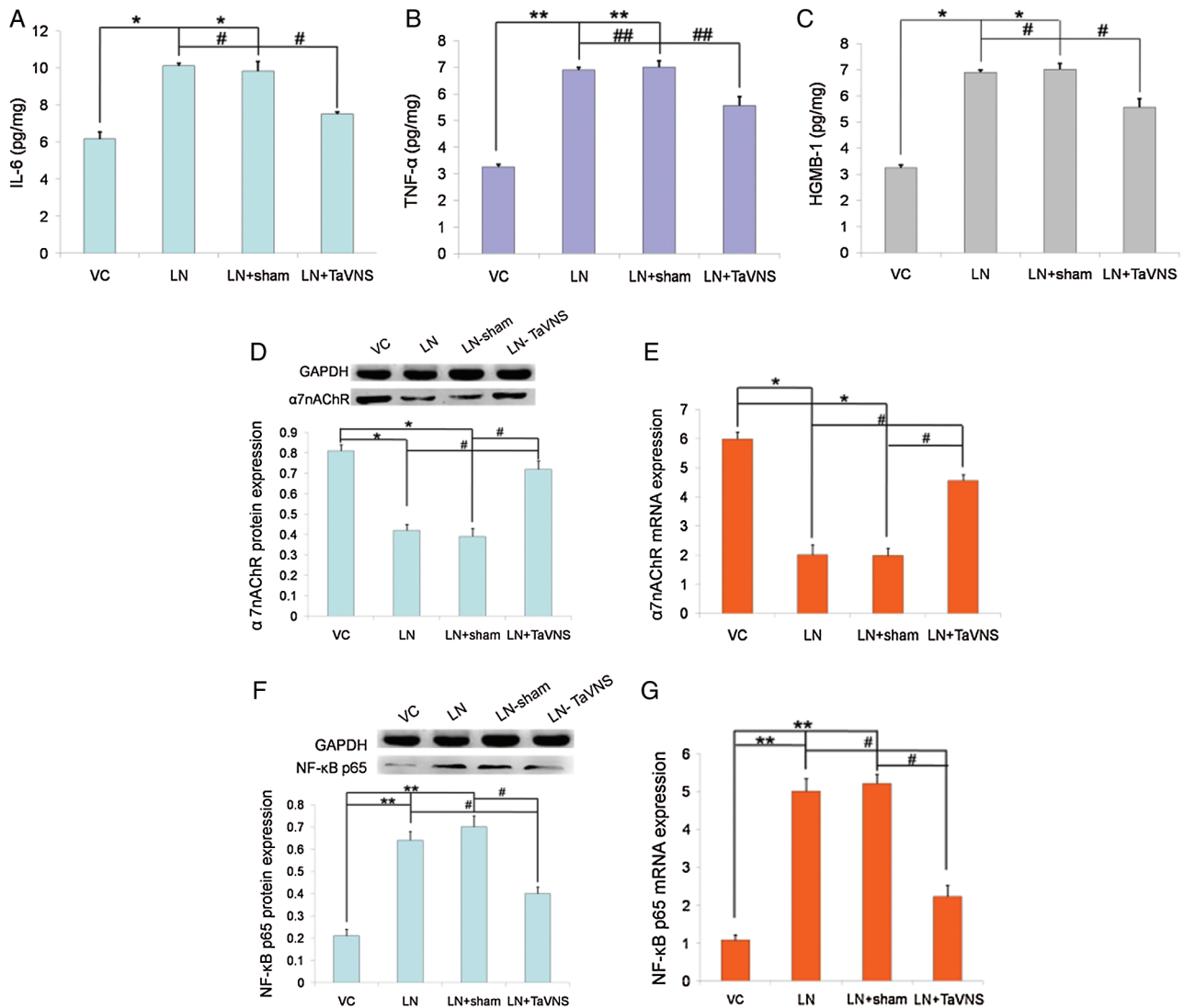
data showed that the protein expression of NF- $\kappa B$  p65 was significantly lower in the LN+TaVNS group ( $0.4 \pm 0.03$ ) than that in the LN ( $0.64 \pm 0.04$ ) and LN+sham ( $0.7 \pm 0.05$ ) groups ( $P < 0.05$ ). In agreement with the results from Western blotting, NF- $\kappa B$  p65 mRNA expression were significantly increased in the LN ( $5.01 \pm 0.33$ ) and LN+sham ( $5.21 \pm 0.25$ ) groups ( $P < 0.05$ ), while TaVNS significantly decreased the elevated levels ( $2.23 \pm 0.3$ ) ( $P < 0.05$ ). There was no statistical significance between the VC group ( $0.21 \pm 0.03$ ;  $1.08 \pm 0.14$ ) and LN+TaVNS group in the protein and mRNA levels (both  $P > 0.05$ ).

