ORIGINAL ARTICLE

Carbachol improves secretion in the early phase after rabbit submandibular gland transplantation

L Shi[1], X Cong[2], Y Zhang[2], C Ding[2], QW Ding[1], FY Fu[2], LL Wu[2], GY Yu[1]

[1]*Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing;* [2]*Department of Physiology and Pathophysiology, Peking University Health Science Center and Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, China*

OBJECTIVES: To investigate the changes in the muscarinic receptor signaling pathway with submandibular gland (SMG) transplantation and whether carbachol improves secretion in transplanted SMGs.
MATERIALS AND METHODS: SMG autotransplantation was performed in a rabbit model. Carbachol (1 μM) was infused into the transplanted glands from postoperative day 1–7. The expression of the M1 and M3 muscarinic receptors, aquaporin-5 (AQP5), and phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2) was measured by RT-PCR, immunoblotting or immunofluorescence. The content of inositol 1, 4, 5-trisphosphate (IP_3) was measured by radioimmunoassay.
RESULTS: Salivary flow of the transplanted SMGs was decreased after transplantation. As well, the expressions of M1 and M3 receptors and their downstream signaling molecules, IP_3, p-ERK1/2 and AQP5, were all reduced. Atrophy of acinar cells was shown in transplanted glands. However, all these alterations were reversed after carbachol treatment for 7 days. Furthermore, carbachol directly increased the mRNA expression of AQP5 and phosphorylation of ERK1/2 in cultured neonatal rabbit SMG cells.
CONCLUSION: A lack of acetylcholine and downregulation of the muscarinic receptor signaling pathway is involved in the early hypofunction of transplanted SMGs. Carbachol treatment could be a new therapeutic strategy to improve secretion and prevent the obstruction of Wharton's duct in the early phase after SMG transplantation.
Oral Diseases (2010) **16**, 351–359

Correspondence: Li-Ling Wu, MD, Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing 100191, China. Tel: +86 10 82802403, Fax: +86 10 82802403, E-mail: pathophy@bjmu.edu.cn; Co-corresponding author: Guang-Yan Yu, MD, PhD, Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing 100081, P.R.China.
Tel: +86 10 62191099, Fax: +86 10 62173402, E-mail: gyyu@263.net

Introduction

Keratoconjunctivitis sicca is a common ophthalmological disease characterized by reduced or lack of tears and has serious complications, including corneal damage and even loss of sight (Sieg *et al*, 2000). Microvascular autotransplantation of submandibular glands (SMGs) into the temporal fossa with insertion of Wharton's duct into the upper conjunctival fornix provides a continuous, endogenous source of ocular lubrication that can substantially improve clinical manifestations and reduce the corneal damage (Murube-del-Castillo, 1986; Yu *et al*, 2004; Jacobsen *et al*, 2008). However, in almost all patients, secretion in the transplanted glands is reduced at 5 days to 3 months after surgery and may lead to obstruction of Wharton's duct in some patients (Yu *et al*, 2004). Such patients require reoperation to relieve the obstruction, otherwise the transplantation is considered a failure. Therefore, investigating the secretory mechanism of the transplanted SMGs is critical to reveal a therapeutic strategy to improve secretory function early after transplantation.

Salivary secretion is controlled by both sympathetic and parasympathetic autonomic nerves. Fluid and electrolyte secretion from salivary glands is primarily evoked by the action of acetylcholine on muscarinic receptors and norepinephrine on α-adrenoceptors, whereas protein secretion is mainly evoked by adrenergic agonists acting through β-adrenoceptors (Baum and Wellner, 1999; Melvin *et al*, 2005). Parasympathetic stimulation leading to muscarinic cholinergic receptor activation is linked to the formation of inositol 1, 4, 5-trisphosphate (IP_3), which subsequently increases intracellular calcium level and water secretion (Baum and Wellner, 1999). However, transplanted SMGs are completely disconnected from the normal nerve supply,

352

and their secretory mechanism is poorly understood. In a previous study, we showed that phenylephrine, a selective α_1-adrenoceptor agonist, could upregulate α_1-adrenoceptor expression, increase secretion, inhibit apoptosis, and ameliorate atrophy of acini in the transplanted glands (Xiang *et al*, 2006, 2008). Use of sialoscintigraphy revealed good response with long-term SMG transplantation to administration of parasympathomimetic drugs (Geerling *et al*, 2008; Jacobsen *et al*, 2008). Therefore, we have hypothesized that the short hypofunction of transplanted SMGs was due to lack of neurotransmitters and alteration of the related receptors. The muscarinic receptor signaling pathway plays an essential role in salivary fluid secretion (Baum, 1993). Although the expression and function of muscarinic receptor subtypes of SMGs have been examined in different species (Dorje *et al*, 1991; Tobin, 1995; Ryberg *et al*, 2008), their alteration in transplanted SMGs is still unknown.

We aimed to investigate the change in the muscarinic receptor signaling pathway early after SMG transplantation and to evaluate the possible protective effect of carbachol, an agonist of muscarinic receptor, on the structure and function of transplanted SMGs in a rabbit model.

Materials and methods

Animals
Healthy male New Zealand white rabbits weighing 2.4 ± 0.3 kg were used. All experimental procedures were approved by the Committee of Animal Research, Peking University Health Science Center and were in accordance with the Guidance of the Ministry of Public Health for the care and use of laboratory animals.

Reagents and antibodies
Carbachol was purchased from Sigma (St. Louis, MO, USA). Antibodies to M1 and M3 muscarinic receptors, aquaporin-5 (AQP5), extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphorylated ERK1/2 (p-ERK1/2), actin, and horseradish peroxidase-, TRITC-, and FITC-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents were of analytical grade.

