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Repeated restraint stress modifies fatty acid and amino acid metabolism in the mouse skin

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ABSTRACT. In modern society, stress caused by relationships and emotions is one of the greatest social problems. Similar to humans, domestic and captive animals live under various stresses. Several stresses have been associated with skin disorders, such as atopic dermatitis, but there is a lack of reliable and objective indicators for the characterization of this association. This study aimed to define the changes in fatty acid composition and amino acid concentration in the skin following repeated restraint stress in ICR mice. Mice subjected to 30 min of daily restraint stress for 8 days showed changes in the composition of saturated fatty acids, such as an increase in palmitic acid content, which are the substrates of Δ-9 desaturase. Conversely, unsaturated fatty acids decreased with stress treatment, which appeared to be a result of these fatty acids being the substrate of Δ-6 desaturase. Changes in fatty acid composition after stress treatment may be one of the factors that cause skin inflammation. The water-retention capacity may have been lowered by stress treatment because histidine and leucine, which are natural moisturizing factors, were significantly decreased. The collagen content in the skin gradually decreased after repeated stress treatment. Our results indicate that repeated restraint stress may impact skin health through changes in both the fatty acid composition and amino acid concentration in mice.

KEYWORDS: amino acid, collagen, fatty acid, repeated restraint stress, skin

The number of individuals suffering from atopic dermatitis (AD), one of the most common chronic allergic skin inflammatory diseases, is increasing [19]. AD is a common skin disease in dogs [29]. Although the connection between skin diseases such as AD and psychological stress has been explored, there is a lack of reliable and objective indicators for the characterization of this association [24]. Stress has been suggested to negatively affect barrier permeability and homeostasis in the skin [4]. Mental stress has been shown to reduce lipogenesis and delay recovery of the skin barrier [1]. We believe that it is necessary to clarify the relationships between stress and skin conditions to reduce the number of patients with skin diseases.

The skin comprises three layers: the epidermis, dermis, and subcutaneous tissue. The epidermis plays an important role in providing protection from external environmental factors, such as ultraviolet radiation [3]. The outermost layer of the epidermis, the stratum corneum, plays a key role in maintaining skin barrier functions [17]. The stratum corneum stores moisture in the skin and protects the skin from drying and external abrasion.

Three important factors contribute to the barrier function. The first is sebum. The sebum forms a sebaceous membrane, which is a natural protective film that covers the surface of the stratum corneum and prevents the evaporation of water. The second is the presence of intercellular lipids, such as ceramide, cholesterol, and fatty acids, between the stratum corneum cells [2]. Fatty acids are associated with sebum and other intercellular lipids and are essential for maintaining the permeability barrier. Studies have shown that elongation and desaturation of fatty acids are also required for barrier homeostasis [6]. The cutaneous permeability barrier is significantly affected by the lipid composition of the skin [30]. The third factor important for barrier function is the natural moisturizing factor (NMF). NMF contributes greatly to the moisturizing function of the stratum corneum. NMF is composed of free amino acids, organic acids, and mineral salts. Free amino acids that have been reported to be present in NMF are glycine, alanine, serine, proline, threonine, leucine, isoleucine, and histidine [23]. Reduced levels of NMF in the stratum corneum

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have been shown to be associated with more severe AD symptoms [7]. However, the effects of psychological stress on free amino acids in the skin have not been elucidated.

Collagen, irrespective of its origin, contains 19 amino acids, including hydroxyproline, which does not occur in other proteins [9]. Collagen accounts for approximately 70% of the dermis, provides elasticity and firmness to the skin, and keeps it fresh and healthy. Stress can change the quantity and integrity of the skin collagen [15].

The present study aimed to investigate the changes in fatty acid composition and amino acid concentration in the skin following repeated restraint stress. By comprehensively understanding the mechanism by which stress affects the skin, nutrients could be used for the treatment of skin diseases caused by stress. We applied restraint stress as an alternative to social stress in this study, as chronic restraint stress causes physiological and behavioral responses, such as depression [31]. However, physical stress was also loaded simultaneously by this method. Therefore, to reduce the physical effect as much as possible, we adopted a short restraint time of 30 min and gently restrained the subjects using a wire mesh. Furthermore, we investigated the effect of repeated restraint stress on collagen in the skin based on the concentration of hydroxyproline, a collagen-specific amino acid. By clarifying the effect of subchronic stress on skin conditions, we believed that it would be possible to prevent skin diseases caused by chronic stress.

MATERIALS AND METHODS

Animals and stress treatments

ICR mice (male, 7 weeks old) were obtained from Japan SLC. We attempted to eliminate the differences between individuals other than restraint stress as much as possible. Male mice were selected to reduce the effect of sex hormones, as female mice have a short sexual cycle (4–5 days). Mice were kept at 23 ± 1°C under 12 hr dark-12 hr light cycle conditions, housed individually, and had free access to food (MF, Oriental Yeast, Tokyo, Japan) and water. Food intake was monitored for each mouse.

