

# **Hyperoxia reduces salivary secretion by inducing oxidative stress in mice**

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Running title: Hyperoxia induces hyposalivation

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### **Acknowledgements**

The present study was financially supported by Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (Okayama, Japan).

### **Conflict of interest statement**

No conflicts of interest have been declared

The present study was previously presented, in part, at 93th General Session & Exhibition of the International Association for Dental Research in Boston, USA on March 13, 2015

# **Abstract**

## **Objective**

The aim of this study was to determine the effects of prolonged hyperoxia on salivary glands and salivary secretion in mice.

## **Design**

Male C57BL/6J mice were kept in a 75% oxygen chamber (hyperoxia group) or a 21% oxygen chamber for 5 days. We measured the secretion volume, protein concentration, and amylase activity of saliva after the injection of pilocarpine. In addition, we evaluated the histological changes induced in the submandibular glands using hematoxylin and eosin and Alcian blue staining and assessed apoptotic changes using the TdT-mediated dUTP nick end labeling (TUNEL) assay. We also compared the submandibular gland expression levels of heme oxygenase-1 (HO-1), superoxide dismutase (SOD)-1, and SOD-2 using the real-time polymerase chain reaction.

## **Results**

In the hyperoxia group, salivary secretion was significantly inhibited at 5 and 10 minutes after the injection of pilocarpine, and the total salivary secretion volume was significantly decreased. The salivary protein concentration and amylase activity were also significantly higher in the hyperoxia group. In the histological examinations,

enlargement of the mucous acini and the accumulation of mucins were observed in the submandibular region in the hyperoxia group, and the number of TUNEL-positive cells was also significantly increased in the hyperoxia group. Moreover, the expression levels of HO-1, SOD-1, and SOD-2 were significantly higher in the hyperoxia group.

### **Conclusion**

Our results suggest that hyperoxia reduces salivary secretion, and oxidative stress reactions might be involved in this.

Keywords: hyperoxia, saliva, hyposalivation, oxidative stress

## Introduction

Recently, it was demonstrated that oral care contributes not only to the maintenance of oral functions, but also to improving patients' general health, e.g., it helps to prevent pneumonia (Yoneyama, Yoshida, Matsui, Sasaki & the Oral Care Working Group, 1999). Moreover, it was reported that oral care during the perioperative period reduces the risk of postoperative pneumonia (Akutsu et al., 2010). These findings suggest that among patients that undergo surgery the maintenance of oral function during the perioperative period might contribute to improving patient prognosis, and oral healthcare during the perioperative period has received a lot of attention in recent years.

Saliva is a digestive fluid containing digestive enzymes and is secreted during the ingestion of foods. It also has other roles, e.g., it has lubricating, anti-bacterial/bactericidal, buffering, protective, anti-demineralizing, and washing effects, and these roles are very important for helping to maintain a suitable oral environment and oral functions (Frenkel & Ribbeck, 2015; Humphrey & Williamson, 2001). In other words, among patients that undergo surgery suitable salivation is very important for ensuring that oral functions are maintained during the perioperative period.

In the clinical setting, patients are exposed to high concentrations of oxygen during perioperative care and/or intensive medical care; i.e., oxygen therapy, which involves

the prolonged administration of high concentrations of oxygen, is routinely used to ensure that the organs are sufficiently oxygenated. However, the use of high-concentration oxygen therapy is being reviewed in the light of oxygen's noxious effects (Habre & Petak, 2014) because hyperoxia is assumed to be a common cause of oxidative stress. The adverse effects of hyperoxia have been reported in previous studies. In the lungs, hyperoxia induces alveolar congestion, pulmonary bleeding, inflammatory cell infiltration, and cell death, and it has severely adverse effects on lung functions (Nagato et al., 2012; Pagano & Barazzzone, 2006; Yu et al., 2015). In the brain, hyperoxia induces neuronal apoptosis and inhibits brain development (Felderhoff-Mueser et al., 2004). In the eyes, hyperoxia also induces cell death in retinal photoreceptors (Natoli, Provis, Valter & Stone, 2008). With regard to saliva, it was reported that short-term exposure (30 minutes) to 100% oxygen had anti-stress effects and decreased amylase activity in human saliva (Kawada, Fukusaki, Ohtani & Kobayashi, 2009). However, it is unknown whether prolonged exposure to high concentrations of oxygen has beneficial or noxious effects because no previous studies have evaluated the effects of prolonged exposure to hyperoxia on the salivary glands or saliva secretion.

We are concerned that prolonged high-concentration oxygen therapy might have adverse effects on salivation, as it does in the lungs, brain, and eyes. It is very important that all healthcare workers who are engaged in oral healthcare understand the effects of prolonged hyperoxia on salivary function. So, the aim of this study was to evaluate the influence of prolonged hyperoxia on salivary function in mice.

## **Materials and Methods**

This study was carried out in strict accordance with the recommendations outlined in the Guide for the Care and Use of Laboratory Animals at Okayama University. The protocol of the present study was approved by the Animal Care and Use Committee of Okayama University (No. OKU-2014139).

### **1. Animals**

We used male C57BL/6J mice (age: 30 weeks old), which were obtained from SHIMIZU Laboratory Co., Ltd. (Kyoto, Japan). The mice were housed under a 12-h day/night cycle and provided with food and water ad libitum.

### **2. Hyperoxia protocol**

The mice were randomly assigned to hyperoxia and control groups. The hyperoxia group was exposed to 75% oxygen for 5 days, and the control group was exposed to 21% oxygen for 5 days. We used a dedicated animal chamber (Biospherix, Ltd., Parish, NY) for the oxygen exposure and constantly controlled the oxygen concentration using a ProOx110 oxygen controller (Biospherix, Ltd., Parish, NY). We placed GRACE

SODASORB® LF (Smiths Medical, Ltd., Hythe, England) in the chamber to remove any surplus carbon dioxide. All of the mice were given free access to standard rodent food and tap water during the hyperoxic period.

