Identification of the optimal method for removing the capsule from the acinus of the rat’s mandibular glands when preparing specimens for superficial morphology examination

By

Mamoru UEMURA1, Takamitsu ARAKAWA2, Rieko KOMINAMI3, Satoru HONMA3, Akimichi TAKEMURA1

1Department of Anatomy, Osaka Dental University, 8-1, Kuzuhahanazono-cho, Hirakata, Osaka 573-1121, Japan
2Department of Rehabilitation Science, Kobe Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma-ku, Kobe, Hyogo 654-0142, Japan
3Department of Anatomy, School of Medicine, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Kahoku, Ishikawa 920-0293, Japan

–Received for Publication, September 1, 2017–

Key Words: mandibular gland, acinar capsule, scanning electron microscope, removing method of capsule

Summary: The superficial morphology of the acinus of the mandibular gland in rats, which corresponds to the submandibular gland in humans, is very difficult to observe under scanning electron microscope due to a closely adherent capsule. Therefore, we evaluated the most effective protocol for removing this capsule from the acinus using various solutions, at different temperatures and for different durations of soaking. Based on the data for 50 male Wistar rats, the most effective method was soaking in an 8 N hydrochloric acid solution at 60°C for 70 min, in a water bath, followed by soaking in a 0.1–0.2% collagenase solution at 37°C for 330–350 min.

Introduction

The superficial morphology of the acinus of the mandibular gland in rats, which corresponds to the human submandibular gland1, is very difficult to observe under scanning electron microscope due to a closely adherent capsule. Our aim in this study was to evaluate the most appropriate method for removing the capsule from the acinus using various solutions, at different temperatures and for different durations. Adequate removal of the capsule allowed us to perform a morphological study of the mandibular gland in a rat model of type 2 diabetes mellitus2.

Materials and Methods

Experimental animals

Ninety mandibular glands were harvested from 45 Wistar rats (8-weeks-old; body weight, 220–180 g; Shimizu Laboratory Supplies, Kyoto, Japan). Our methods were approved by the Osaka Dental University Animal Research Committee (approval number 10-07002, 11-03015, 12-06001, and 13-02031) and complied with the guidelines for animal research.

Harvesting of the mandibular glands

Under isoflurane inhalation anesthesia (Forane®, Abbott Japan, Tokyo, Japan), heparin sodium (1000 units; Novo Heparin Injection 5000®, Mochida Pharmaceutical, Tokyo, Japan) was administered via intraperitoneal injection. After a 30-min resting period, animals were euthanized with an intraperitoneal injection of sodium pentobarbital (Nembutal®, Dainippon Sumitomo Pharma, Osaka, Japan). We inserted a cannula through the left ventricle and into the ascending aorta, and infused physiological saline into the ascending aorta. The right atrium of the heart was then drained from its blood. Following this procedure, a physiological saline solution, containing a 2.5% (W/V) glutaraldehyde solution (Kishida Chemical, Osaka, Japan) and a 2% paraformaldehyde solution (Formaldehyde Solution®, Kishida Chemical), was in-
fused into the ascending aorta. The mandibular glands were then harvested and fixed by soaking in the same solution at 4°C for 24 h. The specimens were washed with an ultrasonic cleaner (UT-105HS®, Sharp, Osaka, Japan) and the cleaned specimens were used to evaluate the effectiveness of different techniques to remove the capsule from the acinus portion of the mandibular glands.

**Test protocols to remove the capsule from the acinus**

The different combinations of solutions, durations and temperatures tested are summarized in Table 1, and described below.

1) **4N sodium bromide**

Specimens were soaked in 4N sodium bromide, at 25°C (room temperature) for 720 min.

2) **6N sodium hydroxide**

Specimens were soaked in 6N sodium hydroxide at 60°C for 10 and 20 min, in a water bath (Thermo Regulator, CTR-320, Iwaki, Tokyo, Japan).

3) **8N hydrochloric acid**

Specimens were soaked in 8N hydrochloric acid at 60°C for 20, 30, 50, 90, and 120 min, in a water bath.

4) **1N hydrochloric acid and 0.1% collagenase**

Specimens were soaked in 1N hydrochloric acid at 60°C for 50 min, in a water bath. Following this first soaking, specimens were soaked in a 0.1% collagenase solution (Sigma-Aldrich Japan, Tokyo, Japan) at 37°C for 360 and 480 min.

