DEVELOPMENT OF LIP TREATMENT ON THE BASIS OF DESQUAMATION MECHANISM

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Summary

Lip chapping is a serious cosmetics problem, though remedies other than moisturizing have not been proposed. We investigated changes in the surface configurations of lip corneocytes and

activities of desquamation-regulating proteinases associated with lip chapping. Using scanning electron microscopy, villus-like projections were observed on the inner surfaces of most corneocytes from normal lips, whereas those with flatter surface were predominant in chapped lips. Further, cell surface area increased with the severity of lip chapping. Cathepsin D (CD)-like and chymotrypsin-like proteinase, which are also present in skin as desquamation-regulating proteinases, were detected in lip corneocytes, though only CD activity was found to decrease in severely chapped lips. Hydration was also lower in areas of lip chapping. Sequential topical application of apricot extract essence increased CD activity and improved chapping severity. Our results suggest that lip chapping can be characterized as similar to senile xerosis rather than dry skin such as winter xerosis, as it shows a delayed transition of corneocytes through the stratum corneum, and the reduced CD activity may be one of the mechanisms that is further decreased by low hydration. We propose that an enhancement of both CD activity and lip moisture may be effective to improve lip chapping.

Introduction

The lips are an area of transition between the mucous membrane and skin, with physiological characteristics that are considered different from those of skin, as lip cells have a faster rate of turnover [1], their stratum corneum is thin, and pre-mature cells are exposed to the outer environment. Thus, as compared to skin, lips readily lose their moisture content and a higher incidence of chapping has been reported [2], which is often considered to be a serious cosmetic problem. One of the unique characteristics of lip chapping is abnormal desquamation along with scaling, which is frequently observed in the transitional area from the lower lip to the mucous membrane [3]. We previously reported changes in the surface configurations of the stratum corneum of the lips [4] [5] and also compared physiological parameters with those of skin[6]. However, many unanswered questions remain regarding the mechanism of the development of this condition and moisturizing is the only currently available remedy.

Contrary to lip chapping, the mechanisms of dry skin have been extensively studied and one of its characteristics is known to be dry flaky scaling. In normal conditions, corneocytes in the outermost stratum corneum are detached and shed from the skin surface through the degradation process of desmosomes, which are adhesion molecules that tightly bind individual corneocytes. Desmosomes

have been found to persist in the outmost layers of dry skin due to an impaired degradation process [7] [8], resulting in abnormal desquamation that can be observed as detached corneocytes or stratum corneum flakes with irregular arrangements [9] [10]. It is considered that proteinases such as trypsin-like and chymotrypsin-like proteinase regulate the degradation process of desmosomes [11] which is affected by hydration [12] and the lipid structure [13] of the stratum corneum. Thus, dry skin is likely to be aggravated by low humidity or aberrant cornification of epidermal cells causing an imbalance of lipid composition, as well as impaired maturation and barrier function of the stratum corneum, though the production of these proteinases has been reported to be unchanged even in dry skin. Based on these findings, various skin care methods have been proposed for dry skin which include moisturizing, correction of lipid composition[14], correction of the balance between proliferation and differentiation of epidermal cells[15] [16]., and inhibition of the plasminogen cascade [17].

Since lip chapping is frequently associated with abnormal desquamation, it is likely that it can be relieved by proper regulation of the desquamation function of the lip stratum corneum. Recently, we reported the involvement of cathepsin D-like proteinase in desquamation of the stratum corneum of skin, in addition to chymotrypsin-like proteinase [18] [19] [20] However, in the lips, which are a

transitional area from the mucous membrane to the skin, the presence of these enzymes has not been confirmed and their involvement in the regulation of desquamation in the lip stratum corneum remains obscure.

In an attempt to find an effective treatment method appropriate for lip chapping, we examined detailed changes in the surface configuration of the outermost corneocytes from lips along with the severity of lip chapping using scanning electron microscopy. We also investigated the involvement of cathepsin D-like and chymotrypsin-like proteinases in lip chapping, and the improvement of this condition by regulation of proteinase activity.

Materials and Methods

Morphological characteristics of lip corneocytes

- ***Subjects***

Lip corneocytes were taken from the lower lips of 35 healthy adult males ranging in age from 27 to 56 years old.

- ***Collection of corneocytes and observation of surface configurations***

Corneocytes were taken from the lips (mucosal part, red margin, and skin part) by pressing a piece of adhesive tape (Kanebo, Japan) onto the lower lip of each subject. With this method of collection, the inner sides of the cells was exposed and the outer side (the area exposed to the outer environment) adhered to the tape. The inner sides of the cells were used for observation of the surface configuration without additional treatment. However, since the outer sides were contaminated by the tape adhesive, they were prepared with the following additional treatment [21]. A thin film of vinyl chloride resin (Konishi Inc., Japan) was applied to a polyethylene terephthalate sheet and each piece of tape with the outer side of the cells was placed firmly onto the film. Each sample was incubated with ethanol for 10 minutes, followed by xylene for 2 hours, after which the adhesive tape was removed from the sample, which remained mounted onto the polyethylene terephthalate sheet, and then each sample was incubated with xylene for 1 hour and air-dried. As a result, samples with the outer side of the cells exposed were obtained and used for observation of the surface configuration.

Both inner and outer side corneocyte samples were coated with platinum palladium vapor (Ion sputter E-1030, Hitachi Inc., Japan), fixed on the specimen stage of an electron microscope (SEM; s-4200, Hitachi Inc., Tokyo, Japan) with a piece of a two-sided tape, and observed.

Changes in lip stratum corneum associated with the chapping

- ***Subjects***

Fifty-seven healthy adult females ranging in age from 21 to 43 years old were used as subjects.

- ***Overall findings of surface configuration in the chapping***

The degree of lip chapping in each subject was classified according to the criteria described in Figure 1. Five subjects was selected for each degree and 100 cells from each individual lip sample were observed using scanning electron microscopy.

