

## Effects of Curcumin and Capsaicin Irradiated with Visible Light on Murine Oral Mucosa

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**Abstract.** *The purpose of this study was to evaluate the histopathological effects of curcumin and capsaicin, with or without visible light (VL) irradiation for 5 min, on the oral mucous membrane in mice. Capsaicin-treated, but not curcumin-treated, buccal epithelium exhibited slight tissue damage; VL irradiation caused excessive tissue damage, particularly when combined with the former treatment. The TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method demonstrated that both capsaicin and curcumin induced apoptosis, with the apoptotic effect of capsaicin appearing at an early stage of application. VL irradiation increased the number of apoptotic cells, particularly those upon in the capsaicin-treated area. Capsaicin and curcumin acted as photosensitizers exposure to VL, in the presence of oxygen. Curcumin and capsaicin with VL irradiation could thus be used for photodynamic therapy in the clinical setting, especially in precancerous oral diseases.*

Curcumin, 1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a compound obtained from the rhizome of the turmeric plant, *Curcuma longa*. Turmeric is used as a colorant, a food preservative and a dye, and is a major ingredient of curry dishes. Curcumin acts not only as a potent antioxidant but also as a pro-oxidant (1, 2), and is of interest as a chemopreventive agent because of its wide variety of medicinal properties, including anti-inflammatory and anticancer effects, such as induction of apoptosis, generation of reactive oxygen species (ROS), and inhibition of cyclooxygenase-2 (1, 3, 4).

Capsaicin, 8-methyl-*N*-vanillyl-6-nonenamide, is the pungent component of a wide variety of red peppers of the genus *Capsicum*, being associated with an oral burning sensation in addition to focal sweating (5, 6). Capsaicin has an active effect on nociceptors and induces central and peripheral hyperplasia,

as well as stimulates small-diameter capsaicin-sensitive nociceptive afferent fibers, a phenomenon known as neurogenic inflammation (7). Capsaicin has been reported to inhibit metabolic activation, as well as to exert antioxidant, antiproliferative and anti-inflammatory effects, and induce tumor cell apoptosis (8).

Photodynamic therapy (PDT) is an experimental cancer treatment modality. When tissues containing a sensitizer are exposed to light of an appropriate wavelength and dose, a photochemical reaction occurs between the sensitizer and light, and the activated photosensitizer reacts with the available oxygen, which subsequently damages cells and may eventually cause tumor necrosis (9, 10). Previously, we have used the phytophenol eugenol, a curcumin-related compound, and its derivative, as photosensitizing agents and have shown that these compounds, particularly the former, show enhanced inflammatory activity when irradiated with visible light (VL), after application to normal oral mucosa (11). These findings suggested that PDT with these compounds might be potentially applicable as an anticancer treatment. As curcumin and capsaicin are phytophenols with wide usage in everyday life, we examined their biological effects on the oral mucosa. In the present study, we investigated the histopathological effects of curcumin and capsaicin on the oral mucous membranes of mice in combination with VL irradiation. Histopathological changes caused in tissue were evaluated using hematoxylin and eosin (HE) staining, transmission electron microscopy (TEM), and the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method (12).

### Materials and Methods

Curcumin and capsaicin, obtained from Wako (Tokyo, Japan), were mixed with glycerin (Wako) to prepare 1 M solutions. Twelve ICR male mice (30~40 g in weight), 8-weeks-old, were anesthetized with pentobarbital sodium (50 mg/kg), then a round filter paper 0.5 cm in diameter (Toyo, Tokyo, Japan) wetted with 0.3 cc of curcumin or capsaicin solution was placed on the oral mucous membrane of each animal. In one group of mice, the filter paper bearing the test agent was applied to the buccal mucous membrane for 1 min and 5 min, and the mice were then released and not subjected to further treatment. In the other group, the filter papers bearing the same agents were similarly

