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Effects of estrogen on STIM1/Orai1 in the sublingual gland of ovariectomized rats

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Summary. Background. Studies have shown that estrogen can protect the function of the sublingual gland, but the specific mechanism is still unclear. Besides, the STIM1/Orai1 pathway is important to secretion in the salivary gland. Here, we explore the possible effects of estrogen on sublingual gland function by observing changes of STIM1 and Orai1 levels in the sublingual glands of ovariectomized rats. Methods. 42 adult female Sprague-Dawley rats were randomly divided into three groups: SHAM, OVX, and OVX+E (n=14 per group). Two weeks after ovariectomy, rats were treated with estrogen (β-estradiol). The expression of STIM1 and Orai1 in the sublingual gland were observed by double label-immunohistochemistry and immunofluorescence. Calcium imaging was conducted to observe changes in cellular Ca^{2+} levels. Results. IHC and IF showed that the levels of both STIM1 and Orai1 decreased following ovariectomy, but increased to SHAM levels after estrogen treatment. By IF, STIM1 and Orai1 exhibited perfect co-localization. Calcium imaging results showed that the Ca^{2+} in the cells decreased after ovariectomy. Estrogen intervention returned levels of these proteins and Ca^{2+} to the same as those in the control group. Conclusion. This study demonstrates that low estrogen status significantly reduced the expression of STIM1 and Orai1 in the sublingual gland of rats, along with cellular Ca^{2+} levels. These data provide insight into the likely mechanisms underlying sublingual gland secretion dysfunction during menopause.

Key words: STIM1, Orai1, Sublingual gland, Estrogen, Ovariectomized rats

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Introduction

The sublingual gland is one of three major salivary glands and is important for saliva secretion. It secretes mucin, salivary amylase, lysozyme, and immunoglobulin, which have important functions in the maintenance of normal oral function. Preliminary findings by our group in rats revealed apoptosis in the sublingual gland following ovariectomy, suggesting that estrogen has important protective effects on this physiological structure (Da et al., 2017). In addition, studies have shown that saliva secretion in women varies with hormone levels, with significant differences in response to the menstrual cycle, pregnancy, and menopause, and high levels of expression of estrogen receptors in salivary glands and the oral mucosa (Tarkkila et al., 2001, 2008). Overall, there is strong evidence that estrogen has an important regulatory role in the function of the sublingual gland in women.

Calcium is an important second messenger involved in salivation, and one of the most important mechanisms of calcium entry in non-excitable cells is the Store Operated calcium Entry (SOCE). SOCE has been implicated in a wide variety of physiological and

pathological processes and is regulated by the Stromal Interaction Molecule 1 (STIM1) and Orai1. STIM1 is a single transmembrane protein distributed on the endoplasmic reticulum membrane and a sensor of Ca^{2+} in the endoplasmic reticulum calcium pool. The associated muscarinic receptor on the acinar cell membrane receives stimulation from external signals to activate phospholipase C, which in turn produces Inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol by hydrolyzing phosphatidylinositol diphosphate. The combination of IP3 and Inositol 1, 4, 5-trisphosphate receptor (IP3R) on the endoplasmic reticulum causes depletion of calcium stores. Once calcium is exhausted, STIM1, located on the endoplasmic reticulum membrane, undergoes position and conformation alteration, resulting in protein multimerization and extension of the C-terminal domain, along with transfer to the cell periphery, where it accumulates at specific endoplasmic reticulum/plasma membrane junctions. At these junctions, STIM1 interacts with Orai1 (Wu et al., 2006; Liou et al., 2007; Muik et al., 2011; Zhou et al., 2013), a cell membrane protein with four transmembrane domains. Each of the four transmembrane domains of Orai1 is rich in amino acids that highly selectively bind and transport calcium ions. When regulated by STIM1, the Orai1 calcium channel mediates extracellular Ca^{2+} influx and increases intracellular Ca^{2+} concentration (Liou et al., 2005; Feske, 2010; Robinson et al., 2012). Abnormal changes in STIM1 and Orai1 expression cause disruption of intracellular Ca^{2+} and consequent changes in cell structure and function (Sun et al., 2015), which may lead to apoptosis or dysfunctional glandular cell secretion. Furthermore, STIM1 and Orai1-mediated calcium influx is also important for salivary gland secretion (Ambudkar, 2012).

