Oral collagen-derived dipeptides, prolyl-hydroxyproline and hydroxyprolyl-glycine, ameliorate skin barrier dysfunction and alter gene expression profiles in the skin

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ABSTRACT

Oral supplementation with collagen hydrolysate (CH) has been shown to improve the condition of the skin in humans and experimental animals. Several hydroxyproline-containing oligo-peptides were previously detected in human peripheral blood after the ingestion of CH, and the two dipeptides, prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG), have been proposed to have beneficial effects on human health. When HR-1 hairless mice were fed a HR-AD diet, which lacked magnesium and zinc, transepidermal water loss (TEWL) increased and water content of stratum corneum decreased. In the present study, we investigated the effects of dietary PO and OG on skin barrier dysfunction in HR-1 hairless mice. Mice were fed a HR-AD diet with or without PO (0.15%) and OG (0.15%) for 35 consecutive days. The administration of PO and OG significantly decreased TEWL, and significantly increased water content of stratum corneum. A DNA microarray analysis of the dorsal skin revealed differences in gene expression between the group administered PO and OG and the control group. We also identified muscle-related Gene Ontology as a result of analyzing the up-regulated genes. These results suggested that the administration of PO and OG improved skin barrier dysfunction and affected muscle-related gene expression.

Keywords: Collagen hydrolysate, Prolyl-Hydroxyproline, Hydroxyprolyl-Glycine, Transepidermal water loss, Water content of stratum corneum, DNA microarray

Abbreviations: CH, collagen hydrolysate; PO, prolyl-hydroxyproline (Pro-Hyp); OG, hydroxyprolyl-glycine (Hyp-Pro); TEWL, transepidermal water loss.

1. Introduction

Collagen is one of the most abundant proteins in animals, and is a major constituent of connective tissues. Gelatin is a denatured form of collagen, and collagen hydrolysate (CH) is produced from gelatin by enzymatic hydrolysis. After the oral administration of radiolabeled CH, radioactivity was shown to accumulate in mouse skin for up to 96h [1]. The administration of CH also reportedly improved the loss of epidermal barrier function and skin elasticity in hairless mice following UV irradiation [2,3]. Proksch et al recently demonstrated that the oral ingestion of CH had beneficial effects on skin hydration, transepidermal water loss, and elasticity in a double-blind, placebo-controlled study [4]. These findings from experimental animals and humans suggest that the administration of CH has beneficial effects on the health of skin.

Several peptides have been detected in human peripheral blood following the oral ingestion of CH. Of these collagen-derived peptides, prolyl-hydroxyproline (PO) was identified as the most prevalent dipeptide [5-7]. On the other hand, previous studies detected not only PO, but also hydroxyprolyl-glycine (OG) in human peripheral blood after the ingestion of CH [8,9]. PO and OG are resistant to intracellular dipeptide hydrolysis and were shown to be transported into intestinal cells via peptide transporter-1 (PEPT-1) [10,11]. Therefore, PO and OG may play an important role in the improving skin barrier dysfunction. However, the effects of the pure peptides, PO and OG, on skin function have not yet been examined.

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The HR-AD diet-fed hairless mouse has dry skin and more prominent transepidermal water loss [12]. The effects of some food ingredients have already been evaluated on skin barrier function in this animal model [13-15].

The aim of the present study was to examine the effects of ingesting PO and OG on skin barrier dysfunction in HR-1 hairless mice fed a HR-AD diet. Furthermore, a gene expression analysis was performed on the skin using DNA microarrays.

2. Materials and methods

2.1. Peptides

The synthetic dipeptides, prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG) , were purchased from Bachem AG (Bubendorf, Switzerland).

2.2. Animals

Male Hos:HR-1 mice (4 wks old) were purchased from Hoshino Experimental Animals Inc. (Ibaraki, Japan). Mice were housed in plastic cages, and were kept in an air controlled room maintained at a temperature of $23 \pm 1°C$, humidity of $50 \pm 10\%$, and 12-h light-dark cycle (light on 7:00-19:00). They had free access to de-ionized water and semi-purified powder diet (Labo MR stock diet, Nosan Corporation, Yokohama, Japan). Animal care and experiments were approved by the Animal Committee of Josai University. After a 1-week acclimatization period, mice were divided into three groups ($n= 6$) and assigned experimental diets.