Rabbit model of SMG autotransplantation
Submandibular gland autotransplantation was performed as described previously (Xiang *et al*, 2006). Briefly, with rabbits under sodium pentobarbital (20 mg kg^{-1} body weight) anesthesia, the right SMGs, along with the Wharton's duct and related blood vessels, were isolated from the submandibular triangle and then transplanted to the left temporal region and revascularized by anastomosing the artery of the gland to the distal part of the external carotid artery and the vein of the gland to the temporal vein. A polyethylene tube (inner diameter 0.5 mm) was inserted into the Wharton's duct and left outside of the temporal skin to measure

secretion of SMG and to deliver carbachol or saline. The rabbits were randomly divided into three groups: (i) control, sham-operated without transplantation ($n = 5$); (ii) transplantation ($n = 5$); and (iii) carbachol ($n = 5$). After transplantation, 100 μl carbachol (1 μM) was retrogradely infused into the transplanted glands for 30 s and kept for 2 min, then saliva was allowed to flow out spontaneously through the cannula inserted into Wharton's duct from postoperative day 1 to 7. The control and transplantated glands received 100 μl normal saline. The SMGs were removed on postoperative day 7 with rabbits under anesthesia.

Measurement of salivary secretion
After daily carbachol or saline infusion, secretion of SMGs from the cannula inserted into the Wharton's duct was measured until the pH value of saliva reached 8.0. SMG salivary flow was detected for 5 min by Schirmer's test (López-Jornet *et al*, 2006) by use of a moistened length of filter paper (35×5 mm). All measurements were taken between 9:00 and 10:00 in resting, conscious animals.

Reverse-transcriptase polymerase chain reaction (RT-PCR)
Total RNA from SMGs was purified with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA preparations were treated with DNase I to remove contamination by genomic DNA before RT-PCR. cDNA was prepared from 3 μg of total RNA with use of M-MLV reverse transcriptase (Promega, Madison, WI, USA) and primed with oligo dT. cDNA was amplified as follows: 1 cycle at 94°C for 5 min, then 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. The sense and antisense primers for M1 (NM_000738), M3 (NM_000740), aquaporin-1 (AQP1, AF495880), AQP5 (AF495879), and β-actin were 5′-GAGGGCTCACCAGAGACTCCT-3′ and 5′-GTCTCGGGAACACAGTCCTTG-3′; 5′-CTTCTCCAAGCTTCCCATCCA-3′ and 5′-TGACCGACTGTCTCTGCTGGT-3′; 5′-CCTCGTCCCTGACCCTGAACT-3′ and 5′-AGGGTTAATGCCACAGCCAGT-3′; 5′-GTTCCTGGCCACCCTCATCT-3′ and 5′-ACAGACAGGCCGATGGACAG-3′; 5′-ATCTGGCACCACACCTTCTACAATGAGCTGGCG-3′ and 5′-CGCCATACTCCTGCTTGCTGATCCACATCTGC-3′, respectively. Amplification of β-actin was the internal standardization. The amplification products were visualized on 1.5% agarose gel with ethidium bromide and sequenced to confirm their identities. The band densities were quantitated by the use of a LEICA550IW image system (LEICA, Manheim, Germany).

Determination of IP$_3$ content
The content of IP$_3$ was determined by the use of a ^{3}H-labeled IP$_3$ assay kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

Immunofluorescence
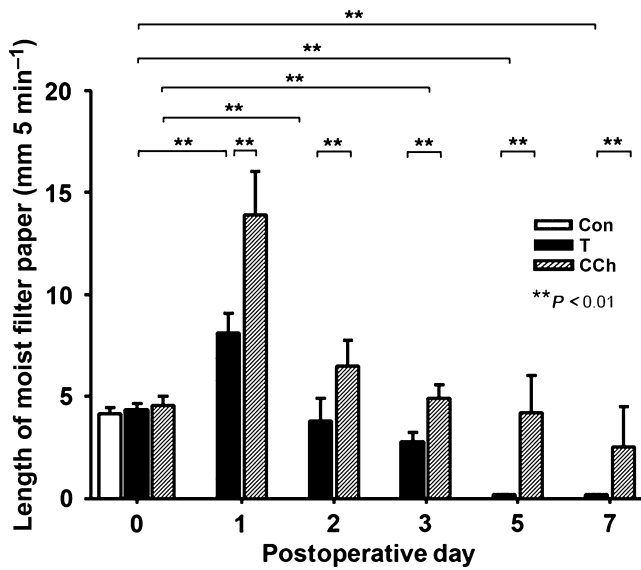Frozen sections of SMGs were immunostained with anti-M1 and anti-M3 receptor or anti-AQP5 antibodies

Figure 1 Effect of carbachol on salivary secretion in submandibular glands (SMGs). Salivary flow was measured by Schirmer's test before and after transplantation. Transplanted SMGs were administered carbachol (1 μM) during postoperative days 1–7. The salivary flow measurements from five individual experiments were used to calculate the mean $\pm$ s.d. Con, control; T, transplantation; CCh, carbachol

at 1:100 dilution, then incubated with FITC- or TRITC-labeled secondary antibodies as described (Xiang *et al*, 2006; Ryberg *et al*, 2008). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma). Fluorescence images were captured under a confocal microscope (Leica TCS SP2).

Immunoblotting
Submandibular gland tissues were homogenized and centrifuged as described previously (Xiang *et al*, 2006). The concentration of proteins was measured by Lowry's method (Lowry *et al*, 1951). Crude protein extracts (60 μg) were separated by 9% sodium dodecylsulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk, and then probed with primary antibodies (p-ERK1/2 and ERK1/2) and horseradish peroxidase-conjugated secondary antibodies. The blots were visualized by the use of enhanced chemiluminescence reagents. The density of bands was scanned and quantified by the use of the LEICA550IW image analysis system. Membranes were stripped and reprobed with anti-actin antibody to assure equal loading.