Figure 1

- ***Hydration state of the stratum corneum***

To understand the physiological characteristics of the subject lips, the hydration state of the stratum corneum of the central lower lip was determined using an impedance meter (Skicon-200, IBS,

Japan) by measuring high-frequency conductance at 3.5 MHz. The same area of each subject was measured 5 times, and the mean value, obtained after excluding the highest and lowest values, was used as the hydration state.

- ***Amount of desquamation index measurement, topological measurement, and observation of surface configuration of lip stratum corneum***

The lip stratum corneum of each subject was stripped twice with a piece of adhesive tape. The first strip was used to measure the amount of exfoliated stratum corneum, analyze its physical characteristics, and observe the surface configurations of the cells, while the second strip was used to determine the activities of desquamation-regulating proteinases in the cells.

An index of the amount of desquamation (DIA) was determined by taking images of the collected samples with a film scanner (LS 3500, Nikon Co Ltd., Japan) that were analyzed with an image analyzer (Nexus 6800, Nexus Co. Ltd., Japan) [22]. To analyze the physical characteristic of the cells, images were taken with a light microscope video system and the surface area of 50 cells was measured using an image analyzer (Nexus 6800, Nexus Inc.), with the mean value used as the surface area of the cells in each sample. Next, the surface morphology of the inner side of the

corneocytes was observed with an electron microscope and the correlation with lip chapping was investigated.

- ***Desquamation-regulating proteinase activities on the lips***

DIA in the second stripped samples was measured using a film scanner before measurement of proteinase activities. The areas (ϕ 6 mm, 2 areas for each sample) were then punched out and used as samples to measure the activities of cathepsin D-like proteinase (CD) and stratum corneum chymotryptic proteinase (SCCE). The punched out samples were reacted with oxidized insulin B chain (Sigma, USA) as a substrate at pH 3.0 for 3 hours and pH 6.0 for 18 hours, for CD and SCCE, respectively. Derivatives degraded from the oxidized insulin B chain were separated on high performance liquid chromatography (HPLC) (Figure 2). Proteinase activities were determined by measuring the peak areas of the derivatives specific for cathepsin D-like or chymotrypsin-like activities, and proteinase activity was expressed as index of the peak area per DIA corresponding to the area punched out and used for measurement of activity.

Figure2

- ***Effect of apricot extract essence and other extracts on cathepsin D-like proteinase activity and improvement of lip chapping***

For *in vivo* examinations, apricot extract essence and other extracts (Esperis Inc., Italy) were dissolved in a 50% ethanol solution to make a final extract concentration of 5%. Each sample was applied onto the flexor aspect of forearm of 5 subjects (1 male and 4 females) twice a day for 2 weeks. To examine the effect of the apricot extract essence on lips, petrolatum was used as a solvent instead of 50% ethanol solution. Ten male subjects were divided into 2 groups and an apricot extract essence-containing sample was applied twice a day for 1 week to the lips of those in the experimental group, while a placebo sample was applied to the lips of the control group. Cathepsin D-like proteinase activity before and after the experiment was determined as described above. Improvement of lip chapping was also examined by clinical assessment.

Data analysis

All results are presented as the mean and standard deviation. Statistical comparisons were made using Student's t test, with significance set at 10%.

Results

Observation of surface configuration of outermost corneocytes of lips using scanning electron microscope

The surface configurations of corneocytes in normal lips were observed using scanning electron microscopy and compared among several parts associated with the lips. In observations of the inner side of the corneocytes, the mucous part was found to consist of flat cells with a smooth surface (Figure 3a). On the other hand, in the red margin, which is the area generally recognized as the lip, most cells showed large villus-like projections over the whole surface (Figure 3b). In the boundary region between the red margin and skin part (Figure 3c), we observed shorter villus-like projections, however, in the skin part around the red margin such villus-like projections were rarely seen, though flattened projections were typically observed (Figure 3d). In a comparison between the inner and outer sides of the corneocytes from the red margins, villus-like projections were particularly noted on the inner side, while the outer side had pore-like depressions corresponding to the projections observed on the inner side (Figure 4).

Figure3

Figure4

Changes in surface configuration and physiological characteristics of corneocytes in lip chapping

- ***Change in surface configuration of corneocytes with lip chapping***

In order to better elucidate the mechanism of lip chapping, changes in surface configuration were examined. Contrary to the corneocytes of normal lips, where villus-like projections were observed on the inner surface, corneocytes from severely chapped lips showed flattened surfaces on both the inner and outer sides of corneocytes, though villus-like and pore-like depressions were also observed in limited areas (Figure 5).

Figure5

- ***Change in distribution of cells with different surface configurations along with severity of lip chapping***

Since obviously different corneocyte surface configurations were seen between normal and chapped lips, the distribution of cells with villus-like projections or flattened surfaces was compared to the severity of lip chapping. Corneocytes were classified into 4 levels based on the appearance of the villus-like projections, according to the criteria listed in Figure 6. In the severity grade 0 group, which consisted of normal lips, most of the corneocytes showed villus-like projections on the inner side (Figure 7). However, the number of cells with villus-like projections (class 3 and 4) decreased as lip chapping increased (severity grades 1 and 2) and cells with flattened surfaces became the major component.

Figure 6

Figure 7

- ***Changes in physiological parameters of corneocytes and stratum corneum associated with lip chapping***

In order to elucidate the physiological changes of the stratum corneum along with lip chapping, individual cell surface area and the amount of tape-stripped corneocytes were measured, in addition

to hydration state. The cell surface area of corneocytes tended to increase in correlation with the severity of lip chapping (Figure 8), while the number of corneocytes collected by tape stripping showed a tendency to decrease (Figure 9). Further, the hydration state of the stratum corneum was significantly decreased in samples from chapped lips (grades 1 and 2) as compared with those from normal lips (grade 0) (Figure 10).

