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**Original Article**

## **Effect of Cevimeline on Radiation-Induced Salivary Gland Dysfunction and AQP5 in Submandibular Gland in Mice**

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### **Abstract**

The aim of this study was to clarify the effects of the muscarinic receptor agonist, cevimeline, on saliva flow and expression of aquaporin5 (AQP5) in submandibular gland after X-ray irradiation. Using a previously established radiation-induced xerostomia model mouse, saliva flow from at 7 days before irradiation to at 28 days after irradiation was investigated in mice that were treated with cevimeline before or after irradiation. Radiation caused a significant decrease in saliva flow compared with nonirradiated salivary glands. Cevimeline post-treatment also caused a significant decrease in saliva flow. In contrast, cevimeline pre-treatment did not significantly decrease saliva flow. Expression of AQP5 fluorescent intensity and mRNA were also analyzed. Irradiation significantly decreased expression of AQP5 in submandibular gland. However, pre-treatment with cevimeline prevented this decrease in AQP5 expression. These data suggest that pre-treatment with cevimeline prevents radiation-induced xerostomia and radiation-induced decrease in expression of AQP5 in submandibular gland.

Key words: Cevimeline—Aquaporin5—Radiation—Xerostomia—Salivary gland

### **Introduction**

Radiotherapy is one of the most important treatments for oral, head and neck cancers. However, by necessity, the treatment field required for irradiation of cancerous tissue includes areas of normal tissue, and thus is associated with subsequent negative effects.

In particular, it has been shown that xerostomia results from decreased saliva flow, and this damage is often irreversible<sup>21,22</sup>. It remains unclear as to why salivary glands, which have a slow turnover (>60 days), have high radiosensitivity<sup>6</sup>. Additionally, radiation damage leads to immediate salivary gland dysfunction. The mechanism behind radiation-induced xeros-

tomia remains to be elucidated.

Cevimeline is a muscarinic agonist that directly stimulates muscarinic acetylcholine receptors in the salivary gland. Because cevimeline has a long-acting sialogogic effect<sup>7,10</sup>, it is predicated to ameliorate radiation-induced xerostomia. Aquaporins (AQPs) are membrane proteins forming water channels, and are widely distributed through the organisms. Of the known AQPs, AQP1, AQP3, AQP4, AQP5 and AQP8 have been identified in mammalian salivary glands<sup>1,13,19</sup>. In muscarinic agonist stimulation studies, Ma *et al.*<sup>15</sup> found that showed no defect in the volume or composition of saliva in AQP1- and AQP4-knockout mice, but did in AQP5-knockout mice. These reports have played an important role in the understanding of AQP5 in saliva flow<sup>13,15,19</sup>. One study investigated the distribution of AQP3, but the mechanism of this distribution remains to be elucidated<sup>1</sup>. For example, they demonstrated that high epithelial cell membrane water permeability is required for active, near-isosmolar fluid transport. Evidence for the expression of AQP8 in the submandibular glands of rats and mice has been reported, although the localization of AQP8 has yet to be established<sup>12,16</sup>.

The present experiment employed irradiation of a xerostomia model mouse irradiated by X-rays to investigate the usefulness of cevimeline in irradiation-induced xerostomia. Both immunofluorescence and qRT-PCR were used to investigate expression of AQP5.

## Materials and Methods

### 1. Animals

Five-week-old (body weight 25–31.5g) female ICR mice (Clea Japan Inc., Tokyo) were used in all experiments. They were housed in polycarbonate cages under a 12-hour light-dark cycle. Food and water were given *ad libitum*. After a week of acclimatization, the study was started. The study was carried out according to “The guideline for the treatment of experimental animals in Tokyo Dental College” (1823-03).

