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Original Article

Effects of ovariectomy of rat on tongue neuropathy: Relevance to burning mouth syndrome

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KEYWORDS
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Abstract *Background/purpose:* Burning mouth syndrome (BMS) is common in postmenopausal women, indicating that sex hormones are involved in disease pathogenesis. Recent studies have shown that the decreased nerve innervation of the papillae of the tongue may be related to neuropathic pain conditions in BMS. However, it remains unknown whether sex hormones contribute to changes in the nerve fiber density in the tongues of patients with BMS. Therefore, the aim of this study was to examine changes in pain intensity and nerve fiber density in the tongue of ovariectomized (OVX) rats subjected to surgical menopause to elucidate one aspect of the mechanism of pain onset in BMS.
Materials and methods: Two groups of 9-week-old female Sprague–Dawley (SD) rats (ovariectomy; OVX and sham treatment; Sham) were established. Water containing capsaicin was provided at defined intervals to investigate oral capsaicin avoidance over a 2-week period. Serum circulating estrone (E1) and estradiol (E2) levels were measured in OVX and Sham rats. The protein gene product 9.5 immunoreactivity and transient receptor potential vanilloid 1 (TRPV1) in the tongues were also analyzed.
Results: Two weeks after OVX treatment, intake of water containing capsaicin and serum levels of E1 and E2 significantly decreased. The nerve fiber density in fungiform papillae and connective tissue layer below the filiform papillae was also decreased. However, an abundant TRPV1-positive expression was observed in the tongue of OVX rats.
Conclusion: These results indicate that decreased estrogen signaling causes neuropathy in tongue, resulting in tongue pain hypersensitivity in BMS.

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Introduction

Burning mouth syndrome (BMS) is a chronic pain syndrome involving the tongue, lips, and other areas of the oral mucosa without any clinically evident causative lesions revealed upon thorough clinical examination and investigation.[1] In addition to persistent oral pain, patients with BMS are known to exacerbate oral pain by ingesting irritants, which forces them to change their diet and significantly reduce their quality of life.[2] Various targeted therapies are currently used to relieve pain; however, they are often ineffective for patients with BMS. The global prevalence of BMS is 0.7 %—2.6 %.[3] It is significantly more prevalent in women, with a sex ratio of 7:1,[4] especially in menopausal and postmenopausal women.[5] Therefore, it is estimated that the decrease in sex hormone such as estrogen may be one of the most important etiologic factors in BMS. Several clinical and animal studies have indicated that estrogen plays an essential role in chronic pain mechanisms.[6–8] Estrogen replacement therapy in perimenopausal women relieved symptoms and increased estrogen receptor expression in some patients with BMS,[9] thereby indicating the involvement of estrogen in BMS.

Another possible cause of BMS is axonal neuropathy. Laura et al. reported that decreased nerve fibers density and axonal degeneration of small epithelial and subpapillary fibers observed in the tongue of patients with BMS were involved in sensory neuropathy.[10] Furthermore, nerve fibers penetration into the tongue epithelium is reduced,[11,12] and the expression of transient receptor potential vanilloid 1 (TRPV1) is significantly increased in the epithelial nerve fibers of patients with BMS.[11] Nerve fibers density in the vagina is significantly decreased in ovariectomized (OVX) rats subjected to surgical menopause.[13] The decreased nerve fibers density was restored to normal levels following sustained estrogen administration. These results indicate that a decrease in female hormone levels and axonal neuropathy may be closely associated with BMS. Whether estrogen is involved in neurodegeneration in the tongue of BMS patients is an intriguing question, which remains to be addressed.

Therefore, in the present study, changes in tongue pain sensitivity to capsaicin and nerve ending density in OVX rats with decreased female hormone levels were examined. The aim was to assess the role of female hormones in triggering tongue neuropathy and to clarify the mechanisms underlying the development of BMS.

