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Nitric oxide in enteric nervous system mediated the inhibitory effect of vasopressin on the contraction of circular muscle strips from colon in male rats

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Abstract

Background *Arginine vasopressin (AVP) is widely used in the treatment of critical diseases with hypotension, but the reports about its effect on gastrointestinal motility are controversial. The purpose of this study was to characterize the role of AVP in the regulation of colonic motility and the underlying mechanism.* ***Methods*** *The contraction of the circular muscle strips (CM) of colon in male rats was monitored by a polygraph. The expressions of cytoplasmic inducible nitric oxide synthase (iNOS), I-κB, and the nuclear P65 in proximal colon were measured by Western blot. The* V_1 *receptors (*V_1Rs*) and iNOS were localized by immunohistochemistry. The content of nitric oxide (NO) in the colon was measured by Griess reagent at the absorbance of 560 nm.* ***Key Results*** *Arginine vasopressin (*10^{-10}*–*10^{-6} *mol* L^{-1}*) caused a concentration-dependent inhibition on CM contraction. Pretreatment with one of the following chemicals, including V-1880 (*10^{-7} *mol* L^{-1}*), TTX (*10^{-5} *mol* L^{-1}*), L-NAME (*10^{-4} *mol* L^{-1}*), NPLA (*10^{-7} *mol* L^{-1}*), SMT (*10^{-3} *mol* L^{-1}*), and PDTC (*10^{-3} *mol* L^{-1}*), attenuated the inhibitory effect of AVP on CM contraction. Arginine vasopressin increased the expression of iNOS and the content of NO in proximal colon. These effects were attenuated by pretreatment with PDTC (*10^{-3} *mol* L^{-1}*). Following AVP administration, the amount of cytoplasmic I-κB decreased, but that of nuclear P65 increased. Double immunofluorescence labeling revealed that* V_1Rs *and iNOS were co-localized on the cells of myenteric plexus in proximal colon.* ***Conclusions & Inferences*** *Arginine vasopressin inhibited the contraction of CM in proximal colon. This effect was mediated by NO produced from NF-κB–iNOS pathway and neuronal NOS activation in myenteric plexus.*

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INTRODUCTION

Arginine vasopressin (AVP) is a non peptide secreted from neurohypophysis. It is traditionally recognized as a vasoconstrictor and antidiuretic hormone. Arginine vasopressin receptors are the members of G-protein coupled receptors on plasma membrane. They are divided into three subtypes, including V_1 receptors (V_1Rs), V_2 receptors (V_2Rs) and V_3 receptors (V_3Rs).[1,2] Exogenous V_1Rs agonists induce vasoconstriction, so they are widely used as alternative non-adrenergic vasopressors for hemodynamic support of patients with critical illness, such as during the shock states,[3–8] epidural anesthesia-induced arterial hypotension,[9] and perioperative catecholamine-refractory arterial hypotension.[10] However, the vasoconstrictive effect of AVP might impair tissue perfusion and induce the genesis of ischemic tissue injury.[11]

Arginine vasopressin analog was also used clinically to treat the gastrointestinal (GI) hemorrhage,[12] but in animal experiment it was demonstrated that AVP treatment might induce the accumulation of polymorphonuclear neutrophilic granulocytes in the intestinal muscularis layer of shocked rats and impairment of smooth muscle contractility.[13] As we know, a huge amount of bacteria, both pathogenic and non-pathogenic, are hosted in the GI tract. The decreased GI transit might result in the bacterial overgrowth,[14] and the subsequent bacterial translocation might be the reason of systemic sepsis and multiple organ failure

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(MOF) during shock.[14–16] So we believe that, besides the hypoperfusion and mucosal changes in the gut, it is also meaningful to pay attention to the GI dysmotility during hemorrhagic shock.

Although it has been demonstrated that AVP receptors were widely expressed in the human GI tract,[17] the effect of AVP on GI motility seemed to be dose-dependent and species different. In overnight fasted, hydrated human, the plasma vasopressin level was 4.7 pg mL^{-1}.[18] In the early phase of septic shock, the vasopressin level was increased to about 10 pg mL^{-1},[19] and in animals, the level was often above 500 pg mL^{-1} in dogs and above 300 pg mL^{-1} in baboons.[20] Infusion of low doses of AVP (0.01–0.03 U min^{-1}), the recommended dose for the treatment of septic shock, increased the serum level of AVP up to 60–130 pg mL^{-1}.[21,22] Although the effect of AVP at physiological level might not influence colonic motility of the rat and human,[23,24] the effect at high level has not been clearly understood. Ward *et al.* reported that lower concentration of AVP (10^{-12}–10^{-9} mol L^{-1}) increased the slow waves and phasic contraction of colonic muscle strips from dogs *in vitro*, but higher concentration of AVP (>10^{-8} mol L^{-1}) caused an inhibitory effect.[25] We also found that intravenous injection of AVP (0.03–3 μg kg^{-1}) decreased the intracolonic pressure of ovariectomized female rats.[26] Because V_1Rs are located in the myenteric neurons in enteric nervous system(ENS)[27] and nitric oxide (NO) is the major relaxant of GI muscle, we hypothesized that exogenous AVP might inhibit colonic contraction in rats via activation of neuronal NO synthase (nNOS) and increase of NO production. AVP increased the inducible NOS (iNOS) expression and NO content in cardiac fibroblasts via activation of nuclear factor-kappa B (NF-κB),[28] the possibility that NF-κB and iNOS in colon were involved in this process was investigated.

MATERIALS AND METHODS

Experimental animals

In order to exclude the effect of endogenous estrogen on the colonic motility,[26] in this study, only male Wistar rats (280–320 g) were used. The rats were provided by the Animal Center of Shandong University. Before the experiments, the rats were fasted for 12 h with free access to tap water. All the procedures in this study were approved by the Ethics Committee for Experimental Animals, Shandong University School of Medicine.

Muscle strips preparation

The method has been described in our recent study.[26] In brief, after each rat was sacrificed, a segment of proximal colon (1 cm from cecum) was cut and opened along the mesenteric border. After rinsed with Krebs solution, the rectangular sheet from the proximal colon was pinned flat (mucosa up) in a silica gel dish which was filled with Krebs solution and bubbled with 95% O_2 and 5% CO_2. The mucosa was carefully removed by fine forceps (DUMONT #5; FST, Heidelberg, Germany). Muscle strips (8×3 mm) were cut parallel to the circular fibers and were designated as circular muscle strips (CM).