5) **8N hydrochloric acid and 0.1% collagenase**

The specimens were soaked in 8N hydrochloric acid at 60°C for 70 min, in a water bath, followed by a second soaking in a 0.1% collagenase at 37°C for 300, 330, 345, and 350 min.

6) **8N hydrochloric acid and 0.2% collagenase**

Specimens were soaked in 8N hydrochloric acid at 60°C for 70 min, in a water bath, followed by a second soaking in a 0.2% collagenase solution at 37°C for 330, 345, 350, and 360 min.

**Assessment of the relative effectiveness of test protocols to remove the capsule from the acinus**

After each test protocol, specimens were washed with a 0.1 mol phosphate buffer solution at room temperature (25°C) for 48 h. Following this initial washing, specimens were soaked in a 1% tannic acid solution (Kishida Chemical) at 25°C for 2 h, and subsequently washed again in a 0.1 mol phosphate buffer for 2 h. Specimens were then soaked in 1% osmium tetroxide solution (Kishida Chemical) at 25°C for 2 h. After conductive staining, specimens were dehydrated with ascending alcohol and subsequently soaked in t-butyl alcohol (Kishida Chemical) at 25°C for 12 h. Specimens were then frozen and dried (JFD-310®, JEOL, Tokyo, Japan). Each specimen was mounted on a metal stage with conductive tape (NEM TAPE®, Nisshin EM, Tokyo, Japan) and the silver paste (Dotite®, Fujikura Kasei, Tochigi, Japan). Digital images of the superficial morphological specimens of the mandibular glands were obtained with a scanning electron microscope (JSM-5500®, JEOL), at an acceleration voltage of 5 kV and a working distance of 47 mm.

---

**Table 1. Various conditions of removing capsule of the submandibular glands.**

| 1st Solution | Temperature (°C) | Time (min) | 2nd Solution | Temperature (°C) | Time (min) | Result | Fig. |
|--------------|------------------|------------|--------------|------------------|------------|--------|------|
| 4N sodium bromide | 25 | 720 | - | - | - | R | 1 |
| 6N sodium hydroxide | 60 | 10 | - | - | - | D | - |
| | | 20 | - | - | - | D | - |
| 8N hydrochloric acid | 60 | 20 | - | - | - | R | 2a |
| | | 30 | - | - | - | R | 2b |
| | | 50 | - | - | - | R | 2c |
| | | 90 | - | - | - | D | - |
| | | 120 | - | - | - | D | - |
| 1N hydrochloric acid | 60 | 50 | 0.1% collagenase | 37 | 360 |
| | | | | 480 | R | 3a |
| | | | | | R | 3b |
| 8N hydrochloric acid | 60 | 70 | 0.1% collagenase | 37 | 300 |
| | | | | 330 | R | 4a |
| | | | | 345 | * | 4b |
| | | | | 350 | * | 4c |
| | | | | | D | 4d |
| 8N hydrochloric acid | 60 | 70 | 0.2% collagenase | 37 | 330 |
| | | | | 345 | * | 5a |
| | | | | 350 | * | 5b |
| | | | | 360 | * | 5c |
| | | | | | D | 5d |

B: breaking of the acinus, D: dissolution of the acinus, R: remaining of the capsule, *: success in removing of capsule.
Results

The most effective method for removing the adhesive capsule from the acinus portion of the mandibular gland was by soaking in an 8 N hydrochloric acid solution at 60°C for 70 min, in a water bath, followed by soaking in a 0.1–0.2% collagenase solution at 37°C for 330–360 min.

