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ORIGINAL RESEARCH ARTICLE

The Anti-Wrinkle Efficacy of Argireline, a Synthetic Hexapeptide, in Chinese Subjects

A Randomized, Placebo-Controlled Study

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Abstract

Background Argireline is a synthetic peptide that is patterned from the N-terminal end of the protein SNAP-25 and has been shown to reduce the degree of facial wrinkles. It is reported to inhibit vesicle docking by preventing formation of the ternary SNARE complex and by interfering in catecholamine release. The anti-wrinkle efficacy of argireline has not been studied in Chinese subjects.

Objective The objective of the study was to evaluate the safety and efficacy of argireline in the treatment of peri-orbital wrinkles in Chinese subjects.

Methods A total of 60 subjects received a randomized treatment of argireline or placebo in a ratio of 3:1. Argireline or placebo was applied to their peri-orbital wrinkles twice daily for 4 weeks, and then evaluations were made for the improvements in wrinkles. In the subjective evaluation, Daniell's classification and Seeman's standard were applied to make a global assessment of changes in the appearance of peri-orbital lines. In the objective evaluation, silicone replicas of the skin at the application area were made before and after the treatment, which were analyzed by a wrinkle-analysis apparatus.

Results In the subjective evaluation, the total anti-wrinkle efficacy in the argireline group was 48.9 %, compared with 0 % in the placebo group. In the objective evaluation, the parameters of roughness were all decreased in the argireline group ($p < 0.01$), while no decrease was obvious in the placebo group ($p > 0.05$).

Conclusions This study showed that argireline had a significant anti-wrinkle effect in Chinese subjects.

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1 Introduction

Nowadays, the desire to maintain a youthful appearance has driven the development of dermatologic cosmetics designed to rejuvenate the aging face. Argireline, a synthetic hexapeptide, is one of the new popular options to treat aging skin. It is a unique peptide that is used to reduce existing wrinkles, especially in the forehead and around the eyes.

The synthetic hexapeptide is acetyl hexapeptide-3 (AC-gly-glu-met-gln-arg-arg-NH_2), patterned from the N-terminal end of the protein SNAP-25. The identification of argireline is the result of efforts to find an effective but less toxic synthetic version of botulinum neurotoxin type A (BoNTA) [1, 2]. It has been found that this peptide can inhibit vesicle docking by preventing formation of the ternary soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (a vesicular fusion complex required to drive Ca^{2+}-dependent exocytosis). It also interferes in catecholamine release, which is involved in synaptic vesicle exocytosis [3, 4]. These effects closely relate to the basic biochemical mechanisms of wrinkle formation. The hexapeptide is called argireline [5]. Argireline is currently marketed in China by McEit (Tianjin) International Trade Co. Ltd.

Argireline inhibits the repetitive contraction of the intrinsic muscles of facial expression and thereby reduces hyperkinetic facial lines [6]. One open-label trial in which ten women received twice-daily applications of 5 % argireline cream demonstrated a 27 % improvement in peri-orbital lines after 30 days, as measured by a silicone replica analysis [1]. In another study, with healthy American women volunteers, argireline solution reduced the depth of wrinkles up to 17 % after 15 days, and 30 % after 30 days [5]. Theoretically, argireline may mimic the effects of

BoNTA injection, by reducing hyperkinetic lines associated with muscles of facial expression. However, currently only BoNTA has been approved for subcutaneous, intradermal, and intramuscular injection for facial wrinkles by the US FDA [7].

The objective of this study is to test the efficacy and tolerability of argireline applied to peri-orbital wrinkles in Chinese subjects.

2 Materials and Methods

The study design was a prospective, double-blind, randomized, placebo-controlled, parallel-group, comparative study in China to investigate the effects of argireline in subjects with moderate to severe peri-orbital lines as assessed at natural expression. A total of 60 subjects were randomized to receive a single treatment of argireline or placebo in a ratio of 3:1. A total sample size of 40 patients was sufficient to have an 85 % chance of detecting a 60-percentage point difference between the treatment groups in the proportion of patients reporting a global assessment score of 2 or greater, significant at the 0.05 level and with a randomization ratio of 3:1. We enlarged the number of patients to 60. Participants applied argireline or placebo to their peri-orbital wrinkles twice daily for 4 weeks, subjective and objective evaluations were made for the improvements in wrinkles. The study was approved by the Ethical Committee of the College of Medicine of Xi'an Jiaotong University, Xi'an, China.

The patient population of 60 Chinese volunteers with different degrees of peri-orbital wrinkles was randomly selected from outpatients in the Department of Dermatology from The Second Hospital of Xi'an Jiaotong University, Xi'an, China. All of them desired to reduce their wrinkles. Participants ranged from 25 to 60 years of age, whose natural expression showed peri-orbital lines of at least moderate severity. Exclusion criteria included known allergy or sensitivity to the medication or its components, infection, or other skin disease at the treatment region. The subjects were advised not to use any other facial cosmetic around the peri-orbital area or undergo any aesthetic medical treatment (e.g. face lift surgery, resurfacing, or filler treatment) during the study period. They were also requested to complete the entire course of the study and to comply with study instructions. All of the volunteers gave their consent before enrollment and they had the right to withdraw at any time during the study if they had any complaint.

Each vial contained 10 % argireline in an oil and water (O/W) emulsion without preservatives. The placebo solution was a non-active O/W emulsion alone, without argireline. Vials of argireline and placebo with identical investigational labels, which prevented identification of the contents, were all prepared by McEit (Tianjin) International Trade Co. Ltd.

For the subjective evaluation, investigators applied Daniell's [8] classification to evaluate the peri-orbital wrinkles at natural expression before use, and after the first, second, third, and fourth week (graded on a 4-point wrinkle severity scale: none, mild, and moderate to severe). After 4 weeks, investigators made a global assessment of changes in the appearance of peri-orbital lines by Seeman's [9] standard, graded on a 5-point scale ranging from 0 (no change) to 4 (100 % improved). The total anti-wrinkle efficacy was calculated as the percentage of subjects who were graded 3 or 4 on the global assessment of improvement scale after 4 weeks.

For the objective evaluation, silicone replicas of the skin at the application area were made before and after the treatment period. These were analyzed by a wrinkle-analysis apparatus (Skin-Visioline VL 650®; Courage+Khazaka Electronic GmbH, Germany). The silicone replicas were processed by confocal laser scanning microscopy to assess the evolution of the wrinkles and to record gray level images of the wrinkles. Confocal microscopy in reflection mode and three-dimensional analysis were used to assess the different parameters of roughness. Then, the relevant parameters of roughness obtained were analyzed by statistical analysis.

The statistical software used in this study was Windows SPSS 17.0.

3 Results

3.1 Subjective Evaluation

Patient demographics and baseline wrinkle evaluations are shown in Table 1. After 4 weeks, none of the subjects discontinued the study and no one experienced any adverse effect. After evaluation and classification of the wrinkles before and after treatment, the total anti-wrinkle efficacy in the argireline group was 48.9 % (22/45), compared with 0 % in the placebo group (Fig. 1). The improvement in the appearance of wrinkles in two patients is shown in Figs. 2 and 3.

3.2 Objective Evaluation

Two subjects' gray level images of peri-orbital wrinkles are shown in Figs. 4 and 5. It can be concluded from Fig. 4 that in this subject the wrinkles count is 14 before treatment, while it is 7 after treatment. In another subject, the wrinkles count is 13 before treatment but 9 after treatment

Table 1 Patient characteristics and baseline peri-orbital wrinkles at natural expression

Variable	Argireline ($n = 45$)	Placebo ($n = 15$)	p-Value
Patient characteristics			
Age, mean (y)	43.7	41.3	0.07
Sex, n (%)			
Female	38 (84.4)	12 (80)	0.08
Male	7 (15.6)	3 (20)	
Baseline severity of peri-orbital wrinkles at natural expression, n (%)			
None	0 (0)	0 (0)	0.13
Mild	14 (31.1)	4 (26.7)	
Moderate	19 (42.2)	8 (53.3)	
Severe	12 (26.7)	3 (20)	

in Fig. 5. Furthermore, the average depth of wrinkles, deepest wrinkle, total wrinkle volume, total wrinkle area, total form factor wrinkles, and total length of wrinkle are all decreased after the treatment in the two subjects.