Detailed study of the biological effects of and mechanisms underlying STIM1 and Orai1 function indicate that estrogen is one of the regulators of the normal physiological roles of these proteins. In chronic lung disease, estradiol inhibits STIM1 phosphorylation by a non-genetic pathway, reducing STIM1 oligomerization and interaction with Orai1 (Sheridan et al., 2013). Moreover, in osteoblasts, estradiol promotes the expression of STIM1 and Orai1 via the Phosphatidylinositol 3 kinase/Protein kinase B-mammalian target of rapamycin (PI3K/AKT-mTOR) pathway (Han et al., 2016); however, studies of the effects of estrogen on the STIM1/Orai1 pathway in the sublingual gland have not been reported. The aim of this study was to investigate the effects of low estrogen status on STIM1 and Orai1 in the sublingual gland of rats and to explore the possible mechanism of action of estrogen on the sublingual gland.

Materials and methods

Reagents

Rabbit anti-STIM1 (HPA012123, lot NO. B115778) polyclonal antibody and Mouse anti-Orai1 (SAB3500126, lot NO. PM52050902) monoclonal antibody were from Sigma (Merck; Darmstadt, Germany). As for immunofluorescence, we purchased DyLight 594 donkey anti-mouse IgG (E032411-01), DyLight 488 donkey anti-rabbit IgG (E032221-01), and donkey serum (S010190-01) from EarthOx (EarthOx; San Francisco, United States). For immunohistochemistry, we used avidin-biotin complex labeling kits (SP-9001, SP-9002, Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd; Beijing, PR China). Anti-fluorescent attenuating sealer containing DAPI and Fluo-3/AM were from Solarbio (Solarbio life science; Beijing, China).

Experimental animals

Specific-pathogen-free (SPF) level female Sprague-Dawley rats (8 weeks old; weight, 210-230 g) were purchased from the Department of Laboratory Animal Science of Peking University Health Science Center. 42 rats were randomly divided into three groups: sham operation (SHAM), ovariectomy (OVX), and estradiol treatment (OVX+E) groups (n=14 per group), 6 rats were used for immunohistochemistry and immunofluorescence, and 8 rats were used in Ca^{2+} imaging. The rats were maintained in the laboratory and ovariectomized after 2 days of adaptation to the environment. After recovering for two weeks, the rats were treated for one month as described below (Establishment of the ovariectomized rat model), then their sublingual glands removed.

Establishment of the ovariectomized rat model

Rats in OVX and OVX+E groups underwent bilateral ovariectomies under aseptic conditions, while SHAM group rats underwent a sham operation (i.e., the pelvic cavity was opened, the ovaries were located, and peri-ovarian adipose tissue equivalent to ovarian volume was removed). Abdominal cavities were closed following surgery. After a post-surgical recovery period of 2 weeks, estradiol was injected subcutaneously into OVX+E rats, while SHAM and OVX group rats were injected with sesame oil as described below (Da et al., 2017).

Medication

First, 17-β estradiol (20 mg, Sigma E8875) was dissolved in 4 ml of anhydrous ethanol. A 200 μl volume of the mixture was then mixed into 20 ml of aseptic sesame oil, forming a 50 μg/ml mother solution, which was stored at 4°C. This mixture was hypodermically injected into rats at 25 μg/kg.

Sublingual gland preparation

The rats underwent cardiac perfusion with 4%

paraformaldehyde solution after anesthesia with intraperitoneal administration of 80 mg/kg sodium pentobarbital, the sublingual gland was removed using scissors. After fixing with 4% paraformaldehyde, glands were embedded in OCT and stored at -80°C until IF and IHC analyses. Then, tissues were stored at -80°C for western blotting.