### **3. Measurements of salivary secretion**

We used a previously described method to obtain the salivary secretion measurements (Gautam et al., 2004; Iida, Ono, Inagaki, Hosokawa & Inenaga, 2011; Matsui et al., 2000; Nakamura et al., 2004). The mice were fasted for the night before the saliva was collected. After the induction of general anesthesia via the intraperitoneal injection of pentobarbital (40.0 mg/kg) (Kyoritsu Seiyaku Corporation, Tokyo, Japan), pilocarpine (1.0 mg/kg) (Santen Pharmaceutical Co., Ltd., Osaka, Japan) was injected subcutaneously. We collected saliva from the oral cavity using pre-weighed pieces of cotton every 5 minutes for a total of 30 minutes. We then weighed the pieces of cotton again, and the increase in weight was calculated as a measure of salivary secretion and normalized to the weight of each mouse.

### **4. Biochemical analysis of saliva**

Mice were anesthetized and injected with pilocarpine as described above. Then, saliva was collected from the oral cavity using a micropipette. The total protein concentration of the saliva was measured using the Coomassie plus (Bradford) assay kit (Thermo Fisher Scientific, Inc., Waltham, MA), according to the manufacturer's instructions. Amylase activity was also measured using the salivary  $\alpha$ -amylase kinetic enzyme assay kit (Salimetrics, Inc., Carlsbad, CA), according to the manufacturer's instructions.

## **5. Histology**

After the submandibular glands had been harvested, they were immediately placed in 4% paraformaldehyde and embedded in paraffin. Paraffin blocks were sectioned at a thickness of 4  $\mu$ m and stained with hematoxylin and eosin (H-E) and Alcian blue, according to the standard pathology department protocols. The TdT-mediated dUTP nick end labeling (TUNEL) assay was performed using the in situ apoptosis detection kit (TAKARA BIO, Inc., Shiga, Japan), according to the manufacturer's instructions. All sections were imaged using a binocular microscope system (microscope: OLYMPUS BX53; digital camera: OLYMPUS DP70; imaging software: OLYMPUS

cellSens, Tokyo, Japan). To evaluate the area of Alcian blue staining, one image (160  $\mu\text{m}$  x 220  $\mu\text{m}$ ) of each section was randomly captured and opened in the software ImageJ (available at <https://imagej.nih.gov/ij/index.html>, developed by Wayne Rasband, National Institutes of Health). Then, the area of blue staining was determined by the measuring function of ImageJ. The number of TUNEL-positive cells was also evaluated using ImageJ, and images of 4 areas (160  $\mu\text{m}$  x 220  $\mu\text{m}$ ) in each section were randomly captured. The images were opened in the software ImageJ, and the number of positive cells per 1000  $\mu\text{m}^2$  was calculated and averaged. The investigator who measured the area of Alcian blue staining and counted the number of positive cells was blinded to the source of the sections.

## **6. Quantitative real-time PCR**

Mice were euthanized via cervical dislocation under isoflurane-induced anesthesia (AbbVie, Inc., Chicago, IL), and then the submandibular glands, brain cortex, lungs, tongue, liver, and kidneys were harvested. Each tissue was homogenized with TRIzol reagent (Life Technologies, Ltd., Carlsbad, CA) on ice, and chloroform was added to the homogenate. After the homogenate was centrifuged at 12000 rpm, the clear top

layer was extracted, and then isopropanol and glycogen were added (Roche Diagnostics K. K., Basel, Switzerland). The resultant RNA pellet was rinsed with 80% ethanol and then dissolved with nuclease-free water (Promega Corporation, Fitchburg, WI). The RNA was transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen N.V., Venlo, The Netherlands), according to the manufacturer's instructions.

The quantitative real-time polymerase chain reaction (PCR), which was performed using the real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA), was used to analyze the mRNA expression levels of heme oxygenase-1 (HO-1), superoxide dismutase-1 (SOD-1), SOD-2, interleukin-6 (IL-6), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). The reactions were performed with SYBR Premix Ex Taq II (TAKARA BIO, Inc., Shiga, Japan), according to the manufacturer's instructions.  $\beta$ -actin was used as an internal standard. The forward and reverse primers used in this study are listed in Table 1.

## **7. Statistical analysis**

All statistical analyses were performed using statistical software (GraphPad Prism ver. 6; GraphPad Software, Inc., La Jolla, CA). We employed the Student's t-test for

comparisons of continuous data. We employed two-way analysis of variance (ANOVA) (with salivary secretion as the main factor and time as the repeated measure) and Bonferroni's post-hoc test for the comparisons of salivary secretion at each time point. Significance was defined as a P-value of  $<0.05$  in the Student's t-test and two-way ANOVA, and a P-value of  $<0.01$  in Bonferroni's post-hoc test.

## Results

### 1. Hyperoxia-induced hyposalivation and changes in the levels of salivary components

At first, we examined whether hyperoxia influenced salivary secretion in mice. No significant difference in the body weights of the mice was observed between the two groups (hyperoxia group:  $27.22 \pm 2.0$  g, control group:  $25.44 \pm 1.9$  g). In the hyperoxia group, the salivary secretion induced by the subcutaneous injection of pilocarpine was significantly decreased compared with that seen in the control group at 5 and 10 minutes after the injection (two-way ANOVA with Bonferroni's post-hoc test, the interaction between the main factor and time was significant) (Fig. 1A). The total volume of saliva released during the 30-minute test period was also significantly decreased in the hyperoxia group (hyperoxia group:  $13.3 \pm 2.1$  mg/g, control group:  $17.6 \pm 0.9$  mg/g) (Fig. 1B). Next, we measured the protein concentration and amylase activity of saliva to evaluate the changes in the levels of salivary components induced by hyperoxia (Figs. 1C and 1D). The protein concentrations of the pooled saliva from 1 to 30 minutes did not differ significantly between the two groups. However, during the first 15 minutes the pooled saliva of the hyperoxia group (1-15 minutes) had a higher

protein concentration than that of the control group. Regarding amylase activity, the pooled saliva of the hyperoxia group exhibited significantly higher amylase activity during the first 15 minutes (1-15 minutes) and from 1-30 minutes than that of the control group. However, there were no significant intergroup differences in the protein concentration or amylase activity of the pooled saliva from 15 to 30 minutes.