2.3. Diets

Labo MR stock powder diet was used for the normal (N) group. A HR-AD diet (Nosan Corporation) was used for the control (C) group. The ingredients of this diet have been described in detail previously [16]. In the PO and OG group (PO+OG), PO and OG were added to the HR-AD diet at 0.15% each (total 0.30%). Mice received the diets and

drinking water *ad libitum* for 35 days.

2.4. Measurement of transepidermal water loss, water content of stratum corneum, and skin

   viscoelasticity

Transepidermal water loss (TWEL) in the dorsal skin was assessed once every week using VAPOSCAN AS-VT100 RS (Asahi Techno Labo. Ltd., Kanagawa, Japan). Water content of stratum corneum and skin viscoelasticity were measured with a Skikon 200EX (IBS Co. Ltd., Shizuoka, Japan) and Cutometer MPA580 (Courage & Khazawa Electronics, Cologne, Germany), respectively. Skin viscoelasticity (R7) indicated the percent return at 0.1 second after the skin is subjected to reduced pressure at 300 mb for 4 seconds with a probe. To avoid applying stress to the skin, water content of stratum corneum and skin viscoelasticity were measured before the final day of the experimental period. Skin condition was assessed in triplicate at each dorsal skin spot. All skin condition measurements were performed on conscious animals, and the room was kept at a temperature of 23 ± 1°C and humidity of $50 \pm 10\%$.

2.5. Histology of dorsal skin samples

After the 35-day experimental period, mice were sacrificed under diethyl ether anesthesia, and skin samples were obtained. A portion of the skin was fixed with 10% neutral buffered formalin, and skin sections (5μm) were stained with hematoxylin and eosin.   Images were

recorded using the Moticam Pro 282B microscope digital system (Shimadzu Rika Corporation, Tokyo, Japan) at 200 × magnification. Ten sites were randomly selected in sections from each mouse, and measurements of the thickness of the epidermis were performed using Motic Images Plus Software (Shimadzu Rika Corporation).

2.6. Isolation of total RNA and DNA microarray analyses

Total RNA from the dorsal skin was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. In each group, an equal amount of total RNA was pooled into one sample to normalize individual differences. Pooled total RNA was labeled by Cy3 using the Agilent Low RNA Input Liner Amplification Kit, one-color (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions. Cy3-labeled cRNA were purified using the RNeasy mini kit (QIAGEN, Venlo, Netherlands). The concentration and quality of the labeled cRNA were assessed using the Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bio Analyzer (Agilent Technologies), respectively. After the fragmentation step, labeled cRNA was hybridized to the Agilent Whole Mouse Genome Oligo DNA microarray (4×44K, Product No. G4122F) according to the manufacturer's instruction. Microarray slides were analyzed using an Agilent Microarray Scanner (Model G2565BA). The fluorescence intensities of microarray images were digitized using Agilent Feature Extraction software (version 10.7).

GeneSpring GX (version 12.6, Agilent Technologies) was used to compare array data. After the elimination of flagged, empty, and control spots, the normalization of signals was carried out using the 75th percentiles of all measurements as a positive control for each sample. Since some genes had multiple probes, gene expression levels were calculated using the "Gene-level experiment" function in GeneSpring GX. The functional clustering of genes whose expression was at least two-fold higher or lower than the control group was assigned according to the Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) in order to extract Gene Ontology (GO) terms at "high" stringency.

2.7. Statistical Analysis

Results are expressed as means ± standard deviations (SD). Statistical analyses between the C group and PO+OG group was performed using the Student's t-test. A probability of less than 0.05 was considered significant.

3. Results

3.1. Food intake, water intake, and final body weight

Table S1 shows the final body weights, food intakes, and water intakes for the 35 days of the experimental period.   No significant differences were observed in food intake or final body weight between the C group and PO+OG group.