Recording of the contraction of muscle strips

Circular muscle strips was suspended in a chamber containing 5 mL of oxygenated (95% O_2 and 5% CO_2) Krebs solution (37 °C). One end of CM was tied to a hook at the bottom of the chamber. The other end was connected to an isometric force transducer (JH-2B; Instrument Company of Chengdu, Chengdu, China). The tension of CM was recorded by a polygraph (SMUP-PC; Jia Long Educational Instrument Factory, Shanghai, China). Before any chemical administration, the CM with 1 g preload was equilibrated in Krebs solution for at least 60 min. Each CM was exposed to AVP only once. The tension of CM was recorded continuously for 30 min after AVP administration. In some experiments, the strips were pretreated with one of the six specific inhibitors for 10–30 min before AVP application.[27,29] These inhibitors include [deamino-Pen1, O-Me-Tyr2, Arg8]-Vasopressin (V-1880, the antagonist of V_1Rs), Pyrrolidine dithiocarbamate (PDTC, the inhibitor of NF-κB), *N* (G)-nitro-L- arginine methyl ester (L-NAME, the inhibitor of NOS), *S*-methylisothioure (SMT, the specific inhibitor of iNOS, tetrodotoxin (TTX, the inhibitor of the voltage dependent Na^+ channel on neuron membrane) and *N*-Propyl-L-Arginine (NPLA, a specific inhibitor for nNOS[30]).

Immunohistochemistry

Four-micron-thick sections were prepared from 4% paraformaldehyde fixed, paraffin-embedded colonic segments that were removed from the proximal colon of male rats. Sections were de-waxed and hydrated. After antigen retrieval, the endogenous peroxidase was quenched for 10 min using 3% hydrogen peroxide in a Two-Step IHC Detection Reagent (ZSGB-BIO, Beijing, China). Following three rinses in phosphate-buffered saline (PBS) and treated by 5% bovine serum for 1 h, the sections were incubated with rabbit anti-iNOS antibody (1 : 200, sc-651; Santa Cruz, CA, USA) or goat anti-V_1R receptor polyclonal antibody (1 : 100, sc-18096; Santa Cruz) overnight at 4 °C. After washing with PBS, the sections were incubated with biotinylated secondary antibodies (ZSGB-BIO) for 30 min at 20 °C. The sections were then washed and treated with horse radish peroxidase (HRP) labeled streptavidin-complex (ZSGB-BIO) for 30 min at 20 °C. After three rinses, the peroxidase was revealed by a 3,3′-diaminobenzidine tetrahydrochloride substrate kit (ZSGB-BIO). At the final step, the sections were counterstained with hematoxylin. The sections of negative control were incubated with PBS instead of primary antibody.

Immunofluorescence staining

V_1receptors and neuronal nuclei (NeuN) were co-located by dual-immunoflurescence staining on paraffin sections of the proximal colon. After the endogenous immunoglobulins was blocked by bovine serum, the sections were incubated in anti-V_1Rs polyclonal antibody (1 : 100, sc-18096; Santa Cruz) and the anti-NeuN monoclonal antibody (1 : 100, MAB377; Chemicon International, Temecula, CA, USA) overnight at 4 °C. After washed with PBS, the sections were incubated for 1 h at 20 °C with tetramethyl-

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rhodamine isothiocyanate (TRITC) (rhodamine)-conjugated rabbit anti-goat IgG (1 : 50, ZSGB-BIO) and fluorescein isothiocyanate (FITC) (fluorescein)-conjugated rabbit anti-mouse IgG (1 : 50, ZYMED Laboratories of Invitrogen, San Diego, CA, USA).

In order to co-locate V_1Rs and iNOS in the proximal colon, paraffin sections from the proximal colon were incubated in anti-V_1Rs polyclonal antibody (1 : 100, sc-18096; Santa Cruz) and anti-iNOS polyclonal antibody (1 : 200, sc-651; Santa Cruz) overnight at 4 °C. After washed with PBS, the sections were incubated at 20 °C for 1 h with FITC (fluorescein)-conjugated donkey anti-goat lgG (1 : 50, sc-2024; Santa Cruz) and TRITC (rhodamine)-conjugated donkey anti-rabbit lgG (1 : 50, sc-2095, Santa Cruz).

The immunopositive cells on the sections were detected by a fluorescence microscope (80i; Nikon, Tokyo, Japan).

Extraction of nuclear and cytoplasmic protein

After incubating with AVP or vehicle for 5 min, the segments of proximal colon without mucosa were homogenized in cytoplasmic extraction reagent A CERA (0.2 mL) (BioTeke Corporation, Beijing, China). After the vortex and centrifugation at 4 °C, the supernatant containing cytoplasmic proteins was collected. The sediment was dissolved in phenylmethanesulfonyl fluoride buffer (0.05 mL), diluted by same volume of nuclear extraction reagent B, and rotated for 30 min at 4 °C. After centrifuged at 21 130 ***g*** for 10 min at 4 °C, the supernatant was collected as nuclear extracts.

Western blot

The amount of protein in the cytoplasmic or nuclear extracts was quantified by Protein Quantitative Analysis kit (k3001-BCA; Shenergy Biocolor, Shanghai, China). The supernatant was electrophoresed and transferred to nitrocellulose membrane. The membrane was incubated in blocking buffer [5% non-fat dry milk in tween/tris-buffered salt solution (TTBS)] for 1 h at 20 °C, washed in TTBS, and incubated overnight with one of the three primary antibodies, including rabbit anti-iNOS antibody (1 : 500, sc 651; Santa Cruz), rabbit anti-NF-κB (P65) antibody (1 : 500, sc 8008; Santa Cruz), and rabbit anti-I-κB antibody (1 : 800, sc 371; Santa Cruz). After multiple washes, the membranes were incubated at 20 °C for 1 h with secondary antibodies (1 : 20 000, A0208; Beyotime, Nantong, China) conjugated with HRP. The immunopositive proteins on the membrane were detected by ECL plus (Millipore, Bedford, USA).

