Original Contribution

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A comparative study of the effects of retinol and retinoic acid on histological, molecular, and clinical properties of human skin

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Abstract

Background All-trans retinol, a precursor of retinoic acid, is an effective anti-aging treatment widely used in skin care products. In comparison, topical retinoic acid is believed to provide even greater anti-aging effects; however, there is limited research directly comparing the effects of retinol and retinoic acid on skin.

Objectives In this study, we compare the effects of retinol and retinoic acid on skin structure and expression of skin function-related genes and proteins. We also examine the effect of retinol treatment on skin appearance.

Methods Skin histology was examined by H&E staining and *in vivo* confocal microscopy. Expression levels of skin genes and proteins were analyzed using RT-PCR and immunohistochemistry. The efficacy of a retinol formulation in improving skin appearance was assessed using digital image-based wrinkle analysis.

Results Four weeks of retinoic acid and retinol treatments both increased epidermal thickness, and upregulated genes for collagen type 1 (COL1A1), and collagen type 3 (COL3A1) with corresponding increases in procollagen I and procollagen III protein expression. Facial image analysis showed a significant reduction in facial wrinkles following 12 weeks of retinol application.

Conclusions The results of this study demonstrate that topical application of retinol significantly affects both cellular and molecular properties of the epidermis and dermis, as shown by skin biopsy and noninvasive imaging analyses. Although the magnitude tends to be smaller, retinol induces similar changes in skin histology, and gene and protein expression as compared to retinoic acid application. These results were confirmed by the significant facial anti-aging effect observed in the retinol efficacy clinical study.

Keywords: retinol, retinoic acid, epidermal thickness, collagen, wrinkle reduction, *in vivo* confocal microscopy

Introduction

Retinoids, such as retinoic acid and retinol, have been widely used in the treatment of skin aging. Retinoic acid was first shown to be an effective treatment for photoaging in both a mouse model and a clinical study

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in the mid-1980s.[1,2] Topical application of retinoic acid resulted in histological improvements including increased dermal collagen synthesis.[3] Retinoic acid was later shown to play an important role in blocking collagenase activity, thus, preventing collagen degradation, which appears to be the molecular basis of its anti-aging clinical efficacy.[4,5] Despite its anti-aging effects, retinoic acid treatment caused skin irritations such as burning, scaling, and dermatitis, limiting its acceptance by some patients.[6,7]

All-trans retinol, a precursor of endogenous retinal and retinoic acid, was recognized as an effective anti-aging treatment after it was reported to induce epidermal thickening and enhance expression of cellular retinoic acid-binding protein II (CRABPII) and cellular retinol-binding protein (CRBP) mRNA and protein, similar to the effects of retinoic acid.[8] Retinol treatment also resulted in fewer signs of erythema and skin irritation compared to retinoic acid.[8,9]

Retinol anti-aging effects include the inhibition of UV-induction of matrix metalloproteinases, and the promotion of collagen synthesis in photoaged skin.[5,10] In clinical studies, topical retinol treatment significantly improved fine wrinkles.[11] and affected markers of photoaging, including matrix metalloproteinase, collagenase, and collagen.[12] Retinol was effective in producing retinoid-mediated histological changes, such as keratinocyte proliferation.[13]

It is generally assumed that retinol is not as efficacious as retinoic acid, due to the additional step needed to convert retinol to retinoic acid;[8,14] however, very few studies have been reported directly comparing the two compounds. In this study, we compare the effects of topically applied retinol and retinoic acid formulations on histological skin changes, and expression levels of select genes and proteins involved in skin functions. A 12-week clinical study was also conducted to evaluate the effect of retinol on facial skin, especially its effect on wrinkle reduction.