## **2. Histological changes induced in the submandibular gland by hyperoxia**

In both groups, H-E staining of the submandibular glands showed a lobular structure with acini and ducts. In the control group, serous acini accounted for a large part of the submandibular gland; however, in the hyperoxia group the mucous acini were enlarged and accounted for a greater proportion of the submandibular gland than the serous acini. In addition, we evaluated the levels of acidic polysaccharides, such as mucins, in the submandibular gland using Alcian blue staining. Significantly greater amounts of mucins accumulated in the submandibular gland in the hyperoxia group than in the control group (Fig. 2). Moreover, we also assessed the apoptotic changes induced in the submandibular gland by hyperoxia. TUNEL assays showed that the number of apoptotic

cells was significantly greater in the hyperoxia group than in the control group (hyperoxia group:  $1.89 \pm 0.73/1000 \mu\text{m}^2$ , control group:  $0.67 \pm 0.57/1000 \mu\text{m}^2$ ) (Fig. 3).

### **3. Hyperoxia-induced oxidative stress in the submandibular gland in mice**

To clarify the etiology of the changes seen in our model, we evaluated the effects of hyperoxia on the submandibular gland using oxidative stress and inflammatory markers, such as SOD-1, SOD-2, IL-6, TNF- $\alpha$  and HO-1 (Fig. 4 and Fig. 5). As a result, it was found that the SOD-1, SOD-2 and HO-1 mRNA levels of the hyperoxia group were significantly higher than those of the control group. However, there was no significant difference in IL-6 or TNF- $\alpha$  mRNA expression between the two groups. This suggested that exposure to hyperoxia induces oxidative stress, but not inflammation, in the submandibular gland.

Finally, we also evaluated the effects of hyperoxia on other organs, such as the brain cortex, lungs, tongue, liver, and kidneys, to compare the susceptibility of each organ to oxidative stress (Fig. 5). In the submandibular glands, lungs, and kidneys, the HO-1

mRNA levels of the hyperoxia group were significantly higher than those of the control group. This suggested that the submandibular gland is highly sensitive to hyperoxia.

## Discussion

The present study demonstrated that hyperoxia induced hyposalivation and increased the protein concentration and amylase activity of saliva, the accumulation of mucins, and apoptotic changes in the submandibular gland. Moreover, hyperoxia induced increases in HO-1, SOD-1, and SOD-2 mRNA expression in the submandibular gland.

It was reported that similar hyposalivation is caused by aging, Sjögren's syndrome, and radiation-induced disorders. Choi, Park, Kim, Lim and Kim (2013) examined age-related changes in the functional morphology of the salivary gland using 10-, 30-, and 90-week-old mice. In the latter study, acinar cell atrophication, cytoplasmic vacuolization, lymphocyte infiltration, small mucin components, and increased periductal fibrosis were seen in the 90-week-old mice. TUNEL assays also revealed that compared with the younger mice apoptotic salivary epithelial cells were significantly more numerous, the salivary lag time was significantly greater, and the flow rate of saliva was significantly lower in the 90-week-old mice. Acinar cell destruction, lymphocyte infiltration, and increased numbers of TUNEL-positive cells were found to be histological features of Sjögren's syndrome (Fox, 2005; Ishimaru et al., 2000). Moreover, cytoplasmic vacuolization, increased numbers of TUNEL-positive cells, and

lymphocyte infiltration were observed in radiation-damaged salivary glands (Muhvic-Urek et al., 2006). Hyposalivation was also reported in an experimental periodontitis model (Nakamura-Kimura et al., 2014), and an increased number of TUNEL-positive cells, cytoplasmic vacuolization, and greater amounts of 8-hydroxy-guanosine (8-OHdG) (a marker of oxidative damage) were detected in this model (Ekuni et al., 2010). Thus, all of these conditions exhibit cytoplasmic vacuolization, increased numbers of TUNEL-positive cells, and lymphocyte infiltration. In our hyperoxia study, we detected changes in the morphology of the salivary gland, such as enlarged mucous acini, the accumulation of mucins, and an increased number of TUNEL-positive cells. However, no acinar cell atrophication, cytoplasmic vacuolization, or lymphocyte infiltration was observed. We measured the levels of IL-6 and TNF- $\alpha$  in the salivary glands using PCR analysis to evaluate the changes in the concentrations of these inflammation-associated molecules, but no significant changes in the levels of these molecules were seen, and the inflammation was not related to the etiology by the hyperoxia exposing.

In the current study, enlarged mucous acini and the accumulation of mucins were observed in the hyperoxia group, and these histological findings were suggestive of changes in the levels of salivary components. Inoue et al. (2008) reported that the viscosity of saliva was positively correlated with the total protein concentration, and the

protein concentration of saliva was related to the composition of saliva. Therefore, we examined the changes in salivary components induced by hyperoxia by measuring the protein concentration and amylase activity of saliva, and both of these parameters were increased in the hyperoxia group, especially during the first 15 minutes after the injection of pilocarpine. However, there are two possible explanations for this result. One is that the amounts of protein and amylase being discharged in saliva had not changed (or had decreased slightly), but the protein concentration and amylase levels of the saliva had increased in relative terms due to a reduction in the water content of the saliva. The other is that the amounts of protein and amylase in the saliva had truly increased in response to stress. It is well known that a number of stressors activate sympathetic neuronal systems and increase salivary protein output (Keremi et al., 2017; Racz et al., 2018). In our study, the increases in the protein concentration and amylase activity of saliva seen in the hyperoxia group were synchronized with the reduction in salivary secretion observed in the early period after the injection of pilocarpine. These results support the former explanation for the increases in amylase activity and the protein concentration of saliva and suggest that hyperoxia might suppress the secretion of serous saliva and mainly affect the ion and water content of saliva, rather than its

protein concentration. However, no definitive conclusions regarding this issue can be drawn from our findings.

To clarify the mechanisms underlying the changes seen in our model, we evaluated HO-1, SOD-1, and SOD-2 mRNA expression in the submandibular gland. HO-1 breaks heme down into biliverdin, which has antioxidant effects. Therefore, increased HO-1 expression is associated with resistance to hyperoxia, and HO-1 probably plays a protective role against hyperoxic damage (Mantell & Lee, 2000). SOD is an intracellular enzyme that breaks superoxide down, and it has a protective effect against hyperoxia-induced damage (Zaghloul et al., 2012). Therefore, HO-1, SOD-1, and SOD-2 expression are related to oxidative stress, and hence, are used as oxidative stress markers.

