IFSCC 2025 full paper (IFSCC2025-674)

"Investigation of Novel SIRT-1-Activating Cosmetic Ingredients and Evaluation of Their Effects on Human Skin through Clinical Trials"

Kanako Minami[1], Hideyo Watanabe[1], Marie Nishikawa[1], Taiki Tsubaki[2], Yoshinori Katakura[3], Tsutomu Kamata[1]

[1] R&D Dept, AIR WATER REALIZE INC., Tokyo, Japan
[2] Graduate School of Systems Life Science, Kyushu University, Fukuoka, Japan
[3] Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University, Fukuoka, Japan

1. Introduction

The skin is an important organ that acts as a barrier to prevent the entry of pathogens and harmful substances into the body, while also reducing water loss. From exterior to interior, the skin comprises three layers: epidermis, dermis, and subcutaneous tissue. The stratum corneum, as the outermost epidermal layer, makes a particularly large contribution to the skin's barrier function. Intercellular lipids, including ceramides, play an important role in the barrier function of the stratum corneum [1]. Given that the synthesis of intercellular lipids decreases with age, the barrier function of the stratum corneum also declines with age. Skin with reduced barrier function is associated with early aging signs, such as dryness and flakiness, as well as more severe aging-related features such as spots and wrinkles [2]. Reduced barrier function is also known to accelerate skin aging because it makes the skin less able to retain moisture and more susceptible to damage from exposure to ultraviolet (UV) rays.

Therefore, suppressing the decline in skin barrier function should ultimately reduce the appearance of skin aging. Conventionally, moisturizing with natural moisturizing factors (NMF), glycerin, and oils has been employed as a means to suppress the decline of skin barrier function. However, in recent years, the production of ceramides and involucrin by the activation of mitochondrial function via the redox reaction of nicotinamide adenine dinucleotide (NAD) has emerged as a novel way of improving skin barrier function [3].

The longevity genes called sirtuins (SIRT) have been found to act as histone deacetylases with NAD^+ as a coenzyme [4], attracting attention in the fields of medicine and food because they improve intracellular metabolic function and extend cell lifespan. SIRT have been shown to extend lifespan in *Caenorhabditis elegans* (roundworm) [5] and *Drosophila* (fruit fly) [6], and it has been reported that they may also be involved in antiaging in mammals [7]. Seven SIRT have been identified in humans, namely, SIRT-1 to SIRT-7, which differ in localization and function. SIRT-1 is localized in the nucleus and cytoplasm and has been reported to improve intracellular metabolic function by deacetylating proteins [8]. SIRT-1 is the human ortholog of the yeast silent information regulator 2 (Sir2) and is involved in lifespan regulation in various

life forms by mediating the effect of caloric restriction [9]. Additionally, SIRT-1 has been shown to activate mitochondria via peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) [10] and promote intercellular lipid synthesis via acetyl coenzyme A (acetyl CoA) [11], suggesting its potential application in the field of cosmetics. However, despite the promising effects of SIRT-1 on the skin, the SIRT-1-activating substances reported to date have not yet obtained International Nomenclature of Cosmetic Ingredients approval, and there are issues with their application in cosmetics in terms of safety and stability. Although many *in vitro* studies have examined the effects of SIRT-1 on the skin, few clinical trials on this issue using human skin have been reported.

In the present study, we searched for cosmetic ingredients that activate SIRT-1 from among those whose safety has been confirmed and evaluated their effects on human skin through a clinical trial. Plant extracts and plant oils containing polyphenols were selected as the cosmetic ingredients for this screening because polyphenols, represented by resveratrol, have SIRT-1-activating effects [12]. In addition, a 4-week human continuous-use trial was conducted using a cream containing SIRT-1-activating cosmetic ingredients, and the results were evaluated on the basis of parameters that measure improvement in the epidermal stratum corneum.

