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Original Paper

Effects of visible light-irradiated camphorquinone and 9-fluorenone on murine oral mucosa

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The purpose of this study was to evaluate the histopathological effects of camphorquinone (CQ) and 9-fluorenone (9F) with or without visible light (VL) irradiation on the oral mucous membranes of mice. VL irradiation resulted in a higher degree of tissue damage after CQ or 9F application, particularly the latter. Necrosis and apoptosis were responsible for the tissue damage after application of either agent in the presence of VL irradiation.

Key words: Camphorquinone, Visible light, Cytotoxicity

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INTRODUCTION

Camphorquinone (CQ) is widely used as an initiator in modern dental visible light (VL)−cured resin systems[1]. At the same time, CQ is also characterized as a potential allergenic compound[2]. However, studies on the adverse effects of CQ in resin systems have focused mainly on photoinitiators[3-8], while studies on VL−irradiated systems have been scarce.

The cytotoxicity of aliphatic CQ and its aromatic counterparts — such as 9-fluorenone (9F), benzyl, and benzophenone — upon exposure to VL has been studied previously using a human submandibular gland carcinoma cell line and human gingival fibroblasts[3,5,7,8]. The findings showed that CQ was a promising photosensitizer, as it was cytocompatible[8]. Similarly, CQ at a concentration ≦0.01% w/w was reported to be cytocompatible for NIH/3T3 fibroblasts in the presence of VL. Moreover, it has been reported that the cytotoxicity of CQ against human pulp fibroblasts is significantly lower than that of 9F, despite the greater radical production by CQ[9-11].

Although the cytotoxic effects of CQ and 9F at the cellular level have been reported previously, it has not been studied at the tissue level. In this respect, we have previously evaluated the cytotoxicity of a dental material, eugenol, and its dimer in the absence or presence of VL at the tissue level[12,13].

In the present study, we investigated the cytotoxic effects of CQ and 9F on the oral mucous membranes of mice in the absence or presence of VL irradiation. Cytotoxicity was evaluated by examining the histopathological changes in tissues, using both hematoxylin and eosin (H&E) staining and the TdT−mediated dUTP−biotin nick end−labeling (TUNEL) method.

MATERIALS AND METHODS

CQ and 9F obtained from Tokyo Kasei (Tokyo, Japan) were dissolved in dimethyl sulfoxide (Wako, Japan; DM) at a concentration of 100 mM. Six adult ICR mice (8 weeks old) were anesthetized with diethyl ether (Wako, Japan), and a round filter paper 0.5 cm in diameter (Toyo Filter Paper, Tokyo, Japan) was immersed in 0.5 ml CQ or 9F solution. For one group of mice, the filter paper bearing the test agent was applied to the cheek mucous membrane for 1 minute. The mice were then released and not treated further. For the other group, the filter paper with the test agent was similarly applied to the mucous membrane for 1 minute. Subsequently, the treated area was exposed to VL irradiation for 5 minutes from an Astral VL lamp (Litema Astra Dental, Germany; λ=400−500 nm, radiation dose: 400 mW/cm^2<). The control group was treated with the DM vehicle only.

In accordance with the guidelines for animal experimentation of Meikai University School of Dentistry, the mice were sacrificed, and the cheek mucosa was excised and fixed in 20% formalin solution immediately for 2 hours at room temperature. The specimens were studied histologically by HE staining, and TUNEL method was used to detect apoptosis *in situ*.

RESULTS

The oral mucosa did not show any changes in histological architecture after application of DM (Fig. 1). The buccal epithelium following the application of CQ for 5 minutes showed a marked thickening of the prickle cell layer due to cellular edema, but the

nuclei were intact residually, followed by a decrease in staining capacity. The granular layer disappeared and hyperchromatic basal cells were accumulated to a thickness of about three or four cells, indicating acute inflammation (Fig. 2A). After CQ-treated mucosa was irradiated with VL, more severe cellular edema was found in the prickle cell layer than that after CQ treatment alone. In addition, hazy cell boundaries and vesicle formation were evident, and the nuclei were partly disintegrated (Fig. 2B).