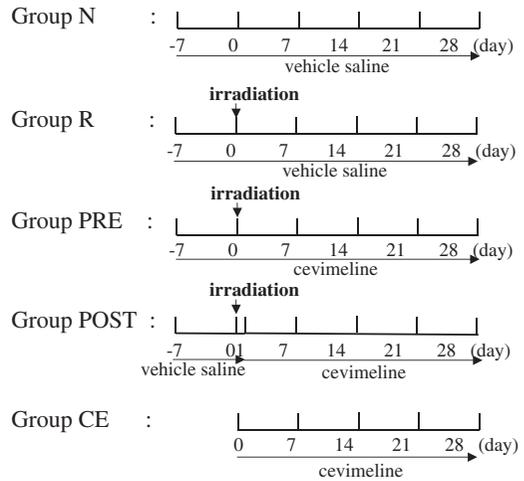


Fig. 1 Experimental group

A line chart showing optimal design for irradiation and treatment with cevimeline protocol.

### 2. Experimental group

Cevimeline was dissolved in saline at 10 mg/kg bw and administered orally with a feeding tube once daily during the experimental periods as follows: 1) from 7 days before to 28 days after irradiation (R+PRE group); 2) only 28 days after irradiation (R+POST group); 3) 28 days for non-irradiation (CE group).

Vehicle saline (2ml/kg bw) was administered orally with a feeding tube for 35 days to the non-irradiated animals (N group) and irradiated animals (R group), or supplied for without cevimeline administration to the irradiated animals (Fig. 1).

### 3. Irradiation

All mice were treated with cevimeline or vehicle saline orally with a feeding tube 1 hour before irradiation. Irradiation was performed according to the study of Takeda *et al.*<sup>25</sup>. Briefly, all animals were anesthetized with an intraperitoneal injection of 60 mg/kg bw pentobarbital sodium. In the R, R+PRE and R+POST groups, the bilateral submandibular glands were irradiated with a single dose of 15 Gy delivered by the X-ray unit

MEVATORON74 DX40 (Toshiba Medical System, Tokyo) at a dose rate of 3 Gy/min.

#### 4. Collection of saliva

Saliva was collected at 7 days before and at 1, 3, 5, 7, 14 and 28 days after irradiation ( $n = 5$ ). The mice were fasted during the 6 hours before the experiment, but were given access to water. All mice were weighed and anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). Ten minutes after pentobarbital injection, pilocarpine (0.5 mg/kg) was injected intraperitoneally to stimulate saliva flow. Saliva was collected from the floor of the mouth with a capillary micropipette during the first 15 minutes after pilocarpine injection, and normalized to total body weight on the day of saliva collection. Mice did not receive cevimeline or normal saline on the day they were stimulated with pilocarpine.

#### 5. Immunofluorescence analysis

After treatment with cevimeline for 10 minutes, submandibular glands were dissociated ( $n = 3$ ). The tissue specimens were embedded in OCT compound (Tissue-tek, Miles, IN) and frozen in liquid nitrogen. Frozen sections were cut into 4  $\mu$ m slices. After washing in phosphate buffered saline (3 times for 5 minutes each time), the sections were fixed in cold acetone for 5 min, and then washed. The sections were blocked in 10% fetal bovine serum for 1 hour. The sections were incubated overnight at 4°C with primary antibodies, goat polyclonal anti-AQP5 antibodies (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and then washed. Washed sections were incubated for 1 hour with FITC-Donkey anti-Goat IgG antibodies (1:100 dilution; The Jackson Laboratory, Bar Harbor, ME). Sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA). To evaluate the amount of AQP5, tissues showing deposition of fluorescence to FITC (The Jackson Laboratory) were observed under a fluorescent microscope, BX50 (Olympus, Japan). These were measured at a magnification of  $\times 400$ . The images were then analyzed with

the Color Image Analyzer (Mac scope, Mitani, Fukui, Japan) at 20 randomly selected sites each (0.016 mm<sup>2</sup>) on the apical plasma membrane of the acinar cells.