2. Materials and Methods

2.1 Evaluation of SIRT-1 promoter activity

2.1.1 Cell line and treatment

In this study, a human epidermal keratinocyte cell line (HaCaT cells) was used. HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Nissui, Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum (FBS; Life Technologies, CA, USA) in cell culture dishes (Grenier Bio-one, Tokyo, Japan) at 37°C under conditions with 5% CO_2. DMEM was prepared by dissolving 4.7 g of DMEM powder in 470 mL of Milli-Q water and adding 1 mL of 100 mg/L streptomycin (Meiji Seika Pharma, Tokyo, Japan), 1 mL of 100,000 U/L penicillin (Meiji Seika Pharma, Tokyo, Japan), 10 mL of 0.2 mol/L L-glutamine (FUJIFILM Wako, Osaka, Japan), and 10 mL of 10% $NaHCO_3$ (FUJIFILM Wako, Osaka, Japan). The test samples, resveratrol, and various cosmetic ingredients are shown in Tables 1 and 2. The test samples were dissolved in 100% dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS) to 10 mM and 30 mg/mL, respectively. These were used as stock test solutions, which were diluted 1000-fold with DMEM and added to HaCaT cells. Therefore, in the comparison group, 100% DMSO and PBS were added to HaCaT cells to make a 1000-fold dilution. Then, resveratrol and various cosmetic ingredients that were diluted 1000-fold with DMEM were adjusted to final concentrations of 10 µM and 30 µg/mL, respectively, and applied to the cells. In the present study, resveratrol was used as a positive control for SIRT-1 promoter activity in HaCaT cells.

Table 1. Classification of test samples and solvent extraction
The substances listed represent the active components of the cosmetic raw materials.The solvent was selected on the basis of the characteristics of the ingredients, as either phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO).

Classification	Extracting solvent	Ingredients	Solvent
Control	-	DMSO, PBS	-
Positive	-	Resveratrol	DMSO
Sample 1	Ethanol, water	*Paeonia Albiflora* Root Extract	PBS
Sample 2	BG, water	*Paeonia Albiflora* Root Extract	PBS

Table 2. Classification of test samples
The substances listed represent the active components of the cosmetic raw materials.The solvent was selected on the basis of the characteristics of the ingredients, as either phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO).

Classification	Ingredients	Solvent	Concentration (μg/mL)
Control	DMSO, PBS	-	-
Positive	Resveratrol	DMSO	10
Sample 3	Resveratrol (cosmetic grade)	DMSO	30
Sample 4	*Paeonia Suffruticosa* Root Extract	DMSO	30
Sample 5	*Saxifraga Sarmentosa* Extract	PBS	30
Sample 6	*Isodonis Japonicus* Leaf/Stalk Extract	PBS	30
Sample 7	*Salvia Officinalis* (Sage) Leaf Extract	PBS	30
Sample 8	*Prunus Yedoensis* Leaf Extract	PBS	30
Sample 9	*Eriobotrya Japonica* Leaf Extract	PBS	30
Sample 10	*Foeniculum Vulgare* (Fennel) Fruit Extract	PBS	30
Sample 11	*Cnidium Officinale* Rhizome Extract	PBS	30
Sample 12	*Camellia Sinensis* Leaf Extract	PBS	30
Sample 13	*Angelica Acutiloba* Root Extract	PBS	30
Sample 14	*Salix Alba* (Willow) Bark Extract	PBS	30
Sample 15	*Glycyrrhiza Glabra* (Licorice) Leaf Extract	PBS	30
Sample 16	*Acorus Calamus* Root Extract	DMSO	30
Sample 17	*Rosa Multiflora* Fruit Extract	DMSO	30
Sample 18	*Citrus Junos* Fruit Extract	DMSO	30
Sample 19	*Argania Spinosa* Kernel Oil	DMSO	30
Sample 20	*Lithospermum Officinale* Root Extract	DMSO	30
Sample 21	Meadowfoam Estolide	DMSO	30

2.1.2. SIRT-1 promoter reporter assay
The human SIRT-1 promoter (−1539 to −1) was amplified by PCR using the DNA of the human gene as a template, and the sequence was confirmed by DNA sequence analysis. The amplified human SIRT-1 promoter was cloned into pEGFP-C (Takara, Shiga, Japan) from

which the cytomegalovirus (CMV) promoter had been removed using restriction enzymes (AseI, NheI). The resulting plasmid (hSIRT1p-EGFP) (1 µg) was transfected into HaCaT cells [HaCaT(hSIRT1p-EGFP)] using Hily Max (Dojindo, Kumamoto, Japan), in accordance with the manufacturer's protocol. Stable transformants were selected using G418. Changes in hSIRT1p-EGFP-derived EGFP fluorescence were evaluated using IN Cell Analyzer 2200 (GE Healthcare, Little Chalfont, UK). HaCaT(hSIRT1p-EGFP) cells were cultured in the same way as described above for HaCaT cells.