Measurement of NO content in proximal colon

The method was described in our recent report.[29] Simply, after incubating with AVP (10^{-8} mol L^{-1}) or vehicle for 5 min, the segments of proximal colon without mucosa layer were homogenized. After centrifuging at 94 ***g*** for 5 min at 4 °C, supernatant (50 μL) was mixed with an equal volume of Griess reagents I and II (Beyotime, Jiangsu, China) at room temperature. The amount of nitrite production was measured at the absorbance of 560 nm by a Universal Microplate Spectrophotometer (Multiskan MK3; Thermo Electron Corporation, Waltham, MA, USA).

Chemicals and solutions

Arginine Vasopressin, V-1880, L-NAME, SMT, TTX, NPLA and PDTC were purchased from Sigma-Aldrich Corp (St Louis, MO, USA).

Because all these reagents were diluted into normal saline (NS) (0.9% NaCl solution, Shandong Provincial Hospital, Jinan, China), tissues were treated with NS as the vehicle (control) group.

The Krebs solution was composed of the following reagents (m mol L^{-1}): NaCl 120.6, KCl 5.9, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 15.4, and glucose 11.5. Phosphate-buffered Saline was composed of 0.01 mol L^{-1} NaH_2PO_4–Na_2HPO_4 and 0.14 mol L^{-1} NaCl, pH = 7.2. TTBS was composed of 0.1% Tween-20, 50 m mol L^{-1} Tris, and 150 m mol L^{-1} NaCl.

Data analysis

Both the tonic and phasic contraction of the muscle strips were simultaneously recorded during the experiments. In order to facilitate the quantification of the muscle contractions, the recording trace was integrated with an interval of 10 s. The mean tension in 1 min was calculated by dividing the summarized integration value with the corresponding period (60 s). The mean tension of the muscle strips before AVP treatment was defined as reference value. The average tension for a period after each chemical treatment was normalized to a standardized ratio (R) where the reference value for each experiment was equal to one. The R value was taken as the change in muscle contraction due to each AVP treatment.

The Western blot band was quantified by Scion Image software (Scion Corp., Frederick, MD, USA). The band was expressed as relative protein amounts compared to c-jun (nuclear protein) or β-actin (cytoplamic protein).

All the values in these experiments were presented as mean ± SEM. Significant differences between several treatment groups and one control group were determined by one way ANOVA on ranks followed by Dunn's test, and that between two groups was determined by student's t-test. $P < 0.05$ was considered to be a significant difference.

RESULTS

Effects of exogenous AVP on the contraction of CM of proximal colon in male rats

First of all, we investigated the effect of AVP on the spontaneous contraction of CM from the proximal colon in male rats by muscular tension recording method. Arginine Vasopressin (10^{-8} mol L^{-1}) inhibited the contraction of CM (Fig. 1A). The spontaneous contraction of the strips decreased immediately after AVP administration, reached the lowest level at 3–5 min, and returned to normal at 12 min (Fig. 1A, B). Pretreatment with V-1880 (10^{-7} mol L^{-1}), a potent V_1Rs antagonist, abolished this effect (Fig. 1B). The inhibitory effect of AVP on muscle contraction was in a dose-dependent manner. Higher concentration of AVP (10^{-10}–10^{-6} mol L^{-1}) significantly decreased the muscle contraction, whereas, the effect was not observed in lower level (1×10^{-12} and 1×10^{-11} mol L^{-1}) (Fig. 1C). Five minutes after AVP (10^{-10} mol L^{-1}) administration, R value decreased from 1 (control) to 0.86 ± 0.02, significantly lower than that of the control group ($P = 0.004$, $n = 22$) (Fig. 1C). The maximal inhibition was reached when the

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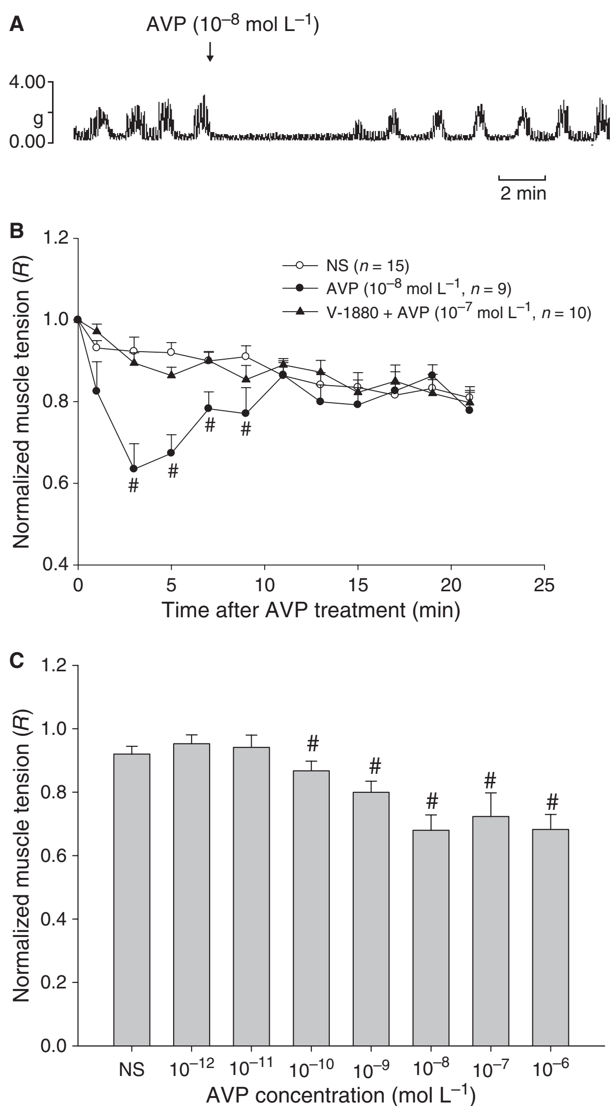

Figure 1 Effect of exogenous arginine vasopressin (AVP) on the contraction of circular muscle (CM) of proximal colon in male rats. (A) The representative recording of the effect of AVP (10^{-8} mol L^{-1}) on the contraction of the muscle strips. Arrow indicates the AVP treatment. The spontaneous contraction of CM was inhibited immediately following AVP administration. This effect was maintained for more than 5 min. (B) shows the time course of the CM in response to AVP (10^{-8} mol L^{-1}) with and without pretreatment of V-1880 (10^{-7} mol L^{-1}). $^{\#}P < 0.05$ *vs* NS group (vehicle control). The mean value of the normalized muscle tension decreased immediately following AVP administration, reached the nadir at 3 min and returned to normal level at 12 min. Pretreatment of muscle strips with V-1880 completely abolished this change. (C) The summarized data of the change in mean muscle tension at 5 min following different doses of AVP (10^{-12}–10^{-6} mol L^{-1}) administration. $^{\#}P < 0.05$ *vs* NS group (vehicle control). Low doses of AVP (10^{-12}–10^{-10} mol L^{-1}) did not influence CM tension.

