



Effects of age, sex, and breed on the composition of free extractable ceramides in the stratum corneum of healthy dogs

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Abstract

Ceramide (CER), an important component of the extracellular lamellar lipids in the stratum corneum (SC), plays a critical role in maintaining the cutaneous barrier function. This study aimed to determine whether the quantity of free extractable SC CERs in dogs was affected by the age, sex, or breed. Fifty-eight dogs from the breeds Shiba Inu, beagle, miniature dachshund, shih tzu, and golden retriever, without any history of skin problems, were enrolled in this study. Lipid extracts from the SC were subjected to high-performance thin-layer chromatography to quantify the free extractable CERs. There were weak negative correlations between the age and the amount of free extractable CERs, CER [NP] (non-hydroxy fatty acids linked to phytosphingosines), CER [AS/NH] (α -hydroxy fatty acids linked to sphingosines/non-hydroxy fatty acids linked to 6-hydroxysphingosines), and CER [AP] (α -hydroxy fatty acids and phytosphingosines). There were no significant sex- or breed-related differences in the amounts of free extractable SC CERs in the dogs. These findings imply that aging causes a decline in the amount of free extractable SC CERs in dogs, similar to that observed in humans. The sex or breed of the dogs investigated in this study did not influence the amount of free extractable SC CERs.

Keywords Ceramide · Skin · Dog · Breed · Age · Sex

Introduction

In the stratum corneum (SC), the corneocytes are embedded in extracellular lamellar lipids (ELL), which are mainly composed of ceramides (CERs), cholesterol, and free fatty acids (Nishifuji and Yoon 2013). Of these three key lipid constituents, the CERs constitute an appreciable component of the ELL, accounting for up to 50% of the ELL (Feingold and Elias 2014; Yoon et al. 2013). The amphiphilic structures and long carbon chains of the CERs in the ELL are important factors in maintaining the barrier function of the SC (Feingold and Elias 2014).

CERs are composed of sphingosine molecules linked to fatty acids. To date, 12 classes of free extractable CERs have been identified in the human SC (Masukawa et al. 2008), namely CER[EOS] (ω -hydroxy fatty acids linked to sphingosines), CER[EODS] (ω -hydroxy fatty acids linked

to dihydrosphingosines), CER[NDS] (non-hydroxy fatty acids linked to dihydrosphingosines), CER[NS] (non-hydroxy fatty acids linked to sphingosines), CER[EOP] (ω -hydroxy fatty acids linked to phytosphingosines), CER[NP] (non-hydroxy fatty acids linked to phytosphingosines), CER[EOH] (ω -hydroxy fatty acids linked to 6-hydroxysphingosines), CER[AS] (α -hydroxy fatty acids linked to sphingosines), CER[ADS] (α -hydroxy fatty acids linked to dihydrosphingosines), CER[NH] (non-hydroxy fatty acids linked to 6-hydroxysphingosines), CER[AP] (α -hydroxy fatty acids linked to phytosphingosines), and CER[AH] (α -hydroxy fatty acids linked to 6-hydroxysphingosines). In addition, liquid chromatography and mass spectrometry analyses have indicated that the canine SC contains 11 classes of free extractable CERs with molecular masses and polarities that are equivalent to those in humans (Yoon et al. 2011). The free extractable CERs in humans (Farwanah et al. 2005; Ponc et al. 2003) and canines (Popa et al. 2010; Yoon et al. 2011) can be separated into at least eight fractions using high-performance thin-layer chromatography (HPTLC).

In humans, differences in the profiles of the SC CERs between healthy and diseased states have been demonstrated.

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In addition, it has been reported that the amount of CER can be influenced by ethnicity and age. Jungersted et al. (2010a, b) have found that the CER/cholesterol ratio was significantly different among different ethnic groups. Asian participants had the highest CER/cholesterol ratio, followed by Caucasian participants, and African participants had the lowest ratio. The amount of the total free extractable SC CERs in Negroid individuals was approximately 50% less than that observed in Caucasian and Hispanic individuals (Rawlings 2006). Imokawa et al. (Imokawa et al. 1991) have reported that the amount of total free extractable CERs in the human SC was significantly decreased with aging. Conversely, Jungersted et al. (2010a, b) have reported that there was no significant correlation in the proportion of free extractable CER with age.