The labial epithelium after application of 9F alone was characterized by nuclei bursting into cytoplasmic granules around the area of cellular edema. Accumulation of hyperchromatic basal cells to a thickness of about four or five cells suggested that the basal membrane worked to resist inflammation (Fig. 3A). After 9F−treated mucosa was irradiated with VL, all intercellular boundaries as well as nuclei disappeared, which could be due to

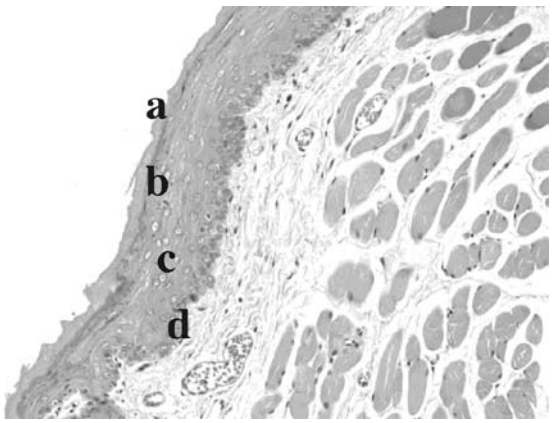

Fig. 1 Normal histological architecture of mouse oral mucosa with H&E staining. a: corium; b: granular layer; c: prickle layer; d: basal layer. Original magnification: ×50.

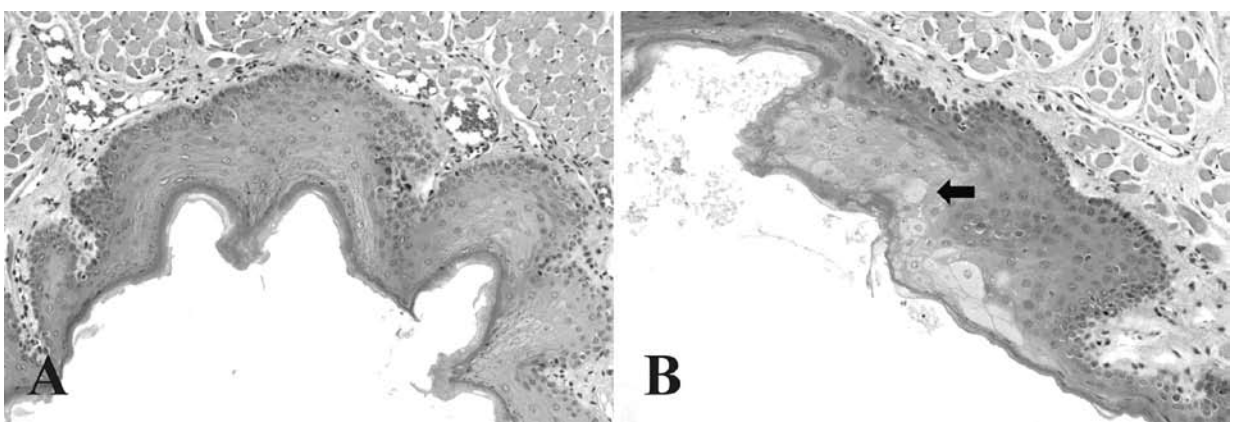

Fig. 2 Buccal epithelium after CQ application and with H&E staining. A: Expansion of intercellular bridges and low staining capacity indicated mild edema, while the corium and granular layer appeared normal. B: After VL irradiation for 5 minutes, there was severe intercellular edema (arrow) and intercellular bridges in the prickle cell layer showed partial degradation. Original magnification: ×50.