3.2. Changes in TEWL

To clarify the influence of the administrated PO+OG on the barrier function of the skin, TEWL was measured weekly. Figure 2A shows changes in TEWL following the administration of the PO+OG diet. Figure 2B shows TEWL after 35 days. TEWL in the N group did not vary throughout the study, but had increased after 21 days in the C and PO+OG groups. TEWL was significantly lower in the PO+OG group than in the C group after 35 days.

3.3. Water content of stratum corneum and skin viscoelasticity

Water content of stratum corneum and skin viscoelasticity were measured to clarify the influence of the PO+OG diet on the water holding capacity and flexibility of the skin, respectively. Figure 3 (A) and (B) show water content of stratum corneum and skin viscoelasticity after 34 days, respectively. Water content of stratum corneum was significantly higher in the PO+OG group than in the C group. No significant difference was

observed in skin viscoelasticity between the C group and PO+OG group.

3.4. Appearance and histopathological findings of the skin

HE-stained dorsal skin sections from HR-1 hairless mice were shown in Figure 3 (A), (B), and (C). The thickness of the epidermis was shown in Figure 3 (D). Epidermal thickening was noted in the C and PO+OG groups. Although the thickness of the epidermis was higher in the C group and PO+OG group, no significant differences were detected.

3.5. DNA microarray analysis

We detected 18,225 genes that were expressed in all groups by a microarray analysis. The expression of 242 genes was higher and that of 22 genes was lower by two-fold or greater in the PO+OG group than in the C group. However, we could not identify the gene related to skin function among these genes. Therefore, we analyzed the genetic characterization of these genes using the functional annotation clustering program, DAVID [22]. Table 1 shows the top 3 ranked annotation clusters analyzed by Functional Annotation Clustering. Annotation cluster 1 included myofibril (GO: 0030016), contractile fiber (GO: 0043292), sarcomere (GO: 0030017), and contractile fiber part (GO 0044449), and all GOs were related to muscle function.

On the other hand, in the analysis of the down-regulated genes, DNA binding (GO: 003677) was found in annotation cluster 1; however, it was impossible to identify genes

related to skin function (Table S2).

4. Discussion

Previous studies have examined the efficacy of the oral ingestion of CH on skin health. We hypothesized that adsorbed PO and OG reached the skin and improved skin barrier dysfunction. In the present study, we investigated the effects of the administration of PO+OG on diet-induced skin barrier dysfunction in HR-1 hairless mice. The results obtained demonstrated for the first time that the administration of PO+OG significantly improved skin barrier dysfunction.

However, the mechanism by which PO+OG improved skin barrier function currently remains unclear. It has been suggested that PO may have bioactively improved barrier function. Kawaguchi et al. reported that some orally administrated PO reached the skin [17]. PO was previously found to stimulate cell proliferation and induce the synthesis of hyaluronic acid in mouse skin fibroblasts and human dermal fibroblasts [18,19]. The mRNA expression of hyaluronic acid synthase was suppressed and the content of hyaluronic acid was decreased in mouse skin chronically irradiated with ultraviolet B [3,20]. In the present study, we could not identify any skin function-related genes, such as hyaluronic acid synthase, in genes whose expression was changed by 2-fold or greater. Hyaluronic acid is a component of the extracellular matrix, and has been shown to play crucial roles in the skin barrier, elasticity, and hydration [21]. Improvements in skin barrier function in the present study may be related to the synthesis of hyaluronic acid by PO and the slightly altered expression of its gene.

On the other hand, OG has been detected at a relatively high concentration in human peripheral blood following the ingestion of CH [8,9]. Shigemura et al. reported that OG enhanced the proliferation of fibroblasts derived from mouse skin more than PO [8]. These findings suggest that not only PO, but also OG modulated cells and the extracellular matrix in the skin. However, information regarding the function of OG is limited. Further studies are needed to investigate the effects of PO and/or OG on skin barrier function. The findings of these studies will provide an insight into whether PO and/or OG play an important role in the function of CH, and also if these peptides are candidates as bioactive peptides.