AVP concentration was 10^{-8} mol L^{-1}, and higher concentration of AVP (10^{-7}–10^{-6} mol L^{-1}) did not induce further decrease of R value (Fig. 1C).

Effect of NOS inhibitors on AVP-induced inhibition on CM contraction

As NOS is generally involved in GI motor dysfunction,[31,32] we further investigated whether they also played a role in the inhibition of CM contraction following AVP administration. The NOS inhibitors used in this study included L-NAME (non-selective NOS antagonist), SMT (selective iNOS antagonist) and NPLA (selective nNOS antagonist). Both L-NAME (10^{-4} mol L^{-1}) and NPLA (10^{-7} mol L^{-1}) increased the tension of CM while SMT (10^{-3} mol L^{-1}) did not exert any effect. At 5 min following L-NAME administration, the normalized CM tension was increased from 1 (before L-NAME administration) to 1.02 ± 0.06, (n = 15, $P < 0.05$) (Fig. 2A).

Pretreatment with L-NAME (10^{-4} mol L^{-1}) significantly attenuated the inhibitory effect of AVP (10^{-8} mol L^{-1}) on the contraction of the muscle strips. At 3 min following AVP administration, the normalized CM tension of L-NAME + AVP group was 0.91 ± 0.05, significantly higher than that of vehicle + AVP group (Fig. 2B). Similar results were found in the groups pretreated with SMT or NPLA (Fig. 2B).

The inhibitory effect of L-NAME was greater than that of NPLA at 3 min following AVP administration. At that time point, the normalized muscle tension in NPLA + AVP group was 0.79 ± 0.02, significantly lower than that of L-NAME + AVP group. There was no difference between L-NAME + AVP group and SMT + AVP group. So at 3 min, when the CM tension reached the lowest level, the NO produced from iNOS pathway seemed to be the predominant contributor for the inhibitory action of AVP on CM (Fig. 2B).

Involvement of enteric nervous system

Nitric oxide could be produced in the neurons in the ENS through both nNOS and iNOS pathway,[29] so we hypothesized that ENS might be involved in the inhibition of CM contraction following AVP administration. In order to test this hypothesis, TTX was used to block the excitation of neuron in ENS. Tetrodotoxin itself increased the CM contraction. Five minutes following TTX (10^{-5} mol L^{-1}) administration, CM tension was increased from 0.91 ± 0.02 (control group) to 1.07 ± 0.04 (Fig. 3). Pretreatment of TTX (10^{-5} mol L^{-1}) significantly reversed the inhibitory effect of AVP on CM contraction. Five minutes following AVP administration, the normalized CM tension in TTX + AVP group was 0.90 ± 0.02, significantly higher than that of NS + AVP group (Fig. 3).

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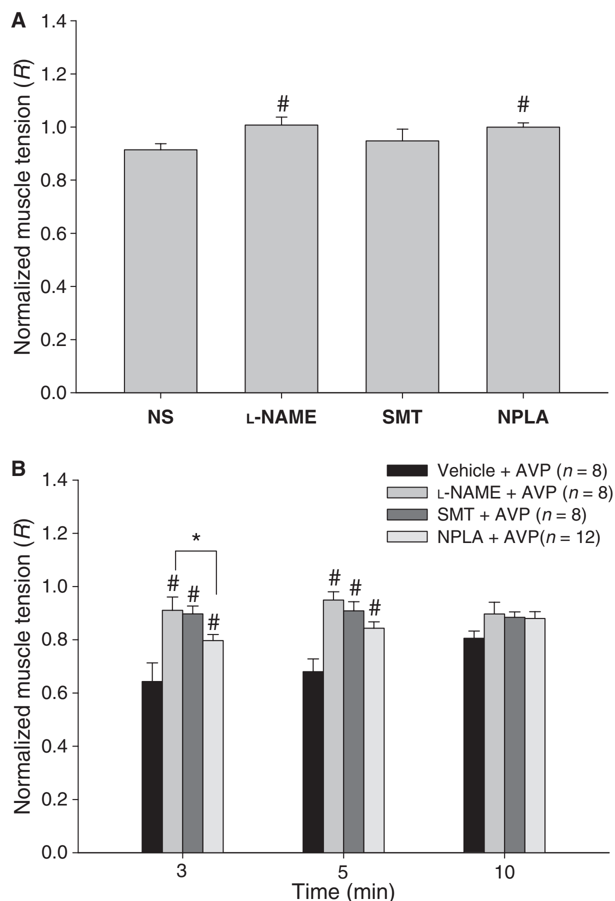

Figure 2 Effect of nitric oxide synthase (NOS) inhibitors on circular muscle (CM) tension and on the inhibition exerted by arginine vasopressin (AVP) on CM contraction. (A) The normalized CM tension at 5 min following administration of three kinds of NOS inhibitors, including *N* (G)-nitro-L- arginine methyl ester (L-NAME) (non-selective NOS inhibitors), *S*-methylisothioure (SMT) (selective inducible NOS inhibitors) and *N*-Propyl-L-Arginine (NPLA) (selective neuronal NOS inhibitors). $^{\#}P < 0.05$ *vs* NS group (vehicle control). L-NAME (10^{-4} mol L^{-1}) and NPLA (10^{-7} mol L^{-1}) increased CM tension but SMT had no effect on it. (B) The pharmacological manipulation of the inhibitory effect of AVP on CM tension. $^{\#}P < 0.05$ *vs* vehicle + AVP. $^{*}P < 0.05$ *vs* L-NAME + AVP. The muscle strips were incubated with one of the three inhibitors for 30 min before AVP administration. Pretreatment with L-NAME (10^{-4} mol L^{-1}), SMT (10^{-3} mol L^{-1}) or NPLA (10^{-7} mol L^{-1}) significantly blocked the AVP (10^{-8} mol L^{-1}) inhibitory action on CM at 3 and 5 min following AVP administration. At 3 min, the normalized muscle tension of NPLA + AVP group was lower than that of the L-NAME + AVP group, although it was higher than that of vehicle + AVP group. It indicated that at 3 min, the effect of L-NAME might be mainly through inhibition of iNOS but not nNOS. At 10 min, when the muscle tension of vehicle + AVP group returned to near the reference value (the normalized tension is 1), there was no significant among the four groups.