After 7 days of acclimation, the mice were separated into two groups: the 1) control group (n=6 for each day) and 2) stress treatment group (n=6 for each day). The stress treatment group was subjected to a daily stress session for up to 8 days. The mice in this group were wrapped in mesh and fixed in place for 30 min every morning on each sampling day. The control group was left undisturbed in their cages. The mice were euthanized under anesthesia with isoflurane (Escain®, Mylan, Osaka, Japan) immediately after 30 min of stress treatment, and trunk blood and dorsal skin samples were collected. The start date of the stress treatment was defined as 0 day. Sampling was performed four times at 0, 1, 4, and 7 days. The control group was sampled according to a similar schedule. After sampling, blood samples were immediately centrifuged at 3,000 × *g* for 15 min at 4°C (MX-307, Tommy, Tokyo, Japan). Plasma and skin samples were stored at −80°C until analysis.

This study was performed in accordance with the guidelines for animal experiments of the Faculty of Agriculture of Kyushu University and complied with Law No. 105 and Notification No. 6 of the Japanese Government. The registration number of this study permitted by Kyushu University is A30-054-4.

Analysis of corticosterone

Plasma samples were analyzed for total corticosterone concentrations using a corticosterone enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol.

Analysis of fatty acids

Total lipid extraction was performed using the modified Folch method [8]. To 0.5 g of the skin, 6 ml each of methanol and chloroform was added and then the tissue was homogenized. Next, 6 ml of chloroform was added, and the mixture was homogenized again and heated at 40°C for 30 min. The mixture was then cooled to 20–25°C and passed through a filter paper. Then, 2 ml of a mixture of chloroform and methanol (2:1) was poured into the sample, and the mixture was filtered again. After filtration, 3.6 ml of distilled water was added to the filtrate, followed by gentle stirring. After leaving the samples at 4°C overnight, the upper layer was discarded with a Pasteur pipette, and the lower layer was dried under nitrogen gas flow. These residues were used as total lipids. The composition of fatty acids present in the total lipids was analyzed. Fatty acid esterification was performed as described by Kamegai *et al*. [16]. Finally, a fatty acid methyl ester solution dissolved in 100 µl hexane was used for the analysis. The measurement conditions for gas chromatography (GC2025, Shimadzu Co., Kyoto, Japan) were as follows; column: Omegawax 320 (30 m × 0.32 mm × 0.25 µm film), column temperature: 120°C (1 min) →4.0°C/min →205°C (20 min) →4.0°C/min →240°C (2 min) →10.0°C/min →250°C (3 min), inlet: 205°C, detector: 250°C, detector type: FID, carrier gas: helium, sample injection volume: 1 µl, split ratio: 1:20, flow rate: 1 ml/min. This analysis was performed at the Food Science Laboratory, Department of Humanities and Sociology Life and Health Course, Nagasaki University.

The fatty acids identified were C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (n-7) (palmitoleic acid), C18:0 (stearic acid), C18:1 (n-9) (oleic acid), C18:2 (n-6) (linoleic acid), C18:3 (n-6) (γ-linolenic acid), and C18:3 (n-3) (α-linolenic acid).

Analysis of free amino acids

Free amino acid levels in the plasma and skin were analyzed using liquid chromatography-mass spectrometry (LC-MS/MS). Plasma samples were filtered through an ultrafiltration tube (Millipore, Bedford, MA, USA). The skin samples were homogenized in ice-cold 0.2 M perchloric acid solution and left for deproteinization on ice. After 30 min, the mixtures were centrifuged at 20,000 × *g* for 15 min at 4°C and filtered through a 0.20-µm filter. The pH of the filtrate was adjusted to approximately 7.0, with 1 M sodium hydroxide. All the samples were processed on the same day.

The separation and quantification of free amino acids were performed according to a previous report [22]. Product ion spectra were obtained for each compound (Table 1), and the concentration of free amino acids in the skin samples was expressed as pmol/mg of wet tissue.

Table 1. MS/MS conditions in the multiple reaction monitoring transitions employed for the determination of each amino acid

Compound	Precursor ion (m/z)	Cone voltage (V)	Product ion (m/z)	Collison energy (V)
Alanine	90.1	20	44.1	10
Arginine	175.1	24	70.1	24
Aspartic acid	131.9	44	86.0	12
Citrulline	177.0	20	114.1	10
Glutamic acid	148.1	46	84.1	24
Glycine	75.8	10	30.1	10
Histidine	156.1	18	110.1	8
Hydroxyproline	133.9	20	74.1	20
Isoleucine	132.1	44	86.2	10
Leucine	132.1	44	86.2	10
Lysine	147.0	20	84.1	20
Ornithine	133.1	20	74.0	10
Phenylalanine	166.1	38	120.1	10
Proline	116.1	66	70.1	12
Serine	106.1	48	60.2	6
Threonine	120.1	30	74.0	10
Tyrosine	182.1	24	136.3	12
Valine	118.1	60	72.1	16

Analysis of hydroxyproline of collagen

To hydrolyze the skin samples, 500 mg of the samples and 2 ml of 6 N hydrochronic acid were mixed in a glass tube. Hydrochloric acid (HCl) was purchased from Wako (Osaka, Japan). Hydrolysis was then performed for 10 hr using a block heater heated to 120°C. The samples were passed through filter paper (5A 185 mm) and diluted with 20 ml distilled water. Each 1 ml sample was evaporated to dryness, and distilled water was added to reconstitute the samples. The hydroxyproline content was analyzed using LC-MS/MS. The total collagen content of the skin was calculated by multiplying the hydroxyproline content by 7.52 [5], because skin collagen contains 13.3% hydroxyproline.