In humans with atopic dermatitis (AD) (Imokawa et al. 1991; Ishikawa et al. 2010) and psoriasis (Motta et al. 1994), a significant negative correlation has been demonstrated between the amount of CERs and transepidermal water loss (TEWL); TEWL is considered to be a valuable parameter that reflects the permeability of the barrier function of the skin. Similar to the case in humans, the proportions of some CER classes have been reported to be decreased in dogs with AD (Yoon et al. 2011; Reiter et al. 2009). In addition, significant negative correlations have been shown between the amount of total SC CERs and TEWL in dogs with AD (Shimada et al. 2009). Therefore, in dogs with AD, the CER-associated cutaneous barrier function is thought to be impaired, similar to that in human patients with AD. However, the effects of age, sex, and breed on the amount and composition of the free extractable CERs in the canine SC have not yet been fully elucidated. Therefore, in the present study, we aimed to determine whether the age, sex, or breed affected the free extractable CERs in the SC of healthy dogs.

Materials and methods

Study subjects

A total of 46 client-owned dogs and 12 experimental dogs [32 castrated males and 26 spayed females, median age: 8 years (0.5 to 15 years)] without any history of skin problems were enrolled in this study. All animals were fed regular commercial food, and no topical products were used on the skin during sampling. Sampling of all dogs was conducted in autumn. The breeds of the dogs enrolled in this study were as follows: Shiba Inu [n = 15; 11 castrated males and 4 spayed females, median age: 8 years (0.5 to 14 years)], beagles [n = 12; 6 castrated males and 6 spayed females, median age: 7 years (2 to 10 years)], miniature dachshunds [n = 11; 4 castrated males and 7 spayed females, median age: 7 years (3 to 12 years)], shih tzus [n = 11; 5 castrated

males and 6 spayed females, median age: 10 years (0.5 to 15 years)], and golden retrievers [n = 9; 6 castrated males and 3 spayed females, median age: 4 years (2 to 12 years)]. The All procedures involving animals were in compliance with good clinical practice guideline issued by the Japanese Ministry of Health, Labor and Welfare on 29th February 2008, and all participating dog owners provided informed consent. Animal Research Committee of the Tokyo University of Agriculture and Technology approved for the study as a research project (No. 21–21).

Collection of the SC and lipid extraction

The SC was collected by tape stripping from the inguinal area of the dogs as has been previously reported (Imokawa et al. 1991; Ishikawa et al. 2010). Briefly, polyphenylene sulfide film tape with an area of 2 × 4 cm glued with silicon (#695–25, Nichiban, Tokyo, Japan) was pressed against the skin and subsequently stripped. This procedure was repeated five times in the same area. Then, the five consecutive tapes were immersed in *n*-hexane (Sigma, St. Louis, MO, USA) and sonicated for 10 min. The SC extracts were weighed, transferred to glass tubes, and dissolved in 5 mL of chloroform/methanol (2:1, v/v; Sigma) at 20–25 °C with shaking for 30 min to obtain lipid extracts. After centrifugation (1000 × g for 5 min), the supernatants containing the lipid extracts were transferred to new glass tubes, dried using a nitrogen stream at 38 °C, reconstituted in 50 µL of chloroform/methanol (2:1, v/v), and stored at –20 °C until HPTLC analysis.

HPTLC

Lipid extract (5 µL) was applied to an HPTLC plate (Merck, Darmstadt, Ger-many). Lipids were size fractionated in chloroform/methanol/acetic acid (190:9:1, v:v:v). Bovine-derived CER[NS] and CER[AS] (Matreya, Pleasant Gap, PA, USA) were used as standards to determine the polarities and amounts of the HPTLC bands in the SC extracts. The plates were sprayed with 10% CuSO₄ (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 8% H₃PO₄ (Wako) aqueous solutions and then heated at 180 °C for color development. The HPTLC bands were scanned and subjected to density plot analysis using Bio1D-ver.12.11 software (Vilber Lourmat, Marne-la-Vallée, France). The quantity of each class of free extractable CER was determined by comparing the density plots with those of serially diluted CER standards and calculated as the amount of each CER class in 1 mg of the SC from healthy dog skin. The total free extractable CER was calculated as the sum of all the CER classes in 1 mg of canine SC.

Statistical analysis

The unpaired Student's t-test was used to compare the amount of total free extractable CERs and CER classes between male and female dogs. One-factor analysis of variance followed by the Tukey–Kramer method as a post-hoc test was used to compare the amounts of total free extractable CERs and different CER classes among the different breeds. Statistical significance was set at $P < 0.05$. The Pearson correlation test was used to analyze the correlation between the amount of total free extractable CERs and age. Correlation coefficients (r) of < -0.2 and > 0.2 indicate weak negative and positive correlations, respectively, and r values < -0.4 and > 0.4 indicate significant negative and positive correlations, respectively.