The 4N sodium bromide solution, at 25°C for 720 min, did not sufficiently dissolve the many thick fibers (arrow head) in the capsule (Fig. 1). In contrast, the 6N sodium hydroxide solution at 60°C, with either a 10- or 20-min soaking duration, caused a collapse of the acinus portion of the mandibular gland, which subsequently dissolved. The specimen evaporated with no opportunity for observation. Similarly, soaking in 8N hydrochloric acid at 60°C for 90 or 120 min also caused the acinus to collapse, and subsequently the acinus was dissolved. A shorter duration of soaking in 8N hydrochloric acid at 60°C (namely, 20, 30 or 50 min) was insufficient to fully remove the capsule (arrow head; Figs. 2a–c). Similarly, soaking in 1N hydrochloric acid at 60°C for 50 min with a subsequent soaking in a 0.1% collagenase solution at 37°C for 360 or 480 min hardly removed the capsule, with parts of the acinus not available for observation (Figs. 3a–b). In contrast, combining soaking in an 8N hydrochloric acid solution at 60°C for 70 min followed by soaking in a 0.1% collagenase solution at 37°C for 300, 330, 345, or 350 min was efficient in removing the capsule, with the extent of removal increasing with the duration of soaking, from 300 min (in which a small amount of capsule remained adherent) to 350 min (at which point the acinus was dissolved (Figs. 4a–d). When the initial soaking in the 8N hydrochloric acid was followed by soaking in a 0.2% collagenase solution, peeling of the capsule was further facilitated (Figs. 5a–c), with the acinus dissolving at a soaking duration of 360 min (Fig. 5d).

Fig. 1. Scanning electron micrographs of the surface morphology of the mandibular gland for specimens soaked in a 4 N sodium bromide solution at 25°C for 720 min.

Fig. 2. Scanning electron micrographs of the surface morphology of the mandibular glands for specimens soaked in an 8 N hydrochloric solution at 60°C for 20 min (a), 30 min (b) and 50 min (c), in a water bath.
Discussion

We evaluated our optimal protocol to remove the adherent capsule of the acinus of the mandibular gland in rats to previously used protocols. Sodium bromide has been previously used to remove the mucosal epithelium of the dorsum of the tongue with excellent results obtained in unfixed samples. The poor effectiveness of sodium bromide in our study might have resulted from the fact that we fixed the tissue in a physiological saline solution.

Fig. 3. Scanning electron micrographs of the surface morphology of the mandibular glands for specimens soaked in a 1 N hydrochloric solution at 60°C for 50 min, in a water bath, followed by soaking in a 0.1% collagenase solution at 37°C for 360 min (a) and 480 min (b).

Fig. 4. Scanning electron micrographs of the surface morphology of the mandibular glands for specimens soaked in an 8 N hydrochloric solution at 60°C for 70 min, in a water bath, followed by soaking in a 0.1% collagenase solution at 37°C for 300 min (a), 330 min (b), 345 min (c), and 350 min (d).
Removing the acinar capsule from the mandibular gland

solution, containing glutaraldehyde and paraformaldehyde, after the initial soaking. That sodium hydroxide dissolved the acinus likely results from its strong alkalinity. The difficulty with using 8N hydrochloric acid was the high density of the solution that made it difficult for us to accurately adjust the appropriate duration of soaking. To address this issue, we used 1N hydrochloric acid, at 60°C for 50 min, followed by soaking in a 0.1% collagenase solution at 37°C. However, even with a long duration of soaking (360–480 min), the capsule remained largely adherent. Consequently, 8N hydrochloric acid was needed and, therefore, we adjusted the subsequent soaking using either a 0.1% or 0.2% collagenase solution, at 37°C, with a soaking duration of 340-350 min in the 0.2% collagenase solution allowing the capsule to be easily peeled in its entirety. The acinus did dissolve at a duration of soaking of 350 min in the 0.1% collagenase solution and 360 min in the 0.2% solution.

Acknowledgements

We would like to thank the staff of the facilities of Osaka Dental University for their support with the animal experimentations and image processing.

References

1) Hayakawa T, Yamashita H, Iwaki T: A Color Atlas of Sectional Anatomy of Rat. Chikusan Publishing Tokyo, 1997; 29 (in Japanese).
2) Morishita A, Uemura M, Suwa F: Morphological study of the submandibular gland in the type 2 diabetes mellitus model rat. J Osaka Dent Univ 2014; 48:1–8.
3) Nakamura M, Okada S: Microvascular architecture of the lingual papillae in the Japanese monkey (Macaca fuscata fuscata) Okajimas Folia Anat Jpn 1992; 69:183–198.

Fig. 5. Scanning electron micrographs of the surface morphology of the mandibular glands for specimens soaked in an 8 N hydrochloric solution at 60°C for 70 min in a water bath, followed by soaking in a 0.2% collagenase solution at 37°C for 330 min (a), 345 min (b), 350 min (c), and 360 min (d).