Sublingual gland immunohistochemistry

Frozen sections were incubated with anti-STIM1 (1:500) or anti-Orai1 (1:200) antibodies overnight at 4°C. The next day, sections were processed using an IHC kit (SP9001/SP9002). DAB was used as a color developmental reagent. The IHC reaction product was brown. IHC sections were not counterstained with hematoxylin or other stains. Finally, sections were observed under light microscopy (Q550CW, Leica) at a magnification of 200x. Image Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, Md., USA) was used to quantify area and integral optical density (IOD). Measured IOD minus background IOD were established as parameters to isolate measured objects from the background. Average optical density was calculated as (AOD)=Σ IOD/Σ area (Da et al., 2017).

Immunofluorescence double labelling

Frozen sections (20μm) were incubated with rabbit anti-mouse STIM1 (1:25) and mouse anti-mouse Orai1 (1:50) antibodies at 4°C for 2 days. Next, sections were treated with anti-mouse Dylight594 (EarthOx) or anti-rabbit Dylight488 (EarthOx) secondary antibodies (1:300) at room temperature for 3 h in the dark. Sections were preserved in anti-fluorescence attenuating mounts containing DAPI. Images were acquired under confocal microscopy at 488 nm and 594 nm (TCS SP5 II, Leica; Wetzlar, Germany). Images were edited using AXON DIGIATA 1430 (Zhang et al., 2017a).

Calcium imaging

Rats were anesthetized with intraperitoneal sodium pentobarbital (80 mg/kg) and placed in the supine position. Sublingual glands were isolated from surrounding tissue and excised. Tissue samples were placed in 10 ml of DMEM medium containing 5% BSA and 0.125 mg/ml mixed digestive enzyme preparation (Liberase TL enzyme, Roche Applied Science; Indianapolis, IN, USA) and digested in a water bath at 37°C for 10 min. After centrifugation at 3000 rpm for 3 min, cells were re-suspended in DMEM supplemented with 1% BSA, rinsed, and incubated with Fluo-3/AM fluorescent probes to detect free intracellular Ca^{2+} levels. Cells were loaded with 5 μm Fluo-3/AM for 40 min at room temperature and imaged under confocal laser microscopy at 488 nm (Leica, TCS NT) (Zhang et al., 2017b).