2.1.3. Test evaluating activation of SIRT-1 promoter expression

SIRT-1 promoter activity was evaluated by measuring EGFP fluorescence intensity in HaCaT(hSIRTp-EGFP) cells using IN Cell Analyzer 2200 (GE Healthcare, Little Chalfont, UK). HaCaT(hSIRTp-EGFP) cells (6.0×10^4 cells/mL) were seeded into 96-well plates (Grenier Bio-one, Tokyo, Japan) and cultured for 24 h. Resveratrol and various cosmetic ingredients were then added to final concentrations of 10 µM and 30 µg/mL, and cultured for 48 h. Thereafter, 8% (w/v) paraformaldehyde was added to a final concentration of 4% (w/v) at 100 µL/well, and the solution was left to stand at room temperature for 15 min for fixation. In addition, 8% (w/v) paraformaldehyde was prepared by diluting paraformaldehyde (Wako, Osaka, Japan) with 1× PBS, adding 5 µg/mL of 2N sodium hydroxide solution, and dissolving the mixture in a 60°C water bath. After removing the paraformaldehyde and washing twice with 1× PBS, CellstainR-Hoechst 33342 solution (Dojindo, Kumamoto, Japan) diluted 500-fold was added at 100 µL/well, and the solution was left to stand at room temperature for 20 min for nuclear staining. After removing the CellstainR-Hoechst 33342 solution and washing twice with 1× PBS, PBS was added at 100 µL/well, and EGFP fluorescence intensity was measured using IN Cell Analyzer 2200.

2.1.4. Statistical analysis

All tests were conducted at least three times and corresponding data are presented. Results are expressed as mean ± standard deviation (mean ± SD), and statistical significance was determined using two-tailed Student's t-test. Differences were considered significant at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

2.2. Clinical trial

*2.2.1. Ethics review committee and approva*l

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Nikoderm Research Inc. Written informed consent was obtained from all participants.

2.2.2. Trials and measurement methods

The formulation of Cream A containing resveratrol (cosmetic grade) and *Argania spinosa* kernel oil (hereinafter referred to as SIRT-1-activating ingredients) as active ingredients for SIRT-1 activation is shown in Table 3. Approximately 0.02 g of Cream A was applied by the subjects themselves to a designated area of 9.6 cm^2 on the inside of their left and right upper arms twice a day, in the morning and evening for 4 weeks. Comparative trials were conducted with Cream B (Table 3), which does not contain SIRT-1-activating ingredients, and without application of any cream. Cream B was applied continuously for 4 weeks to a different designated area than that for Cream A under the same conditions as those for Cream A.

Table 3. Formula of cream for clinical trials
Cream A contains *Argania spinosa* kernel oil and resveratrol as active ingredients.
Cream B does not contain any active ingredients.

Ingredients	Weight percentage (wt%)	
	Cream A	Cream B
Argania Spinosa Kernel Oil	0.3	-
Resveratrol	0.3	-
Polyols	16.0	16.0
Oil	25.2	25.2
Emulsifier	5.2	5.2
Preservatives	q.s.	q.s.
Thickening agents	q.s.	q.s.
pH adjusters	q.s.	q.s.
Water	add to 100	add to 100

2.2.3. Measurement
The trial period was from May to July 2022, with measurements taken three times: before use, and 2 and 4 weeks after use. All three measurements were performed at the same time of the day. After wiping the measurement sites, the test sites were acclimated for 15 min at 20 ± 1°C and 55 ± 5% humidity, and the stratum corneum water content was measured using MPA6 Corneometer CM825, whereas transepidermal water loss (TEWL) was measured using MPA6 Tewameter TM300. In addition, images of skin texture were taken using VHX-6000 (VH-Z20T).

