Gingival absorption of \( \alpha \)-tocopherol acetate and 18\( \beta \)-glycyrrhetinic acid: in vitro evaluation in reconstructed gingival tissue

Yun-Sun Kim, Ju-Ae Kim, Aram You, Hosong Cho, Jae Young Shin, Sanghwa Lee
Research Park, LG Household & Healthcare Ltd., Daejeon, Korea

Objectives: To assess the absorption of \( \alpha \)-tocopherol acetate and 18\( \beta \)-glycyrrhetinic acid, which are used as active ingredients in toothpaste, into a reconstructed gingival tissue.

Methods: EpiGingival\textsuperscript{TM} tissues were treated with a 25% slurry of toothpaste containing 2% \( \alpha \)-tocopherol acetate and 0.3% 18\( \beta \)-glycyrrhetinic acid, for 2 minutes. The treatment was repeated up to 6 times, with 1 hour intervals. After completion of all treatments, the active ingredients in the tissue extracts and receiver solutions were measured by high performance liquid chromatography.

Results: Although \( \alpha \)-tocopherol acetate was not detected, \( \alpha \)-tocopherol was detected in the tissue extracts, indicating that \( \alpha \)-tocopherol acetate was bioconverted to \( \alpha \)-tocopherol after absorption. We could detect 18\( \beta \)-glycyrrhetinic acid both in the tissue extracts and in the receiver solutions, with a positive correlation to the number of treatments.

Conclusions: We found that our toothpaste effectively delivered \( \alpha \)-tocopherol acetate and 18\( \beta \)-glycyrrhetinic acid to a reconstructed gingival tissue in vitro.

Key Words: Absorption, \( \alpha \)-Tocopherol acetate, 18\( \beta \)-Glycyrrhetinic acid, Periodontal disease, Toothpaste

Introduction

The human inflammatory periodontal diseases are the most common chronic diseases. Periodontal diseases can affect the periodontal tissues including alveolar bone, periodontal ligament, cementum and gingiva\textsuperscript{11}. It is believed that the primary etiological agent in periodontal diseases is bacteria. The majority of periodontal tissue destruction is caused by an inappropriate host response to bacteria and their products\textsuperscript{20,21}. More specifically, host immune responses play an important role in the induction and progression of the diseases\textsuperscript{24,25}, and reactive oxygen species (ROS) are also believed to be responsible\textsuperscript{26}. Therefore, the regulation of microorganism, inflammation, and ROS in the periodontal tissue is a possible preventive and therapeutic strategy for periodontal diseases.

\( \alpha \)-tocopherol acetate has been used due to its antioxidant activities and additional gum health benefits\textsuperscript{6-11}. It is readily hydrolysed to \( \alpha \)-tocopherol, also known as vitamin E, which...
is generally regarded as the most important and effective lipid soluble antioxidant in vivo. It is vital to maintain the cell membrane integrity against lipid peroxidation by peroxyl radical scavenging. α-tocopherol also inhibits platelet aggregation and improves blood circulation. In addition, in vitro and in vivo experiments have shown that treatments with 18β-glycyrrhetinic acid have potentially beneficial effects, such as anti-inflammatory, anti-bacterial effect, and alveolar bone loss inhibition in the periodontal tissue. Thus, the toothpaste containing α-tocopherol acetate and 18β-glycyrrhetinic acid may contribute to the prevention and treatment of periodontal diseases. However, for the toothpaste to be effective, it is crucial that these ingredients are delivered to and retained at the target sites after brushing.

Reconstructed oral tissue has been used for pharmacological and toxicity test as a substitute for human or animal models. Yang et al. reported that reconstructed oral tissue was also suitable for assessing retention of ingredients following topical application of toothpaste. Keratinized oral epithelium-EpiGingival tissue was used to assess the retention of ingredients on gingival tissue. Four consecutive 2-minute topical applications of toothpaste slurries with an hour interval were well tolerated by the tissue, and the ingredients were detected after repeated application on the tissue. Thus, the reconstructed gingival tissue model is an appropriate tool to estimate the gingival delivery of the toothpaste ingredients.

We recently developed toothpaste containing 2% α-tocopherol acetate and 0.3% 18β-glycyrrhetinic acid. Gingival absorption of these ingredients was assessed by using the EpiGingival tissue. Due to their water-insoluble characteristics, a considerable amount of α-tocopherol acetate and 18β-glycyrrhetinic acid retained on the gingival tissue even after washing with water. The bioconversion of α-tocopherol acetate into its bioactive form, α-tocopherol, was also measured.

