Therapeutic Effects of Green Tea Catechin Ointment on Oral Leukoplakia and Its Penetration into the Oral Mucosa

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Introduction
Studies over time have shown that green tea catechin (GTE) from tea extracts possesses various bioactivities, including anti-carcinogenic activity (anti-tumor-promoting activity, suppression of cancer cell growth, induction of apoptosis), anti-mutagenicity (anti-initiation activity, anti-cancer agent, suppression of DNA adduct formation), and inhibition of angiogenesis. This study aimed to investigate the therapeutic effects of applying GTE as a topical agent for oral leukoplakia and the subsequent histopathological changes. We examined the absorption of GTE in rat oral mucosa. In addition, we examined 30 patients diagnosed with oral leukoplakia between April 2009 to March 2011. We administered an oral ointment composed of 1% GTE kneaded into a base material. A single dose (0.5 g) was applied to the affected area thrice a day for 90 days. Macroscopic observations and serum catechin levels were examined before, during, and after administration. The comparative histological effects and Ki-67-positive cell rates were examined using hematoxylin and eosin (H&E) staining and Ki-67 immunohistochemical staining. Nine patients in the treatment group exhibited disappearance, shrinkage, or thinning of the leukoplakia. After administration, effective cases exhibited histopathological normalization of the mucosa, and Ki-67 immunohistochemical staining suggested normalization of epithelial growth dynamics. GTE ointment applied to the dorsum of the rat tongues were examined for tissue permeability of GTE in frozen sagittal sections. Imaging of the rat tongue sections showed GTE penetrating the epithelium over time. Together, our results indicate that topical administration of GTE may be effective against oral leukoplakia.

Keywords: oral leukoplakia, catechin, green tea, ointment

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Tea extract (green tea catechin, GTE) has been shown to possess various bioactivities, including anti-carcinogenic activity (anti-tumor-promoting activity, suppression of cancer cell growth, induction of apoptosis), anti-mutagenicity (anti-initiation activity, anti-cancer agent, suppression of DNA adduct formation), and inhibition of angiogenesis. This study aimed to investigate the therapeutic effects of applying GTE as a topical agent for oral leukoplakia and the subsequent histopathological changes. We examined the absorption of GTE in rat oral mucosa. In addition, we examined 30 patients diagnosed with oral leukoplakia between April 2009 to March 2011. We administered an oral ointment composed of 1% GTE kneaded into a base material. A single dose (0.5 g) was applied to the affected area thrice a day for 90 days. Macroscopic observations and serum catechin levels were examined before, during, and after administration. The comparative histological effects and Ki-67-positive cell rates were examined using hematoxylin and eosin (H&E) staining and Ki-67 immunohistochemical staining. Nine patients in the treatment group exhibited disappearance, shrinkage, or thinning of the leukoplakia. After administration, effective cases exhibited histopathological normalization of the mucosa, and Ki-67 immunohistochemical staining suggested normalization of epithelial growth dynamics. GTE ointment applied to the dorsum of the rat tongues were examined for tissue permeability of GTE in frozen sagittal sections. Imaging of the rat tongue sections showed GTE penetrating the epithelium over time. Together, our results indicate that topical administration of GTE may be effective against oral leukoplakia.
verify its inhibitory effect on cell growth in human oral squamous cell carcinoma lines (HSC-4, Ca9-22). After confirming a decrease in cell activity, we examined its application as a therapeutic agent for oral leukoplakia. The immunohistochemical results suggested that the GTE ointment is absorbed locally into the oral mucosa where it acts on cell growth-related factors (5). We also reported that GTE ointment may have a clinical effect on oral leukoplakia (5).

In the present study, we examined the effects of topical application of the GTE ointment on oral leukoplakia and determined the local absorption of GTE in the oral mucosa of rat tongues.

**Materials and Methods**

**Subjects and administration methods**

The subjects included 30 patients (15 men and 15 women; mean age 69.0 years), who were examined between April 2009 to March 2011 at the departments of oral surgery at Tokyo Dental College’s Chiba Dental Center or Suidobashi Hospital, and diagnosed with oral leukoplakia via biopsy; the included patients consented to participate in the study. Seventeen patients (group A) were administered with a topical ointment containing 10% GTE (THEA-FLAN 30 ARG) and 4.17% tea polyphenols. Table 1 shows the composition of the GTE ointment. The remaining thirteen patients comprised the control group (group B) and were administered the base material ointment (only excluding GTE). The patients used a dedicated measuring spoon to obtain 0.5 g of the ointment (containing 50 mg of GTE) and applied it to the affected area three times per day between meals for 90 days. The experiment was a double-blind study, and the administration of the drug and extraction of sample from patients were performed according to the trial protocol. Informed consent was obtained from all the participants.