Cell culture and carbachol stimulation
Neonatal (1-day-old) rabbits were anesthetized with sodium pentobarbital (20 mg kg^{-1} body weight). Primary rabbit SMG cells were prepared by enzymatic digestion and seeded in six-well plates at 10^5 cells per well. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C as described (Zhang *et al*, 2006). For stimulation experiments, cells were serum starved in DMEM for 24 h and then treated with or without
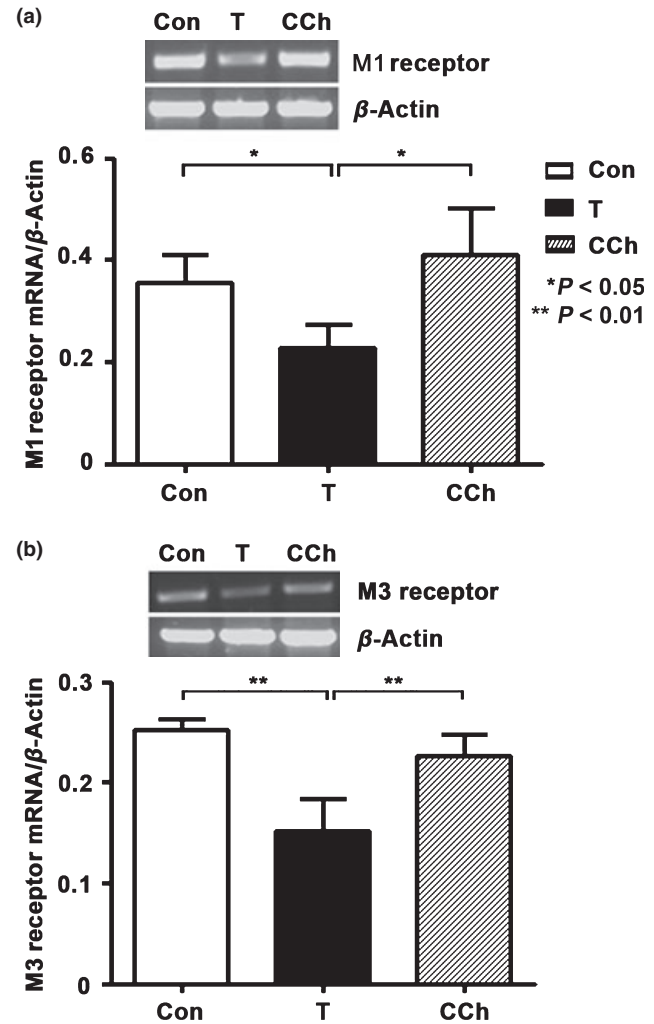

Figure 2 mRNA expression of muscarinic receptor subtypes M1 and M3 in submandibular glands (SMGs). RT-PCR analysis of mRNA expression of M1 (**a**) and M3 (**b**) receptors. The mRNA expression was normalized to that of β-actin. Data are mean $\pm$ s.d. of five independent experiments. Con, control; T, transplantation; CCh, carbachol

carbachol (10 μM) for the indicated times. After treatment, cells were analyzed on RT-PCR or immunoblotting as described.

Histological examination
Morphologic changes of SMGs were observed in paraffin sections stained with hematoxylin and eosin (H&E) on light microscopy (Q550CW, Leica). Other gland specimens were fixed in 2% paraformaldehyde-1.25% glutaraldehyde. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate and observed on transmission electron microscopy (H-7000 electron microscope; HITA-CHI, Tokyo, Japan).

Morphometric analysis
Hematoxylin and eosin-stained sections were used for microscopic microscopy with the Leica Qwin software program. Data were reported as mean acinar and duct
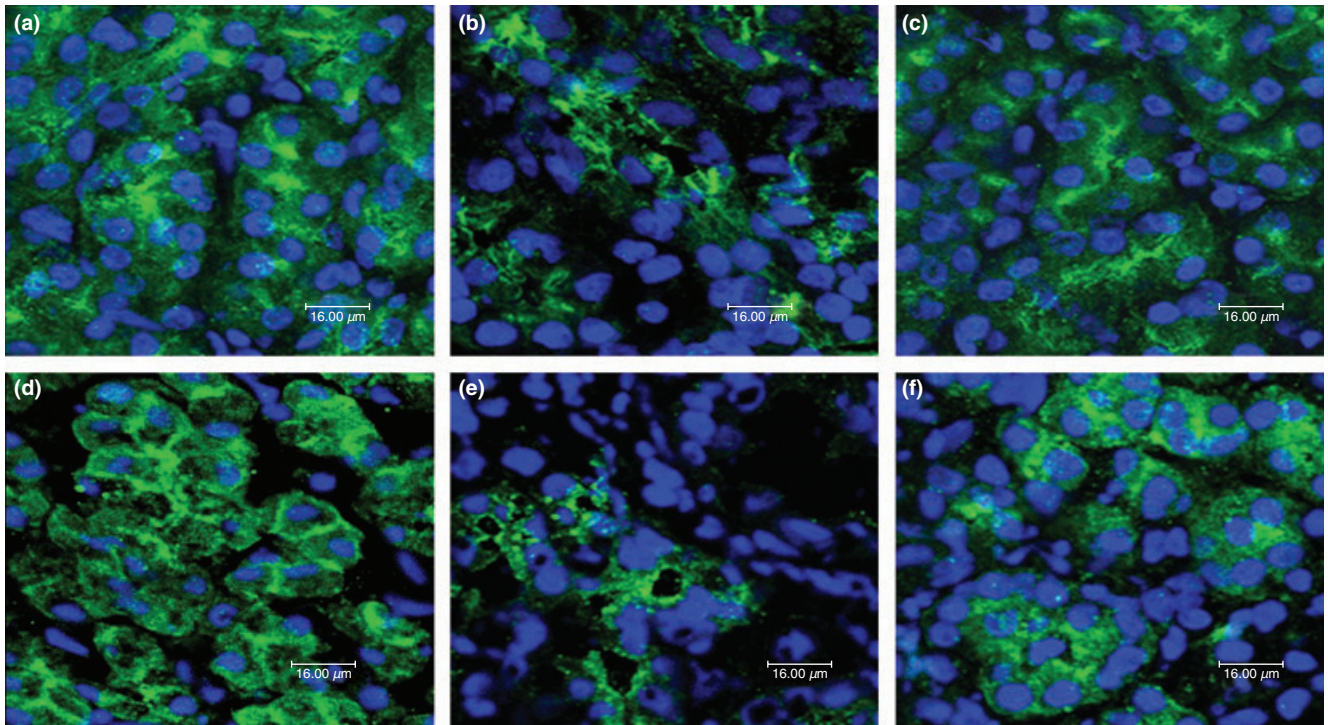
Figure 3 Localization of muscarinic receptor subtypes in submandibular glands (SMGs). Representative immunofluorescence images of M1 receptor in control (**a**), transplanted (**b**), and carbachol-treated SMGs (**c**). Representative immunofluorescence images of M3 receptor in control (**d**), transplanted (**e**), and carbachol-treated SMGs (**f**). M1 or M3 receptors were labeled as green and nuclei were labeled as blue. Bar indicates 16 μm

