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Parasympathectomy increases resting salivary secretion in normal and irradiated submandibular glands of rats

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Fluid and ion secretion from the submandibular gland (SMG) is mainly regulated by parasympathetic nerves. This study evaluated the effect of parasympathectomy on salivary secretion from normal and irradiated rat SMGs from 1 to 24 wk after denervation. Although stimulated salivary secretion was significantly lower in denervated SMGs compared with contralateral self-controls, the resting salivary flow rates were markedly higher in the denervated SMGs at 1, 12, and 24 wk after denervation. The levels of muscarinic acetylcholine M1 and M3 receptors, as well as of aquaporin 5, were up-regulated. Notably, although irradiated SMGs showed significantly lower resting and stimulated salivary secretion rates than non-irradiated SMGs, the resting salivary secretion rates of the irradiated and denervated SMGs were markedly higher than seen in the irradiated self-control SMGs at 1, 12, and 24 wk after parasympathectomy, and were even higher than seen in the non-irradiated sham-operated rats. The expression of M1 and M3 receptors was similarly elevated. Taken together, our results suggest that parasympathetic denervation increases resting salivary secretion of both normal and irradiated SMGs. This approach might provide a potential modality for relieving radiation-induced xerostomia, which is a common complication following treatment of head and neck cancer.

Head and neck cancer, which is one of the most common malignancies worldwide, with a 5-yr survival rate of approximately 60% (1–3), is typically treated with surgery and radiation (4). However, salivary glands are in the radiation field and become severely damaged; consequently, radiation-induced xerostomia (dry mouth) is a frequent and serious treatment complication characterized by dryness of oral mucosa and eating and speaking difficulties, and could lead to dental cavities and oral infections (5–7). Although several methods are available to alleviate the symptoms, severe xerostomia still lacks an effective treatment. Thus, it is pivotal to investigate the precise secretory mechanism in salivary glands and explore an effective therapy to treat radiation-induced xerostomia.

Under resting conditions, approximately 60–65% of saliva is produced by the submandibular gland (SMG) (8,9). It is well known that the SMG is innervated by both sympathetic and parasympathetic nerves (10). Parasympathetic stimulation, which mainly controls the secretion of water and ions, contributes to production of a larger amount of saliva compared with sympathetic stimulation, which is predominantly responsible for the secretion of proteins (11). As reviewed by Garrett & Kyriacou (12), Bernard had noticed already, in 1864, that dog SMGs deprived of innervation from chorda and cervical sympathetic nerves enter into a state of continuous resting secretion, named 'paralytic' secretion (12). A few years later, Heidenhain showed that section of the chorda tympani alone induced 'paralytic' secretion and suggested that this could be caused by changes in the gland after denervation (12). In the early 1950s, Emmelin *et al.* (13–15) found that 'paralytic' secretion by parasympathectomy is attributed to circulating catecholamines released from opioid anaesthetics on supersensitive salivary cells. By using non-opioid anaesthesia, Garrett & Kyriacou again observed 'paralytic' secretion in parasympathetic denervated rabbit SMGs and found that this secretion was completely inhibited by atropine, a muscarinic acetylcholine receptor antagonist (12). In children with familial dysautonomia, the flow rates of submandibular and sublingual glands are significantly increased compared with healthy controls, and these changes may be the result of parasympathetic denervation (16). Moreover, it has been reported that parasympathectomy causes significant decreases in stimulated saliva secretion and induces degradation of aquaporin 5 (AQP5) in

rat SMGs (17,18). Morphological observation has further revealed that short-term parasympathetic denervation causes atrophy in acinar cells and reduction in gland wet weight (19). These results suggest that the acetylcholine released from terminals of parasympathetic nerves plays an essential role in 'paralytic' secretion after denervation. However, it is notable that the salivary flow rate of the denervated glands is higher than that of the contralateral glands under resting conditions (12). As the salivary flow into the mouth under resting conditions is normally sufficient to ensure that the protective roles of salivary glands are fulfilled, we propose that parasympathetic denervation could be a potential way to attenuate radiation-induced xerostomia. The present study was therefore designed to evaluate the effect of parasympathectomy on the secretory function of both normal and irradiated rat SMGs and to explore the possibility of relieving radiation-induced xerostomia by parasympathetic denervation.

Material and methods

Reagents and antibodies

Antibodies to muscarinic acetylcholine M1 subtype receptor, to AQP5, and to actin were purchased from Bioworld Technology (Minneapolis, MN, USA). Antibody to M3 subtype receptor was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies – horseradish peroxidase (HRP)-conjugated IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other chemicals and reagents were of analytical grade.

Animals

Eighty, healthy, adult male Sprague-Dawley rats (8–10 wk of age, weighing 200–300 g) were kept in a temperature- and humidity-controlled environment (12-h light/12-h dark cycle) and had free access to water and standard laboratory chow. All experimental procedures were carried out under aseptic conditions, were approved by the Ethics 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.