Statistical analysis

Food intake was analyzed using repeated measures two-way analysis of variance (ANOVA) test. The plasma corticosterone concentration, fatty acid composition, amino acid concentration, and collagen content were analyzed using a two-way factorial ANOVA test. The Tukey–Kramer test was applied when the effects of days as well as interactions were significant ($P<0.05$). The analysis was performed using StatView version 5 (SAS Institute, Cary, NC, USA, 1998). Outlier data were rejected at $P<0.01$ by Thompson's test criteria for outlier observations.

RESULTS

Corticosterone concentrations in plasma

On all days, significantly ($P<0.0001$) higher corticosterone concentrations than those in the control group were induced by stress treatment (Fig. 1A).

Food intake

Although some changes were observed depending on the day, no significant difference in food intake was detected between the control and stress treatment groups (Fig. 1B).

Effect of repeated restraint stress on the individual fatty acid composition

The effects of repeated restraint stress on the composition of individual fatty acids are shown in Fig. 2. Significant effects of days of treatment ($P<0.01$) and stress ($P<0.01$) on the levels of palmitic acid (Fig. 2B) were detected. A significant ($P<0.05$) interaction between stress and days suggested that the palmitic acid content gradually increased when the stress treatment was prolonged. The oleic acid content significantly changed with the days of treatment (Fig. 2D). Although not significant, the change in oleic acid due to stress treatment had a P-value very close to 0.05 ($P=0.0567$). Significant effects of the days of treatment on the levels of myristic acid (Fig. 2A) and palmitoleic acid (Fig. 2C) were detected, suggesting that they increased over time. However, a reverse was observed for linoleic acid (Fig. 2E). γ-Linolenic acid levels fluctuated significantly between consecutive days (Fig. 2F).

Effect of repeated restraint stress on saturated fatty acid and unsaturated fatty acid composition and the involvement of desaturases

The changes in saturated and unsaturated fatty acids are shown in Fig. 3. The stress treatment group showed higher total levels of saturated fatty acids (Fig. 3A). These changes were almost explained by the changes in palmitic and stearic acid, which were both desaturated by Δ-9 desaturase (Fig. 3E). However, the stress treatment group showed lower levels of total unsaturated fatty acids (Fig. 3B). These changes were also explained by the changes in the total levels of palmitoleic, oleic, linoleic, and α-linolenic acid, which were desaturated by Δ-6 desaturase (Fig. 3F).

Monounsaturated fatty acids changed over time (Fig. 3C). The total content of polyunsaturated fatty acids with two double bonds significantly decreased over time (Fig. 3D).

Effect of repeated restraint stress on individual amino acid and NMF concentrations

The changes in the free amino acids are shown in Table 2. Significant changes were found for the effect of days on the levels of arginine, aspartic acid, citrulline, glutamic acid, glycine, isoleucine, ornithine, phenylalanine, proline, threonine, tyrosine, and

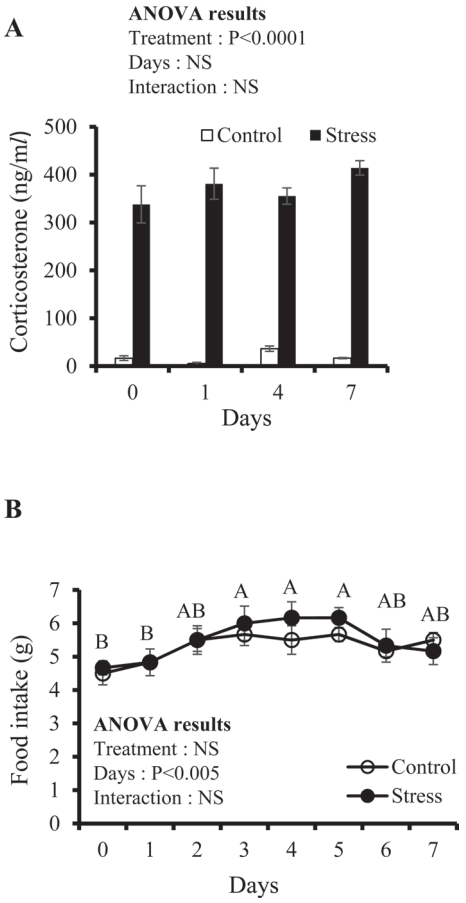

Fig. 1. (**A**) Effect of repeated restraint stress on plasma corticosterone concentration. Values are expressed as means ± SEM; n=6 in each the control and stress groups (each day). ** $P<0.0001$ vs. Control. (**B**) Effect of repeated restraint stress on food intake. Values are expressed as means ± SEM. The number of mice in both groups was 24 (day 0), 18 (day 1), 12 (days 2–4), and 6 (days 5–7). Different letters (A–C) among days indicate significant differences at $P<0.05$. NS, not significant.

valine. The difference in the valine content between days could not be clarified by the Tukey–Kramer test, but the values tended to be higher on days 1 and 4. However, no significant effects of stress treatments and interactions between stress treatments and days were detected for these amino acids. Histidine, leucine, and lysine changed with time, and the stress treatment induced low concentrations of these amino acids over time. The effects of days and interaction between stress treatment and days were significant for alanine, serine, and NMF, suggesting that the levels of the amino acids were increased by stress treatment on day 1, and the reverse was almost true for the responses on day 4.