Data collection and statistics

The data were calculated as means ± standard

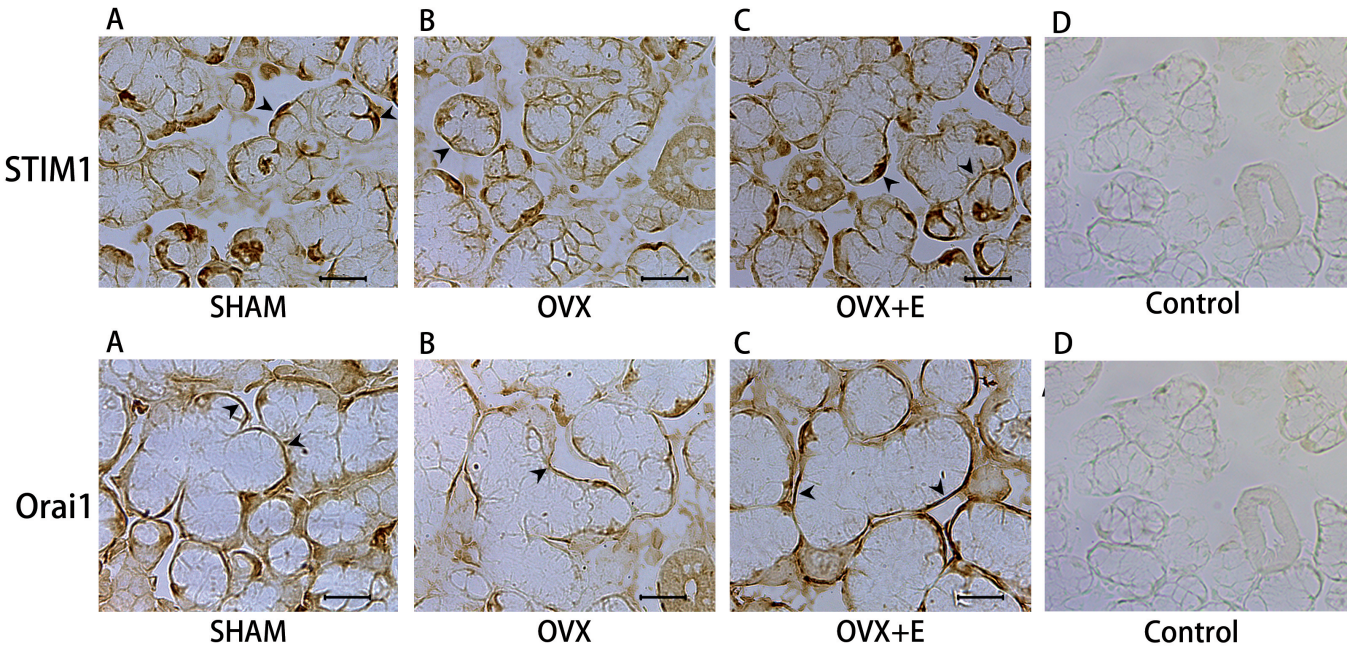

Fig. 1. IHC of STIM1 and Orai1 in the sublingual gland. Representative images from the SHAM **(A)**, OVX **(B)**, OVX+E **(C)**, and Control groups **(D)**. 1st horizontal, STIM1, location (➤); 2nd horizontal, Orai1, location (➤). Scale bars: 30 μm.

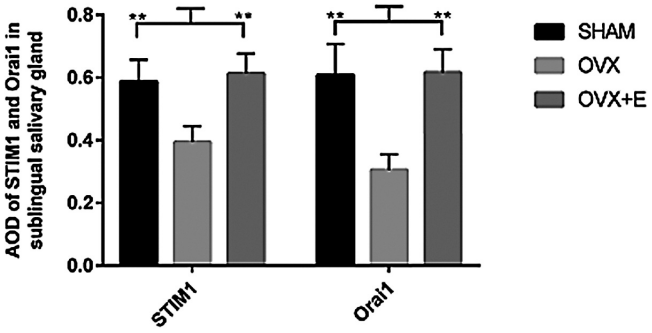

Fig 2. AOD (Average Optical Density) of STIM1 and Orai1 in the sublingual gland. Values are means±SD from six independent experiments. N=6, **P<0.01.

deviations (x±SD) and analyzed using SPSS 17.0 (IBM, Chicago, IL, USA). Post-hoc multiple comparisons were used to compare the differences between the groups (one-way ANOVA was performed, followed by the Student-Newman-Keuls test). All graphs were drawn using GraphPad Prism 5 software. P<0.05 and P<0.01 were considered statistically significant.

Results

Localization and distribution of STIM1 in the sublingual gland

IHC assay revealed that STIM1 was abundantly expressed in serous demilune cells of the sublingual gland (Fig. 1). The AOD of STIM1 in OVX group was