#### Effect of TaVNS on placental levels of $\alpha 7nAChR$ and NF- $\kappa B$ p65 by immunohistochemistry

Expression of  $\alpha 7nAChR$  and NF- $\kappa B$  p65 in the placenta was also assessed by immunohistochemistry (Fig. 5). The locations of the expression of the  $\alpha 7nAChR$  in experiment groups were mainly in the cell membrane, and partly in the cytoplasm. Low but detectable levels of  $\alpha 7nAChR$  were observed in the LN and LN+sham groups. Intense  $\alpha 7nAChR$  staining was seen after TaVNS treatment on GD20 (Fig. 5A). NF- $\kappa B$  p65 immunohistochemical staining was predominantly observed in the nuclei. The level of NF- $\kappa B$  p65 was significantly elevated in the LN and LN+sham groups, whereas TaVNS treatment significantly lowered the number of positive immunostaining cells (Fig. 5B).

## Discussion

This study demonstrates, for the first time, that TaVNS treatment alleviated some of the pre-eclampsia-like symptoms, such as adverse hypertension, proteinuria, and fetal growth restriction in a pre-eclampsia rat model. Furthermore, this study showed that the effect of TaVNS treatment was exerted by promoting  $\alpha 7nAChR$  expression via activating the CAP activity and by inhibiting the activity of NF- $\kappa B$  p65 and other pro-inflammatory cytokines in the placenta. Chronic activation of the immune system and persistently high levels of pro-inflammatory cytokines are some of know pathological pathways of pre-eclampsia. The abnormal changes in cytokines can result in chronic systemic inflammation as well as local inflammation in

