



# Changes in skin structure of the *Zip13*-KO mouse by Makomo (*Zizania latifolia*) feeding

Takeshi YAMAUCHI<sup>1,2)</sup>, Takuya HIROSE<sup>1)\*</sup>, Kaho SATO<sup>1)</sup>, Kenji IWAI<sup>3)</sup>,  
Naoki TAKAHASHI<sup>1)</sup>, Jun MINAGUCHI<sup>1)</sup>, Takeshi UENO<sup>4)</sup>,  
Prasarn TANGKAWATTANA<sup>5)</sup> and Kazushige TAKEHANA<sup>1)</sup>

<sup>1)</sup>Laboratory of Microanatomy, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

<sup>2)</sup>The Maple Foundation, Chuou-ku, Tokyo 103-0014, Japan

<sup>3)</sup>Japan Institute of Leather Research, Toride, Ibaraki 302-0017, Japan

<sup>4)</sup>Laboratory of Mathematics, School of Food Science and Human Wellness, Rakuno Gakuen University Ebetsu, Hokkaido 069-8501, Japan

<sup>5)</sup>Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand

**ABSTRACT.** Ehlers-Danlos syndrome (EDS) is a group of disorders caused by abnormalities in the extracellular matrix (ECM). Transforming growth factor- $\beta$  (TGF- $\beta$ ) plays a crucial role in formation of the ECM by the SMAD (Sma-and Mad-related protein, mothers against decapentaplegic homolog) pathway. It has been reported that loss of function of zinc transporter ZRT/IRT-like protein 13 (ZIP13) is the cause of the spondylocheiro dysplastic form of EDS (SCD-EDS: OMIM 612350). Our previous study suggested that TGF- $\beta$ 1 has a relationship with the skin pathological condition in the *Zip13*-Knockout (KO) mouse, which is a model of SCD-EDS. Thus far, effective treatment based on modern medicine for this syndrome has not yet been established. According to an approach of traditional Chinese medicine, the present study investigates the medicinal effects of Makomo (*Zizania latifolia*) on certain aspects of SCD-EDS, such as skin morphology and plasma TGF- $\beta$ 1, in *Zip13*-KO mice. Increases in densities of collagen fibers and fibrils without a significant change in thickness of the dermal layer were observed in the group of mice fed a Makomo-containing diet. No change in the amount of collagen suggests that Makomo feed does not elevate collagen synthesis, but changes the length of glycosaminoglycan chains and decreases the distance between collagen fibrils. In conclusion, the changes of the skin structure suggest that Makomo can increase the mechanical strength of skin.

**KEY WORDS:** Chinese medicine, collagen fiber, Makomo, skin structure, *Zip13*-KO mouse

*J. Vet. Med. Sci.*

79(9): 1563–1568, 2017

doi: 10.1292/jvms.17-0206

Received: 16 April 2017

Accepted: 20 July 2017

Published online in J-STAGE:  
4 August 2017

Ehlers-Danlos syndrome (EDS) is a group of intractable diseases caused by abnormality of extracellular matrix (ECM) components, such as collagen. Characteristics of this syndrome include hyperextension of the skin, hypermobility of joints and fragility of blood vessels [1, 3]. Thus, EDS patients are likely to have easily cracked skin, slow wound healing and bleeding [3]. Based on symptoms and causes, EDS cases are categorized into six major types: classical, hypermobility, vascular, kyphoscoliosis, arthrochalasia and dermatosparaxis types [3, 12]. The spondylocheiro dysplastic form of EDS (SCD-EDS: OMIM 612350) has become recognized as a new EDS type after mutation in SLC39A13/ZIP13 (ZIP13) was found [7]. Our previous study suggested that transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) has a relationship with the skin pathological condition in the *Zip13*-knockout (KO) mouse, which is a model of SCD-EDS [10].