After silicone replicas were processed by Skin-Visioline VL 650®, various parameters of roughness were obtained and analyzed by SPSS 17.0 statistical software. The parameter Sa represents the average wrinkle height in one place, the parameter Smax is the difference from a peak to the lowest point of all the wrinkles in the region, and the parameter St represents the average wrinkle height over all the wrinkles in the region. These parameters were all decreased in the argireline group ($p < 0.01$), while they were not obviously decreased in the placebo group ($p > 0.05$) [Table 2].

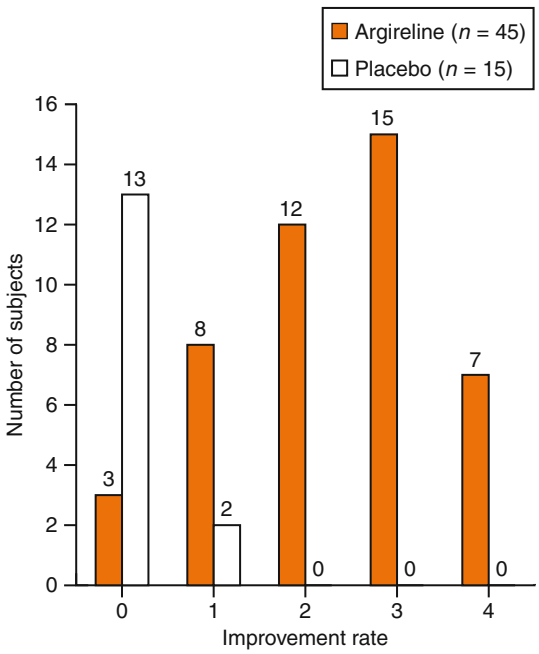

Fig. 1 The number of subjects with different improvement rates. 0 = no improvement; 4 = 100 % improvement

4 Discussion

BoNT brought about a revolution in cosmetic science because of its remarkable and long-lasting anti-wrinkle activity. BoNT rejuvenates the aging face by reducing hyperkinetic lines associated with muscles of facial expression and is the most popular aesthetic product. In 1992, Carruthers and Carruthers [10] investigated selectively injecting BoNTA to treat glabellar wrinkles. Since then, other studies have continuously corroborated their results [11–16]. In 2006, injection of BoNTA was the most frequently performed cosmetic procedure in the USA, with over 3 million patients receiving injections [17].

Physiologically, the formation of wrinkles appears to be due, at least partly, to the excessive stimulation of the muscle fibers in the face, which pull the skin inwards giving rise to the well known wrinkle [18, 19]. Thus, a useful strategy to reduce the intensity of wrinkles is to downregulate muscle action either directly or by attenuating the activity of the innervating neuron [20, 21]. In support of this tenet, treatment with BoNTA significantly reduces the intensity of wrinkles. BoNTA strongly inhibits the Ca^{2+}-dependent neurotransmitter release in neurons. These proteins are metalloproteases that specifically cleave synaptic proteins essential for regulated neuronal exocytosis, specifically the vesicular protein VAMP (a vesicle-associated membrane protein, which is essential for the docking and fusion of the synaptic vesicle to the presynaptic membrane for the release of acetylcholine) and the membrane proteins syntaxin and SNAP-25. As a result, the critical protein fusion complex assembled by these proteins, known as the SNARE complex, is destabilized preventing vesicle fusion with plasma membrane, and consequently abrogating Ca^{2+}-triggered exocytosis [22].

Although botulinum toxins, especially BoNTA, have been extensively used to attenuate facial signs of aging, their use is seriously limited because of their high toxicity

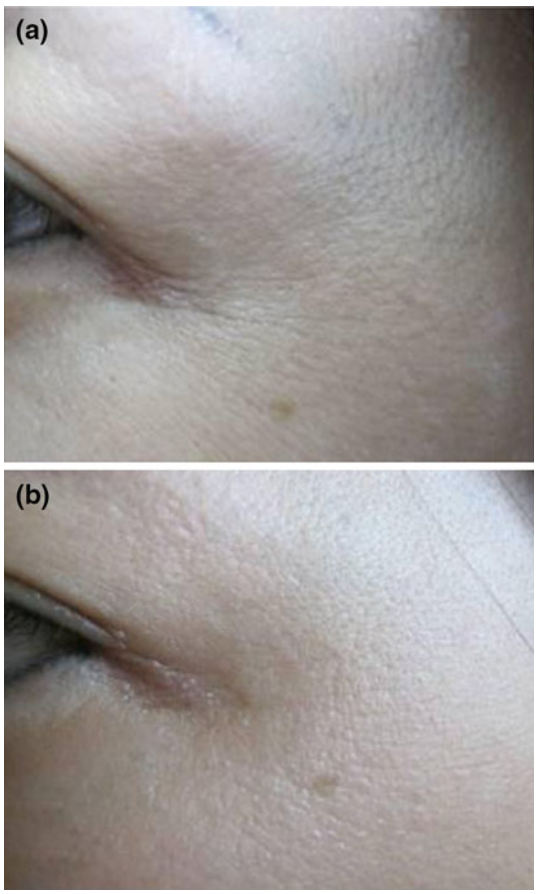

Fig. 2 Changes seen in a subject (female, aged 40 years), (**a**) before and (**b**) after treatment with argireline

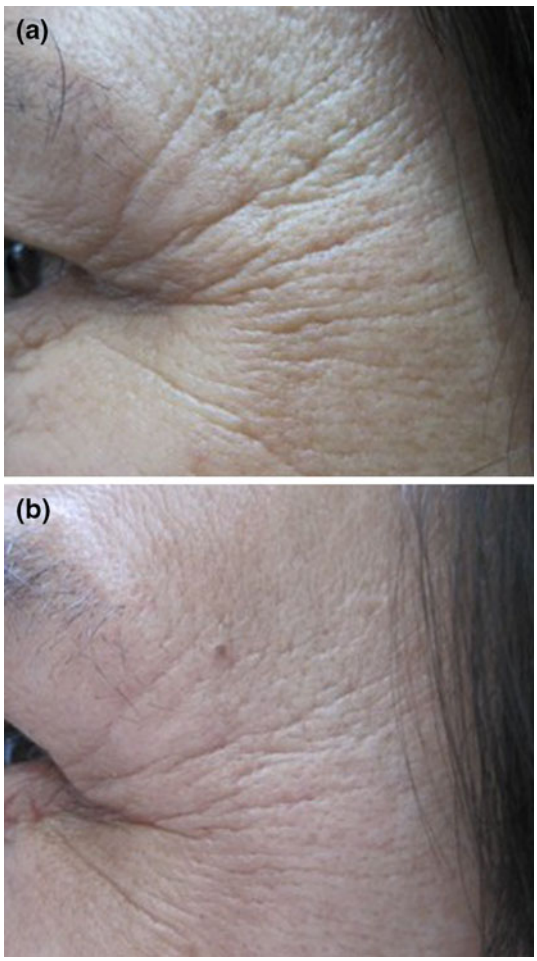

Fig. 3 Changes seen in a subject (male, aged 49 years), (**a**) before and (**b**) after treatment with argireline

(human lethal dose, 50 % [LD_{50}] $\approx$ 2,500 biologic mouse units) [20]. Thus, there is a need to design and validate non-toxic molecules that mimic the action of BoNTA [1, 2]. In this regard, a 6-mer peptide (Ac-EEMQRR-NH_2) that emulates the amino acid sequence of the synaptic protein SNAP-25 is shown to be a specific inhibitor of neurosecretion at micromolar concentrations. It is patterned after the N-terminal domain of SNAP-25 (aa 12–17) and exhibits a significant capacity to permeate through the skin. Toxicologic and primary irritation data indicate that it is well tolerated. This hexapeptide is called argireline [5].

Analysis of the mechanism of action showed that argireline significantly inhibited neurotransmitter release with a potency similar to that of BoNTA. Inhibition of neurotransmitter release was due to the interference of the hexapeptide with the formation and/or stability of the SNARE ternary complex that is required to drive Ca^{2+}-dependent exocytosis. Notably, this peptide did not exhibit in vivo oral toxicity or primary irritation at high doses [5]. Therefore, this hexapeptide represents a biosafe alternative to BoNTA in cosmetics to attenuate facial wrinkles.