Effect of repeated restraint stress on hydroxyproline and collagen concentrations

Figure 4A–D shows the effect of repeated restraint stress on the (A) free hydroxyproline, (B) total hydroxyproline, and (C) collagen contents, and (D) the free hydroxyproline ratio (free/total). The free hydroxyproline content (Fig. 4A) and ratio (Fig. 4D) changed over time, and low values for the stress treatment were maintained during the experiment. Significant interactions between stress and days for both parameters were due to the difference on day 1. Significant ($P<0.01$) effects of days and significant ($P<0.05$) interactions between stress and days were confirmed for the collagen content (Fig. 4C), and this response was similar to that of total hydroxyproline (Fig. 4B). The responses between stress and days were not constant. No significant effects of the stress treatment were observed.

DISCUSSION

Although significant changes in the plasma corticosterone levels were observed, food intake did not change between the two groups in this study. ICR mice loaded daily with restraint stress for 2 hr had significantly reduced food intake from the day after the start of the stress load to the sixth day [13]. Therefore, the repeated restraint stress loaded here may be a mild stress model.

This study evaluated whether repeated restraint stress affected the fatty acid composition and amino acid concentration in the skin. Corticosterone is a type of glucocorticoid secreted by the adrenal cortex, and its secretion is increased during periods of stress, making it a reliable indicator of stress. We found that the corticosterone concentration was significantly increased by stress treatment, suggesting that stress was properly loaded (Fig. 1A). Although the fatty acid composition and amino acid concentration of the skin can be affected by dietary ingredients and/or the amount of food consumed, food intake between the control and stress treatment groups was not significantly different (Fig. 1B). Thus, changes in fatty acid composition and amino acid concentration in this experiment were most likely caused by the stress response rather than diet.

We found an increase in the total saturated fatty acids and a decrease in the total unsaturated fatty acids after stress treatment. Saturated fatty acids, such as palmitic acid, do not have direct pro-inflammatory effects but have been found to amplify the immune response to exogenous stimuli [12]. Previous studies have also shown that mental stress increases the levels of saturated fatty acids and reduces the antimicrobial barrier of the skin [14]. These findings suggest that an increase in the level of palmitic acid or saturated fatty acids is one of the factors causing inflammation in the skin. In contrast, oleic acid exerts anti-inflammatory effects by reducing the levels of cytokines, including interleukin (IL)-6 and tumor necrosis factor-α, while increasing the levels of the anti-inflammatory cytokines IL-10 and adiponectin. Oleic acid prevents palmitic acid-induced inflammation [27]. Therefore, it has been suggested that increasing the level of palmitic acid may correspond with a decrease in the level of oleic acid, and this decrease may cause weakening of the skin barrier. We considered two possible causes for this dynamic. First, the unsaturation could be inhibited owing to the stress load. Second, when stress is loaded, catabolic pathways that produce energy from fat accumulated in the body are promoted to generate energy. In our study, the corticosterone concentration in the stress group increased, which should have promoted catabolism. Because we found that saturated fatty acids increased and unsaturated fatty acids decreased in the skin, it may be that unsaturated fatty acids were utilized in catabolism. The recruitment of fatty acids from white adipocytes has been shown to be selective, and unsaturated fatty acids are mobilized more than saturated fatty acids [28]. Significant effects of stress treatment were also found to increase the total levels of palmitic acid and stearic acid, which are desaturated by Δ-9 desaturase, and to decrease the total levels of palmitoleic acid, oleic acid, linoleic acid, and α-linoleic acid, which are desaturated by Δ-6 desaturase. These results indicate that stress treatment may decrease the activity of Δ-9 desaturase and increase the activity of Δ-6 desaturase. Stearoyl-CoA desaturase 1 (SCD1) is a family of enzymes