#### 6. Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from submandibular glands ( $n = 3$ ) was isolated using the EZ1 RNA Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five micrograms extracted RNA was used as a template for cDNA synthesis using River Script II (Nippon Gene, Tokyo, Japan) and random primer according to the manufacturer's instructions. Quantification of AQP5 mRNA was performed using RT-PCR with the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and qPCR Mastermix Plus for SYBER Green I (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. The sequence of the primer to amplify mouse AQP5 was designed as follows: forward primer, 5'-GCC TTATCCATTGGCTTGTC-3'; reverse primer, 5'-CCCAGAAGACCCAGTGAGAG-3'. The product size amplified with this primer set was 138 bp. The sequence of the primer to amplify mouse actin was designed as follows: forward primer, 5'-CATTGCTGACAGGAT GCAGAA-3'; reverse primer, 5'-GCTGATCCA CATCTGCTGGA-3'. The product size amplified with this primer set was 150 bp. These primers were designed using Primer Express software (Applied Biosystems). PCR conditions included an initial incubation at 95°C for 10 min followed by 40 cycles comprising 15 s at 95°C–60 s at 60°C. The relative quantities of different mRNA transcripts were calculated after normalization of the data against the level of actin using the comparative CT method. Expression was calculated as relative to that of day 1 of group N, defined as 1.

#### Statistical Analysis

The experiments were repeated two times. Values given are the means of all measure-

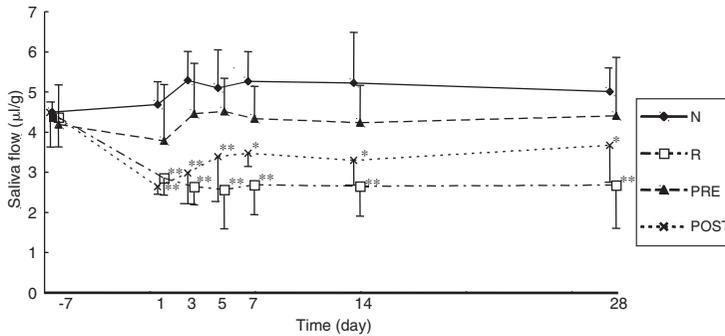


Fig. 2 Effects of cevimeline on saliva flow rate during first 15 min after pilocarpine (0.5 mg/kg) stimulation.

Mice saliva flow in non-irradiated (N), irradiated (R), irradiated + pre-treatment with cevimeline (R+PRE), and irradiated + post-treatment with cevimeline (R+POST) groups. Five mice in each group were analyzed. Values represent mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$  compared with group N.

ments. The student's *t*-test was used to determine significant differences between groups. Statistical analysis of mRNA expression data was performed using one-way analysis of variance (ANOVA). The relative expression of AQP5 mRNA in the submandibular gland was calculated based on the  $\Delta\Delta C_t$  method.

## Results

### 1. Effects of cevimeline on saliva flow

Figure 2 shows the effects of cevimeline on saliva flow rate ( $n = 5$ ). In the R group, irradiation significantly decreased saliva flow rate at 1, 3, 5, 7, 14 and 28 days ( $2.85 \pm 0.42$ ,  $2.64 \pm 0.46$ ,  $2.57 \pm 0.97$ ,  $2.68 \pm 0.74$ ,  $2.66 \pm 0.74$ ,  $2.68 \pm 1.08 \mu\text{l/g}$ ) compared with the N group ( $4.68 \pm 0.57$ ,  $5.29 \pm 0.72$ ,  $5.10 \pm 0.95$ ,  $5.26 \pm 0.74$ ,  $5.22 \pm 1.26$ ,  $5.00 \pm 0.59 \mu\text{l/g}$ ) ( $p < 0.01$ ). In the R+POST group, saliva flow rate was also significantly decreased at 1, 3, 5, 7, 14 and 28 days ( $2.65 \pm 0.20$ ,  $2.98 \pm 0.73$ ,  $3.40 \pm 1.12$ ,  $3.47 \pm 0.32$ ,  $3.29 \pm 0.61$ ,  $3.67 \pm 0.92 \mu\text{l/g}$ ) compared with the N group ( $p < 0.01$ ). Although a slight recovery in saliva flow rate was observed at 7 days after irradiation compared with in the R group, none of these increases were significant compared with in the R group ( $p < 0.05$ ). In contrast, in the R+PRE group, saliva flow rate showed no significant decrease