In this study, after 4 weeks of application of argireline on peri-orbital wrinkles, none of the human subjects experienced any adverse effect, the total anti-wrinkle efficacy was 48.9 %, and the wrinkle parameters were all decreased in the argireline group. All the findings demonstrated that the anti-wrinkle activity of argireline was significant, in agreement with its cellular activities.

Although our study identified that argireline had a significant anti-wrinkle effect, there were some limitations. Most importantly, the best way to demonstrate changes in the skin is to do pre- and post-biopsies and then to compare the histologic changes, which is the gold standard to determine effectiveness in improving quality of the skin. Unfortunately, we were unable to persuade the volunteers to do biopsies because of concerns they had regarding postoperative scarring. Potential errors with silicone impressions include that tissue edema caused by the study medication or even mild rubbing can produce better than deserved results, although these errors can also impact on

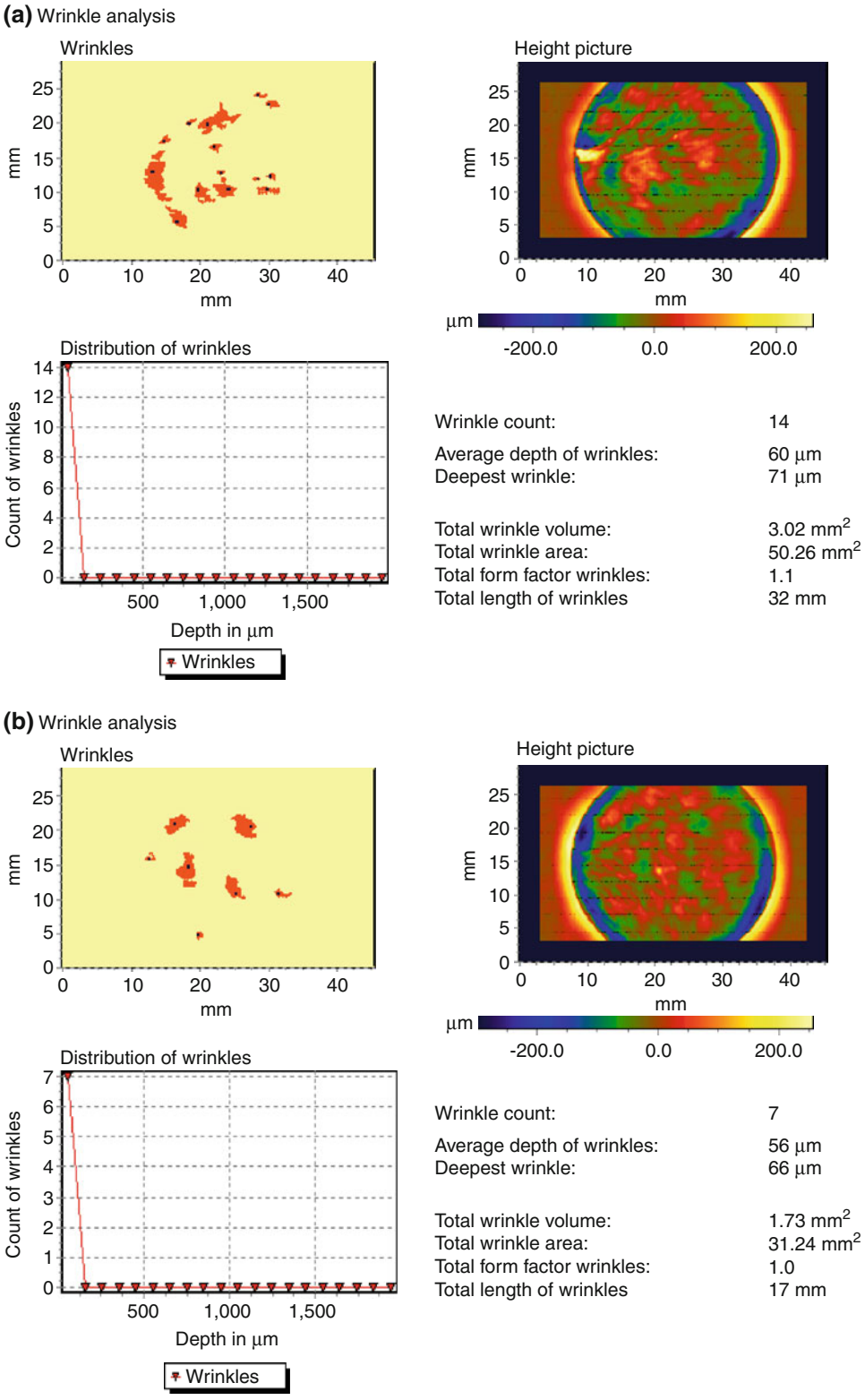

Fig. 4 A subject's (female, aged 45 years) gray level images of peri-orbital wrinkles processed by Skin-Visioline VL 650®, (**a**) before and (**b**) after treatment

placebo results. However, silicone impressions provide non-invasive results that are far better than photography, and this method is relatively mature having been used in many previous studies [1, 5]. For these reasons, it was selected for the objective measurement in this study, and our best efforts made to avoid potential errors.

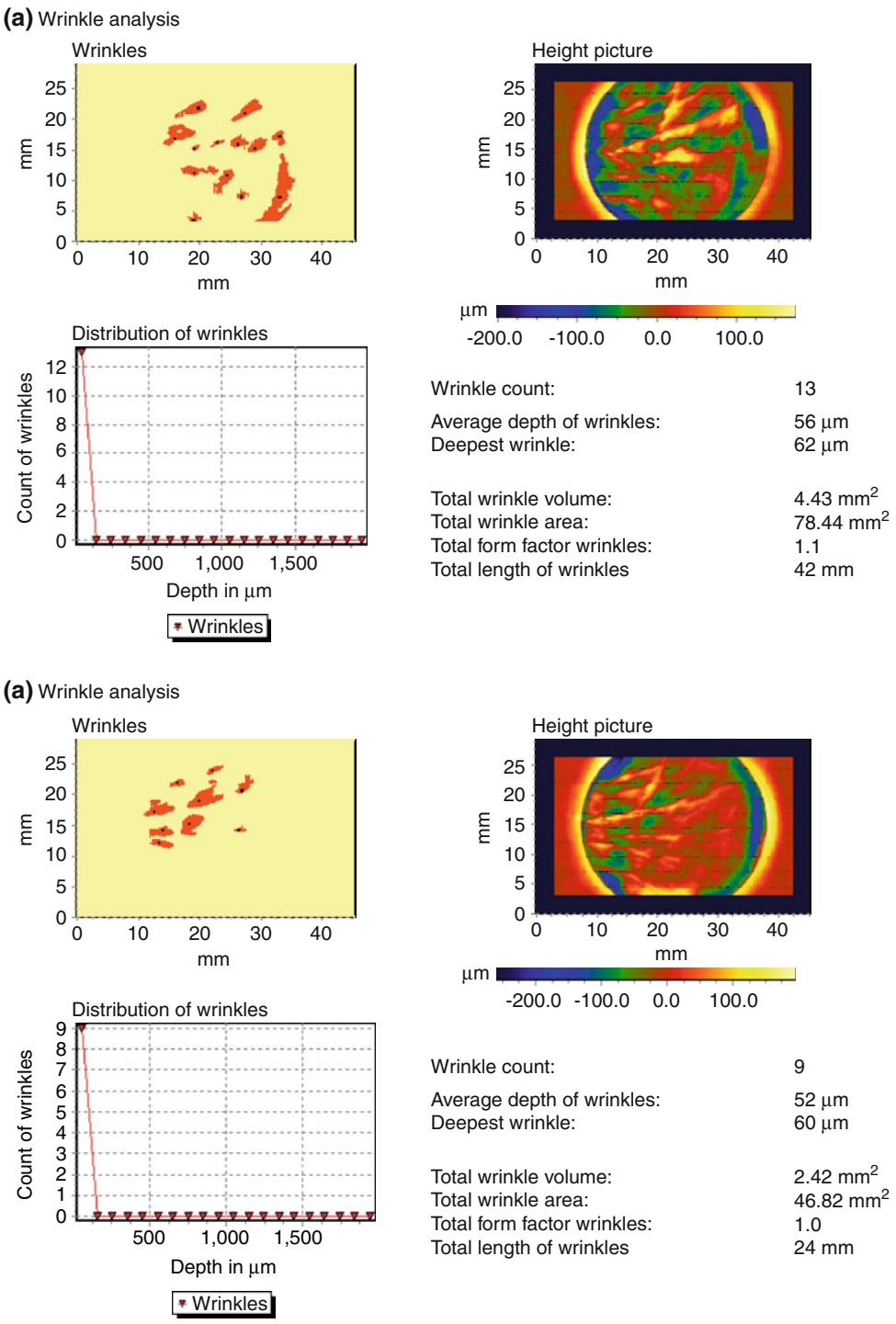

Fig. 5 A subject's (male, aged 55 years) gray level images of peri-orbital wrinkles processed by Skin-Visioline VL 650®, (**a**) before and (**b**) after treatment

5 Conclusions and Prospects

Applying argireline around the eyes for up to 4 weeks was an effective treatment for reducing the severity of peri-orbital wrinkles in Chinese people with moderate and severe peri-orbital lines, and argireline was safe and well tolerated in this study group.