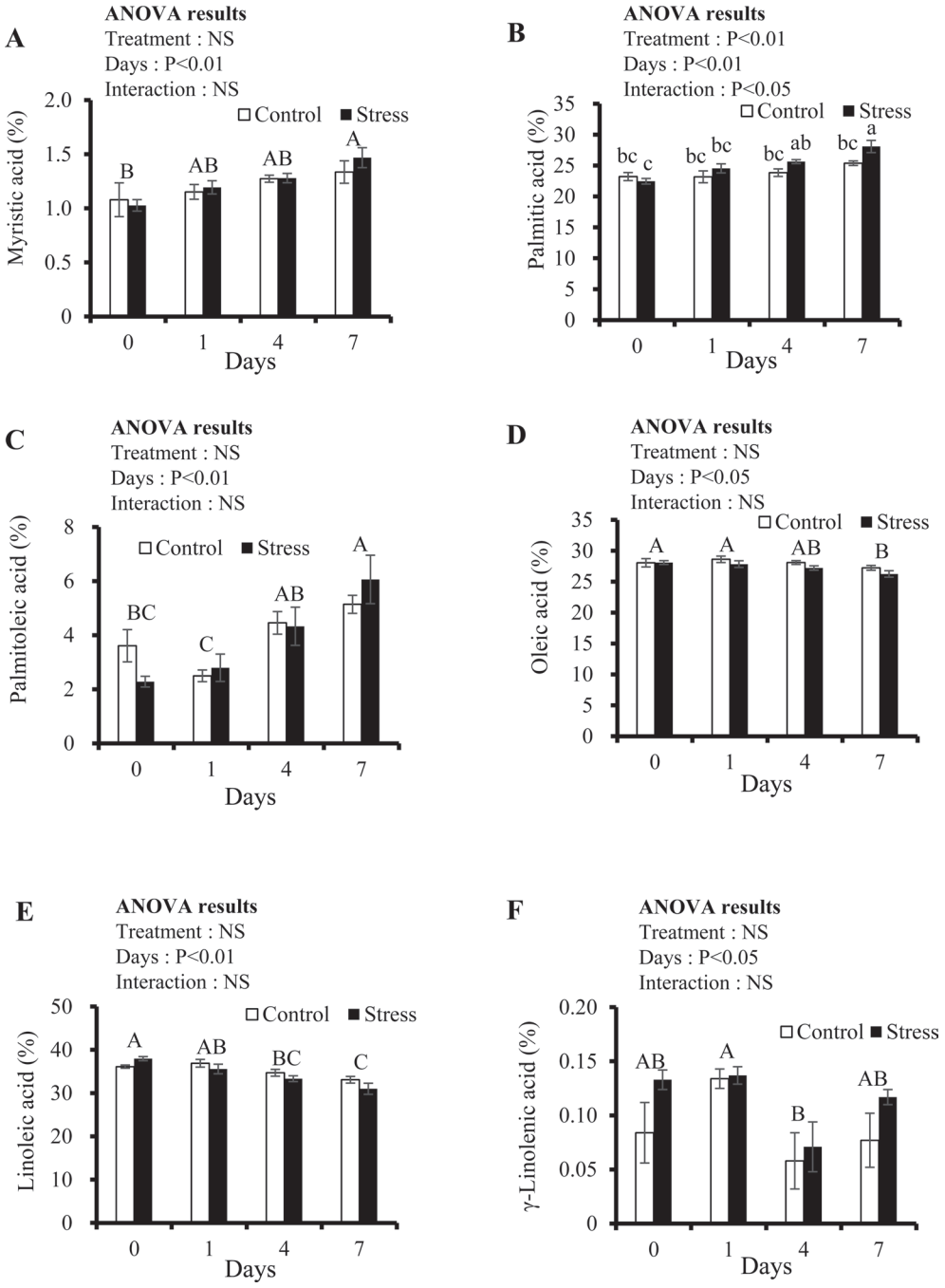

Fig. 2. Effects of repeated restraint stress on individual fatty acid composition. Values are expressed as means ± SEM; n=6 in each the control and stress groups (each day). Different letters (A–C) among days indicate significant differences at $P<0.05$. Different letters among values (a–c) indicate significant differences at $P<0.05$. NS, not significant.

that are Δ-9 fatty acid desaturases. SCD1 has emerged as a regulator of inflammation and stress-related diseases [21]. Based on the involvement of desaturase in skin diseases, it has been suggested that fatty acid metabolism-related genes, such as desaturase, may be altered by stress treatment, and these changes lead to dermatitis. In addition, the fatty acid composition of the skin can be easily modified by changing the dietary fat sources [25, 26]. Considering that stress may change the fatty acid composition of the skin, it is suggested that, in times of stress, attention should be paid to the composition of fatty acids in the diet. For many fatty acids, significant changes with days were confirmed, but the cause was not clear. The changes in the fatty acid composition confirmed in this experiment were relatively small. Further research is needed in the future, such as extending the period and applying another type of stress.

We found that the concentrations of several amino acids in the skin were influenced by stress treatment. The histidine, leucine, and lysine levels decreased significantly over the eight days. Histidine and leucine are elements of NMF, which suggests that the