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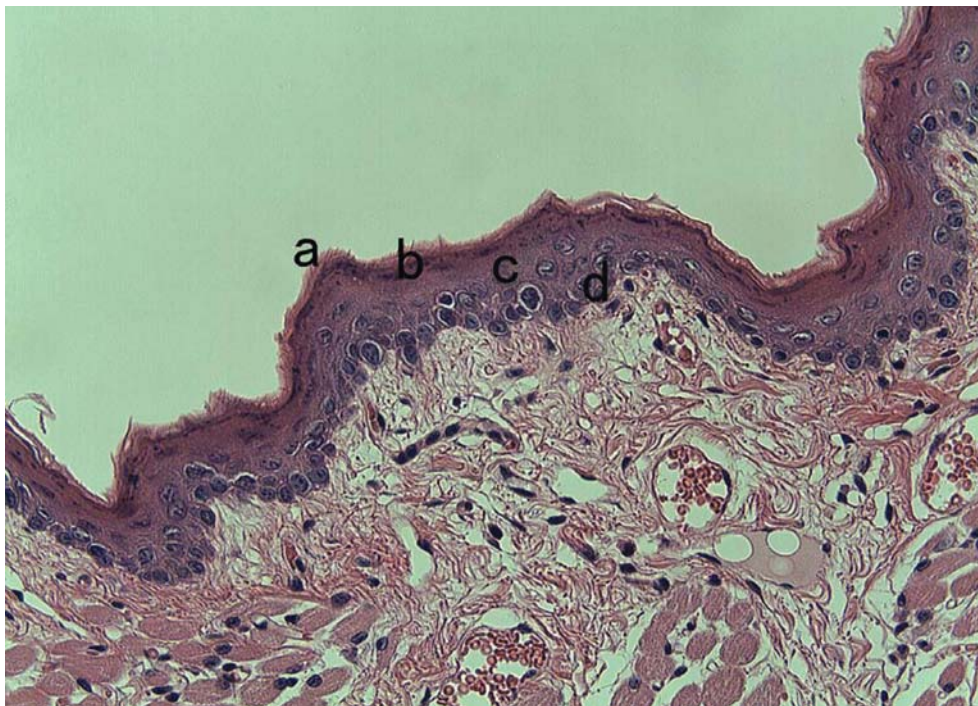


Figure 1. Normal histological architecture of mouse buccal epithelium (a: corium; b: granular layer; c: prickle layer; d: basal layer) evident after visible light irradiation for 5 min. Original magnification  $\times 50$ .

applied to the mucous membrane, but afterwards the treated area was irradiated with VL from an Astral VL lamp [Litema Astra Dental, Germany;  $\lambda=400-500$  nm, ( $\lambda_{\max}$ , approx. 470 nm), radiation dose 400 mW/cm<sup>2</sup>] for 1 min and 5 min. The control group was untreated, treated with the glycerin vehicle only or treated with the glycerin vehicle in the presence of 5 min VL irradiation. The histological findings for the group with the glycerin vehicle containing capsaicin or curcumin in the presence of VL irradiation for 1 min or 5 min were compared with those in the absence of VL irradiation. For each experiment, 3 subjects were used.

In accordance with the guidelines for animal experimentation of the Meikai University School of Dentistry, the mice were sacrificed immediately after treatments and the cheek mucosa was excised. Then half of the specimens were immediately fixed in 20% formalin solution for 2 h at room temperature. The other half were fixed in 2.5% glutaraldehyde for 2 h, and post-fixed in 1% osmium tetroxide. The samples were embedded in paraffin. Sections were cut at 5  $\mu$ m thickness. The specimens were studied histologically after HE staining, or observed using TEM (JEM-100cx, JEOL, Akishima, Japan), as previously described (11). The TUNEL method was also used to detect apoptosis *in situ* (12). The TUNEL kit was purchased from Takara Bio Inc. (Otsu, Japan).

## Results

The oral mucosal epithelium did not exhibit any changes regarding its histological architecture after application of glycerin, or upon VL irradiation alone, for 5 min. A typical example is shown in Figure 1.

The buccal mucosal epithelium treated with curcumin for 5 min showed slight thickening, but the shape and arrangement of the cells were normal, without any disruption or disintegration of the nuclei (Figure 2A). The curcumin-treated mucosal epithelium without VL irradiation exhibited no significant histopathological changes. However, when the curcumin-treated mucosal epithelium was irradiated with VL for 5 min, the cuboid basal cells became flattened on the supraspinous layer. Transition from spinous-type cell layers to granular layers was also observed, with elongation of the rete pegs and an increase in the staining capacity of the epithelial cells, accompanied by hyperactivity, indicating slight inflammation (Figure 2B).

The buccal epithelium treated with capsaicin exhibited marked thickening of the epithelium, with hyperkeratosis and a prominent granular layer, and a number of the spinous-type cells, blood vessels and cuboid-type cells in the basal layer changed to a columnar form, suggesting the presence of inflammation (Figure 3A). When capsaicin-treated mucosa was irradiated with VL for 5 min, cracking of the corium, with expansion of the intercellular bridges and cytoplasmic swelling were observed. There was also disruption and disintegration of nuclei in the spinous cell layer and a halo cell formation on the basal cell layer, indicating more severe tissue damage than observed following capsaicin treatment alone (Figure 3B).