Many numerical genes are related to skin function. We hypothesized that PO+OG up-regulated gene expression related to the maintenance and repair of skin. Therefore, we adopted a DNA microarray analysis to investigate changes in gene expression in the skin. We extracted genes whose expression levels were higher or lower by 2-fold or greater in the PO+OG group than in the C group. To clarify the extracted genes, up- or down-regulated genes were obtained by searching against the Gene Ontology (GO) using the functional annotation clustering program, DAVID [22]. However, the results obtained showed that no gene was related to skin function. The present study demonstrated that PO+OG did not improve skin barrier function by altering gene expression. The top 3 ranked clusters of up-regulated genes were muscle-related genes. In this study, we used dorsal skin as a sample for the DNA microarray, and mice did not perform exercise. The results of this experiment suggested that PO and/or OG have the potential to enhance muscle function.

Further studies are required to investigate the effects of PO and/or OG on muscle and search for new food-derived bioactive peptides.

In conclusion, the results of the present study demonstrated that the daily administration of PO+OG improved skin barrier dysfunction in HR-1 hairless mice. Furthermore, PO+OG affected muscle-related gene expression. These results demonstrated that PO and/or OG may have important beneficial effects on skin and muscle. Foods containing PO and/or OG have the potential to be used as food supplements for skin and muscle.

References

[1] S. Oesser, M. Adam, W. Babel, et al., Oral administration of ^{14}C labeled gelatin hydrolysate leads to an accumulation of radioactivity in cartilage of mice (C57/BL), J. Nutr.129 (1999) 1891-5.

[2] M. Tanaka, Y. Koyama, Y. Nomura, et al., Effects of collagen peptide ingestion on UV-B-induced skin damage, Biosci. Biotechnol. Biochem. 73 (2009) 930-2.

[3] C. Oba, H. Ohara, M. Morifuji, et al., Collagen hydrolysate intake improves the loss of epidermal barrier function and skin elasticity induced by UVB irradiation in hairless mice, Photodermatol. Photoimmunol. Photomed. 29 (2013) 204-11.

[4] E. Proksch, D. Segger, J. Degwert, et al., Oral supplementation of specific collagen peptides has beneficial effects on human skin physiology: a double-blind, placebo-controlled study, Skin Pharmacol. Physiol. 27 (2014) 47-55.

[5] K. Iwai, T. Hasegawa, Y. Taguchi, et al., Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates, J. Agric. Food Chem. 53 (2005) 6531-6.

[6] H. Ohara, H. Matsumoto, K. Ito, et al., Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources, J. Agric. Food Chem. 55 (2007) 1532-5.

[7] S. Ichikawa, M. Morifuji, H. Ohara, H. Matsumoto, et al., Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral

administration of gelatin hydrolysate, Int. J. Food. Sci. Nutr. 61 (2010) 52-60.

[8] Y. Shigemura, S. Akaba, E. Kawashima, et al., Identification of a novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate, Food Chem. 129 (2011) 1019-24.

[9] F. Sugihara, N. Inoue, M. Kuwamori, et al., Quantification of hydroxyprolyl-glycine (Hyp-Gly) in human blood after ingestion of collagen hydrolysate, J. Biosci. Bioeng. 113 (2012) 202-3.

[10] M. Aito-Inoue, D. Lackeyram, M.Z. Fan, et al., Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane, J. Pept. Sci. 13 (2007) 468-74.

[11] C. Liu, K. Sugita, K. Nihei, et al., Absorption of hydroxyproline-containing peptides in vascularly perfused rat small intestine in situ, Biosci. Biotechnol. Biochem. 73 (2009) 1741-7.

[12] M. Fujii, T. Nabe, J Tomozawa, et al., Involvement of skin barrier dysfunction in itch-related scratching in special diet-fed hairless mice, Eur. J. Pharmacol. 530 (2006) 152-6.

[13] K. Tsuji, S. Mitsutake, J. Ishikawa, et al., Dietary glucosylceramide improves skin barrier function in hairless mice, J. Dermatol. Sci. 44 (2006) 101-7.

[14] N. Ikarashi, W. Sato, T. Toda, et al., Inhibitory Effect of Polyphenol-rich fraction from the bark of Acacia mearnsii on itching associated with allergic dermatitis, Evid. Based Complement Alternat. Med.(2012) 120389.

