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Arecoline Alters Taste Bud Cell Morphology, Reduces Body Weight, and Induces Behavioral Preference Changes in Gustatory Discrimination in C57BL/6 Mice

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Abstract

Arecoline, a major alkaloid in areca nuts, is involved in the pathogenesis of oral diseases. Mammalian taste buds are the structural unit for detecting taste stimuli in the oral cavity. The effects of arecoline on taste bud morphology are poorly understood. Arecoline was injected intraperitoneally (IP) into C57BL/6 mice twice daily for 1–4 weeks. After arecoline treatment, the vallate papillae were processed for electron microscopy and immunohistochemistry analysis of taste receptor proteins (T1R2, T1R3, T1R1, and T2R) and taste associated proteins (α-gustducin, PLCβ2, and SNAP25). Body weight, food intake and water consumption were recorded. A 2-bottle preference test was also performed. The results demonstrated that 1) arecoline treatment didn't change the number and size of the taste buds or taste bud cells, 2) electron microscopy revealed the change of organelles and the accumulation of autophagosomes in type II cells, 3) immunohistochemistry demonstrated a decrease of taste receptor T1R2- and T1R3-expressing cells, 4) the body weight and food intake were markedly reduced, and 5) the sweet preference behavior was reduced. We concluded that the long-term injection of arecoline alters the morphology of type II taste bud cells, retards the growth of mice, and affects discrimination competencies for sweet tastants.

Key words: electron microscopy, gustatory discrimination, morphological alteration, sweet preference, taste bud cell

Introduction

Areca nut (*Areca catechu*, palm seed) chewing is a key factor in taste sensation defects and oral cancer, producing euphoria, sweating, and palpitation (Wang et al. 1997). Areca nut is the fourth most common addictive substance after alcohol, tobacco, and caffeine (Winstock 2002), and arecoline is the major alkaloid of areca nut present in the saliva of areca nut chewers (Guh et al. 2007). Arecoline is absorbed in the buccal cavity and is detected in the blood in dose- and time-dependent manners during fasting in humans (Strickland et al. 2003). Nair et al. (1985) reported that the detected concentration of arecoline in the saliva of areca nut chewers was approximately 0.3 mM or 0.1–10 μg/mL, with a sudden peak concentration of approximately 140 μg/mL. At these concentrations, arecoline induces oral carcinogenesis (Jeng et al. 2003), inflammation (Hendricks et al. 2004) and reactive oxygen species production (Thangjam and Kondaiah 2009). The clinical features associated

with chewing areca nut include excessive salivation, absent gustatory sensation, juxtaepithelial hyalinization, and muscle fibrosis of the oral cavity (Alshadwi and Bhatia 2012).

Mammalian taste buds are the basic structural and functional units for the detection of different taste stimuli, and these structures are primarily distributed on the dorsal surface of the tongue and throughout the mucosal oral cavity. Each taste bud harbours 50–100 taste bud cells, including gustatory cells (type II, III), supporting cells (type I), and basal cells (Finger 2005). Taste receptor cells express the molecular machinery for detecting taste compounds (tastants) and transmitting the signals, either directly or indirectly, via other taste bud cells, to the peripheral gustatory nerves that innervate the taste buds. Taste abnormalities not only decrease the quality of life but also lead to anorexia, weight loss, malnutrition, and certain diseases and conditions, and the normal aging process might be associated with taste disorders (Hamamichi et al. 2006; Cohn et al. 2010; Perea-Martinez et al. 2013). Aberrations in taste bud homeostasis, such as abnormal or suboptimal cell renewal, differentiation and degeneration, are likely contributors to taste dysfunction (Wang et al. 2009; Kim et al. 2012; Shin et al. 2012).

The results of a recent study (Rooban et al. 2006) indicated that the salivary flow rate and pH are altered in areca nut chewers, rendering the oral mucosa vulnerable to the toxic effects of areca nut. In addition, Mirlohi et al. (2015) claimed that study of salivary constituents might provide information concerning taste dysfunction, which is relevant in health and disease. To the best of our knowledge, the effects of arecoline on the taste bud morphology have not been reported; therefore, we selected C57BL/6 mice as an animal model to examine the effects of arecoline on 1) the morphological and immunohistochemical alterations of taste bud cells, 2) animal growth, and 3) the preference changes in gustatory discrimination.

Materials and methods

Animals and arecoline treatment

Adult C57BL/6 mice (6–8 weeks old, ~20 g body weight) were purchased from National Taiwan University Animal Center and maintained in polythene cages under controlled conditions (photoperiod, 12 L/12 D, room temperature: 22 °C, relative humidity: 55%) with adequate food and water for 2 weeks for acclimatization prior to the experiments. All experiment protocols were approved by the Laboratory Animals Committee, College of Medicine, National Taiwan University. Arecoline-hydrobromide (methyl 1-methyl-1,2,5,6-tetrahydronicotinate hydrobromide) (A-6134, Sigma–Aldrich) was dissolved in 0.1 M phosphate-buffered saline (PBS, pH 7.4). For the experimental groups, 2 injections were administered IP into each mouse at a dose of 2 mg/kg body weight daily at 9 AM and 9 PM for 7, 14, 21, and 28 days, and the control group did not receive treatment (normal control). The body weight, food intake, and water intake were measured and recorded every other day during the arecoline treatment period. At the completion of arecoline treatment, all animals were sacrificed, and the following experiments were performed.

Tongue epithelium isolation and scanning electron microscopy

The animals were deeply anaesthetized and sacrificed with an overdose of Nembutal (sodium pentobarbital, 60–80 mg/kg body weight). The entire tongue containing vallate papilla was immediately removed prior to death. The lingual epithelium was isolated as previously described (Huang et al. 2005). The peeled epithelium was pinched flat on a piece of cork, fixed in 4% paraformaldehyde, and post-fixed in 1 % OsO_4. After ethanol dehydration and critical point drying (HCP-2 Critical Point Drier, Hitachi, Tokyo, Japan), the specimens were mounted on metal stubs, coated with gold and examined in a scanning electron microscope (JEOL-1100, JEOL). Because mice only have 1 vallate papilla (2 trenches per papilla) and more than 95% of the taste pores represent one taste bud, the number of taste buds per vallate papilla can be expressed as the total number of taste pores on the exposed trench surfaces from each mouse vallate papilla ($n = 6$, per group).