at 1, 3, 5, 7, 14 or 28 days ( $3.78 \pm 1.40$ ,  $4.46 \pm 1.26$ ,  $4.51 \pm 0.83$ ,  $4.33 \pm 0.81$ ,  $4.23 \pm 0.93$ ,  $4.40 \pm 1.46 \mu\text{l/g}$ ) compared with the N group ( $p < 0.05$ ). A recovery in saliva flow rate was seen at 3 days after irradiation compared with in the R group. These increases were significant compared with R group during days 1–28 ( $p < 0.01$ ).

### 2. Immunofluorescence

An example of distribution of AQP5 in mouse submandibular gland by immunofluorescence with affinity-purified antibodies to mouse AQP5 is shown in Fig. 3. AQP5 is normally expressed in the apical plasma membrane of acinar (Fig. 3A) and duct cells (Fig. 3B). In this study, comparative experiments were performed in acinar cells. Immunofluorescence detection of AQP5 protein in mouse submandibular glands in the R, R+PRE and R+POST groups at 0, 1, 7, 14 and 28 days after irradiation are shown in Fig. 4A ( $n = 3$ ). Immunolocalization of AQP5 protein, which was analyzed by fluorescent intensity with the Color Image Analyzer, is shown in Fig. 4B. In the R group, fluorescence intensity in the acinar cells was significantly decreased at 1, 3, 5, 7, 14 and 28 days ( $35.72 \pm 9.36$ ,  $33.72 \pm 8.04$ ,  $31.42 \pm 6.68$ ,  $30.82 \pm 8.5$ ,  $30.56 \pm 8.43$ ,  $30.64 \pm 8.43$ ) compared with the N group ( $70.62 \pm 12.5$ ,

71.01 ± 12.8, 69.74 ± 12.31, 71.14 ± 13.08, 69.26 ± 14.25, 69.38 ± 13.97) ( $p < 0.01$ ). In the R+POST group, fluorescence intensity in the acinar cells was significantly decreased at 1, 3, 5, 7, 14 and 28 days (35.42 ± 10.67, 36.99 ± 10.22, 41.11 ± 9.54, 47.04 ± 13.04, 54.31 ± 11.79, 59.89 ± 17.14) compared with the N group ( $p < 0.01$ ). However recoveries were observed at 3 days after irradiation compared with the R group. In contrast, in the R+PRE group, fluorescence intensity in the acinar cells was increased significantly at 0 day (96.01 ± 17.25) compared with the N group (70.69 ± 10.33) ( $p < 0.01$ ). However, fluorescence intensity decreased significantly after 3, 5 and 7 days (55.21 ± 14.05, 52.65 ± 10.71, 57.93 ± 17.82) ( $p < 0.01$ ), increasing again at 7 days compared with the R group. After 14 days, fluorescence intensity showed no significant decrease (62.19 ± 12.26) compared with the N group ( $p < 0.05$ ), and recoveries were observed compared with the R group.

In the CE group, fluorescent intensity at 7, 14, 21 and 28 days after treatment is shown in Fig. 5A. Immunolocalization of AQP5 protein, which was analyzed via fluorescent intensity with the Color Image Analyzer, is shown in Fig. 5B. Fluorescence intensity showed a significant increase in the acinar cells at 7, 14, 21 and 28 days (96.48 ± 17.25, 96.31 ± 13.19, 105.36 ± 21.46, 105.85 ± 17.00) compared with the N group ( $p < 0.01$ ).