[15] K. Kawano, K. Umemura, Oral intake of beet extract provides protection against skin barrier impairment in hairless mice, Phytother. Res. 27 (2013) 775-83.

[16] M. Fujii, J. Tomozawa, N. Mizutani, et al., Atopic dermatitis-like pruritic skin inflammation caused by feeding a special diet to HR-1 hairless mice, Exp. Dermatol. 14 (2005), 460-8.

[17] T. Kawaguchi, P.N. Nanbu, M. Kurokawa, Distribution of prolylhydroxyproline and its metabolites after oral administration in rats, Biol. Pharm. Bull. 35 (2012) 422-7.

[18] Y. Shigemura, K. Iwai, F. Morimatsu, et al., Effect of Prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin, J. Agric. Food Chem. 57 (2009) 444-9.

[19] H. Ohara, S. Ichikawa, H. Matsumoto, et al., Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts, J. Dermatol. 37 (2010) 330-8.

[20] G. Dai, T. Freudenberger, P. Zipper, et al., Chronic ultraviolet B irradiation causes loss of hyaluronic acid from mouse dermis because of down-regulation of hyaluronic acid synthases, Am. J. Pathol. 171 (2007) 1451-61.

[21] L. Wiest, M. Kerscher, Native hyaluronic acid in dermatology - results of an expert meeting, J. Dtsch. Dermatol. Ges. 6 (2008) 176-80.

[22] W. Huang, B.T. Sherman, X. Zheng, et al., Extracting biological meaning from large gene lists with DAVID, Curr. Protoc. Bioinformatics. Chapter 13 (2009) Unit 13.11.

Figure and Table legends

Figure legends

Fig. 1. Effects of prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG) on transepidermal water loss (TEWL). HR-1 hairless mice were fed experimental diets (A), and after 35 days (B). Symbols and bars represent the mean and standard deviation (n=6). N, normal; C, control; PO+OG, PO plus OG. *p<0.01 vs. the C group (Student's t-test).

Fig. 2. Effects of prolyl-hydroxyproline (PO) and hydroxyprolyl-glycine (OG) on water content in the stratum corneum (A) and viscoelasticity (B) after 34 days in HR-1 hairless mice fed the experimental diets. Data represent the mean and standard deviation (n=6). *p<0.01 vs. the C group (Student's t-test).

Fig. 3. Hematoxylin-eosin (HE)-stained sections from the dorsal skin of HR-1 hairless mice fed the experimental diets. (A) Normal, (B) Control, (C) PO+OG. The scale bar represents 100μm.   (D) The thickness of the epidermis (μm). Data represent the mean and standard deviation (n=6).

Table legends

Table 1

Functional annotation cluster (top 3 ranked) of Gene Ontology (GO) terms found in genes whose expression levels were up- regulated 2-fold or more by PO+OG intake compared to the C group. Altered genes, the number of genes up-regulated by PO+OG intake. The total genes, the number of genes belonging to GO category. P values indicate Fisher's extract P value in DAVID functional clustering.

Supplemental Table legends

Supplemental Table 1

Effect of PO+OG on food intake, water intake and final body weight. HR-1 hairless mice were fed experimental diets for 35 days.

Supplemental Table 2

Functional annotation cluster (top 3 ranked) of Gene Ontology (GO) terms found in genes whose expression levels were down- regulated 2-fold or more by PO+OG intake compared to the C group. Altered genes, the number of genes down-regulated by PO+OG intake. The