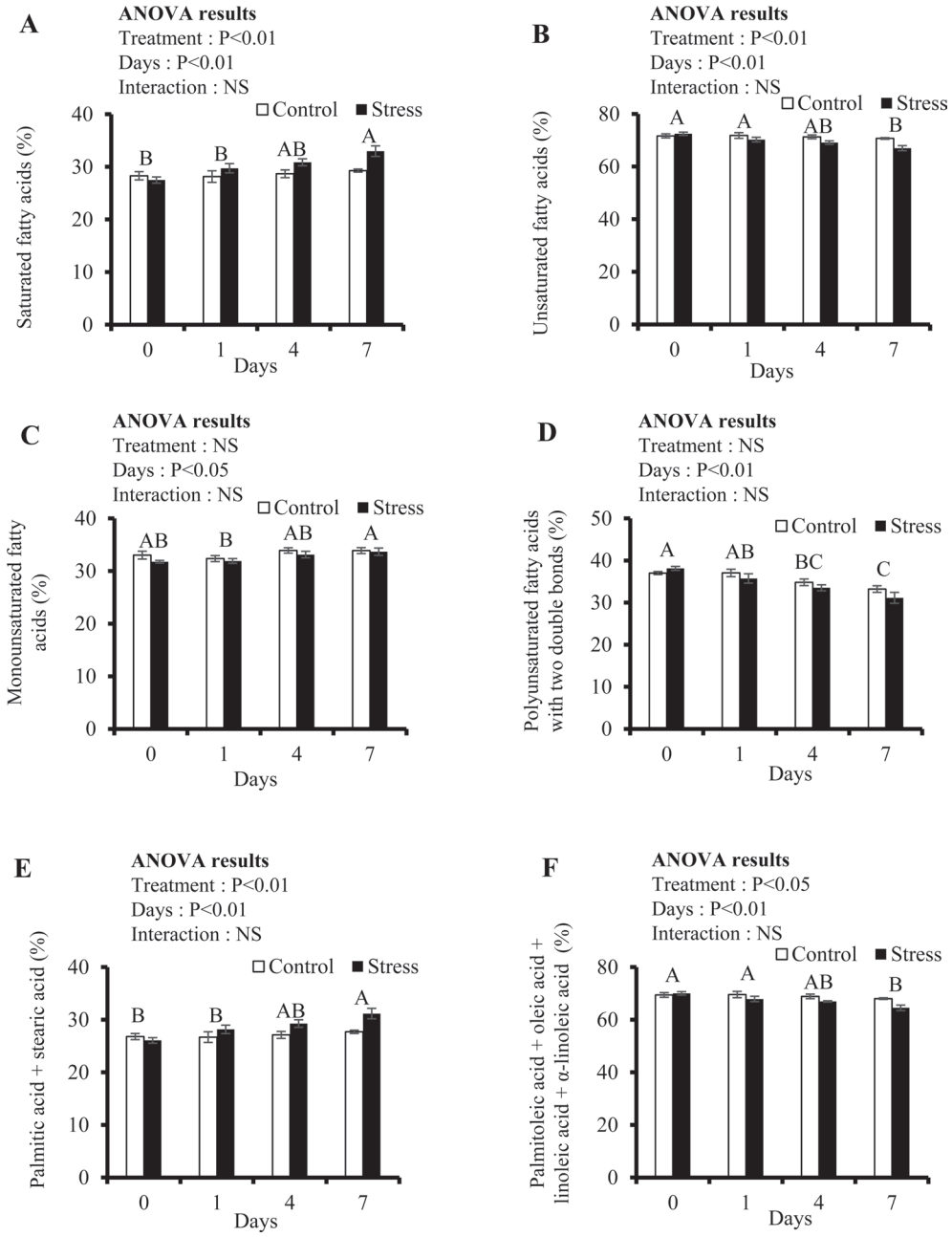

Fig. 3. Effects of repeated restraint stress on fatty acid composition. (**A**) Saturated acids. (**B**) Unsaturated fatty acids. (**C**) Monounsaturated fatty acids. (**D**) Polyunsaturated fatty acids with two double bonds. (**E**) Total fatty acids that are desaturated by Δ-9 desaturase. (**F**) Total fatty acids that are desaturated by Δ-6 desaturase. Values are expressed as means ± SEM; n=6 in each the control and stress groups (each day). Different letters (A–C) among days indicate significant differences at $P<0.05$. NS, not significant.

water-retention capacity of the skin may be lowered by stress treatment. Histidine supplementation would be a safe approach to augment the NMF, enhance the skin barrier function, and reduce the AD severity [10]. Therefore, it was considered that the decrease in histidine may have had an adverse effect on the skin. Sestrin2 is a leucine sensor for mTOR complex 1, which is a master growth controller [33]. When the leucine concentration is high in the presence of Sestrin2, wound healing is improved [20]. If leucine increases with stress treatment, it may be helpful to heal wound inflammation caused by stress loading on the skin. However, in the present study, leucine levels were decreased by stress treatment, which might impair wound healing. Moreover, leucine and lysine produce acetyl-CoA when degraded. Because acetyl-CoA is also used for fatty acid biosynthesis, it is possible that leucine and lysine are catabolized to allow for the biosynthesis of fatty acids. The levels of alanine, serine, and NMF were altered over the course of the experiment, and the effect of stress was altered depending on the day of treatment. Alanine and serine are also elements of NMF. Therefore, altering the levels of alanine and serine may also affect skin moisture. Taken together, our data suggest that animals under stressful conditions may require additional amino acid supplementation, such as histidine

Table 2. The effects of chronic psychological stress on individual amino acids and natural moisturizing factor (NMF) in the dorsal skin