Although lacking effective modern therapeutics, treatments of EDS using traditional Chinese medicine (TCM) become promising alternative approaches for this ailment [24]. Upon the Basic Theories of TCM [21], kidney, liver and spleen are indicated to be responsible organs for the major EDS symptoms, including congenital abnormalities of the skeleton and cartilage, skin fragility, dental hypoplasia, deep-set eyeballs and growth retardation. Accordingly, “essence” stored in the kidneys is a fundamental substance to maintain good conditions of teeth, bones and marrow for the well-beings of body condition, vital activity, growth and reproduction, and familial characteristics [17]. Liver is believed to be the organ that governs nails, tendons and eyes. Its functions are ascribed in the capability to dynamical movement, “Flow of qi” and balanced distribution of “qi” throughout the body [16]. Moreover, the essential substances and acquired qi (alimentary qi) that regulate the transportation and transformation

\*Correspondence to: Hirose, T.: s20903053@g.rakuno.ac.jp

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**Table 1.** Nutritional composition of diets for the *Zip13*-KO mice in the control and Makomo feeding (MF) groups. The diet for the MF group contains lower energy and higher carbohydrate and fiber

Component	Control	MF
Energy (kcal)	329.1	260.4
Crude protein (g)	18.8	19.0
Crude fat (g)	3.9	3.3
Crude carbohydrate (g)	54.7	39.3
Crude fiber (g)	6.6	15.9

(digestion and absorption) of nutrients to maintain vital energy after birth are believed to be generated by spleen [15]. The organs and tissues being under the regulation of spleen are muscles and limbs [15]. Thus, promoting healthy conditions of these organs leading to the curation of their various disorders are one of the most important principles of TCM.

*Zizania latifolia*, also known as Munchurian wild rice, is a native perennial gramineous plant of Asia. Its stem, root, leave and grain are suitable for human consumption either as food, supplement or medicine. In the TCM approaches, all parts of this plant have been used as medicines for promoting good health, treating various disorders of kidney, liver and spleen, inhibiting lipid accumulation in the liver, diuresis, febrifuge and preventing arterogenesis [9, 11, 20]. Moreover, leaf of this wild rice is dried, blended and used as a sole raw material of Makomo which has been used wildly as supplements for the promotion of good and balance flow of qi and essence in human. Upon the TCM theory, major clinical symptoms of SCD-EDS mentioned above could be counted in the medicinal spectrum of Makomo. Thus, the present study gave Makomo-containing feed to *Zip13*-KO mice having SCD-EDS with aims to investigate the effects of Makomo on major aspects of SCD-EDS, such as skin morphology and plasma TGF- $\beta$ 1.

## MATERIALS AND METHODS

### *Animals and experimental design*

The animal experiments were approved by the Committee on the Ethics of Animal Experiments of Rakuno Gakuen University (#VH15A6, June 15, 2015). Six *Zip13*-KO mice were weaned at 4 weeks of age and equally divided into two groups: a control group and Makomo feeding (MF) group. The control group was fed a 100% basic powder diet (MR Stock, Oriental Yeast Co., Ltd., Tokyo, Japan), and the MF group was fed a diet of 30% Makomo (MACOMO Co., Ltd., Tokyo, Japan) + 70% basic powder diet (Table 1). At the age of 12 weeks, all animals were euthanized by exsanguination after being anesthetized by an intraperitoneal administration of 65 mg/kg pentobarbital (Somnopentyl®, Kyoritsu Pharmaceutical, Tokyo, Japan). Blood samples were collected by cardiac puncturing for analysis of plasma TGF- $\beta$ 1. Immediately after the euthanasia, pre-scapular and dorsal skin samples were collected for the analyses of morphology (light and electron microscopy with osmium tetroxide or cupromeronic blue staining), glycosaminoglycan (GAG) and amino acid.

### *Light microscopy*

Samples were fixed in Bouin's fixative for 24 hr at room temperature. After dehydration through an ethanol and xylol series, the samples were embedded in paraffin. Eight- $\mu$ m-thick sections were cut perpendicularly in the epidermal-dermal direction and finally stained with hematoxylin and eosin. Dermal thickness was measured at 5 points from 8 areas in each sample. In addition, the number of all cell per 0.01 mm<sup>2</sup> was counted.