**Assessment of effects**

Macrosopic evaluation: Macrosopic observations, measurements of major and minor axes, and recordings of the leukoplakia with a digital camera were performed prior to the administration of the ointment and after 30, 60, and 90 days of administration. The assessments followed the therapeutic effect assessment criteria in the "General Rules for Clinical Studies on Head and Neck Cancer (4th edition)". Two oral surgeons (qualified oral surgery specialists) assessed the concentration of leukoplakia with definitions as follows: complete response (CR) = complete clinical resolution of lesion; partial response (PR) = 50% or greater diminution of bi-dimensional lesion; no change (NC) = less than 50% diminution of bi-dimensional lesion; progressive disease (PD) = more than 25% increase in lesion (6).

Histopathological evaluation: All patients underwent biopsy prior to the ointment administration. Patients whose lesions did not disappear underwent total resection or biopsy of the lesions within 10 days of the end of the treatment. All samples were subjected to hematoxylin and eosin (H-E) staining and Ki-67 immunohistochemical staining for microscopic examination. Immunohistochemical staining by Ki-67 was performed by creating 4 µm paraffin slices, which were subjected to deparaffinization, microwave treatment (0.01 M citrate buffer, 90°C, 15 min), endogenous peroxidase blocking with 3% H2O2-methanol, washing, and blocking with 10% normal goat serum. The primary antibody was an anti-Ki-67 mouse monoclonal MIB-1 antibody (1:200, Dako, Glostrup, Denmark) that was reacted at 4°C for 12 h, and then washed thrice with PBS for 5 min each. Antibody reactions were performed with Simple Stain (MAX PO (MULTI), Nichirei Biosciences Inc.) for DAB and nuclear staining (Mayer’s hematoxylin). The assessments were performed using an optical microscope at 400× magnification. One hundred randomly selected cells were examined, and those with brown-stained nuclei were counted as positive cells. Positive cell rates (LI) were calculated in three layers: the basal layer (layer directly above the basa-

| Table 1. Composition of ointment included 10% GTE(30ARG) |
|-----------------|------------------|
| Ingredients     | Contents         |
| GTE(TEAFURAN30ARG) | 10.0g           |
| CMC sodium      | 28.8g            |
| Liquid Paraffin | 10.8g            |
| White Vaseline  | 28.8g            |
| Platinum base   | 21.6g            |
| **Total**       | **100.0g**       |
al lamina), parabasal layer (layer above the basal layer), and prickle layer (layer with obvious spinous cells). The two-sample t-test was used for statistical analysis.

**Measurement of blood GTE levels and regular blood test results**

Serum catechin (epicatechin, EC) levels were measured before GTE administration and after 30, 60, and 90 days. The measurements were performed by connecting an electrochemical detector to a liquid chromatograph using the column-switching method.

Regular blood testing and biochemical analysis were performed immediately before administration and after 30, 60, and 90 days to examine changes in liver and kidney function, electrolytes, and blood components.

**Verification of local GTE absorption in rat tongues**

GTE ointment (0.5 g) was applied to the lingual prominence on the dorsum of Sprague-Dawley (S-D) rat tongues, which were euthanized after 1 and 3 h of GTE administration. The tongues were then removed, and frozen sagittal sections were prepared to examine the tissue permeability of the GTE ointment as described below. Laser Micro Dissection (AS IMD Leica, Leica Microsystems, Germany) was used to selectively divide the epithelium into three layers to perform mass analysis of the GTE in each layer.

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, SHIMADZU): The penetration of ointment into the tissue was examined in three dimensions. We estimated the penetrating substances in the tissue by analyzing the molecular weights and examined the changes in their distribution over time.

Mass spectrometry of GTE using Laser Micro Dissection (AS IMD Leica): The epithelium was divided into three layers, extracted with an organic solvent, and analyzed using a mass spectrometer (high-frequency linear ion trap qualitative analysis) to investigate the presence of catechins in each layer.

**Declarations**

The present study was approved by the ethics committee of Tokyo Dental College (No. 95).

**Results**

**Clinical effects of the GTE ointment**

All patients continuously applied the ointment for 90 days. Table 2 A–B shows the breakdown of cases by site and a summary of the macroscopic evaluations. In group A, CR was assessed in one case (6%), PR was assessed in eight cases (47%), and NC was assessed in eight cases (47%) (Fig. 1A–C). In control group B, no patients were assessed as CR, while four patients (33%) were assessed as PR, and nine patients (67%) as NC (Fig. 2). The lesions did not advance or worsen in any patient from both groups A or B, and therefore none were assessed as PD.

