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Pre- and Post-synaptic Effects of Botulinum Toxin A on Submandibular Glands

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Abstract

Intraglandular injection of botulinum toxin type A (BoNT/A) is an effective treatment for sialorrhea. Despite numerous experimental and clinical studies on inhibition of saliva section by BoNT/A, the proteolysis of synaptosomal-associated protein 25 (SNAP-25) following BoNT/A treatment has not yet been confirmed in the salivary gland after injection of BoNT/A. More important, it is not known whether BoNT/A exerts a direct effect in acinar cells. Here, we show that injection of BoNT/A into the rat submandibular gland (SMG) decreased salivary flow in a dose-dependent manner; the inhibitory effect lasted at least 4 wk, and salivary flow recovered to normal levels by 12 wk. During the inhibitory period, SMG neurons and synapses expressed lower levels of full-length SNAP-25, and cleavage of SNAP-25 was observed, as indicated by detection of reduced molecular weight SNAP-25 using Western blotting. In addition, the water channel aquaporin 5 (AQP5) was downregulated and abnormally distributed in rat SMG after injection of BoNT/A. The direct effects of BoNT/A on AQP5 expression and distribution were assessed in vitro to exclude the influence of BoNT/A-induced inhibitory neurotransmission. In stable *GFP-AQP5*–transfected SMG-C6 cells, treatment with BoNT/A reduced the cell surface protein level of AQP5 in a dose- and time-dependent manner without affecting total AQP5 protein expression. Cell surface biotinylation and immunofluorescence demonstrated translocation of AQP5 from the membrane to the cytoplasm, which was confirmed by decreased levels of AQP5 protein in the membrane fraction and increased levels in the cytoplasmic fraction, suggestive of AQP5 redistribution. Taken together, these results indicated that BoNT/A reversibly decreased saliva secretion in rat SMGs through not only the presynaptic SNAP-25 cleavage but also the postsynaptic AQP5 redistribution. These data provide the first evidence for a direct effect of BoNT/A on the salivary gland.

Keywords: sialorrhea, secretion, aquaporin 5, synaptosomal-associated protein 25, proteolysis, transfection

Introduction

Saliva is critical for maintaining a stable oral environment and lubrication, digestion, and oral mucosal immunity (Humphrey and Williamson 2001). Sialorrhea is commonly observed in neurological and systemic conditions, such as infant cerebral palsy, Parkinson disease, and amyotrophic lateral sclerosis (Suskind and Tilton 2002; Young et al. 2011; Srivanitchapoom et al. 2014). Moreover, more than 40% of patients who undergo submandibular gland (SMG) transplantation to relieve severe dry eye syndrome experience epiphora caused by excess saliva-tear production 3 to 6 mo posttransplantation (Yu et al. 2004; Qin et al. 2013). Sialorrhea and epiphora are both caused by salivary gland hypersecretion and induce physical and psychosocial complications that significantly affect quality of life (Kalf et al. 2007). The most common therapy is anticholinergic drugs, which are effective but of limited use due to side effects (Hockstein et al. 2004). Therefore, it is essential to seek an effective therapeutic strategy to treat hypersalivation and epiphora after autologous SMG transplantation.

Intraglandular injection of botulinum toxin A (BoNT/A) can effectively treat sialorrhea (Lipp et al. 2003; Jongerius et al. 2004). BoNT/A is a metalloprotease that blocks synaptic transmission via cleaving synaptosomal-associated protein 25 (SNAP-25), a component of the neural soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors (SNAREs) that mediate exocytotic release of neurotransmitters at synapses

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(Schiavo et al. 2000; Rossetto et al. 2006; Tafoya et al. 2006). The classical mechanism for BoNT/A is to inhibit acetylcholine (ACh) release from cholinergic nerves. Recently, several studies suggested the possibility of other mechanisms of action for BoNT/A, as it can also directly inhibit antral muscular contractility (Park et al. 2012), and its mechanism of action in the urinary bladder also involves urothelial sites of action (Hanna-Mitchell et al. 2015). These results indicate BoNT/A has direct inhibitory effects on muscle and bladder. However, SNAP-25 proteolysis has not been studied in salivary gland injected with BoNT/A, and more important, it is not known whether BoNT/A has direct effects on the salivary gland, in addition to blocking the release of ACh from nerves.

The apical plasma membrane water channel aquaporin-5 (AQP5) plays an important role in transporting water across the apical surface of the salivary gland epithelia (Ishikawa et al. 2006a; Ishikawa et al. 2006b). Proper expression and subcellular localization of AQP5 are required to maintain water homeostasis (Krane et al. 2001; Steinfeld et al. 2001). ACh acts on M3 muscarinic acetylcholine receptor (M3R) to induce AQP5 translocation in rat parotid glands (Ishikawa et al. 1998), whereas cholinergic denervation suppresses AQP5 expression in the rat SMG (Xiang et al. 2006), suggesting parasympathetic innervation is essential for the expression and distribution of AQP5 in salivary glands. We previously showed BoNT/A decreased AQP5 messenger RNA (mRNA) expression and membrane distribution in the rabbit SMG, and this process was linked to BoNT/A-induced chemical denervation (Shan et al. 2013). However, based on the available data, the possibility that BoNT/A exerts a direct effect on AQP5 expression and distribution in acinar cells cannot be excluded.