Oxygen is essential for human survival because it is required for oxidative phosphorylation, which is involved in the production of adenosine triphosphate (ATP) in biological tissues. After oxygen is taken up into the body, some of it is converted to reactive oxygen species (ROS), such as the hydroxyl radical ( $\bullet\text{OH}$ ) and the superoxide radical ( $\text{O}_2\bullet^-$ ). Regarding the physiological roles of ROS in vivo, they are involved in defending the body against foreign substances, such as microbes; controlling signaling; and inhibiting transcription. On the other hand, ROS are highly reactive and readily

induce cell damage and functional disturbances. Normally, antioxidant defense systems protect individuals from oxygen's noxious effects (Kregel & Zhang, 2007), but if the activity of ROS exceeds the capacity of the antioxidant defense systems, oxidative stress, such as lipid peroxidation, protein peroxidation, DNA damage, apoptosis, and/or necrosis, is induced (Mantell & Lee, 2000; Yu et al., 2015). We found that the expression levels of HO-1, SOD-1, and SOD-2 in the submandibular gland of hyperoxia group were significantly increased, which suggested that oxidative stress had been induced in the submandibular gland by hyperoxia. Moreover, our findings indicated that the susceptibility of organs to hyperoxia differs, and that the submandibular glands are highly susceptible to hyperoxia. It is well known that radiation sensitivity differs among organs (Busch, 1993). Generally, cells that exhibit faster cell cycles and/or display more undifferentiated forms and functions are more susceptible to radiation, and hence, suffer more severe radiation damage. The cell cycle does not operate very quickly in the salivary glands; however, the salivary glands are susceptible to radiation (Konings, Coppes & Vissink, 2005). Oxidative stress, such as ROS production, is closely related to the pathology of radiation-induced disorders (Lee, Lee & Park, 2005). Based on these findings, it is suggested that the salivary glands might be more susceptible to oxidative stress than other organs, as was indicated in this study.

Saliva includes constituents of non-salivary origin derived from gingival crevicular fluid, expectorated bronchial secretions, serum, or blood cells from oral wounds, as well as bacteria and bacterial products, viruses, fungi, desquamated epithelial cells, and food debris. Therefore, it is considered that saliva is a good diagnostic tool for assessing the oral environment, various systemic conditions, psychological states, and stress (Ghallab, 2018; Keremi et al., 2017). This means that saliva reflects not only local pathological changes affecting the salivary glands, but also general pathological changes. Therefore, when attempting to determine the mechanism responsible for hyperoxia-induced hyposalivation, we have to consider both local and general mechanisms. In order to further investigate our findings, we examined aquaporin-5 (AQP5) expression in the submandibular gland (see the supplementary information). AQP5 is a water channel protein, which plays a role in the regulation of water secretion in the salivary glands (Krane et al., 2001). In addition, transcellular transport mainly depends on AQP5 water channels (Keremi et al., 2017). AQP5-knockout mice exhibited hyposalivation, more viscous saliva, and enlarged acinar cells, and these phenotypes were very similar to those of our hyperoxia model (Kawadia et al., 2007; Ma et al., 1999). Thus, we hypothesized that hyperoxia might affect AQP5 expression in the salivary glands, so we examined it via immunostaining and Western blotting. However, no significant

difference in AQP5 expression was observed between the two groups (see supplementary figure). As for other mechanisms that might be responsible for hyperoxia-induced hyposalivation, circulatory changes in the salivary glands or changes in the susceptibility of the receptors that regulate salivary secretion to hyperoxia are possibilities. It is known that hyperoxia induces vasoconstriction in many organs (Rousseau, Steinwall, Woodson & Sjoberg, 2007), and it also reduces blood flow in the salivary glands. Many previous studies have found that a close relationship exists between salivary secretion and blood flow (Fazekas, Pósch & Zelles, 1983; Shimizu & Taira, 1980), and the drop in blood flow induced by hyperoxia might lead to a reduction in salivary secretion. Moreover, it is well known that the salivary glands are subjected to dual regulation by the sympathetic and parasympathetic nervous systems. Sympathetic stimulation acts on the  $\beta$ -adrenergic receptors, which results in the production of saliva with a higher protein content and lower water volume. On the other hand, parasympathetic stimulation acts on muscarinic receptors and leads to the secretion of saliva with greater amounts of water and electrolytes (Romero, Bergamaschi, de Souza & Nogueira, 2016). Bickford et al. (1999) indicated that both  $\beta$ -adrenergic and muscarinic receptor function were inhibited by hyperoxia. These findings suggest that hyperoxia might influence the local and general neural regulation

of saliva secretion. Another issue that we have to discuss is why saliva secretion was mainly decreased during the early period (5 to 10 minutes) after the initiation of hyperoxia. Similar results were reported in a study involving spontaneously hypertensive rats (Zhang et al., 2017). Pilocarpine-induced saliva secretion was decreased in the hypertensive rats compared with the control rats during the early period after the injection of pilocarpine, as was found in the present study, and AQP5 expression was markedly decreased in the hypertensive rats. As mentioned previously, AQP5 plays a role in the regulation of water secretion in the salivary glands. This means that the water content of saliva might be selectively reduced in spontaneously hypertensive rats. Although the expression level of AQP5 was not changed in our hyperoxia model, our findings suggest that hyperoxia might only affect the salivary secretion of ions and water, which might explain the temporal changes in saliva secretion. However, our results did not clarify the mechanism responsible for hyperoxia-induced hyposalivation, and so further studies will be necessary to elucidate this mechanism.

In conclusion, we evaluated the effects of hyperoxia on the salivary gland. The results of the present study demonstrated that hyperoxia reduced salivary secretion, and the secreted saliva included more protein and amylase. In addition, enlargement of the

mucous acini, the accumulation of mucins, and an increase in the number of TUNEL-positive cells were seen in the salivary glands. Moreover, hyperoxia significantly increased HO-1, SOD-1, and SOD-2 mRNA expression. These findings suggest that the salivary gland is highly sensitive to hyperoxia, and hyperoxia-induced oxidative stress might cause hyposalivation.

### **Acknowledgements**

The present study was financially supported by Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (Okayama, Japan).

### **Conflicts of interest statement**

No conflicts of interest exist.