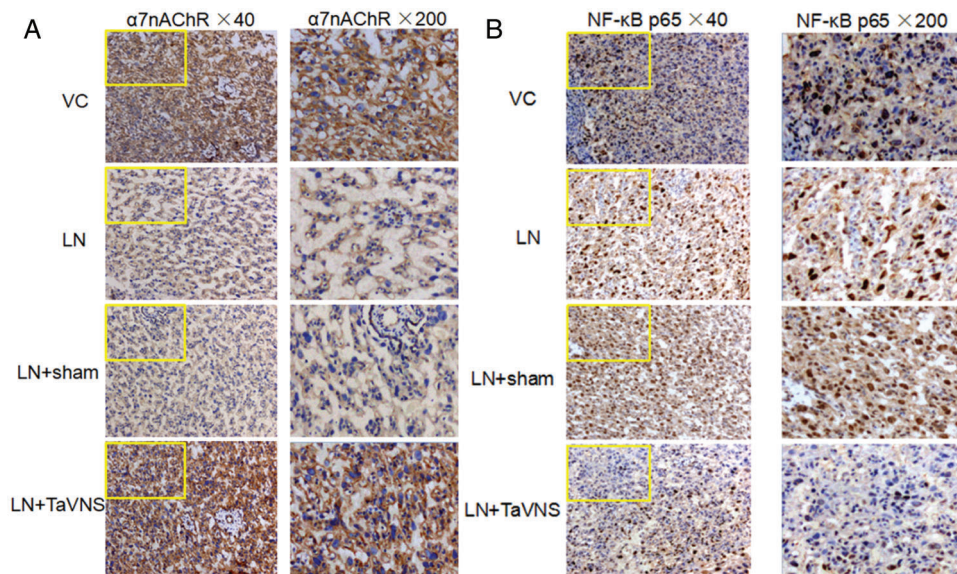


**FIGURE 4.** Graphical representations of (A) IL-6, (B) TNF- $\alpha$ , and (C) HGMB-1 expression in the placentae of pregnant dams on GD20. (D and F) Representative Western blot analysis of  $\alpha 7nAChR$  and NF- $\kappa B$  p65 with GAPDH as a loading control. (E and G) Relative level of  $\alpha 7nAChR$  and NF- $\kappa B$  p65 mRNA expression. \* $P < 0.05$  vs. VC, \*\* $P < 0.01$  vs. VC, # $P < 0.05$  vs. LN+TaVNS, ## $P < 0.01$  vs. LN+TaVNS.

placenta, thus contributing to the symptoms of pre-eclampsia (Baumann *et al.*, 1990; Gadonski *et al.*, 2006). Previous reports have revealed that the maternal exposure to L-NAME, an inhibitor of NO synthesis, could stimulate the production of pro-inflammatory cytokines, which in turn triggered severe systemic inflammatory responses during pregnancy (Soobryan *et al.*, 2017; Yallampalli and Garfield, 1993). Therefore, a rat model of L-NAME-induced pre-eclampsia was used in this study to observe the effects of TaVNS on placental inflammation.

In this study, a rat model of pre-eclampsia was successfully established by the infusion of L-NAME into pregnant rats. Subsequently, maternal hypertension and proteinuria were observed. Results from this study were consistent with those in previous reports demonstrating that NOS inhibition could result in a pre-eclampsia-like syndrome (Tian *et al.*, 2016; Zhu *et al.*, 2017; Zou *et al.*, 2014). The present study showed no effect of L-NAME on litter size in the rat model. In contrast, a few previous studies have shown reduced the size of litter in L-NAME-induced rats (Celadilla *et al.*, 2005; Isler *et al.*, 2003;

Mayr *et al.*, 2005). Furthermore, in the present study, the administration of L-NAME showed an approximately 20% decrease in pup weight, suggesting that pre-eclampsia might cause intrauterine growth retardation. These findings were in line with the results of other studies reporting lower fetal weights in L-NAME treated rats (Kiliç *et al.*, 2003; Tsukimori *et al.*, 2008). In contrast, some studies reported higher fetal weights (Tanir *et al.*, 2005) or no changes in fetal weights (Yang *et al.*, 2011) among L-NAME treated rats. In addition, although no significant difference was observed in terms of fetal crown-rump length between the TaVNS treated L-NAME group and the VC group in this study, previous studies have shown that human fetuses suffered from asymmetrical growth restriction under chronic hypoxia conditions (Chauhan *et al.*, 2006). Moreover, this study also demonstrated that the placental weight in L-NAME-treated rats was approximately 20% lower than that in the VC group. This result was consistent with the studies reported by Ramesar *et al.* (2010) and Ma *et al.* (2010) who also found a dramatic reduction in the placental weight in the Sprague Dawley rats treated with



**FIGURE 5.** In the placental tissue, the expression of NF- $\kappa$ B p65 and  $\alpha$ 7nAChR was significantly up- and down-regulated in the LN and LN+sham group, respectively ( $P < 0.01$ ), as compared with the VC control.

Treatment with TaVNS resulted in a substantially lower expression of NF- $\kappa$ B p65 and higher expression of  $\alpha$ 7nAChR in the LN+TaVNS rats as compared to the L-Name treated rats.