Results

Free extractable SC CERs decline with age in dogs

Free extractable CERs in the canine SC were separated into eight fractions according to the polarity using HPTLC, as has been reported previously (Yoon et al. 2011). The correlation between the amount of total free extractable CERs in the SC and the age of the dogs is shown in Fig. 1. The r value between the total free extractable CERs and age of the dogs was -0.262 ($p = 0.0473$), indicating a weak negative correlation between the amount of total free extractable CERs and age. In addition, weak negative correlations were observed between the amounts of CER[NP] ($r = -0.267$, $p = 0.036$), CER[AS/NH] ($r = -0.351$, $p = 0.007$), and CER[AP]

($r = -0.378$, $p = 0.004$) and the age of the dogs. No significant correlations were identified between the amounts of CER[EOS], CER[NDS/NS], CER[EOP], CER [EOH], and CER[AH] and the age of the dogs (Supplemental Fig. 1).

The sex and breed did not affect the amount of free extractable SC CERs in dogs

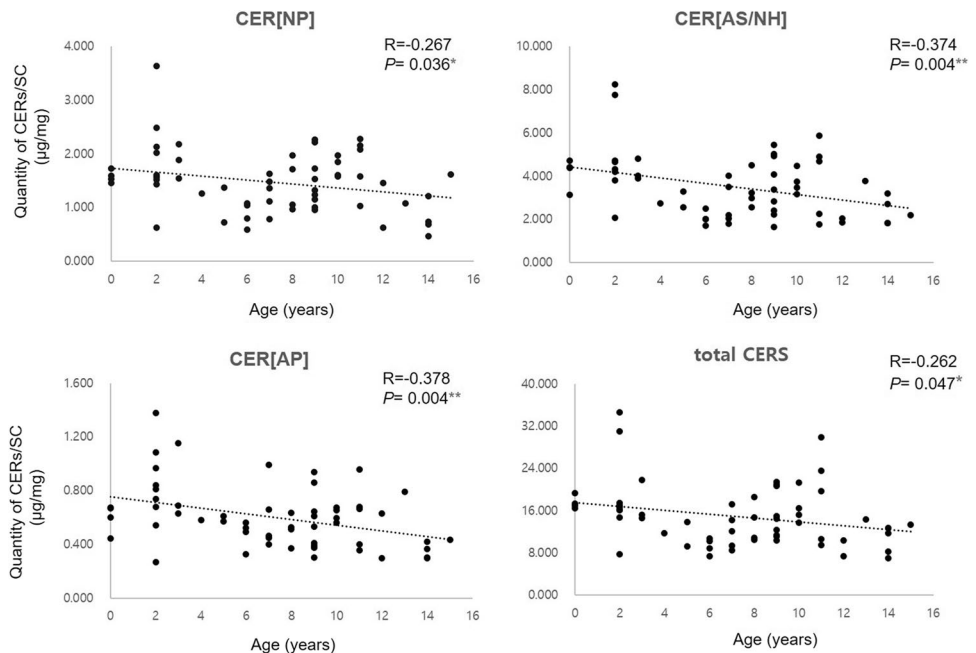
The amounts of free extractable SC CERs in the canine SC in relation to the sex and canine breeds were further analyzed. There were no significant differences in the amount of total free extractable CERs, or amounts of any CER fractions, between the sexes of the dogs examined (Fig. 2, Supplemental Table 1).

The amounts of free extractable SC CERs in Shiba Inus, beagles, miniature dachshunds, shih tzus, and golden retrievers were also compared. There were no significant differences in the amounts of total free extractable CERs, or amounts of any CER fractions, among the five breeds (Fig. 3, Supplemental Table 2).

Discussion

Previously, we have demonstrated that the proportion of free extractable CERs in the SC was associated with the permeability barrier function of the skin in canine atopic dermatitis (CAD) (Shimada et al. 2009). However, our previous study did not investigate any relationships between the quantity of free extractable CERs in the SC and the sex, age, or breed of the dogs.

Fig. 1 The correlation between the quantities of CERs and the age of the dogs. Weak negative correlations between the quantities of total CERs and the CER classes, CER[NP], CER[AS/NH], and CER[AP], and the age of the dogs were observed ($*p < 0.05$, $**p < 0.01$)