In the present study, we investigated the precise effects of BoNT/A on saliva secretion and further confirmed SNAP-25 proteolysis in BoNT/A-treated SMGs. Most important, we elucidated the direct effects of BoNT/A on AQP5 expression and distribution in cultured acinar cells.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (230 to 250 g) were supplied by the Laboratory Animal Service Center, Peking University Health Science Center. All studies involving animals are reported in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al. 2010). All procedures were approved by the Ethics Committee for Animal Research, Peking University Health Science Centre, and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

BoNT/A Injection

Rats were randomly divided into control and BoNT/A groups. Rats were anesthetized with an intramuscular injection of 100 $mg \cdot kg^{-1}$ ketamine in combination with 5 $mg \cdot kg^{-1}$ xylazine. The SMGs were exposed by a median cervical incision. BoNT/A was intraglandularly injected into SMGs. The left gland was injected with 1, 3, 5, and 10 U BoNT/A (Lanzhou Biochemical Co., Gansu, China) reconstituted in 0.1 mL physiological saline for the BoNT/A groups (6 rats per dosage), whereas the left gland was injected with 0.1 mL physiological saline for the control group (6 rats). At postoperative week 2, salivary flow was measured. To observe the long-term effect of BoNT/A on SMGs, the rats injected with 3 U BoNT/A were randomly divided into the 5 groups. At postoperative weeks 1, 2, 4, 12, and 24, salivary flow was measured (6 to 8 rats per time point). The SMGs were removed for further investigation.

Measurement of Saliva Secretion

Salivary flow was measured in resting, conscious rats between 9:00 and 10:00 a.m. The length of filter paper (35 mm × 5 mm) moistened within 5 min by saliva produced by the SMG and released into a cannula inserted into Wharton's duct was assessed using Schirmer's test, as previously described (Lopez-Jornet et al. 2006).

Western Blotting

Crude protein was isolated from the SMGs and quantified as described previously (Zhang et al. 2006). Proteins (40 μg) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels; transferred to polyvinylidene difluoride membranes; blocked with 5% nonfat milk; probed with primary antibodies against the C-terminus of SNAP-25 (Synaptic Systems, Göttingen, Germany), N-terminus of SNAP-25 (BD Biosciences, San Jose, CA, USA), AQP5 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or Na^+-K^+-ATPase (Bioworld Technology, Minneapolis, MN, USA) at 4 °C overnight; and incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies, and immunoreactive bands were visualized by enhanced chemiluminescence. Actin (Santa Cruz Biotechnology) was used as a loading control.

Immunohistochemistry

Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 4-μm-thick sections were subjected to staining following standard protocols using a primary antibody against the C-terminus of SNAP-25 (1:100; at 4 °C overnight) and HRP-conjugated secondary antibodies. Sites of immunoreactivity were visualized using 3,3′-diaminobenzidine tetrahydrochloride. Staining was evaluated semi-quantitatively, as previously described (Ellies et al. 2000).

Immunofluorescence

Frozen sections of SMGs (5-μm thick) were fixed in cold acetone for 15 min, blocked with 10% goat serum, and immunostained with primary antibodies against AQP5 (1:100), and C-terminus of SNAP-25 (1:100) overnight at 4 °C followed by

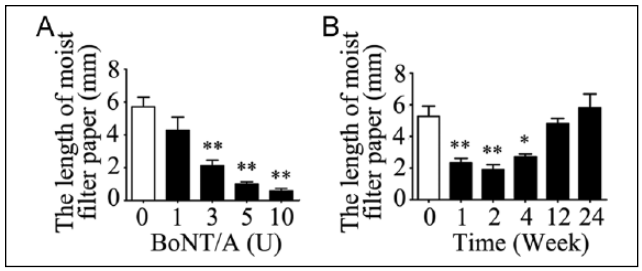

Figure 1. Effect of botulinum toxin type A (BoNT/A) on rat submandibular gland (SMG) salivary flow. BoNT/A was injected into the rat SMG, and salivary flow was assessed by measuring the length of filter paper (35 × 5 mm) moistened in 5 min by a cannula inserted into Wharton's duct. (**A**) Dose-response curve for BoNT/A at 2 wk postinjection. Values are mean ± SEM of 6 independent animals. $^{**}P < 0.01$ vs. control animals (0 U). (**B**) Time-response curve for BoNT/A (30 U·mL^{-1}, 0.1 mL). Values are mean ± SEM of 6 independent animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (0 wk).

FITC-labeled and DyLight 594-labeled secondary antibodies (Molecular Probes, OR, USA). The SMG-C6 cells were plated on coverslips and fixed in 4% paraformaldehyde for 10 min. Following incubation with 1% bovine serum albumin (BSA) for 30 min at room temperature, the cells were stained with rhodamine-labeled phalloidin (Life Technologies, Carlsbad, CA, USA) and AQP5 antibody for 2 h at 37 °C. Nuclei were stained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich, St. Louis, MO, USA). Fluorescence images were captured by confocal microscopy (Leica TCS SP8, Frankfurt, Germany). Quantitative assessment of fluorescence intensity was performed using ImageJ 1.44 software (National Institutes of Health, Bethesda, MD, USA) and the Leica 550IW system for 9 randomly chosen acini per section.

Cell Culture and Transfection

The rat SMG cell line SMG-C6 was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) as described previously (Quissell et al. 1997). The AQP5 complementary DNA (cDNA) was constructed in pCMV6-AC-GFP vectors and synthesized by OriGene Technologies (Rockville, MD, USA). Cells were transfected with cDNA of green fluorescent protein (GFP)–AQP5 by use of MegeTran 1.0 (OriGene) according to the manufacturer's instructions. Selective medium containing G418 (800 μg·mL^{-1}) was used to obtain cell clones stably expressing GFP-AQP5.