Transmission electron microscopy

The mice were perfused with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) under deep anaesthesia (40–50 mg/kg bw). At the completion of perfusion, the lingual tissues containing vallate papillae were immediately dissected and immersed in the same fixative for 4 h at 4 °C. After cutting into 100-µm-thick sections with a vibratome, the specimens were osmicated, dehydrated, and subsequently embedded in Polybed-Araldite mixture (EMS-Grivory). Thin 70-nm sections were cut and stained with uranyl acetate and lead citrate before examining in a Hitachi H-7100 transmission electron microscope equipped with a Gatan 832 digital camera (Gatan, Inc.).

Determination of the taste bud size and taste bud cell number

Ribbons containing 10 longitudinal serial plastic semithin sections (1 µm) from each resin-embedded block were collected prior to thin sectioning onto a glass slide and stained with toluidine blue (T3260, Sigma–Aldrich). Images of the taste buds were obtained from every other section in a light microscope (DMR HC, Leica) equipped with a Nikon D70 digital camera with Nikon Capture 4.0 software. For the taste bud size determination, the maximal taste bud profile area was measured. As shown in Figure 1B, the long axis (a) of a taste bud was measured from the taste pore to the basement membrane of the of the oral epithelium, the short axis (b) was measured by tracing the widest line perpendicular to the long axis, and the maximal profile area of taste bud was calculated as $\pi \times a \times b/4$. To determine the number of cells in each taste bud, the numbers of taste bud cells were quantified according to the modified method of Ohtubo and Yoshii (2011). Using Adobe Photoshop CS4 (Adobe), we compared every 2 adjacent images of an identified taste bud with a maximal diameter not shorter than the average of the short axis (ca. 32 µm) to ensure each taste bud was counted once. The number of taste cells in a taste bud was the sum of the taste cell border in every other image in the series.

Cryostat section, immunostaining, and quantitative analysis of immunoreactive cells

The lingual tissues containing vallate papillae were dissected from the mouse immediately after perfusion with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Tissue blocks were cryoprotected after submerging in 10% and 20% sucrose in PBS for 2 h and then overnight in 30% sucrose in PBS. The next day, the tissue samples were embedded in OCT compound (Sakura Finetechnical Co.) and flash-frozen in a liquid nitrogen bath. The sections (9 µm thick) were cut in cryostat (model CM3050S; Leica Microsystems) and stored at −80 °C until further use.

For immunostaining, the stored cryostat sections of vallate papillae were thawed and rehydrated, followed by incubation in blocking buffer for 2 h at room temperature, with subsequent incubation for

24 h at 4 °C in the primary antisera at the indicated working concentration (Table 1). After several rinses in PBS, the sections were incubated with AlexaFluor 488 (green fluorescence)-conjugated secondary antibody (1:200; Invitrogen) for 1 h in the dark at 37 °C. The sections were subsequently washed again and mounted with antifading mounting medium (Vecta Shield H-1000, Vector Laboratories) and photographed with a Leica TCS SP5 confocal microscope. The staining specificity was assessed after treating the sections in the absence of primary antibodies.

Confocal images, representing single optical sections (1024 × 1024 pixels), were collected in a TCS SP5 confocal microscope equipped with an oil-immersion objective lens (PL APO 40X 1.25-0.75 NA). Approximately 100–180 taste buds per animal were analyzed, and the taste bud cells were scored as immunoreactive only when a nuclear profile was present in the cell. The total number of taste buds in each section was counted, and the average number of immunoreactive taste cells expressed in single taste bud was calculated by dividing the number of immunoreactive taste cells by the total number of taste buds.

Preference behavior test

After 4 weeks of arecoline treatment, the animals were subjected to a 2-bottle preference test between deionized water and 1 % sucrose in a random schedule. The amount of liquid consumed was measured every 48 h. Each test solution was presented for 2 consecutive days, 1 day on the left and 1 day on the right side of the cage to control for bottle position habits.

Statistical analysis

All the numerical data are expressed as the mean ± SEM (standard error of mean), and the mean values of different experimental groups were analyzed with ANOVA. A P value less than 0.05 was considered statistically significant.

Results

Morphological alterations of the taste buds in arecoline-treated mice

Figure 1A shows the distribution and arrangement of taste pores on the exposed trench wall surface of vallate papilla in a control mouse with scanning electron microscopy. There were no significant differences in the distribution, arrangement, and total number of taste pores between the control group and the arecoline-treated groups (Table 2).

As shown in Table 2, the numerical data for the long (*a*) and short (*b*) (Figure 1B) taste bud axes, the maximal taste bud profile area (MTBPA, πab/4; n = 24) and the average number of taste bud cells per cross-sectional vallate taste buds profile (n = 24) (Figure 1C) showed no statistically significant difference between the control and arecoline-treated groups. Thus, the number, size and arrangement of taste buds were not affected in arecoline-treated mice at the light microscopy level.

Ultrastructure alterations

After arecoline treatment, significant ultrastructural changes were primarily detected in type II cells, whereas type I, type III, and basal cells appeared relatively normal compared with the control. Therefore, the following description focused on the ultrastructural changes of type II cells.