### 3. Expression of AQP5 mRNA

Figure 6 shows the expression of AQP5 mRNA in mouse submandibular glands by qRT-PCR. Figure 6A shows the CE and N groups at 0, 7, 14, 21 and 28 days after treatment with cevimeline, and Fig. 6B shows the R, PRE and POST groups at -7, 1, 7, 14 and 28 days after irradiation.

Expression of AQP5 mRNA was highest at 7 days in the CE group. However, expression of AQP5 mRNA was slightly higher in the CE group than in the N group. In the R and R+POST groups, expression of AQP5 mRNA was lower than in the N group at -7, 1, 7, 14 and 28 days, but not significantly so. In the R+POST group, slight recoveries were

observed at 7 days after irradiation. In contrast, in the R+PRE group, expression of AQP5 mRNA at 1 day (1.78 ± 0.23) was significantly higher than that in the N group (0.99 ± 0.38). Expression of AQP5 mRNA in the R+PRE group was similar to that in the N group during days 14–28. In addition, in the CE group, expression of AQP5 mRNA at 7 and 14 days (1.82 ± 0.36, 1.51 ± 0.20) was significantly higher than that in the N group (0.99 ± 0.38, 1.06 ± 0.21). In all groups, RT-PCR products of AQP5 and actin were obtained with a single band (data not shown).

## Discussion

Irradiation did not decrease body weight in any group. In contrast, in previous studies<sup>2,14</sup>, irradiated animals have been shown to display a reduction in body weight. These changes were probably caused by changes in the amount of food and water intake caused by anesthesia and irradiation. In this study, the oral cavity was not included in the X-ray field, which may have minimized the side effects (ulcer, mucositis etc.) of irradiation.

We observed that irradiation significantly decreased the saliva flow rate. Post-treatment with cevimeline also significantly decreased the saliva flow rate, although slight recoveries were observed at 3 days after irradiation. Pre-treatment did not significantly decrease the saliva flow rate. These data suggest that salivary flow is significantly decreased by irradiation. However, pre-treatment prevents irradiation-induced decrease in saliva flow rate.

Coppes *et al.*<sup>5,6</sup> reported that pre-treatment with muscarinic acetylcholine receptor and alpha-adrenoceptor agonists protected salivary glands from radiation damage. Moreover, they suggested that indication of protection by muscarinic acetylcholine receptor and alpha-adrenoceptor agonists was mediated by PLC/PIP2 second messenger pathways. Tapp and Trowell<sup>26</sup> suggested that pre-treatment with the muscarinic acetylcholine receptor agonist pilocarpine caused the formation of watery vacuoles. They hypothesized that this vacuole

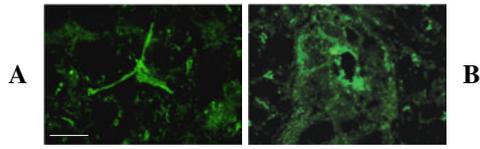


Fig. 3

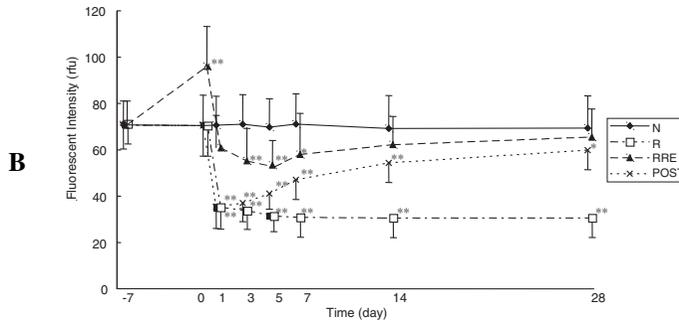
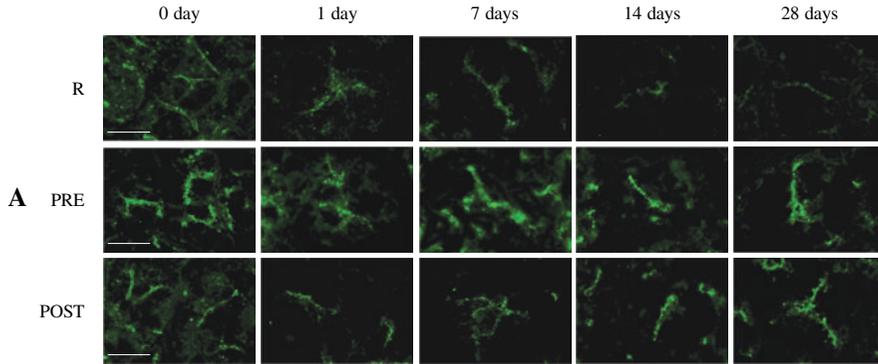