Animal parasympathetic denervation experiment

To explore the sole effect of parasympathetic denervation on the secretory function of SMGs, 16 rats were anaesthetized with chloral hydrate (400 mg kg^{-1} of body weight). A vertical skin incision was made in the neck and the underlying muscles were bluntly dissected to expose the mylohyoid muscle, with the help of an operating microscope. The right chorda-lingual nerve was exposed to the mylohyoid muscle and excised as extensively as

Table 1

Experimental design of the parasympathetic denervation model

Number of rats	Subgroups (wk)	Number of rats	Right-side SMG	Left-side SMG	Comparisons
16	1	4	Sham operation	Sham operation	B vs. A:
	4	4	A	A	unpaired Student's *t*-test
	12	4	'Control'	'Control'	
	24	4			
16	1	4	Operation with denervation	Sham operation	C vs. B:
	4	4	C	B	paired Student's *t*-test
	12	4	'Denervation'	'Self-control'	
	24	4			

SMG, submandibular gland.

Table 2

Design of the irradiation and denervation experiments

Number of rats	Subgroups (wk)	Number of rats in each subgroup	Right-side SMG	Left-side SMG	Comparisons
16	1	4	Sham operation	Sham operation	B vs. A:
	4	4	A	A	unpaired Student's *t*-test
	12	4	'Control'	'Control'	
	24	4			
16	1	4	IR + Sham operation	IR + Sham operation	C vs. B:
	4	4	B	B	unpaired Student's *t*-test
	12	4	'IR'	'IR'	
	24	4			
16	1	4	IR + Denervation	IR + Sham operation	D vs. C:
	4	4	D	C	paired Student's *t*-test
	12	4	'IR + denervation'	'IR + selfcontrol'	
	24	4			

IR, irradiation 4 wk before denervation; SMG, submandibular gland.

possible so that the entire preganglionic and partial postganglionic secretory motor nerve fibres were sectioned (20). The denervated rats were randomly allocated to one of four subgroups ($n = 4$ for each time point), in which they were killed and evaluated at 1, 4, 12, or 24 wk after parasympathectomy. The denervated SMGs were designated the 'denervation' group, and the contralateral SMGs, which had the sham operation without denervation, were designated 'self-control'. To assess the possible effect of one-side parasympathectomy on the salivary secretion of the contralateral SMG, another 16 rats underwent a sham operation, were designated 'controls', and had their SMGs collected as described as above ($n = 4$ for each time point). The experimental design is shown in Table 1.

Animal irradiation and denervation experiment

A total of 32 rats were irradiated, 4 wk before parasympathectomy, by a single acute exposure of 10 Gy in the field of bilateral SMG areas with an output of 100 cGy min^{-1} at 50 cm and operating with an energy of 600 kV (X-ray apparatus AC-MLC2000B; HaiTai, Chengdu, China). The bodies of the rats were covered by lead sheets. Of these 32 irradiated rats, 16 underwent bilateral sham operations and served as the 'irradiation' (IR) group, while the remaining 16 underwent right-side parasympathectomy (IR + denervation) and left-side sham operation (IR + self-control), as described above. Another 16 rats underwent a sham operation, without irradiation or parasympathectomy, and were designated 'controls'. The SMGs from each group were collected at 1, 4, 12, or 24 wk after parasympathectomy ($n = 4$ for each time point). The experimental design is shown in Table 2.

Measurement of salivary secretion

Both resting and chewing-induced stimulated salivary secretions from SMGs in each group were detected using filter paper (50 mm × 5 mm) moistened by a cannula inserted into Wharton's duct as oral Schirmer's test (21). All collections were taken twice (5 min each time) between 10.00 am and 12.00 am in conscious rats, and the average values were calculated.

After taking the salivary measurements, rats were killed by injection with a high dose of anaesthetic, and the SMGs were collected and weighed before histological examination and further analyses.

Histological examination

Submandibular gland tissues (2 mm × 2 mm) were fixed in 4% paraformaldehyde, and routine paraffin sections (5 μm) were stained with haematoxylin and eosin (H&E). The morphology of SMGs was evaluated from 10 randomly selected fields in each section by two blinded examiners. The images were captured under light microscopy (Q550CW; Leica, Mannheim, Germany).