NO content in colon following AVP treatment

Next, we tested the effect of AVP on the production of NO in CM. Arginine vasopressin (10^{-8} mol L^{-1}) significantly increased the production of NO in proximal colonic segments. Five minutes following AVP administration, the concentration of NO in supernatant was 23.76 ± 0.9 μ mol L^{-1}, significantly higher than that of the control groups. With the pretreatment of SMT, the increase of NO content following AVP (10^{-8} mol L^{-1}) administration was significantly attenuated (Fig. 4).

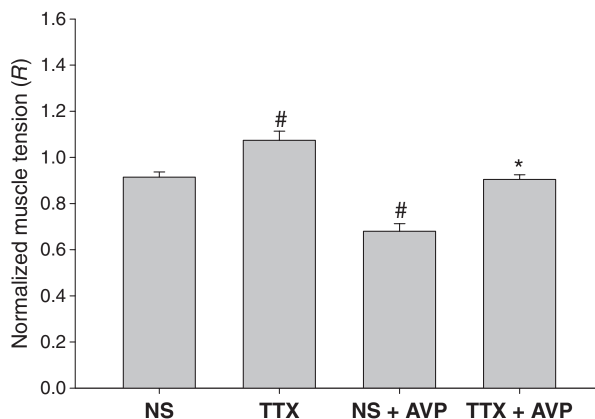

Figure 3 Effect of tetrodotoxin (TTX) on the circular muscle (CM) contraction and arginine vasopressin (AVP) induced decrease of CM tension. The first column shows the mean normalized muscle tension at 5 min following NS administration, which acts as a vehicle control. Subsequent columns represent the data at 5 min following the administration of TTX alone, NS + AVP and TTX + AVP. Note that TTX significantly increased the muscle tension. Pretreatment of TTX significantly reversed the inhibitory effect of AVP on muscle tension. $^{\#}P < 0.05$ *vs* NS; $^{*}P < 0.05$ *vs* NS + AVP.

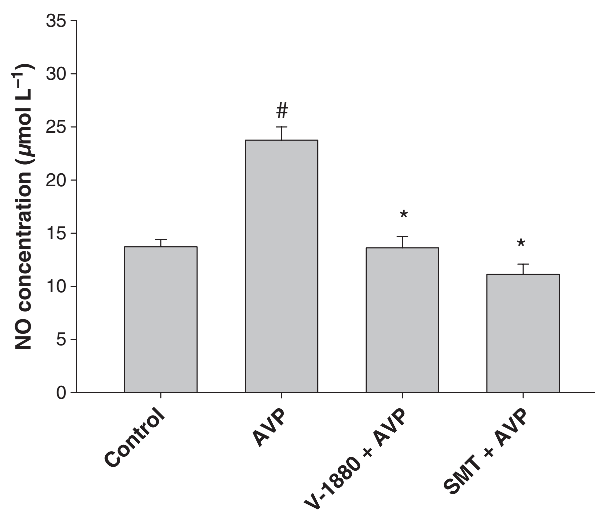

Figure 4 Effect of arginine vasopressin (AVP) on the production of nitric oxide (NO) in colon and the involvement of V_1 receptors (V_1Rs) and inducible NOS. The first column shows the concentration of NO in the supernatant of colon at 5 min following NS administration, which acts as a vehicle. Subsequent columns represent the data taken from the supernatants at 5 min after AVP treatment. Note that AVP (10^{-8} mol L^{-1}) significantly increased NO concentration. Pretreatment of V-1880 (10^{-7} mol L^{-1}) or SMT (10^{-3} mol L^{-1}) significantly reversed this effect. $^{\#}P < 0.05$ *vs* control, $^{*}P < 0.05$ *vs* vehicle + AVP.

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Effect of PDTC on increase of iNOS expression and inhibition of CM contraction following AVP administration

It has been demonstrated that NF-κB is a critical component of iNOS gene transcriptional activation in many cells.[33,34] We speculated that AVP might increase iNOS expression through activation of NF-κB. Several experiments were conducted to test this hypothesis.

Arginine vasopressin increased the amount of iNOS in the colon. Five minutes following AVP (10^{-8} mol L^{-1}) administration, the amount of iNOS in proximal colon was 2.09 ± 0.6 times more than that of the control group ($P < 0.05$, $n = 4$) (Fig. 5A). Pretreatment of PDTC (10^{-3} mol L^{-1}), the inhibitor of NF-κB, significantly reversed this increase in iNOS expression following AVP administration (Fig. 5A). Same dose of PDTC attenuated the inhibitory effect of AVP (10^{-8} mol L^{-1}) on CM contraction (Fig. 5B).

Effect of AVP administration on the expression of cytoplasmic I-κB and nuclear NF-κB (P65) in proximal colon

During the resting state, the inactive state of NF-κB (P65) in plasma combines with I-κB. After activation, NF-κB dissociates with I-κB and translocates into the nucleus.[29] In order to test the hypothesis that NF-κB was activated following AVP administration, the extracts of NF-κB in nucleus and I-κB in plasma were assayed by Western blot. Arginine vasopressin (10^{-8} mol L^{-1}) significantly decreased the amount of cytoplasmic I-κB and increased the nuclear P65 in colon (Fig. 6). Five minutes following AVP administration, the amount of I-κB in plasma decreased by 26.3 ± 5.36% ($P < 0.05$, $n = 4$) (Fig. 7A), while that of NF-κB in nucleus increased by 4.3 ± 0.4 times ($P < 0.05$, $n = 6$) (Fig. 6B). Pretreatment of PDTC (10^{-3} mol L^{-1}) partly reversed these changes (Fig. 6A,B).