Argireline, one of the cosmeceutical peptides, is a new popular option to treat aging skin. It is not considered as a drug and is therefore not regulated by the FDA [1, 2]. Although much less potent than BoNTA (12 vs. 0.003 assigned amount units), this small peptide exhibits the great advantage of insignificant acute toxicity (≥2000 mg/kg) as compared with BoNTA (20 ng/kg). Furthermore, the

Table 2 The parameters of the different groups ($\bar{x} \pm$ sd)

Parameter	The argireline group	The placebo group
Sa	9.2000 ± 2.28576*	5.3000 ± 2.22919
Smax	138.000 ± 30.69133*	99.3000 ± 14.05754
St	131.5000 ± 41.66569*	108.0000 ± 17.01732

Sa the average wrinkle height in one place, *Smax* the difference from a peak to the lowest point of all the wrinkles in the region, *St* the average wrinkle height over all the wrinkles in the region

* $p < 0.01$ for differences between before and after the treatment

hexapeptide does not show primary skin irritation in an intracutaneous test or genotoxicity as determined by the Ames test, thus making its use safe and physician independent [5]. Therefore, peptides that mimic the action of BoNTA, such as argireline, represent the next generation of biosafe products with anti-wrinkle activity that could be extensively used in cosmetic preparations.

Most studies used to assess the incorporation of these ingredients into skin care products are in vitro. In this study, clinical data are presented to suggest that argireline may have a place in a comprehensive skin care protocol for aging skin. A study with larger samples is planned that will provide more comprehensive data to support the present study.

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Immediate and Long-term Clinical Benefits of a Topical Treatment for Facial Lines and Wrinkles

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ABSTRACT

Objective: To evaluate the efficacy and tolerance of a novel line treatment for periocular and perioral wrinkles. The line treatment was formulated with multiple growth factors, antioxidants, and a collagen-building peptide—ingredients that have been shown to increase collagen levels and provide long-term aesthetic benefits. To help provide immediate smoothing effects, hyaluronic acid filling spheres and a muscle contraction-inhibiting peptide were also included in the formulation. **Design:** Three-month, single-center, open-label, clinical study with clinical assessments at Baseline, Minutes (within 15 minutes of initial application), Month 1, and Month 3. **Treatment:** Subjects treated periocular and perioral wrinkles twice daily for three months with the line treatment. **Participants:** Thirty-seven females, 33 to 45 years of age, with mild-to-moderate, fine and coarse periocular and perioral wrinkles, were enrolled in the study. **Measurements:** Investigator assessments of fine and coarse periocular and perioral wrinkles, digital photography, and tolerance assessments were conducted at all visits. Subject self-assessment questionnaires were conducted within 15 minutes of initial application and at Month 3. **Results:** Investigator assessments of both periocular and perioral wrinkles showed statistically significant improvements over Baseline within minutes of initial application; these positive findings continued to improve through Months 1 and 3 (all $P \leq 0.0003$). No treatment-related adverse events were reported. **Conclusions:** The results from this study demonstrate that this uniquely formulated line treatment was well tolerated and provided both immediate and long-term improvements in the appearance of fine and coarse wrinkles.
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One of the most prominent signs of skin aging is the development of fine lines and wrinkles caused by both intrinsic and environmentally induced aging processes.[1] Intrinsic aging is a naturally occurring process relating to chronological age; whereas, environmentally induced aging results from external factors, the most notable of which is ultraviolet (UV) exposure. The main structural changes resulting from both types of aging are characterized by a reduction in collagen and elastin and a loss in hydration; all of which contribute to the appearance of lines and wrinkles. Reactive oxygen species (ROS), a by-product of both environmentally induced and intrinsic aging, cause a cascade of biochemical reactions within the skin, which results in the production of matrix metalloproteinases (MMPs) and proinflammatory cytokines. MMPs, secreted by fibroblasts and keratinocytes, decrease collagen formation and enhance collagen degradation, contributing to the breakdown of the dermal matrix.[2–4] Proinflammatory cytokines lead to the degradation of elastin and also cause the production of additional ROS.

In addition to these structural changes in collagen and elastin, facial areas associated with expression movement, such as the periocular and perioral areas, are especially vulnerable to wrinkle formation. As a result, it can be challenging for physicians and patients to find a topical treatment that produces visible improvement in these areas.

DISCLOSURE: Drs. Trookman and Rizer received grant payments from SkinMedica as clinical investigators for the study. Ms. Ford and Ms. Ho are employed by SkinMedica. Mr. Gotz is a consultant for SkinMedica. Financial support for this study was provided by SkinMedica, Inc.
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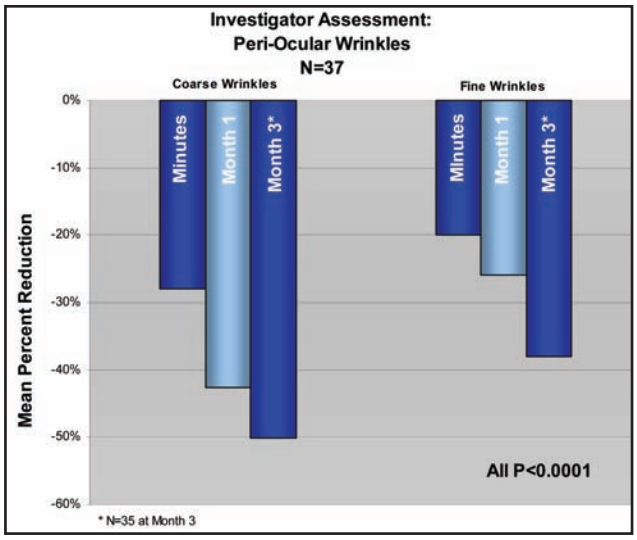


Figure 1. Mean reductions from baseline in periocular wrinkles

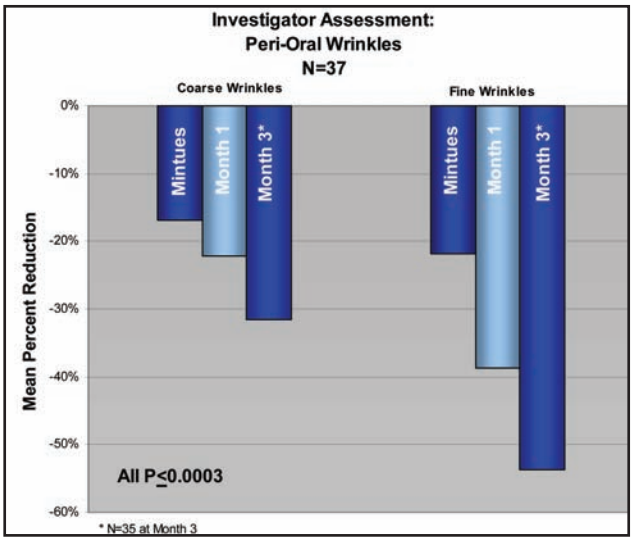


Figure 2. Mean reductions from baseline in perioral wrinkles

To address the challenge of treating expressive facial areas, a topical line treatment containing a combination of ingredients was formulated to provide immediate improvements in the visible signs of aging as well as long-term anti-aging benefits. Multiple growth factors, peptides, and antioxidants were incorporated into the formulation for their ability to increase collagen levels by targeting various biochemical processes involved in collagen production and degradation. In addition, to affect immediate physical improvements in wrinkle appearance, a muscle contraction-inhibiting peptide and dehydrated hyaluronic acid (HA) filling spheres were included in the formulation. The hygroscopic properties of HA and its ability to create and fill space have been well documented.[5,6] The filling spheres, which penetrate into superficial folds of the skin, take advantage of HA's properties and swell when hydrated, exerting pressure in the direction of the skin surface resulting in a smoothing effect on the skin. To soften the appearance of fine lines, light-diffusing ingredients, such as specialized polymeric microspheres, were also included in the line treatment.

