Cutaneous Application of Royal Jelly Inhibits Skin Lesions in NC/Nga Mice, a Human-Like Mouse Model of Atopic Dermatitis

Junki Miyamoto, Mariko Kiyomi, Yuuki Nagashio, Takuya Suzuki, and Soichi Tanabe

Abstract—Anti-allergic effects of royal jelly were evaluated in a human-like mouse model of atopic dermatitis. NC/Nga mice were cutaneously applied with royal jelly for 6 weeks. Royal jelly-treated mice exhibited lower levels of serum total immunoglobulin E in comparison with controls. We found that the treatment decreased (11% to the control) expression of mRNA for aquaporin-3, which is involved in the modulation of epidermal hydration. Microarray analysis revealed more than 10-fold changes in the expression of several genes, such as transglutaminase 2, repetin, and keratins. In normal human epidermal keratinocytes, royal jelly extract suppressed interleukin-8 elevation induced by TNF-α and interferon-γ, suggesting direct anti-inflammatory activity in keratinocytes. Collectively, topical application of royal jelly may be useful for amelioration of lesions and inflammation in atopic dermatitis.

Keywords—Aquaporin 3, immunoglobulin E, NC/Nga, royal jelly.

I. INTRODUCTION

ATOPIC dermatitis is a chronic inflammatory skin disorder characterized by the elevation of immunoglobulin E (IgE). The patients often suffer from severe itching; a serious problem that not only causes emotional stress but also further exacerbates the disease [1]. Topical steroids and oral anti-histamines are generally used as treatment for atopic dermatitis. Some improvements in the symptoms achieved using natural products are currently attracting much attention, as patients increasingly wish to avoid the long-term use of steroids and anti-histamines. In this study, we examined the efficacy of royal jelly (RJ) as a natural alternative remedy for atopic dermatitis.

RJ is the exclusive food of the honeybee (*Apis millifera*) queen larva. This substance is produced by the hypopharyngeal and mandibular glands of worker honeybees. It contains water (50-60%), proteins (18%), carbohydrates (15%), lipids (3-6%), minerals (1.5%), and vitamins. It also contains a large number of bioactive substances, and it has been used as a dietary supplement [2]. Previous studies have reported that oral administration of RJ ameliorates allergic reactions in mice [3], [4]. However, the effects of topical application of RJ to allergic skin remain to be examined. In this study, we evaluated the anti-allergic effects of RJ in NC/Nga mice.

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II. MATERIAL AND METHODS

A. Animal Procedures

Four-week-old female NC/Nga mice (ca. 17-18g) were purchased from Japan SLC (Shizuoka, Japan) and housed in a room with controlled temperature (24°C) and a 12-h light/12-h dark cycle under conventional conditions. Animals were given free access to commercial diet (MF, Oriental Yeast, Tokyo, Japan) and tap water and maintained according to the Hiroshima University Guide for the Care and Use of Laboratory Animals. All animal experimental protocols were approved by the Animal Care Committee, Graduate School of Biosphere Science, Hiroshima University. Mice were divided into 3 groups (n = 3 in each); each animal had its forehead and part of the back shaved at 6 weeks of age. In group 1 (control group) and group 2 (powder group), PBS solution and corn starch (0.67 g), respectively, was applied to shaved area. Group 3 (RJ group) received a topical application of the 1:1 mixture (0.67 g) of RJ and corn starch. RJ (enzymatic hydrolyzate in a freeze-dried powder form) was provided by Yamada Bee Farm (Okayama, Japan). All mice were treated every other day for 6 weeks (until 12 weeks of ages). There was no significant difference in body weight between the three groups during the experimental period (data not shown).

B. Cell Culture

Normal human epidermal keratinocytes (NHEK) and the growth medium were purchased from DS Pharma Biomedical (Osaka, Japan). Cells were cultured at 37°C under humidified 5% CO^2 atmosphere. The cells were seeded into 24-well plates (500 μl) at a density of 5×10^4 cells/well. They were stimulated with both tumor necrosis factor-α (20ng/mL, Wako Pure Chemical Industries, Osaka, Japan) and interferon-γ (20ng/mL, Thermo Fisher Scientific, Kanagawa, Japan). At the same time, 80% ethanol-soluble fraction of RJ (to a final concentration of 0.5 mg/ml) was added to the culture.