Figure 8

Figure 9

Figure 10

Changes in desquamation-regulating enzyme activities and improvement of lip chapping by enhancing its activity

- ***Cathepsin D-like and chymotrypsin-like proteinases in lip stratum corneum***

Cathepsin D-like (CD) and chymotrypsin-like (SCCE) proteinases are both considered to regulate desquamation of the outermost stratum corneum of the skin. In corneocytes stripped by tape from normal lip surfaces, these activities were also confirmed (Figure. 11, Grade 0), indicating their

involvement in desquamation of the lip stratum corneum. In heavily chapped lips (grade 2), CD activity was significantly lower than in normal lips (grade 0), whereas there was no difference between normal lips (grade 0) and mildly chapped lips (grade 1) (Figure 11a). Contrary to CD activity, there was no difference observed in SCCE activity among any of the groups (Figure11).

Figure11

- ***Improvement of lip chapping by enhancement of CD activity in the stratum corneum***

Since the CD activity of corneocytes decreased as the severity of lip chapping increased, we attempted to improve lip chapping by enhancing CD activity with cosmetic ingredients. Several types of ingredients were dissolved in 50% ethanol solutions and applied continuously onto the flexor aspect of forearms of subjects for 2 weeks, and changes in cathepsin D-like proteinase activity before and after were determined. Among the different types, a significant increase in CD activity was observed only with the apricot extract essence, as compared with the control group (Figure 12). In order to confirm the same effect of apricot-extract essence on lips, either petrolatum containing 5% apricot extract essence or petrolatum alone was applied continuously for 1 week, and a significant

increase in CD activity was observed in those who received the apricot extract essence formulation (Figure 13). We also examined the improvement of lip chapping using clinical assessment and found that the severity of lip chapping was significantly decreased in the group who received apricot-extract essence (Figure 14), confirming that chapping was improved by enhancement of CD activity with the apricot-extract essence formulation.

Figure13

Figure14

Discussion

Configuration of lip corneocytes and its change in chapping

Corneocytes in the stratum corneum are eventually shed from the outermost layer of the skin after premature cells in the bottom part of the stratum corneum grow and move toward the outermost layer, and then change their morphology to become flat and stronger. Using electron microscopy, previous observations of the inner side of skin corneocytes obtained from the outermost layer of stratum corneum in the upper arm found cells with a flattened surface, while relatively smooth cells with few

villi were observed in those from the facial area [23] [24] [25]. However, cells from diseased skin, where cells were actively proliferating, had villus-like projections on the inner side of the corneocytes and pore-like depressions corresponding to those projections on the outer side, with surfaces that were quite rough [21] [23] [26] [27]. This condition can be explained by the fact that premature cells move up quickly to the surface of the stratum corneum, before they become flat and fully mature. Thus, observation of surface configuration is a useful tool to distinguish and classify abnormal skin conditions. However, there is only one other known study of the surface morphology of lip corneocytes [28], which used tissue collected from corpses, and none regarding correlations observed between the physiological characteristics of the lips and surface configuration of lip corneocytes.

In the present study, we investigated the morphological characteristics of corneocytes from normal lips, and observed villus-like projections and pore-like depressions on the inner and outer cells, respectively, from the red margin, which were similar to those found in actively proliferating skin (Figure 3, 4). In contrast, shorter villus-like projections and smoothly curved projections were observed on the boundary of the skin and red margins, as well as from the skin portion (Figure 3). Moreover, a comparison of the red margin between normal and chapped lips showed that the number

of cells with these villus-like projections were decreased, while cells with flattened surfaces increased to become the major component in chapped lips (Fig. 5, 7). Thus, normal lips had corneocytes with villus-like projection like those seen in immature cells associated with skin disease and an acceleration of cell proliferation, whereas the chapped lips had cells characterized by flattened surfaces, which resembled mature corneocytes in skin. This was considered to occur because the lip stratum corneum turnover is as fast as 3 to 4 days [1]. Further, since cell surface area, an indicator of turnover rate [29], was increased in chapped lips, the delayed rate of the transition of cells through the stratum corneum accompanied by full maturation may be a characteristic of lip chapping. In addition, chapped lips are known to have a hard texture and less moisture in the stratum corneum. We previously reported that trans-epidermal water loss (TEWL) in the stratum corneum was decreased with lip chapping [6]. Together, these results strongly suggest that the stratum corneum in lip chapping resembles that of senile xerosis and not of dry skin such as winter xerosis, as a delayed transition rate and decreased TEWL were demonstrated.

Changes in activities of desquamation-regulating proteinases in chapped lips

Our configuration study showed a delayed turnover of the stratum corneum in lip chapping

(Figure 8). Further, we confirmed that cell adhesion near the surface of the stratum corneum was stronger in chapped lips (Figure 9). These results led us to examine the involvement of desquamation-regulating proteinases in lip chapping. We report here for the first time the activities of cathepsin D-like (CD) and chymotrypsin-like proteinases in the lips (Figure 11, Grade 0), which are considered to regulate desquamation of the stratum corneum in skin. Further, we found a significant decrease in CD in severely chapped lips (Figure 11-A). Previously, we reported a declined expression of cathepsin D in psoriasis [30], which shows hyper-accumulation of stratum corneum though aberrant proliferation, and CD activity has been reported to decrease with aging [31]. Thus an accumulation of stratum corneum might be caused by a reduction of CD activity. On the other hand, a decrease in hydration of the stratum corneum was observed in lip chapping (Fig. 10). In our experiment, we measured CD activity after the cell samples were dipped into a solution where proteinase showed full activity. In *in vivo* conditions, the water content of the stratum corneum is thought to directly influence proteinase activity [16], therefore, reduced CD activity might be further decreased by low water content, resulting in the promotion of a greater amount of irregular desquamation.

Proposed lip care to improve chapping

In order to improve lip chapping, we believe that appropriate lip care must be based on the particular characteristics of lips and not on those of skin. A low level of hydration in the stratum corneum was observed in slight (grade 1) and severe lip chapping (grade 2), while a low level of CD activity was observed in severely chapped lips (Figure 11). For improvement of slightly chapped lips, moisturizing may be effective, however, to improve those that are severe, the reduced CD activity must be increased. We found that an apricot extract essence preparation caused an increase of CD activity and confirmed that lip chapping was improved by its application (Figure 13, 14). The increase in CD activity in the outermost stratum corneum observed in our experiment was considered to be due to an increase in the production of CD in the upper granular layer of epidermis. Therefore, enhancement of CD activity by topical application of an effective ingredient may provide fundamental improvement of lip chapping by boosting the reduced self-regulating ability of lips. Therefore, we propose that enhancement of both CD activity and lip moisture could be effective to improve lip chapping.