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## Figure legends

### **Figure 1. The changes in salivary secretion and composition induced by hyperoxia**

(A) Time courses of the changes in salivary secretion seen after the injection of pilocarpine

Data represent the mean  $\pm$  SD (n=5 in each group; data were obtained from 2 independent experiments). \*Adjusted P-values (\*\*P<0.01, \*\*\*P<0.0001) are for between-group comparisons made at the specified timepoints according to two-way ANOVA and Bonferroni's post-hoc test.

(B) Total salivary secretion during the 30 minutes after the injection of pilocarpine

Data represent the mean  $\pm$  SD (n=5 in each group; data were obtained from 2 independent experiments). \*\*P<0.01, according to the Student's t-test

(C) Total protein concentrations of saliva during the whole collection period (1-30 minutes), the first 15 minutes (1-15 minutes), or the latter 15 minutes (16-30 minutes)

(D) Amylase activity detected in saliva during the whole collection period (1-30 minutes), the first 15 minutes (1-15 minutes), or the latter 15 minutes (16-30 minutes)

Data represent the mean $\pm$ SD (control: n=9, hyperoxia: n=7-9; data were obtained from 7 independent experiments). \*P<0.05, \*\*P<0.01, according to the Student's t-test

**Figure 2. Histological analysis of the influence of hyperoxia on the submandibular gland**

(A) H-E staining

(B) Alcian blue staining

(C) Quantitative evaluation of the area of Alcian blue staining

In the hyperoxia group, the mucous acini were enlarged and were more common than serous acini. In addition, the hyperoxia group exhibited significantly greater accumulation of mucins (blue) than the control group.

Data represent the mean  $\pm$  SD (n=4 in each group; data were obtained from 2 independent experiments). \*\*P<0.01, according to the Student's t-test

**Figure 3. Apoptotic changes induced in the submandibular gland by hyperoxia**

(A) TUNEL staining

(B) High-magnification image of the boxed area shown in A

The cells indicated by the arrows are TUNEL-positive cells.

(C) Quantitative evaluation of the number of TUNEL-positive cells

Data represent the mean  $\pm$  SD (n=5 in each group; data were obtained from 4 independent experiments). \*P<0.05, according to the Student's t-test

**Figure 4. The effects of hyperoxia on the mRNA expression levels of SOD-1, SOD-2, IL-6, and TNF- $\alpha$  in the submandibular gland**

(A) SOD-1, (B) SOD-2, (C) IL-6, (D) TNF- $\alpha$

The data are expressed as the mean  $\pm$  SD ratios of SOD-1, SOD-2, IL-6, or TNF- $\alpha$  expression to  $\beta$ -actin expression (control: n=4-6, hyperoxia: n=4-8; data were obtained from 4 independent experiments). \*P<0.05, \*\*P<0.01, according to the Student's t-test

**Figure 5. The effects of hyperoxia on the mRNA expression levels of HO-1 in various organs**

(A) Brain cortex, (B) lungs, (C) submandibular glands, (D) liver, (E) tongue, (F) kidneys

The data are expressed as mean  $\pm$  SD ratios of HO-1 expression to  $\beta$ -actin expression (n=5-6 in each group; data were obtained from 4 independent experiments). \* P<0.05, \*\*P<0.01, \*\*\*P<0.001, according to the Student's t-test