Cell Surface Biotinylation and Western Blotting

Cell surface biotinylation was carried out using the Pierce Cell Surface Protein Isolation Kit (Pierce, Rockford, IL, USA). Briefly, SMG-C6 cells were washed and incubated with cell-impermeable sulfo-NHS-SS-biotin at 4 °C for 30 min, excess biotin was quenched, the cells were lysed and centrifuged, the cleared supernatant was reacted with NeutrAvidin gel slurry, and the immobilized biotinylated surface proteins were washed, eluted using sample buffer containing dithiothreitol (DTT), and analyzed by Western blotting.

Cell Surface Biotinylation and Immunocytofluorescence

Confluent monolayer of cells were grown in Transwell chambers (polycarbonate membrane, filter pore size: 0.4 μm; Corning Costar, Cambridge, MA, USA) for 5 to 7 d. Cells were washed, fixed with 4% paraformaldehyde for 20 min, incubated with 50 mM NH_4Cl for 15 min, blocked with 1% BSA for 1 h at room temperature, reacted with cell-impermeable sulfo-NHS-LC-biotin at room temperature for 40 min, incubated with 50 mM NH_4Cl to quench excess biotinylation reagent, and incubated with Cy3-conjugated streptavidin (1:200; Sigma-Aldrich), and fluorescent images were captured using confocal microscopy.

Preparation of Cytoplasm and Membrane Fractions

Cytoplasm and membrane fractions were prepared as described previously (Kawedia et al. 2008). Briefly, cells were lysed in ice-cold 0.5% Triton X-100, 100 mM NaCl, 10 mM Tris-HCl (pH 7.4), and 300 mM sucrose for 20 min at 4 °C. Then the detergent-soluble (cytoplasm fraction) protein was removed and the remaining insoluble residue was again lysed (membrane fraction) in 50 mM Tris (pH 6.8), 2% SDS, and 10% glycerol.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons with GraphPad Prism software (GraphPad Prism, La Jolla, CA, USA). Differences were considered statistically significant if $P < 0.05$.

Results

BoNT/A Decreases Rat SMG Salivary Flow

Basal SMG salivary flow for control rats (5.27 ± 0.65 mm) was not significantly different from the BoNT/A group before injection (5.41 ± 0.97 mm). Two weeks postinjection of BoNT/A, salivary flow reduced significantly in a dose-dependent manner (Fig. 1A). In addition, salivary flow decreased markedly at 1 and 2 wk (both $P < 0.01$) after injection of BoNT/A (30 U·mL^{-1}, 0.1 mL), gradually recovered at 4 wk ($P < 0.05$), and was not significantly different from the control group at 12 and 24 wk (Fig. 1B).

BoNT/A Cleaves SNAP-25 in the Rat SMG

The effects of BoNT/A on SNAP-25 expression and distribution were examined using an anti–SNAP-25 C-terminus antibody that mainly recognizes full-length SNAP-25. SNAP-25 immunostaining was localized around the gland parenchyma but absent within acini and ducts (Fig. 2A). SNAP-25 was observed as discontinuous linear or punctiform expression in the control SMGs. In glands injected with BoNT/A (30 U·mL^{-1}, 0.1 mL), both the area and intensity of SNAP-25 immunostaining were