C. ELISA

Serum total IgE titer (as a marker of atopic condition) was determined once every alternate week, using a sandwich ELISA kit (Bethyl Laboratories, Montgomery, USA) according to the manufacturer's instructions. Also *in vitro* assay, after 24-h incubation, the medium was collected and interleukin (IL)-8 concentrations in the supernatants were measured using a DuoSet sandwich ELISA (R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

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D. Real-time RT-PCR and Microarray

Skin samples were excised from the area where PBS or powder had been applied and immediately immersed in RNAlater solution (Life Technologies, California, USA). Samples were ground in liquid nitrogen and total RNA was isolated using RNeasy Mini kit (Qiagen, Tokyo, Japan). The RT reaction was conducted with Multiscribe™ reverse transcriptase using a high-capacity cDNA reverse transcription kit (Life Technologies), at 25°C for 10min and at 37°C for 120 min. The reaction was terminated by heating at 85°C for 5s followed by cooling at 4°C. Real-time RT-PCR was performed using KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Boston, USA) and Step One Real-Time PCR system (Life Technologies).

Primer sequences are as follows: mouse aquaporin-3, sense 5'-CTCTGCCCATTAGGGCATTA-3', and anti-sense 5'-TTGAAAACTTGGTCCCTTGC-3'; mouse hypoxanthine phosphor ribosyl transferase 1 (HPRT1), sense 5'-GGTGGTGATTAGCGATGATG-3', and anti-sense 5'-ACAGAGGGCCACAATGTGAT-3'.

There action was per formed at 95°C for 2min, followed by 40 cycles of 95°C for 5sec and 60°C for 30s. The dissociation stage was analyzed at 95°C for 15s, followed by one cycle of 60 °C for 15s and 95°C for 15s. The fluorescence of the SYBR® green dye was determined as a function of the PCR cycle number, giving the threshold cycle number at which amplification reached a significant threshold. Data were analyzed by generating a standard curve using a dilution series and presented as fold-change in gene expression, after normalization against the HPRT1 gene.

To comprehensively examine the changes in gene expression in the skin, we performed microarray analysis, a method for studying complex processes in which many genes are involved. Total RNA of 3 mice from groups 2 and 3 was pooled and biotin-labeled cRNA was synthesized using an Illumina® RNA Amplification kit (Illumina, San Diego, USA). Labeled cRNA was hybridized to a BeadChip (Sentrix Mouse WG-6, Illumina) at 58°C for 18h. Forty-eight thousand transcripts representing six whole-genome samples can be analyzed on a single BeadChip. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 (GE Healthcare Biosciences, Buckinghamshire, UK) and scanned using Illumina BeadStation 500GX-WG system (Illumina). Background-corrected values for each probe on the BeadChip array were obtained using BeadStudio (Illumina). These results were expressed as arbitrary fluorescence units (AFUs) and analyzed using GeneViewer (ver 1.0, Moritex, Tokyo, Japan). Genes with the expression higher than 1,000 AFUs (a total of 1,663 genes) were chosen and further analyzed.

E. Statistical Analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. All data are expressed as the mean ± standard error (S.E.).

III. RESULTS

NC/Nga mice have been shown to develop atopic dermatitis-like lesions when kept in conventional surroundings [5]. In this study, serum IgE levels in groups 1 and 2 markedly increased until 10 and 12 weeks of age, respectively. However, the IgE level of the RJ-treated group was lower than that of the control groups (groups 1 and 2) at 8-12 weeks (Fig. 1). These results suggest that cutaneous application of RJ inhibits the peripheral IgE elevation associated with the atopic dermatitis-like lesions.
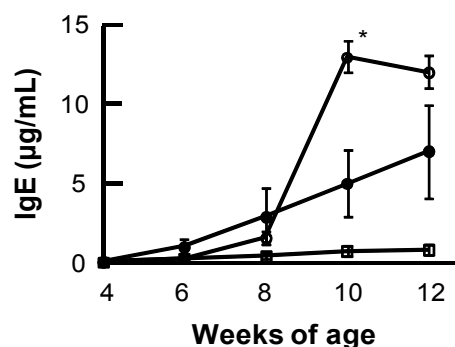

Fig. 1 The effect of cutaneous application of royaljelly (RJ) on serum total IgE levels in NC/Nga mice

Mice received cutaneous application of PBS (group 1, ○), corn starch (group 2, ●), or 1:1 mixture of RJ and corn starch (group 3, □) every other day for 6 weeks (from 6-12 weeks of ages). Total serum IgE titers were determined by enzyme-linked immune-sorbent assay (ELISA). Data are means ± SE, n=3 in each group. *$P < 0.05$ between group 1 and 3.