Reverse-transcription–polymerase chain reaction

Total RNA was purified with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was prepared from 5 μg of total RNA with M-MLV reverse transcriptase (Promega, Madison, WI, USA). The sense and antisense primers for M1 receptor (NM_080773), M3 receptor (NM_012527), AQP5 (NM 012779) and actin were 5′-CAGCAGCAGCTCAGAGAG GTC-3′ and 5′-GGTGCCTGTGCTTCAGAATCT-3′, 5′-CCAAGCTTCCCATCCAGTTAG-3′ and 5′-GTGTTCA CCAGGACCATGATG-3′, 5′-GGGATCTACTTCACCG GCTGT-3′ and 5′-CCGTCAGCTCGATGGTCTTCT-3′, and 5′-ATCTGGCACCACACCTTCTACAATGAGCTG GCG-3′ and 5′-CGCCATACTCCTGCTTGCTGATCC ACATCTGC-3′, respectively. Actin served as the internal standard. The amplification products were visualized after electrophoresis on 1.5% agarose gels stained with ethidium bromide and were sequenced to confirm their identities. The band densities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

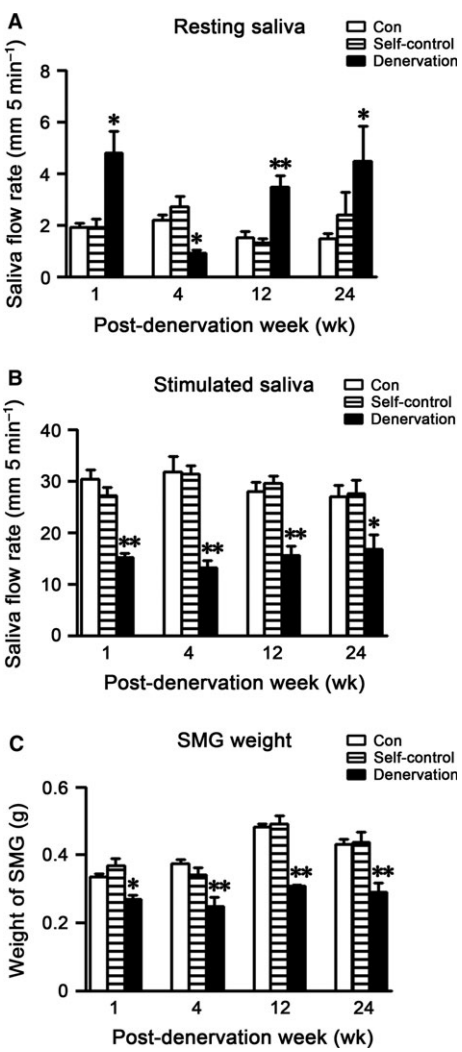

Fig. 1. Resting salivary secretion (A), stimulated salivary secretion (B), and submandibular gland (SMG) weight (C) of rat SMGs at the 1-, 4-, 12-, and 24-wk study time points. Data are presented as mean ± standard error of the mean. Con, sham-operated control; Denervation, the denervated SMGs; Self-control, the contralateral glands of the denervated SMGs. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with self-controls. $n = 4$ per group.

Western blot analysis

Submandibular gland tissues were homogenized in lysis buffer (containing 50 mM Tris–HCl, pH 7.2, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate) by use of a polytron homogenizer. The homogenate was centrifuged at 1,000 ***g*** for 10 min at 4°C, and the concentration of protein in the supernatant was determined using the Bradford method (22). Equal amounts of protein (40 μg) were separated by electrophoresis on a 12% SDS-polyacrylamide gel, electrotransferred to polyvinylidene difluoride membranes, blocked with 5% non-fat milk, probed with antibodies against M1 receptor (1:500 dilution), M3 receptor (1:500 dilution) and AQP5 (1:500 dilution), at 4°C overnight, and then incubated with HRP-conjugated secondary antibodies. The bands of target proteins were detected by an enhanced chemiluminescence reagent (Thermo Scientific Pierce, Rockford, IL, USA). Membranes were reprobed with antibody against actin (1:2,000 dilution) after being stripped to ensure equal loading of the lanes.

Statistical analysis

Data were expressed as mean ± standard error of the mean. Statistical analysis was performed using the unpaired Student's *t*-test for the comparisons among groups from different rats and the paired Student's *t*-test for the comparisons between denervated and contralateral glands from the same rats (Tables 1 and 2) using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Values of $P < 0.05$ were considered statistically significant.

Results

Salivary secretion and gland weight

The secretion of saliva from sham-operated control SMGs, including both resting and chewing-induced stimulated secretion of saliva, did not show significant differences compared with secretion of saliva from the self-control glands (the contralateral glands of denervated SMGs) at any time point. However, the resting salivary flow rate of the parasympathetic denervated SMGs was significantly higher at 1, 12, and 24 wk ($P < 0.05$), but lower at 4 wk ($P < 0.05$), after parasympathectomy, compared with that of the self-controls (Fig. 1A). In contrast, the stimulated secretion of saliva was markedly reduced by parasympathectomy at each time point (Fig. 1B).

The weights of the parasympathetic denervated SMGs were significantly lower than those of self-controls at 1, 4, 12, and 24 wk after parasympathectomy (Fig. 1C). There was no difference between the controls and self-control SMGs in SMG weight at any time point.