total genes, the number of genes belonging to GO category. *P* values indicate Fisher's
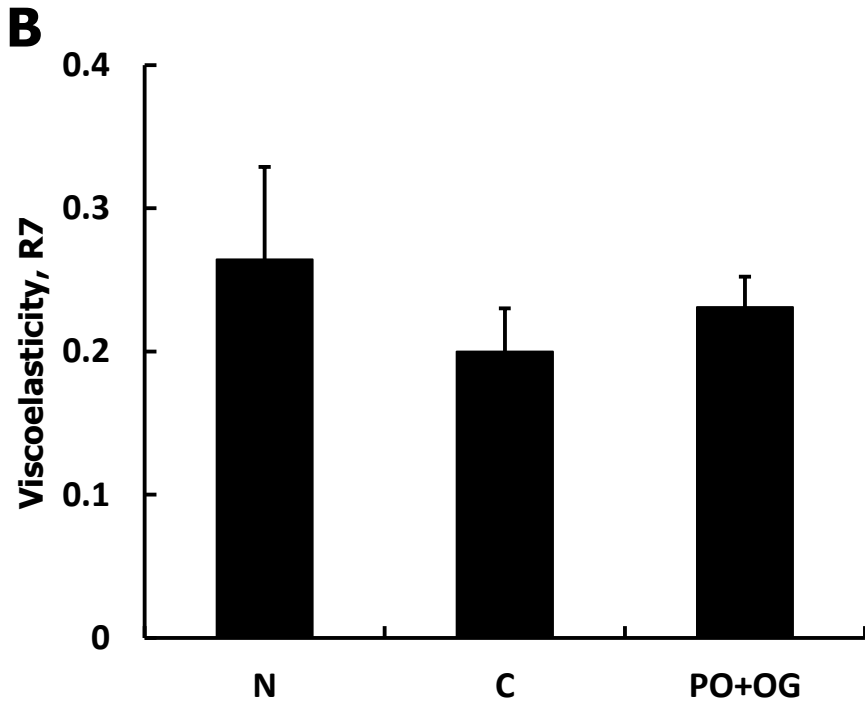
extract *P* value in DAVID functional clustering.

A
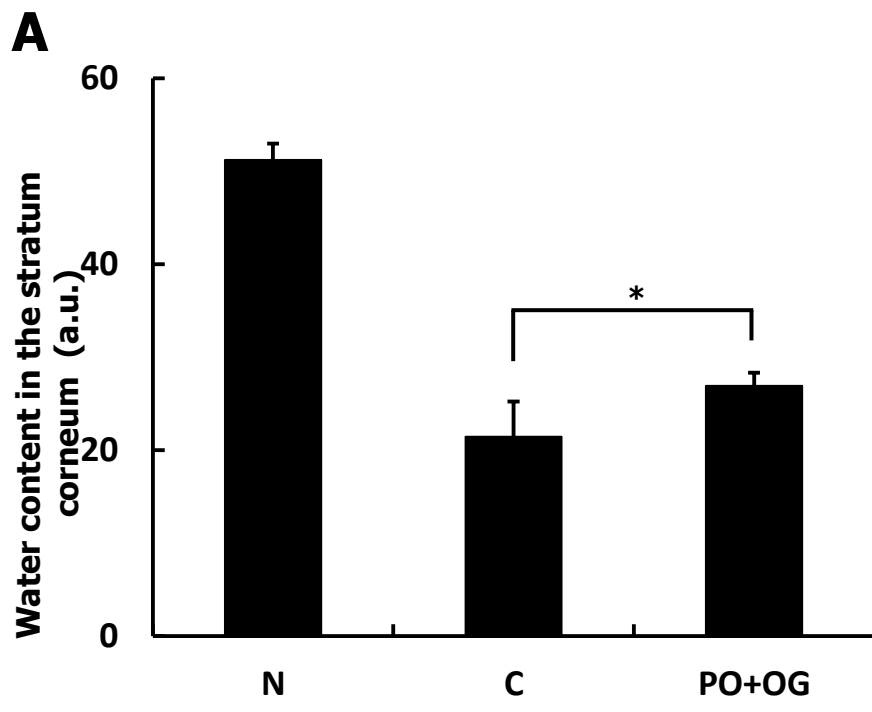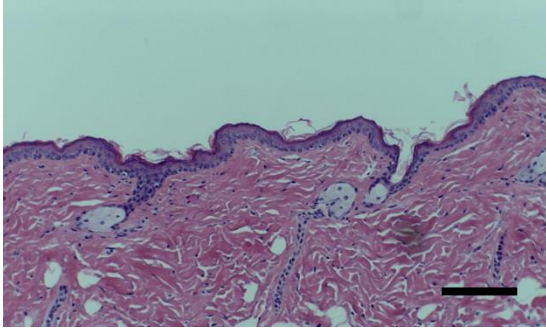
B