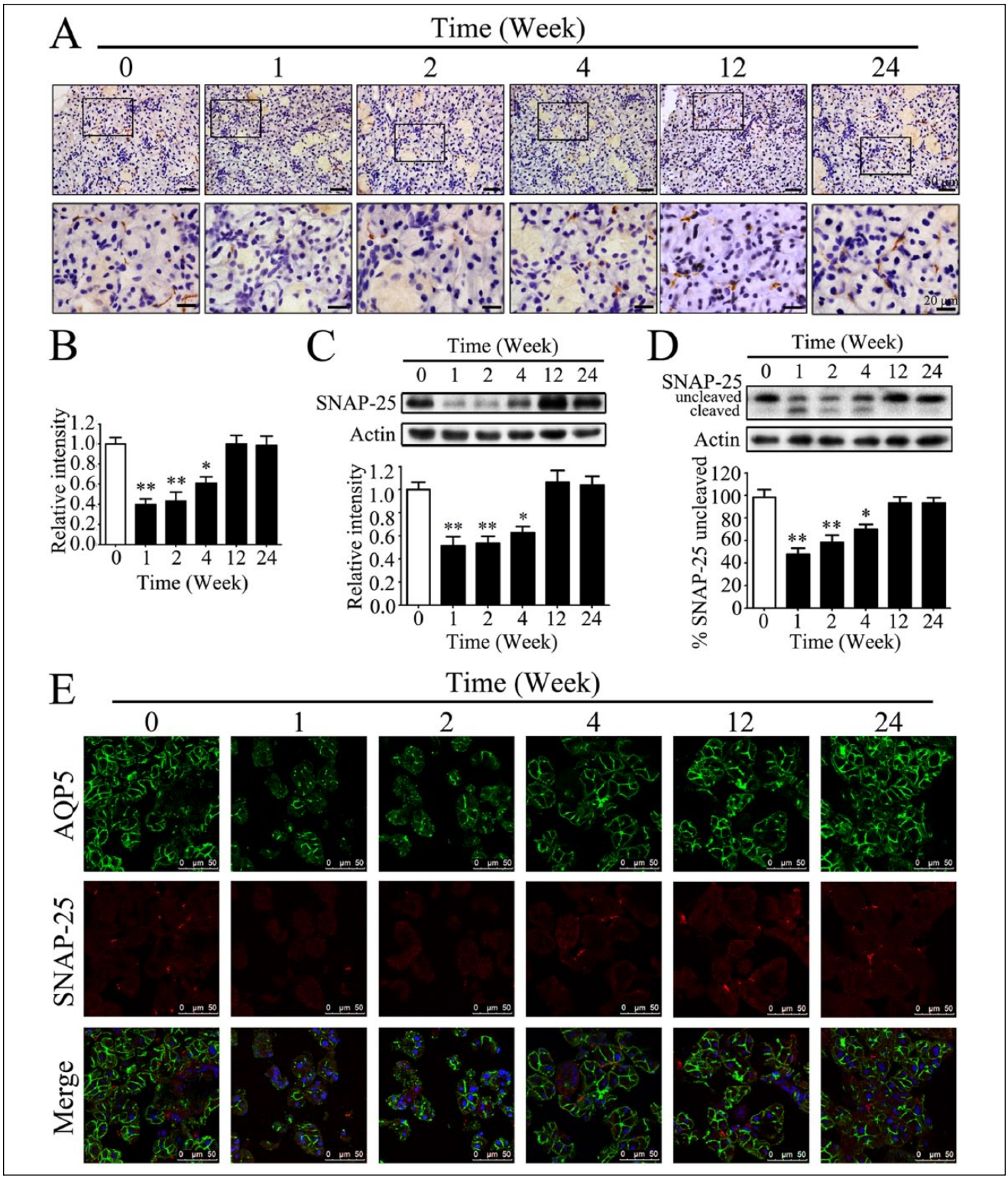

Figure 2. Botulinum toxin type A (BoNT/A) cleaves synaptosomal-associated protein 25 (SNAP-25) in the rat submandibular gland (SMG). (**A**) Immunohistochemical staining of SNAP-25 in control (0 wk) and BoNT/A (30 $U \cdot mL^{-1}$, 0.1 mL)–treated SMGs, with the boxed areas in the top panels (scale bar, 50 μm) presented at higher magnification in the bottom panels (scale bar, 20 μm). (**B**) Quantitative assessment of relative SNAP-25 staining intensity at different time points after injection in 9 randomly chosen fields from each section. (**C**) Representative SNAP-25 immunoblotting using an antibody specific for the C-terminus of SNAP-25; staining intensity is expressed relative to actin. (**D**) Western blot analysis and quantification of SNAP-25 cleavage (average %). Cleavage of SNAP-25 was detected by immunoblotting using an antibody specific for the N-terminus of SNAP-25. The presence of 2 bands in blots from BoNT/A (30 $U \cdot mL^{-1}$, 0.1 mL)–treated glands indicates BoNT/A-induced cleavage, with the lower band representing cleaved SNAP-25. (**E**) Costaining of SNAP-25 with AQP5 in control and BoNT/A (30 $U \cdot mL^{-1}$, 0.1 mL)–treated SMGs. Scale bar, 50 μm. All values are mean ± SEM of 6 animals. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control (0 wk).

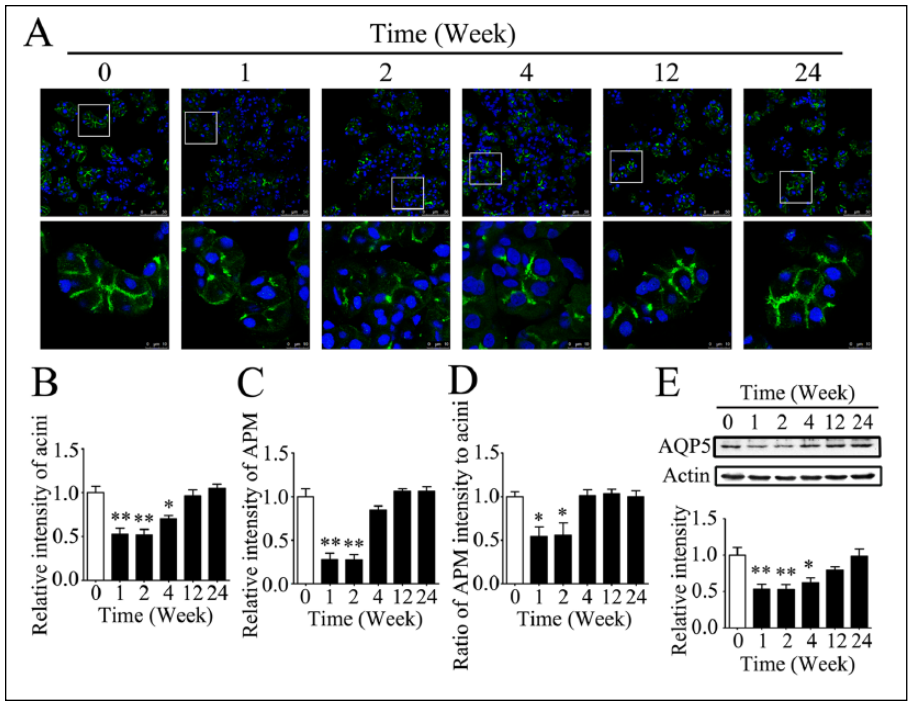

Figure 3. Botulinum toxin type A (BoNT/A) decreases expression of aquaporin 5 (AQP5) in the submandibular gland (SMG). (**A**) Immunofluorescent localization of AQP5 (green) in control and BoNT/A (30 U·mL^{-1}, 0.1 mL)–treated SMGs by confocal microscopy. The boxed areas in the top panels (scale bar, 50 μm) are presented at higher magnification in the bottom panels (scale bar, 10 μm). Nuclei were stained with DAPI (blue). (**B**) Total fluorescence intensity for AQP5 in each acinus. (**C**) AQP5 fluorescence intensity in the apical and lateral membrane (APM) of acinar cells. (**D**) Ratio of AQP5 fluorescence intensity in the apical and lateral membranes of acinar cells relative to total AQP5 fluorescence intensity. (**E**) Western blot analysis of AQP5 expression in control and BoNT/A (30 U·mL^{-1}, 0.1 mL)–treated glands; expression is normalized to β-actin. Values are mean ± SEM of 6 animals. *P < 0.05 and **P < 0.01 vs. control (0 wk).

significantly decreased within 2 wk postinjection and then recovered slowly after 4 wk. Semi-quantitative assessment demonstrated the SNAP-25 staining intensity decreased within 4 wk and returned to normal at 12 wk postinjection of BoNT/A (Fig. 2B). Similar results were obtained in immunoblotting using the same antibody (Fig. 2C).