Localization of V_1Rs in colon

V_1 receptors-positive cells were located in myenteric plexus of proximal colonic sections (Fig. 7A1). No immunopositive protein was detected if the primary antibody was replaced by PBS (Fig. 7A2).

In order to further verify the hypothesis that V_1Rs were located on the neurons in ENS, a double staining was performed for V_1Rs and NeuN and the result indicated that both V_1Rs and NeuN were expressed in myenteric plexus (Fig. 7B,C). After overlapping Fig. 7B,C, it was clear that V_1Rs was expressed on the NeuN positive cells in the myenteric plexus of the proximal colon (Fig. 7D). Inducible NOS was detected in the myenteric plexus (Fig. 7E1,E2) and V_1Rs and iNOS were also co-located in the cells in myenteric plexus (Fig. 7F–H).

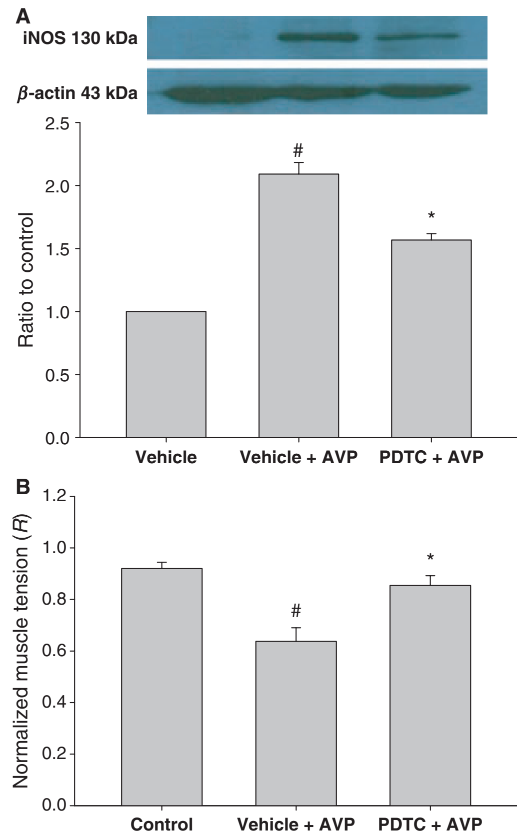

Figure 5 Effect of AVP on the iNOS expression in colon and the pharmacological manipulation of PDTC (10^{-3} mol L^{-1}) on AVP induced iNOS increase and CM tension decrease. The upper panel of (A) is the representative immunoblots for iNOS (130 kDa) and β-actin (43 kDa, loading control) in three groups, including vehicle, vehicle + AVP and PDTC + AVP. The lower panel of (A) represents the data taken from the three groups. The first column is the data from the colon at 5 min with NS treatment, which acts as vehicle. The data of this group acts as control (reference value) and normalized to 1. The subsequent columns represent the data taken from the colons at 5 min after AVP administration. Note that AVP (10^{-8} mol L^{-1}) significantly increased the iNOS amount in colon (the second column) and pretreatment of PDTC (10^{-3} mol L^{-1}) reversed this effect (the third column). $^{\#}P < 0.05$ *vs* control, $^{*}P < 0.05$ *vs* vehicle + AVP. (B) shows the change of CM tension at 5 min following various chemicals administration. The first column represents the data following NS treatment, which acts as vehicle control. Note that AVP (10^{-8} mol L^{-1}) significantly decreased muscle tension (the second column). Pretreatment of PDTC (10^{-3} mol L^{-1}) reversed this effect (the third column). $^{\#}P < 0.05$ *vs* control, $^{*}P < 0.05$ *vs* vehicle + AVP.

DISCUSSION

It is indubitable that vasopressin plays a critical role in the regulation of physiological functions in body,

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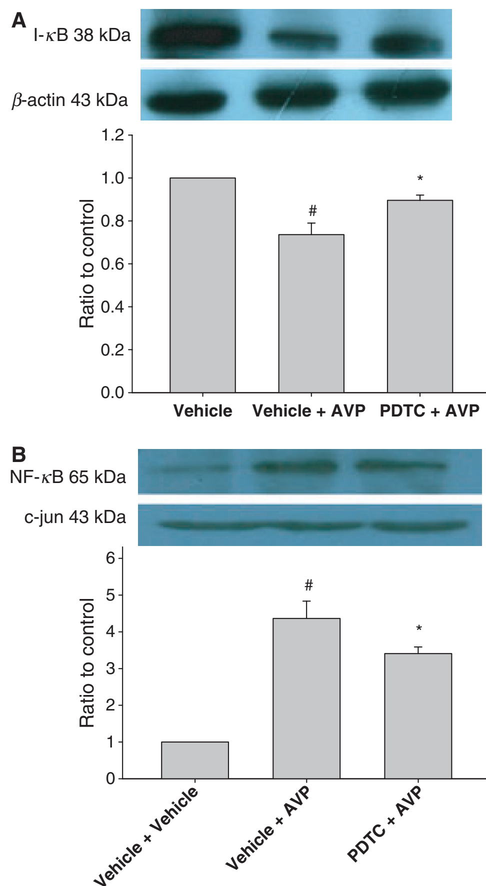

Figure 6 Activation of NF-κB in colon at 5 min following AVP administration. The upper panel of (A) shows the representative immunoblots of cytoplasmic I-κB (38 kDa) and β-actin (43 kDa, loading control) in three groups, including vehicle, vehicle + AVP and PDTC + AVP. The lower panel is the summarized data taken from solution of cytoplasmic protein of proximal colon at 5 min following chemicals administration. The first column represents the data of NS treatment, which acts as vehicle control. Note that AVP (10^{-8} mol L^{-1}) significantly decreased the amount of cytoplasmic I-κB (the second column) and pretreatment of PDTC significantly reversed this effect (the third column). $^{\#}P < 0.05$ *vs* vehicle, $^{*}P < 0.05$ *vs* vehicle + AVP. The upper panel of (B) shows the representative immunoblots of nuclear NF-κB (65 kDa) and c-jun (43 kDa, loading control) in three groups, including vehicle + vehicle, vehicle + AVP and PDTC + AVP. The lower panel is the summarized data taken from solution of nuclear extraction at 5 min following chemical administration. The first column represents the data of NS + NS treatment, which acts as vehicle control. Note that AVP (10^{-8} mol L^{-1}) significantly increased the amount of nuclear NF-κB (the second column) and pretreatment of PDTC significantly reversed this effect (the third column). $^{\#}P < 0.05$ *vs* vehicle + vehicle, $^{*}P < 0.05$ *vs* vehicle + AVP.