2.2.4. Evaluation of skin texture
The skin texture images obtained as described in section 2-3 were evaluated by an expert evaluator using a visual score. Scores are on a 7-point scale, with higher scores indicating a smoother and clearer texture. The skin texture scoring criteria are shown in Figure. 1.

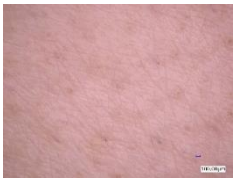
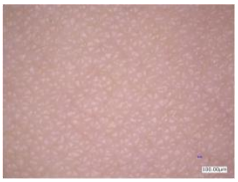
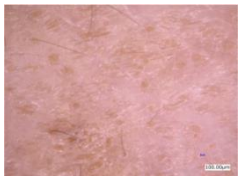
	Score = 7	Score = 4	Score = 1
Skin surface texture			

Figure 1. Representative images of skin surface texture.
Images captured with a digital microscope VHX-6000 (VH-Z20T) were visually evaluated on a 7-point scale.

2.2.5. Statistical tests
Measurements were taken three times at each of the three test sites. Results are expressed as mean ± standard deviation (mean ± SD), and statistical significance was determined using Tukey's HSD test. Differences were considered significant at $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Results

3.1. Screening for cosmetic ingredients that activate SIRT-1

3.1.1. Differences in activation effects of plant extracts depending on the extraction solvent

Among *Paeonia Albiflora* Root Extracts shown in Table 1, Sample 1, for which *Paeonia albiflora* root had undergone extraction using ethanol, strongly activated the SIRT-1 promoter in HaCaT cells compared with Sample 2, for which extraction had been performed using butylene glycol (BG) (Figure 2).

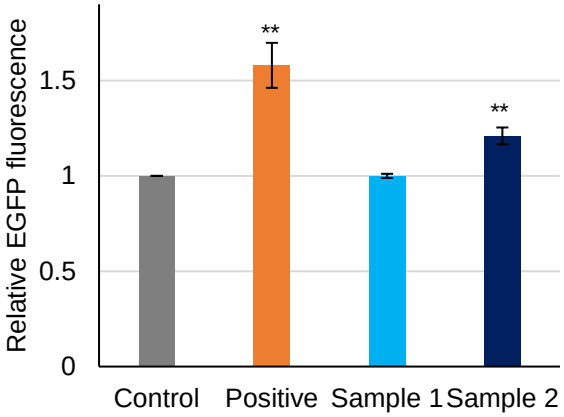


Figure 2. Differences in SIRT-1 activation rates between Sample 1 and Sample 2. Ingredients (30 µg/mL) were added to HaCaT (hSIRTp-EGFP) cells followed by culture for 48 h, and changes in EGFP fluorescence were monitored. The significance of differences was calculated using Student's *t*-test. Values are presented as means ± SD. $^{**}p < 0.01$.

3.1.2. Effect of test samples on activating SIRT-1 promoter

Among the cosmetic ingredients shown in Table 2, resveratrol (cosmetic grade) and the following plant extracts were extracted using ethanol-containing solvents: *Paeonia Suffruticosa* Root Extract, *Saxifraga Sarmentosa* Extract, *Isodonis Japonicus* Leaf/Stalk Extract, *Salvia Officinalis* (Sage) Leaf Extract, *Prunus Yedoensis* Leaf Extract, *Eriobotrya Japonica* Leaf Extract, *Foeniculum Vulgare* (Fennel) Fruit Extract, *Cnidium Officinale* Rhizome Extract, *Camellia Sinensis* Leaf Extract, *Angelica Acutiloba* Root Extract, *Acorus Calamus* Root Extract, *Rosa Multiflora* Fruit Extract, *Citrus Junos* Fruit Extract, *Argania Spinosa* Kernel Oil, and *Lithospermum Officinale* Root Extract. These extracts significantly activated the SIRT-1 promoter in HaCaT cells compared with the findings in the comparison group (Figure 3).