area, and percentage parenchyma area (representing the acini and ducts), determined by five experimental measurements as described (Dayan *et al*, 2000). Briefly, video microscopy was used to outline the acinar and ductal cells, and then area measurements were calculated by use of an image software program (Q550CW, Leica). Acinar cell area and percentage parenchyma area of 10 randomly selected fields in each section were averaged.

Statistical analysis
Data are presented as mean $\pm$ s.d. Differences among groups were analyzed by one-way ANOVA, and then Bonferroni testing. A $P < 0.05$ was considered statistically significant.

Results

Carbachol increased the secretion of transplanted SMGs
Before transplantation, the basal salivary flow of SMGs did not significantly differ among control, transplantation, and carbachol groups (4.14 $\pm$ 0.34 mm, 4.37 $\pm$ 0.26 mm, 4.53 $\pm$ 0.53 mm, respectively; Figure 1). The salivary flow from transplanted glands was increased by 85.4% on postoperative day 1 ($P < 0.01$), was reduced significantly on postoperative days 2–3, and was barely detected on postoperative days 4–7 compared with that before transplantation. The preliminary experiment showed that daily saline infusion (100 μl) did not change the salivary flow of control and transplanted

SMGs. Carbachol treatment markedly increased the secretion of transplanted glands on postoperative days 1–7 (Figure 1).

Carbachol increased the expression of M1 and M3 muscarinic receptors in transplanted SMGs
PCR products were the expected sizes of 449 and 470 bp for M1 and M3 receptor subtypes, respectively. The mRNA expression of M1 and M3 receptors in transplanted glands was markedly lower than that in controls ($P < 0.05$, $P < 0.01$, respectively), but were recovered to the control level after carbachol treatment for 7 days (Figure 2a, b). Immunofluorescence to further evaluate the expression and distribution of M1 and M3 receptor proteins revealed the expression of both M1 and M3 receptors in the membrane and cytoplasm of acini of control SMGs (Figure 3a, d) but significantly diminished in transplanted acini (Figure 3b, e). The fluorescent intensities and distribution of M1 and M3 receptors in the carbachol-treated group appeared similar to those in controls (Figure 3c, f).

Carbachol elevated the content of IP$_3$ in transplanted SMGs
The content of IP$_3$, an important signaling molecule located downstream of the muscarinic receptors, was significantly lower by 62.4%, in transplanted SMGs than in controls ($P < 0.01$); the reduced level of IP$_3$ was reversed in part but substantially in carbachol-treated SMGs (Figure 4a).
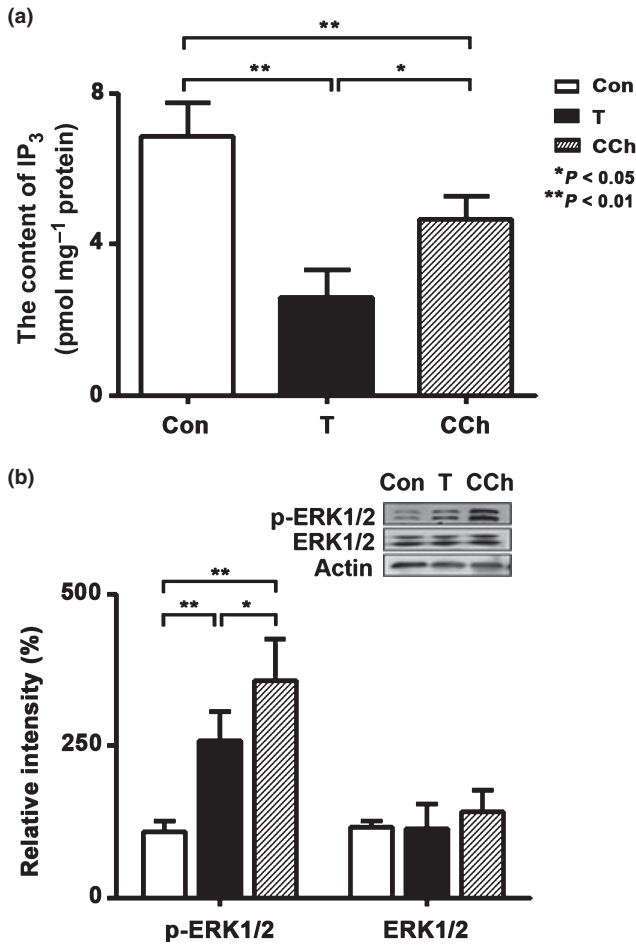
(a)

(b)

Figure 4 Effect of carbachol on the content of IP$_3$ and ERK1/2 phosphorylation. (**a**) Content of IP$_3$ determined by radioimmunoassay. (**b**) Levels of p-ERK and ERK1/2 detected by immunoblotting. Data are mean ± s.d. of five independent experiments. Con, control; T, transplantation; CCh, carbachol

(a)

(b)

Figure 5 Effect of carbachol on mRNA expression of aquaporin 5 (**a**) and AQP1 (**b**) in submandibular glands (SMGs). Normalized was to β-actin. Data are mean ± s.d. of five independent experiments. Con, control; T, transplantation; CCh, carbachol