Fig. 1

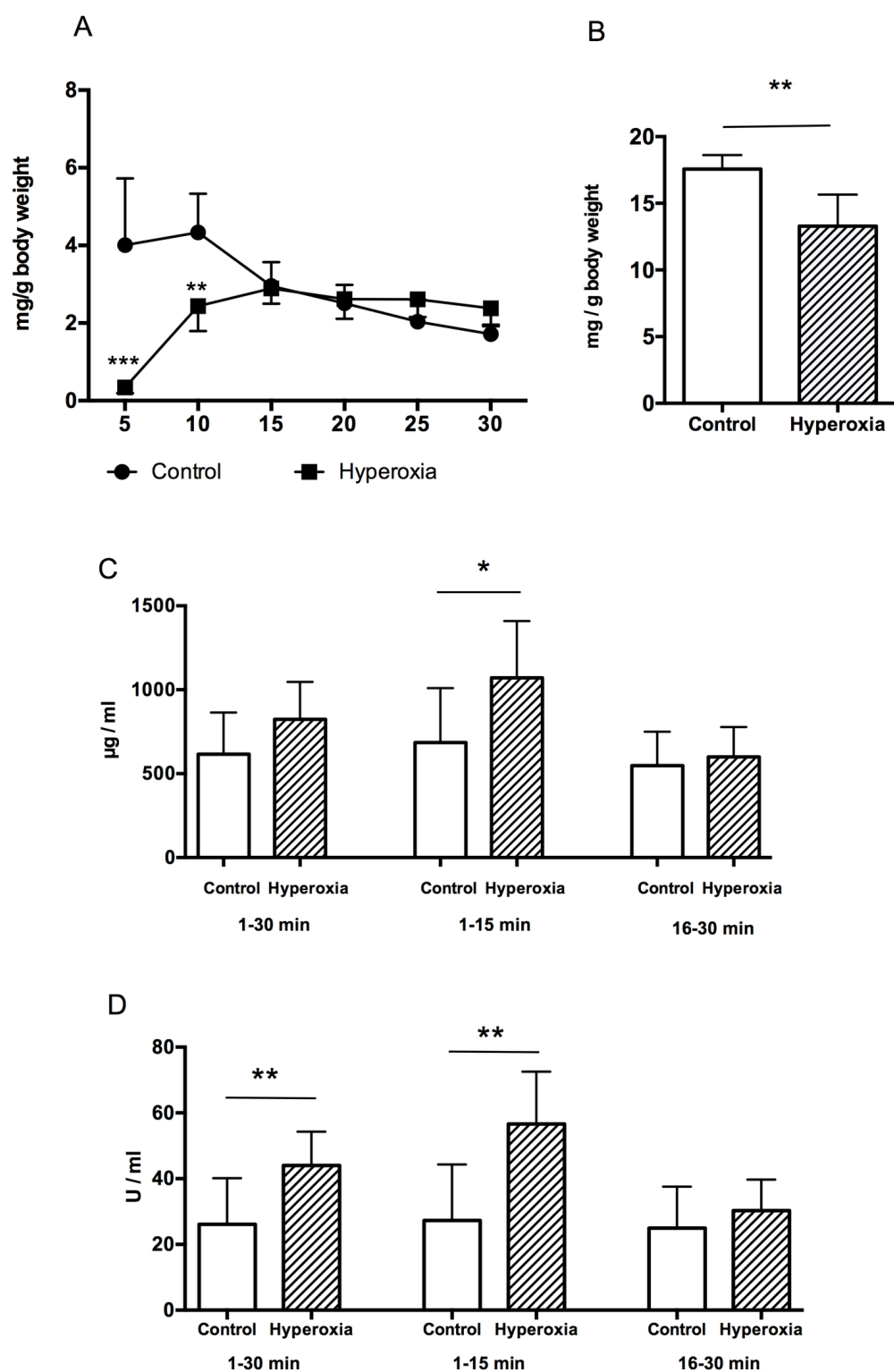
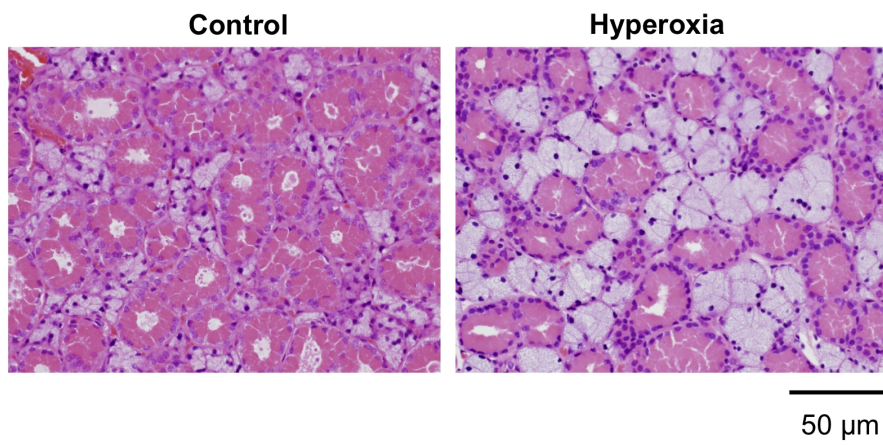
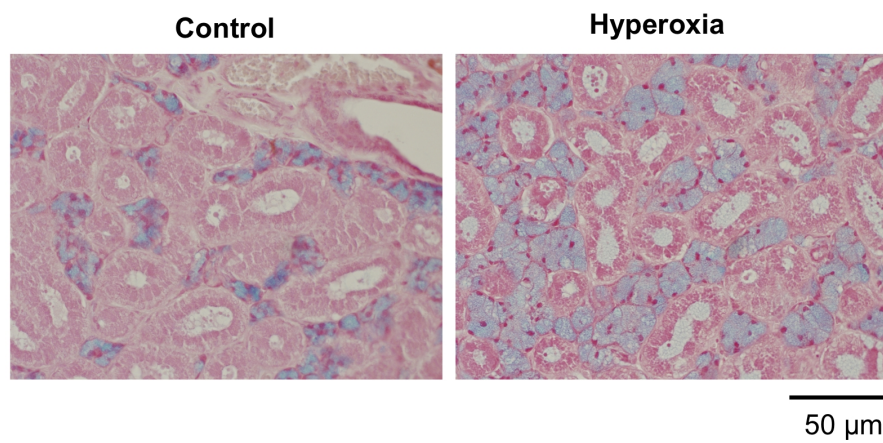