VL-irradiated curcumin-treated mucosa was positively

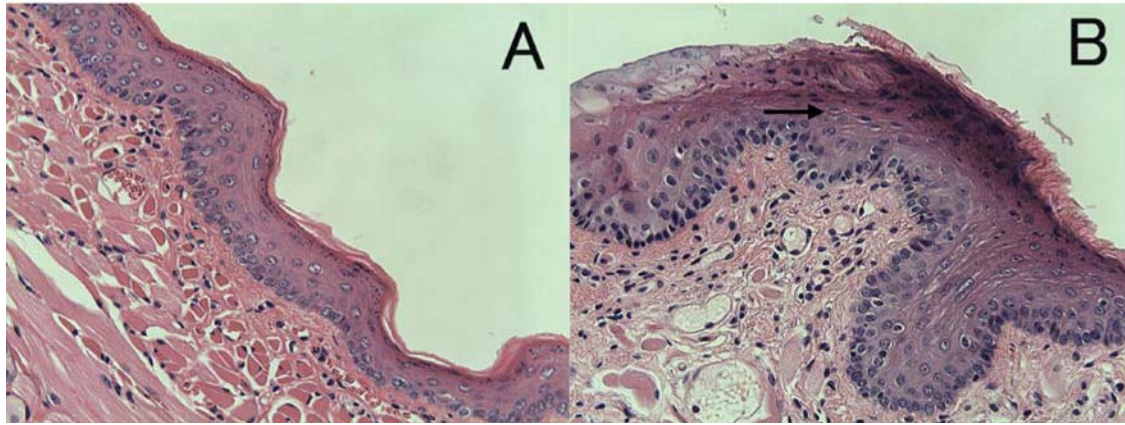


Figure 2. Buccal epithelium following application of curcumin appears normal (A). Visible light irradiation for 5 min elicited hyperactivity involving a transition from a cuboid-type to a flattened-type epithelial form (arrow), induced elongation of the rete pegs and an increased staining capacity, indicative of slight inflammation (B). Original magnification  $\times 50$ .

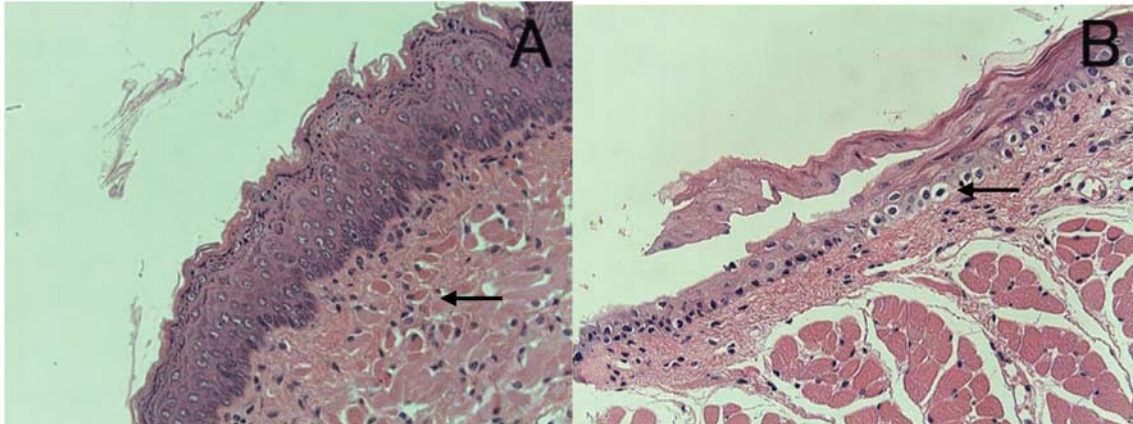


Figure 3. Buccal epithelium treated with capsaicin, showing an increase in the thickness of the spinous layer, numerous blood vessels (arrow) and a prominent granular layer, indicative of moderate inflammation (A). Additionally, visible light irradiation for 5 min led to expansion of intercellular bridges due to edema and formation of halo cells (arrow) in the basal layer, indicative of strong inflammation (B). Original magnification  $\times 50$ .