### *Transmission electron microscopy (TEM)*

Fresh skin samples with a dimension of 0.5 × 0.5 × 0.5 mm were fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr at room temperature. The samples were post-fixed in 1.0% osmium tetroxide in 0.1 M phosphate buffer for 1 hr at room temperature and then washed with distilled water. Another fresh skin samples were cut into sections with approximately 1 mm in thickness, and immediately fixed and stained with staining buffer containing 0.05% (w/v) cupromeronic blue, 0.1 M MgCl<sub>2</sub>, 25 mM sodium acetate (pH 5.8) and 2.5% glutaraldehyde (w/v) for 5 days at 4°C. The specimens were further incubated with 0.034 M Na<sub>2</sub>WO<sub>4</sub> for one hr at room temperature [19]. Both groups of samples were then processed similarly through dehydration in a graded ethanol series and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Sections of approximately 60 nm in thickness were cut with a Reichert Supernova system (Leica, Vienna, Austria) equipped with a diamond knife. The sections were mounted on a copper grid and stained with 0.2% tannic acid + 10% ethanol in water for 15 min and then with 1.0% uranyl acetate for 5 min and 1.0% lead citrate for 10 sec, consecutively. A TEM (JEM-1220; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV was used for the investigation. Diameters of 500 collagen fibrils randomly selected from each sample with osmium tetroxide were measured. Collagen Fibril Index (CFI, ratio between collagen's square surface and the sum of collagen and non-collagen's square surfaces) in the extracellular matrix was analyzed by Image J software (version 1.48v; National Institutes of Health, Bethesda, MD, U.S.A.). Length of GAG chain was measured in the samples with cupromeronic blue staining. The analysis was also performed by Image J software.

### *Analysis of GAG*

Frozen samples were cut into small blocks on ice. After dehydration and degreasing by stirring in acetone 3 times (30 min

each time) at room temperature and then in diethyl ether 3 times (30 min each time) at room temperature, dry weight of these samples was determined. Removal of GAG from proteoglycan core proteins was performed by stirring the samples in 20 volumes of 0.5 N NaOH at 4°C for 15 hr. Neutralization was performed in 1 N HCl with a similar condition. Proteins in the mixture were denatured by heating at 100°C for 10 min. pH of the mixture was adjusted to 8.0 with 1 M Tris-HCl buffer (pH 7.8) prior to being digested with 1 mg/ml pronase (actinase E; Seikagaku Kougyo, Tokyo, Japan) at 50°C for 24 hr. Trichloroacetic acid was then added to a final concentration of 10%. After incubating for 1 hr, the mixture was centrifuged at 1,600 ×g for 15 min in order to remove the precipitated proteins, and the supernatant was dialyzed against distilled water at 4°C for 3 days. The dialyzed sample was freeze-dried and subjected to two-dimensional electrophoresis on a cellulose acetate membrane. GAG was stained with a solution containing 0.1% alcian blue 8GX (Sigma, St. Louis, MO, U.S.A.) and 0.1% acetic acid. GAG content was quantified by an assay for hexosamine. In this study, only hyaluronic acid (HA) and dermatan sulfate (DS) were examined. HA, DS, heparin and chondroitin-6-sulphate (Nacalai Tesque, Kyoto, Japan) were used as GAG standards.

#### *Analysis of amino acid*

After the addition of 6 N HCl, skin samples were punched using a trephine with a diameter of 4 mm and treated in a heat block at 110°C for 24 hr. The solvent was evaporated in an evaporator. The remaining amino acid was dissolved in 0.02 M HCl. The solution obtained from the filtration was analyzed for amino acid contents. The amount of collagen per unit area was calculated from the amount of hydroxyproline.

#### *Plasma concentration of TGF-β1*

Fresh blood samples were immediately placed in ice for 10–60 min before being centrifuged in a refrigerated centrifuge (4°C) at 1,000 ×g for 30 min for plasma collection. The plasma samples were stored at –80°C. A TGF-β1 assay kit (Quantikine Mouse/Rat/Porcine/Canine; R & D Systems, Minneapolis, MN, U.S.A.) was used for this purpose.