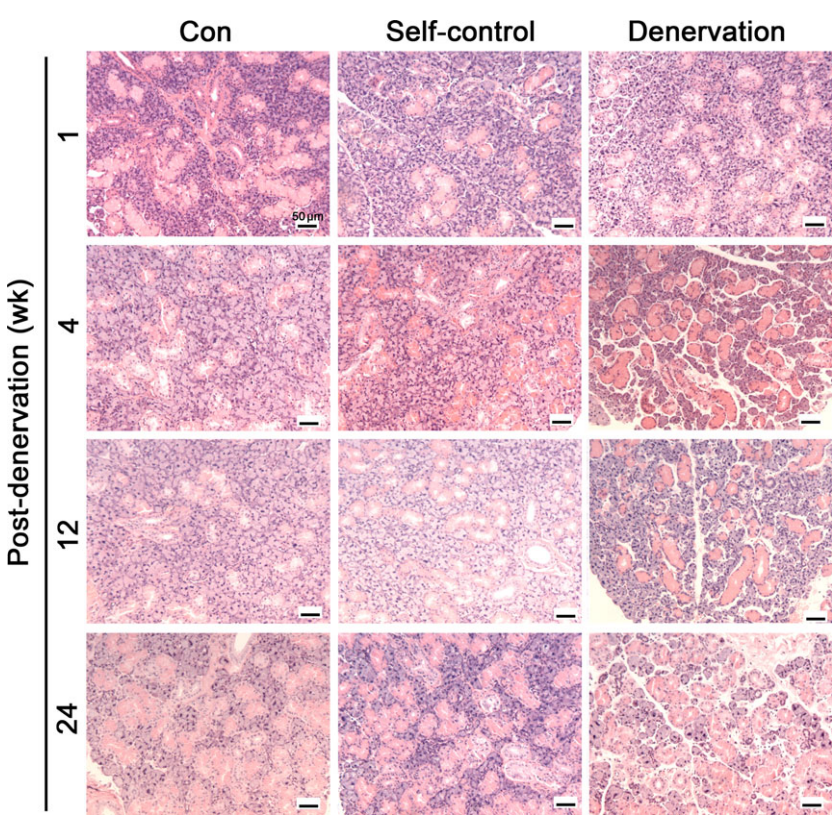

Fig. 2. Representative histological images of rat submandibular glands (SMGs) after parasympathectomy at the 1-, 4-, 12-, and 24-wk study time points. Con, control; Denervation, the denervated SMGs; Self-control, the contralateral glands of the denervated SMGs. Bars, 50 μm.

SMG morphology

Normal mucosal acinar cells were observed in control SMGs at all study time points, and the morphology of the self-control SMGs did not show significant differences compared with control glands at any time point (Fig. 2). Atrophy in mucosal acinar cells was observed in parasympathetic denervated glands at 4 and 12 wk postoperatively, and this atrophy was more pronounced at 24 wk. Moreover, denser connective tissue fibres within or around the glandular lobule, proliferation of small ducts, and a widened interacinar space were observed in denervated glands from 4 wk after parasympathectomy (Fig. 2).

M1/M3 receptors and AQP5 expression

The level of M1 receptor mRNA expressed in the denervated SMGs was similar to that of the self-controls at all time points, while the amount of M3 receptor mRNA expressed showed a small, but significant, elevation at 24 wk after denervation (Fig. 3A–C). The expression of both M1 receptor and M3 receptor proteins was higher in the denervated SMGs than in self-controls at 1, 12, and 24 wk, and significantly lower at 4 wk after denervation (Fig. 3D–F).

The expression of AQP5 mRNA in the denervated SMGs was similar to that of the self-controls at 1, 4, and 12 wk, but significantly higher at the 24-wk time point. The content of AQP5 protein after parasympathectomy was lower at 4 wk, higher at 12 wk, and significantly higher at 24 wk, compared with self-controls (Fig. 3G–I).

Salivary secretion of irradiated SMGs

The resting salivary flow rates of IR groups (SMGs irradiated only) were significantly lower compared with control groups (sham-operated SMGs without irradiation or parasympathectomy) for all four time points after surgery. Notably, the resting salivary flow rates of IR + denervation groups (the irradiated and denervated SMGs) were markedly higher compared with those of IR + self-control groups, and were even