Fig. 1

A

B
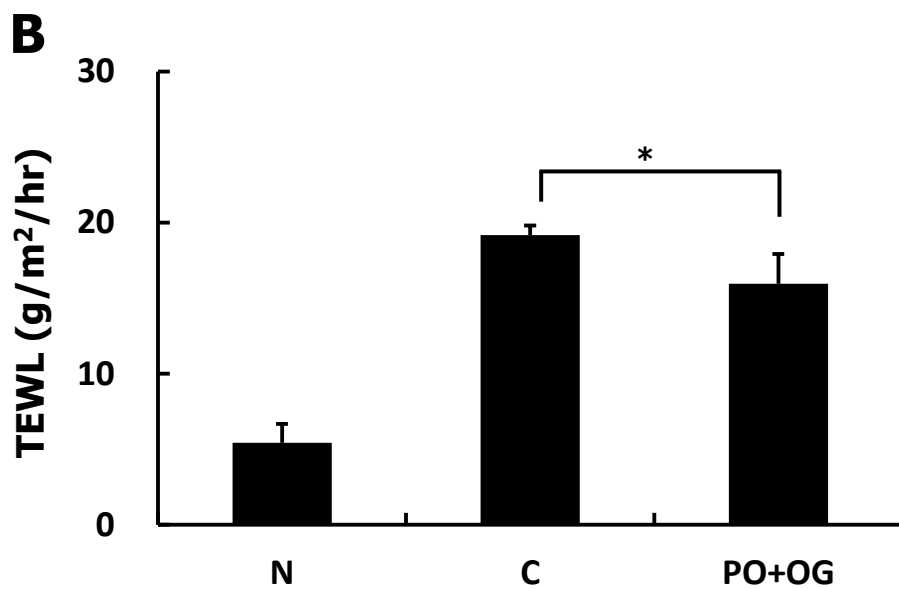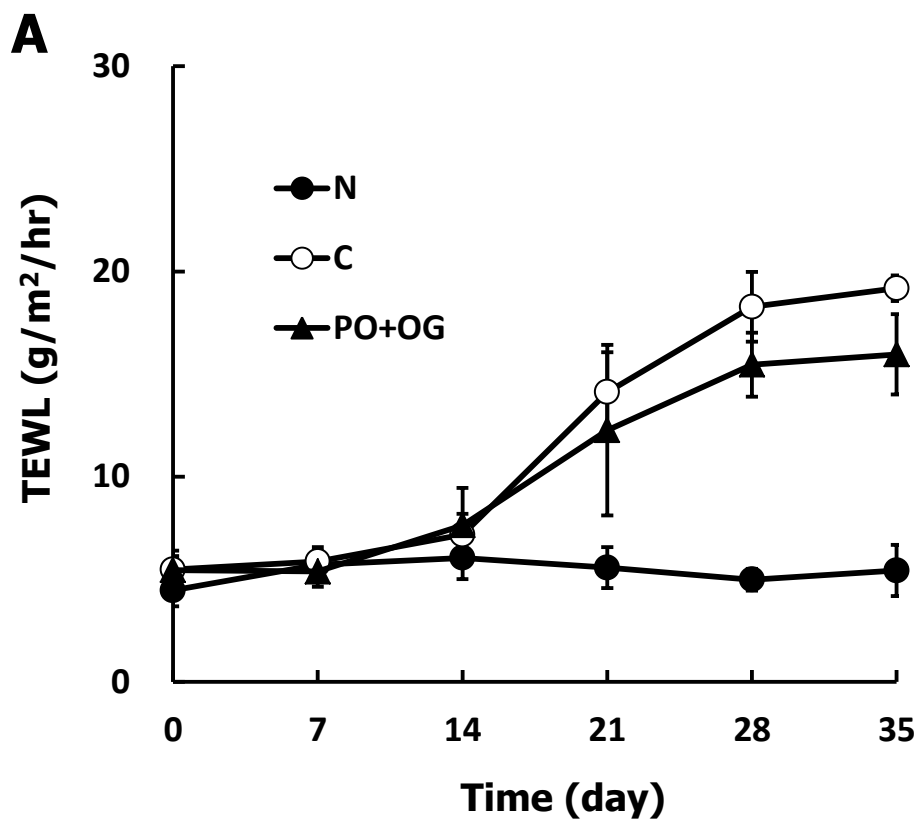
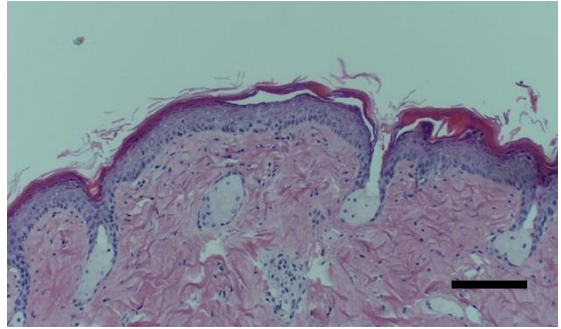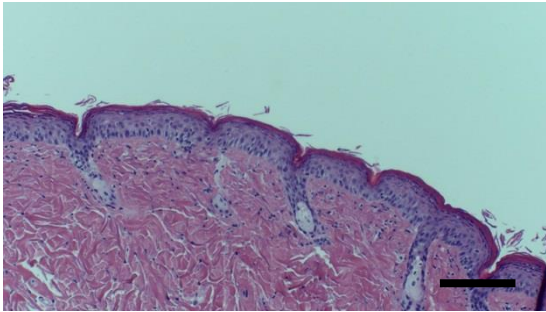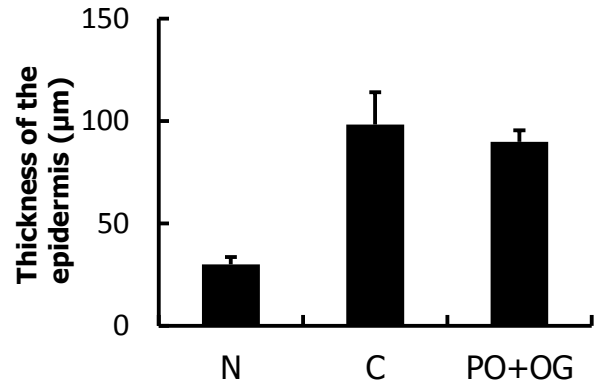**Fig. 2**