#### *Statistical analysis*

All of the data were initially tested for the normal distribution using Kolmogorov-Smirnov test. Statistical significance in diameter of collagen fibrils and plasma concentration (TGF-β1) was determined at  $P < 0.05$ . Differences in median diameters of collagen fibrils and plasma concentration (TGF-β1) between groups were tested by Mann-Whitney *U* test (non-parametric). The results were determined statistically significant when  $P < 0.05$ . Student's *t*-test (parametric) was applied to determine the differences among the means of thickness of dermis, density of cells, CFI, length of GAG chain, ratio of GAG and amount of collagen at a significance level of  $P = 0.05$ .

## RESULTS

#### *Skin morphology and collagen fibril index*

In the control group, rough collagen fibers were present predominantly in the reticular dermis (Fig. 1). The dermal thickness in the control group was  $142.2 \pm 11.18 \mu\text{m}$  (mean  $\pm$  standard error,  $N=3$ ), while that in the MF group was  $151.2 \pm 6.86 \mu\text{m}$  (Table 2). Density of the cellular population in the control group was  $32.4 \pm 1.77$  cells/ $0.01 \text{ mm}^2$ , and that in the MF group was  $34.3 \pm 2.40$  cells/ $0.01 \text{ mm}^2$  (Table 2). There was no significant difference between the two groups in dermal thickness ( $P=0.54$ ) or density of the cellular population ( $P=0.57$ ).

Spaces between bundles of collagen fibers were apparent in the control group. However, such spaces were very limited in the MF group (Fig. 1). Median diameters of the collagen fibrils were  $119.9 \pm 3.08 \text{ nm}$  in the control group and  $111.7 \pm 4.73 \text{ nm}$  in the MF group (Table 2). There was no significant difference between median diameters of the collagen fibrils in the two groups ( $P=0.4$ ). Collagen Fibril Index in the control group ( $66.0 \pm 0.59\%$ ) and that in the MF group ( $67.9 \pm 0.50\%$ ) were significantly different ( $P=0.006$ ) (Table 2).

#### *Length of GAG chains*

We observed GAG chains of decorin in the skin samples stained with cupromeronic blue stain for GAGs of proteoglycans. The GAG chains bound collagen fibrils into collagen fiber. The length of GAG chain in the control group was  $36.5 \pm 9.59 \text{ nm}$ , while that in the MF group was  $14.2 \pm 2.43 \text{ nm}$ . In the MF group, the GAG chain length tends to be shorter than control group ( $P=0.15$ ) (Fig. 1 and Table 2).