L-NAME (Ma *et al.*, 2010; Ramesar *et al.*, 2010). In addition, a higher percentage of fetal resorption was observed in pre-eclampsia rats both in this study and other studies (Xue *et al.*, 2015).

Recently, most studies have focused on the inhibition of inflammatory responses in pre-eclampsia (Amaral *et al.*, 2015; Lin *et al.*, 2012). Low carbon monoxide (CO) exposure could suppress the production of pro-inflammatory cytokines and attenuate the severity of pre-eclampsia in a rat model (Venditti *et al.*, 2014). In addition, the anti-inflammatory cytokine IL-10 secreted during gestation could ameliorate the symptoms of pre-eclampsia and decrease the IFN- $\gamma$  level in pregnant DOCA/saline treated (PDS) rats (Tinsley *et al.*, 2010). Furthermore, LXA4 weakened inflammation and alleviated the clinical symptoms in endotoxin administrated pregnant rats (Lin *et al.*, 2012).

As a mixed nerve, the vagus nerve (VN) plays a key role in the neuroendocrine-immune axis and provides an important first-line innate defense against inflammation (Johnston and Webster, 2009). The VN is sensitive to pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and TNF- $\alpha$ , which are released by macrophages and other immune cells (Hosoi *et al.*, 2005; Werner *et al.*, 2003). Vagus nerve stimulation (VNS) achieved via a surgically implanted device has been approved as an adjunctive epilepsy and depression therapy for more than 20 years (Fan *et al.*, 2019; Flesler *et al.*, 2017), and its anti-inflammatory properties is currently investigated in a potential therapy for a range of inflammatory disease, such as inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (Bonaz *et al.*, 2017; Koopman *et al.*, 2016). VNS could reduce the serum levels of IL-6 and increase the serum levels of IL-10 (Aalbers *et al.*, 2012; Majoie *et al.*, 2011). In addition, VNS could reduce the production of TNF, IL-1B, IL-6 and IL-8 in isolated peripheral blood mononuclear cells (De Herdt *et al.*, 2009; Koopman *et al.*, 2016). Yang *et al.* (2000) found vagus nerve dysfunction in pre-eclampsia women. In all previous cases, the safety of VNS was confirmed through its use during pregnancy, labor, and delivery, and no morphological abnormalities of

the fetus were reported (Salerno *et al.*, 2016). However, surgical operation risks, adverse effects and expensive treatment have limited the application of VNS (Fitzgerald, 2013; Ventureyra, 2000).

To deal with obstacles limiting the application of invasive VNS (iVNS) or non-invasive transcutaneous VNS (tVNS) has been started to appear in clinic studies. According to the report in a neural anatomy study, the auricular branch of the VN is mainly distributed on the meatus acusticus externus (Peuker and Filler, 2002). Therefore, TaVNS may increase the discharge of Nucleus TractusSolitarii (NTS) (Frangos *et al.*, 2015). In addition, the stimulation on the auricular branch of the VN may produce a modulation effect similar to that of iVNS (Carreno and Frazer, 2016; Hein *et al.*, 2013). TaVNS was used to treat many disorders, such as epilepsy (Rong *et al.*, 2014), chronic tinnitus (Shim *et al.*, 2015) and diabetic neuropathy development (Li *et al.*, 2018), which also boosts associative memory (Jacobs *et al.*, 2015). In experiments conducted on non-pregnant rats, Zhao *et al.* (2012) found that TaVNS inhibited LPS-induced inflammation through CAP mediated by  $\alpha$ 7nAChR. The present study illustrated that L-NAME administration significantly elevated the placenta levels of several pro-inflammatory cytokines, such as TNF- $\alpha$ , HMGB-1 and IL-6, whereas the TaVNS treatment during pregnancy significantly inhibited the expression of above pro-inflammatory cytokines. It is important to note that no changes in the fetal number and no evidence of malformation were observed in all groups. It was also found that the maternal behavior and mental status of rats were not significantly affected by TaVNS, indicating that the applied parameters of TaVNS in this study could be considered as in the safe range.

