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Visual Imaging of Calcium Ion Distribution in Acetone and Tape Stripping Damaged Canine Epidermis

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Abstract : The purpose of this study is to establish experimental canine skin barrier disruption model, the study was designed to observe calcium ion in skin frozen tissue of canine skin and also the modulation of calcium ion distribution of normal skin with disrupted skin such as clipping, acetone, tape stripping damages according to time. To compare the changes of calcium ion gradient after damages, the distribution of calcium ion in the canine epidermis was visualized by blotting to gel containing chemical indicator (Calcium Green-1) with fluorescent microscope and the effects of skin barrier damages were examined according to time. Three mins and 1hr after acetone damage, the gradations of epidermis and hair follicle showed more radiant and disappeared after 48 hrs. On the contrary, 3mins and 1hr after tape stripping damage, the gradations showed more radiant than those of acetone damage, and these gradations were stabilized after 48 hrs. The method we presented here could show the visual image of the calcium ions in frozen tissue without further preparation, and it might be useful to investigate the role of calcium ion in the canine epidermal barrier recovery, however, it might be need further methodological improvement to get accurate quantitative information.

Key words **:** calcium; canine; skin barrier; acetone; tape stripping.

Introduction

The calcium distribution in the epidermis demonstrates a characteristic pattern, with little calcium within the basal and spinous layers and a gradual increase toward the outer Stratum Granulosum (SG) (14,16). However, calcium largely disappears from the Stratum Corneum (SC) above the SG-SC interface. During fetal epidermal development the epidermal calcium gradient develops late in gestation, parallel to the emergence of a competent permeability barrier in fetal mouse and rat skin (9). Ionic signals such as calcium and potassium play an important role on the homeostatic mechanism of the epidermal barrier function (13,16). Thus, observation of these ions would provide us important information to understand epidermal homeostasis, however, image analysis of ions in the skin is technically difficult (6). One study demonstrated a new method of visualization of magnesium, calcium, potassium, sodium, and hydrogen ions and the modulation of the distribution pattern by tape stripping in skin frozen tissue of human skin (6). However, there has been few study on epidermal ions in veterinary dermatology research. The purpose of this study is to establish experimental canine disrupted skin model for veterinary skin research. The study was designed to study calcium ion in skin frozen tissue of canine skin and also the modulation of calcium ion distribution of normal skin with disrupted skin such as clipping, acetone, tape stripping damages according to time.

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Materials and Methods

Experimental animals and environment

Six clinically normal, 2-4-year-old, male Beagles without dermatological problems and abnormal blood health profiles were chosen for this study. The dogs were fed Natural Choice® Adult chicken and rice (Nutro Co., USA) for 12 weeks prior to testing and were kept in wire-floor cages in a room held at 27-32°C and 40-57% relative humidity. Clipping of the hair coat was performed on the shoulder back of each dog with a standard pair of shaving clippers (Oyster pro76®, No 40, Oyster Co., USA). Loose hair was brushed away before the probe was applied. The clipped shoulders back of each dog were divided into four parts for this experiment.

Acetone damage

Acetone damage was performed 48h after clipping. One spot out of 2 spots on shoulder back was brought into contact twice with 5 ml acetone (Merck, Darmstadt, Germany) for 2.5 mins. No rubbing occurred. Skin contact was made by fixing Pyrex tubes (Extrelut® 15 ml, Merck) filled with acetone on the skin while the dogs were gently moving. The acetone was then discarded. Evaluations were done before ($t_1 = 0$) and after (t_2 = acetone) acetone treatment.

Tape stripping damage

Tape stripping damage was performed using Scotch-tape (3M. USA). An area of 40 × 40 mm was marked and 20 squares of exactly fitting pieces of adhesive tape were consecutively applied to the skin. The tape was homogeneously pressed on to the skin for 5s. The tapes were then slowly removed at an angle of 170° with the skin (22). Tape stripping was carried out 20 times. At that time, most of stratum corneum removed but still some remained.

Skin biopsy

Samples were obtained from the shoulders divided into four parts with punch biopsy method. The samples were biopsied from untreated skin and 3 min, 1 hr and 48 hrs after acetone and tape stripping. To avoid the misobservation of artifacts, at least three samples were taken from one region. Samples were immediately frozen in isopentane- filled metal jar which kept in liquid nitrogen to prevent artifactual redistributions (3). The frozen samples were kept at -80°C until sectioning.

Visualization of calcium ion

Calcium Green-1 (cell permeable) was purchased from Molecular Probes™ (Invitrogen. USA). Agarose (Sigma. USA) gel (final 2%) contained indicator was spread on the slide glass with 50 mm thickness. For calcium observation, 10 mg/ml of calcium green 1 was mixed before the formation of the membrane. First agarose gel membrane was formed and then was spread over the gel membrane. A frozen section, 10 μm in thickness, was put on the gel membrane and within a couple of hours, the whole picture was taken. Within 12h after the preparation, the clear images were disappeared. Nikon Fluorescent microscope (SMZ-1B. Nikon-co. Japan) was used for observation of the present study. For Calcium Green 1, the wavelength of the excitation light was 546 nm. For this observation, at least five sections were observed to find common features. The modulation of ion distribution were compared normal skin with disrupted skin such as clipping, acetone, tape stripping damages according to time to study of the skin recovery processing of calcium ion for comparing of recovery process.