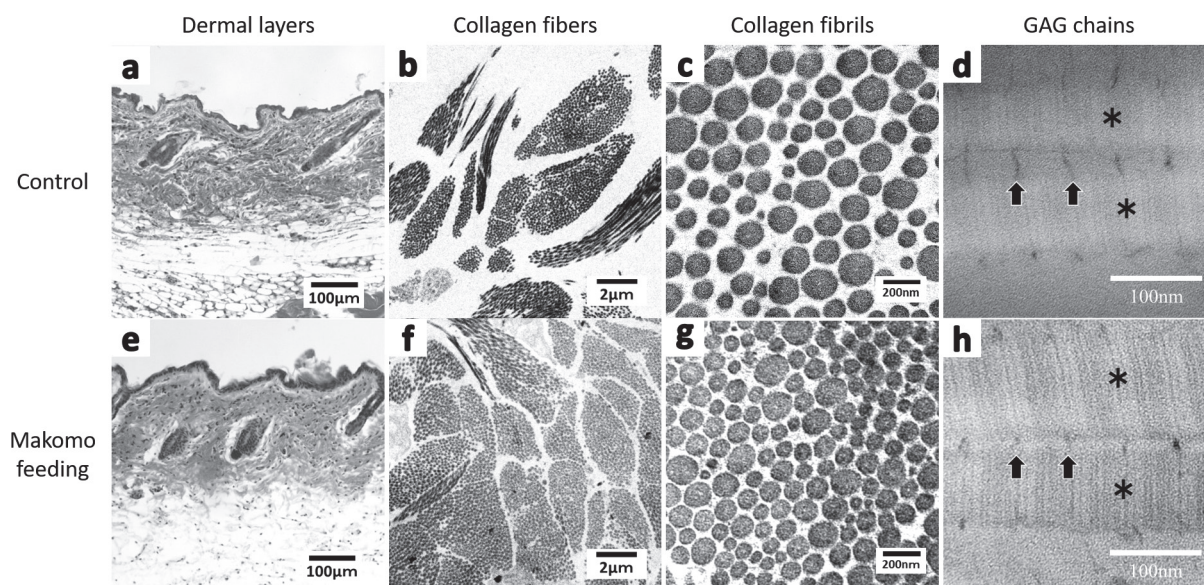
#### *Analysis of GAG*

Although there are many types of GAGs, only HA and DS in the skin samples were measured. HA and DS ratios in the control group were 46.3 and 53.7%, respectively, and those in the MF group was 46.8 and 53.2%, respectively (Table 2). There was no significant difference between the ratios of GAG in the control and MF groups ( $P=0.88$ ).

#### *Analysis of amino acid*

The amount of collagen in the control group ( $33.3 \pm 2.09 \mu\text{g}/\text{mm}^2$ ) and that in the MF group ( $26.7 \pm 2.38 \mu\text{g}/\text{mm}^2$ ) were not significantly different ( $P=0.11$ ) (Table 2).





**Fig. 1.** Morphological analysis of the control group (a–d) and MF group (e–h). (a, e): Light microscopic observation of the H&E-stained dermal layers. There was no significant difference between the dermal thicknesses in the control and MF groups ( $P < 0.05$ ). (b, c, f, g): Transmission electron microscopic observation of collagen fibers and collagen fibrils in the reticular layers. Increment in densities of collagen fibers and collagen fibrils in the MF group was detected. (d, h): Transmission electron microscopic observation of cupromeronic blue stained collagen fibrils in the reticular layers. The glycosaminoglycan chains (arrows) bridge collagen fibrils (\*). Length of the glycosaminoglycan chains in the MF group tends to be shorter than in the control group ( $P < 0.05$ ).

**Table 2.** Morphological and biochemical analyses of the dermal layer and plasma concentration of TGF- $\beta$ 1 in the control and Makoto feeding (MF) groups

Analysis	Control group	MF group
Thickness of dermis ( $\mu\text{m}$ )	142.2 $\pm$ 11.18	151.2 $\pm$ 6.86
Density of cells (cells/0.01 $\text{mm}^2$ )	32.4 $\pm$ 1.77	34.3 $\pm$ 2.40
Diameter of collagen fibrils (nm)	119.9 $\pm$ 3.08	111.7 $\pm$ 4.73
Collagen Fibril Index (CFI) (%)	66.0 $\pm$ 0.59	67.9 $\pm$ 0.50 <sup>a)</sup>
Length of glycosaminoglycan chains (nm)	36.5 $\pm$ 9.59	14.2 $\pm$ 2.43
Ratio of glycosaminoglycan components		
Hyaluronic acid (%)	46.3	46.8
Dermatan sulfate (%)	53.7	53.2
Amount of collagen ( $\mu\text{g}/\text{mm}^2$ )	33.3 $\pm$ 2.09	26.7 $\pm$ 2.38
Serum concentration of TGF- $\beta$ 1 (ng/ml)	35.1 $\pm$ 6.39	46.8 $\pm$ 19.96

Mean  $\pm$  Standard error, a) Significantly different ( $P < 0.05$ ). Significant difference was found only in the CFI.

### Plasma concentration of TGF- $\beta$ 1

Plasma concentration of TGF- $\beta$ 1 in the control group was 35.1  $\pm$  6.39 ng/ml, and that in the MF group was 46.8  $\pm$  19.96 ng/ml (Table 2). There was no significant difference between TGF- $\beta$ 1 concentrations in the control and MF groups ( $P = 0.63$ ).