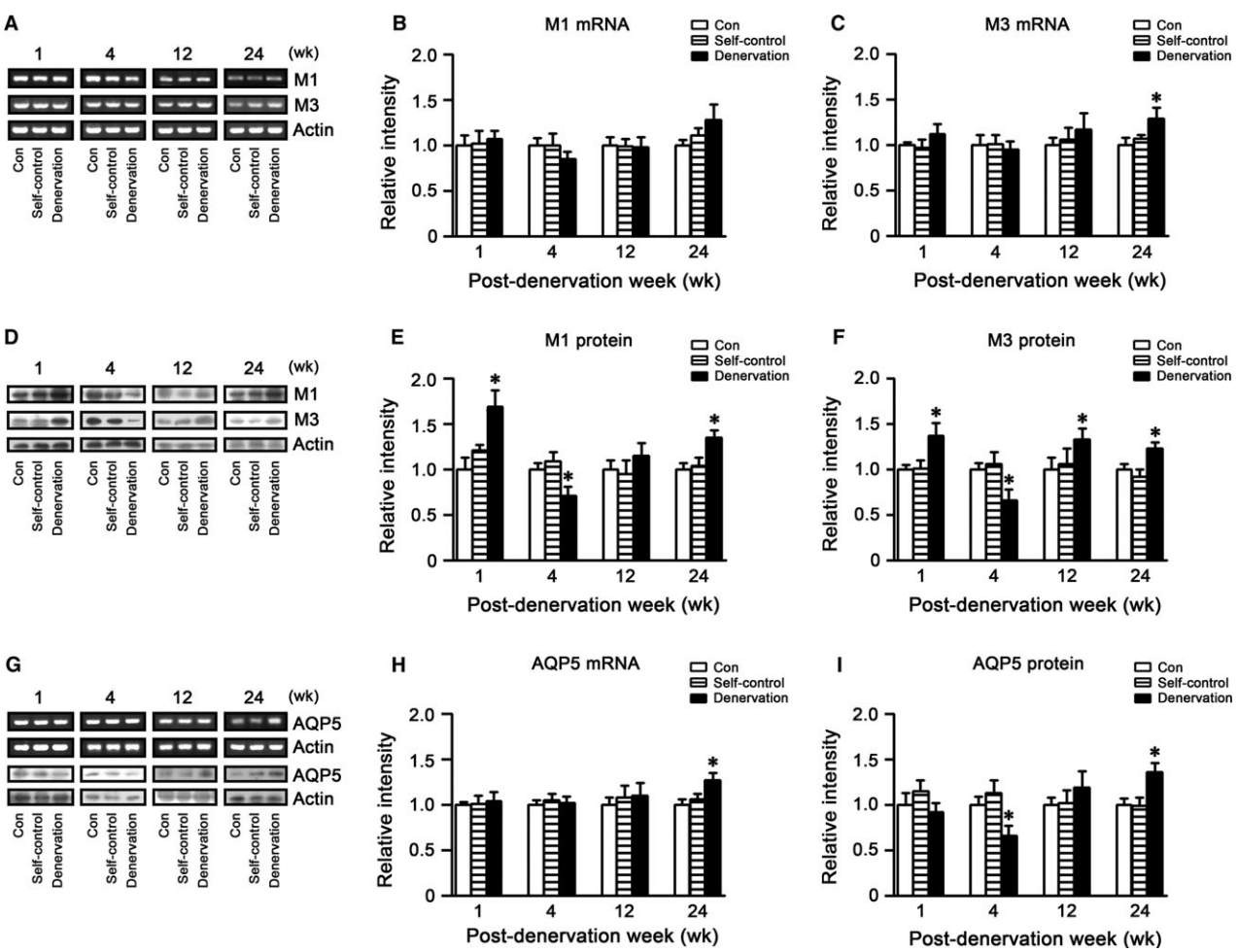

Fig. 3. Expression of M1 and M3 mRNA (A–C), M1 and M3 protein (D–F), and aquaporin 5 (AQP5) mRNA and protein (G–I) in rat submandibular glands (SMGs) at the 1-, 4-, 12-, and 24-wk study time points. All data are presented as mean ± standard error of the mean. Con, control; Denervation, the denervated SMGs; Self-control, the contralateral glands of the denervated SMGs. *$P < 0.05$ compared with self-controls. $n = 4$ per group.

higher than for control SMGs, at 1-, 12-, and 24-wk time points ($P < 0.05$, Fig. 4A). However, the resting salivary flow rate of the IR + denervation group was lower than found for the IR + self-control group at 4 wk after denervation. In addition, compared with the control group, the stimulated salivary flow rates were significantly lower in IR groups at all study time points. The stimulated secretions of IR + denervation groups were lower compared with those of IR + self-control groups at all four study time points after surgery (Fig. 4B).