Carbachol increased the phosphorylation of ERK1/2

On immunoblotting, the level of p-ERK1/2 was higher in transplanted SMGs than in controls (Figure 4b; $P < 0.01$) and highe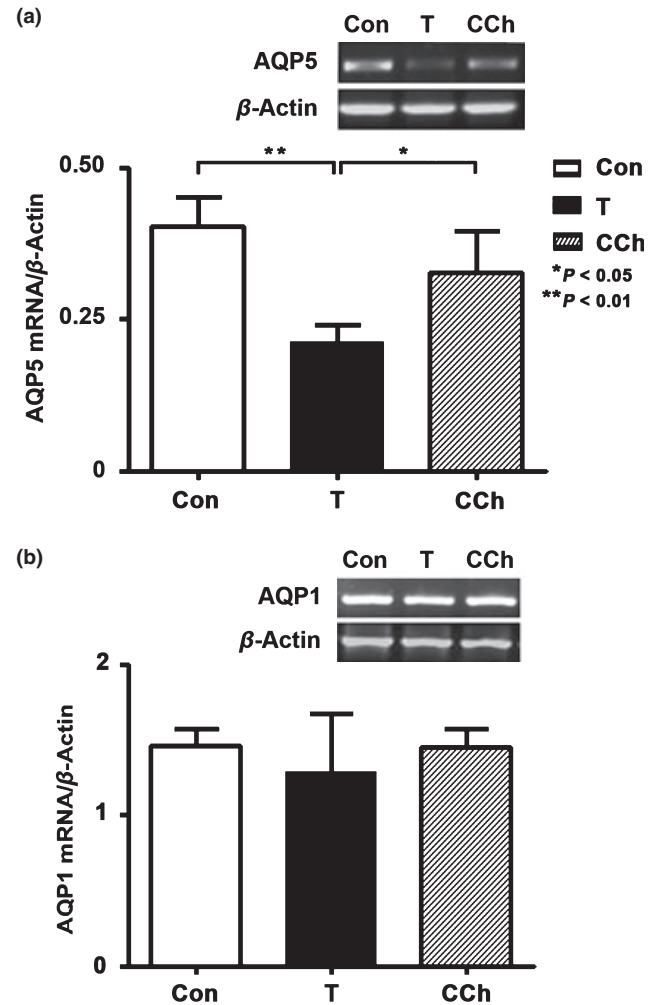r in carbachol-treated SMGs than in transplanted SMGs alone ($P < 0.05$). However, the expression of ERK1/2 was not changed in transplanted and carbachol-treated glands.
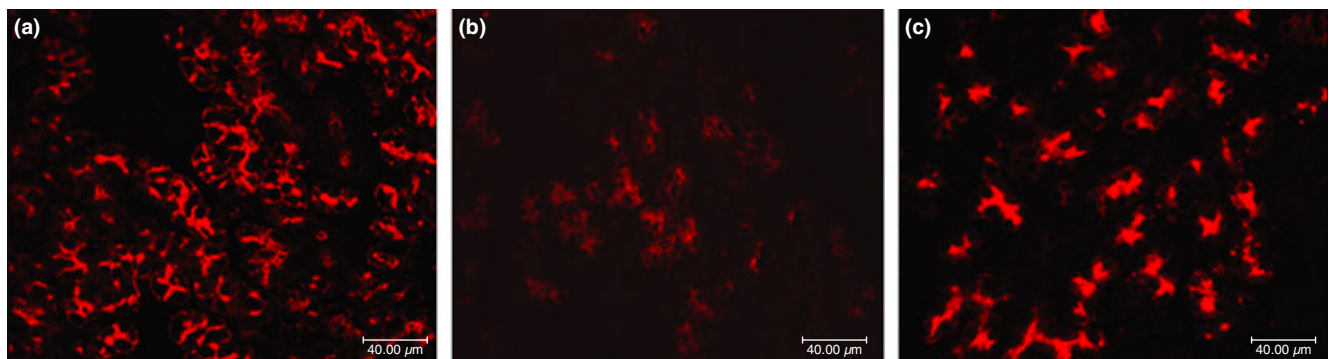

Figure 6 Effect of carbachol on the distribution of AQP5 in submandibular glands (SMGs). Representative immunofluorescence images of AQP5 in control (**a**), transplanted (**b**), and carbachol-treated SMGs (**c**). Bar indicates 40 μm

(a)

CCh (μM)   0   10

p-ERK1/2

ERK1/2

Actin

☐ Con
▨ CCh

*P < 0.05
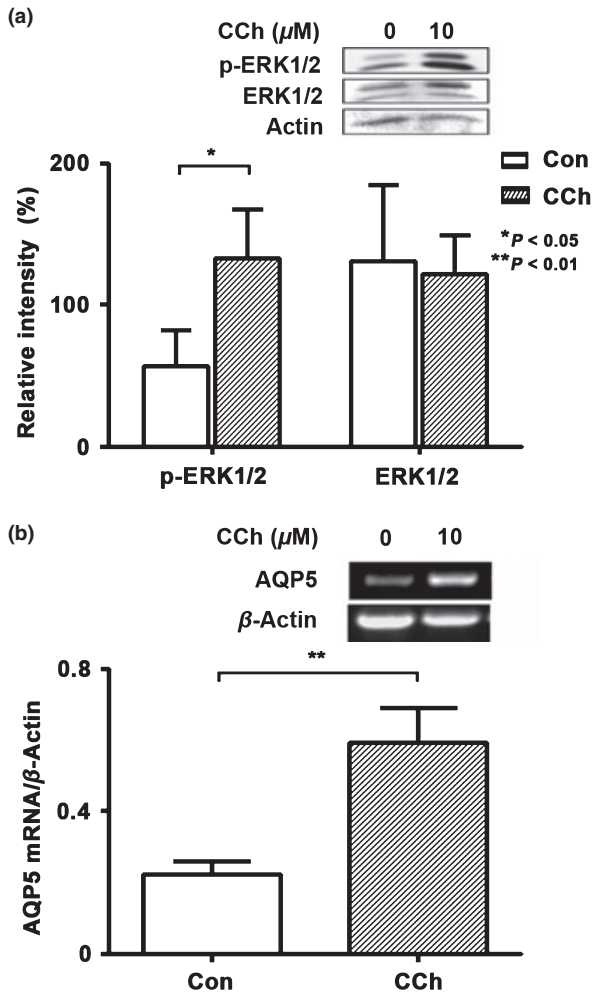**P < 0.01

(b)