## DISCUSSION

In clinical practice, certain EDS symptoms, such as skin fragility, are the major causes of easily torn suturing and delayed wound healing [1, 3]. Skin fragility has also been reported in patients with SCD-EDS and in the *Zip13*-KO mouse, which is generally accepted as an SCD-EDS mouse model [7, 8]. Based on the results of previous studies, skin structure of the *Zip13*-KO mouse is characterized by thinning of the dermal layer and decreases in density and diameter of collagen fibrils [7, 10]. Normal dermis usually contains thick collagen fibers with a small but important amount of elastic fibers. Good ratio of these two fiber types would provide strength and elasticity of the skin. Dermal thinning would not only affect the profile of collagen fibers as shown in the results, but also elastic fibers. Such imbalance would convey a certain impact to the skin.

Even Makomo is generally used as a supplement or tonic to promote general health condition through the good flow and strength of qi upon the practices in TCM, this study was the first investigation of therapeutic property of Makomo in SCD-EDS mice. The

maximum level of Makomo that can be used in the feed ratio for these mice is 30%. At this concentration, their daily nutritional requirements would not be lacking. Upon comparison with the basic diet, Makomo contains lower energy and carbohydrate, but with higher crude fiber (Table 1). This study used free-feeding system, because the daily energy and carbohydrate intake was not the factors affecting the results. Since the daily amount of feed that the animals usually eat is determined by their daily energy requirements, the indifferent final body weight of both groups was the best proof of this statement (data not shown). In addition, difference in amount of dietary fiber through eating diet with higher fiber may change the intestinal microbial profiles [5]. Nevertheless, there is no evidence whether the change of intestinal microbial profile shall convey any impact on the skin structures.

From the result, an increase in density of collagen fibers without a significant change in thickness of the dermal layer was observed in the MF group. Such morphology has a relationship with the strength of skin. Since mechanical strength of the skin is related to the number of collagen fibers per unit area [2], our results suggest that Makomo feeding can increase the mechanical strength of skin. Electron micrographs clearly showed that there was an increase in collagen fibril density without a change in shape or diameter of collagen fibrils in each collagen fiber. Hence, it is thought that the strength of the skin against tension was increased. These changes without change in the amount of collagen indicate increases in other constituents rather than collagen. One of the other constituents, decorin, which carries DS as a GAG chain, is known to bind collagen fibrils [18, 19, 22]. In addition, TGF- $\beta$ 1 is stored in the ECM through binding with decorin [4, 23]. Since there was no change in the plasma level of TGF- $\beta$ 1 or ratio of GAG, it is not clear whether decorin is related to the increases in densities of collagen fibers and collagen fibrils. However, the GAG chains of decorin form a ring-mesh structure and change the length [13, 22]. As a result of cupromeronic blue stain, it seems likely that the GAG chains can change their lengths and decrease the distance between collagen fibrils without changing the ratio of GAG in the MF group. TGF- $\beta$ 1 is inactive when combined with decorin, and it becomes active when it separates from decorin [23]. The active form of TGF- $\beta$ 1 binds to its receptor component, and SMAD (Sma- and Mad-related protein, mothers against decapentaplegic homolog) transcription factors are subsequently phosphorylated by the activated receptors. SMAD proteins then move into the nucleus and promote transcription of target genes leading to the synthesis and accumulation of the extracellular matrix [14]. This SMAD pathway is involved in the skin fragility in the *Zip13*-KO mouse [6, 7, 10].

In the meantime, there is no direct proof whether the changes in skin structure of the *Zip13*-KO mice fed with Makomo in this present study were promoted merely by improvement of the SMAD pathway. However, the increase in mechanical strength of the skin in these SCD-EDS mice with Makomo feeding suggests that there should be other factors other than the SMAD pathway involved in this improvement. Further studies to elucidate this question are essential.

**ACKNOWLEDGMENTS.** We wish to thank Dr. Hideyuki Asaoka for technical assistance in measurement of TGF- $\beta$ 1. This work was partially supported by The Maple Foundation.

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