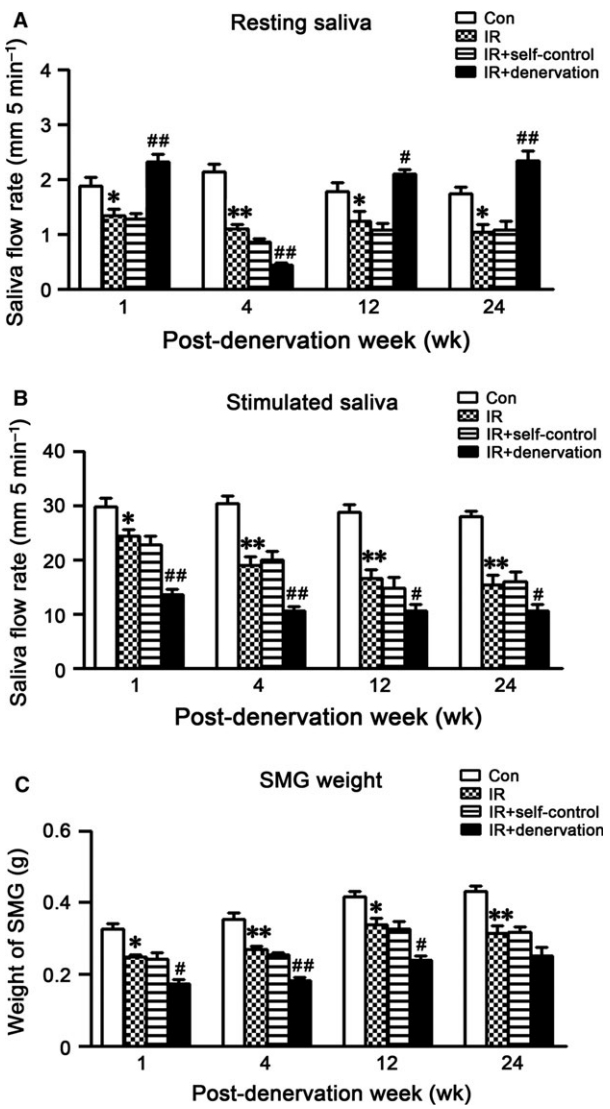

Fig. 4. Resting salivary secretion (A), stimulated salivary secretion (B), and submandibular gland (SMG) weight (C) of rat irradiated SMGs at the 1-, 4-, 12-, and 24-wk study time points. All data are presented as mean ± standard error of the mean. Con, control; IR, irradiation; IR + self-control, the contralateral glands of the irradiated denervated SMGs (with irradiation but no denervation); IR + denervation, the irradiated and denervated SMGs. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with Con; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with IR + self-controls. $n = 4$ per group.

The weights of IR + denervation glands were much lower at 1, 4, 12, and 24 wk after denervation compared with those of the IR + self-control group (Fig. 4C). The SMG weights were not different between IR and IR + self-control SMGs at any time point.

Morphology of irradiated SMGs

Irradiated SMGs showed increased gaps between neighbouring acini, as well as atrophy in acinar cells, and these phenomena were much more obvious at the 12- and 24-wk time points. Moreover, parasympathetic denervation induced acinar atrophy to a much greater extent in IR + denervation glands at all time points (Fig. 5).

M1/M3 receptors and AQP5 expression in irradiated SMGs

The expression of mRNA for both M1 and M3 receptors was not significantly different among the four groups at 4, 12, and 24 wk after denervation (Fig. 6A–C). The expression of M1 and M3 receptor proteins was lower at 4 wk in the IR + denervation SMGs compared with IR + self-controls. By contrast, the level of M1 receptor protein was significantly higher at 24 wk, and the level of M3 receptor protein was higher at both 12 and 24 wk after denervation in the IR + denervation glands. There was no difference in the levels of M1 and M3 receptor proteins in the IR group or the IR + self-control glands compared with controls (Fig. 6D–F).

The expression of AQP5 mRNA was significantly lower in IR, IR + self-control, and IR + denervation SMGs at the 4-wk time point (Fig. 6G,H). However, the level of AQP5 mRNA was similar to that of the control in these three groups at 12- and 24-wk time points. The expression of AQP5 protein showed changes that were consistent with the expression of mRNA (Fig. 6G,I).

Discussion

In this study, we found that parasympathetic denervation resulted in a significantly higher resting salivary secretion rate, but a lower stimulated salivary secretion rate and a lower gland weight than seen for glands with intact innervation. Moreover, in this radiation-induced xerostomia model, parasympathetic denervation promoted the resting secretion from the irradiated hypofunctional SMGs. The up-regulation of M1 and M3 receptors and AQP5 proteins may be involved in the increased and sustained resting secretion induced by parasympathectomy. Our study indicates that parasympathetic denervation could be a potential method for relieving radiation-induced xerostomia.

Although parasympathectomy increases the secretory flow rate of denervated SMGs in a few weeks (12–15),