Materials and methods

Animals

This study was performed in accordance with the approved by the National Research Council's Guide for the Care and Use of Laboratory Animals and Animal Experimentation Committee of the Nihon University (approval no. AP24DEN030). All procedures followed the guidelines of the International Association for the Study of Pain.[14] Female Sprague—Dawley (SD) rats (9-weeks old; mean weight: 210 g) were used in all experiments, and one animal was housed per cage at temperatures ranging 22—24 °C with a 12-h light—dark cycle under specific pathogen-free (SPF) conditions and fed food and water *ad libitum*. All experiments were blindly performed.

Ovariectomy

The rats were randomly divided into two groups: OVX and Sham. In the OVX group, procedures were performed as described previously.[15] Briefly, the rats were intraperitoneally anesthetized with a mixture of three anesthetics (medetomidine hydrochloride 0.15 mg/kg, midazolam 2 mg/kg, and butorphanol tartrate 2.5 mg/kg), and bilateral ovaries encased in adipose tissue were exposed and removed. After OVX treatment, the peritoneum and skin were sutured, and skin incisions were disinfected with iodophor. In the Sham group, the treatment was identical to that given to the OVX rats except for bilateral ovary removal.

Capsaicin intake avoidance test

The experimental schedule is shown in Fig. 1. Capsaicin intake avoidance was examined using the 1-bottle test with modifications to the previous study.[16] The animals were individually housed and provided standard rat chow and water with 0.1 % ethanol vehicle *ad libitum* for acclimatization for 7 d. Water supply bottles (SN—950H, AS-ONE, Tokyo, Japan) were used to measure the intake of capsaicin-dissolved drinking water. The tip of the tube of the bottle was equipped with a ball to ensure no spillage, thus enabling accurate measurement of the liquid. After acclimatization, rats were allowed free access to 1.0 μM drinking water with capsaicin (Sigma—Aldrich, St. Louis, MO, USA) for 3 h after 24 h of restricted access to drinking water.[17] Separate bottles were used to measure water and capsaicin solutions. To measure capsaicin consumption, each bottle was weighed before and after the testing and the difference was determined. The animals were then randomly divided into OVX and Sham groups and provided with free access to water after OVX or Sham treatment. On days 3, 5, 7, and 14 after the treatment, the capsaicin intake avoidance tests were performed again. Water was provided *ad libitum* between the capsaicin intake avoidance tests. Body weight was measured before treatment and on days 3, 5, 7, and 14 after treatment before the capsaicin intake avoidance test. Weight gain ratios were calculated with respect to the pre-treatment baseline.

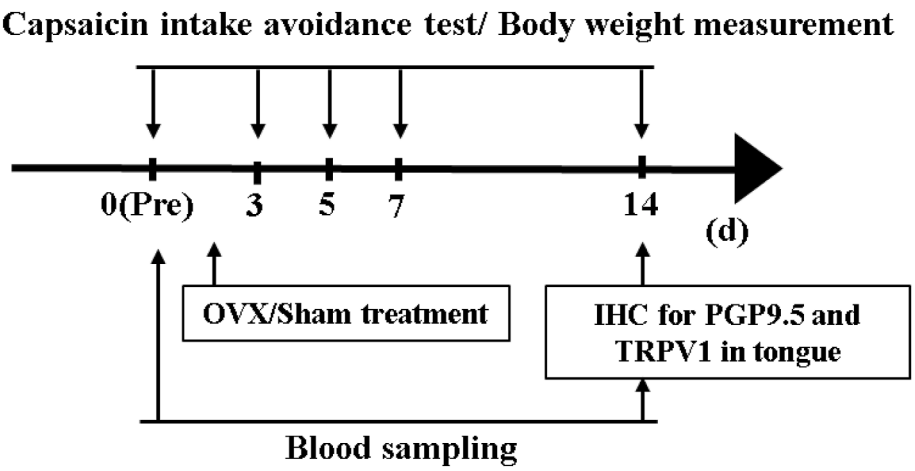

Figure 1 Schematic illustration of the time course of the OVX/Sham treatment, capsaicin intake avoidance test, body weight measurement, blood sampling, and IHC. pre, pre-treatment; OVX, ovariectomy; IHC, immunohistochemistry; PGP9.5, protein gene product 9.5.