Furthermore, we confirmed proteolysis of SNAP-25 in the BoNT/A-treated SMG by immunoblotting using an anti–SNAP-25 N-terminus antibody that recognizes both full-length and truncated SNAP-25 (Fig. 2D). SNAP-25 was detected as a single uncleaved band in control SMGs, whereas a second lower-molecular-weight band (cleaved SNAP-25) was detected at 1, 2, and 4 wk in SMGs injected with BoNT/A (30 U·mL^{-1}, 0.1 mL). The proportion of uncleaved SNAP-25 at 1, 2, and 4 wk postinjection of BoNT/A was 47.88% ± 5.34% (P < 0.01), 58.39% ± 6.35% (P < 0.01), and 70.02% ± 4.49% (P < 0.05), respectively. These results confirm BoNT/A cleaves SNAP-25 in the SMG.

To further characterize the potential correlation of SNAP-25 and AQP5, we conducted the dual staining of SNAP-25 with AQP5. Results showed that SNAP-25 localized around the acini and duct, without colocalization with AQP5 (Fig. 2E). In glands injected with BoNT/A (30 U·mL^{-1}, 0.1 mL), both the SNAP-25 staining and AQP5 staining markedly decreased at 1 and 2 wk, slightly recovered at 4 wk, and returned to nearly normal levels at 12 wk postinjection.

BoNT/A Decreases Expression and Membrane Distribution of AQP5 in the SMG

Immunofluorescent analysis demonstrated AQP5 was mainly localized to the apical and lateral plasma membranes of the acinar cells in control SMGs (Fig. 3A). In animals injected with BoNT/A (30 U·mL^{-1}, 0.1 mL), AQP5 staining markedly decreased at 1 and 2 wk, slightly recovered at 4 wk, and returned to nearly normal levels at 12 wk postinjection. Morphometric studies revealed that, compared with control SMGs, the mean AQP5 fluorescence intensity in acini declined markedly at weeks 1 and 2 (P < 0.01), recovered slightly at 4 wk (P < 0.05), and returned to control levels at 12 wk postinjection (Fig. 3B). Compared with control SMGs, the intensity of AQP5 staining on the apical and lateral plasma membranes of acinar cells decreased to 27.97% ± 7.53% (P < 0.01) and 27.68% ± 6.30% (P < 0.01) at 1 and 2 wk postinjection, respectively (Fig. 3C). The ratios of AQP5 fluorescence intensity in the apical and lateral membranes of acinar cells relative to total AQP5 fluorescence intensity were reduced to 54.48% ± 10.74% (P < 0.05) and 55.85% ± 14.29% (P < 0.05) at 1 and 2 wk postinjection, respectively (Fig. 3D). Western blotting confirmed AQP5 protein expression reduced within 4 wk and returned to similar levels as control SMGs at 24 wk postinjection of BoNT/A (Fig. 3E).

BoNT/A Promotes Redistribution of AQP5 in AQP5-Transfected SMG-C6 Cells

To clarify whether BoNT/A exerts a direct effect on AQP5 in acinar cells, stable *GFP-AQP5*–expressing SMG-C6 cells were established. AQP5 was costained with F-actin, and *z*-stacks of the cell monolayers were captured between the apical and basal region at 8 different *z*-steps. AQP5 was predominantly localized to the cell membrane, with faint, diffuse cytoplasmic distribution. F-actin mainly localized at the basal region (Fig. 4A). BoNT/A (25, 50, 100, and 200 U·mL^{-1}) did not induce apoptosis in SMG-C6 cells (Appendix Figure). In cells treated with 50 U·mL^{-1} BoNT/A for 12 h, AQP5 staining

reduced at the membrane, and AQP5-bearing vesicles appeared in the cytoplasm close to the cell membrane. Treatment with higher doses of BoNT/A (100 and 200 U·mL^{-1}) induced a higher degree of AQP5 redistribution, as indicated by a greater reduction in plasma membrane AQP5 staining and increased number of cytoplasmic vesicles with enhanced fluorescence. The distribution of F-actin did not change obviously, thus excluding the possibility of a nonspecific toxic effect exerted by BoNT/A (Fig. 4B). Morphometric studies revealed that AQP5 fluorescence intensity decreased at the membrane and increased in the cytoplasm after 100 and 200 U·mL^{-1} BoNT/A treatment (Fig. 4C). The protein level of cell surface AQP5, as assessed using biotin labeling, decreased significantly as the BoNT/A concentration increased. A typical dose-response curve using total AQP5 expression as an internal control is illustrated in Figure 4D. These results indicate BoNT/A decreased cell surface AQP5 in a dose-dependent manner.

We also examined the time-dependent effects of 100 U·mL^{-1} BoNT/A on AQP5 expression and distribution. At 6 h, AQP5 membrane staining clearly reduced, while the number of cytoplasmic AQP5-bearing vesicles significantly increased. Redistribution of AQP5 was more obvious after 12 and 24 h (Fig. 5A). Morphometric studies revealed that AQP5 fluorescence intensity decreased at the membrane and increased in the cytoplasm after BoNT/A treatment (Fig. 5B). In addition, membrane AQP5 decreased in a time-dependent manner following BoNT/A treatment (Fig. 5C), whereas total GFP-AQP5 remained unchanged (Fig. 5D). Western blotting confirmed a significant decrease of AQP5 in the membrane fraction and corresponding increase in the cytoplasmic fraction in cells treated with BoNT/A (Fig. 5E, F). These results clearly indicate that BoNT/A induced translocation of AQP5 from the membrane to the cytoplasm.