Fig. 4

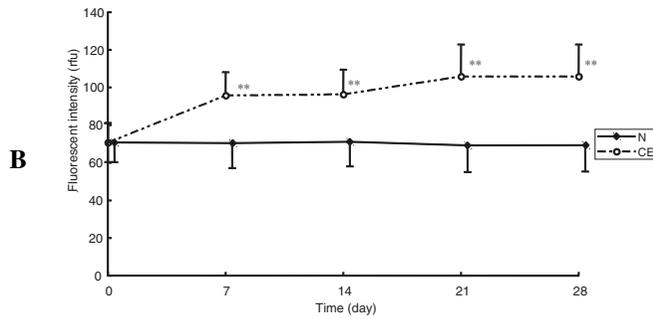
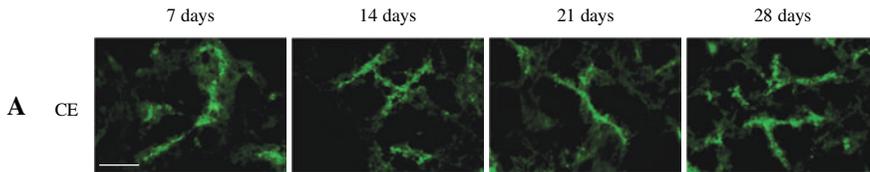


Fig. 5

formation was the main prophylactic mechanism of muscarinic acetylcholine receptor agonists against irradiation damage.

Previous reports have shown that in the early stage, there is no correlation between apoptosis and salivary gland dysfunction<sup>6,20</sup>. Therefore, radiation-induced apoptosis alone cannot explain the mechanisms of salivary gland dysfunction. Moreover, free radicals are also important factors for radiation. It is known that hydroxyl radicals indirectly injure DNA<sup>18</sup>. An earlier study showed the pathway by which nitric oxide affects the pathogenesis of radiation-induced salivary gland dysfunction<sup>25</sup>.

Irradiation decreased AQP5 fluorescence intensity and expression of AQP5 mRNA in the submandibular glands. Post-treatment with cevimeline also decreased AQP5 fluorescence intensity and expression of AQP5 mRNA, although slight recoveries were observed 3–7 days after irradiation. In contrast, pre-treatment increased AQP5 fluorescence intensity and expression of AQP5 mRNA in submandibular glands at 7 days compared with non-irradiated glands. Li *et al.*<sup>14</sup> reported that irradiation decreased AQP5 significantly in rat submandibular gland. Our data are consistent with their results. Moreover, they also reported that AQP5 was possibly more radiosensitive than other proteins. Our data suggest that irradiation significantly decreases AQP5 in submandibular glands and that AQP5 is essential in saliva flow. Using immunofluorescence analysis and qRT-PCR analysis, we clarified that daily treatment with Cevimeline

increased AQP5 expression. This suggests that pre-treatment prevents radiation-induced salivary gland dysfunction.