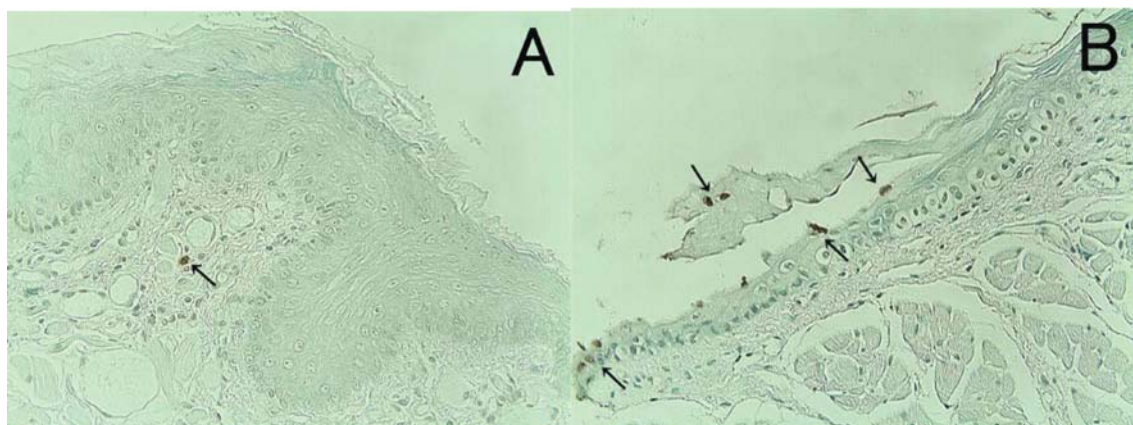


Figure 4. Buccal epithelium following application of curcumin with visible light irradiation for 5 min, showing TUNEL-positive cells (arrow) in connective tissue (A). Buccal epithelium following application of capsaicin with visible light irradiation for 5 min, showing numerous TUNEL-positive cells (arrows) in the spinous layer and the corium (B). Original magnification  $\times 50$ .

stained in the TUNEL method (Figure 4A). On the other hand, in the VL-irradiated capsaicin-treated area, TUNEL-positive nuclei were dispersed in the epithelium, and the presence of many positive nuclei in the spinous cell layer was considered likely to indicate capsaicin-mediated apoptosis (Figure 4B).

TEM observation of the curcumin-treated area, with (Figure 5A) and without (Figure 5B) VL irradiation, revealed transition and accumulation of mitochondria in the spinous layer, indicative of mitochondrial hyperactivity during apoptosis. In the capsaicin-treated area, the corium was closely compacted. Intercellular bridges showed evident destruction and cells had disappeared from the spinous layer, possibly due to the induction of autolysis (Figure 6A). The corium was loosely packed and cracked, partly destroyed, with liquefaction of intercellular bridges or tonofilaments, resulting in cell edema. The area of tissue damage containing TUNEL-positive cells for VL-irradiated capsaicin-treated mucosa was greater than that for the corresponding curcumin-treated mucosa (Figure 6B).

## Discussion

Capsaicin, but not curcumin, is soluble in olive oil. We chose glycerin as a base for application of capsaicin and curcumin powder, and the resulting homogeneous pastes were capable of being closely packed onto the oral mucous membrane. The pastes were retained on the surface of the membrane for an appropriate experimental time without being washed away by saliva. In addition, there was no chemical reaction upon combining the phytophenols with glycerin. Therefore, we used these pastes for capsaicin and curcumin in the absence and presence of VL irradiation. Also, it was found that VL irradiation had no adverse effects on oral mucosa in mice when compared to the corresponding ones without VL irradiation.

Curcumin is a hydrophobic polyphenolic derivative from the rhizome of turmeric, which has been pharmacologically shown to be a safe compound, with no dose-limiting toxicity when administered at doses of up to 10 g/day. Curcumin, as an antioxidant and anticancer agent, can suppress tumor initiation and metastasis (13). Previously, we conducted *in vitro* studies to examine the cytotoxicity of curcumin, eugenol and its derivatives against human gingival fibroblasts and human submandibular carcinoma cells, and found that curcumin generated ROS upon VL irradiation, which highly enhanced its cytotoxicity (3). The absorption wavelength of curcumin is known to lie within the range 410-450 nm. Furthermore, it was recently reported that curcumin in combination with VL inhibited tumor growth in a tumor model using mice xenografted with human epithelial carcinoma A-431 cells (14). These findings seem very consistent with those obtained in the present study conducted at the tissue level. Curcumin-treated oral mucosa had almost normal profiles on 1 or 5 min of application, but when irradiated with VL, the epithelial thickness increased by about 30-40%, indicating that