In recent years, growth factors have emerged as novel anti-aging agents due to their active role in dermal wound repair.[7] The significance of the wound-healing process relates to the similarities between the biochemical pathways involved in the aging process and wound formation.[8] Both processes stimulate the release of growth factors, which affect a variety of biochemical pathways critical to repair of the dermal matrix. In addition, topical growth factors have been shown in clinical studies to decrease the appearance of wrinkles and to stimulate collagen synthesis.[8–11]

Peptides are another group of novel ingredients included in the line treatment that play an important role in collagen synthesis and degradation. These protein building blocks are used in the body to signal between cells and influence the up- and down-regulation of various cellular functions. One of the peptides included in the treatment, palmitoyl tripeptide-5, is a synthetic peptide that mimics the sequence found in the protein thrombospondin 1 (TSP-1). TSP-1 has been shown to activate latent tissue growth factor β (TGF-β), a growth factor involved in the stimulation of collagen production.[12,13] To aid in the prevention of collagen degradation, the peptide dipalmitoyl hydroxyproline was incorporated for its ability to promote the production of tissue inhibitors of metalloproteinases (TIMPs), thereby suppressing the synthesis of MMPs.[14]

Another peptide, unrelated to collagen synthesis and degradation, was formulated into the line treatment to target neuromuscular activity. Dipeptide diaminobutyroyl benzylamide diacetate was developed to mimic Waglerin-1, a compound found in temple viper snake venom. Waglerin-1 has been shown to block the nicotinic acetylcholine receptors at the neuromuscular junction, thus inhibiting muscular movement.[15] The function of the Wagerlin-1-mimicking peptide is important for the treatment of lines since facial areas associated with repeated muscle movement are especially susceptible to wrinkle formation.

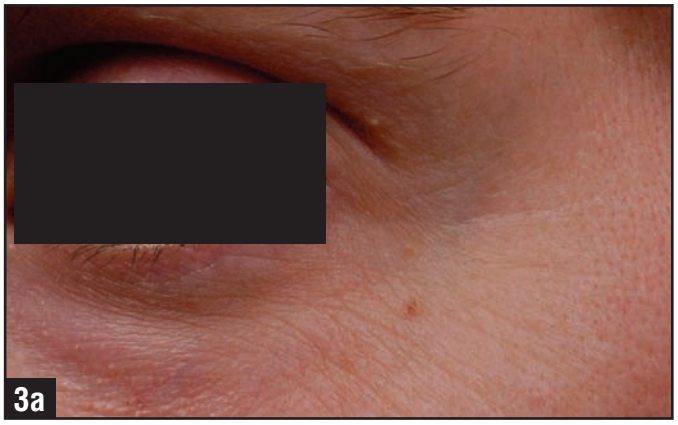
Lastly, the potent antioxidants, ubiquinone and vitamins C and E were incorporated to prevent and reduce oxidative damage, thereby minimizing the effects caused by UV exposure and intrinsic aging.[16–18]

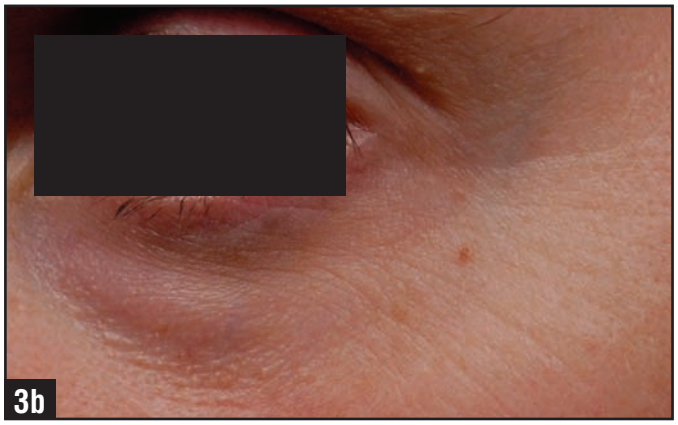
The purpose of this three-month, open-label, single-center study was to evaluate the efficacy and tolerance of this line treatment for periocular and perioral wrinkles.

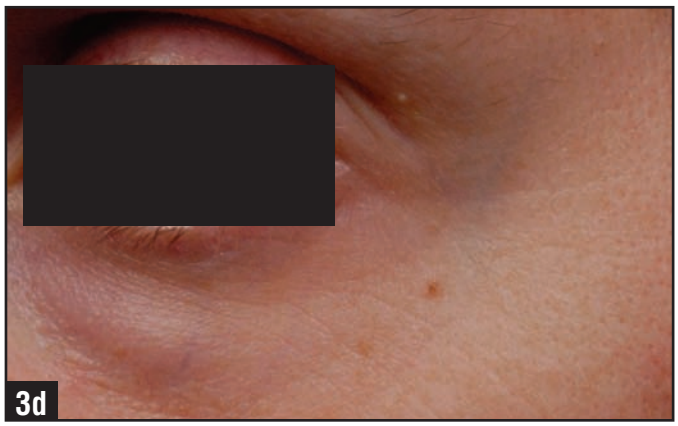
METHODS

Study population. The criteria for study participation included females 30 to 45 years of age with Fitzpatrick skin types I–III with mild-to-moderate fine and coarse periocular wrinkles (scores of 1–6.5) as determined by the clinical investigator. Although not a requirement for study participation, subjects who also had mild-to-moderate fine and coarse perioral wrinkles (scores of 1–6.5) were included in the study.

Subjects could not have used any facial products containing alpha- or beta-hydroxy acids, retinoids,







Figures 3a–3d. Thirty-four-year-old female periocular area at Baseline, Minutes, Month 1, and Month 3

benzoyl peroxide, and salicylic acid within 30 days of study start. In addition, subjects could not have had botulinum toxin, facial fillers, a facial chemical peel, or any other resurfacing procedure within six months of study start. Subjects were instructed not to use any of the above facial products nor begin use of any new facial products or procedures during the study.

Study design. During the three-month, open-label study, eligible subjects applied the product to their periocular and perioral wrinkles twice daily for three months. Investigator assessments of fine and coarse periocular and perioral wrinkles and tolerance assessments were conducted at all visits including Baseline, Minutes (within 15 minutes of initial application), Month 1, and Month 3. Digital photographs of the left and right sides of the face were also taken at all visits.

Study endpoints. *Efficacy.* Investigator evaluations of fine and coarse wrinkles in the periocular and perioral areas were assessed at Baseline, Minutes, Month 1, and Month 3. For the periocular assessments, the left and right sides were graded separately. The wrinkles were graded using a 10-point scale with half-point scores allowed, where 0 = none, 0.5 to 3.5 = mild, 4 to 6.5 = moderate, and 7 to 9 = severe.

Safety. Tolerance was assessed at all visits by objective and subjective irritation parameters and the reporting of adverse events. The investigator assessed objective irritation, including overall erythema, edema, and scaling. For subjective irritation, subjects rated overall burning/stinging, itching, and tingling. Both objective and subjective irritation parameters were assessed on a four-point scale where 0 = none, 1 = mild, 2 = moderate, and 3 = severe. Half-point scores were allowed.

Subject self assessment. Subjects completed a self-assessment questionnaire asking them to rate their experience using the line treatment within minutes of initial application and at Month 3.

Statistical analysis. All scores at each visit were statistically compared to Baseline scores using a paired *t* test. Changes from baseline were considered significant at the $P<0.05$ level. Mean percent change from baseline and incidence of positive responders were reported for the Subject Self-Assessment Questionnaire.

RESULTS

Demographics. Of the 37 female subjects that were enrolled, 35 completed the three-month study. The majority of subjects were Caucasian (92%) and the remaining were Hispanic and Native American (8%). Subjects were 33 to 45 years of age with mild or moderate, fine and coarse periocular and perioral wrinkles. Mean baseline fine and coarse periocular scores were 3.55 and 2.03, respectively; mean baseline fine and coarse perioral scores were 2.25 and 1.36, respectively.