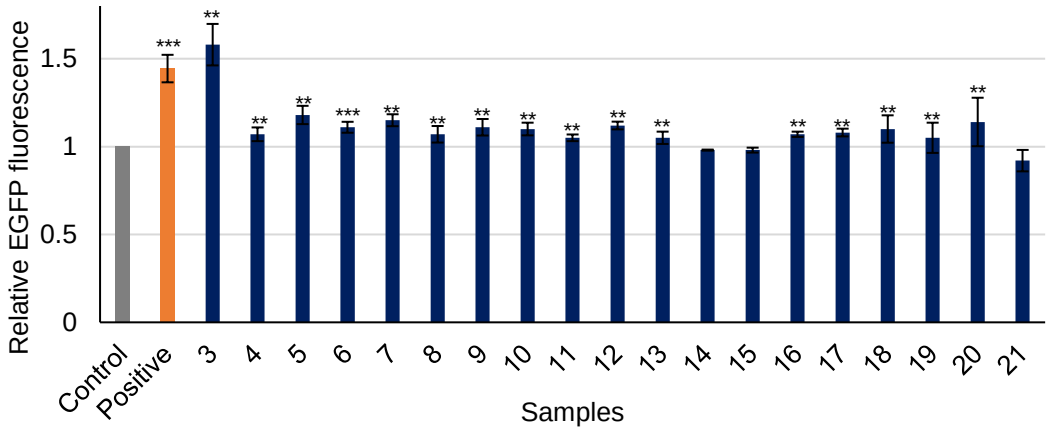


Figure 3. SIRT-1 activation rates of each sample.
Ingredients (30 µg/mL) were added to HaCaT (hSIRTp-EGFP) cells followed by culture for 48 h, and changes in EGFP fluorescence were monitored. The significance of differences was calculated using Student's *t*-test. Values are presented as means ± SD. ***p* < 0.01.

3.2. Clinical trials

The results of the continuous-use trial showed that the stratum corneum water content increased significantly after 4 weeks of using Cream A compared with the level before its use (Figure 4(a)). In addition, it was confirmed that using Cream A for 2 weeks significantly reduced TEWL compared with Cream B and no application of any cream (Figure 4(b)). The skin texture score improved after using Cream A for 4 weeks compared with that before use, and also showed a trend toward improvement compared with using Cream B (Table 4) (Figure 5).

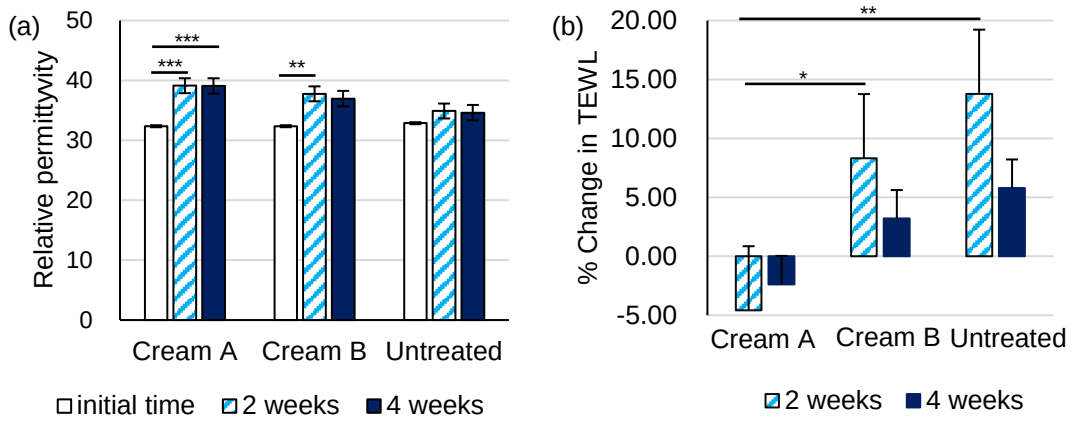


Figure 4. (a)Changes in epidermal moisture content over 4 weeks.
Stratum corneum water content was measured using MPA6 Corneometer CM825 before use, and at 2 and 4 weeks. The significance of differences was calculated using Tukey's HSD test. Values are presented as means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$.
(b) Changes in transepidermal water loss (TEWL) ratio over 4 weeks compared with the value at the initial time point.