Materials and Methods

1. Reconstructed gingival tissue

The EpiGingival tissues (GIN-100), purchased from MatTek, USA, were placed in 6 well plates and added 900 μl of GIN-100 Assay medium (mattek, USA). The tissues were equilibrated in a 37°C 5% CO2 incubator, overnight. All treatments added to the tissues were applied on top of the culture insert.

2. Test toothpastes

Toothpaste containing 2% α-tocopherol acetate (DSM Nutritional Products Asia Pacific Pte Ltd, Singapore) and 0.3% 18β-Glycyrrhetic acid (PHARMAPIA, Korea) and toothpaste containing 0.3% dipotassium glycyrrhizinate (Wha Costech Inc, Korea) with the same base formulation were manufactured.

3. Repeated application of the toothpastes

The test toothpastes were diluted with water in 1:3 ratio and 100 μl of these 25% toothpaste slurries were applied on the 0.6 cm² EpiGingival surfaces for 2 minutes followed by a single washing. The toothpaste slurries were aspirated and the tissues were washed with 300 μl of water. This treatment was applied two, four, or six times hourly. Between treatments, 100 μl of phosphate buffered saline (PBS) was applied. Following all treatments, the surfaces of the tissues were washed with PBS three times and then the tissues were extracted.

4. Tissue extraction

Receiver solutions (assay media) were saved to investigate whether the ingredients were penetrated through the membrane. To detect the substances in the tissues, treated EpiGingival tissues were extracted. The tissues were placed into vials followed by addition of 1 ml of extraction solution. Methanol was used as extraction solution for α-tocopherolacetate and 18β-glycyrrhetinic acid and DI water for dipotassium glycyrrhizinate. The samples were stored at 4°C until required for analysis. Before analysis, the samples were homogenized by using hard tissue homogenizer (Bertin Precellys 24 homogenizer, USA) and were sonicated for 1 hour. The clear supernatant was analyzed.

5. HPLC analysis

Analysis was performed using Agilent 1200 infinity series equipped with high performance liquid chromatography (HPLC) system consisting of an autosampler, a quaternary gradient pump system and DAD detector (Agilent Technologies, USA).

5.1. Analysis of α-tocopherol acetate and α-tocopherol

Tissue extracts and assay media were diluted by half with methanol and passed through PTFE membrane filter (0.45 μm for methanol) before being injected into HPLC. α-tocopherol and α-tocopherol acetate, purchased from Sigma Aldrich, USA, were used as standard. Chromatographic separation of α-tocopherol and α-tocopherol acetate was performed using a CAPCELL PAC C18 column (150 mm × 4.6 mm, 5 μm) (Shiseido, Japan). The flow rate of the mobile phase was set to
1.0 mL/min, operated at 25°C. UV/VIS chromatograms were acquired at 292 nm. Methanol was used for mobile phase in isocratic mode for 20 min. Quantification of α-tocopherol and α-tocopherol acetate was determined from standards of known concentration. α-tocopherol acetate was not detected in samples (Fig. 1A), on the other hand, the detection limit of α-tocopherol was 1.6 ppm (Fig. 1B).

5.2. Analysis of 18β-Glycyrrhetinic acid

Tissue extracts and assay media were diluted by half with methanol and passed through PTFE membrane filter before injected into HPLC. Chromatographic separation of 18β-glycyrrhetinic acid was carried out utilizing a SHISEIDO CAPCELL PAC C18 column (150 mm × 4.6 mm, 5 µm). The flow rate was set at 1.0 mL/min, kept at 30°C. UV/VIS chromatograms were acquired at 250 nm. Mobile phase used in gradient mode was composed of distilled water acidified with 0.2% phosphoric acid (A) and acetonitrile (B). A gradient elution was performed as follow: The elution started from 30% of eluent B up to 80% in 10 min and was maintained for 5 min. It was readjusted to the initial conditions for 5 min and re-equilibrated for 5 min. Quantification of 18β-glycyrrhetinic acid was determined from standards of known concentration, and the detection limit was 0.1 ppm (Fig. 1C).