inflammation may be associated with a photoaffinity. It was interesting that application of curcumin affected mitochondria in the supra-spinous cell layer, and TEM observation revealed loss of mitochondrial cristae. It was reported that curcumin induced an increase of mitochondrial membrane permeability in rat liver, resulting in swelling, loss of membrane potential and inhibition of ATP synthesis (15). Curcumin significantly reduced the intracellular level of glutathione in human gingival fibroblasts derived from explant culture of clinically healthy gingiva after VL irradiation (16). Several *in vitro* studies have indicated that curcumin-induced apoptosis is associated with ROS production. Apoptosis induction is also associated with DNA fragmentation, cell shrinkage, externalization of cell membrane phosphatidylserine, and mitochondrial disruption, which are preceded by an increase in the intracellular ROS level (16-19). In the present study, the histopathological features of curcumin-treated oral mucosa subjected to VL irradiation suggested induction of apoptosis. It has been reported that induction of apoptosis is mediated through permeability transition pores, and that curcumin induces oxidation of membrane thiols (20). In contrast, capsaicin, which contains 4-hydroxy-3-methoxy benzoic rings, has been reported to have a photoaffinity. Capsaicin induces long-lasting stimulation of calcium uptake after ultraviolet (UV) irradiation when in contact with the cells, but this does not occur if samples are kept in the dark (21). The results obtained with a reaction time of 1 min were comparable to those after 5 min in the capsaicin-treated area. Capsaicin was thus suggested to be an acute stimulant, possibly producing neurogenic inflammation.

The pro-oxidant and antioxidant activities of capsaicin in UV-induced liposomal lipid peroxidation have been investigated; high-dose capsaicin had antioxidant activity, whereas low-dose capsaicin had pro-oxidant activity (22). The absorbance of capsaicin has a peak at 206 nm, followed by a peak at 280 nm, and is therefore sensitive in the UV spectrum range. Moreover, with its deep red color, capsaicin absorbs at a peak of 460 nm and may therefore have mild sensitivity to VL irradiation. In the present study, capsaicin in combination with VL irradiation induced more severe tissue damage than capsaicin alone, and this may have been associated with ROS production, derived from capsaicin-induced pro-oxidant activity upon VL irradiation. Similarly, curcumin without VL irradiation produced no significant histopathological changes, but application of VL irradiation resulted in slight damage. It has been reported that capsaicin suppresses the growth of leukemia cells due to apoptosis, associated with elevated production of intracellular ROS (23). Capsaicin-induced apoptosis has also been reported in human hepatoma HepG2 cells (24). A number of reports have indicated that capsaicin inhibits metabolic activation, has anti-inflammatory, antioxidant and antiproliferative effects, and induces apoptosis in tumor cells (8). The present study, by use of the TUNEL, method demonstrated that capsaicin-induced apoptosis occurred at the tissue level, particularly upon VL