Materials and methods

Skin biopsies and retinoid treatment

Skin punch biopsies were obtained from six healthy adult human volunteers (three males and three females, aged 35–55 years), without current or prior skin disease and with Fitzpatrick skin type III. Formulations of retinoic acid (0.1%), retinol (0.1%), and a base formulation as a vehicle control were applied topically on the forearms. The applications were performed under occlusion for 1 day to prevent loss and exposure to light. The applications were renewed weekly for 4 weeks following the same procedure. After the 4-week treatment, full-thickness punch biopsies (4 mm) were obtained from each site, embedded in optimal cutting temperature (OCT) compound, snap-frozen in liquid nitrogen, and stored at −70 °C for cryostat sectioning. All procedures involving human volunteers were approved by the University of Michigan Institutional Review Board, and signed informed consent was obtained from each volunteer prior to start of the study.

Retinol clinical efficacy testing

To determine the clinical efficacy of the retinol treatment, we studied 41 healthy women (aged 35–55 years), without current or prior skin disease. Subjects applied the 0.1% retinol formulation to the full face every other day for 2 weeks and then, every day for the next 10 weeks. Full face images were taken at baseline and every 4 weeks post-treatment using a Canfield VISIA-CR system (Fairfield, NJ, USA). Facial wrinkles were evaluated using the Facial Analysis Computer Evaluation System (F.A.C.E.S.), a proprietary facial image analysis software program. The computer analysis quantifies the extent of facial wrinkling using both number and severity of wrinkles. The facial images were analyzed and assigned a wrinkle score based on the quantitative analysis, as previously described.[15]

Histology and immunohistochemistry

Hematoxylin and eosin (H&E) stains of frozen sections were performed according to established protocols. Immunohistochemistry was performed as previously described.[16] In brief, skin OCT-embedded cryo-sections (7 μm) were incubated with anti-procollagen Type I or anti-procollagen Type III polyclonal antibodies (EMD Millipore, Billerica, MA,USA), and the bound antibody visualized using a secondary antibody horseradish peroxidase complex. To verify antibody specificity, the primary antibodies were substituted with a rat IgG1 isotype for procollagen I and a mouse IgG1 isotype for procollagen III. There was no visible staining for either isotype control antibody.

In vivo confocal microscopy

In vivo confocal images were taken of both forearm treatment sites and an untreated site using a VivaScope-1500 multilaser microscope (CaliberID, Rochester,

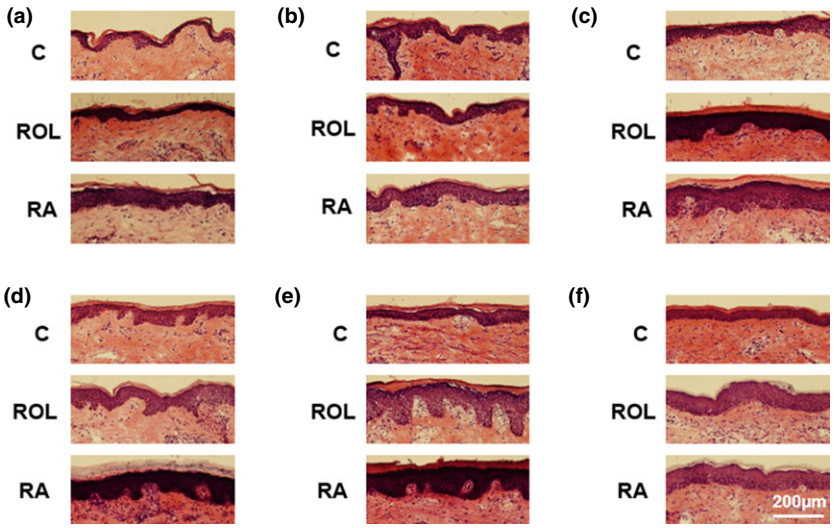

Figure 1 Epidermal thickening after retinol and retinoic acid treatment. Skin biopsy tissue sections from six different subjects (a–f). All subjects showed a thickened epidermis after retinol or retinoic acid treatment. One subject (e) showed a change from flattened to undulating dermaepidermal junction after retinol or retinoic acid treatment. C: vehicle control; ROL: retinol; RA: retinoic acid.