Figure 2 shows the electron micrographs of the vallate taste buds from normal control (Figure 2A,D,G) and arecoline-treated

Table 1. Primary antibodies used in the study

Antibody	Working dilution	Source	Cat No.	Reference
Rabbit polyclonal anti-α-gustducin	1:500	MyBiosource	MBS421805	
Rabbit polyclonal anti-PLCβ2	1:1000	Santa Cruz	Sc-206	Kataoka et al. (2008)
mouse polyclonal anti-SNAP25	1:1000	Chemicon	MAB331	Yang et al. (2000)
Rabbit polyclonal anti-TAS1R1	1:500	Alpha Diagnostic	TR11-A	Daly et al. (2013)
Rabbit polyclonal anti-TAS1R2	1:200	Abcam	AB79229	Elliott et al. (2011)
Rabbit polyclonal anti-TAS1R3	1:200	Abcam	AB74732	Sothilingam et al. (2011)
Goat polyclonal anti-T2R6	1:200	Santa Cruz	Sc-34286	

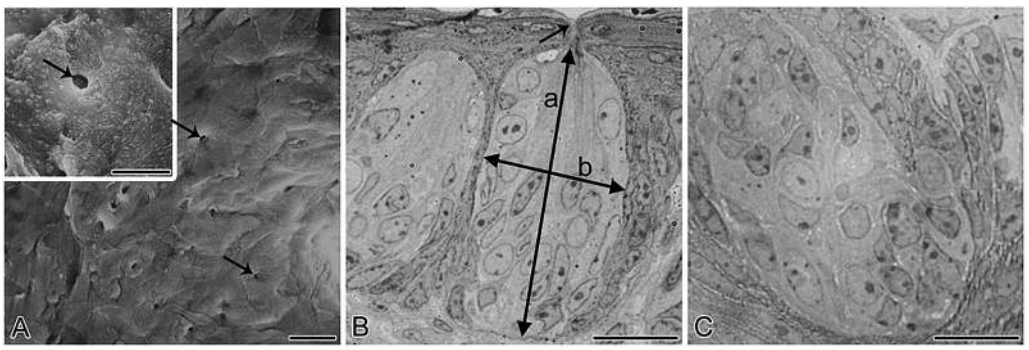

Figure 1. Morphology of the taste bud. (**A**) Scanning electron micrograph showing numerous taste pores (arrows) on the exposed trench surface of the mouse vallate papilla. Inset: higher magnification image of a taste pore. (**B**, **C**) Light micrographs of toluidine blue-stained semithin plastic sections of the taste buds in vallate papilla from control mice. In B, the long axis (*a*) of a taste bud is measured from the taste pore (arrow) to the basement membrane of the oral epithelium, and the short axis (*b*) is measured by tracing the widest line perpendicular to the long axis. (**C**) Transverse sections through the middle portion of the taste buds. The taste buds appear round in shape and surrounded by keratinocytes. The number of the taste cells per profile of taste bud in each section is quantified (the details are described in the text). Each scale bar represents 10 μm.

Table 2. MTBPA, number of taste bud per animal, and number of cells per taste bud profile in mice with or without arecoline treatment

Condition of mice	No. of animals	MTBPA (n = 24, in μm^2)	Average long axis-a (n = 24, in μm)	Average short axis-b (n = 24, in μm)	No. of taste buds/ per animals	No. of cells per taste buds profile (n = 24)
CTL	6	1273.43 ± 212.6	68.83 ± 7.1	31.12 ± 8.9	192 ± 14.4	22.1 ± 9.9
W1	6	1021.66 ± 314.1	63.56 ± 12.8	30.91 ± 15.1	201 ± 30.5	26.1 ± 6.2
W2	6	1216.18 ± 167.1	65.75 ± 9.4	31.76 ± 7.7	181 ± 23.4	20.9 ± 7.9
W3	6	1191.76 ± 191.7	63.39 ± 15.1	31.48 ± 5.7	193 ± 21.7	24.3 ± 3.6
W4	6	1208.18 ± 289.1	66.57 ± 12.2	31.02 ± 12.1	203 ± 16.2	20.2 ± 7.3

Effect of arecoline on the taste bud size and number in mouse vallate papilla. The taste bud size was measured using the formula $\pi ab/4$, where a and b are the long and short axes of the taste bud, respectively. The number of taste buds in each CV papilla was determined after counting taste pores using scanning electron microscopy. The taste bud cell number was quantified according to the modified methods of Ohtubo and Yoshii (2011) as described in the text. MTBPA = $\pi \times a \times b / 4$.