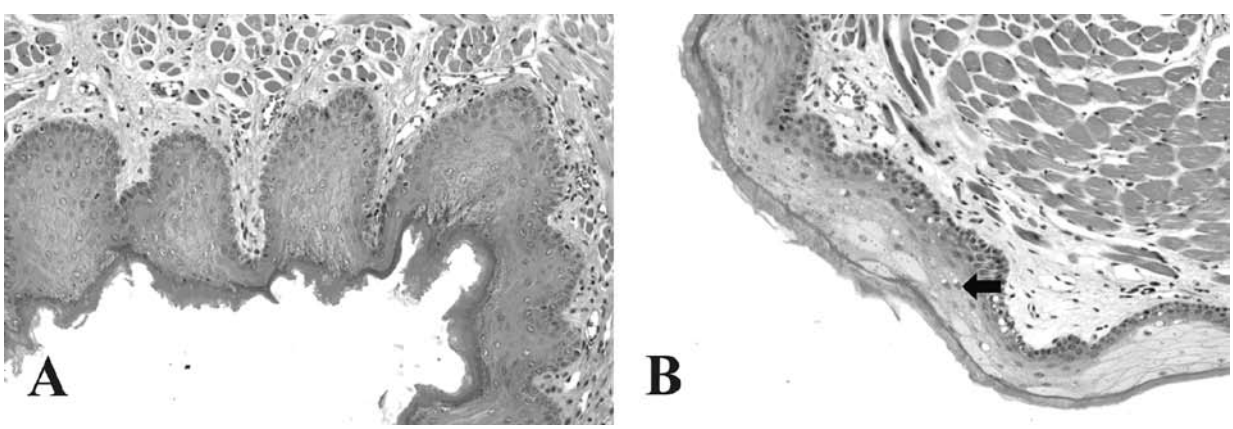

Fig. 3 Buccal epithelium after 9F application and with H&E staining. A: Disintegrated nuclei were evident in the prickle cell layer, with disappearance of some nuclei. Moderate edema was noted. B: After VL irradiation for 5 minutes, there were interrupted intercellular bridges, hazy cell boundaries (arrow), and almost complete disappearance of nuclei, thus indicating severe inflammation. Original magnification: ×50.

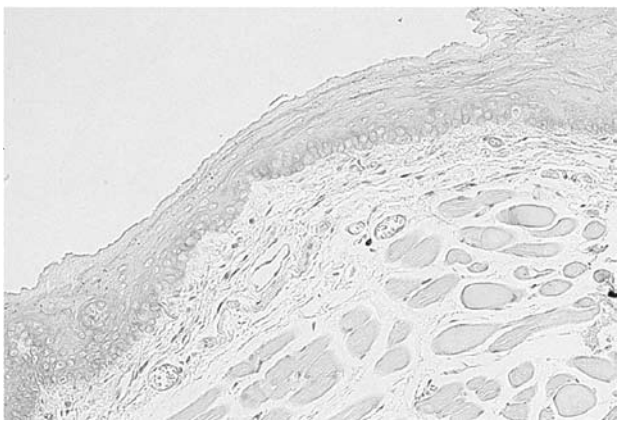

Fig. 4 Normal histochemical appearance of mouse oral mucosa with TUNEL staining. Original magnification: ×50.

severe inflammation. The appearance of coagulation and the increase in granular cells suggested that the acute stage had passed, followed by an onset of the recovery process (Fig. 3B).

The TUNEL method showed that the uppermost layer of the corium was stained partially but positively with a normal appearance (Fig. 4). Specifically, a few typical nuclei with positive staining were evident in the CQ-treated area. However, the number of positive nuclei was too small to allow judgment of whether apoptosis was present (Fig. 5). On the other hand, 9F−treated and VL-irradiated mucosa indicated several TUNEL positive cells in the subprickle cell layer, thereby allowing us to judge it as apoptosis (Figs. 6 and 7).

In the case of CQ, it was unclear whether this photosensitizer indeed induced apoptosis. The main mechanism involved might be necrosis, which could have occurred through photogenesis. On the