NY, USA) as previously described.[17–20] Representative images (image stacks) were taken every 1.52 μm from the stratum corneum to the papillary dermis. Three image stacks were taken at each treatment site.

Quantitation of epidermal thickness

Images taken by the *in vivo* confocal microscope were reconstructed into 3-D images using the NIH ImageJ software package (http://rsb.info.nih.gov/ij/), and virtual optical biopsies were obtained showing the structure across the skin depth. A mathematical method based on signal intensity was used to determine the distance between the stratum corneum and basal layer.[21,22] Epidermal thickness is defined as the minimal distance between the stratum corneum and basal layer as measured from the *in vivo* confocal images.

Images taken from H&E stained biopsy sections were analyzed by first identifying the stratum corneum and basal layers based on algorithms developed in the ImageJ software, and the epidermal thickness calculated as average distance between the stratum corneum and basal layer across the entire image.

Quantitation of gene expression

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using custom primers and probes for CRABPII (forward - 5′- CAAGACCTCGT GGACCAGAGA-3′, reverse - 5′- ACCCTGGTGCACACA ACGT-3′); filaggrin (FLG; forward - 5′-CCAGGATGAAG CCTATGACA-3′, reverse - 5′-TAACTCTGGATCCCCTAC GC-3′); corneodesmosin (CDSN; forward - 5′-CACCCCT TACAATTCCCTCT-3′, reverse - 5′-GGTGGGTTGACTAG ATGTCG-3′); protein-glutamine gamma-glutamyltransferase K (TGM1; forward - 5′-ACTACGGCCAGTTTGAC CAC-3′, reverse - 5-TCGGGAGTAATCACCAGACC-3′); protein-glutamine gamma-glutamyltransferase E (TGM3; forward - 5′-GGTAACCACGCTGAGAGAGA-3′, reverse - 5′-CACATCAGTAGCAGCGTCAC-3′); Acetyl-Coenzyme A carboxylase alpha (ACACA; forward - 5′-CCCAGATTCTGCGTTTAAGA-3′, reverse - 5′-CATCCA CAATGTAAGCACCA-3′); collagen 1 (COL1A1; forward - 5′-TCTTGGTCAGTCCTATGCGGATA-3′, reverse - 5′-C ATCGCAGAGAACGGATCCT -3′); collagen 3 (COL3A1; forward - 5′-TCTTGGTCAGTCCTATGCGGATA-3′, reverse - 5′-CATCGCAGAGAACGGATCCT -3′); Human 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR; forward - 5′-CTTGGTTTTTGGCTCTTTCA-3′, reverse - 5′-GTCAATTGCACTGATCACCA-3)′; ceramide synthase 3 (LASS3; forward - 5′-GCTATATGACTTATG GGAGG-3′, reverse - 5′-AAGATCATGAGCTGTAGGTT -3′); ceramide synthase 4 (LASS4; forward - 5′-GTTTCA ACGAGTGGTTTTG-3′, reverse - 5′-TGAATCTCTCAAA GGCAAG -3′); fibrillin1 (FBN1; forward - 5′-CACACAA CTGTGGCAAACAT-3′, reverse - 5′-CCCATGGTATTCT TGCAGTC-3′);and the housekeeping gene 36B4 (forward - 5′-ATGCAGCAGATCCGCATGT-3′, reverse - 5′-T TGCGCATCATGGTGTTCTT -3′).