Amino Acids (μmol/g)	Day 0		Day 1		Day 4		Day 7	
	Control (n=6)	Stress (n=6)	Control (n=6)	Stress (n=6)	Control (n=6)	Stress (n=6)	Control (n=6)	Stress (n=6)
Alanine	1.37 ± 0.11^{b}	1.50 ± 0.16^{ab}	1.35 ± 0.02^{b}	1.93 ± 0.14^{a}	1.48 ± 0.16^{ab}	1.25 ± 0.05^{b}	1.12 ± 0.09^{b}	1.15 ± 0.11^{b}
Arginine	0.341 ± 0.024 AB	0.410 ± 0.047	0.507 ± 0.079 A	0.519 ± 0.070	0.381 ± 0.078 B	0.210 ± 0.009	0.305 ± 0.015 B	0.223 ± 0.022
Aspartic acid	0.368 ± 0.047 A	0.403 ± 0.060	0.499 ± 0.020 A	0.534 ± 0.068	0.181 ± 0.068 B	0.068 ± 0.005	0.094 ± 0.040 B	0.619 ± 0.026
Citrulline	0.235 ± 0.023 B	0.207 ± 0.012	0.239 ± 0.026 B	0.289 ± 0.039	0.480 ± 0.059 A	0.403 ± 0.054	0.393 ± 0.044 A	0.454 ± 0.041
Glutamic acid	1.86 ± 0.16 B	2.24 ± 0.28	2.09 ± 0.17 AB	2.54 ± 0.11	3.31 ± 0.27 A	2.55 ± 0.10	2.00 ± 0.15 B	2.11 ± 0.22
Glycine	1.48 ± 0.12 AB	1.57 ± 0.24	1.50 ± 0.10 A	1.95 ± 0.26	1.39 ± 0.10 BC	1.20 ± 0.07	1.00 ± 0.04 C	0.872 ± 0.057
Histidine	0.115 ± 0.010 AB	0.115 ± 0.018	0.143 ± 0.010 A	0.145 ± 0.016	0.120 ± 0.021 BC	0.065 ± 0.006	0.078 ± 0.013 C	0.044 ± 0.003
Isoleucine	0.134 ± 0.022 B	0.179 ± 0.040	0.280 ± 0.030 A	0.292 ± 0. 041	0.195 ± 0.059 BC	0.061 ± 0.031	0.068 ± 0.032 C	0.008 ± 0.004
Leucine	0.315 ± 0.028 AB	0.350 ± 0.051	0.496 ± 0.043 A	0.427 ± 0.033	0.390 ± 0.091 B	0.203 ± 0.044	0.272 ± 0.046 B	0.187 ± 0.042
Lysine	0.573 ± 0.040 AB	0.566 ± 0.057	0.773 ± 0.089 A	0.620 ± 0.021	0.596 ± 0.079 B	0.425 ± 0.008	0.466 ± 0.031 B	0.398 ± 0.020
Ornithine	0.151 ± 0.007 A	0.152 ± 0.015	0.187 ± 0.014 A	0.183 ± 0.021	0.090 ± 0.020 B	0.039 ± 0.004	0.063 ± 0.015 B	0.036 ± 0.005
Phenylalanine	0.178 ± 0.014 AB	0.203 ± 0.030	0.261 ± 0.020 A	0.239 ± 0.018	0.223 ± 0.040 B	0.126 ± 0.016	0.143 ± 0.016 B	0.122 ± 0.015
Proline	0.349 ± 0.027 AB	0.379 ± 0.066	0.431 ± 0.016 A	0.521 ± 0.058	0.532 ± 0.062 A	0.374 ± 0.039	0.303 ± 0.033 B	0.298 ± 0.038
Serine	1.13 ± 0.10^{abc}	0.96 ± 0.09^{bc}	1.14 ± 0.04^{abc}	1.54 ± 0.24^{a}	1.48 ± 0.09^{ab}	1.07 ± 0.08^{abc}	0.94 ± 0.10^{bc}	0.91 ± 0.08^{c}
Threonine	0.476 ± 0.031 AB	0.478 ± 0.060	0.561 ± 0.032 A	0.595 ± 0.050	0.484 ± 0.057 BC	0.347 ± 0.024	0.387 ± 0.036 C	0.322 ± 0.025
Tyrosine	0.233 ± 0.004 B	0.241 ± 0.028	0.290 ± 0.022 A	0.313 ± 0.034	0.233 ± 0.034 BC	0.175 ± 0.016	0.166 ± 0.013 C	0.164 ± 0.014
Valine	0.562 ± 0.054	0.547 ± 0.064	0.626 ± 0.014	0.746 ± 0.072	0.853 ± 0.068	0.583 ± 0.083	0.538 ± 0.085	0.509 ± 0.079
NMF	5.37 ± 0.40^{ab}	5.54 ± 0.68^{ab}	5.85 ± 0.15^{ab}	7.46 ± 0.79^{a}	6.00 ± 0.64^{ab}	4.53 ± 024^{b}	4.26 ± 0.38^{b}	3.81 ± 0.33^{b}

Different letters (A–C) among days indicate significant differences at $P<0.05$. Different letters among each value (a–c) indicate significant differences at $P<0.05$. The results of the ANOVA are shown in Supplementary Table 1.