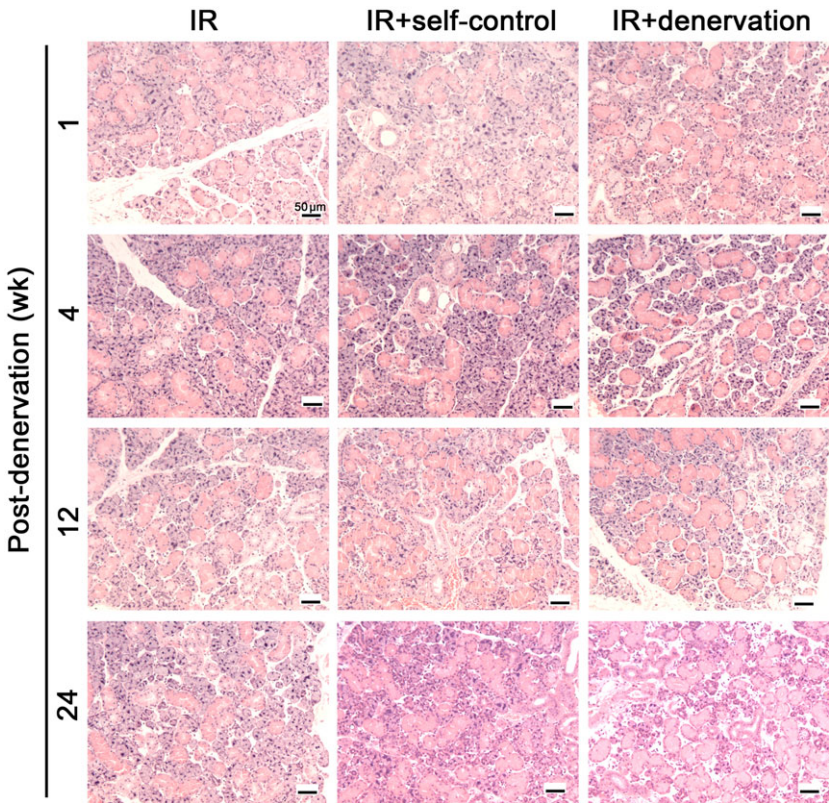

Fig. 5. Representative histological images of rat irradiated submandibular glands (SMGs) after parasympathectomy at the 1-, 4-, 12-, and 24-wk study time points. IR, irradiation; IR + self-control, the contralateral glands of the irradiated denervated SMGs (with irradiation but no denervation); IR + denervation, the irradiated and denervated SMGs. Bars, 50 μm.

the long-term effect of parasympathectomy on secretion of SMGs has not been evaluated. Our observation, that the resting salivary secretion rate was elevated 1 wk after parasympathectomy, is in accordance with the 'paralytic' secretion reported previously (12–15). This suggests that the temporary hypersecretion may be a result of the release of excess neurotransmitters from degenerating terminal axons from the damaged nerve end. At 4 and 12 wk after denervation, the resting secretion became lower, which might be a result of the depletion of neurotransmitters, resulting in a decrease in the resting secretion. In a rabbit model of SMG autotransplantation, an effective therapy for severe keratoconjunctivitis sicca, we found that the secretion from transplanted SMGs was substantially decreased and barely detectable 4–7 days postoperatively, while the application of carbachol significantly increased the secretion on postoperative days 1–7 (23). As transplantation of the SMG is performed with the anastomosis of vascular vessels, but not of nerves (24–26), these results again imply that denervation can lead to decreased secretion in the early phase after parasympathectomy.

Interestingly, although the weights of the denervated SMGs were lower and acini were obviously atrophic compared with those of sham-operated controls, the resting secretion rates of SMGs were significantly higher at 12 and 24 wk after denervation. Similar changes in resting secretion over time have been reported in patients who have undergone SMG autotransplantation. After 4 months, the salivary secretion gradually recovered, and more than 40% of the patients experienced epiphora at 6 months after transplantation, resulting in some patients needing additional surgical intervention to remove part of the transplanted SMGs (24–26). These results suggest that phenotypic changes occur in the glandular cells in 'long-term' denervated salivary glands. Even so, the mechanisms that are involved in the late stage of parasympathectomy-induced increased resting salivary secretion are not fully understood.

Muscarinic acetylcholine receptors, particularly M1 and M3 subtypes, play dominant roles in regulating the fluid and ion secretion from SMGs (23, 27, 28). Activation of M1 and M3 receptors induces the formation of inositol 1,4,5-trisphosphate, which subsequently increases intracellular calcium levels and water secretion through AQP5 (27). In this study, we found that increased secretion of saliva was paralleled by up-regulation of M1 and M3 receptor proteins and their downstream target, AQP5, in the denervated SMGs. Similar phenomena have been observed in the epiphora of human SMGs, where expression of M1 and M3 receptor proteins are up-regulated and the presence of calcium further indicates that these receptors are hypersensitive (29). These data suggest that the up-regulation of M1