5.3. Analysis of dipotassium glycyrrhizate

Tissue extracts and assay media were diluted by half with distilled water and passed through PVDF membrane filter (0.45 µm for DI water) before being injected into HPLC. Dipotassium glycyrrhizate was separated by using a J’sphere ODS-H80 column (250 mm × 4.6 mm, 4 µm) (YMC, USA). The flow rate of mobile phase was set at 0.8 mL/min, kept at 30°C. UV/VIS chromatograms were acquired at 254 nm. Mobile phase used in gradient mode was composed of distilled water acidified with 0.2% phosphoric acid (A) and acetonitrile (B). A gradient elution was performed as follow: The elution started from 30% of eluent B up to 80% in 10 min and was maintained for 5 min. It was readjusted to the initial conditions for 5 min and re-equilibrated for 5 min. Quantification of dipotassium glycyrrhizate was determined from standards of known concentration, and detection limit was 0.9 ppm (Fig. 1D).

6. Statistical Analysis

The student’s t-test was used to compare the differences between two groups. Values of $P<0.05$ were considered significant at a 95% confidence interval.

**Results**

A 25% of slurry of the toothpaste containing 2% α-tocopherol acetate and 0.3% 18β-glycyrrhetinic acid was prepared to reflect the dilution with saliva in the mouth\(^1\). The toothpaste slurries were treated on the EpiGingival\(^\text{TM}\) tissues for 2 minutes, a general brushing time. In order to explore the relationship between numbers of topical applications and absorption of the ingredients, the toothpaste slurry was applied repeat-

---

Fig. 1. HPLC analysis. (A) α-tocopherol acetate (B) α-tocopherol (C)18β-glycyrrhetinic acid and (D) dipotassium glycyrrhizate. The top figures represent standards and the bottom ones represent the test samples. Arrows are the peaks of the target materials.
Firstly, we assessed the absorption of the major ingredient, \( \alpha \)-tocopherol acetate. \( \alpha \)-tocopherol acetate was not detected in the tissue extracts and in the assay media (Fig. 1A). However, the metabolite, \( \alpha \)-tocopherol, was detected in the tissue extracts suggesting that \( \alpha \)-tocopherol acetate was absorbed into the reconstructed gingival tissue. An equivalent distribution of \( \alpha \)-tocopherol was found in the tissues regardless of the repeated treatments, 2.3 \( \mu g \)/tissue seems to be a maximum value of bioconversion (Fig. 2). \( \alpha \)-tocopherol was not detected in the receiver solutions (data not shown).

Another ingredient, 18\( \beta \)-glycyrrhetinic acid, was detected both in the tissue extracts and the receiver solutions (Fig. 3). We also found a positive correlation between the number of applications and the amount of absorbed ingredients: the amount of 18\( \beta \)-glycyrrhetinic acid detected in the tissue extracts and the assay media increased with the number of treatments.

Component from licorice root such as dipotassium glycyrrhizate has been known to have anti-inflammatory effect and used as ingredients of toothpastes and mouth rinses\(^\text{19} \). Instead of dipotassium glycyrrhizate, we used 18\( \beta \)-glycyrrhetinic acid due to its hydrophobic property. We expected hydrophobic 18\( \beta \)-glycyrrhetinic acid to be more absorbable into the gingival tissue than hydrophilic dipotassium glycyrrhizate. To prove our hypothesis, we compared the absorption of 18\( \beta \)-glycyrrhetinic acid and dipotassium glycyrrhizate into the EpiGingival\(^\text{TM} \) tissues. Toothpaste containing 0.3\% dipotassium glycyrrhizate in the same base formulation was manufactured. After treatments with 25\% toothpaste slurries with 18\( \beta \)-glycyrrhetinic acid or dipotassium glycyrrhizate six times, the amount of 18\( \beta \)-glycyrrhetinic acid detected in the tissue extracts was higher than that of dipotassium glycyrrhizate (Fig. 4A). In the receiver solutions, more 18\( \beta \)-glycyrrhetinic acid was detected than dipotassium glycyrrhizate, but it was not statistically significant (Fig. 4B).

**Discussion**

Many oral care products have been developed for the prevention and treatment of periodontal diseases. However, no study has shown whether the active compounds of the products can be delivered to the inside of the gingival tissue. Yang et al.\(^\text{18} \) have developed an *in vitro* model to investigate the retainment of the ingredients on the gingival tissue.