Results

Visualization of calcium ion

Images of calcium in the skin are shown in Fig 1. These are the representatives of each observation. Calcium localized in the epidermal granular layer in normal skin. Before clipping, and 3 mins, 1 hr and 48 hrs after clipping, the gradations showed the almost similar tendency and a homogeneous distribution around the whole epidermis which was not affected by clipping. Three mins and 1hr after acetone damage, the gradations of epidermis and hair follicle showed more radiant,

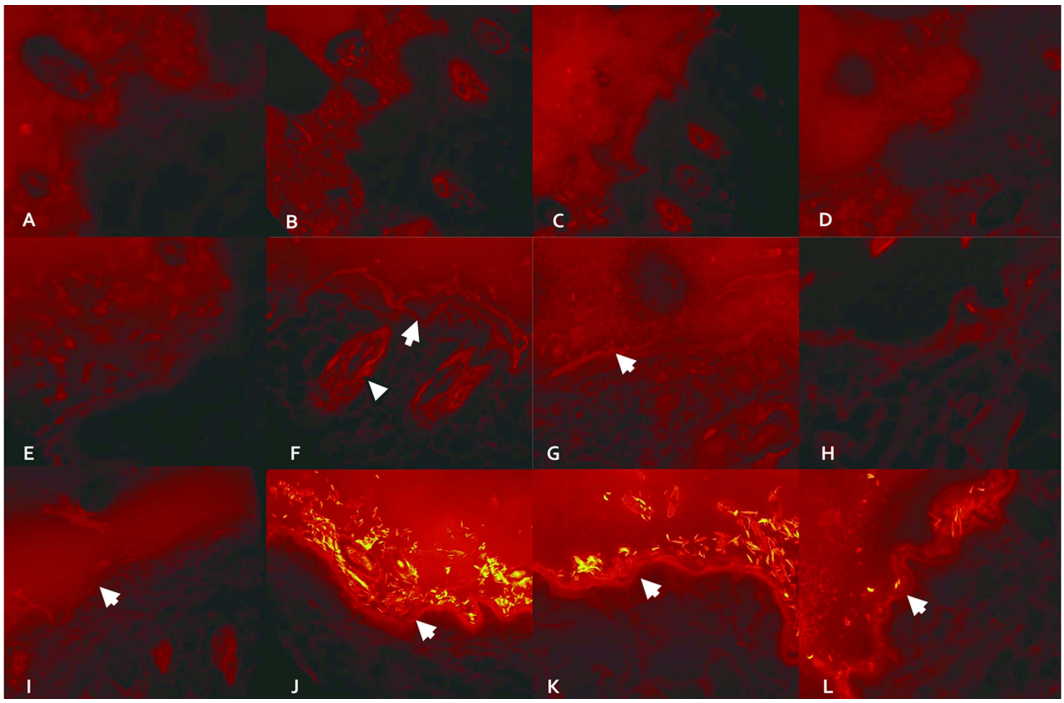

Fig 1. Localization of calcium in the epidermal granular layer in normal canine skin. Before clipping (A) and 3mins, 1hr, 48hrs after clipping (B, C, D), the gradations showed the almost similar tendency and a homogeneous distribution around the whole epidermis which was not affected by clipping. Before acetone damage (E) and 3mins, 1hr, 48hrs after acetone damage (F, G, H), the gradations of epidermis (arrow) and hair follicle (arrow head) showed more radiant in F. These gradations disappeared in H. On the contrary, before tape stripping damage (I) and 3mins, 1hr, 48hrs after tape stripping damage (J, K, L), the gradations (arrow) showed especially radiant in J, K, L. These gradations were stabilized in L.

and these gradations were disappeared after 48 hrs. On the contrary, 3mins and 1hr after tape stripping damage, the gradations showed especially radiant, and these gradations were stabilized after 48 hrs.

Discussion

In human studies, the calcium gradient was first demonstrated using ultrastructural cytochemistry and then later by several quantitative methods in adult human and murine epidermis and by using ion capture cytochemistry and PIXE, all of which methods provided similar results (11,14,18). Recently, Denda *et al* (6) demonstrate a new method of visualization of magnesium, calcium, potassium, sodium, and hydrogen ions in skin frozen tissue of human. Both localization and distribution of calcium and magnesium ions play a crucial role in regulation of barrier homeostasis, whereas the role of potassium, sodium, chloride, and phosphate ions has still not been investigated well (6). There is a Ca^{2+} gradient in the epidermis with low concentrations in the basal layer and progressively higher concentrations towards the upper epidermis (2). Thus, observation of these ions would provide us important information to understand epidermal homeostasis.