Immunoreactivity for protein gene product 9.5 (PGP 9.5) and transient receptor potential vanilloid 1 (TRPV1)

Cytoplasmic PGP 9.5 is considered a reliable marker of intraepidermal nerve fibers.[18] Rats were deeply anesthetized with 5 % isoflurane inhalation 2 weeks after Sham or OVX treatment. The animals were transcardially perfused with saline, followed immediately by 4 % paraformaldehyde (PFA; pH 7.4). The tongues were dissected out, post-fixed in the same fixative for 24 h at 4 °C, and transferred to 20 % sucrose (w/v) in phosphate buffer for 24 h for cryoprotection. The specimens were embedded and stored until cryosectioning at −80 °C. Serial 30-μm-thick coronal sections were obtained from the bilateral tongue 1 cm posterior to the apex of the tongue and more than 2 mm lateral to the midline using a freezing microtome (SM2010R; Leica, Wetzlar, Germany). Immunostaining of these sections was performed using rabbit anti-cytoplasmic PGP 9.5 (1:500, Invitrogen, Carlsbad, CA, USA) or rabbit anti-TRPV1 polyclonal antiserum (1:500, Alomone, Jerusalem, Israel). The images were acquired using a confocal microscope. Negative control was subject to the same treatment with the primary antibody replaced with 0.01 M PBS.

Quantitative analysis of nerve fibers density in tongue

Five sections of fungiform and filiform papillaes from both groups were randomly selected from each PGP 9.5 immunostaining set and assessed using a microscope. The densities of the PGP 9.5- immunoreactive nerve fibers in area (140×60 μm^2) within the fungiform papillae were measured using ImageJ software (version 1.54m; NIH, Bethesda, MD, USA). The average densities of all fungiform papillae from five sections in each group were calculated. For PGP 9.5-immunoreactive nerve fibers below the filiform papillae, the average density in five sections was calculated in a 200×40 μm^2 area in the connective tissue layer below the filiform papillae, under the most distended portion of the bilateral tongues using ImageJ software.

Measurement of serum hormone levels

Blood (1—1.5 mL) was collected in a 2.0-mL centrifuge tube from the tail vein under isoflurane anesthesia before and 14 d after Sham or OVX treatment. Immediately, the blood was centrifuged at $2000 \times g$ for 10 min at −4 °C, and the supernatant was stored at −8 °C for serum analyses. Estrone (E1) and estradiol (E2) in serum after OVX or Sham treatment were measured using liquid chromatography-mass spectrometry at a cooperating extramural laboratory (AZ Science, Nagano, Japan).[19]

Statistical analysis

The data are expressed as the mean ± standard error of the mean (SEM) or median and interquartile range (25—75 %). The normality and homogeneity of variances of the sample data were assessed using the Shapiro—Wilk test and Levene test, respectively. The Mann—Whitney test was used for the analysis of the capsaicin intake avoidance test because they were not considered to be normally distributed and satisfy homoscedasticity. The unpaired Student's *t*-test or one-way repetitive measures analysis of variance (ANOVA) followed by the Tukey—Kramer test was used in body weight, serum estrogen levels and immunohistochemistry (IHC; Prism version 9, GraphPad Software, Boston, MA, USA). Statistical significance was set at $P < 0.05$.

Results

Effect of OVX for capsaicin intake avoidance test and body weight

The capsaicin intake was significantly decreased 14 d after OVX compared to that of pre-OVX and Sham treatment. On the other hand, after Sham treatment, capsaicin intake