In control mice, atopic dermatitis-like lesions gradually developed between 8 and 9 weeks of age, with dermatitis particularly prominent around the eyes and ears at 10-12 weeks. However, no skin lesions were observed in the RJ-treated mice. Particularly, skin dryness symptoms substantially improved in this experimental group. Therefore, we examined the effect of topical RJ treatment on the expression of mRNA for aquaporin-3, a protein involved in the modulation of epidermal hydration [6], using real-time RT-PCR. We found that RJ treatment decreased the aquaporin-3 mRNA expression in the skin (11 ± 5.6% to the control group (group 2 = powder alone); means of n = 3 ± standard error).

Microarray analysis revealed that gene expression was substantially changed after topical RJ treatment for 6 weeks (group 3). We observed that 9 genes were upregulated and 22 downregulated more 15 than 10-folds in comparison with group 2 (powder group) mice (Table I).

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TABLE I
CHANGES IN mRNA EXPRESSIONS IN MICE SKIN AFTER ROYAL JELLY-TREATMENT

(A) Nine upregulated genes (more than 10-fold change)

gene	GenBank accession no.	RJ (-)	RJ (+)	change fold
-	NM_008972	278.5	7103.8	25.5
Myh1 (myosin, heavy polypeptide 1)	XM_354615	280.6	6960.4	24.8
Rps11 (ribosomal protein S11)	NM_013725	110.8	2463.5	22.2
-	NM_009093	540.8	7803.4	14.4
Gbas (glioblastoma amplified sequence)	NM_008095	124.4	1773.6	14.3
LOC638133	XM_134291	471.9	6112.6	13.0
Tgm2 (transglutaminase 2)	NM_009373	121.4	1561.8	12.9
Atp1a2	NM_178405	530.9	5829.9	11.0
(ATPase, Na+/K+ transporting, alpha 2 polypeptide)				
Hnrpf	NM_133834	237.5	2399	10.1
(heterogeneous nuclear ribonucleoprotein F)				

(B) Twenty-two downregulated genes (more than 10-fold change)

gene	GenBank accession no.	RJ (-)	RJ (+)	change fold
Krt71 (keratin 71)	NM_019956	3053.1	14.3	0.005
Krt25 (keratin 25)	NM_133730	9611.1	49.6	0.005
Krt27 (keratin 27)	NM_010666	6392.9	37.3	0.006
Krt33a (keratin associated protein 33a)	NM_027983	1924.1	17.2	0.009
Krtap8-1 (keratin associated protein 8-1)	-	3039.5	41.7	0.014
Krtap16-1(keratin associated protein 16-1)	NM_130870	3884.6	60.5	0.016
Rptn (repetin)	NM_009100	2084.4	34.5	0.017
Krtap16-8 (keratin associated protein 16-8)	NM_130856	4219	90.9	0.022
Krt31(keratin 31)	NM_010659	1519.6	33.1	0.022
Krtap21-1 (keratin associated protein 21-1)	NM_130875	1158.2	26.3	0.023
Krtap16-5 (keratin associated protein 16-5)	NM_130857	2918.7	71	0.024
Krtap8-2 (keratin associated protein 8-2)	NM_010676	1540.5	40.3	0.026
Stfa2l1 (stefin A2 like 1)	XM_148531	2917.2	91.5	0.031
-	NM_030005	1049.7	34.2	0.033
LOC640643	-	4142.9	138.8	0.034
Krtap16-9 (keratin associated protein 6-9)	NM_130876	1324.9	52.8	0.040
5430433J05Rik	-	5397.6	217.5	0.040
A030004J04Rik	NM_175424	4043.8	176.8	0.044
Krtap5-4 (keratin associated protein 5-4)	NM_015809	1987.5	100.2	0.050
S100a8	NM_013650	1586.7	133.9	0.084
(S100 calcium binding protein A8 (calgranulin A))				
S100a3 (S100 calcium binding protein A3	NM_011310	1777.6	156.3	0.088

Also RJ treatment drastically (60-fold change) downregulated the gene expression of gene for repetin, which is associated with the keratin intermediate filaments and necessary for the formation of the cornified envelope [9].

Interestingly, among the strongly downregulated genes (more than 10-fold change), the most represented group was formed by the keratin genes (keratin 25, 27, 31, 33a, and 71) and keratin-associated protein genes (keratin-associated protein 5-4, 8-1, 8-2, 16-1, 16-5, 16-8, 16-9, and 21-1).