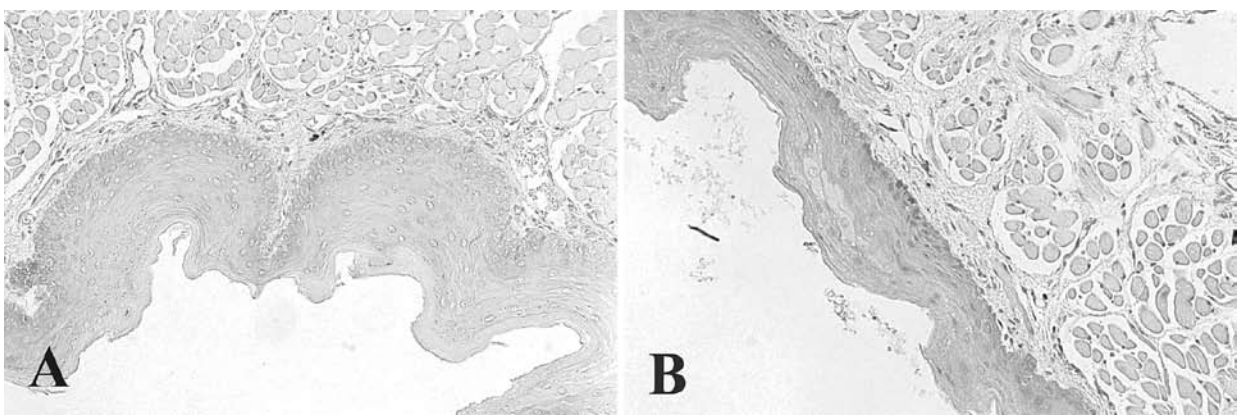

Fig. 5 Buccal epithelium after CQ application and with TUNEL staining. A: Nuclei with positive stain of apoptosis could not be observed. B: After VL irradiation for 5 minutes, deep staining of the basal layer was evident but nuclei showing positive TUNEL staining could not be observed. Original magnification: ×50.

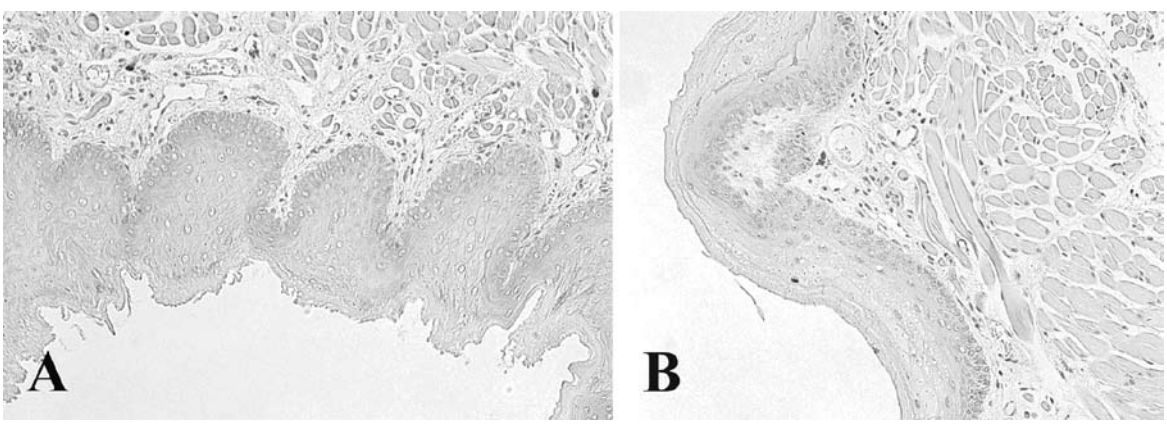

Fig. 6 Buccal epithelium after 9F application and with TUNLE staining. A: Nuclei with positive stain of apoptosis could not be observed. B: After VL irradiation for 5 minutes, several nuclei in prickle cell layer showing positive TUNEL staining were evident. Original magnification: ×50.