Efficacy. Statistically significant reductions in mean scores for fine and coarse periocular wrinkles were achieved within minutes of initial product application with continued improvements observed through Months 1 and 3 (all $P<0.0001$). The mean percent changes in

wrinkle severity at all visits compared to Baseline are presented in Figure 1, with negative values indicating improvements. In addition, fine and coarse perioral wrinkles also showed significant improvements at all visits (all $P \leq 0.0003$) (Figure 2). Visible improvements in periocular wrinkles are displayed in Figures 3 and 4.

Within minutes of initial application, subjects noticed benefits from using the line treatment, as demonstrated by the percentage of subjects selecting "agree strongly" or "agree" in response to the questionnaire. Most notably, subjects felt the line treatment made their skin feel firmer and tightened, look smoother, feel refreshed and more youthful, and brightened the area around their eyes (all $P<0.05$). After three months of use, subjects reported additional improvements in the appearance of their facial wrinkles. Subject responses to the questionnaire within minutes of initial application and at Month 3 are presented in Table 1. Overall the line treatment was highly rated by subjects with 83 percent rating their overall satisfaction at Month 3 as "excellent" or "good."

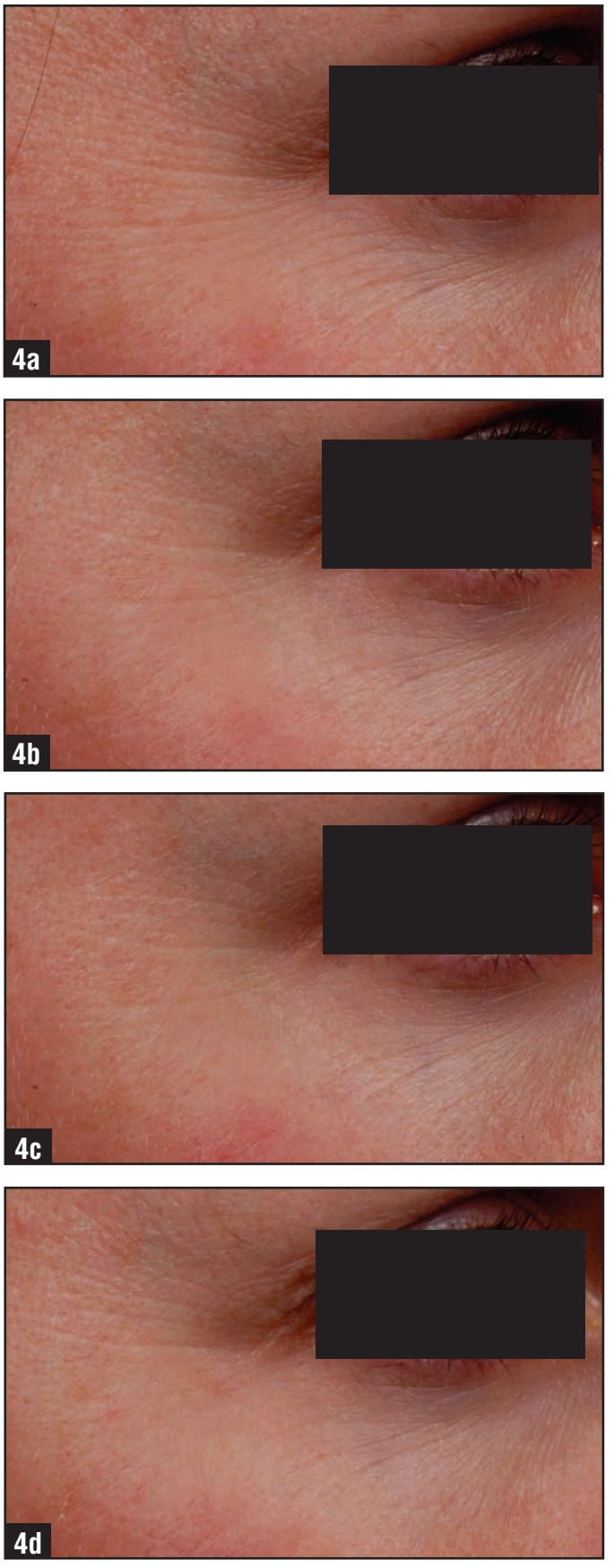
Safety. The line treatment was well tolerated as demonstrated by low mean scores (<0.05) for edema, scaling, burning/stinging, itching, and tingling tolerance parameters. Mean scores for erythema decreased at Month 3 from Baseline (0.78 to 0.57, respectively). Mean scores for all objective and subjective tolerance parameters are presented in Table 2. No treatment-related adverse events were reported.

DISCUSSION

In this clinical study, the topical line treatment demonstrated both immediate and long-term improvements in the appearance of mild and moderate, fine and coarse periocular and perioral wrinkles, as confirmed by both clinical and subject assessments. These results are of particular note since the wrinkles in these facial regions tend to pose a treatment challenge for patients and physicians alike.

The early onset of efficacy observed within minutes of application suggests that this unique combination of HA filling spheres and a muscle contraction-inhibiting peptide may work synergistically to promote rapid reductions in wrinkle appearance. The ability of HA to attract and retain moisture may contribute to the observed smoothing effect on lines and wrinkles. The HA in the spheres acts like a molecular sponge, causing the filling spheres to swell and exert pressure in the direction of the surface, resulting in a smoothing effect on the skin. The immediate improvement in wrinkle appearance may also be due to the muscle-relaxing effects of the Waglerin-1 mimicking peptide as wrinkles in the periocular and perioral areas are often associated with repeated muscle movement. In addition, the light-diffusing polymeric microspheres in the line treatment may also soften the appearance of fine lines in these facial areas.

The line treatment demonstrated continued improvement in both fine and coarse wrinkles over the course of the study. Results observed at Months 1 and 3 may be attributed to the



Figures 4a–4d. Thirty-four-year-old female periocular area at Baseline, Minutes, Month 1, and Month 3

TABLE 1. Subject self-assessment questionnaire: Minutes and Month 3
Percent of subjects (agree strongly or agree)

	MINUTES (N=37)	MONTH 3 (N=35)
Softened the fine lines/wrinkles around my eyes	65%	86%
Made my skin look smoother	68%	83%
Brightened the area around my eyes	62%	80%
Made my skin feel refreshed	81%	80%
Made my skin feel firmer and tightened	86%	80%
Softened the fine lines/wrinkles around my upper lip	58%	71%
Is convenient to use as part of my daily skin care regimen	Not asked	86%
Is comfortable to use under my makeup	Not asked	88%
Is easy to use	Not asked	86%

TABLE 2. Mean objective and subjective tolerance scores

	BASELINE	MINUTES	MONTH 1	MONTH 3
Erythema	0.78	0.77	0.76	0.57
Edema	0.00	0.00	0.00	0.00
Scaling	0.00	0.00	0.00	0.00
Burning/stinging	0.00	0.01	0.03	0.00
Itching	0.00	0.03	0.00	0.00
Tingling	0.00	0.05	0.00	0.00

long-term effects of the growth factors and peptides. These ingredients have been shown to prevent MMP-induced damage to the dermal matrix by targeting various biochemical processes involved in collagen production and degradation.[8–10,12–14] In addition, the antioxidants in the line treatment may provide protection from UV-induced ROS, preventing the cascade of reactions that would ultimately lead to the structural changes associated with intrinsic and environmentally induced aging.[16–18]

Frequently, invasive or surgical procedures, such as injections and lasers, are chosen for the immediate and significant improvements they produce in facial wrinkles. However, these procedures are also associated with certain disadvantages, such as a potential risk for complications and a period of recovery time.[19,20] The line treatment produced significant improvements in facial wrinkles and provides a well-tolerated, no-downtime alternative to invasive procedures.