Total RNA was extracted using an RNeasy Mini kit (Qiagen, Chatsworth, CA, USA), and reverse transcribed by TaqMan reverse transcription (Applied

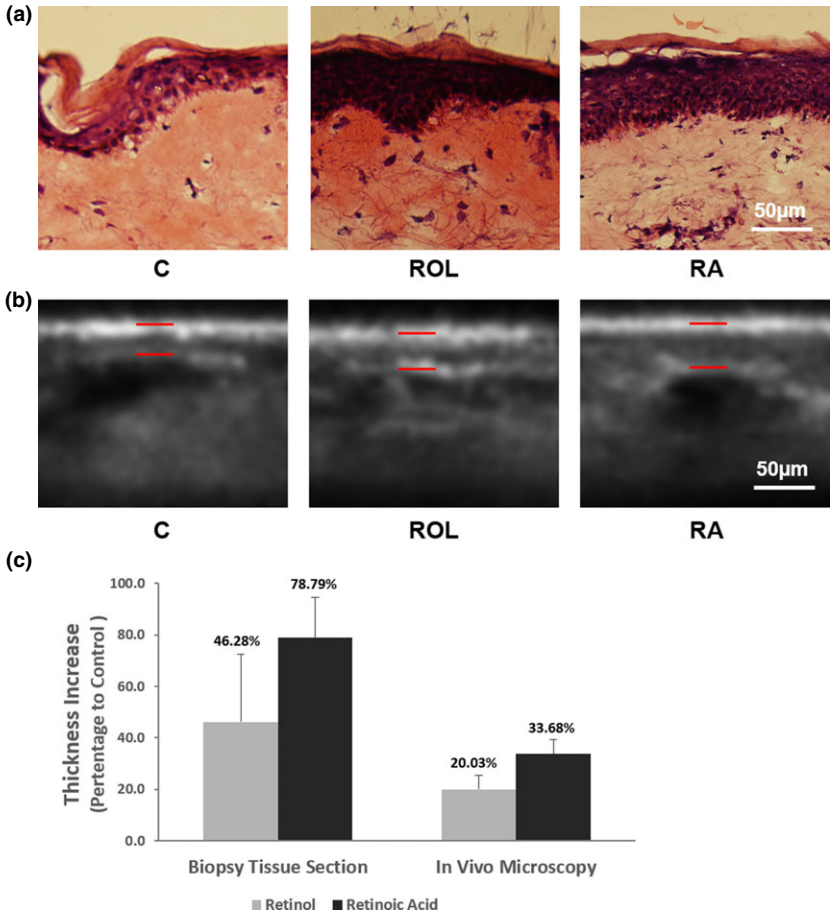

Figure 2 Epidermal thickness measurement. Epidermal thickness is measured from H&E staining (a) and noninvasive confocal imaging (b). The thickness of the epidermis from the *in vivo* confocal images is determined by the two boundaries noted in red. Results from both measurement methods (c) demonstrate significant epidermal thickening after retinol and retinoic acid treatment ($P < 0.05$). C: vehicle control; ROL: retinol; RA: retinoic acid.

Biosystems, Foster City, CA, USA). RT-PCR reactions were performed in duplicate for all genes of interest using SYBR Green PCR Mix (Applied Biosystems) and an internal control (36B4 gene) on a 7300 sequence detector system (Applied Biosystems). Results are presented as fold change in treated *vs.* vehicle control (normalized to transcript levels of 36B4).

All liquid handling procedures were performed with a calibrated robotic workstation (Biomek 2000; Beckman Coulter, Inc., Hialeah, FL, USA) to ensure accuracy and reproducibility.

Statistics

Data were analyzed using a paired t-test for comparison between treatment groups. When appropriate, logarithmic transformation of data was performed to achieve normality. For data with a small sample size, normality was assumed. Data are presented as means ± SEM.

Results

Epidermal thickening following retinol and retinoic acid treatments

Hematoxylin and eosin-stained sections from biopsy samples obtained from the six study subjects following the 4-week treatment period were analyzed for thickening of the epidermis. All subjects had a thickened epidermis following retinol and retinoic acid treatment as compared to the vehicle control (Fig. 1). At least one subject showed a change from a flattened dermaepidermal junction (DEJ) to an undulating DEJ with prominent rete ridges after retinol as well as retinoic acid treatment (Fig. 1e).