Previous studies have proposed that radiation-induced dysfunction such as loss of AQP5 and other membrane fusion proteins in acinar cells may cause dysfunction<sup>14,24</sup>. In addition, the higher radiosensitivity of AQP5 compared with other proteins has also been reported<sup>14</sup>. However, these studies did not investigate the biological activity of AQP5. AQP5 localizes in lipid raft and induced transport to the apical plasma membrane in interlobular duct cells of rat parotid glands by activation of M3 muscarinic acetylcholine receptors<sup>9</sup>. Recent studies have demonstrated that a defect AQP5 transport might contribute to decreased lacrimation<sup>27</sup> and saliva flow<sup>11,23</sup> in patients with Sjögren's syndrome. Although a senescent xerostomia rat model showed normal transport of AQP5, a streptozotocin-induced diabetic xerostomia rat model revealed disordered trafficking<sup>8</sup>. The translocation of AQP5 by cevimeline is mediated by elevation of intracellular Ca<sup>2+</sup> concentrations<sup>9</sup>. Radiation impairs mobilization of Ca<sup>2+</sup> from intracellular Ca<sup>2+</sup> stores (such as endoplasmic reticulum<sup>4</sup>). It is possible that radiation-induced disorders in mobilization of Ca<sup>2+</sup> might impair transport of AQP5. Therefore, AQP5 trafficking must be investigated in future studies.

Clinically, saliva flow rate decreases during the first week of radiation with a fractionation protocol of Gy/dose 1 day<sup>3,17</sup>. Therefore, we cannot rule out the possibility of regulation of

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- Fig. 3 Distribution of AQP5 in submandibular gland  
Distribution of AQP5 in submandibular gland in acinar cells (A) and duct cells (B) by immunofluorescence with affinity-purified antibodies to mouse AQP5. Scale bar = 20  $\mu$ m.
- Fig. 4 Immunofluorescence detection of AQP5 and expression of AQP5 analyzed by fluorescent intensity  
Immunofluorescence detection of AQP5 in mouse submandibular glands of (A) irradiated (R), irradiated + pre-treatment with cevimeline (R + PRE), and irradiated + post-treatment with cevimeline (R + POST) groups at 0, 7, 14, 21 and 28 days after irradiation. Scale bar = 20  $\mu$ m. (B) Twenty randomly selected sites (0.016 mm<sup>2</sup>) on apical plasma membrane in acinar cells were analyzed. Non-irradiated (N), irradiated (R), irradiated + pre-treatment with cevimeline (R + PRE), and irradiated + post-treatment with cevimeline (R + POST) groups, at -7, 0, 1, 3, 5, 7, 14 and 28 days after irradiation. Values represent mean  $\pm$  SD. \**p* < 0.05, \*\**p* < 0.01 compared with group N. (n = 3)
- Fig. 5 Immunofluorescence detection of AQP5 and expression of AQP5 analyzed by fluorescent intensity  
Immunofluorescence detection of AQP5 in mouse submandibular glands of (A) non-irradiated + treatment with cevimeline (CE) groups at 7, 14, 21 and 28 days after treatment with cevimeline. Scale bar = 20  $\mu$ m. (B) Twenty randomly selected sites (0.016 mm<sup>2</sup>) on apical plasma membrane in acinar cells were analyzed. Non-irradiated + treatment with cevimeline (CE) and non-irradiated (N) groups at 0, 7, 14, 21 and 28 days after treatment with cevimeline. Values represent mean  $\pm$  SD. \**p* < 0.05, \*\**p* < 0.01 compared with group N. (n = 3).