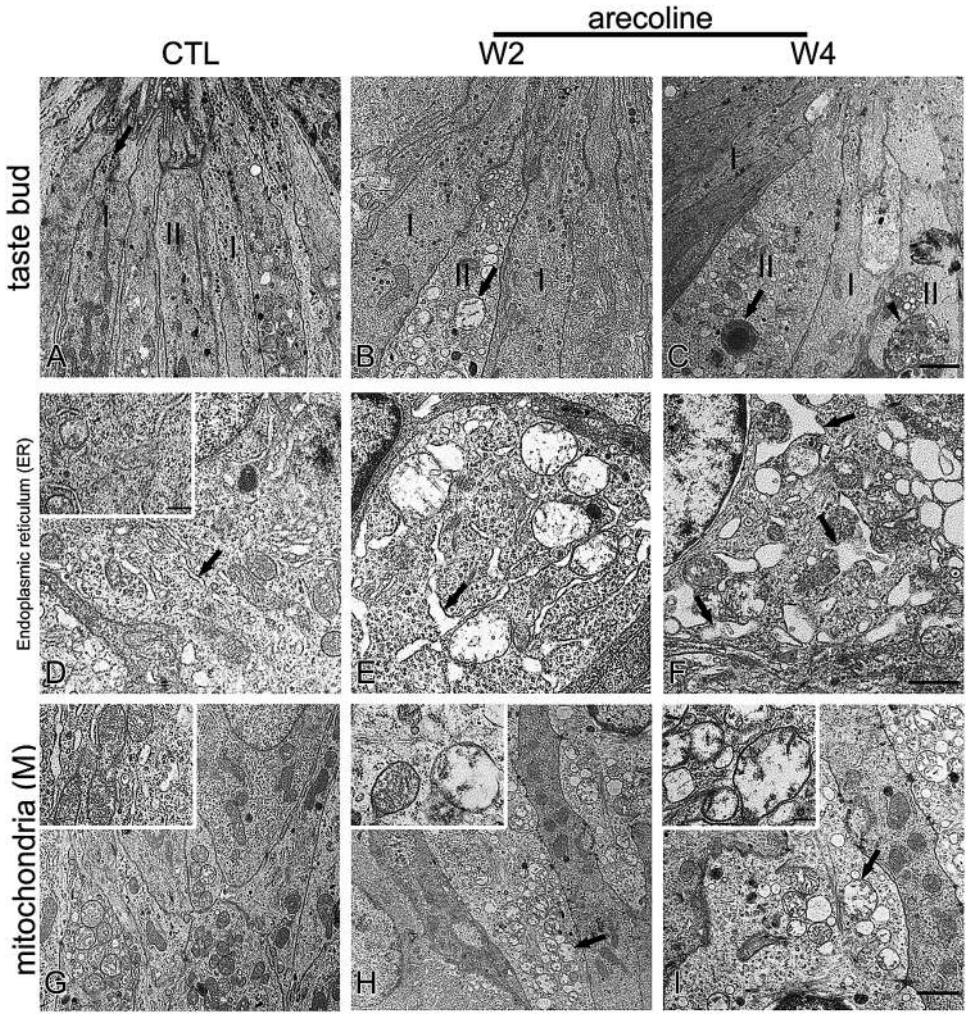

Figure 2. Ultrastructural analysis of taste bud cells with or without exposure to arecoline. (**A**) Apical part of the vallate taste bud from a normal control mouse. Type I cell (I) displays dark cytoplasm and contains numerous dense secretory granules (arrows). (**B**) Apical portion of the vallate taste bud from a mouse after arecoline treatment for 2 weeks. Type II (II) cell containing pale cytoplasm filled with dense bodies and swollen mitochondria (arrow). (**C**) Apical portion of the vallate taste bud from a mouse after arecoline treatment for 4 weeks. The number of dense granules in the apical cytoplasm of type I taste cells is decreased, but the dense bodies (arrow) and autophagic vacuoles (arrow head) are increased in size and number, particularly in type II taste cells. A–C, scale bars = 1 μm. (**D**) For normal control mice, the juxta-nuclear cytoplasm of the type II taste bud cells contains regular slender ER cisternae and oval to round mitochondria. (**E**) After arecoline treatment for 2 weeks, the type II cell exhibited mildly dilated ER cisternae (arrow) and swollen mitochondria. (**F**) Dilated vesicular form of ER cisternae and swollen mitochondria were observed in the type II cells of mice after arecoline treatment for 4 weeks. D–E, scale bars = 500 nm. (**G**) The cytoplasm of normal taste bud cells was rich in mitochondria with cristae alignment. (**H**) After arecoline treatment for 2 and (**I**) 4 weeks, the cytoplasm of some type II cells contained swollen mitochondria (arrow). Inset: high magnification of swollen mitochondria with cristae loss. G–I, scale bars = 2 μm and for high magnification scale bars = 500 nm.

mice (Figure 2B,C,E,F,H,I). The mouse taste buds of control or normal mice (Figure 2A) showed characteristic nuclear morphologies and cytoplasmic electron densities, rendering 4 different cell types: dark cells (type I), light cells (type II), intermediate cells (type III), and basal cells. Type I cells possess high-electron density cytoplasm (dark cells) and accumulated round to oval dense