Table 1 Expression of skin function-related genes following topical application of retinol and retinoic acid

Genes	Retinol		Retinoic acid	
	Fold change	Upregulation	Fold change	Upregulation
Collagen Type 1 (COL1A1)	1.34**	4/6	2.48*	5/6
Collagen Type 3 (COL3A1)	1.43*	5/6	2.77*	6/6
Cellular retinoic acid-binding protein II (CRABPII)	1.36*	6/6	1.33**	4/6
Filaggrin (FLG)	1.51*	6/6	2.01*	6/6
Protein-glutamine gamma-glutamyltransferase K (TGM1)	1.35*	5/6	1.60**	5/6
Protein-glutamine gamma-glutamyltransferase E (TGM3)	1.57*	5/6	1.58*	4/6
Acetyl-CoA carboxylase 1 (ACACA)	1.01	3/6	1.23	4/6
Ceramide Synthase 3 (LASS3)	2.68	3/6	2.8	3/6
Ceramide Synthase 4 (LASS4)	1.36	3/6	1.22	4/6
Corneodesmosin (CDSN)	1.16	3/6	1.33	3/6
Fibrillin-1 (FBN1)	0.95	2/6	1.06	2/6
HMG-CoA reductase (HMGCR)	1.10	3/6	1.26	3/6

Data are fold changes of gene expression over the vehicle control, and number of subjects out of six showing increased gene expression. *$P < 0.05$; **$P < 0.1$.

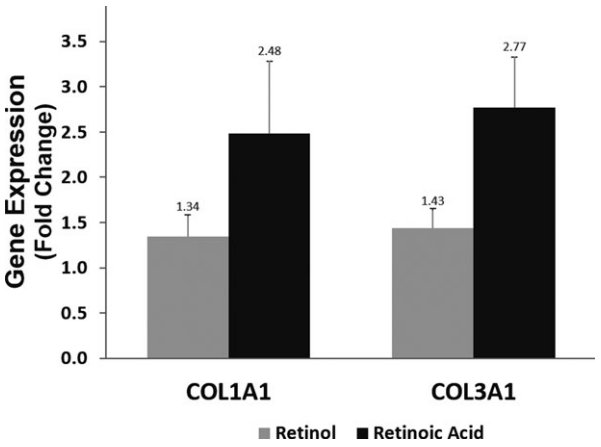

Figure 3 Collagen type 1 (COL1A1) and collagen type 3 (COL3A1) genes were upregulated following retinol and retinoic acid treatment. The results shown are fold change over the vehicle control.

Measurements of epidermal thickness by H&E staining and *in vivo* confocal microscopy are shown in Figure 2. Consistent with visual observations of the H&E-stained samples, quantitative measurement of the epidermal layer of the biopsy tissue sections following treatment with retinol and retinoic acid showed an increase in epidermal thickness over control values of 46.28% and 78.79%, respectively (Fig. 2a,c). The epidermal thickness based on *in vivo* confocal imaging, measured from the stratum corneum to the top of the dermal papillae, increased following retinol and retinoic acid treatments by 20.03% and 33.68% over control values, respectively, (Fig. 2b,c).

Change in gene expression following retinol and retinoic acid treatments

The epidermal thickening following treatment with retinol and retinoic acid suggests underlying molecular changes. To elucidate the molecular mechanism causing skin changes, we quantitated the expression of 12 genes involved in skin functions. Six genes showed statistically significant changes (Table 1) and six others showed no statistical significance, partly due to the small number of subjects. Overall, the results demonstrate a trend toward upregulation of a majority of these genes following both retinol and retinoic acid treatment. For instance, increases in gene expression following retinol and retinoic acid treatments occurred for COL1A1, and COL3A1 (Fig. 3), which encode for procollagen I and procollagen III proteins, respectively. The increase in COL1A1 and COL3A1 gene expression was 1.34- and 1.43-fold over the control following retinol treatment, and 2.48- and 2.77-fold change over the control after retinoic acid treatment, respectively (Fig. 3). Of the six subjects, the majority of them (4–6 subjects in each group) had an increase in COL1A1 and COL3A1 gene expression following retinol and retinoic acid treatment.