unbalance of which brings abnormal changes of target organisms. Previous studies indicated that the production of AVP was increased under several situations, such as ether inhalation,[35] electric stimulation of neuro-intermediate lobe of the hypophysis,[36] immobilization[37] and septic shock.[38,39] Although several studies indicated that exogenous vasopressin influenced GI motility in human and animals,[23,26,40–43] most of these experiments were conducted *in vivo* and could not exclude the possibility that AVP influenced the gut motility by decreasing the blood flow in the organ or by binding to AVP receptor in central nervous system. So, in this study, we investigated the effect of vasopressin on the isolated muscle strips of the proximal colon in male rats. We found that exogenous AVP inhibited the contraction of the CM from proximal colon in rats. It is consistent with results from QI *et al.*, which indicated that vasopressin induced intestinal dysrhythmia and emetic symptoms and inhibited intestinal motility in dogs.[44] V-1880, the specific antagonist of V_1Rs,[45] attenuated the inhibitory effect of AVP on proximal colon contraction *in vitro*. This result indicated that AVP inhibited colonic motility through binding to local V_1Rs.

The result of the present study was consistent with that reported by Ward *et al.*,[25] which indicated that the effect of AVP on colonic motility was dose-dependent and high concentration of AVP decreased the contraction of colonic muscle strips *in vitro*. The difference between the two studies was that the effect of exogenous AVP on the colonic contraction from rat was mainly inhibitory, but that on the colonic traction from dogs was excitatory at low concentration (10^{-12}–10^{-8} mol L^{-1}) and inhibitory at higher concentration (>10^{-8} mol L^{-1}). This might be attributed to the species difference. It is noteworthy that, in this study, we found that AVP at the level of 1×10^{-10} mol L^{-1} inhibited the colonic contraction. The plasma concentration of AVP could reach or near this level at the early stage of septic shock in animals and human, and during the treatment of critical illness with low dose of AVP.[20–22] So it might be possible that the increased level of AVP during septic shock might increase the concentration of AVP in plasma, and then inhibit the colonic traction. The inhibition of colonic contraction and the resulting overgrowth of the bacteria in colon might be one of the reasons of MOF during shock or other critical disease with hypotension. So the results of this study indicate that we should pay attention to the change of GI motility during shock and treatment of critical illnesses with AVP.

Another important finding of this study is that exogenous AVP increased the NO production. This might induce intestinal injury and dysfunction of the gut and decrease of the blood pressure. So during the AVP treatment for hemorrhagic shock or other disease with hypotension, reducing bioavailability of NO may be beneficial to the gut. This finding is consistent with the report of Hierholzer *et al.*, which indicated that

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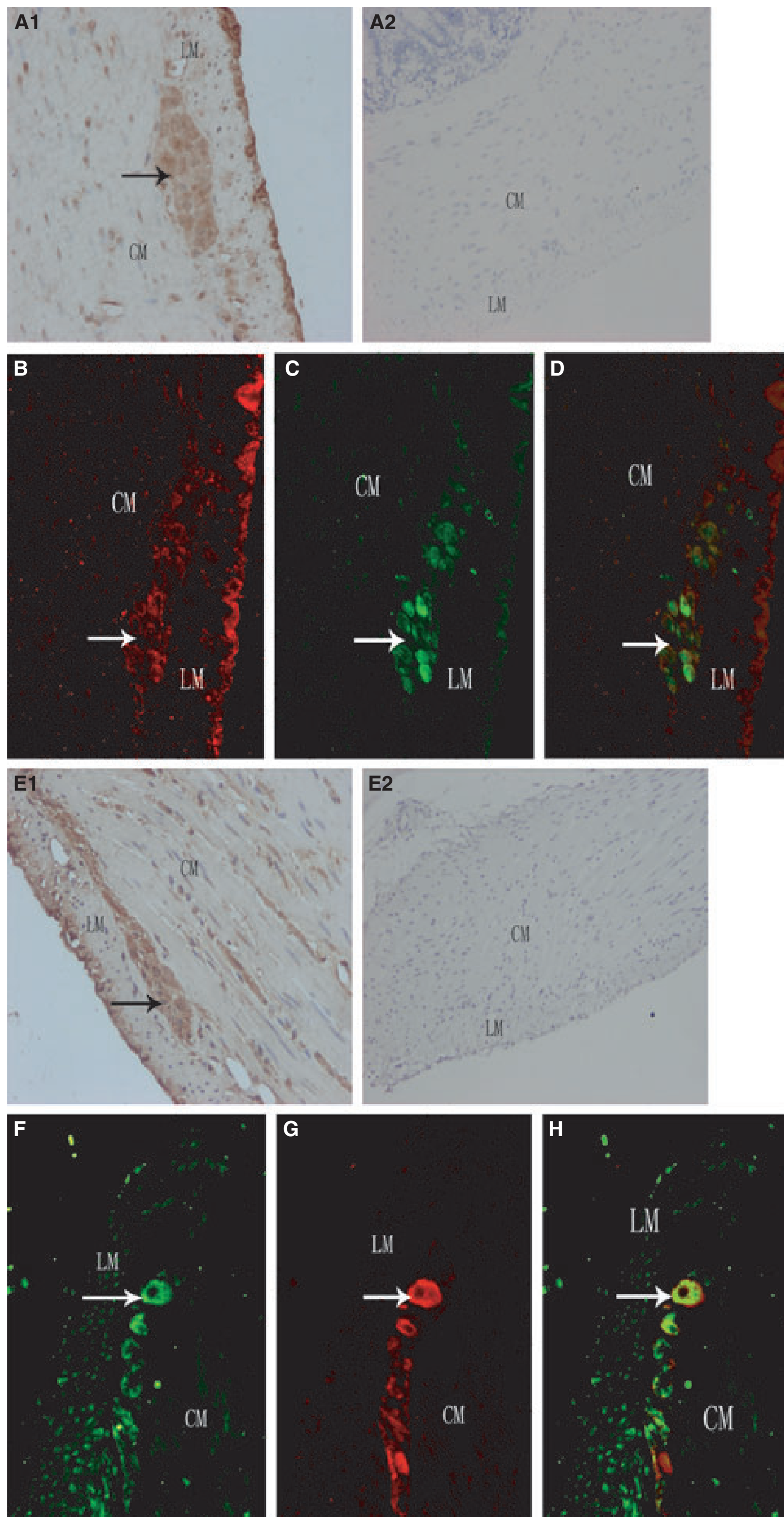