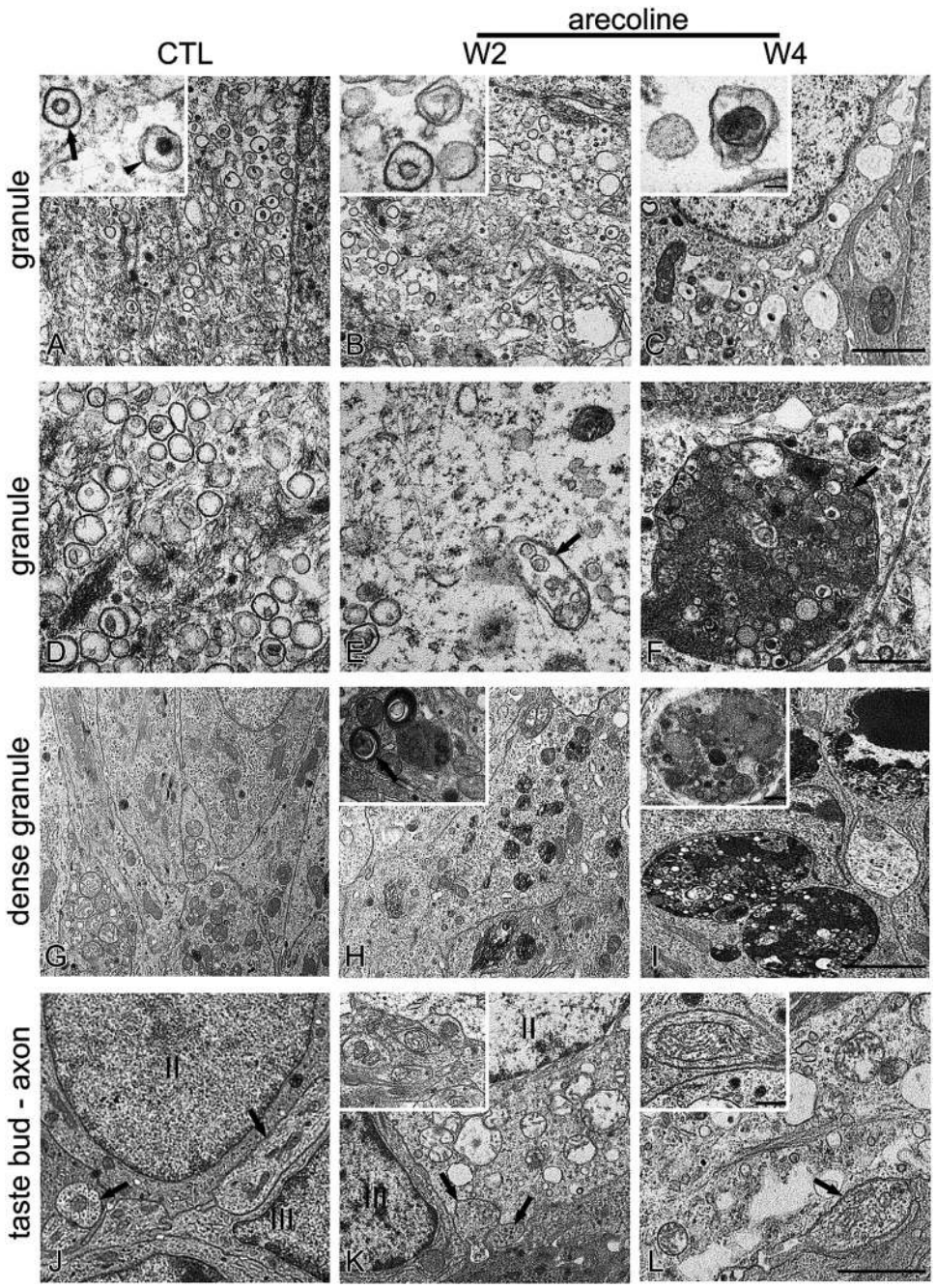

Figure 3. Transmission electron microscope micrographs showing the toxic effects of arecoline on the ultrastructure of type II taste bud cells. (**A**) In control mice, double-ring membrane granules and dense-core granules characterized the type II taste cell cytoplasm. Inset: high magnification of double-ring membrane granules (arrow) and dense-core granules (arrow head). (**B**) After arecoline treatment for 2 weeks, the double-ring membrane granules and dense-core granules remained normal in type II cells. (**C**) The dense-core granules were enlarged and irregularly shaped after treatment with arecoline for 4 weeks. A–C, scale bar = 500 nm, and inset scale bars = 10 nm. (**D**) In control mice, the cytoplasm of type II taste bud cell contains abundant double ring-membrane granules (Dr) and dense-core granules (Dc). (**E**) After arecoline treatment for 2 weeks, few granules encompassed by smooth ER-like structures were observed (arrow). (**F**) A variety of granules were observed and surrounded by a 2-layer isolation membrane to form autophagosomes (arrow) at 4 weeks after arecoline treatment. The dense-core granules were enlarged and irregularly shaped after treating with arecoline for 4 weeks. A–C, scale bars = 300 nm. (**G**) In normal control mice, type I taste bud cells were rich in round, oval, or elongated mitochondria with parallel cristae alignment, and type II taste cells contained rather round or oval mitochondria with pale matrices. (**H**) After arecoline treatment for 2 weeks, the dense bodies were increased in size and number in the cytoplasm of type II taste cells. Inset: part of the cytoplasm of type II taste cell showing heterogeneous dense bodies and myelin-like structures (arrow). (I) Large autophagic vacuoles (or residual bodies) were prominent in type II taste cells at 4 weeks after arecoline treatment. Inset: large autophagic vacuole contains mitochondria, heterogeneous dense bodies and organelle debris. E–F, scale bars = 2 μm and Inset, Scale bars = 1 μm. Intragemmal nerve profiles (arrow) were observed among the taste bud cells and remained intact in control mice (**J**), and animals treated with arecoline for 2 (**K**) or 4 **(L)** weeks. G–I, scale bars = 2 μm and inset scale bars = 500 nm.

secretory granules in the apical cytoplasm. Type II cells displayed a low-electron density cytoplasm with round or oval mitochondria (Figure 2G), characterized by the appearance of double-ring membrane granules (the arrow in the inset of Figure 3A) and dense-core granules (the arrow head in in the inset of Figure 3A) (both are 50–70 nm in diameter, Figure 3A,B). Type III cells

contained indented nuclei and cytoplasm of intermediate density, and basal cells were located at the basal portion of the taste bud with round nuclei and abundant rough endoplasmic reticulum (ER) and ribosomes.

After 2 weeks of arecoline treatment, type II cells showed significant intracellular morphological changes. The majority of mitochondria were swollen and associated with a disturbed arrangement of cristae and partial or total cristolysis reflecting the dilation of the intracristal spaces, although a few mitochondria did appear normal with condensed matrices. Cytoplasmic disruption with mildly dilated cisternae of rough ER was also observed (Figure 2E). Most of the dense-core granules or double-ring membrane granules were not affected during this period (Figure 3B). However, some of these structures were surrounded by a smooth ER-like double-layered membrane structure, reflecting the autophagic process, and isolated from the cytoplasmic matrix (Figure 3E). Heterogeneous dense bodies (ca. 0.5 μm diameter) and multivesicular bodies (ca. 0.5 μm diameter) increased in number in the cytoplasm (Figure 3H). Occasionally, these structures became markedly enlarged (0.4–0.5 μm in diameter) and pleomorphic. The nearby heterogeneous dense myelinosomes were infrequently encountered (Figure 3H inset).

At 4 weeks after arecoline treatment, type I cells (Figure 2C) appeared as normal as control cells (Figure 2A), except for a decrease in the number of the apical dense secretory granules. Severe changes continually occurred in type II cells. Type II cells contained large heterogeneous dense bodies and autophagic vacuoles (Figure 2C). Dramatically dilated rough ER cisternae (Figure 2F), swollen mitochondria (Figure 2I) and enlarged and irregularly shaped dense-core granules (Figure 3C) were frequently observed in type II cells. Large heterogeneous dense autophagic vacuoles containing accumulated debris, and dense bodies and dense-core granules forming double membrane-bound autophagic vacuoles were frequently observed in type II cells (Figure 3F,I). In addition, the autophagic vacuoles were larger in size and more conspicuous in the type II taste cells of animals treated with arecoline for 4 weeks compared with those treated for 2 weeks.

The intragemmal nerve profiles and synapses between nerve fibers and taste cells remained intact, and no discernible changes were observed (Figure 3J–L).

Decrease in sweet receptor (T1R2 and T1R3) immunoreactive cells after arecoline treatment

The immunohistochemical expression patterns of taste receptor proteins (T1R2, T1R3, T1R1, and T2R) and taste functional marker proteins (α-gustducin, PLCβ2, and SNAP25) in the vallate taste buds from control and arecoline-treated animals are shown in Figure 4A. It is clear that these taste buds, either in control or arecoline-treated groups, contained cells immunoreactive to α-gustducin, PLCβ2, SNAP25 and taste receptors T1R2 (sweet), T1R3 (sweet, umami) T1R1 (umami), and T2R (bitter). Quantitative analyses of immunoreactive cells per taste bud profile are shown in Figure 4B,C. There was no significant difference in the number of immune-reactive cells labeled with functional taste proteins α-gustducin, PLCβ2, and SNAP25 and the T1R1 umami and T2R bitter taste receptors between the control and arecoline-treated groups (α-gustducin, PLCβ2, SNAP25, T1R1, and T2R, $P > 0.05$). Compared with the control group, the numbers of T1R2- and T1R3-expressing cells were significantly decreased in arecoline treatment for 4 weeks. (T1R2, $P = 0.037$ and T1R3, $P = 0.035$).