SMG-C6 cells provide a well-defined polarized cell model during cultivation on a polycarbonate

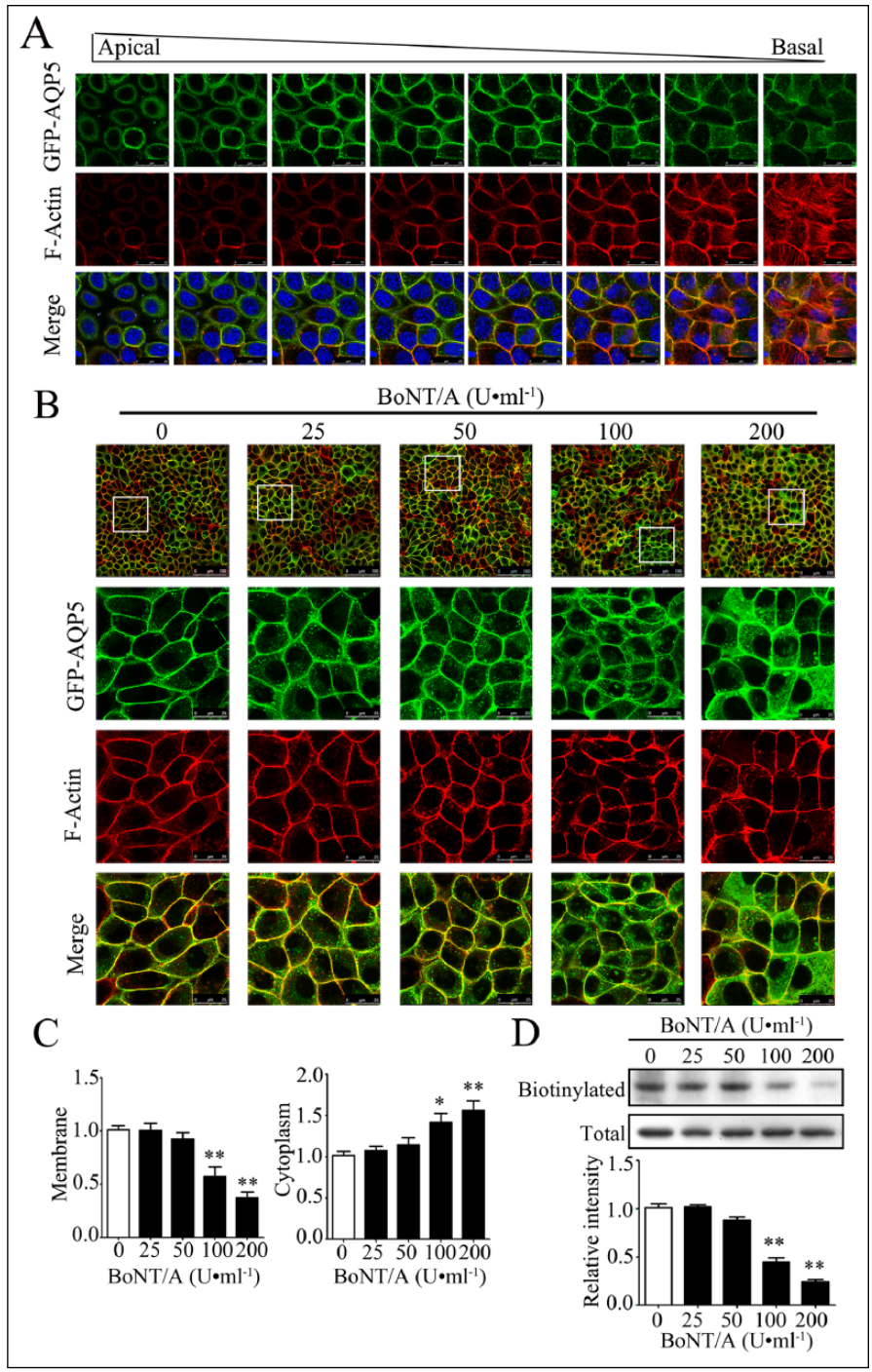

Figure 4. Botulinum toxin type A (BoNT/A) promotes redistribution of aquaporin 5 (AQP5) in a dose-dependent manner in AQP5-transfected submandibular gland (SMG)–C6 cells. (**A**) Fluorescent confocal micrography images of AQP5 (green) and F-actin (rhodamine-labeled phalloidin; red). A *z*-stack of 8 confocal fluorescence micrograph images (*x*-*y* plane) captured at 0.5-μm increments is presented. Apical, apical membrane; basal, basal membrane. Scale bar, 25 μm. (**B**) Dose-effect curve for BoNT/A on green fluorescent protein (GFP)–AQP5 distribution. Cells were treated with different doses (25, 50, 100, or 200 U·mL^{-1}) of BoNT/A for 12 h. Cells expressing GFP-AQP5 (green) were costained with rhodamine-labeled phalloidin (red). Nuclei were stained with DAPI (blue). Images are presented by both lower (scale bars, 100 μm) and higher magnifications (scale bars, 25 μm). (**C**) Quantitative assessment of relative AQP5 staining intensity at the membrane and in the cytoplasm in Figure 4B. (**D**) Dose-effect for BoNT/A on the level of cell surface AQP5. Following biotinylation of the apical cell surface, solubilized membrane proteins were absorbed onto streptavidin-agarose, precipitated, and subjected to Western blotting using an anti-AQP5 antibody. Values are mean ± SEM of 4 independent experiments. *P < 0.05 and **P < 0.01 vs. control.