Conclusions

We investigated changes in the surface configurations of lip corneocytes and activities of desquamation-regulating proteinases associated with lip chapping. Our results suggest that lip chapping can be characterized as similar to senile xerosis rather than dry skin such as winter xerosis, with a delayed transition of corneocytes through the stratum corneum, and the declined CD activity may be one of the mechanisms that is decreased by low hydration. Further, we propose that an enhancement of both CD activity and lip moisture may be effective to improve lip chapping.

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Figure legends

Fig. 1. Criteria used for clinical assessment of lip chapping. Grade0: Normal, Grade1: Slight chapping with mild dryness, Grade2: Severe chapping with heavy dryness and scaling.

Fig. 2. HPLC elution profiles of the oxidized insulin chain B derivatives after reaction with tape-stripped corneocyte samples. (a) Reaction at pH 3 for detection of cathepsin D-like proteinase activity. (b) Reaction at pH 6 for detection of chymotrypsin-like proteinase activity. Arrows indicate peaks used for determination of activity.

Fig. 3. Images of the surface configurations of the inner side of outermost corneocytes, by scanning electron microscopy. (a) Mucous part. (b) Red margin. (c) Boundary region between red margin and skin part. (d) Skin part around red margin.

Fig. 4. Images of surface configurations of the inner and outer sides of outermost corneocytes collected from normal lips, by scanning electron microscopy. (a) Inner side. (b) Outer side.

Fig. 5. Images of surface configurations of the inner and outer sides of outermost corneocytes collected from chapped lips, by scanning electron microscopy. (a) Inner side. (b) Outer side.

Fig. 6. Classification of corneocytes according to degree of villus-like projections (VP) observed on the cell surfaces. Class I: Flattened surface without VP, Class II: slight frequency with short VP, Class III: frequent with short VP, Class IV: frequent with tall VP.

Fig. 7. Distribution of corneocytes classified by degree of villus-like projections in various degrees of lip chapping. □ : Class I corneocytes, ▩ :Class II

corneocytes, ▨ : Class III corneocytes, ■ : Class IV corneocytes. The degree of lip chapping was defined as described in Figure 1.

Fig. 8. Changes in cell surface area associated with severity of lip chapping.

Fig. 9. Changes in the number of corneocytes collected by tape stripping from lip surfaces associated with severity of lip chapping. The number of corneocytes was determined from an image of the tape strip and expressed as amount of desquamation index (DIA).

Fig. 10. Changes in water content of stratum corneum of lips associated with severity of lip chapping*$P<0.05$

Fig. 11. Activities of desquamation-regulating proteinases in various degrees of lip chapping.

(A) Cathepsin D-like proteinase. (B) Chymotrypsin-like proteinase. *$P<0.05$.

Fig. 12. Effect of various kinds of extracts on cathepsin D activity in skin stratum corneum. Each sample contained 5% of the extract in a 50% ethanol solution. +$P<0.10$.

Fig. 13. Effect of apricot extract essence on increase of cathepsin D-like proteinase activity in stratum corneum of lips.

Apricot extract essence (5% in petrolatum) was applied to lip surfaces twice daily for 2 weeks. Cathepsin D-like proteinase activity was measured before and after the experiment. Activity after treatment is expressed as a percentage against that before treatment. *$P<0.10$.

Fig. 14. Improvement of lip chapping after treatment with apricot extract essence (5% in petrolatum). *$P<0.05$.

Figure 1

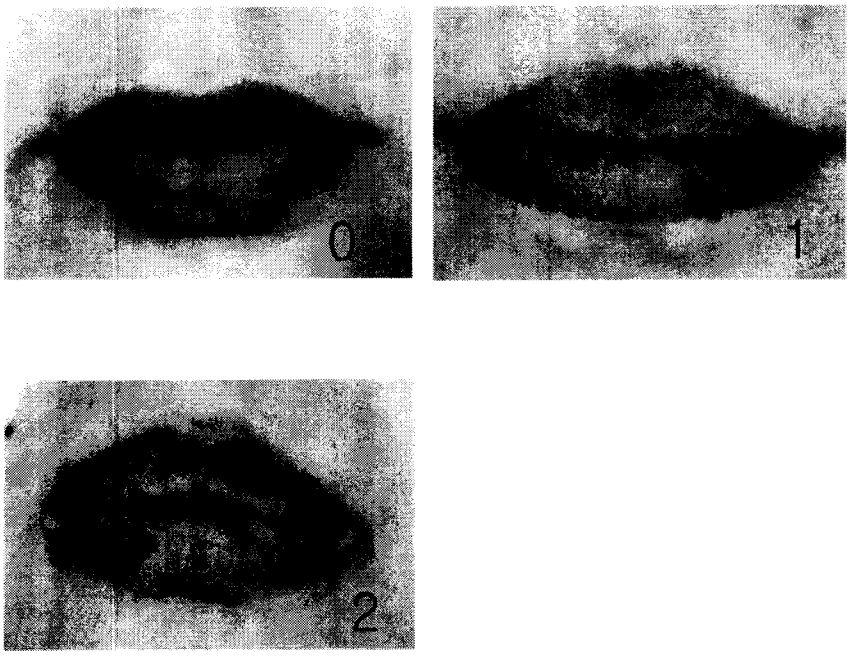
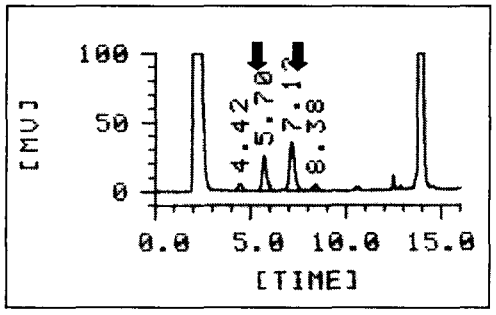