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Hyaluronic acid
A key molecule in skin aging

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Keywords: hyaluronic acid, hyaluronic acid synthases, hyaluronidases, CD44, RHAMM, skin aging

Abbreviations: UV, ultraviolet; ROS, reactive oxygen species; MMP, matrix metalloproteinase; HA, hyaluronic acid; GAG, glycosaminoglycan; ECM, extracellular matrix; HAS, hyaluronic acid synthases; HYAL, hyaluronidases; CD44, cluster of differentiation 44; RHAMM, receptor for HA-mediated motility; TGF, transforming growth factor

Skin aging is a multifactorial process consisting of two distinct and independent mechanisms: intrinsic and extrinsic aging. Youthful skin retains its turgor, resilience and pliability, among others, due to its high content of water. Daily external injury, in addition to the normal process of aging, causes loss of moisture. The key molecule involved in skin moisture is hyaluronic acid (HA) that has unique capacity in retaining water. There are multiple sites for the control of HA synthesis, deposition, cell and protein association and degradation, reflecting the complexity of HA metabolism. The enzymes that synthesize or catabolize HA and HA receptors responsible for many of the functions of HA are all multigene families with distinct patterns of tissue expression. Understanding the metabolism of HA in the different layers of the skin and the interactions of HA with other skin components will facilitate the ability to modulate skin moisture in a rational manner.

Skin Aging

Human skin aging is a complex biological process, not yet fully understood. It is the result of two biologically independent processes. The first is intrinsic or innate aging, an unpreventable process, which affects the skin in the same pattern as it affects all internal organs. The second is extrinsic aging, which is the result of exposure to external factors, mainly ultraviolet (UV) irradiation, that is also referred to as photoaging.[1] Intrinsic skin aging is influenced by hormonal changes that occur with age,[2] such as the gradual decreased production of sex hormones from the mid-twenties and the diminution of estrogens and progesterone associated with menopause. It is well established that the deficiency in estrogens and androgens results in collagen degradation, dryness, loss of elasticity, epidermal atrophy and wrinkling of the skin.[3]

Even though intrinsic and extrinsic skin aging are distinctive processes, they share similarities in molecular mechanisms. For example, reactive oxygen species (ROS), arising from oxidative cell metabolism, play a major role in both processes.[4] ROS in extrinsic or intrinsic skin aging induce the transcription factor c-Jun via mitogen-activated protein kinases (MAPK), leading to overexpression of matrix metalloproteinase (MMP)-1, MMP-3 and MMP-9 and prevention of the expression of procollagen-1.[5] Therefore, elevated levels of degraded collagen and reduced collagen synthesis are pathologies occurring in intrinsically aged as well as photoaged skin.

Skin aging is also associated with loss of skin moisture. The key molecule involved in skin moisture is hyaluronan or hyaluronic acid (HA), a glycosaminoglycan (GAG) with a unique capacity to bind and retain water molecules.[6] HA belongs to the extracellular matrix (ECM) molecules. During the past decades the constituents of the skin have been well characterized. In the beginning, most of the studies focused on the cells that comprise the skin layers, such as the epidermis, the dermis and the underlying subcutis. Recently, it is appreciated that ECM molecules that lie between cells, in addition to providing a constructive framework, they exert major effects on cellular function. These ECM molecules, although they appear amorphous by light microscopy, they form a highly organized structure, comprising mainly of GAG, proteoglycans, growth factors and structural proteins such as collagens. Yet, the predominant component of the skin ECM is HA.

Recent reviews have described the involvement of HA with respect to its role in angiogenesis,[7] reactive oxygen species,[8] chondrocytes,[9] cancer,[10,11] lung injury,[12,13] immune regulation[14,15] and skin.[16] This review presents in brief recent knowledge in HA biology and function and focuses on its involvement in skin aging.

Hyaluronic Acid

Chemistry and physicochemical properties. HA is a non-sulphated GAG and is composed of repeating polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine linked by a glucuronidic β (1→3) bond.[17,18] In aqueous solutions HA forms specific stable tertiary structures.[19] Despite the simplicity in its composition, without variations in its sugar composition or without branching points, HA has a variety of physicochemical properties. HA polymers occur in a vast number of configurations

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and shapes, depending on their size, salt concentration, pH, and associated cations.[20] Unlike other GAG, HA is not covalently attached to a protein core, but it may form aggregates with proteoglycans.[21] HA encompasses a large volume of water giving solutions high viscosity, even at low concentrations.[13]

Tissue and cell distribution of HA. HA is widely distributed, from prokaryotic,[22,23] to eukaryotic cells.[24] In humans, HA is most abundant in the skin,[25-29] accounting for 50% of the total body HA,[30] the vitreous of the eye,[31] the umbilical cord,[17] and synovial fluid,[32,33] but it is also present in all tissues and fluids of the body, such as skeletal tissues,[27] heart valves,[34] the lung,[35-39] the aorta,[40] the prostate,[41] tunica albuginea, corpora cavernosa and corpus spongiosum of the penis.[42] HA is produced primarily by mesenchymal cells but also by other cell types.[34-38,43]

Biological function of HA. Over the past two decades there was considerable evidence presented that unraveled the functional role of HA in molecular mechanisms and indicated the potential role of HA for the development of novel therapeutic strategies for many diseases.

Functions of HA include the following: hydration, lubrication of joints, a space filling capacity, and the framework through which cells migrate.[34] The synthesis of HA increases during tissue injury and wound healing[25,44,45] and HA regulates several aspects of tissue repair, including activation of inflammatory cells to enhance immune response[46-48] and the response to injury of fibroblasts[49,50] and epithelial cells.[51-55] HA also provides the framework for blood vessel formation[7,45] and fibroblast migration,[56,57] that may be involved in tumor progression.[58] The correlation of HA levels on the cell surface of cancer cells with the aggressiveness of tumors has also been reported.[59]

The size of HA appears to be of critical importance for its various functions described above. HA of high molecular size, usually in excess of 1,000 kDa, is present in intact tissues and is antiangiogenic and immunosuppressive, whereas smaller polymers of HA are distress signals and potent inducers of inflammation and angiogenesis.[38,46,60-63]

Biosynthesis of HA

HA is synthesized by specific enzymes called HA synthases (HAS). These are membrane bound enzymes that synthesize HA on the inner surface of the plasma membrane[64] and then HA is extruded through pore-like structures into the extracellular space.[24,65] There are three mammalian enzymes HAS -1, -2 and -3, which exhibit distinct enzymatic properties and synthesize HA chains of various length.[66-68]

Degradation of HA

HA has a dynamic turnover rate. HA has a half-life of 3 to 5 min in the blood, less than a day in the skin and 1 to 3 weeks in the cartilage.[69-71] HA is degraded into fragments of varying size by hyaluronidases (HYAL) by hydrolyzing the hexosaminidic β (1–4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in HA. In humans, six HYAL have been identified so far: HYAL-1, -2, -3, -4, PH-20 and HYALP1.[72] The family of HYAL enzymes received little attention until recently[73,74] because they are found at extremely low concentrations and they are difficult to purify, characterize and measure their activity, which is high but unstable.[16] New procedures have now enabled the isolation and characterization of HYAL.[75,76] HYAL-1 is the major HYAL in serum.[77] Mutations in the HYAL-1 gene are associated with HYAL deficiency and mucopolysaccharidosis type IX.[78] HYAL-2 has very low activity in comparison to plasma HYAL-1 and it hydrolyzes specifically HA of high molecular weight, yielding HA fragments of approximately 20 kDa, which are further degraded to small oligosaccharides by PH-20.[79] HYAL-3 is mainly expressed in bone marrow and testis,[74] but also in other organs, such as the human lung.[37,38] The role of HYAL-3 in the catabolism of HA is not clear and it is suggested that it may contribute to HA degradation by enhancing the activity of HYAL-1.[80]

HA can also be degraded non-enzymatically by a free-radical mechanism[81] in the presence of reducing agents such as ascorbic acid, thiols, ferrous, or cuprous ions, a process that requires the presence of molecular oxygen. Thus, agents that could delay the free-radical-catalyzed degradation of HA may be useful in maintaining the integrity of dermal HA and its moisturizing properties.[16]

Hyaluronic Acid Receptors

There is a variety of proteins that bind HA, called hyaladherins, which are widely distributed in the ECM, the cell surface, the cytoplasm and the nucleus.[15] Those that attach HA to the cell surface constitute HA receptors. The most prominent among these receptors is the transmembrane glycoprotein "cluster of differentiation 44" (CD44) that occurs in many isoforms, which are the products of a single gene with variable exon expression.[82-84] CD44 is found on virtually all cells, except red blood cells, and regulates cell adhesion, migration, lymphocyte activation and homing, and cancer metastasis.