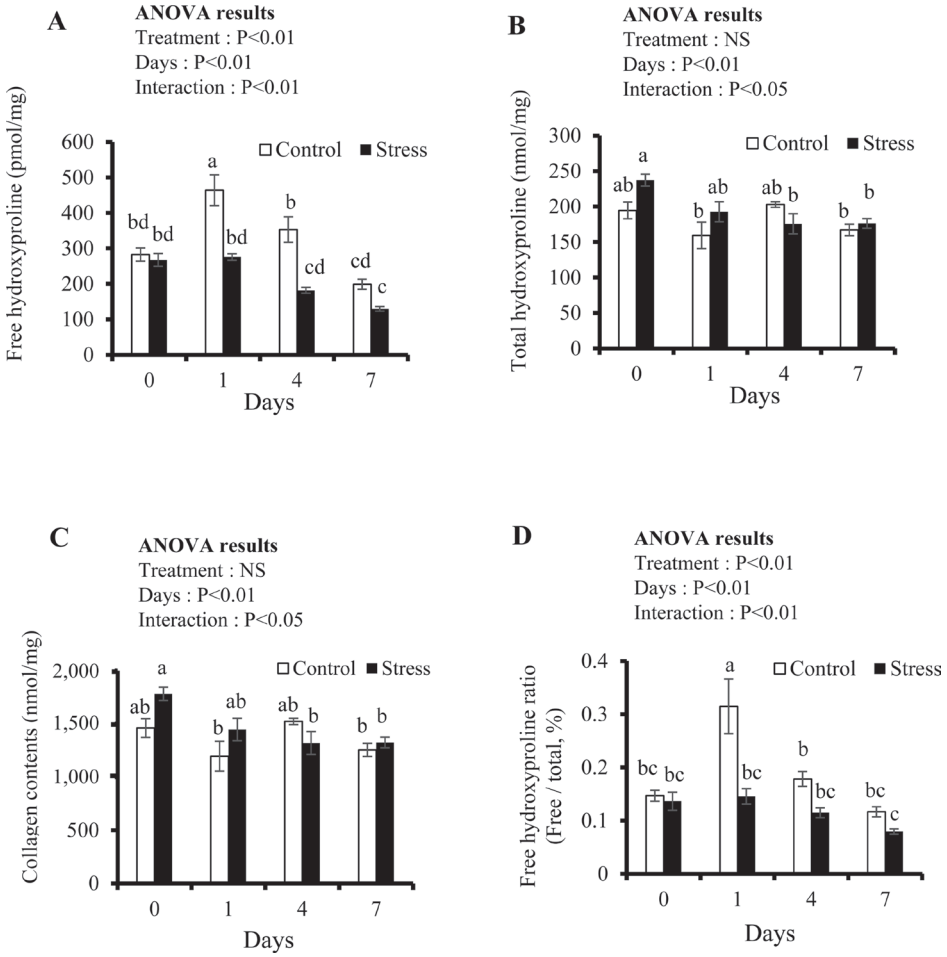

Fig. 4. The effects of repeated restraint stress on hydroxyproline and collagen. (**A**) Free hydroxyproline. (**B**) Total hydroxyproline. (**C**) Collagen contents calculated from hydroxyproline as collagen constituents. (**D**) Free hydroxyproline ratio (Free hydroxyproline/total hydroxyproline) in the skin. Values are expressed as means ± SEM in pmol/mg and nmol/mg wet tissue; n=6 in each the control and stress groups. Different letters (A–C) among days indicate significant differences at $P<0.05$. Different letters among values (a–c) indicate significant differences at $P<0.05$. NS, not significant.

and leucine, to protect the skin. Significant changes with days were confirmed for all amino acids, but the cause was not clear, as mentioned for fatty acids.

The collagen contents (Fig. 4C) were highest on day 0 in the stress group and subsequently decreased significantly over time. This result suggests that the collagen content of the skin was decreased by repeated restraint stress in the stress group. The decrease in the collagen levels in the stress treatment group may have been caused by decreased protein synthesis resulting from stress-induced catabolism. Vitamin C is an essential nutrient for the biosynthesis of collagen through the hydroxylation of proline [18]. In addition, the vitamin C concentrations in the plasma and leukocytes rapidly decline during infection and stress [32]. Therefore, it may be that vitamin C levels were decreased by repeated restraint stress and that collagen could not be fully synthesized. A decrease in the amount of collagen leads to a decrease in skin firmness and elasticity. Therefore, it is possible that the firmness and elasticity of the skin would be reduced by repeated restraint stress for a longer period of time. Significant decreases in free hydroxyproline in the skin were observed in response to stress treatment over time (Fig. 4A). Free hydroxyproline is released into the amino acid pool in the body through the breakdown of collagen. Although the amount of collagen (Fig. 4C) was higher or unchanged in the stress treatment group compared to the control group, the amount of free hydroxyproline was significantly lower in the stress treatment group regardless of day. In addition, we determined the free hydroxyproline ratio in the skin and found that it changed significantly with time and decreased with stress treatment (Fig. 4D). We consider this result to be an indicator of the amount of catabolized collagen. However, the results of this experiment only represented the concentration of free hydroxyproline and did not consider the amount of oxidized hydroxyproline. From these results, we considered that the amount of degraded collagen was also reduced due to the reduced amount of collagen by repeated restraint stress treatment, and free hydroxyproline resulting from collagen degradation in the amino acid pool was used more for stress-promoted energy supply in stress treatment groups. When hydroxyproline is metabolized, it is converted to pyruvate. Pyruvate drives ATP production via oxidative phosphorylation and multiple biosynthetic pathways intersecting the citric acid cycle [11]. Based on these findings, it is possible that free hydroxyproline is metabolized to pyruvate for energy production under stressful conditions.

In conclusion, this study suggested that repeated restraint stress may modify the fatty acid composition and amino acid concentration of the skin and may reduce the barrier function of the stratum corneum. It is also suggested that repeated restraint stress may lower the amount of collagen and reduce the firmness and elasticity of the skin.

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest.

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