Enhanced protein synthesis of procollagen I and procollagen III following retinol and retinoic acid treatments

Following the upregulation of COL1A1 and COL3A1 gene expression, we examined the protein synthesis of procollagen I and procollagen III using immunohistochemical staining. The results demonstrate an increase

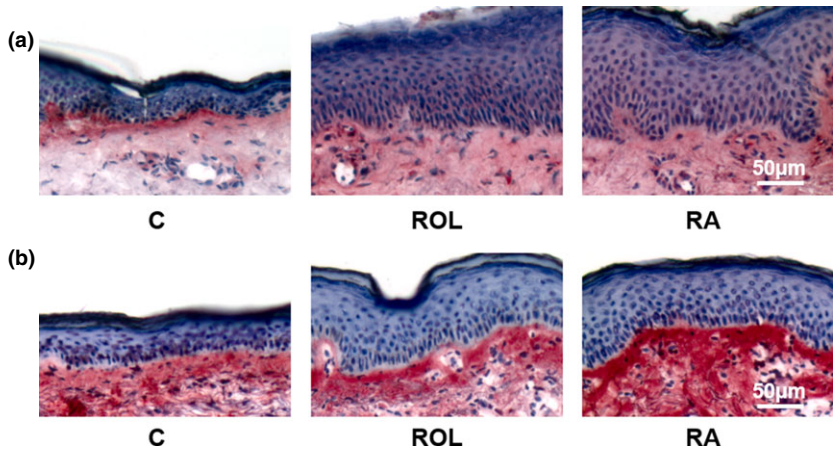

Figure 4 Immunohistochemical staining shows enhanced protein synthesis of procollagen I (a) and procollagen III (b) following retinol and retinoic acid treatment, which are most prominently present in the papillary dermis and stained in red. C: vehicle control; ROL: retinol; RA: retinoic acid.

in both proteins following retinol and retinoic acid treatments, most notably at the papillary dermis (Fig. 4). Retinol and retinoic acid treatment showed similar increases in procollagen I protein synthesis, and greater protein synthesis for procollagen III was observed following retinoic acid treatment as compared to retinol treatment. Overall, both retinol and retinoic acid increased new collagen synthesis in the skin. The immunohistochemical staining also showed epidermal thickening after retinol and retinoic acid treatment similar to that seen with the H&E images.

Effect of retinol treatment on facial skin appearance

The analysis of the skin biopsies demonstrated that both retinol and retinoic acid treatments increased epidermal thickening, upregulated gene expression related to skin functions, and increased collagen synthesis in skin. Based on these results, we examined the effect of a 0.1% retinol formulation on facial wrinkles. Full face images were obtained from the female subjects during the 12-week retinol treatment period (Fig. 5a), and the extent of facial wrinkles were analyzed using the proprietary F.A.C.E.S. software. A wrinkle score was calculated to reflect both wrinkle number and severity.[15] The facial wrinkle analysis showed a significant reduction in wrinkle scores following retinol treatment over the 12-week period (Fig. 5a). Wrinkle reduction was observed as early as 4 weeks, with a wrinkle score reduction of 58.68% at the cheeks and 27.93% in eye areas (Figs 5b and 6). Wrinkle scores were reduced by 63.74% at the cheeks and 38.74% in the eye areas after 12 weeks of treatment.

Discussion

Retinol has been used in cosmetics for several decades, ever since it was shown to have similar anti-aging effects as retinoic acid but with fewer adverse effects. Despite reports of similar efficacy, exactly how well retinol performs as compared to retinoic acid has not been fully elucidated; therefore, a direct comparison of the effects of retinol and retinoic acid on skin histology and molecular markers would be beneficial.

In this study, we demonstrate that retinol treatment caused significant epidermal thickening similar to that seen in the retinoic acid-treated sites. Epidermal thickening is believed to be caused by increased mitotic activity and is an indicator of retinoid effect on skin.[8] These results suggest that both retinol and retinoic acid enhance skin cell proliferation.