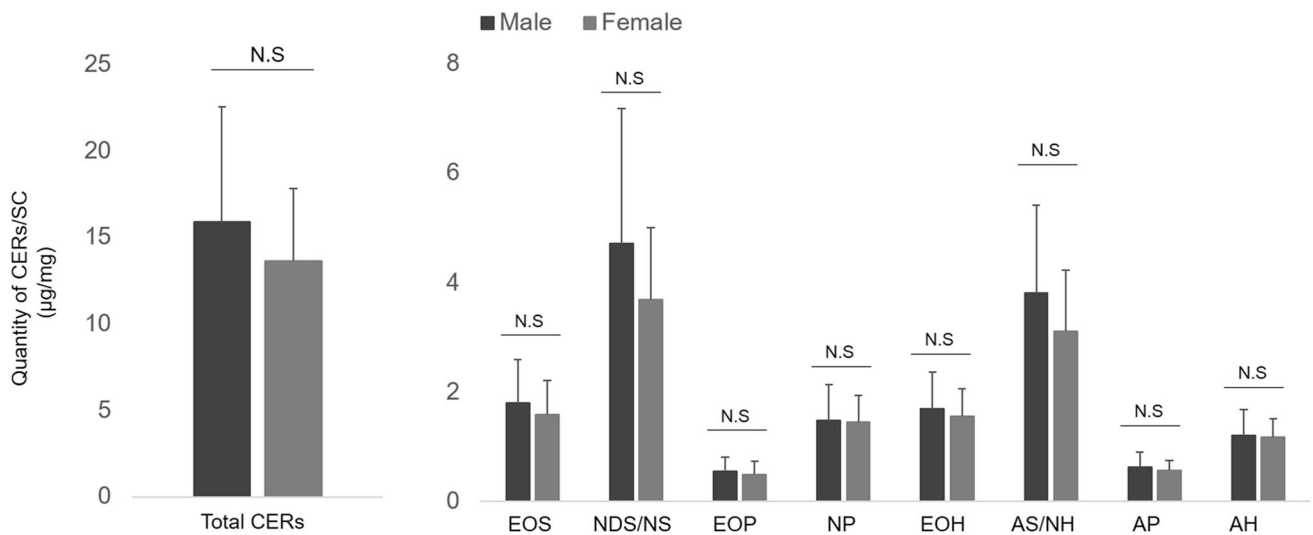


Fig. 2 Comparison of the quantities of total CERs and the CER fractions between the sexes in dogs. N.S. = not significant

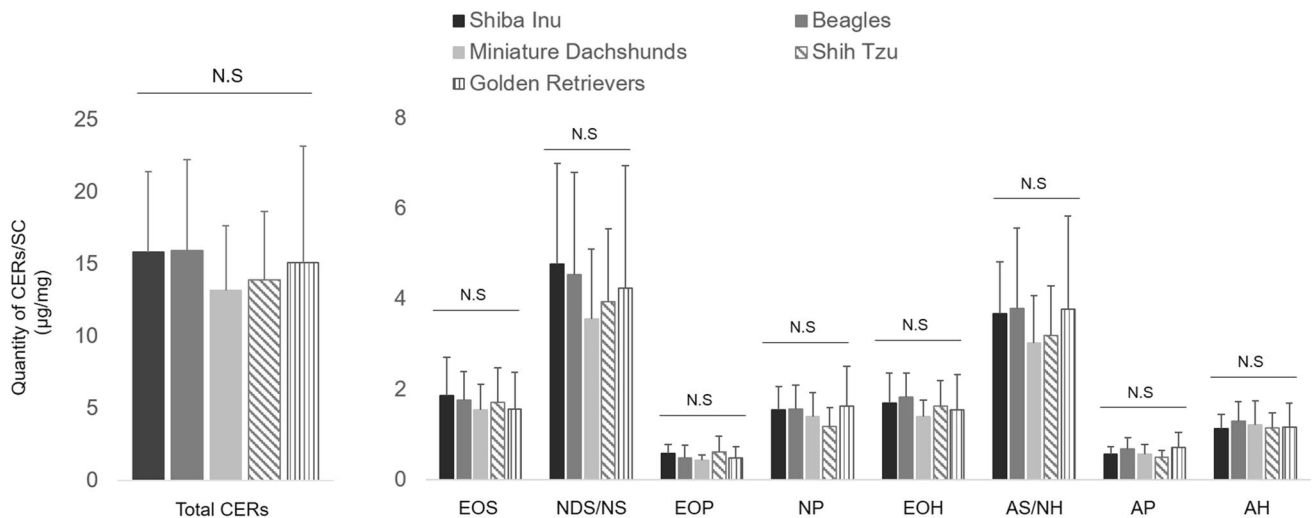


Fig. 3 Comparison of the quantities of the total and different CER classes among Shiba Inu, beagle, miniature dachshund, shih tzu, and golden retrievers dog breeds. N.S. = not significant