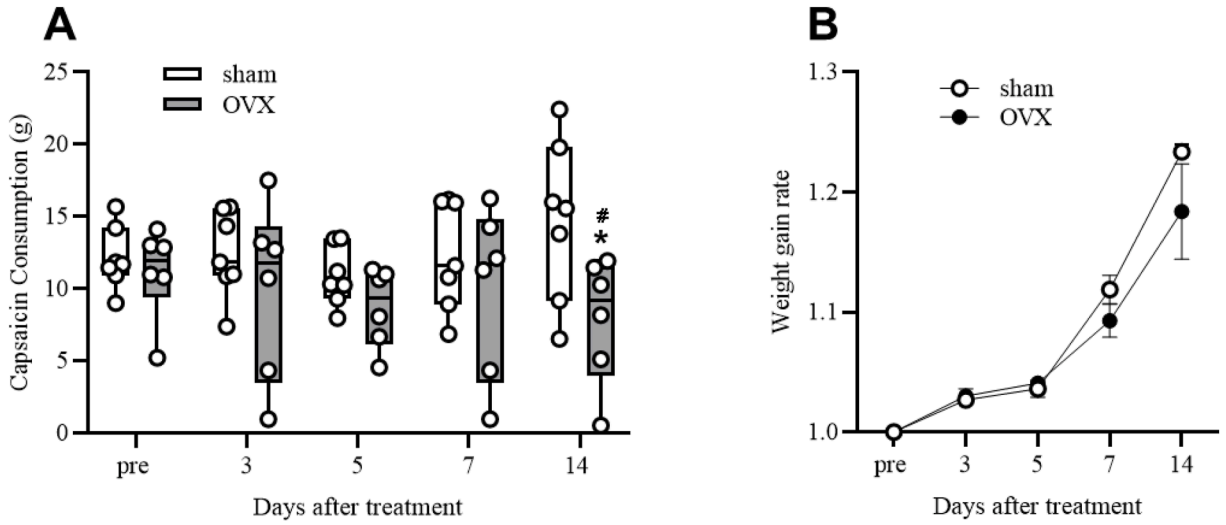

Figure 2 Change of capsaicin intake avoidance and body weight after OVX or Sham treatment. Time course of capsaicin intake volume (A) and body weight (B) both before and after OVX or Sham treatment (OVX group, n = 6; Sham group, n = 7). pre, pre-treatment; OVX, ovariectomy. *: P < 0.05, OVX vs. Sham group; #: P < 0.05, vs. capsaicin consumption on day pre.

slightly increased on day 14, but no significant change (Fig. 2A). In both groups, the rate of weight gain increased over time, but the ratio of gain did not differ between groups from day 3—14 of treatment (Fig. 2B).

Change in serum estrogen levels

We measured serum estrone and estradiol concentrations, the primary components of estrogen. Serum estrone and estradiol concentrations were nearly 0 on day 14 after OVX. These concentrations were almost unchanged and not significantly different from pre-values on day 14 after Sham treatment (Fig. 3A and B). By day 14, OVX rats showed that secretion of both hormones was almost entirely eliminated.

Change in nerve fiber density and TRPV1 expression in tongue

In fungiform papillae in Sham rats, PGP9.5 showed strong staining of nerve bundles from the connective tissue to the tip of the fungiform papillae. On the other hand, in OVX rats, nerve bundles weakly stained with PGP9.5 were observed from the connective tissue to the center of the fungiform papillae (Fig. 4A). The density of PGP9.5-immunoreactive (ir) nerve fibers in the fungiform papillae was significantly lower in OVX rats than Sham rats (Fig. 4B). In addition, the connective tissue layer under the filiform papillae, we observed nerve bundles that were strongly stained with PGP9.5 in the Sham but almost none in the OVX group (Fig. 4C). The density of PGP9.5-ir nerves under the