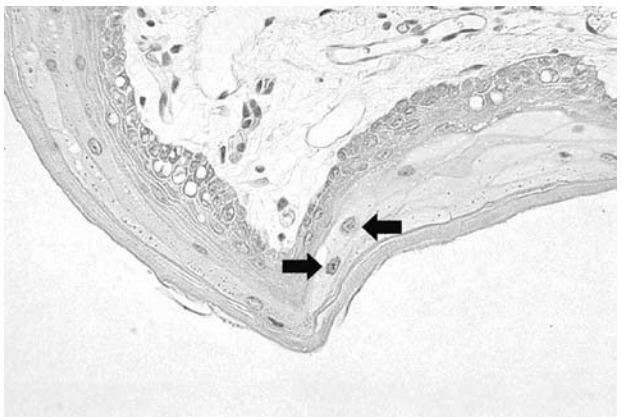

Fig. 7 Typical TUNEL-positive cells (arrows) in subprickle cell layer, after TUNEL staining, are shown in high magnification. Original magnification: ×100.

contrary, in the case of 9F, it was certain that 9F coupled with VL enhanced the occurrence rate of apoptosis.

DISCUSSION

Previously, we conducted a study to examine the cytotoxicity of CQ and 9F toward human pulp fibroblasts and human gingival fibroblasts, and found that the effect of CQ was less marked than that of 9F. Further, 9F in the presence of VL showed markedly greater cytotoxicity than 9F alone in the absence of VL, possibly due to the production of reactive oxygen species (ROS). On the other hand, CQ produced free radicals, but not ROS[10].

These findings were similar to those obtained at the tissue level in the present study. It has been shown that the cell damaging effects of 9F (1 mM-3 minutes) and CQ (10 mM-24 minutes) systems are almost equal, and that additional exposure to VL for 3 minutes decreases the cell survival rate to 40%[11]. We had determined previously that a chemical agent concentration of 100 mM would produce acute inflammation with a reaction time of 1 minute when coupled with VL irradiation for 5 minutes. This period of 6 minutes was enough to allow the chemical agents to work effectively and destructively.

With the expectation and assumption that VL would irradiate the oral mucosa homogenously, the Astral VL lamp (λ=400−500 nm, radiation dose: 400 mW/cm^2<) was employed. Two key reasons led to this choice. First, the beam head of about 10 mm could cover the entire reaction area; second, the Astral lamp is frequently used in dental practice.

It is noteworthy that when we applied CQ or 9F topically, the color of the oral mucosa became slightly paler to the naked eye. Grossly, there was no redness, swelling or ulceration like that seen for inflammation. This was different from the effects of eugenol and related compounds, which penetrated the outer layer of corium and continued their damaging effects inward. In the case of CQ or 9F application, the outer layer of corium was intact. Therefore, we presumed that the test agents infiltrated but did not destroy the corium, although they affected the architecture of the prickle cell layer.

CQ induced a time- and dose-dependent increase in the rates of apoptosis and necrosis in cell lines, and the same could be said when coupled with VL irradiation[14]. In this study, we recognized a few TUNEL-positive nuclei after CQ application. However, their quantity was insufficient to allow a judgment of whether apoptosis had occurred. Nonetheless, we could not rule out the possibility that CQ caused apoptosis. Moreover, against the backdrop that other studies have not demonstrated any TUNEL-positive results with CQ, we adopted the conservative stance that our present experimental system might have preferentially induced necrosis rather than apoptosis.

On the contrary, the same claim could not be applied to 9F. When coupled with VL irradiation, apoptosis was evidently induced — thereby indicating the high cytotoxicity of 9F with VL irradiation on tissue/organ. Apoptosis is a phenomenon lasting an extremely short time. Therefore, apoptotic bodies are often phagocytized and do not remain visible for long. In other words, to clearly demonstrate the existence of apoptosis, further studies involving different time intervals or TUNEL staining coupled with p53 or bcl-2 detection will be needed. Furthermore, these studies can be leveraged to reconfirm the pathological changes after the occurrence of apoptosis, such as epithelial cell dysplasia or functional recovery.

Interestingly, 9F coupled with VL irradiation induced a typical apoptosis within a very short working time. When compared to CQ, 9F produced greater tissue damage, particularly in the presence of VL. To the best of our knowledge, this is the first such report on the cytotoxicity of CQ and 9F at the tissue level.

CONCLUSIONS

At the tissue level, the mildest cytotoxicity was demonstrated by CQ without VL, followed by CQ with VL. 9F alone showed cytotoxicity similar to that of CQ with VL, while 9F with VL showed the strongest cytotoxicity.

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