CCh (μM)   0   10

AQP5

β-Actin

Figure 7 Effect of carbachol on ERK1/2 phosphorylation and mRNA expression of AQP5 in cultured neonatal rabbit submandibular gland (SMG) cells. Cells isolated from neonatal rabbit SMGs were treated with or without l0 μM carbachol. (**a**) Expression of ERK1/2 with 10-min treatment. (**b**) mRNA expression of AQP5 with 12-h treatment. AQP5 mRNA levels were normalized to the β-actin levels. Data are mean ± s.d. of three independent experiments. Con, control; CCh, carbachol

Carbachol enhanced the expression of AQP5 in transplanted SMGs

AQP5 plays an important role in rapid movement of water in salivary glands (Raina *et al*, 1995). The mRNA expression of AQP5 was markedly decreased in transplanted glands (Figure 5a; $P < 0.01$), but was recovered after carbachol treatment. However, the mRNA expression of AQP1 was not changed in transplanted and carbachol-treated glands (Figure 5b).

To further examine the distribution of AQP5, immunofluorescence revealed AQP5 expression predominantly localized in the apical membranes of control acinar cells (Figure 6a). In acinar cells of transplanted SMGs (Figure 6b), the expression of AQP5 was significantly diminished in the plasma membrane and mainly dispersed in the cytoplasm. The fluorescent intensity of AQP5 was increased and mostly located in the apical

membranes of acinar cells after carbachol treatment (Figure 6c).

Carbachol increased the phosphorylation of ERK1/2 and mRNA expression of AQP5 in cultured neonatal rabbit SMG cells

To more closely examine the role of carbachol on ERK1/2 phosphorylation and mRNA expression of AQP5, primary neonatal rabbit SMG cells were cultured. The expression of p-ERK1/2 was significantly increased after stimulation with 10 μM carbachol for 10 min, but the level of total ERK1/2 did not change (Figure 7a). On incubation with 10 μM carbachol for 12 h, the mRNA expression of AQP5 was significantly increased (Figure 7b).

Carbachol diminished the structural injury of transplanted SMGs

Normal acinar and ductal cells in control SMGs were displayed by light microscopy (Figure 8a). On day 7 after transplantation, some acini in transplanted SMGs were replaced by duct-like structures (Figure 8b). Increased connective tissue fibers were seen within or around the glandular lobule. The acinar area and the percentage parenchyma area were reduced in the transplanted SMGs (Figure 8d, e). After carbachol treatment, the parenchyma, including the acini and ducts, had recovered their original shapes, and the morphologic features were closer to those of controls (Figure 8c). The acinar area and the percentage parenchyma area in transplanted SMGs were significantly increased after carbachol treatment (Figure 8d, e).

In parallel, the ultrastructure of SMGs on day 7 after transplantation revealed low matrix-density secretory granules widespread in the cytoplasm of acinar cells in control SMGs on transmission electron microscopy (Figure 8f). However, the proportion of such granules was lower in transplanted SMGs than in controls (Figure 8g). In carbachol-treated glands, abundant low-density secretory granules were shown in acinar cells, with a size similar to that in control glands (Figure 8h). Therefore, carbachol could ameliorate injury of transplanted SMGs in our model.

Discussion

The process of salivary secretion is mainly controlled by acetylcholinic and adrenergic transmitters released from autonomic nerve terminals acting on different receptors. However, transplanted SMGs are completely disconnected from the nerve supply. In our rabbit model, secretion of transplanted SMGs started almost immediately after transplantation, was markedly increased for approximately 1 day, then substantially decreased and barely detected early after transplantation; a pattern similar to that seen in patients with SMG transplantation (Jacobsen *et al*, 2008; Yu *et al*, 2004). Early hypersecretion has been proposed to be attributed to the release of neurotransmitters from degenerating terminal axons (Ekström, 1999), whereas decreased or cessation of secretion of transplanted SMGs is associated with a
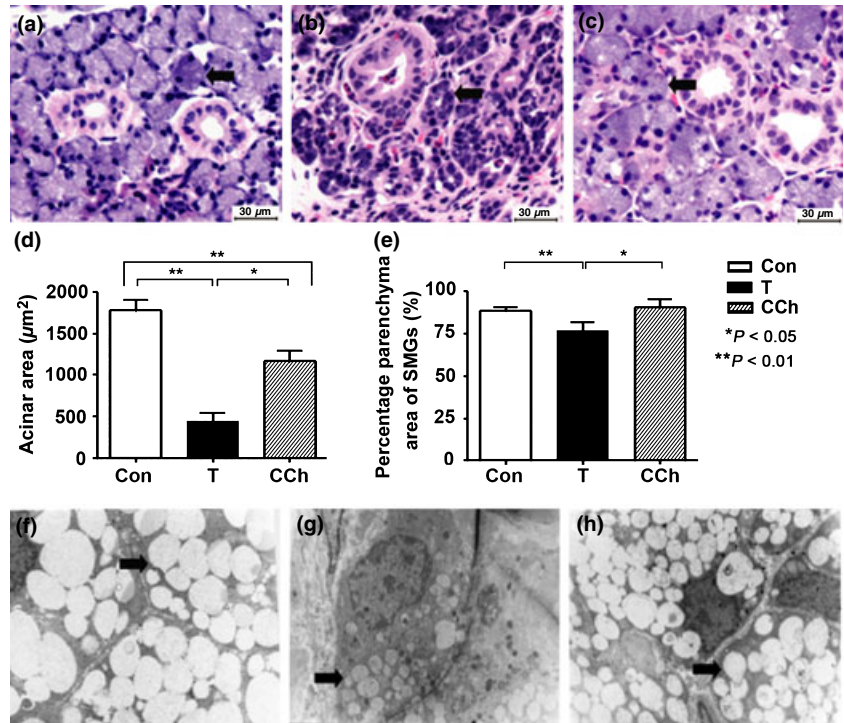
Figure 8 Effect of carbachol on structural injury in transplanted submandibular glands (SMGs) on day 7 after transplantation. Representative histological images in control (**a**), transplanted (**b**), and carbachol-treated SMGs (**c**). Arrows indicate acinar cells. Original magnification 200 ×. (**d**) Acinar area of SMGs. (**e**) Percentage parenchyma area of SMGs. Data are mean ± s.d. of five independent experiments. Con, control; T, transplantation; CCh, carbachol. Ultrastructure of SMGs in control (**f**), transplanted (**g**), and carbachol-treated SMGs (**h**). Arrows show low-matrix-density secretory granule in acinar cell. Magnification 5000 ×