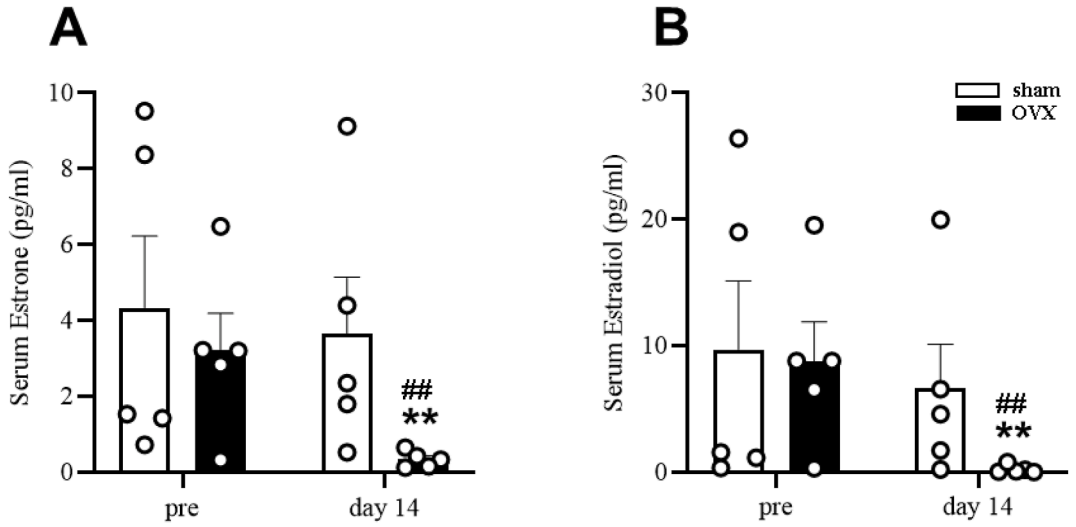

Figure 3 Change of serum estrogen concentrations after OVX or Sham treatment. Serum estrone (A) and estradiol (B) concentrations before and 14 d after OVX or Sham treatment (n = 5 each). pre, pre-treatment; OVX, ovariectomy. **:$P < 0.01$ vs. Sham group; ##: $P < 0.01$, vs. pre.