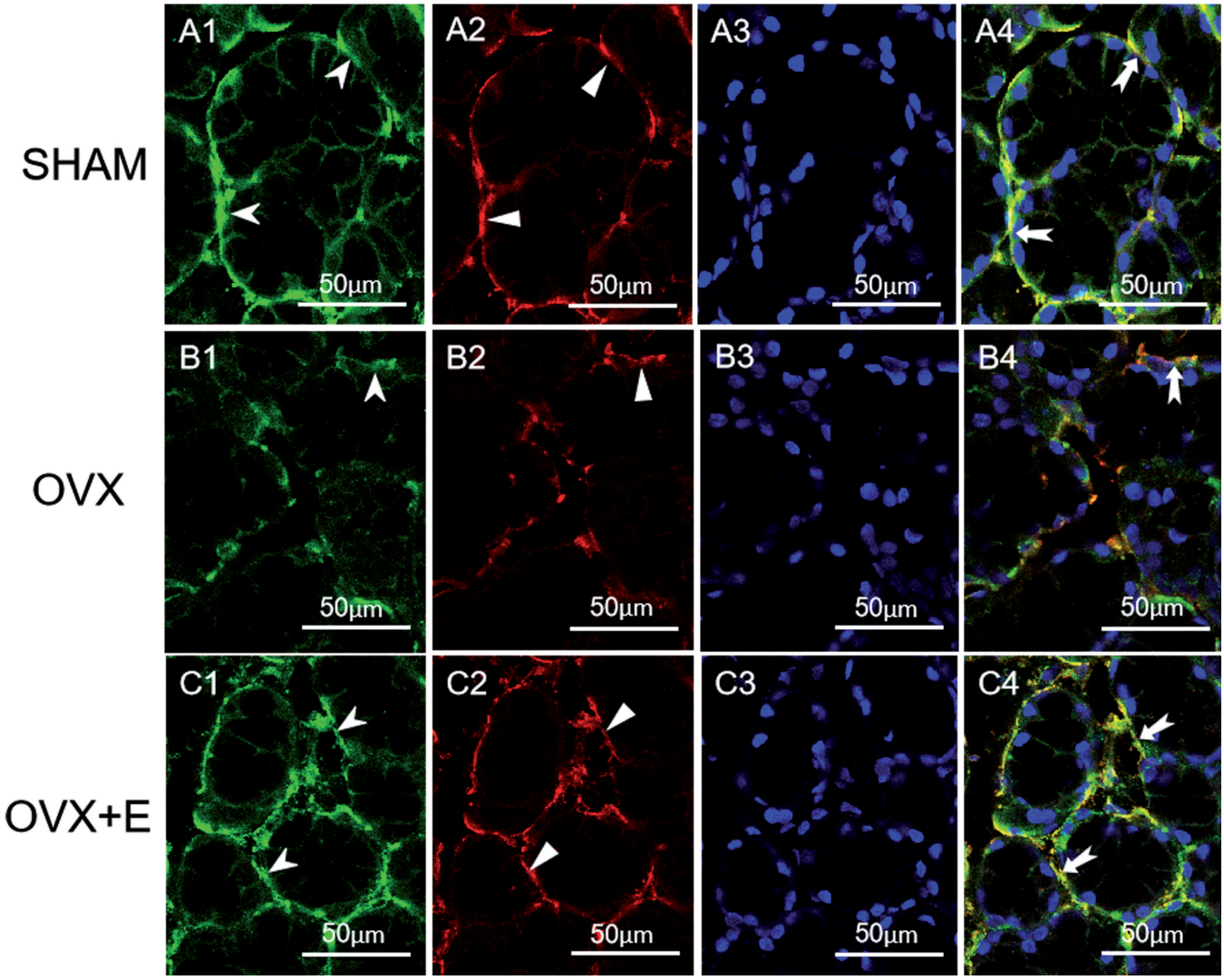

Fig. 3. IF of STIM1 and Orai1 in the sublingual gland. Representative images from the SHAM **(A)**, OVX **(B)**, and OVX+E groups **(C)**. 1st column, STIM1 (green), location (➤); 2nd column, Orai1 (red), location (▲); 3rd column, nuclei (blue); and 4th column, merge, co-location (➧). Scale bars: 50 μm.

significantly lower than that in the SHAM group (P<0.01), while that in the OVX+E group was significantly increased compared with the OVX group (P<0.01), but not the SHAM group (P>0.05).

Localization and distribution of Orai1 in the sublingual gland

IHC analysis showed that Orai1 was abundantly expressed in rat sublingual acinar cells (Fig. 1); there was no expression in the cytosol. And there was moderate reactivity in the serous demilune cells. The AOD of Orai1 in the OVX group was significantly lower than that in the SHAM group (P<0.01), while that in the OVX+E group significantly increased compared with the OVX group (P<0.01), but was not significantly different to the value for the SHAM group (P>0.05) (Figs. 1, 2). The differences in the levels of Orai1 among groups were more significant than those of STIM1.

Double immunofluorescence labelling of STIM1 and Orai1

Green STIM1 and red Orai1 products (Fig. 3) were found to be jointly distributed in sublingual acinar cells (Fig. 3).

Calcium imaging

Calcium imaging showed that the fluorescence intensity of intracellular calcium of the serous demilune cells in the OVX group was significantly lower than that in the SHAM group. Moreover, calcium levels in the OVX+E group were significantly higher than those in the OVX group, which were similar to those in the SHAM group (Fig. 4).