substantial reduction in neurotransmitter release leading to parenchymal atrophy (Geerling *et al*, 2008). Daily saline infusion to retain Wharton's duct patency cannot increase salivary flow of transplanted SMGs, which further indicates that transplanted SMGs are hypofunctional early after transplantation. Previous reports have noted that parasympathetic stimulation evokes a copious flow of saliva, whereas sympathetic stimulation produces a small amount of flow that is rich in protein (Kyriacou *et al*, 1988; Ekström, 1989; Lung, 1994; McCloskey and Potter, 2000), which indicates that acetylcholine plays a predominant role in regulating fluid and electrolyte secretion from salivary glands. We showed that carbachol could stimulate the secretion and reduce the injury of transplanted SMGs early after transplantation. These results provide new evidence that a lack of acetylcholine is involved in the early hypofunctioning of SMGs, and supplementing an exogenous muscarinic receptor agonist might improve secretion of transplanted SMGs and prevent duct obstruction early after transplantation.

The actions of acetylcholine are mediated by the activation of the muscarinic receptor family, which consists of 5 distinct subtypes: M1, M2, M3, M4, and M5 (Nathanson, 1987). M3 receptors are likely to be the principal mediators of cholinergic responses in salivary glands, but functional studies also confirmed that M1 receptors mediate salivary secretion in rabbit and rat SMGs (Tobin, 1995; Gautam *et al*, 2004; Ryberg *et al*, 2008). However, whether muscarinic receptors expression is altered in transplanted organs is unclear. We found the expression and membrane distribution of M1 and M3 receptors significantly decreased after SMG transplantation, and carbachol treatment could recover the level of muscarinic recep-

tors, which suggests that the downregulation of M1 and M3 receptors may contribute to reduced secretion after SMG transplantation.

Muscarinic receptor subtypes connect to various protein kinases and signaling molecules. Stimulation of M1 and M3 receptors classically activates Gq-mediated phospholipase C, promotes IP_3 production, increases intracellular Ca^{2+} level, and subsequently results in electrolyte and water secretion (Baum, 1993). Recent evidence demonstrated that ERK1/2 could be activated by carbachol via an M1 and M3 receptor-mediated protein kinase C pathway in a human salivary cell line (Lin *et al*, 2008). In this study, IP_3 and p-ERK1/2 were reduced in level in transplanted SMGs, which indicates that dysfunction of the M1 and M3 receptors signaling pathway, in addition to decreased in acetylcholine release due to denervation, might also be involved in the hypofunction of transplanted SMGs early after transplantation. Our results showed that carbachol treatment not only recovered the content of M1 and M3 receptors but also improved their function early after SMG transplantation. Carbachol treatment promoted the rapid phosphorylation of ERK1/2 in cultured primary rabbit SMG cells, which further suggests that ERK1/2 activation may contribute to carbachol-mediated protection of transplanted SMGs. However, the ERK downstream targets mediating salivary cell responses to muscarinic receptor agonists need to be further defined.

AQP5 is highly expressed in the apical membranes of salivary acinar cells and plays a major role in salivary fluid secretion (Ma *et al*, 1999), whereas AQP1 is expressed in endothelial cells of salivary glands (He *et al*, 1997). We found the mRNA expression of AQP5, but not AQP1, reduced in transplanted SMGs.

358

Carbachol increased the mRNA expression of AQP5 in both transplanted glands and cultured SMG cells, which suggested that carbachol could specifically regulate the expression of AQP5. Recently, Li *et al* (2008) found the protein expression of AQP5, but not AQP1, significantly decreased in rat SMGs on parasympathectomy, and the M3 receptor-agonist cevimeline could recover the AQP5 protein level. In this study, the alteration in AQP5 mRNA level by transplantation or therapy was associated with changes in fluid secretion early after SMG transplantation, which suggests that carbachol may improve the secretion of the transplanted SMGs by upregulating the mRNA level of AQP5.

In agreement with our previous experiment (Xiang *et al*, 2006), we observed atrophy of acini, an early morphological change in transplanted SMGs. The relative weight of rat SMGs was found to be significantly decreased on parasympathectomy (Li *et al*, 2008). We found that carbachol significantly alleviated the atrophy of acinar cells and increased the number of secretory granules in the cytoplasm of acinar cells in transplanted SMGs, which further confirms that the structure and function of SMGs strongly depends on parasympathetic nerves.

In summary, our study provides new evidence that M1 and M3 receptors and their downstream signaling molecules are reduced in level early after SMG transplantation, which may result in reduced secretion of SMGs after transplantation. Carbachol increased salivary secretion and reduced the functional and structural injuries of the transplanted SMGs by activating muscarinic receptor-mediated signal transduction. Carbachol treatment may be a novel clinical strategy to improve secretion and prevent obstruction of Wharton's duct early after SMG transplantation.

Acknowledgements

Conflicts of interest

There is no conflict of interest.

Author contributions

L Shi participated in research design, acquisition and analysis of data, and writing of the article. X Cong participated in acquisition and analysis of data, and writing of the article. Y Zhang participated in research design, interpretation of data, and writing of the article. C Ding, QW Ding and FY Fu participated in acquisition and analysis of data. LL Wu and GY Yu participated in research design, analysis and interpretation of data, and writing of the article.