Figure 2

[A]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[TIME]

[B]

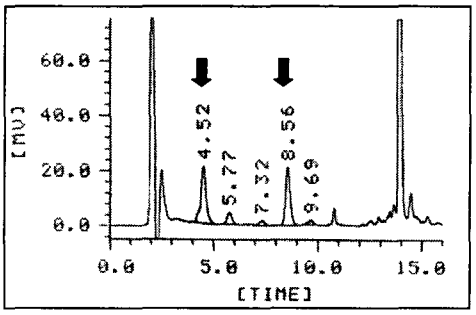

60.0
40.0
20.0
0.0
[MV]
4.52
5.77
7.32
8.56
9.69
0.0
5.0
10.0
15.0
[TIME]

Figure 3

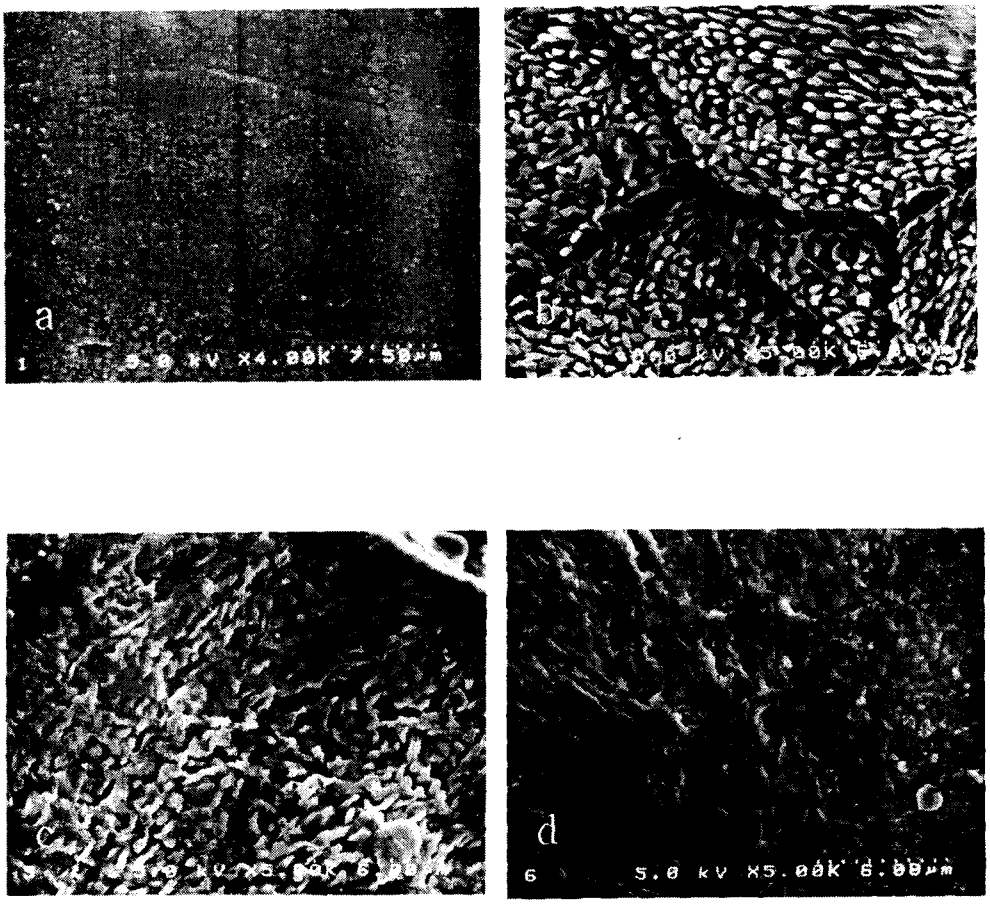


Figure 4