TEWL was measured using MPA6 Tewameter TM300 before use, and at 2 and 4 weeks. Significant differences were calculated using Tukey's HSD test. Values are presented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table 4. Changes in skin surface texture score over 4 weeks.
Skin texture images were taken using VHX-6000 (VH-Z20T) before use and after 4 weeks of use. The images were scored using the evaluation method described in section 2-4.

	Initial time	4 weeks	Difference
Cream A	5.21	6.24	+1.02
Cream B		5.71	+0.49
Untreated		5.12	−0.10

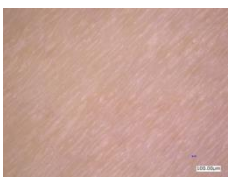
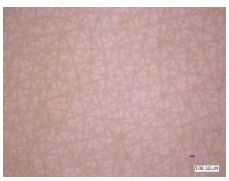
Initial time	4weeks
	

Figure 4. Change in the skin surface texture over 4 weeks.
Skin texture images were taken using VHX-6000 (VH-Z20T) before use and after 4 weeks of use.

4. Discussion

4.1. SIRT-1 activation

In the present study, ethanol-extracted *Paeonia Albiflora* Root Extract activated SIRT-1 more potently than BG-extracted *Paeonia Albiflora* Root Extract did. Because polyphenols in plants are efficiently transferred to ethanol extracts [13], it was inferred that the ethanol-extracted plant extracts in this study also contained high levels of polyphenols, resulting in the activation of SIRT-1. Conversely, *Salix Alba* (Willow) Bark Extract and *Glycyrrhiza Glabra* (Licorice) Leaf Extract, which have been reported to contain polyphenols, did not show any SIRT-1-activating effects. We speculated that the plant extracts lacking SIRT-1-activating effects contained low levels of polyphenols or contained polyphenols that were unable to activate the SIRT-1 promoter.

4.2. Clinical trial

We reported that Cream A, which contains SIRT-1-activating ingredients, improved the moisture content of the stratum corneum and skin texture score with continuous use, and significantly reduced TEWL compared with Cream B, which was a placebo. This can be attributed to the effect of SIRT-1-activating ingredients improving the condition of the epidermis. SIRT-1 activation has been reported to activate mitochondria via PGC-1α [10] [14] [15], promote the synthesis of ceramides and fatty acids by elevating acetyl-CoA synthesis via acetyl-CoA synthetases (AceCSs) [11], and promote hyaluronic acid synthesis via hyaluronic acid synthetases (HAS2) [16]. It is considered that the results obtained here can be explained by these effects. It has also been reported that SIRT-1 activation activates transforming growth factor-β (TGF-β) [17] and myocardin-related transcription factor A (MRTF-A) [18], which are involved in collagen synthesis, and suppresses matrix metalloproteinases-1, 3, 9 (MMP-1, 3, 9), which are collagen-degrading enzymes [19] [20]. This suggests that SIRT-1 activation affects the conditions of the epidermis and dermis.

5. Conclusion

In the present study, SIRT-1-activating effects were confirmed for 17 cosmetic ingredients whose safety has been confirmed. We also reported that the continuous use of a cream containing SIRT-1-activating ingredients improved three parameters of stratum corneum: water content, TEWL, and skin texture. This suggests that SIRT-1 activation promotes the synthesis and suppresses the degradation of components constituting intercellular lipids in the stratum corneum, and can be expected to be effective in improving skin-related problems. Furthermore, it has been reported that β-nicotinamide mononucleotide (NMN) is useful for preventing the functional decline of SIRT-1 caused by UV damage [21], and the SIRT-1-activating ingredients reported in this study are also expected to suppress UV-induced functional decline of SIRT-1, similar to NMN.

Conversely, resveratrol, which has a high capacity to activate SIRT-1, is a poorly soluble substance, and is known to have poor stability in cosmetics, such as becoming discolored over time. The SIRT-1-activating ingredients reported in this study, argan oil and plant extracts, can be used in all cosmetic formulations, and there are no major concerns about their stability.

6. References

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