In human, there are various skin barrier disruption models to study various skin diseases. Acetone damage is the skin barrier disruption by superficial lipid extraction. Barrier disruption has been accomplished with an acute acetone treatment model (2,10,21). Contact with acetone only removed the skin surface lipids, whereas the mode of action of Sodium Lauryl Sulfate (SLS) included the skin barrier disruption of the SC structure by deep lipid extraction (post-inflammatory xerosis model). In the study of Menon *et al* (14,16), between 1 and 24 hours after acetone treatment, calcium-containing precipitates gradually begin to reappear both in the cytosol of upper SG cells and in the extracellular domains, resulting in a parallel restoration of the epidermal calcium gradient. In this study, there were little changes in calcium gradation after acetone damage.

Tape stripping damage is the skin barrier disruption by removal of corneocytes. Barrier disruption has been accomplished with an acute tape-stripping model (in healthy damaged SC model as well as atopic skin model) (1,19,22). The Ca^{2+} gradient disappears immediately after acute barrier disruption such as tape stripping (4). Destroying the Ca^{2+} gradient by sonophoresis perturbs barrier homeostasis (8). In this study, there were much changes in calcium gradation after tape stripping damage, and then the gradient disappeared immediately after tape stripping disruption. The peak of calcium concentration in the epidermal granular layer was increased in 3mins, 1hr after barrier disruption and kept equilibrium in 48hrs after barrier disruption. These results suggest that calcium plays an important role in barrier homeostasis and/or signaling of barrier insults and recovery in the canine skin. Especially, the gradations showed radiant and obvious pattern in 3mins, 1hr after tape stripping damage. Therefore, it seems that calcium is a crucial role in the recovery of disrupted skin and barrier homeostasis.

Two major importances of the calcium ions are signaling the lamellar body secretory response and regulator of keratinocyte differentiation (2,20), which means the calcium ion appears to play a crucial role in skin barrier homeostasis. Denda *et al* (5) reported that influx of calcium and chloride ions into epidermal ketatinocytes regulates exocytosis of epidermal lamellar bodies and skin permeability barrier homeostasis. In the normal epidermis, the calcium concentration is higher in upper epidermis (i.e., granular layer) and lower in the deeper epidermis (i.e., basal layer). Mauro *et al* (15) reported that this calcium gradient in the hairless mice epidermis disappeared immediately after barrier disruption. Calcium is a second messenger, therefore, the changes in the free intracellular calcium level act as an important second messenger in cells, translating external environment signals into internal signals and generating cellular responses (12). Changes in the free intracellular calcium level act as an important second messenger in cells, translating external environment signals into internal signals and generating cellular responses (12). The key event of the calcium ion in the homeostatic response to barrier disruption is lamellar body secretion (7,17). In vitro data indicate that lowered extracellar calcium concentrations promote continued proliferation of keratinocytes, whereas higher calcium concentrations lead to expression of terminal differentiation markers, stratification, and cultures that are differentiating (12).

In conclusion, the observation of calcium ion would provide us information to understand epidermal homeostasis. The method we presented here could show the visual image of the calcium ions in frozen tissue without further preparation, and it might be useful to investigate the role of calcium ion in the canine epidermal barrier recovery, however, it might be need further methodological improvement to get accurate quantitative information. Further study will be required to study magnesium ion and other ions in skin frozen tissue of canine skin as well as the modulation of these ions distribution in acetone and tape stripping damaged canine epidermis according to time.

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개에서 피부손상에 의한 표피내 칼슘이온 분포상

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요 약 : 본 연구의 목적은 개 피부장벽 손상모델을 실험적으로 구현하기 위해 개의 정상피부, 제모 된 피부 그리고 아세톤 및 테잎 스트리립핑에 의한 손상된 피부에서의 표피내 칼슘이온의 분포를 관찰하였다. 피부손상에 따른 표피의 칼슘이온 변화도는 화학적 표시자(Calcium Green-1)가 포함된 gel blotting을 적용하여 피부장벽손상 효과를 시간에 따라 형광현미경으로 관찰하였다. 아세톤 손상 후 3분 및 1시간에 표피와 모낭의 칼슘이온의 차이를 나타내는 형광도차는 보다 밝게 관찰된 후 48시간 후에 소실되었다. 이와는 대조적으로, 테잎스트립핑손상 후 3분 및 1시간의 표피 칼슘이온의 형광도차는 아세톤 손상에서보다 더 밝게 보였다가 48시간 후에 소실되었다. 본 실험상의 방법을 통해 피부손상 방법에 따른 표피 칼슘이온의 가시적 이미지를 관찰할 수 있었고, 표피내 농도차이를 확인할 수 있었다. 따라서 본 화학표시자를 이용한 염색법은 개 피부장벽 복구기전에 대한 칼슘이온의 역할을 규명하는데 유용할 것으로 판단되며, 추후 표피칼슘이온 농도의 정량분석법에 관한 연구가 필요할 것으로 사료된다.

주요어 : 칼슘; 개, 피부장벽, 아세톤, 테잎스트립핑