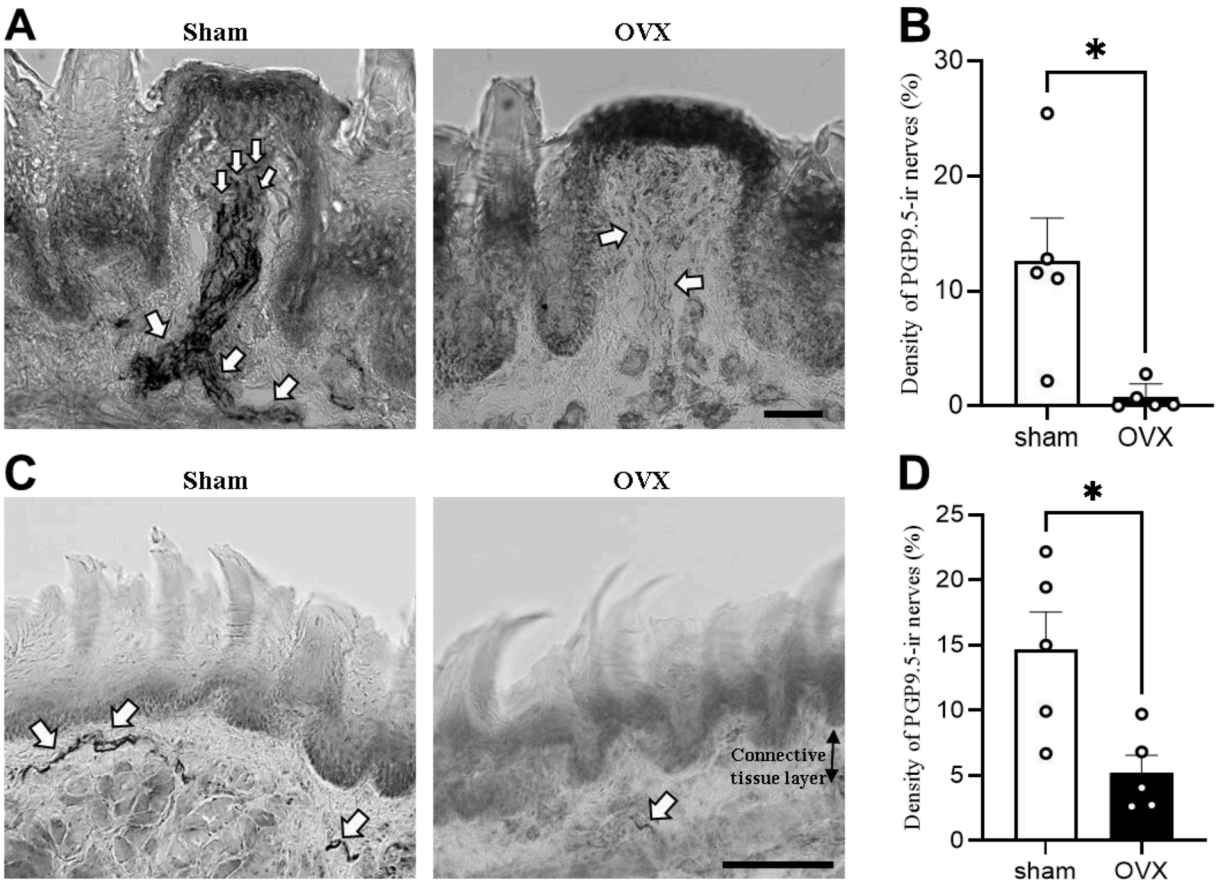

Figure 4 Immunohistological changes of nerve fibers in the tongue after OVX or Sham treatment. PGP9.5-immunoceactive nerve fibers in fungiform papillae of Sham and OVX rats (A) and percentage of their densities (B). PGP9.5-immunoceactive nerve fibers in the connective tissue layer below the filiform papillae of Sham and OVX rats (C) and percentage of their densities (D). n = 5 in each group. Arrows indicate nerve bundles and fibers. OVX, ovariectomy. *: $P < 0.05$. Scale bar in (A) OVX = 20 μm. Scale bar in (C) OVX = 50 μm.

filiform papillae was also significantly lower in OVX than in Sham rats (Fig. 4D).

Numerous TRPV1-positive immunoreactivity was observed in the connective tissue layer under the filiform papillae of OVX rats, although a few TRPV1-positive immunoreactivity was observed in Sham rats (Fig. 5).

Discussion

Patients with BMS are known to experience pain with the ingestion of irritants such as Tabasco sauce, which has capsaicin is a major component.[20,21] In this study, OVX rats showed significant decreasing of capsaicin intake on day 14 after ovariectomy, with a significant decrease compared to Sham rats as well as a decrease in serum estrogen level. This indicated that a decrease in estrogen may cause hypersensitivity of the tongue to capsaicin. The only previous study to examine capsaicin intake avoidance in OVX rats reported a significant decrease in capsaicin intake on day 14 after OVX, which is consistent with our results.[17] Moreover, TRPV1 expression increased in damaged small nerve fibers in patients with BMS.[11] We also confirmed that numerous TRPV1 immunoreactivities were expressed in tongue of OVX rats. It is reported that capsaicin acts as an analgesic in patients with BMS, when taken for longer than 3 months.[22,23] Capsaicin exposure stimulates nociceptor which exist in nerve fibers, causing the local release of neurotransmitters and perception of pain. However, with sustained capsaicin exposure also desensitizes these nerve fibers to other nociceptive stimuli. In this study, capsaicin was used as an irritant to the tongue, but further investigation is needed to determine whether capsaicin acts as an analgesic after long-term administration.

Numerous studies have reported that the escape response threshold of the foot decreases in response to mechanical stimulation in the hind paw plantar region of OVX rats and mice.[24—26] However, some studies have indicated that this threshold is increased in OVX rats.[27,28] These differences can be attributed to factors, including species differences, timing of OVX, and differences in the menstrual cycle of female rats. Sham rats had a normal estrous cycle compared to pseudo-menopausal rats caused by OVX. The age of reproductive stability in SD female rats is considered to be between 8 and 22 weeks, and 9- to 10-week-old rats show the normal estrous cycles, and older rats show a gradual prolonged it.[29] Therefore, we used a 2-week observation period from 9 to 11 weeks of age for