The CAP is a vagal neuro-immune circuit (termed the 'inflammatory reflex') and may lead to inflammatory reactions. This reflex is mediated through the link of Acetylcholine (ACh), the principal vagal neuro transmitter with  $\alpha$ 7nAChR in macrophages could inhibit the synthesis and release of pro-inflammatory cytokines (Borovikova *et al.*, 2000). Many therapies are based on the cholinergic stimulation, which induces the efferent vagal nerve signaling to reduce the activation of immune cells and to suppress the



overproduction of pro-inflammatory cytokines. Pre-treatment with nicotine, a selective agonist of  $\alpha 7$ nAChR, protects rats against LPS-induced inflammatory responses via the CAP activation (Liu *et al.*, 2017). The administration of choline also ameliorated pre-eclampsia-like symptoms and protected against inflammatory symptoms through the functions of  $\alpha 7$ nAChR and CAP (Zhang *et al.*, 2018). In models of sepsis,  $\alpha 7$ nAChR has been repeatedly shown to mediate the beneficial effect of VNS (Rana *et al.*, 2018; Zhao *et al.*, 2013).

In the present study, it was found that TaVNS improved pregnancy outcomes in pre-eclampsia rats, presumably by significantly inhibiting the release of pro-inflammatory cytokines in the placenta. Moreover, the mRNA and protein levels of  $\alpha 7$ nAChR in the placenta of pre-eclampsia rats were elevated by the TaVNS treatment. In summary, these data indicated that TaVNS might exert its anti-inflammatory role in pre-eclampsia placentas by activating the CAP and by regulating the level of  $\alpha 7$ nAChR.

To address the mechanisms by which TaVNS exerts its anti-inflammatory effects in pre-eclampsia rats, the placental NF- $\kappa$ B activation in pre-eclampsia rats was examined. It was found that TaVNS treatment led to significant inhibition of NF- $\kappa$ B activity. Previous studies have shown that NF- $\kappa$ B plays critical roles in the transcriptional regulation of pro-inflammatory genes. A study by Zhao *et al.* (2013) demonstrated that VNS suppressed cytokine release through  $\alpha 7$ nAChR and inhibited NF- $\kappa$ B signaling in a rat model of myocardial ischemia/reperfusion. It was also found in this study that TaVNS improved the placental expression of  $\alpha 7$ nAChR in pre-eclampsia rats and significantly inhibited the activity of NF- $\kappa$ B p65 in the placenta. The stimulation of auricular branch of the vagus nerve (ABVN) ascends to the NTS, where they cause the activation of efferent vagus nerve fibers to inhibit cytokine release through the activation of  $\alpha 7$ nAChR (Zhao *et al.*, 2012).

In conclusion, our data show that TaVNS reversed the impairments resulting from L-NAME induced pre-eclampsia. The underlying mechanisms of TaVNS treatment may include the activation of  $\alpha 7$ nAChR, the inhibition of NF- $\kappa$ B activities, and the downregulation in the expression of pro-inflammatory cytokines. This study provided new insights for TaVNS in the treatment of inflammatory responses during pre-eclampsia without inducing adverse effects. Nevertheless, further studies are needed to optimize the potential use of TaVNS in clinics.

**Acknowledgement:** The authors thank Zhicheng Lu and Fujin Liu for their help with the animal experiments.

**Availability of Data and Materials:** The datasets used and/or analysed during the current study are available upon reasonable request from the corresponding author.

**Author Contribution:** The study was conceived by Lei Shi, 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.

**Ethics Approval:** All animal procedures were approved by the Animal Ethics Committee of Hainan Medical University. (Ratification No. 2020-185; Approval Date Jun. 04, 2020.)

**Funding Statement:** Funding was provided by Provincial Natural Science Foundation of China Hainan (819MS119).

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

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