CERs are present in the SC either as free extractable CERs in the ELL or protein-bound CERs covalently attached to cornified envelopes. The lipid extraction method used in this study can isolate free extractable CERs in the ELL. To extract protein-bound CERs, the methods as reported by Popa et al. (Popa et al. 2010) must be used, which include a saponification procedure. It is well known that ω -hydroxy CERs are mainly present as protein-bound CERs; however, it has been reported that CER[EOS], CER[EOP], and CER[EOH] are also present in free extractable forms in the SC (Rabionet et al. 2014). Moreover, lipid fractions with the same molecular masses and polarities as CER[EOS], CER[EOP], and CER[EOH] have been isolated in human

and canine SC using the same techniques as described in the present study (Masukawa et al. 2008; Yoon et al. 2011). These results are consistent with our findings that free extractable CER[EOS], CER[EOP], and CER[EOH] could be detected and quantified in canine SC.

The present study in healthy dogs revealed that the amount of total free extractable CERs in the SC declined with age, similar to results observed in humans (Imokawa et al. 1991). However, the r values in dogs were lower than those calculated in humans (Imokawa et al. 1991). A possible mechanism underlying the decrease in the amount of CERs in the human SC is the decrease in the activity of sphingomyelin, a CER producing enzyme, in the human

epidermis with age (Jensen et al. 2005). In addition, elevated activity of ceramidase, a CER degrading enzyme, has been reported in aged dry skin (Jin et al. 1994). These mechanisms may also be responsible for the observed decreases in the amounts of CERs in aged dogs and need to be further investigated.

In addition to the total amount of free extractable CERs in the SC, the amounts of CERs in the SC CER classes, CER[NP] and CER[AP], also declined with age in dogs. Because CER[AS] and CER[NH] were observed as a mixed fraction in the HPTLC analysis, it was difficult to clarify which of these CERs declined with age in the present study. The decreased amounts of CER[NP] and CER[AP] in the canine SC imply that the levels of sphingosine might decrease with age in canine skin. The amounts of CER[EOS] (Rogers et al. 1996) and CER[NP] (Denda et al. 1993) have been reported to decrease in human skin with age. However, the reason for this decline is currently unknown and needs to be further elucidated.

There were no significant differences in the amounts of SC free extractable CERs between male and female healthy dogs. Similar findings have been reported for the TEWL value, which is a key indicator of the inside-to-outside barrier function of mammalian cells, including in dogs (Indra and Leid 2011; Ishikawa et al. 2010; Shimada et al. 2009). A previous study has also reported that no significant differences in the TEWL values were observed between female and male dogs (Young et al. 2002).

In the present study, five breeds (Shiba Inu, shih tzu, golden retriever, beagle, and miniature dachshund) were enrolled, most of which are predisposed to CAD. There were no significant differences in the amounts of total free extractable CERs, and CER classes, between the breeds. These findings indicated that the amount of free CERs was not appreciably affected by the type of dog breed, although we did not investigate the amount of CERs in other canine breeds. As a potential limitation of this study, only five different breeds with a small number of dogs from each breed were included in this study. Future studies investigating the amount of CERs in many different dog breeds will provide a better understanding of breed differences in the amount of CERs found in canine skin.

The present study indicated that the age of dogs should be considered in the design of experiments to measure the amount of free extractable CERs in the SC of dogs with skin disorders. Our findings also showed that the sex and breed had a minimal effect on the amount of free extractable CERs. Future studies investigating changes in the expression of CER metabolic enzymes with age will provide a better understanding of the observed decreases in the free extractable CER levels with age in dogs.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11259-021-09835-x>.

Authors' contributions All authors have made considerable contributions to this work. Conceptualization: Toshiroh Iwasaki; Methodology: Ji-Seon Yoon, Ako Sasaki; Formal analysis and investigation: Ji-Seon Yoon, Ako Sasaki, Kenichiroh Shimada, Koji Nishifuji; Writing—original draft preparation: Ji-Seon Yoon, Koji Nishifuji; Writing – review and editing: Ji-Seon Yoon, Ako Sasaki, Kenichiroh Shimada, Kaori Ide, Toshiroh Iwasaki, Koji Nishifuji.

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Data availability All data generated and analyzed during this study are included in this published article and its supplementary information files.

Declarations

Statement of animal ethics Institutional animal ethics guidelines were followed for the experiments. Study animals were subjected to minimum stress. The Animal Research Committee of the Tokyo University of Agriculture and Technology approved the animal experiments (No. 21–21).

Consent to participate All participating dog owners were provided informed consent before inclusion in this study.

Consent for publication All authors gave their consent for research publication.

Conflicts of interest/Competing interests The authors have no conflicts of interests to declare relevant to this manuscript's content.

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