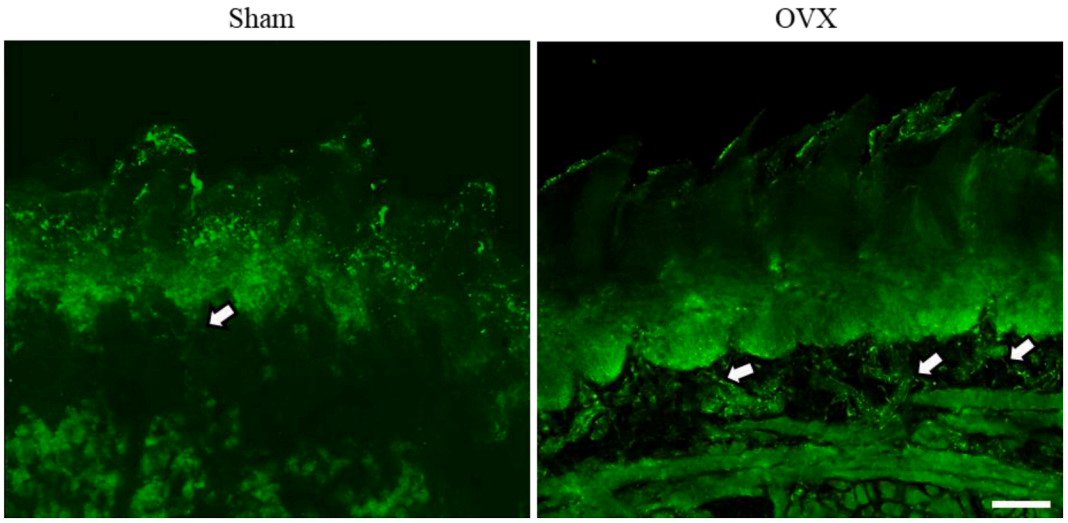

Figure 5 Immunohistological changes of TRPV1-immunoreactivity in the connective tissue layer below the filiform papillae after OVX or Sham treatment. OVX, ovariectomy. Arrows indicate nerve bundles and fibers. Scale bar = 50 μm.

examination, although several studies have used rats that are >8 months old as rats used in the experiment.[17,30]

Lauria et al. showed that epidermal nerve fibers in the tongue with naked axons without Schwann cells play a role similar to that of polymodal nociceptors.[31] Their study using tongue biopsies of patients with BMS revealed atrophy of fine nerve fibers in the tongue epithelium.[10] Therefore, tongue pain in patients with BMS may be because of a neuropathic pain-like neural mechanism. In the present study, the density of PGP9.5-ir nerve fibers observed in the tongue were significantly reduced in OVX rats, indicating possible neuropathy. Burning pain across the tongue is a common complaint in BMS, and nerve fibers below the filiform papillae, which are distributed across most of the tongue, are presumed to be involved. Although there are several studies on nerve fibers distributed in the fungiform papillae, which are few in number, few reports have investigated changes in nerve fibers distributed in the filiform papillae. Sato et al. reported that the base of the filiform papillae had a vertical entry of laminar connective tissue papillae.[32] This layer contains a large number of neurofilament protein (NFP)–positive nerve fibers. In this study, many nerve fibers were found in the connective tissue layer below the filiform papillae as well as within the fungiform papillae of Sham rats. However, in OVX rats, these densities of nerve fibers were reduced, indicating that nerve damage may have occurred over a wide area of the tongue.

Nerve growth factor (NGF), a regulator of TRPV1, was also found to be significantly elevated in the epithelial fibers of the tongue of patients with BMS.[33] Because estrogen decreases NGF signaling to TRPV1, it may play an important role in the development of BMS in postmenopausal women with declining estrogen.[33] The steroid hormone, estrogen is known to exhibit neuroprotective effects, and neurodegeneration due to brain injury has been reported to be inhibited by 17β-estradiol administration.[34–36] Woda et al. reported that ovarian steroid hormones decrease with menopause suppressed the neuroprotective effects of steroids, which may lead to BMS. Thus, as the results of this study indicate, it is possible that OVX-induced estrogen depletion may cause neurodegeneration of the tongue.[37]

In this study, OVX caused hypersensitivity of the tongue in response to capsaicin and a decrease in nerve fibers in the tongue, indicating that female hormones may be responsible for the tongue pain hypersensitivity associated with BMS. These findings may help elucidate etiology and develop treatment strategies for BMS.

Declaration of competing interests

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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