The receptor for HA-mediated motility (RHAMM) is another major receptor for HA, and it is expressed in various isoforms.[85-87] RHAMM is a functional receptor in many cell types, including endothelial cells[88] and in smooth muscle cells from human pulmonary arteries[37] and airways.[38] The interactions of HA with RHAMM control cell growth and migration by a complex network of signal transduction events and interactions with the cytoskeleton.[89] Transforming growth factor (TGF)-β1, which is a potent stimulator of cell motility, elicits the synthesis and expression of RHAMM and HA, and thus initiates locomotion.[90]

Hyaluronic Acid in Skin

The use of biotinylated HA-binding peptide[91] revealed that not only cells of mesenchymal origin were capable of synthesizing HA and permitted the histolocalization of HA in the dermal compartment of skin and the epidermis.[26,92-94] This technique enabled the visualization of HA in the epidermis, mainly in the ECM of the upper spinous and granular layers, whereas in the basal layer HA is predominantly intracellular.[26]

The function of the skin as a barrier is partly attributed to the lamellar bodies, thought to be modified lysosomes containing hydrolytic enzymes. They fuse with the plasma membranes of mature keratinocytes and they have the ability to acidify via proton pumps and partially convert their polar lipids into neutral lipids. Diffusion of aqueous material through the epidermis is blocked by these lipids synthesized by keratinocytes in the stratum granulosum. This boundary effect corresponds to the level of HA staining. The HA-rich area inferior to this layer may obtain water from the moisture-rich dermis, and the water contained therein cannot penetrate beyond the lipid-rich stratum granulosum. The hydration of the skin critically depends on the HA-bound water in the dermis and in the vital area of the epidermis, while maintenance of hydration essentially depends on the stratum granulosum. Extensive loss of the stratum granulosum in patients with burns may cause serious clinical problems due to dehydration.[16]

As mentioned above, skin HA accounts for most of 50% of total body HA.[30] The HA content of the dermis is significantly higher than that of the epidermis, while papillary dermis has much greater levels of HA than reticular dermis.[92] The HA of the dermis is in continuity with the lymphatic and vascular systems. HA in the dermis regulates water balance, osmotic pressure and ion flow and functions as a sieve, excluding certain molecules, enhancing the extracellular domain of cell surfaces and stabilizes skin structures by electrostatic interactions.[16] Elevated levels of HA are synthesized during scar-free fetal tissue repair and the prolonged presence of HA assures such scar-free tissue repair.[95-97] Dermal fibroblasts provide the synthetic machinery for dermal HA and should be the target for pharmacologic attempts to enhance skin hydration. Unfortunately, exogenous HA is cleared from the dermis and is rapidly degraded.[70]

Hyaluronic acid synthases in the skin. In the skin, gene expression of HAS-1 and HAS-2 in the dermis and epidermis is differentially upregulated by TGF-β1, indicating that HAS isoforms are independently regulated and that the function of HA is different in the dermis and the epidermis.[16,98] The mRNA expression of HAS-2 and HAS-3 can be stimulated by keratinocyte growth factor, which activates keratinocyte migration and stimulates wound healing, leading to the accumulation of intermediate-sized HA in the culture medium and within keratinocytes. The migratory response of keratinocytes in wound healing is stimulated by increased synthesis of HA.[99] HAS-2 mRNA is also induced by IL-1β and TNFα in fibroblasts[100] and by epidermal growth factor in rat epidermal keratinocytes.[101]

Dysregulated expression of HA synthases has been reported during tissue injury.[102-104] HAS-2 and HAS-3 mRNA are significantly increased after skin injury in mice, leading to increased epidermal HA.[104] In juvenile hyaline fibromatosis, which is a rare autosomal recessive disease characterized by deposition of hyaline material and multiple skin lesions, there is a significant decreased expression of HAS-1 and HAS-3, accounting for the reduced synthesis of HA in skin lesions.[105] In dermal fibroblasts, where the HAS-2 is the predominant isoform, glucocorticoids inhibit HAS mRNA almost completely, suggesting a molecular basis of the decreased HA in atrophic skin as a result of local treatment with glucocorticoids.[16]

Hyaluronidases in the skin. In the skin, it has not been established which of the various HYAL controls the turnover of HA in the dermis and the epidermis. The elucidation of the biology of HYAL in the skin may offer novel pharmacological targets to confront age related turnover of HA in skin.

HA receptors in the skin. In the dermis and epidermis HA is co-localized with CD44. However, the exact CD44 variants in the different skin compartments have not yet been elucidated. CD44-HA interactions have been reported to mediate the binding of Langerhans cells to HA in the matrix surrounding keratinocytes by their CD44-rich surfaces, as they migrate through the epidermis.[106,107] RHAMM is also expressed in the human skin.[28,29] The TGF-β1 induced stimulation of fibroblast locomotion is mediated via RHAMM,[90] while overexpression of RHAMM can lead to the transformation of fibroblasts.[108]

Hyaluronic Acid and Skin Aging

The most dramatic histochemical change observed in senescent skin is the marked disappearance of epidermal HA, while HA is still present in the dermis.[92] The reasons for this change in HA homeostasis with aging is unknown. As mentioned above, the synthesis of epidermal HA is influenced by the underlying dermis and is under separate controls from the synthesis of dermal HA.[16,98] Progressive reduction of the size of the HA polymers in skin as a result of aging has also been reported.[109] Thus, the epidermis loses the principle molecule responsible for binding and retaining water molecules, resulting in loss of skin moisture. In the dermis, the major age-related change is the increasing avidity of HA with tissue structures with the concomitant loss of HA extractability. This parallels the progressive cross-linking of collagen and the steady loss of collagen extractability with age.[16] All of the above age related phenomena contribute to the apparent dehydration, atrophy and loss of elasticity that characterizes aged skin.

Premature aging of skin is the result of repeated and extended exposure to UV radiation.[110,111] Approximately 80% of facial skin aging is attributed to UV-exposure.[112] UV radiation damage causes initially a mild form of wound healing and is associated at first with an increase of dermal HA. As little as 5 min of UV exposure in nude mice caused enhanced deposition of HA, indicating that UV radiation induced skin damage is an extremely rapid event.[16] The initial redness of the skin following exposure to UV radiation may be due to a mild edematous reaction induced by the enhanced HA deposition and histamine release. Repeated and extensive exposures to UV ultimately simulate a typical wound healing response with deposition of scarlike type I collagen, rather than the usual types I and III collagen mixture that gives skin resilience and pliability.[16]

In the skin, photoaging results in abnormal GAG content and distribution compared with that found in scars, or in the wound healing response, with diminished HA and increased levels of chondroitin sulfate proteoglycans.[111] In dermal fibroblasts this reduction in HA synthesis was attributed to collagen fragments, which activate $\alpha_v\beta_3$-integrins and in turn inhibit Rho kinase signaling and nuclear translocation of phosphoERK, resulting in

reduced HAS-2 expression.[113] We have recently unraveled some of the biochemical changes that may distinguish photoaging and natural aging. Using photoexposed and photoprotected human skin tissue specimens, obtained from the same patient, we have shown a significant increase in the expression of HA of lower molecular mass in photoexposed skin, as compared with photoprotected skin. This increase of degraded HA was associated with a significant decrease in the expression of HAS-1 and an increased expression of HYAL-1, -2 and -3. Furthermore, the expression of HA receptors CD44 and RHAMM was significantly downregulated in photoexposed, as compared with photoprotected skin. These findings indicate that photoexposed skin, and therefore extrinsic skin aging, is characterized by distinct homeostasis of HA.[29] We have also assessed photoprotected skin tissue specimens from adults and juvenile patients and observed that intrinsic skin aging was associated with a significant reduction in the content of HA and downregulation of HAS-1, HAS -2, CD44 and RHAMM.[28] Similar results for photoprotected skin have also been reported for both genders for HA, HAS-2 and CD44.[114]

Conclusion

The available data suggest that HA homeostasis exhibits a distinct profile in intrinsic skin aging, which is totally different of that in extrinsic skin aging. Additional insight needs to be gained in understanding the metabolism of HA in skin layers and the interactions of HA with other skin components. Such information will facilitate the ability to modulate skin moisture in a rational manner and may contribute to the refinement of current drugs and the development of novel treatments for skin aging.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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