We also evaluated the direct anti-inflammatory effect of RJ on normal human epidermal keratinocytes (NHEK). As shown in Fig. 2, the treatment with tumor necrosis factor-α and interferon-γ induced a marked increase in the secretion of IL-8 in NHEK. However, RJ partially, but significantly suppressed IL-8 secretion ($P < 0.01$), which suggested the direct anti-inflammatory activity of RJ in keratinocytes.
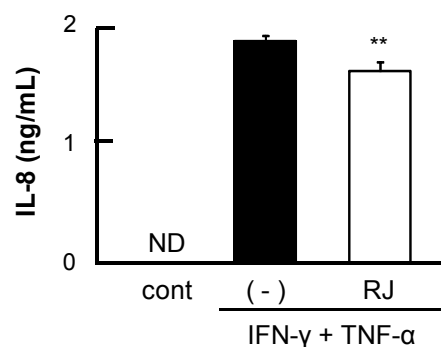

Fig. 2 The effect of royal jelly (RJ) extract on interleukin (IL-8) production induced by tumor necrosis factor-α and interferon-γ in normal human epidermal keratinocytes (NHEK)

NHEK was treated with tumor necrosis factor-α and interferon-γ and incubated with (RJ) or without RJ (-) extract

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for 24h. IL-8 concentrations in the media were determined enzyme-linked immunosorbent assay (ELISA). Data are means ± SE, n=3 in each group. **$P < 0.01$ between (-) and (RJ).

IV. DISCUSSION

We found that RJ treatment decreased the aquaporin-3 mRNA expression in the skin. Aquaporins are a family of transmembrane channels that transport water and in some cases small solutes such as glycerol [6], [7]. Aquaporin-3 is a water/glycerol-transporting protein expressed in keratinocytes of the epidermis. Increased expression of aquaporin-3 has been observed in the skin of patients with atopic eczema [6] and atopic dermatitis [7]. Nakahigashi et al. [7] has reported that epidermal hyperplasia is reduced in aquaporin-3-deficient mice with a decreased number of proliferating keratinocytes. We expected that RJ treatment would at least partially ameliorate water-loss and hyperplasia in NC/Nga mice by downregulating aquaporin-3 gene expression.

We observed that 9 genes were upregulated and 22 downregulated more than 10- folds in comparison with group 2 (powder group) mice (Table I). Among the upregulated genes, transglutaminase 2 gene is particularly worth noting. Transglutaminase catalyzes the post-translational modification of proteins via cross-l inking between primary amines and the γ-carboxamide group of glutamine residues. Transglutaminase 2 is the principal transglutaminase in the dermis; it cross-links the intracellular and extracellular matrix proteins, which are critical for the stabilization of dermal components. Increased transglutaminase 2 expression has been reported in many inflammatory diseases. For example, Kim et al. [8] reported that cystamine, chemical inhibitor of transglutaminase 2, suppresses passive cutaneous anaphylaxis in phorbol myristate acetate-induced BALB/c mouse model. In this study, we found that RJ treatment unexpectedly upregulated the expression of transglutaminase 2 gene. Further experiments, such as Western blotting for transglutaminase 2 protein, are necessary to clarify the function of transglutaminase 2 alterations in RJ-treated mouse skin.

As shown in Table I, B, RJ treatment drastically (60- fold change) downregulated the gene expression of gene for repetin, which is associated with the keratin intermediate filaments and necessary for the formation of the cornified envelope [9]. Interestingly, among the strongly downregulated genes (more than 10-fold change), the most represented group was formed by the keratin genes (keratin 25, 27, 31, 33a, and 71) and keratin-associated protein genes (keratin-associated protein 5-4, 8-1, 8-2, 16-1, 16-5, 16-8, 16-9, and 21-1). It has been reported that the level of keratin 10 and keratin 14, markers of differentiation and proliferation of keratinocytes, respectively, increase in atopic dermatitis in comparison with normal epidermis [10]. Several keratins, such as keratin 14, are also significantly increased in canine atopic dermatitis [11]. However, in this study, the expression levels of keratin 10 and keratin 14 did not change greatly (data not shown). Further investigations are required to elucidate whether downregulation of mRNA expression of keratins 25, 27, 31, 33a, and 71 is closely associated with anti-atopic activity of RJ and is not just the consequence of shaving in our experimental setup. Real-time PCR analyses are also necessary to confirm the changes in mRNA expression observed using microarrays.

In conclusion, we showed that cutaneous application of RJ inhibited IgE elevation and skin lesions in NC/Nga mice and altered mRNA expression of several genes such as aquaporin-3. Thus, topical application of RJ may be useful for amelioration of lesions and inflammation in atopic dermatitis.

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