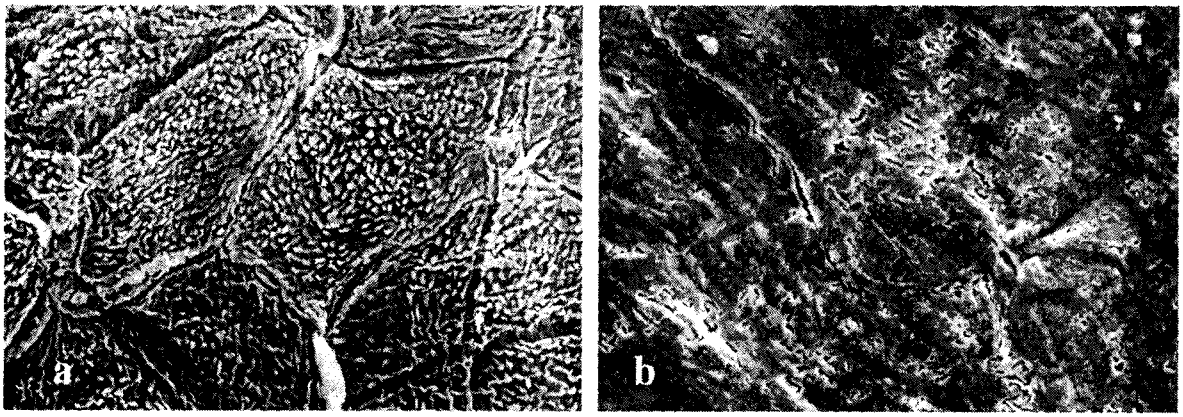


Figure 5

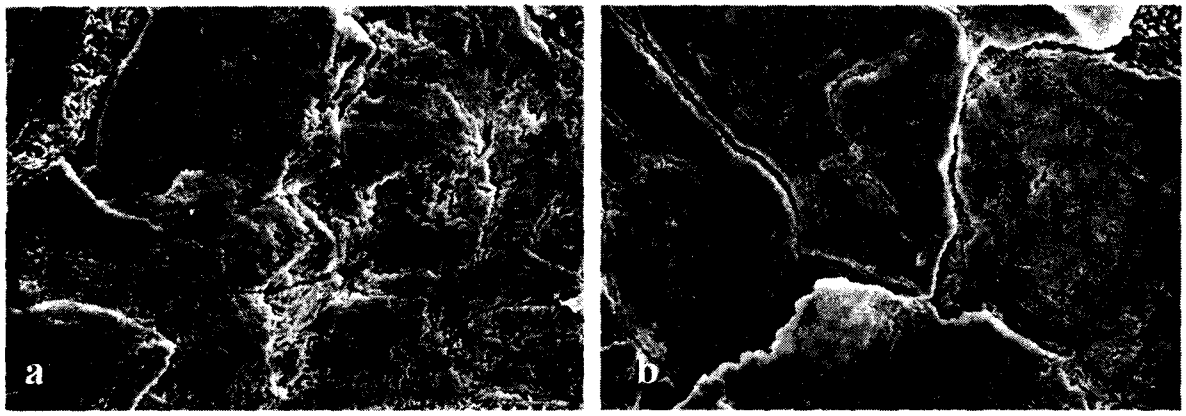


Figure 6

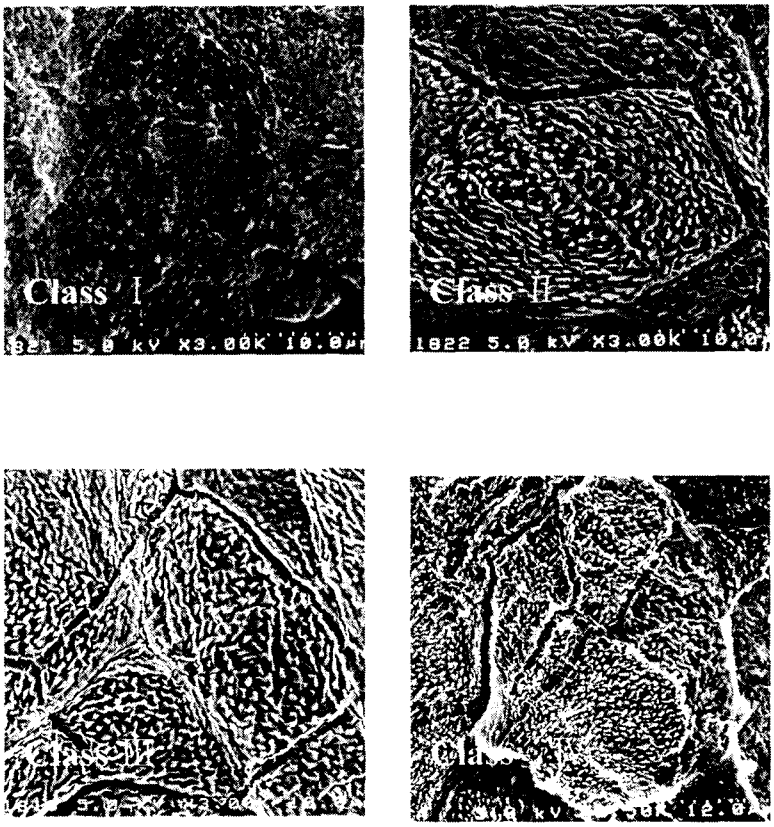


Figure 7

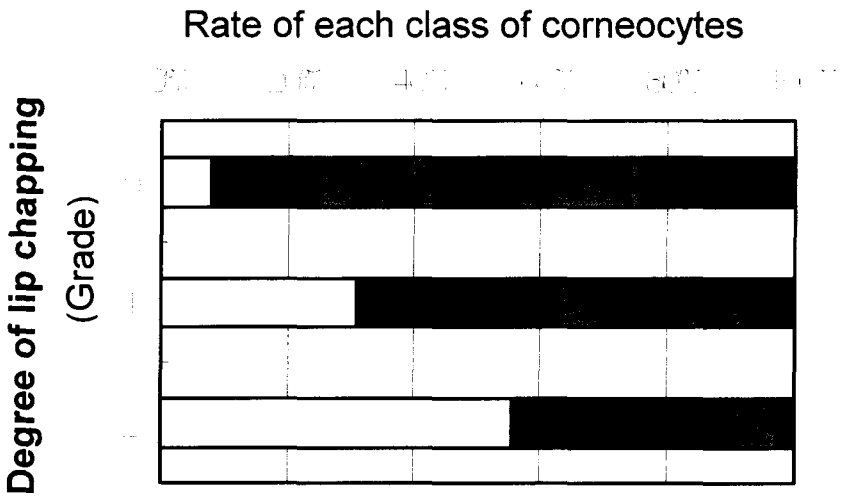

Rate of each class of corneocytes
Degree of lip chapping
(Grade)