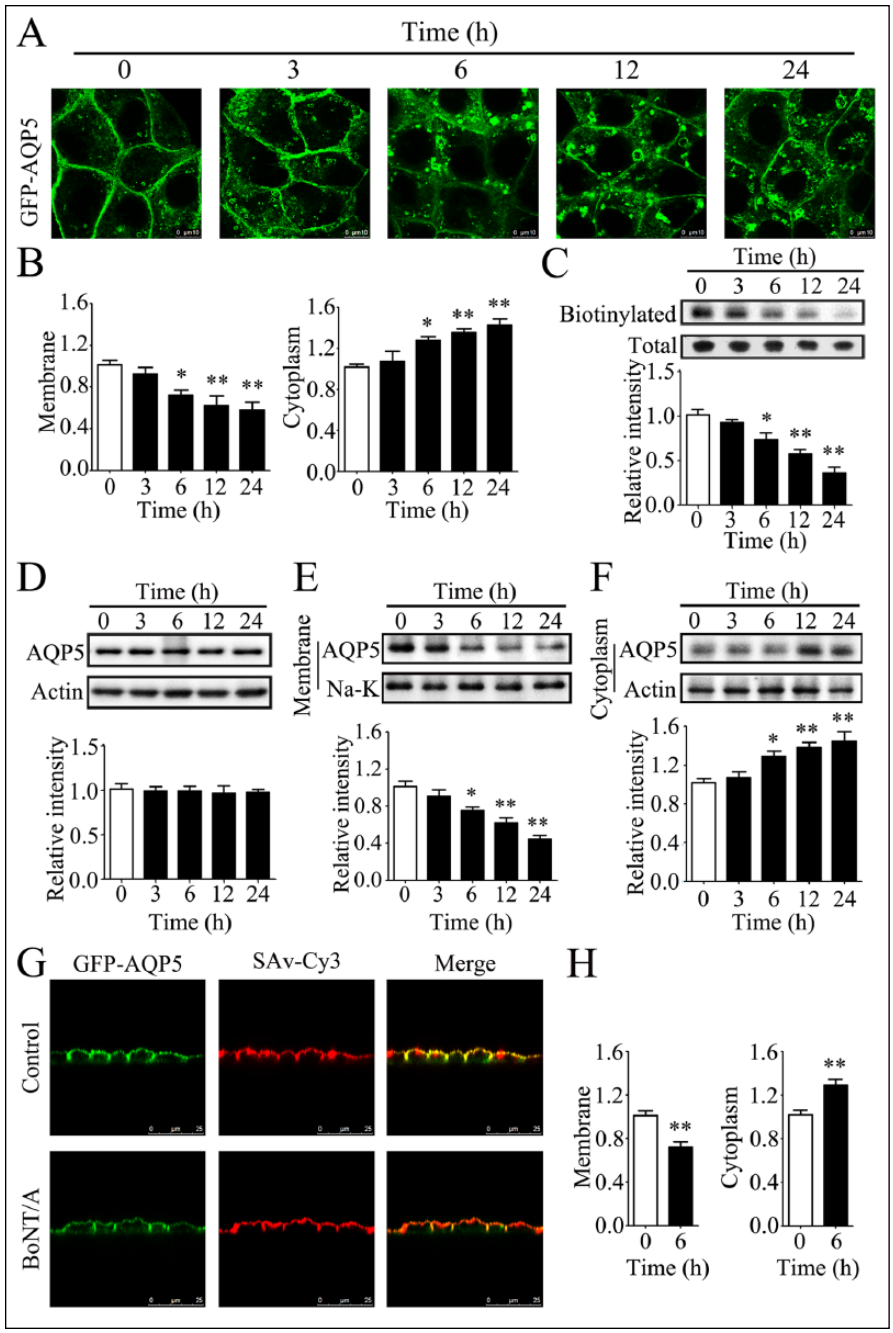

Figure 5. Botulinum toxin type A (BoNT/A) promotes redistribution aquaporin 5 (AQP5) in a time-dependent manner in AQP5-transfected submandibular gland (SMG)–C6 cells. (**A**) Time course of the effect of BoNT/A on the distribution of GFP-AQP5. SMG-C6 cells stably transfected with GFP-AQP5 were treated with 100 U·mL^{-1} BoNT/A for 3, 6, 12, or 24 h and observed by fluorescent confocal micrography; nuclei were stained with DAPI (blue). Scale bar, 10 µm. (**B**) Quantitative assessment of relative AQP5 staining intensity at the membrane and in the cytoplasm in Figure 5A. (**C**) Time-effect curve for the effect of BoNT/A on the level of cell surface AQP5. Following biotinylation of the apical cell surface, solubilized membrane proteins were absorbed to streptavidin-agarose, precipitated, and subjected to Western blotting using an anti-AQP5 antibody. (**D**) Western blot analysis of the effect of BoNT/A on the total level of AQP5 protein. (**E**) Effect of BoNT/A on the level of GFP-AQP5 in the membrane fraction. (**F**) Effect of BoNT/A on the level of GFP-AQP5 in the cytoplasmic fraction. (**G**) Vertical projections of z-stack images of SMG-C6 GFP-AQP5–transfected cell monolayers cultured on polycarbonate membranes to visualize the colocalization of biotinylated cell-surface proteins (biotin/Cy3-conjugated streptavidin; red) and AQP5 (green) in apical cell membranes, which appears yellow in the merged images. Representative images of cells before and after BoNT/A treatment (100 U·mL^{-1}, 6 h) are shown. Na-K, Na^+/K^+-ATPase. Scale bar, 25 µm. (**H**) Quantitative assessment of relative AQP5 staining intensity at the membrane and in the cytoplasm in Figure 5G. Values are mean ± SEM of 4 independent experiments. *$P < 0.05$ and **$P < 0.01$ vs. control.