Changes in body weight, food intake, and water consumption in arecoline-treated mice

Figure 5 depicts the body weight, food intake and water consumption in mice throughout the experiment. The body weight of the control group gradually increased for 4 weeks; however, the body weight of the animals in the arecoline-treated group remained nearly the same throughout the experiment (Figure 5A). At the end of the experiment, the body weight of the control, but not the arecoline-treated group, increased by nearly 7.5%. In addition, as compared with the control group, the arecoline-treated group showed a significant decrease of 30% in daily food intake (Figure 5B). Mouse water intake during the experimental period showed no significant difference among various groups (Figure 5C). Based on these data, it is clear that arecoline caused an acute and sustained reduction of food consumption, with a concurrent decrease in body weight without affecting water intake.

The taste preference was altered in mice treated with arecoline

As indicated in Figure 5C, there was no significant effect of arecoline administration on water intake between control animals and animals treated with arecoline for 1–4 weeks (Figure 5C). At the completion of arecoline treatment, a preference test was conducted with deionized water containing 1% sucrose solution. A high preference ratio of 1% sucrose was observed in the control group (Figure 6, CTL). However, the 1% sucrose preference ratio of arecoline-treated mice was reduced, and significant differences were observed at 3 and 4 weeks after arecoline treatment ($P = 0.024$).

Discussion

The results of the present study demonstrated that arecoline causes morphological changes in taste bud cells, retards the growth, and changes the drinking preference in C57BL/6 mice.

Methodological consideration for arecoline treatment

Several methods, including intragastric intubation, subcutaneous injection, IP injection, surface covering, feeding, drinking water addition, and direct areca nut chewing, have been applied to administer arecoline or areca nut components into various experimental animals with different results. Although the dose and period of arecoline administration were markedly different, with the oral administration (OA) of areca nut, it is not easy to control the exact concentration of arecoline, and it requires a longer time than injection to induce arecoline effects (Bhide et al. 1979; Chiang et al. 2004; Chang et al. 2010). Longer periods of administration induce side effects, such as aging, which might lead to distortions in gustatory function and loss of taste perception (Schiffman 2009). Moreover, previous studies have confirmed that the IP or OA of arecoline induces similar defects in mice (Chatterjee and Deb 1999). In addition, the patterns of metabolism for the OA or IP administration of arecoline are broadly similar (Giri et al. 2006). Furthermore, studies evaluating blood samples showed that chewers had a 25 times higher mean blood arecoline level than nonchewers (Wu et al. 2010). These results suggest that the arecoline concentrations in the blood were highly correlated with cytotoxic properties. To control the exact dose of arecoline, we treated C57BL/6 mice with arecoline through IP injection, although this type of administration in mice does not completely mimic the areca nut chewing habits in humans and might only demonstrate the indirect effects of arecoline on gustatory tissues.