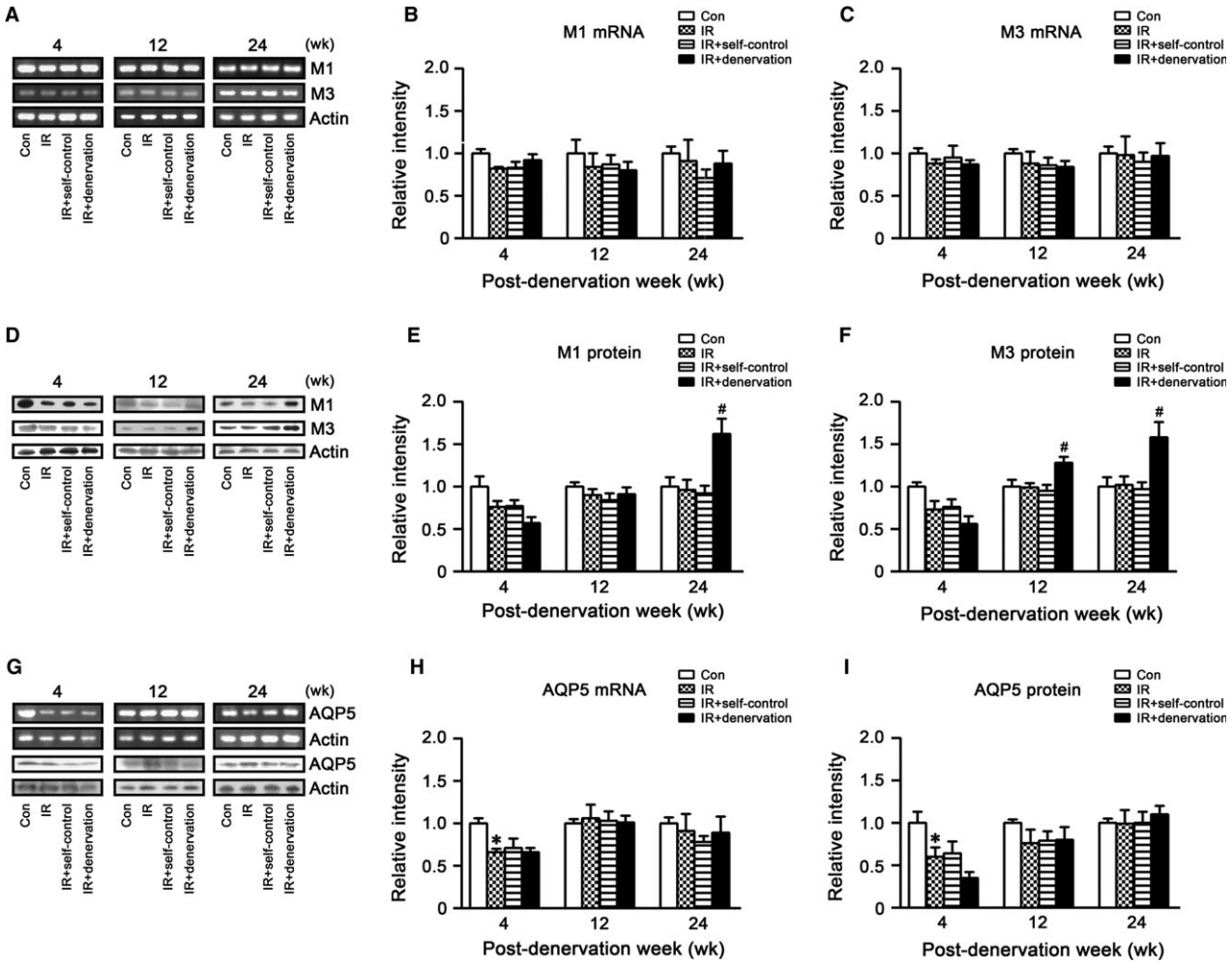

Fig. 6. Effect of parasympathectomy on the expression of M1 and M3 mRNA (A–C), M1 and M3 protein (D–F), and aquaporin 5 (AQP5) mRNA and protein (G–I) in rat irradiated submandibular glands (SMGs) at the 4-, 12-, and 24-wk study time points. All data are presented as mean ± standard error of the mean. Con, control; IR, irradiation; IR + self-control, the contralateral glands of the irradiated denervated SMGs (with irradiation but no denervation); IR + denervation, the irradiated and denervated SMGs. $^{*}P < 0.05$ compared with Con. $^{\#}P < 0.05$ compared with IR + self-controls. $n = 4$ per group.

and M3 receptors, together with AQP5, may be the main mechanism for the hypersecretion in resting conditions after parasympathetic denervation.

More importantly, we found that parasympathetic denervation significantly alleviated the radiation-induced hyposecretion at 12 and 24 wk after denervation, which suggests that denervation might be an effective approach for reducing radiation-induced xerostomia. However, it should be noted that in the IR + denervation SMGs, the expression of AQP5 protein was not higher compared with that in the non-irradiated or denervated self-control SMGs, which is not paralleled with the salivary secretion. The reason for this inconsistency might be the contribution of other factors, such as the apical chloride transporter, which plays a crucial role in the secretion of water and ions by the acinar cells under normal physiological conditions (30–32). As the activity of this channel is under cholinergic control, it is possible that up-regulation of M1 and M3 receptors in IR + denervation SMGs at 12 and 24 wk after denervation might contribute to the increased secretion through the apical chloride transporter. However, more efforts should be made in further studies to identify the underlying mechanism.

In conclusion, our results indicate that parasympathetic denervation can increase resting salivary secretion in both normal and irradiated SMGs. The up-regulation of M1 and M3 receptors, in addition to downstream AQP5, is thought to contribute to this increase and may thereby alleviate the irradiation-induced hyposecretion. Parasympathetic denervation could perhaps become a potential modality for relieving irradiation-induced xerostomia. However, long-term studies and trials are needed before this approach can be used clinically.

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Conflicts of interest – The authors certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, or company that is present in this article.

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