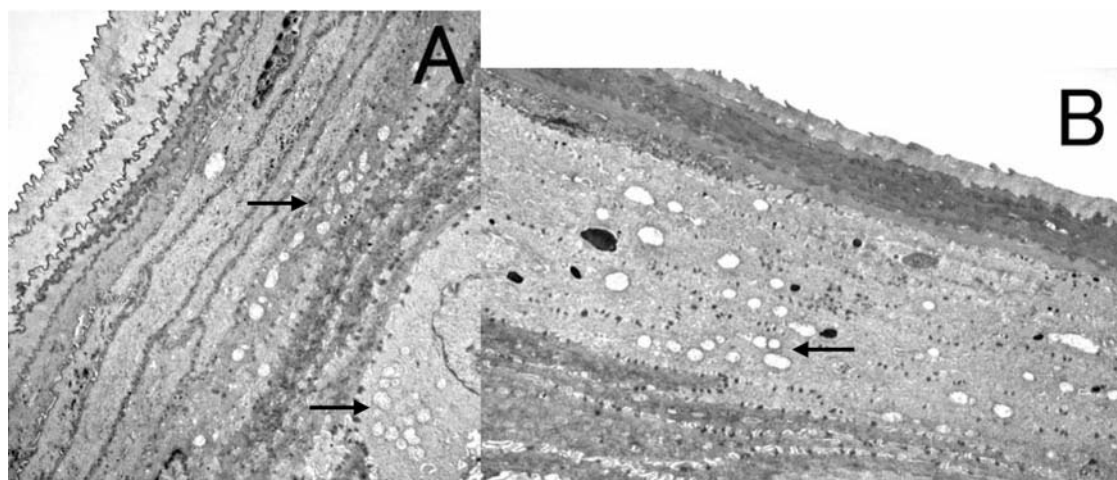


Figure 5. Transmission electron microscopy (TEM) view of buccal epithelium following application of curcumin with visible light irradiation for 5 min. Mitochondria with fewer cristae (arrows) have accumulated in the cells of the spinous and inner layers (A). TEM view of buccal epithelium following application of curcumin, showing characteristic accumulation of mitochondria (arrow) in the spinous layer (B). Original magnification  $\times 2,000$ .

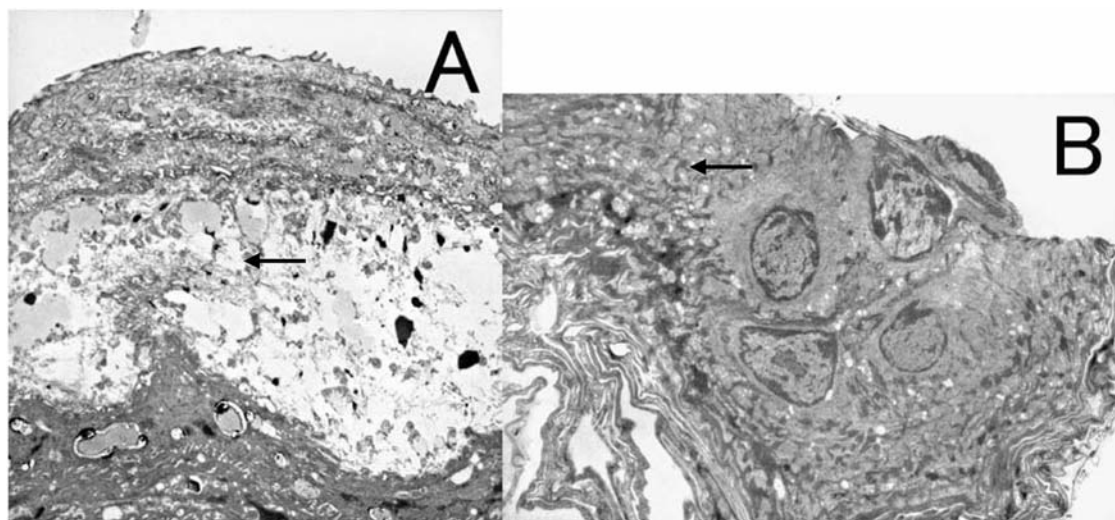


Figure 6. Transmission electron microscopy (TEM) view of buccal epithelium following application of capsaicin (A), showing autolysis (arrow). TEM view following visible light irradiation for 5 min (B), showing intercellular bridge liquefaction as a result of cell edema, and loss of cells (arrow). Original magnification  $\times 2,000$ .

irradiation. In the present study, whether or not capsaicin produces more ROS than curcumin remains unknown, and further studies are needed to clarify this issue.

Leukoplakia is a pre-cancerous lesion that develops on the tongue or the inside of the cheek in response to chronic irritation, and it is considerably difficult to treat. PDT is a well-known experimental cancer treatment (9). PDT has shown potential in the treatment of oral leukoplakia, oral lichen planus, and head and neck cancer (25). Capsaicin and curcumin was activated by exposure to VL in the presence of oxygen, possibly resulting in the formation of toxic oxygen species, such as excessive ROS and free radicals, as well as phenoxyl

radicals. These very reactive chemical species can damage proteins, lipids, nucleic acids, and other cellular components (25). The potent induction of apoptosis for capsaicin and curcumin in the presence of VL irradiation may have been caused by the reactive chemical species derived from VL-irradiation of these compounds. It is of interest that curcumin and capsaicin could be used as a plaster for precancerous lesions treated with PDT. Curcumin and capsaicin combined with VL irradiation using a dental lamp, producing light in the 410-500 nm region of the visible spectrum, for curing of dental composite filling materials (26) may also be applicable for cancer prevention in the PDT jetting.

## Conclusion

VL irradiation of the mucous membranes of mice treated with capsaicin and curcumin, particularly the former, increased tissue damage and TUNEL-positive cells. Curcumin and capsaicin with VL irradiation may thus be applicable for PDT in clinical applications, especially in pre-cancerous oral diseases.

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