In group A, all patients assessed as PR with thinning or shrinkage of leukoplakia exhibited histopathological changes, such as thinning of the cornified layer, normalization of rete ridges, and recovery of polarity of epithelial cells in H-E staining (Fig. 3A, B).

Figure 4 shows the findings of Ki-67 immunohistochemical staining before and after GTE administration in PR cases of group A. Before administration, the Ki-67
Group A (CR case) : Age 47, Female

Lesion completely disappeared macroscopically.

Group A (PR case) : Age 67, Male

Partial disappearance, shrinkage, and overall thinning of lesion.

Group A (NC case) : Age 60, Male

Lesions showed no change macroscopically.

Fig. 1. Clinical effects of green tea catechin (GTE) assessed by macroscopic evaluation. A. Group A (CR case): Age 47, Female. Lesion completely disappeared macroscopically. B. Group A (PR case): Age 67, Male. Partial disappearance, shrinkage, and overall thinning of lesion. C. Group A (NC case): Age 60, Male. Lesions showed no change macroscopically.
**Group B (NC case) : Age 52, Male**

*Lesions showed no change macroscopically.*

Fig. 2. Clinical effects in control patients assessed by macroscopic evaluation. Group B (NC case): Age 52. Male. Lesion showed no change macroscopically.

**Group A (PR case)**

*Thinning of cornified layer and recovery of polarity of epithelial cells confirmed (original magnification x20).*

Fig. 3. Histopathological evaluation post green tea catechin (GTE) treatment using hematoxylin and eosin staining. A. Group A (PR case). Thinning of cornified layer and recovery of polarity of epithelial cells confirmed (original magnification, 20×). B. Group A (PR case). Thinning of cornified layer and recovery of polarity of epithelial cells confirmed (original magnification, 20×).
Group A (PR case)

Positive reactions seen in basal and prickle layers after administration (original magnification x20).

Before
Outside parabasal layer, several cells stained positive, but most positive cells were seen in parabasal layer (original magnification x20).

After

Fig. 4. Immunohistochemical staining of Ki-67 post green tea catechin (GTE) treatment. Group A (PR case). Positive reactions seen in basal and prickle layers after administration (original magnification, 20×). Outside parabasal layer, several cells stained positive, but most positive cells were seen in parabasal layer (original magnification, 20×).

Table 3
Changes in Ki-67-labelling index in each layer before and after GTE administration in PR cases in group A.

|                  | Basal Layer | Parabasal Layer | Prickle Layer |
|------------------|-------------|-----------------|--------------|
| Before-administration | 4.9±3.2     | 18.1±6.9        | 4.5±1.8      |
| After-administration | 4.2±2.1     | 24.4±7.6        | 6.1±1.5      |

n=8
* : p < 0.05

positive cells were observed in the basal and prickle layers, but after administration, most were observed in the parabasal layer. The positive cell rates in the parabasal layer were significantly different before and after GTE administration (p<0.05), indicating that positive cells tended to aggregate in the parabasal layer with catechin administration (Table 3).

Serum EC levels were 10 ng/mL or less during the administration period in all the cases, and thus could not be detected (Fig. 5). No blood chemistry changes associated with the product were observed in patients during the administration period. In addition, none of the patients experienced side effects such as nausea, vomiting, diarrhea, or insomnia due to the application of the product.

Verification of local GTE absorption in rat tongues
Imaging of the tongue tissue sections showed changes in the locations of GTE (penetration into the epithelium) 1 h and 3 h after application (Fig. 6A, B). In addition, MALDI-MS imaging data were used to identify the substanc-
Fig. 5. Changes in serum epicatechin (EC) levels after green tea catechin (GTE) treatment. Serum EC concentrations were less than 10 ng/mL (detection limit) during the administration period in all patients of group A.

Fig. 6. Green tea catechin (GTE) absorption in Sprague-Dawley rat tongues. A. Changes in the location of the GTE (penetration into the epithelium) were observed over time. B. 3D image of the changes in the location of the GTE (penetration into the epithelium).
The substance detected was thought to be caffeine \((m/z = 195, (MH)^+\)), which permeated and diffused into the epithelium over time.

Fig 7. MALDI-MS analysis of substances in the tissue sections after treatment of Sprague-Dawley rat with green tea catechin (GTE). The substance detected was thought to be caffeine [m/z = 195, (MH)+] which permeated and diffused into the epithelium over time.