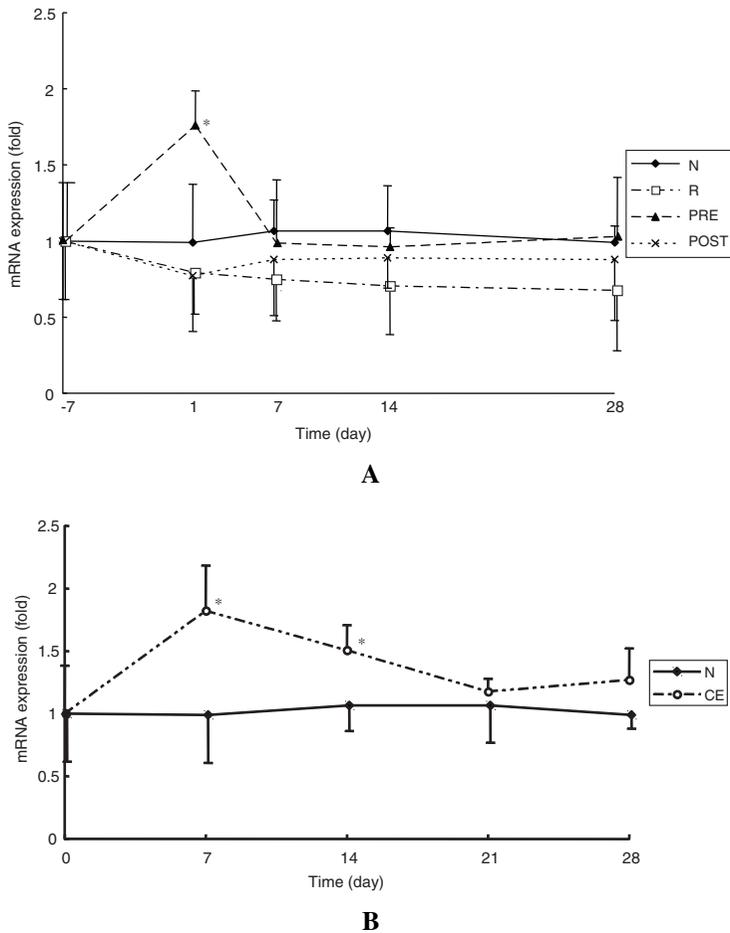


Fig. 6 Comparison of mRNA expression of AQP5

Comparison of mRNA expression of AQP5 in mouse submandibular glands in (A) non-irradiated + treatment of cevimeline (CE) and non-irradiated (N) groups at 0, 7, 14, 21 and 28 days after treatment with cevimeline and (B) non-irradiated (N), irradiated (R), irradiated + pre-treatment with cevimeline (R+PRE), and irradiated + post-treatment with cevimeline (R+POST) groups at -7, 1, 7, 14 and 28 days after irradiation. Three mice in each group were analyzed. Values were normalized to actin as endogenous control. Expression was calculated as relative to that of day 1 in group N, defined as 1. Relative expression of AQP5 mRNA in submandibular gland was calculated based on  $\Delta\Delta C_t$  method. Values represent mean  $\pm$  SD. \* $p < 0.05$  compared with group N.

AQP5 expression. In the epithelial cells of the lung, it has been demonstrated that AQP5 transcription is regulated by cyclic AMP/protein kinase A (PKA) pathways<sup>30</sup>. It is possible that cevimeline causes activation of cyclic AMP-dependent PKA pathways. In fact, other muscarinic receptor agonists, carbachol and isoproterenol, increase saliva flow mediated through cyclic AMP pathways<sup>28</sup>. In contrast to AQP5, it has been demonstrated that signal

transduction through protein kinase C (PKC) plays an important role in the expression of AQP4 and AQP9<sup>29</sup>. More recently, it has been demonstrated that radiation caused a significant decrease in AQP5 expression, with reduction in AQP1 and  $\text{Na}^+/\text{K}^+$ -ATPase proteins in submandibular gland<sup>14</sup>.

In conclusion, pre-treatment with cevimeline prevented radiation-induced salivary gland dysfunction. Furthermore, cevimeline

pre-treatment was more effective than post-treatment. AQP5 may play an important role in radiation-induced salivary dysfunction. Protection of AQP5 may offer a therapeutic strategy for this clinically severe side effect of radiation therapy.

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