membrane (Castro et al. 2000). In control cells, confocal fluorescence micrograph (*x-z* plane) showed that GFP-AQP5 (green) co-localized with Cy3-conjugated streptavidin (red), binding with biotinylated cell-surface protein, at apical cell membrane. The staining appeared yellow lines at apical cell membrane in the merged image (Fig. 5G). After BoNT/A (100 U·mL^{-1}) treatment for 6 h, most GFP-AQP5 proteins appeared in the cytoplasm, resulting in diminished colocalization with surface-labeled biotin. These results suggested that BoNT/A directly altered subcellular localization of GFP-AQP5 in acinar cells. Morphometric studies revealed that AQP5 fluorescence intensity decreased at the membrane and increased in the cytoplasm after 100 U·mL^{-1} BoNT/A treatment for 6 h (Fig. 5H).

Discussion

The present study demonstrates injection of BoNT/A decreased saliva secretion in a dose- and time-dependent manner in rat SMGs. The mechanism includes not only cleaving SNAP-25 at neuroglandular junctions but also decreasing the membrane distribution of AQP5 in SMGs. More important, we provided the evidence that BoNT/A directly modulated AQP5 distribution from the membrane to the cytoplasm in cultured SMG-C6 cells. Accordingly, these results provided new insights into how BoNT/A regulates the secretion of SMGs through the presynaptic and postsynaptic mechanisms.

Clinical studies reported BoNT/A decreases saliva secretion in patients, with the effects lasting several weeks to several months (Jongerius et al. 2004; Truong and Bhidayasiri 2008; Vashishta et al. 2013). However, these studies were limited by small numbers of participants or the absence of control groups. Therefore, additional well-designed, long-term experiments are required to precisely investigate the effects of BoNT/A on salivary flow. Here, we assessed the effect of BoNT/A on the salivary flow rate of the rat SMG at different time points. A significant secretoinhibitory effect was observed within 4 wk, and

salivary flow gradually recovered within 12 to 24 wk. This reversible inhibitory effect indicates injection of BoNT/A may be suitable for temporarily inhibiting salivation without permanently affecting the function of the salivary glands, for example, in patients with temporary epiphora after autologous SMG transplantation.

SNAP-25 is present on presynaptic membranes in neuronal cells, functions as a t-SNARE, and plays an important role in regulating SNARE-dependent exocytosis (McMahon and Sudhof 1995). We confirmed the presence of SNAP-25 in rat SMG and characterized its distribution. SNAP-25 was not expressed in acini or ducts, consistent with previous reports that SNAP-25 was undetectable in parotid acinar cells (Fujita-Yoshigaki et al. 1996; Takuma et al. 2000). SNAP-25 was reported to concentrate in synapses, transport vesicles, and the axonal membrane of nerves (Garcia et al. 1995; Tao-Cheng et al. 2000). Therefore, SNAP-25–positive staining showing as scatted lines and clusters around acini and ducts in SMGs can be identified as nerves and synapses, which form neuroglandular contacts. Moreover, we demonstrated cleavage of SNAP-25 in BoNT/A-treated SMGs using antibodies that bind different regions of SNAP-25. Our results suggest BoNT/A inhibited saliva secretion via chemical denervation by cleaving SNAP-25 at the neuroglandular junctions in the salivary gland. Nonetheless, the degree of secretion inhibition was greater than the extent of SNAP-25 cleavage, suggesting BoNT/A may also suppress saliva secretion via another mechanism.

The proper expression and correct subcellular localization of AQP5 are required to maintain salivary gland function (Krane et al. 2001). Parasympathetic innervation is essential for the expression and distribution of AQP5 in the salivary gland (Ishikawa et al. 2006a; Ishikawa et al. 2006b). We observed AQP5 localized to the apical and lateral plasma membrane of acinar cells in control SMGs, consistent with previous studies (Quissell et al. 1997; Ishikawa et al. 1998). In vivo, BoNT/A significantly reduced protein expression and membrane distribution of AQP5 and induced AQP5 redistribution as indicated by a reduction in the ratio of AQP5 in the apical and lateral membrane relative to total AQP5. We speculated that BoNT/A promoted cleavage of SNAP-25 and thereby induced chemical denervation, which altered AQP5 expression and distribution. In vitro, BoNT/A induced redistribution of AQP5 without affecting AQP5 protein expression, suggesting BoNT/A inhibited acinar cell function by inducing AQP5 redistribution, in addition to cleaving SNAP-25.

In conclusion, BoNT/A reversibly reduced saliva secretion in the rat SMG. The underlying mechanisms of action involved not only a presynaptic effect by which BoNT/A cleaved SNAP-25 at neuroglandular junctions but also a postsynaptic effect as BoNT/A directly induced redistribution of AQP5 in acinar cells. These findings improve our understanding of the mechanism by which BoNT/A reversibly suppresses saliva secretion and may provide new insights into future therapeutics for sialorrhea and epiphora after autologous SMG transplantation.

Author Contributions

H. Xu, contributed to data acquisition, analysis, and interpretation, drafted the manuscript; X.F. Shan, contributed to conception and design, critically revised the manuscript; X. Cong, contributed to design, critically revised the manuscript; N.Y. Yang, contributed to data acquisition and analysis, critically revised the manuscript; L.L. Wu, Y. Zhang, contributed to data analysis and interpretation, critically revised the manuscript; G.Y. Yu, Z.G. Cai, contributed to data analysis and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of work ensuring integrity and accuracy.

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