Discussion

In Japan, 3–5% of oral leukoplakia and potentially malignant oral disorders are reported to develop into squamous cell carcinoma (7). These conditions are often dealt with by removing the risk factors and are followed up with non-invasive treatments such as vitamin A administration. Lesions are often removed when they do not improve and tend to undergo malignant transformation. There are few effective non-invasive treatments for leukoplakia diagnosed in the early stages. GTE is reported to have a variety of biological activities, including anti-carcinogenic, anti-sealing, anti-mutagenicity, inhibition of angiogenesis, and suppression of gene (C-H-ras, C-myc) expression (1, 2, 8). The MD Anderson Cancer Center, USA reported a phase I study wherein GTE was administered orally as a tablet to patients with head and neck cancer (9). In a previous study, we developed an ointment to allow GTE to act directly on the applied areas of leukoplakia and reported its effectiveness (5). There have been no other reports on the preparation and application of GTE as an ointment to treat leukoplakia. The product used in the present study achieves all qualities of an ointment, including good adhesion, low disintegration, sustained release, and flavor, while not compro-
mising the properties of GTE. The control group in this study used only the ointment base material; we experimentally examined the local absorption of GTE into the oral mucosa. Clinically, 9 out of 17 patients (53%) in the GTE group were assessed as CR or PR, and all PR cases exhibited shrinkage or thinning of the lesions. In the control group, 33% of patients were assessed as PR and exhibited thinning of the lesions. We speculate that these reasons were due to the reduction of traumatic irritation by adjusting the denture and polishing the sharp edges of the tooth. Histopathologically, H-E staining showed recovery of polarity, normalization and cornification of basal cells, and thinning of the cornified layer in the PR and few NC cases in group A. Furthermore, Ki-67 immunohistochemical staining showed that the positive cells that were found in all layers before GTE administration, were concentrated in the parabasal layer (second layer above the basement membrane) after administration. The labeling index of the parabasal layer was significantly different before and after administration. While hypertrophy of the stratum corneum was observed in some areas after local application of this product, it is notable that the localization of Ki-67-positive cells was close to normal in some cases. However, these changes were not observed in the control group. It is unclear why thinning of lesions was observed in the control group, however, because this was a macroscopic assessment, the subjective visual sense of the assessor may have been a factor. Ki-67 has been reported to be positive in cells of the parabasal layer, which has high cell proliferative capacity, in normal oral mucosal epithelium (10, 11). Ki-67 is a nucleoprotein that is expressed in the G1, S, G2, and M phases, but does not appear during the dormant phase (12). In addition, when GTE was applied to a human oral leukoplakia cell line for

One hour after the GTE administration, the presence of all four types of catechins (epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate) were confirmed in the first layer.

Fig. 8. Laser micro dissection mass spectrometry analysis of substances in the tissue sections after treatment of Sprague-Dawley rat with green tea catechin (GTE). One hour after the GTE administration, the presence of all four types of catechins (epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate) were observed in the first layer.
24 h, an increase in the cell population in the G1 phase and a decrease in the cell population in the G2 and S phases were observed, which may have been due to Rb protein (pRB) that controls cell turnover (13). Similarly, when GTE was reacted with a human leukemia cell line for 24 h, a decrease in the cell population and an increase in apoptosis were observed in G0/G1, indicating that GTE is involved in cell cycle progression (14). In the present study, the intraepithelial localization of Ki-67 changed before and after application, and some cases exhibited normal localization after administration. This result suggests that the use of GTE as a topical agent for oral leukoplakia may affect the control of cell turnover through pRB, which induces normalization of the oral mucosal epithelium.

The side effects of GTE were reported by the MD Anderson Cancer Center, US from the results of oral administration of GTE (29.6 mg) three times per day, in cases of head and neck cancer. They reported some cases of nausea and gastrointestinal bleeding, with many patients complaining of insomnia (9). However, in the present study, where patients received 50 mg in an ointment three times per day, there were no complaints of unpleasant symptoms such as insomnia or nausea, and a therapeutic effect was elicited without any liver or kidney dysfunction. There may be a need to consider racial differences between the patients in the US and the Japanese people, where the latter regularly drink green tea, to understand the difference in the side effects. In addition, no increase in blood GTE levels was observed, and the experiment showed absorption/stagnation in the epithelial tissue of the oral cavity from 1 to 3 h after administration, which shows that GTE successfully permeated/stagnated in the local area. This indicates that administration of GTE as an ointment with sustained release properties is an effective method to directly act on the lesions and to reduce side effects.

Regarding the tissue penetration of the GTE ointment, we histologically confirmed that, over time, the GTE component diffused and penetrated to a depth of 1 mm from the mucosal surface when applied to the squamous epithelium of rat tongues. This finding supports the results of the clinical investigation.

The above results show that a green tea catechin ointment (THEA-FLAN 30 ARG) may have a clinical effect on oral leukoplakia by shrinking and thinning lesions. Moreover, the results suggested that these effects are achieved by the local absorption of GTE into the oral mucosa and by acting on the cell growth-related factors near the basal cell layer.

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Conflict of Interest
The authors declare that they have no competing interests.

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