Figure 8

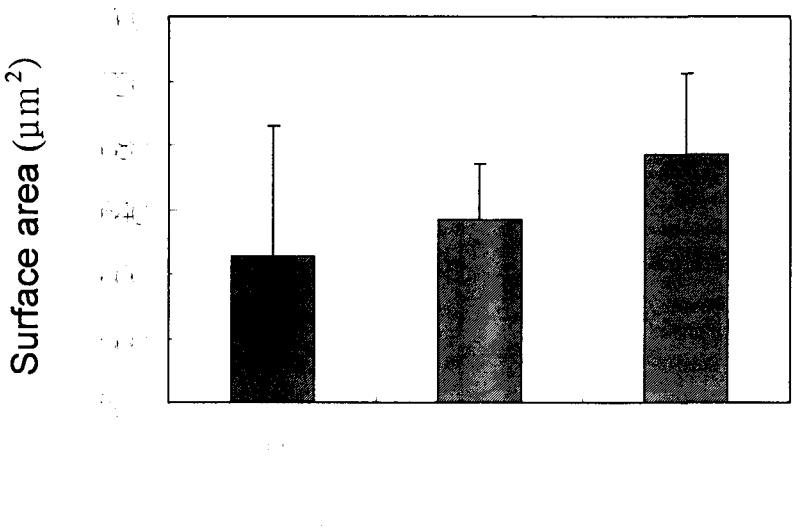


Figure 9

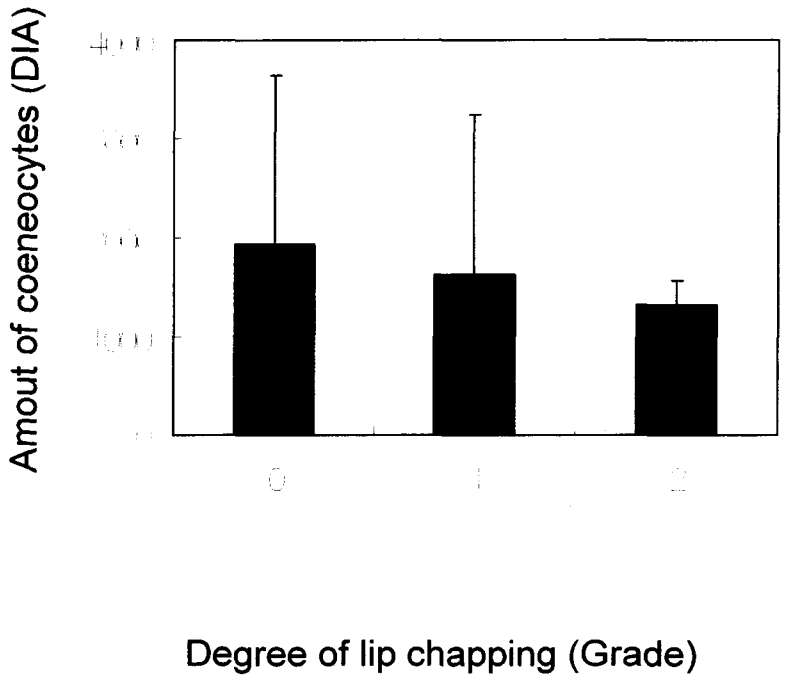


Figure 10

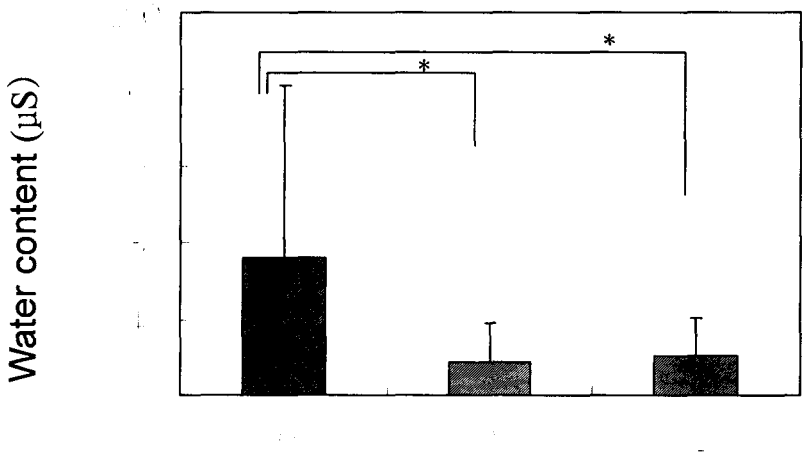


Figure 11

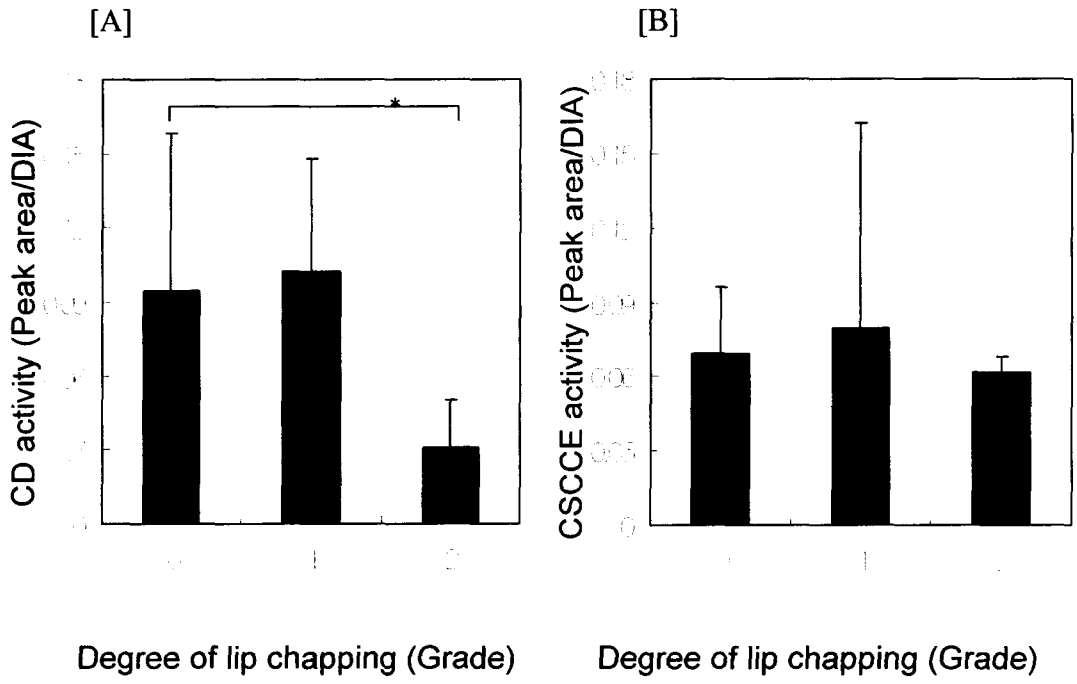

[A]
[B]
CD activity (Peak area/DIA)
CSCCE activity (Peak area/DIA)
*
Degree of lip chapping (Grade)
Degree of lip chapping (Grade)