A

B

C

D

Fig. 3

Table 1

Annotation Cluster 1	Enrichment Score: 15.96	Altered Genes	Total Genes	*P* value
GO ID	GO term			
0030016	myofibril	19	144	8.39E-18
0043292	contractile fiber	19	144	1.90E-17
0030017	sarcomere	17	144	5.06E-16
0044449	contractile fiber part	17	144	1.82E-15
Annotation Cluster 2	**Enrichment Score: 6.53**			
0007517	muscle organ development	14	140	2.92E-08
0014706	striated muscle tissue development	11	140	6.85E-07
0060537	muscle tissue development	11	140	1.29E-06
Annotation Cluster 3	**Enrichment Score: 3.60**			
0008092	cytoskeletal protein binding	14	129	1.77E-04
0003779	actin binding	11	129	4.67E-04

Supplementary Table 1

	N	C	PO+OG
Food intake (g/day)	6.00±0.07	4.65±0.21	4.61±0.13
Water intake (mL/day)	7.89±0.30	9.16±0.99	8.79±0.61
Final body weight (g)	27.9±1.8	26.0±1.5	24.5±1.3

Supplementary Table 2

Annotation Cluster 1	Enrichment Score: 2.27	Altered	Total	
GO ID	GO Term	genes	genes	*P* value
0003677	DNA binding	9	36	0.002
Annotation Cluster 2	**Enrichment Score: 1.63**			
0006355	regulation of transcription, DNA-dependent	7	36	0.012
0051252	regulation of RNA metabolic process	7	36	0.013
0045499	regulation of transcription	7	36	0.076
Annotation Cluster 3	**Enrichment Score: 1.22**			
0034622	cellular macromolecular complex assembly	3	36	0.036
0034621	cellular macromolecular complex subunit organization	3	36	0.045
0065003	macromolecular complex assembly	3	36	0.079
0043933	macromolecular complex subunit organization	3	36	0.092