A consistent histological feature of skin aging has been shown to be a flattening of the DEJ with effacement of rete ridges and decrement of dermal papillary projections.[23,24] In this study, we observed the reversal of this process in several biopsy samples where a flattened DEJ became undulating with prominent rete ridges following retinol treatment (Fig. 1). A similar change in DEJ following retinoic acid treatment has been reported,[25] thus suggesting that retinol has similar anti-aging effects compared to retinoic acid.

Changes in extracellular matrix and especially collagen expression are associated with skin aging.[26–29] In this study, the significant upregulation of COL1A1 and COL3A1 gene expression correlates with an increase in procollagen I and procollagen III protein synthesis following retinol and retinoic acid treatment. These

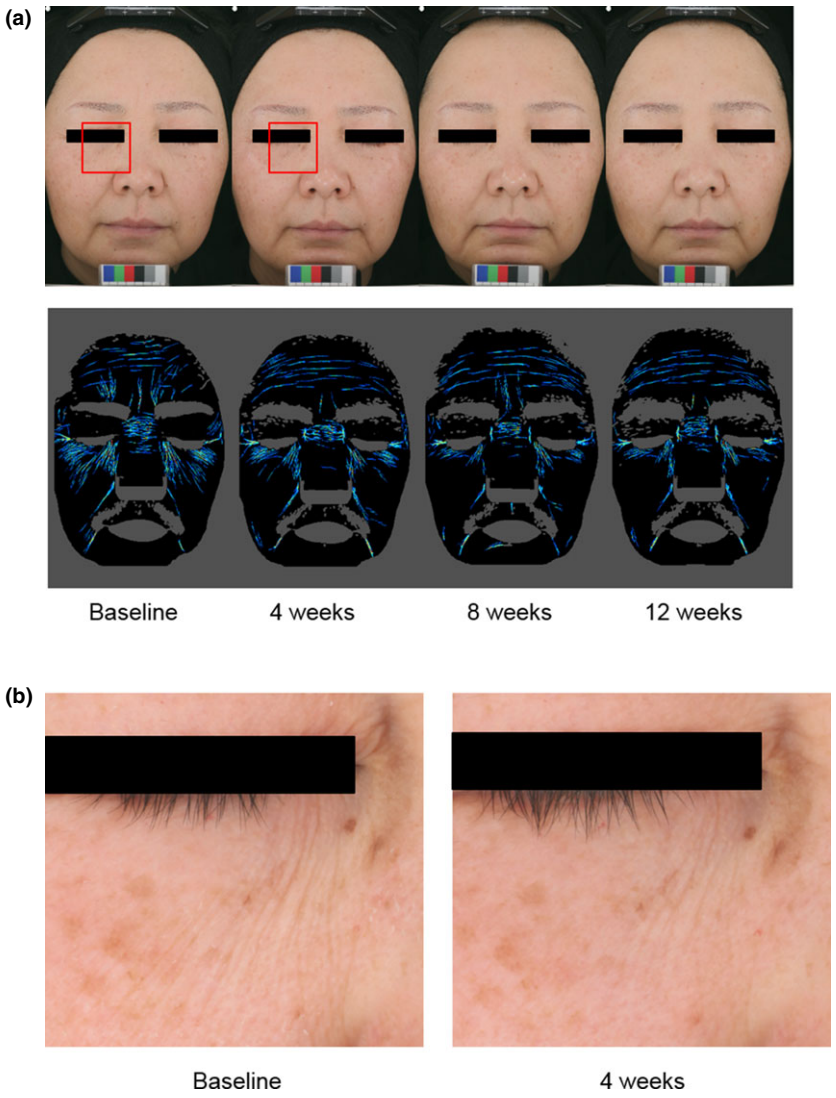

Figure 5 Clinical assessment of wrinkle reduction. (a) Full face images taken at baseline and every 4 weeks post-treatment with a Canfield VISIA-CR system (Top). Analysis output of facial wrinkles using the Facial Analysis Computer Evaluation System (F.A.C.E.S.), a facial image analysis software (Bottom). (b) Close-up images around the eye and cheek areas at the baseline and 4 weeks. These areas are indicated by the red squares in the full face images.