Figure 7 Location of V_1 receptors (V_1Rs) and iNOS in the colon of rats by immunohistochemical and immunofluorescence staining. (A1) The immunohistochemical staining for V_1Rs performed on paraffin sections obtained from proximal colons (200×). V_1R-positive cells were seen in myenteric plexus. (A2) The negative control, in which the primary antibody was replaced by PBS (100×). (B) (200×) and (C) (200×) The immunofluorescence staining with V_1R (red) and NeuN (green) in the colon, and (D) (200×) The merging of the (B) and (C). V_1Rs and NeuNs were co-localized in the myenteric plexus. (E1) (200×) The immunohistochemical staining for iNOS performed on paraffin sections obtained from proximal colons treated with AVP (10^{-7} mol L^{-1}). Inducible NOS-positive cells were located in myenteric plexus. (E2) (100×) The negative control, in which the primary antibody was replaced by PBS. The spatial relationship between V_1R (green) and iNOS (red) were detected by immunohistochemical staining. (H) (200×) The merging of the (F) (immunofluorescence staining of V_1Rs, 200×) and (G) (immunofluorescence staining of iNOS, 200×). It is clear that both V_1Rs and iNOS were expressed in myenteric plexus in colon. CM, circular muscle; LM, longitudinal muscle; the arrows indicate immunopositive cells.

early upregulation of iNOS during the hemorrhagic shock contributed to the inflammatory response in the gut.[46]

Nitric oxide is known to be the main relaxant of the GI tract.[47] Inducible NOS is an inducible member of the three NOS. In a recent study, we found that iNOS

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in myenteric plexus mediated the inhibition of colonic contraction following ethanol administration.[29] In this study, iNOS expression was upregulated and the content of NO increased in the colon following treatment with AVP. Both of L-NAME and SMT reversed the inhibitory effect of AVP on the contraction of CM. So it is clear that the inhibition of CM contraction following AVP administration was mainly mediated by upregulation of iNOS expression and release of NO.

The expression of iNOS is tightly regulated by both transcriptional and post-transcriptional pathways[48] and NF-κB seems to be a central target in the transcriptional pathway.[49] Although it is widely recognized that the process of iNOS synthesis was very complicated, the reports about the time that is necessary for the iNOS induction were controversial, from minutes[50,51] to hours.[28] This controversy might be attributed to the differences of the cell types and species, and most probably, the different mechanisms underlying the iNOS expression regulation.[48] In this study, we found that the expression of iNOS was upregulated at 5 min following AVP administration, and this effect was inhibited by PDTC, the inhibitor of NF-κB. So it might be possible that the early upregulation of iNOS at 5 min following AVP administration is through the NF-κB dependent transcriptional pathway, but the post-transcriptional pathways could not be definitely excluded.

Another interesting finding of this study is that the inhibitory effect of AVP, although partly attributed to the induction of iNOS, was transitory. The contraction of the colonic muscle strips reached the nadir at 3–5 min following AVP administration and returned to normal at about 15 min. This phenomenon is inconsistent with the effect of iNOS, whose activity is not dependent on the intracellular Ca^{2+} and would produce NO as long as substrate available once induced. This might be because of the initiation of the downregulation of iNOS activity through the post-transcriptional pathways,[48] although the mechanism remains to be clearly elucidated. Because active *iNOS* gene was present in the myenteric neurons in basal conditions,[52] the amount and activity of this protein might be more tightly controlled than in other kinds of cells.

The present study indicated that iNOS was located at the myenteric plexus in colon by immunohistochemistry. TTX reversed the inhibitory effect of AVP on the contraction of the colonic muscle strips. So it is clear that the cells in ENS released NO following AVP administration and mediated the relaxant effect of AVP on CM. Immunostaining and immunofluorescence study further supported the results above by indicating that both V_1Rs and iNOS were expressed in the myenteric plexus. This data is consistent with our recent report, which indicated that V_1Rs was localized in the neurons of myenteric plexus on stomach in rat.[27]

The decrease in the contraction of the colonic CM following AVP administration was partly reversed by the pretreatment of iNOS or nNOS inhibitor. So besides iNOS, nNOS was also involved in the effect of AVP on the inhibition of colonic traction. The mechanism underlying this finding is worth investigating in the future.

Nitric oxide is the primary neurotransmitter of inhibitory motoneurons in ENS[53] and exerts tonic inhibition on intestinal motility in child.[54] In this study, we found that both TTX and L-NAME excited CM contraction. This result is consistent with our previous reports[29] and indicated that the tonic inhibitory effect of ENS on colonic contraction might be mediated by NOS motoneuron in ENS. Although both NPLA and SMT reversed the inhibitory effect of AVP on CM contraction, the colonic motility was excited by NPLA but not by SMT. So it is possible that NO produced from NF-κB–iNOS played a predominant effect on AVP induced inhibition on CM contraction, but that from nNOS might play a physiological and tonic control on colonic contraction. The tonic inhibition of ENS on colonic contraction might be through nNOS–NO pathway.

In conclusion, in this study, we found that exogenous AVP inhibited the contraction of CM in rat colon through production of NO via activation of two pathways, including NF-κB–iNOS and nNOS, in myenteric plexus. Because AVP was widely used in the treatment of critical illness with hypotension, these results might provide meaningful information for avoiding its side effect on gut motility.

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AUTHOR CONTRIBUTION

HJ, JQ, MF, and FW performed the experiments; KL provided the technical support; JL and CL organized the project; HJ and CL wrote the manuscript; CL was the principle investigator.

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