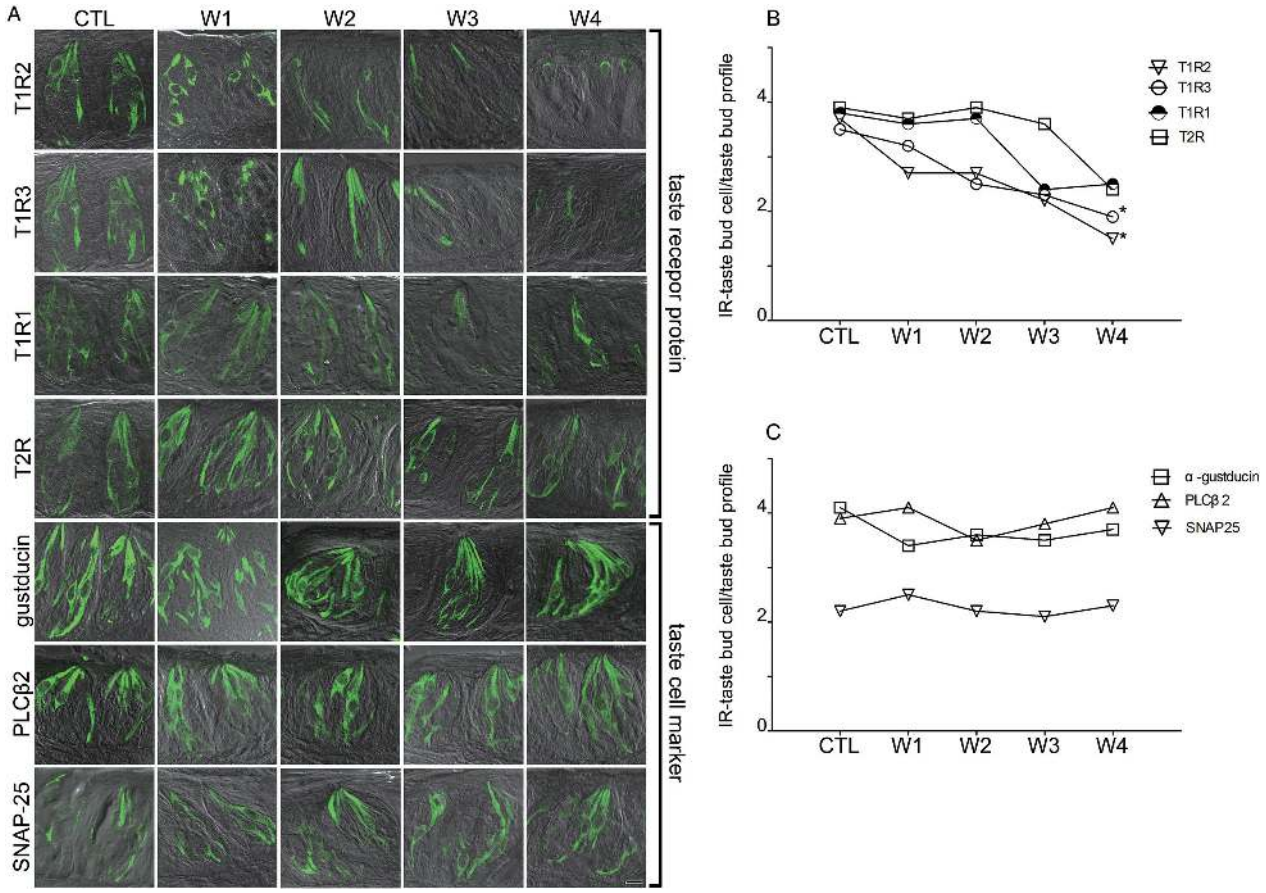

Figure 4. Immunohistochemical staining for taste-associated protein markers in the mouse vallate papilla of the taste buds. **(A)** Confocal images (green immunofluorescence) of taste-associated proteins in the taste buds are overlaid with DIC (differential interference contrast) images. Note that a small subset of taste cells displaying taste-associated proteins in taste buds, including T1R2, T1R3, T1R1, T2R, α-gustducin, PLCβ2, and SNAP25. Scale bars, 20 μm. (**B**, **C**) The number of taste-associated protein labeled cells per taste bud profile in control and arecoline-treated mice. Arecoline treatment did not change the number of α-gustducin-, PLCβ2-, SNAP25-, T1R1-, or T2R-expressing cells in taste buds compared with the control. In contrast, the number of T1R2- and T1R3-expressing cells in taste buds was significantly decreased at 4 weeks after arecoline treatment.

Taste bud number and size alterations in mice after arecoline treatment

In the present study, we observed that the number and size of taste buds in the vallate papilla did not significantly change in arecoline-treated mice. The morphology, size, and number of taste buds were strictly nerve-dependent (Takeda et al. 1996; Mistretta et al. 1999; Nosrat et al. 2012). The number of drugs or chemicals, for example, streptozotocin (STZ) (Pai et al. 2007), hemicholinium-3 (HC-3), and triethylcholine (TEC) (Hui and Smith 1976), could significantly affect the size and number of taste buds. The effects of these chemicals on taste buds are dependent on the innervation of taste bud cells. In the present study, no evidence of nerve injury was detected, synaptic structures in the taste bud remained intact and myelinated axons were also complete in arecoline-treated mice (data not shown). We propose that arecoline, at the concentrations examined, did not affect taste bud innervation, and therefore, the size and total number of taste buds in mice vallate papilla did not significantly change.

Ultrastructural degeneration of mice vallate taste cells

Although the size and number of taste bud cells did not significantly change in the present study, some changes at the electron microscopy level were observed. Arecoline treatment induced morphological degeneration, such as swollen mitochondria, dilated ER cisternae, and enlarged and irregular autophagic vacuoles. No signs of typical apoptotic chromatin condensation or fragmented nuclei were observed. The degenerating features of taste cells in the present study were similar to autophagic degenerating structures (Schweichel and Merker 1973; Clarke 1990), but not typical apoptosis. The electron micrograph displayed numerous autophagosomes abundantly accumulated in the cytoplasm after arecoline treatment for 4 weeks. Recent studies indicate that autophagy is involved in cellular protein and organelle degradation, mediated through the lysosomal pathway (Klionsky and Emr 2000). Autophagocytosis plays a key role in cellular housekeeping through the removal of damaged organelles. During aging, the efficiency of autophagic degradation declines, and intracellular waste products accumulate (Masiero et al. 2009; Salminen and Kaarniranta 2009). These findings suggest that arecoline decreases the taste cell metabolic rate and accelerates cell-aging processes, associated with autophagic degeneration, thereby reducing taste bud activity, as observed in the present study.

Changes in taste receptor immunoreactive cells

The taste buds cells showed morphological alterations; therefore, we assessed the expression of taste functional marker proteins in taste buds. Studies have implicated α-gustducin and PLCβ2 in bitter, sweet, and umami taste transduction (Ruiz et al. 2003; Zhang et al. 2003). SNAP-25-like immunoreactivity has been detected in