results demonstrate that, although a smaller magnitude, the effects of retinol are similar to retinoic acid in promoting collagen synthesis. Analysis of expression of other genes related to skin functions also support the evidence that skin effects of retinol are similar to those of retinoic acid (Table 1). For instance, CRABPII has been shown to be directly involved in the cellular retinoid response[8] and in this study, is significantly upregulated following both retinol and retinoic acid treatment.

Enhanced collagen synthesis is a contributing factor for improved and younger looking skin, in part, through wrinkle reduction.[30–33] A recent study showed that the clinical outcome following retinol treatment did not differ from one using retinoic acid to treat photoaging.[34] The results from our clinical study show similar effects following 12-weeks of retinol treatment as demonstrated by significant wrinkle reduction. These clinical results combined with the histological and molecular changes following retinol treatment are evidence that retinol is as effective in treating aged skin as seen with retinoic acid treatment.[11]

In addition to the comparison of the effects of retinal and retinoic acid on skin, this research also provides a

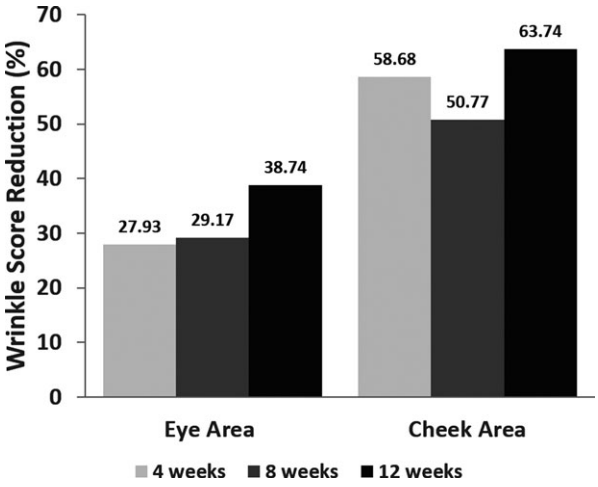

Figure 6 Quantitative analysis of facial wrinkles using the facial image analysis software, Facial Analysis Computer Evaluation System (F.A.C.E.S.) on data collected every 4 weeks of the 12-week clinical study. Data shown are percent wrinkle reduction from baseline as calculated using wrinkle scores.

potential technological improvement in skin histology measurements. H&E-stained histological images from tissue biopsies have long been considered the gold standard for structural analysis. As an emerging technology in the field of clinical and cosmetic dermatology, *in vivo* noninvasive confocal microscopy is gaining popularity as a more convenient alternative to skin biopsies.[35–37] In this study, we measured and compared the epidermal thickening by both skin biopsy and *in vivo* confocal imaging (Fig. 2). Both methods confirmed the observation of epidermal thickening following retinoic acid and retinol treatment. The difference in the percent increase in the epidermal thickness between the two methods is likely only due to the differences in definition of skin thickness, as the ratios between retinol and retinoic acid measurements were almost identical in both methods ($\sim$59%). These results demonstrate that non-invasive *in vivo* imaging technology is capable of capturing many structural changes without taking conventional skin biopsies. *In vivo* confocal imaging provides the researcher with a quick and easy tool to screen and test retinoid effects on skin.

In conclusion, this study demonstrates that topical application of retinol significantly affects cellular and molecular properties of both the epidermis and dermis, as revealed by noninvasive imaging and skin biopsy analyses. Topical retinol treatment induces similar changes in skin histology, and skin-related gene and protein expression as seen with retinoic acid application, and the magnitude is more than half of that from retinoic acid treatment in several structure and gene expression analyses. These results were supported by the significant facial anti-aging effect observed in the retinol efficacy clinical study.

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