Fig.2

A



B



C

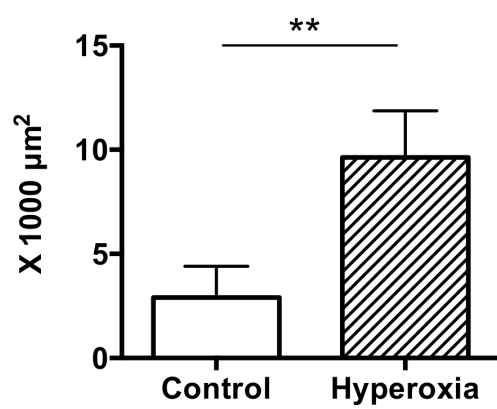


Fig. 3

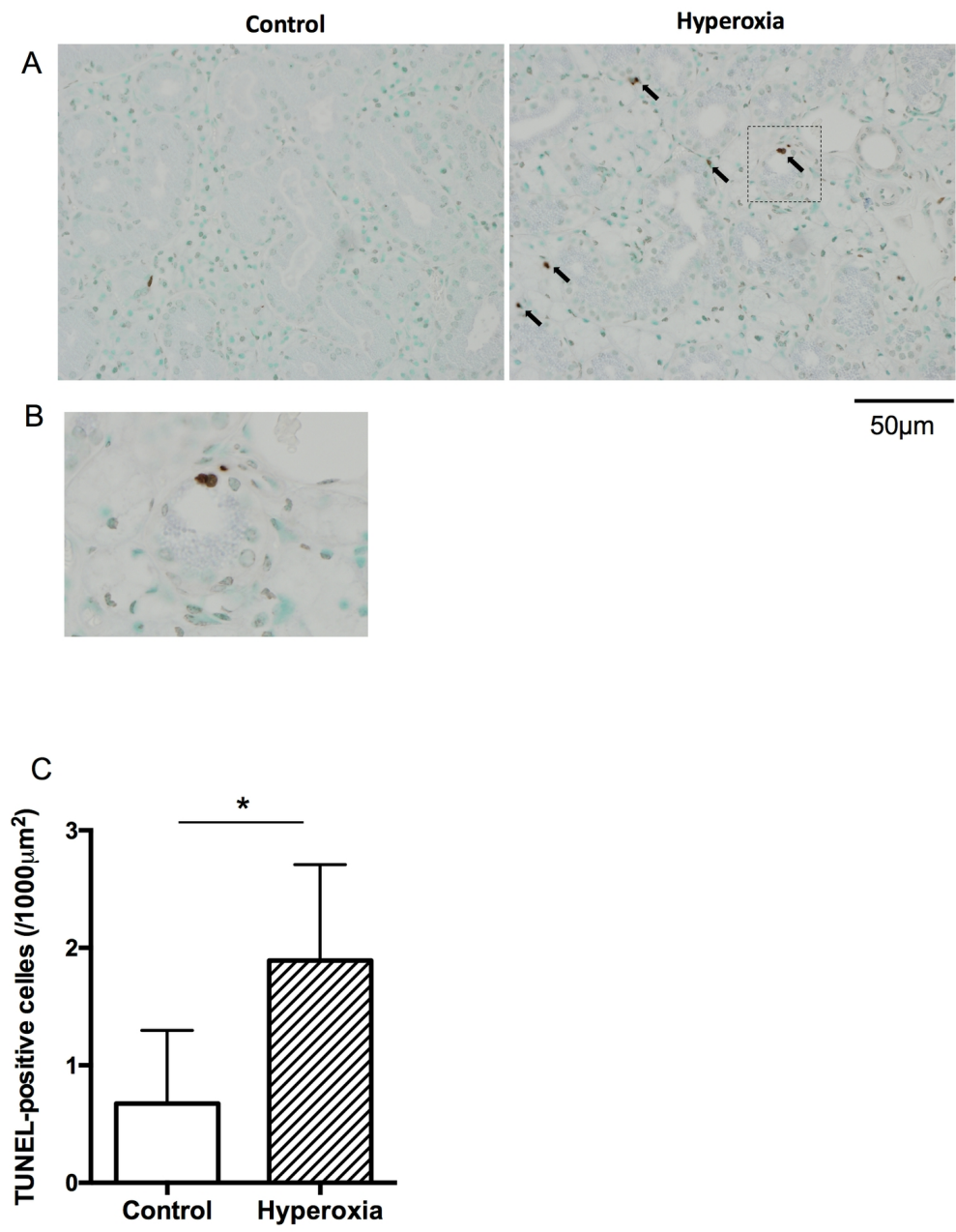


Fig. 4

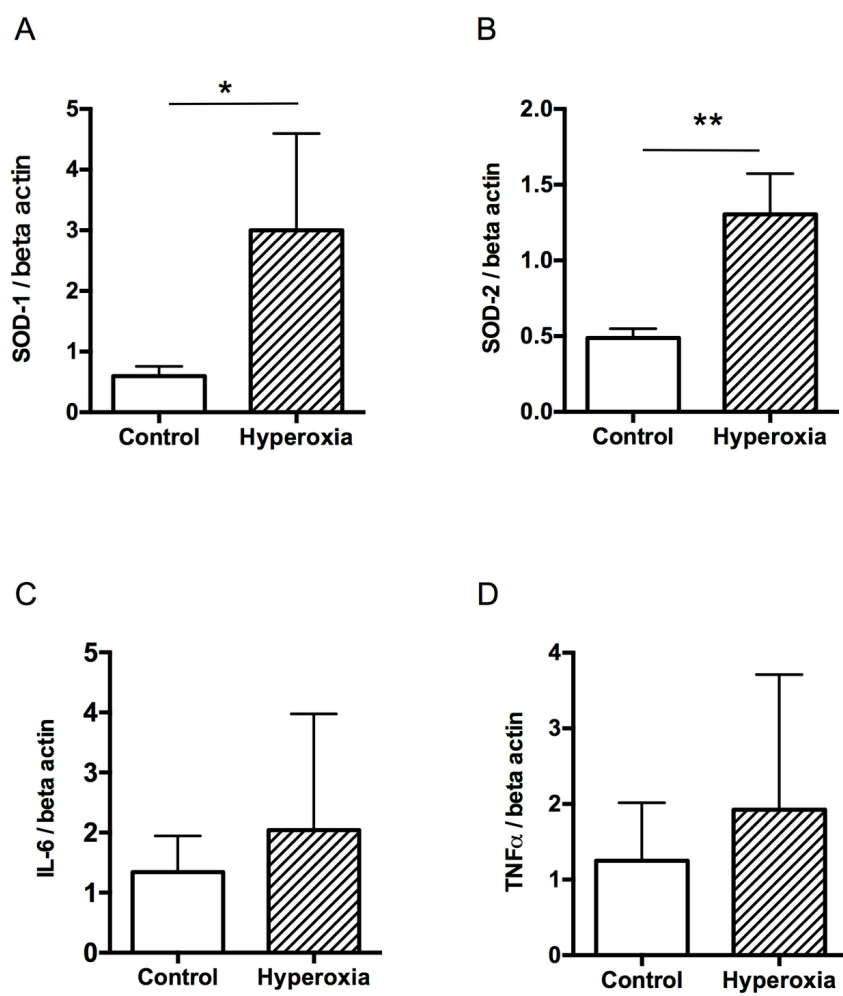


Fig. 5

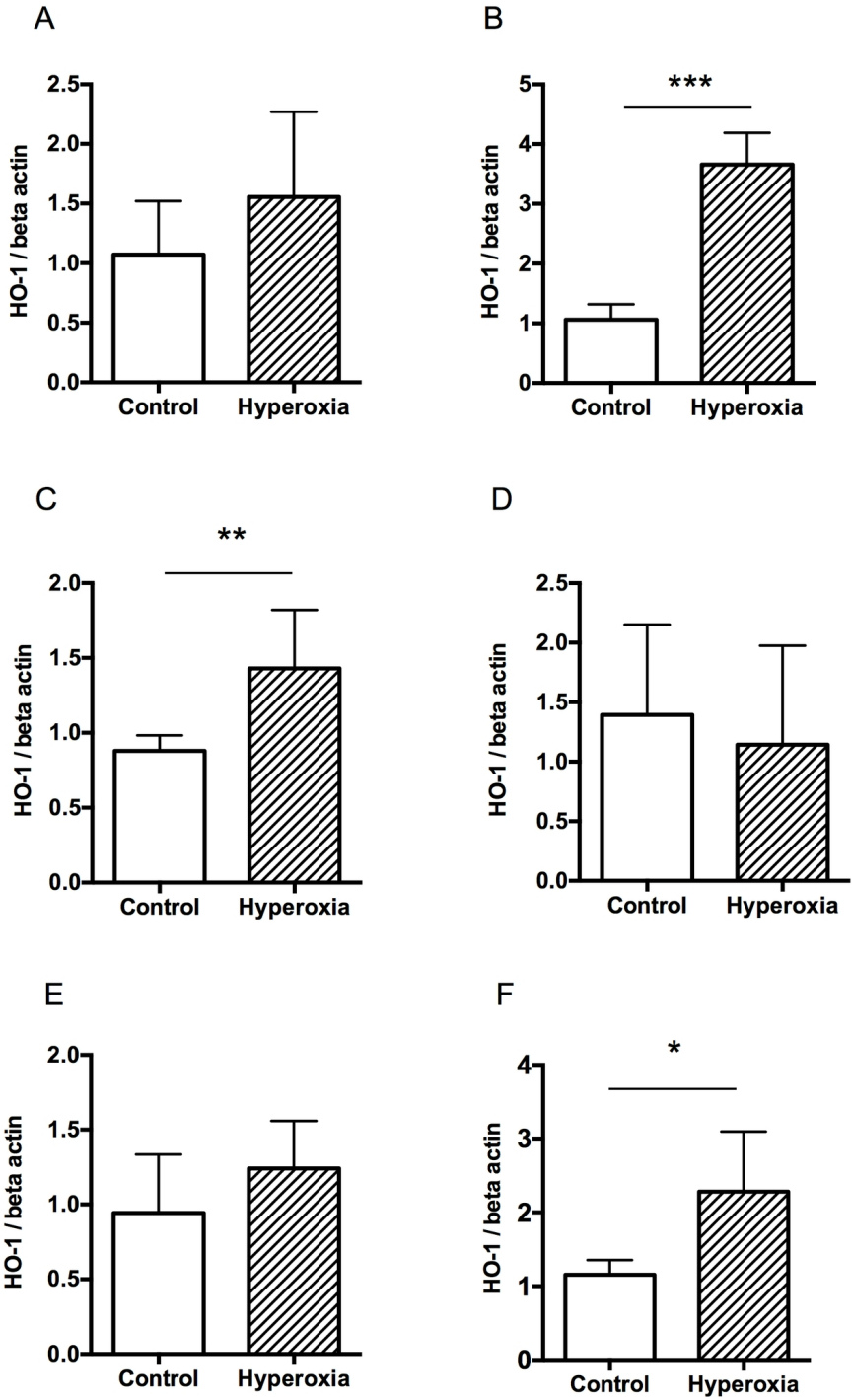


Table 1. PCR primers for Quantitative Real-Time-PCR

| Gene           | Forward and reverse primer sequences                                 |
|----------------|----------------------------------------------------------------------|
| HO-1           | 5'-GTTCAAACAGCTCTATCGTCCTCG-3'<br>5'-GACTAGTCGAAGCTGAGAGTGAGGACCC-3' |
| $\beta$ -actin | 5'-GACCTCTATGCCAACACAGT-3'<br>5'-AGTACTTGCGCTCAGGAGGA-3'             |
| SOD-1          | 5'-TCCCAGCATTTCCAGTCT-3'<br>5'-ACTCTCAGGAGAGCATTCCA-3'               |
| SOD-2          | 5'-AGCTTTCAGATAGTCAGGTC-3'<br>5'-GCAACGTCCCYYACAGAT-3'               |
| IL-6           | 5'-AAGAAAGACAAAGCCAGAGT-3'<br>5'-CTGTTAGGAGAGCATTGGAA-3'             |
| TNF- $\alpha$  | 5'-CTCAAATTCGAGTGACAAGC-3'<br>5'-TTGTCCCTTGAAGAGAACCT-3'             |

## **Supplementary information**

### **Materials and methods for immunostaining and Western blotting**

#### **Immunostaining**

Submandibular glands were fixed with 4% paraformaldehyde for 4 hours at 4°C and then cryoprotected at 4°C in a graded series of sucrose (phosphate-buffered saline [PBS] supplemented with 10% or 20% sucrose for 2 hours or PBS supplemented with 30% sucrose overnight).

The submandibular gland tissue was embedded in optimal cutting temperature compound (OCT) (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and sectioned at a thickness of 7 µm.

For the immunohistochemical analysis of the cryostat sections, the sections were blocked with BlockAid blocking solution (Thermo Fisher Scientific, Waltham, USA), before being incubated overnight at 4°C with an anti-aquaporin5 (AQP5) rabbit polyclonal antibody (Merck Millipore, Darmstadt, Germany) (1:100) and an anti-E-cadherin mouse polyclonal antibody (BD Transduction Laboratories, Tokyo, Japan) (1:50). After being rinsed in PBS, the sections were incubated with the anti-rabbit IgG

Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, Waltham, USA) (1:400) and the anti-mouse IgG Alexa Fluor 568 secondary antibody (Thermo Fisher Scientific, Waltham, USA) (1:400) for 45 minutes at room temperature. Nuclear staining was performed using the NucBlue fixed cell ReadyProbes reagent (Thermo Fisher Scientific, Waltham, USA). All sections were imaged using an LSM780 confocal laser microscope (ZEISS, Oberkochen, Germany).

## **Western blotting**

Cellular proteins were extracted from the submandibular glands using cOMplete lysis-M, ethylenediaminetetraacetic acid-free (Roche Diagnostics K. K., Basel, Schweiz). Protein concentrations were determined using the Coomassie plus (Bradford) assay kit (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions.

Western blotting was performed using the NuPAGE system (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions.

Equal amounts of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10% Bis-Tris gels and antibodies against AQP5 (1:5000) and  $\beta$ -

actin (1:5000). Horseradish peroxidase-conjugated secondary antibodies were employed (Jackson ImmunoResearch, West Grove, PA), and signals were detected with a chemiluminescent reagent (Amersham ECL plus Western blotting detection system, General Electric, Buckinghamshire, UK) and imaged using the ImageQuant LAS 4000 (GE Healthcare, UK).

**Figure legend for supplementary figure**

**The effects of hyperoxia on AQP5 expression in the submandibular gland**

(A) Immunostaining of the submandibular gland

E-cadherin (red) (for detecting acinar and ductal structures) and AQP5 (green) were immunostained in the control and hyperoxia groups.

(B, C) Representative Western blotting bands for AQP5 and  $\beta$ -actin in the submandibular glands of the control and hyperoxia groups and their quantitative evaluation

Data represent the mean  $\pm$  SD (n=6 in each group; data were obtained from 2 independent experiments).

Supplementary Fig.