![Fig. 2. Absorption and metabolism of \( \alpha \)-tocopherol acetate. Toothpaste slurries were treated onto the EpiGingival\(^\text{TM} \) surfaces for 2 minutes. Treatments were repeated 2, 4, or 6 times. After all treatment, \( \alpha \)-tocopherol (\( \alpha \)-Toc) was measured by HPLC.](image)

![Fig. 3. Absorption of 18\( \beta \)-glycyrrhetinic acid. After repeated treatment of 25\% toothpaste slurries, 18\( \beta \)-glycyrrhetinic acid (GA) was measured in the tissue extracts (A) and the assay media (B).](image)
their data was not clear on whether the ingredients were retained on the tissue surface or penetrated through the membrane. To clarify this point, we measured the ingredients not only in the tissue extracts but also in the receiver solutions. Since 18\(\beta\)-glycyrrhetic acid and dipotassium glycyrrhizinate were detected in the receiver solutions, we concluded that these were absorbed into the reconstructed gingival tissues. However, the amount of the compounds detected in the tissue extracts might include both those on the tissue surface and those absorbed into the tissue. Although \(\alpha\)-tocopherol acetate was not detected in the tissue extracts and the assay media, \(\alpha\)-tocopherol was detected in the tissue extracts, suggesting that \(\alpha\)-tocopherol acetate was absorbed into the reconstructed gingival tissues and bioconverted into the active metabolite.

In case of reconstructed human epidermis, it is known to have lower barrier function than human skin\textsuperscript{20}. The TEWL of reconstructed human epidermis is higher than that of human skin. The penetration rate of caffeine in the reconstructed human epidermis was reported to be 10 times higher than in the human skin\textsuperscript{20}. Moreover, due to the thinness of the gingival epithelium, the rate of absorption of \(\alpha\)-tocopherol acetate and 18\(\beta\)-glycyrrhetic acid might be overestimated. In spite of these limitations, the reconstructed gingival tissue is a useful model to estimate gingival delivery of effective components of oral care products\textsuperscript{17,18}. Also, our data indicate that \(\alpha\)-tocopherol acetate and 18\(\beta\)-glycyrrhetic acid might be delivered into the gingival tissue.

\(\alpha\)-tocopherol has several gum health benefits such as anti-oxidant effect and blood circulation\textsuperscript{6-11}, but it is highly unstable to oxidation\textsuperscript{21}. Therefore, we used \(\alpha\)-tocopherol acetate instead. Since \(\alpha\)-tocopherol acetate is a non-active form, it is important to be converted to \(\alpha\)-tocopherol in the body\textsuperscript{21}. Our data show that \(\alpha\)-tocopherol acetate contained in toothpaste was delivered to the inside of the gingival tissue and metabolized into \(\alpha\)-tocopherol. As Shiratori have suggested that skin might be an important storage site and play a major role in distribution and metabolism of \(\alpha\)-tocopherol\textsuperscript{22}, the gingival tissue might be a storage site for \(\alpha\)-tocopherol. Thus, \(\alpha\)-tocopherol might stay in the gingival tissue and provide gum health benefits.

We used 18\(\beta\)-glycyrrhetic acid instead of dipotassium glycyrrhizinate, which has been widely used as ingredient of toothpastes and mouse rinses\textsuperscript{19}. Both materials are originated from licorice extract and have anti-inflammatory effect\textsuperscript{12,13,19}, but their properties are different, dipotassium glycyrrhizinate being hydrophillic while 18\(\beta\)-glycyrrhetic acid hydrophobic. We expected the hydrophobicity of 18\(\beta\)-glycyrrhetic acid could enhance the rate of absorption into the gingival tissue and, as expected, found higher absorption of 18\(\beta\)-glycyrrhetic acid than dipotassium glycyrrhizinate.

**Conclusions**

We verified that the active ingredients of our toothpaste, 2% \(\alpha\)-tocopherol acetate and 0.3% 18\(\beta\)-glycyrrhetic acid, could be absorbed into the gingival tissue using EpiGingival\textsuperscript{TM} tissue model. The active components of toothpaste might be expected to work inside the gingival tissue for gum health. However, this investigation limits to the absorption of the ingredient in an artificial model. Therefore, further study about clinical relevance of this model would be necessary.
Acknowledgement

The work described in this manuscript was funded by LG Household & Healthcare Ltd.

References