Discussion

STIM1 and Orai1 are important for salivation (Ong et al., 2007; Cheng et al., 2008, 2013a). We judged the success of our rat model based on serum estradiol content and exfoliated cell smears (see Zhang et al., 2017a,b for details). We found that STIM1 and Orai1 expression levels were significantly decreased after ovariectomy in rats, and returned to the levels in the control group after estrogen treatment, suggesting that estrogen may regulate the secretion function of the sublingual gland through STIM1/Orai1.

The main function of the salivary glands is the secretion of saliva, which consists of water, electrolytes and proteins. The secretion of these substances involves transport processes using intracellular calcium ions as a

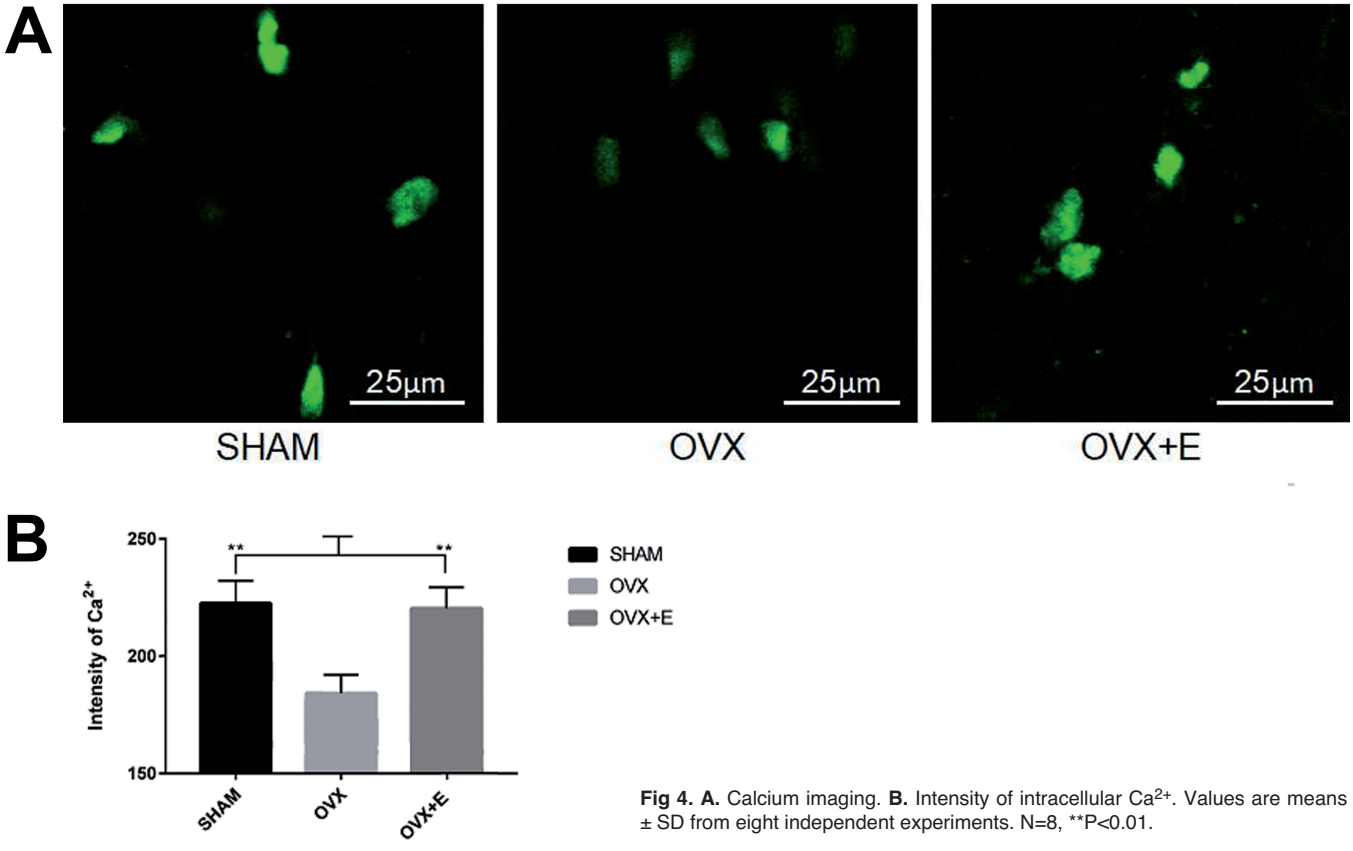

Fig 4. A. Calcium imaging. **B.** Intensity of intracellular Ca^{2+}. Values are means ± SD from eight independent experiments. N=8, **P<0.01.