References

Baum BJ (1993). Principles of saliva secretion. *Ann N Y Acad Sci* **694**: 17–23.

Baum BJ, Wellner RB (1999). Receptors in salivary glands. In: Garret JR, Ekström J, Anderson LC, eds. *Frontiers of oral biolog, Vol 11, Neural mechanisms of salivary gland secretion*. Karger: Basel, pp. 44–58.

Dayan D, Vered M, Paz T, Buchner A (2000). Aging of human palatal salivary glands: a histomorphometric study. *Exp Gerontol* **35**: 85–93.

Dorje F, Levey AI, Brann MR (1991). Immunological detection of muscarinic receptor subtype proteins (m1–m5) in rabbit peripheral tissues. *Mol Pharmacol* **40**: 459–462.

Ekström J (1989). Autonomic control of salivary secretion. *Proc Finn Dent Soc* **85**: 323–331.

Ekström J (1999). Degeneration secretion and supersensitivity in salivary glands following denervations and the effects on choline acetyltransferase activity. In: Garrett JR, Ekström J, Anderson LC, eds. *Frontiers of oral biolog, Vol 11, Neural mechanisms of salivary gland secretion*. Karger: Basel, pp. 166–184.

Gautam D, Heard TS, Cui Y, Miller G, Bloodworth L, Wess J (2004). Cholinergic stimulation of salivary secretion studied with M1 and M3 muscarinic receptor single- and double-knockout mice. *Mol Pharmacol* **66**: 260–267.

Geerling G, Garrett JR, Paterson KL *et al* (2008). Innervation and secretory function of transplanted human submandibular salivary glands. *Transplantation* **85**: 135–140.

He XJ, Tse CM, Donowitz M *et al* (1997). Polarized distribution of key membrane transport proteins in the rat submandibular gland. *Pflugers Arch* **433**: 260–268.

Jacobsen HC, Hakim SG, Lauer I, Dendorfer A, Wedel T, Sieg P (2008). Long-term results of autologous submandibular gland transfer for the surgical treatment of severe keratoconjunctivitis sicca. *J Craniomaxillofac Surg* **36**: 227–233.

Kyriacou K, Garrett JR, Gjortrup P (1988). Structural and functional studies of the effects of sumpathetic nerve stimulation on rabbit submandibular salivary glands. *Arch Oral Biol* **33**: 271–280.

Li X, Azlina A, Karabasil MR *et al* (2008). Degradation of submandibular gland AQP5 by parasympathetic denervation of chorda tympani and its recovery by cevimeline, an M3 muscarinic receptor agonist. *Am J Physiol Gastrointest Liver Physiol* **295**: G112–G123.

Lin AL, Zhu B, Zhang W *et al* (2008). Distinct pathways of ERK activation by the muscarinic agonists pilocarpine and carbachol in a human salivary cell line. *Am J Physiol Cell Physiol* **294**: C1454–C1464.

López-Jornet P, Camacho-Alonso F, Bermejo-Fenoll A (2006). A simple test for salivary gland hypofunction using Oral Schirmer's test. *J Oral Pathol* **35**: 244–248.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with folin phenol reagent. *J Biol Chem* **193**: 265–275.

Lung MA (1994). Mechanisms of sympathetic enhancement and inhibition of parasympathetically induced salivary secretion in anaesthetized dogs. *Br J Pharmacol* **112**: 411–416.

Ma TH, Song YL, Gillespie A, Carlson EJ, Epstein CJ, Verkman AS (1999). Defective secretion of saliva in transgenic mice lacking aquaporin-5 water channels. *J Biol Chem* **274**: 20071–20074.

McCloskey MJ, Potter EK (2000). Sympathetic and parasympathetic interaction in vascular and secretory control of salivary glands in anaesthetised dogs. *Auton Neurosci* **84**: 50–57.

Melvin JE, Yule D, Shuttleworth T, Begenisich T (2005). Regulation of fluid and electrolyte secretion in salivary gland acinar cells. *Annu Rev Physiol* **67**: 445–469.

Murube-del-Castillo J (1986). Transplantation of salivary gland to the lacrimal basin. *Scand J Rheumatol Suppl* **61:** 264–267.

Nathanson NM (1987). Molecular properties of the muscarinic acetylcholine receptor. *Annu Rev Neurosci* **10:** 195–236.

Raina S, Preston GM, Guggino WB, Agre P (1995). Molecular cloning and characterization of an aquaporin cDNA from salivary, lacrimal, and respiratory tissues. *J Biol Chem* **270:** 1908–1912.

Ryberg AT, Warfvinge G, Axelsson L, Soukup O, Gotrick B, Tobin G (2008). Expression of muscarinic receptor subtypes in salivary glands of rats, sheep and man. *Arch Oral Biol* **53:** 66–74.

Sieg P, Geerling G, Kosmehl H, Lauer I, Warnecke K, von Domarus KH (2000). Microvascular submandibular gland transfer for severe cases of keratoconjunctivitis sicca. *Plast Reconstr Surg* **106:** 554–560.

Tobin G (1995). Muscarinic receptor subtypes in the submandibular gland and the urinary bladder of the rabbit: in vivo and in vitro functional comparisons of receptor antagonists. *J Auton Pharmacol* **15:** 451–463.

Xiang B, Zhang Y, Li YM *et al* (2006). Effects of phenylephrine on transplanted submandibular gland. *J Dent Res* **85:** 1106–1111.

Xiang B, Zhang Y, Li YM *et al* (2008). Phenylephrine protects autotransplanted rabbit submandibular gland from apoptosis. *Biochem Biophys Res Commun* **377:** 210–214.

Yu GY, Zhu ZH, Mao C *et al* (2004). Microvascular autologous submandibular gland transfer in severe cases of keratoconjunctivitis sicca. *Int J Oral Maxillofac Surg* **33:** 235–239.

Zhang Y, Xiang B, Li YM *et al* (2006). Expression and characteristics of vanilloid receptor 1 in the rabbit submandibular gland. *Biochem Biophys Res Commun* **345:** 467–473.