Figure 12

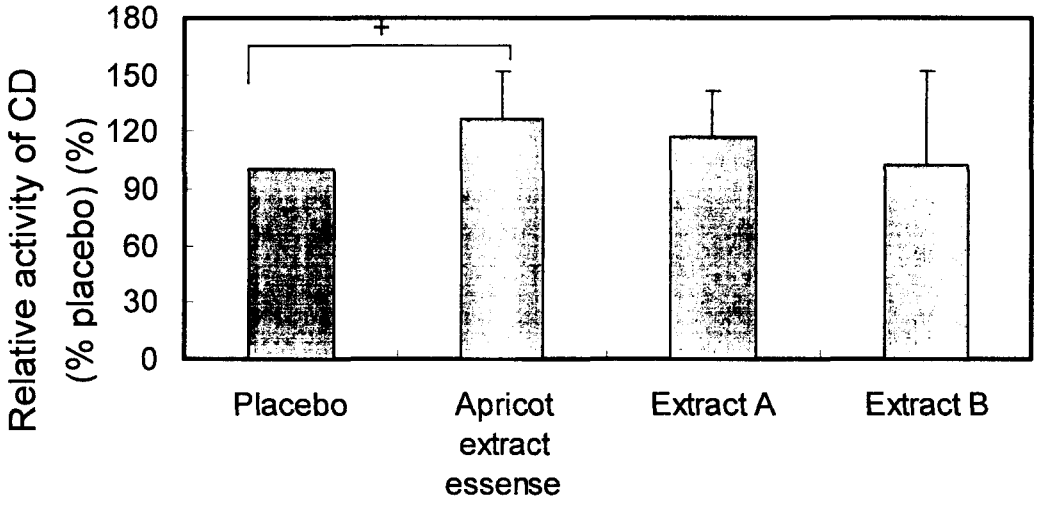

Relative activity of CD
(% placebo) (%)
180
150
120
90
60
30
0
+
Placebo
Apricot extract essense
Extract A
Extract B

Figure 13

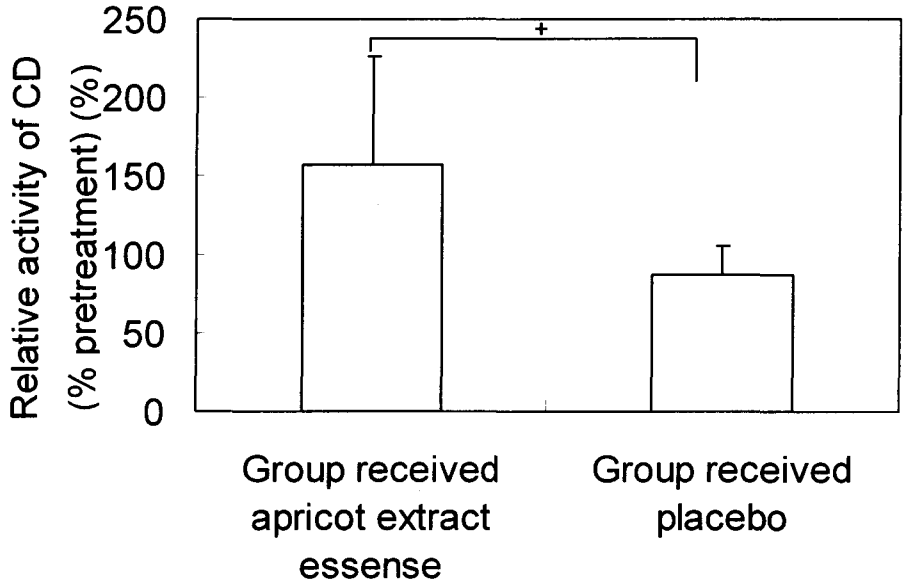
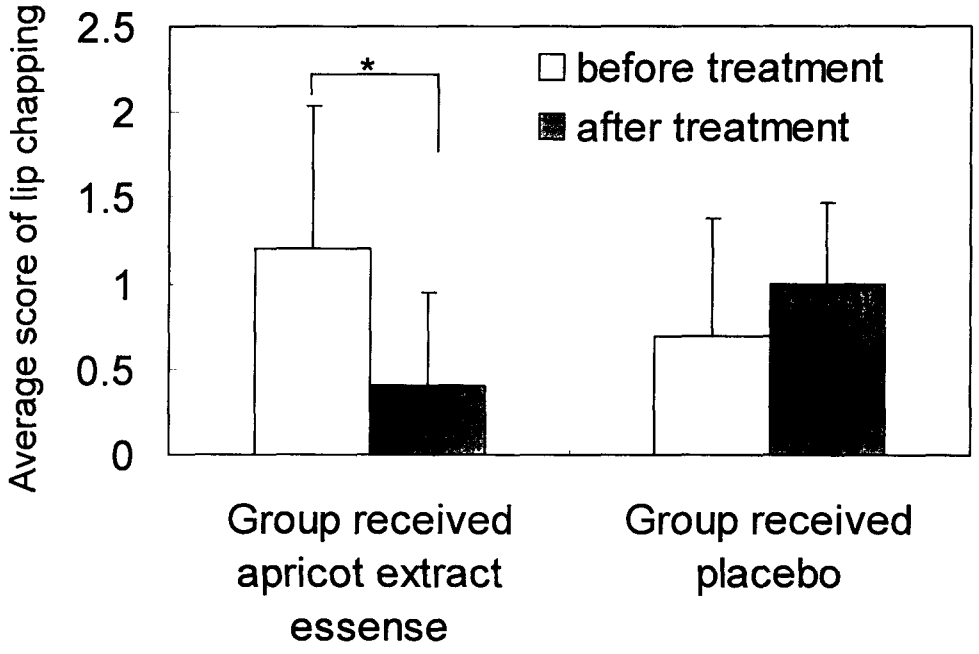

Relative activity of CD
(% pretreatment) (%)
250
200
150
100
50
0
+
Group received apricot extract essense
Group received placebo

Figure 14


Average score of lip chapping
2.5
2
1.5
1
0.5
0
*
before treatment
after treatment
Group received apricot extract essense
Group received placebo