second messenger. The process of stimulated saliva secretion involves STIM1 and Orai1-mediated cellular Ca^{2+} influx, which maintains salivary secretion (Cheng et al., 2013a; Prakriya 2013). The use of calcium antagonists can cause a decline in salivary secretion (Hattori and Wang, 2007). Besides, rat salivary glands are oestrogen-responsive. Purushotham et al., reported that ovariectomy increased sublingual gland weight by 26% (Laine and Tenovuo, 1983; Purushotham et al., 1993). In this study, we found that, following ovariectomy, the expression levels of STIM1 and Orai1 decreased, and that intracellular calcium concentration was also significantly decreased. Based on these data, we speculate that low estrogen status may directly effect Ca^{2+} levels in the sublingual gland cells through the STIM1/Orai1 pathway, thereby affecting the secretion function of the sublingual gland.

STIM1/Orai1 are also the main drivers of Ca^{2+} influx to acinar cells in the stimulated state, and may indirectly affect the function of the sublingual gland by disrupting cell structure, via apoptotic stress and other pathways. STIM1 and Orai1 regulate physiological and pathological cell processes through various pathways. When STIM1 or Orai1 expression is decreased, Ca^{2+} homeostasis is disrupted, inducing various disease states (Irvine, 1990; Rosado et al., 2010). Activation of caspase-3 also leads to the dysfunction of store operated calcium entry, causing reduced Ca^{2+} influx and disrupting intracellular Ca^{2+} homeostasis (Liu et al., 2017). As described above, STIM1/Orai1 have important roles in apoptosis. In our previous study we found that, in rats with low estrogen status following ovariectomy, the expression of caspase-3 increased significantly in the sublingual gland, alongside atrophy and apoptosis of sublingual acinar cells and organelle lesions (mitochondria rupture and endoplasmic reticulum stress). Estrogen inhibits apoptosis and protects glands (Da et al. 2017). Here, we found that during low estrogen status after ovariectomy expression levels of rat STIM1 and Orai1 were significantly decreased in the sublingual gland, and that estrogen could reverse this phenomenon, suggesting that estrogen may affect the function of the sublingual gland through the STIM1/Orai1 pathway. In a low estrogen state, intracellular Ca^{2+} homeostasis may be disrupted by the inhibition of the STIM1/Orai1 pathway, while endoplasmic reticulum stress and mitochondrial apoptosis are induced, causing disordered cell structure and function.

We also found that STIM1 is more widely expressed in the sublingual gland than Orai1, and other researchers have reported similar results for the submandibular gland. STIM1 is co-expressed with Transient receptor potential channel 1 (TRPC1) outside the region in which it is co-expressed with Orai1, and Orai1 and TRPC1 each form two independent calcium influx pathways with STIM1 (Pani et al., 2009). In addition, Orai1 regulates TRPC1 in salivary cells, and the function of TRPC1 is completely dependent on Orai1 (Cheng et al., 2008; Cheng et al., 2013b). In the pancreas the effects of STIM1 and TRPC1 on Ca^{2+} influx are significantly reduced when Orai1 function is inhibited (Ferris et al., 1991). In this study we found that the effect of low estrogen on Orai1 was significantly greater than that on STIM1. We suspect that this may be attributable to the interaction of Orai1 with STIM1, while other mechanisms affect cell function via Orai1.

In summary, estrogen appears to be important for the regulation of secretion from the sublingual gland through the STIM1/Orai1 pathway. This maybe because of the effects of STIM1/Orai1 on intracytoplasmic Ca^{2+} concentration, leading to direct disruption of sublingual gland secretion function. These data provide a new avenue through which to further investigate menopausal salivary dysfunction.

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Conflict of interest. The authors confirm that this article has no conflict of interest.

Ethical approval. All procedures performed in studies involving animal experiments ratified by Peking University Biomedical Ethics Committee for Animal Use and Protection and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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