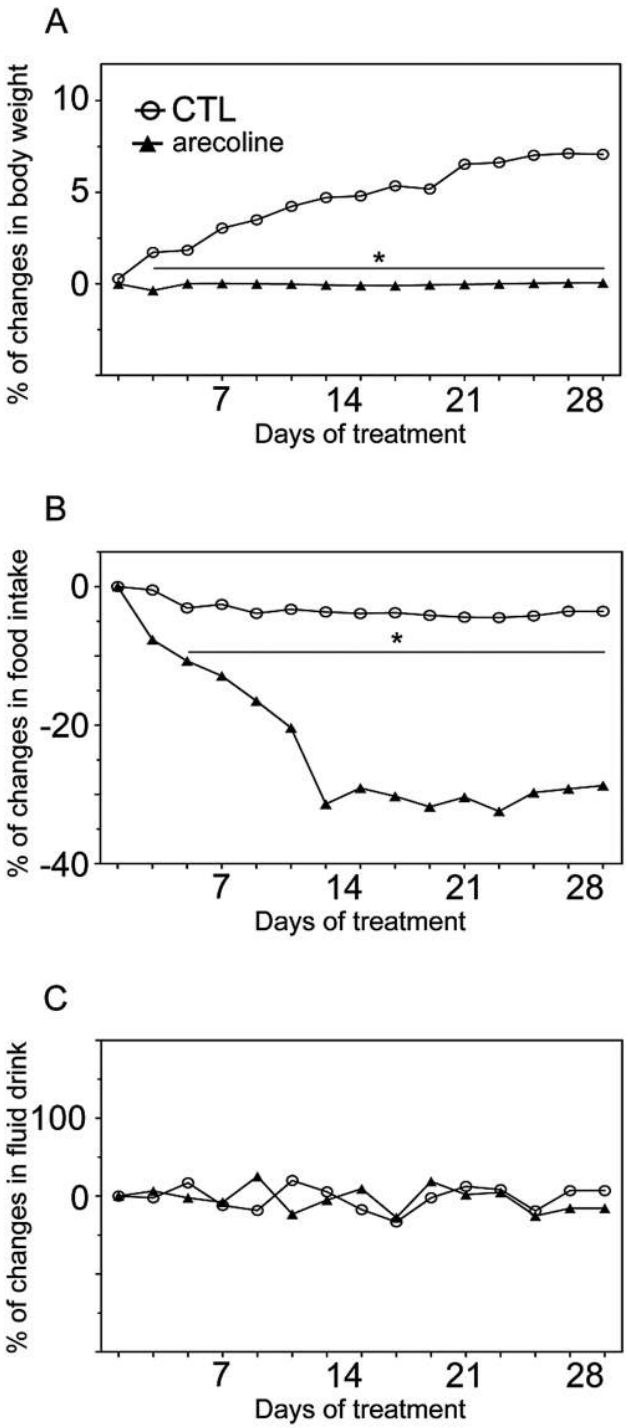

Figure 5. The percentage change in body-weight gain (**A**), food intake (**B**) and water consumption (**C**) in control (○) and arecoline-treated (▲) mice was recorded every other day after the completion of arecoline treatment (20 mg/kg bw, twice daily for 1–4 weeks). Note that from day 5, the body weight and food intake were significantly higher in the control animals than in arecoline-treated mice ($n = 6$/group. $^*P < 0.05$), and no difference in fluid consumption between the control and arecoline-treated groups was observed.

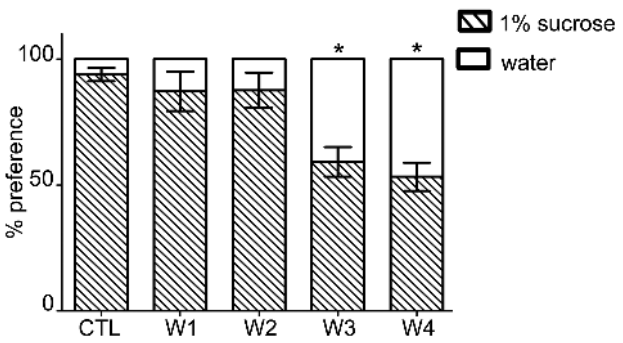

Figure 6. The 2-bottle preference test with 1% sucrose and water between the control and arecoline-treated mice. Note that after arecoline treatment for 3 weeks or longer, significant differences in the fluid intake of the 2 fluids were observed. The preference ratio was defined as the ratio of the volume of 1% sucrose or water ingested to the total fluid intake ($n = 6$/group and $^*P < 0.05$).

a small subset of taste cells, and this protein is a marker for type III cells, responsible for signaling sour and salty tastes (Kataoka et al. 2008; Vandenbeuch et al. 2008). In addition, 2 families (TR gene) of mammalian taste receptors, T1Rs and T2Rs, have been implicated in sweet, umami, and bitter detection. The T1R family contains three specific G protein-coupled receptors, T1R1, T1R2, and T1R3, including the sweet responsive T1R2–T1R3 heterodimer and the umami responsive taste heterodimer T1R1–T1R3 (Nelson et al. 2001, 2002). T2Rs are bitter responsive (Chandrashekar et al. 2000). Behavioral and physiological studies on T1R2/T1R3 double knockout mice have confirmed that T1R2/T1R3 double knockout mice completely lost the preference for sweeteners (Zhao et al. 2003). T1R knockout mice also showed no significant effect on either physiological or behavioral responses to citric acid, sodium chloride, or bitter tastants (Zhao et al. 2003). In the present study, the numbers of T1R2- and T1R3-expressing type II taste cells were significantly reduced after arecoline treatment. However, the evidence of morphological changes was not sufficient to explain the alterations **in** functional activity. Therefore, many studies have used 2-bottle choice tests to investigate rodent preferences for nutrients and taste solutions (Tordoff and Bachmanov 2003). Herein, the results from 2-bottle preference tests confirmed that the functional activity was indeed altered after arecoline treatment.

Alterations in sucrose consumption in arecoline-treated mice

In the present study, the numbers of T1R2- and T1R3-expressing cells were significantly reduced after arecoline treatment for 3–4 weeks. However, no significant alterations in T2R bitter and T1R1 umami taste receptor expression were observed between the control and arecoline-treated groups. Therefore, a 2-bottle preference behavior test was conducted to investigate the fluid intake patterns and preference between sweet tastants (1% sucrose solution) and water. In the present study, the arecoline-treated groups consumed more water and less sucrose solution when provided equivalent choices between the 2 tastants. Thus, the preference ratio for sucrose was exclusively lower than that of the control group. Hence, arecoline treatment might inhibit mice from choosing quality food and drink, and it is likely that arecoline decreased the number of T1R2- and T1R3-immunoreactive cells through alterations in taste discrimination, indicating changes in the selective preference behavior between deionized water and 1% sucrose.

Reduced body weight and food intake in mice treated with arecoline

Chewing areca nut leads to nutritional deficiencies in humans (Weegels et al. 1984). Feeding male and female Swiss mice diets

containing 2.5% and 5% *pan masala*, a popular quid in India, lowered the body weight of experimental animals (Bhisey et al. 1999). Kumar et al. (2000) also showed that the administration of areca nut extract through intragastric intubation to rats for 4 weeks also impairs the activity of alkaline phosphatase and the enzymatic digestion of sucrose on the brush border membrane (Kumar et al. 2000). Drewnowski (1997) reported that sensory responses to taste, smell, and food textures determine food preferences and eating habits (Drewnowski 1997). In the present study, we observed that the food intake and body weight in arecoline-treated groups were lower compared with the control groups. Therefore, we proposed that arecoline might alter the preference for taste sensation, thereby influencing food intake and decreasing the body weight. The taste receptor cell damage induced through arecoline could cause weight loss, but the arecoline effect on body weight resulting from an impaired digestive system cannot be excluded.

Taken together, these results suggest that arecoline induces morphological alterations of taste bud cells, increases stress in taste cells and modulates the gustatory discrimination preference. We also speculated that autophagic degeneration leads to morphological alterations and changes in taste cell functions.

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