2020: FORMULATION AND COMBINATION THERAPIES

TOPIX

PHARMACEUTICALS, INC

CLINICAL STUDY AND POSTER COMPILATION

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MISSION STATEMENT

Partners in advancing the commitment to healthy, beautiful skin.

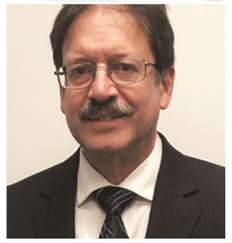
Topix Pharmaceuticals is focused on developing physician dispensed, therapeutic skin care products of the highest quality, innovation and value. We are dedicated to being the physician premier choice in custom branded skin care and elevating the patient experience through efficacious formulas and compliance to improve overall skin health.

FORMULA STATEMENT

Product ingredient grade level distribution, medical channel dispensed products.

The Topix line of skin care is only available through a licensed Physician. Topix product formulations contain a rich combination of innovative active ingredients at high concentrations. Clinical studies demonstrate Topix formulations contribute to improving the appearance of wrinkles, lines, brown spots, hydration and overall repair.

Steve Hernandez
SVP; Research & Development



Topix
Anti-Aging Final Report
December 23, 2019

A Double-Blind, Comparative Clinical Study of Topix Retinol Serums vs. Retin-A Cream in Escalating Doses

STUDY NUMBER: DCS-65-19

INVESTIGATOR: ZOE DIANA DRAELOS, M.D.

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SPONSOR: Topix

PRODUCTS: Retinol Serums (3 strengths: 0.25%, 0.5%, and 1.0%), Retin-A cream (0.025%, 0.05%, 0.1%), Lipid Infused Cream, CeraVe Moisturizing Cream, Topix Sheer Physical Sunscreen SPF 50+, Topix Ultra Gentle Cleanser

STUDY MONITOR: Yohini Appa, PhD

1. PROTOCOL SYNOPSIS

Title of Study:	A Double-Blind, Comparative Clinical Study of Topix Retinol Serums vs. Retin-A Cream in Escalating Strengths
Study Period:	12 weeks
Test Product and Application Instructions:	Study Period 1 Cell 1: 30 subjects applied 0.25% retinol serum for 4 weeks. The 0.25% retinol serum was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. A Topix Test Moisturizer (Lipid Infused Cream) was placed on top of the retinol serum at each application. The test moisturizer was also used in the morning. Cell 2: 15 subjects applied 0.025% Retin-A cream for 4 weeks. The 0.025% Retin-A cream was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. CeraVe Moisturizing Cream was placed on top of the Retin-A cream at each application. CeraVe cream was also used in the morning. Study Period 2 Cell 1: 30 subjects stepped up from 0.25% retinol serum to 0.5% retinol serum every night at week 4 followed by a second step up to 1% retinol every night at week 8. The Test Moisturizer (Lipid Infused Cream) was placed on top of the retinol serum at each application. The test Moisturizer was also used in the morning. At each step up, subjects who were experiencing difficulty at the current concentration, introduced the higher strength 2x a week, while continuing on the current strength for the first week. Those subjects were asked to use the higher strength every other day for the second week, before moving to daily use of the higher strength. Data calculations were made with and without this modified use subjects. Cell 2: 15 subjects stepped up from 0.025% Retin-A cream to 0.05% Retin-A cream every night at week 4 followed by a second step up to 0.1% Retin-A cream every night at week 8. At each step up, subjects who were experiencing difficulty at the current concentration were introduced the higher strength 2x a week, while continuing on the current strength for the first week. Those subjects were asked to use the higher strength every other day for the second week, before moving to daily use of the higher strength. CeraVe Moisturizing cream was placed on top of the Retin-A cream at each application. The CeraVe cream was also used in the morning.

Ancillary Skin Care Products:	Topix Ultra Gentle Cleanser Product applied to entire face for cleansing twice daily for 12 weeks. Topix Sheer Physical Sunscreen SPF 50+ applied to entire face every morning, after cleansing and using the Test Moisturizer, or the CeraVe Moisturizing cream, respectively for Cells 1 and 2. All test products (Retinols, Retin-A and the Lipid Infused Cream) were blind labeled or covered with blinding tape.
Objectives:	Objective 1: To investigate the efficacy, tolerability, and consumer acceptance of anti-aging retinol serums in 3 strengths (0.25%, 0.5%, and 1.0%) in combination with the Test Moisturizer for 12 weeks. Objective 2: To compare the efficacy and tolerability and consumer acceptance of anti-aging retinol serums in 3 strengths (0.25%, 0.5%, and 1.0%) used with the Test Moisturizer, versus an escalating dose of Retin-A cream (0.025%, 0.05% and 0.1%) used with CeraVe Moisturizing Cream.
Design:	45 photoaged females ages 35-65, Fitzpatrick skin types I-IV, with moderate wrinkling (Glogau III), who are not habitual retinoid users were enrolled in this double-blind controlled retinoid study. Subjects who signed consent and met all inclusion criteria and none of the exclusion criteria were enrolled at the baseline visit (visit 1). Subjects were asked to continue their self-selected colored cosmetics unchanged throughout the 12-week study. Subjects used the provided gentle cleanser and sunscreen along with the study products. No other skin care products were used. The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance (rosy glow), lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe). In addition, the dermatologist investigator and the subjects assessed overall clinical improvement from base line at week 12, using a 5-point scale (1= much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The investigator and subjects referred to baseline images (front, right and left side images) of the subjects in completing this evaluation. Tolerability was assessed in terms of the following parameters: itching, stinging, burning, redness, swelling. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR4.3 camera system. Transepidermal Water Loss (TEWL) measurements were taken from the left face. Skin biopsies were

	obtained from 6 subjects in cell 1 and 4 subjects in cell 2. Subjects were randomized by the study coordinator to one of two balanced groups, including the distribution of male subjects between the two groups. The study coordinator maintained the blind. The assignments were allocated as follows for study period 1: Cell 1: 30 subjects applied 0.25% retinol serum for 4 weeks. The 0.25% retinol serum was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. A Topix Test Moisturizer (Lipid Infused Cream) was placed on top of the retinol serum at each application. The test moisturizer was also used in the morning. Cell 2: 15 subjects applied 0.025% Retin-A cream for 4 weeks. The 0.025% Retin-A cream was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. CeraVe Moisturizing Cream was placed on top of the Retin-A cream at each application. CeraVe cream was also used in the morning. Subjects were provided with a daily compliance diary and asked to return to the research center at week 4, bringing diary and the test products. A compliance text was sent at week 2 to ask subjects to contact the research center if they were experiencing any difficulties with the study products and to encourage subjects to be consistent with treatments and continue to maintain daily diaries. Subjects returned to the research center at week 4 (visit 2). The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Subjects completed a self assessment questionnaire (SAQ). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR 4.3 camera system. Diaries were checked for compliance and collected. The test products were visually inspected for compliance and returned to the subjects. The subjects were provided new study product by the study coordinator, who maintained the blind. The study product was assigned as follows for study period 2: Cell 1: 30 subjects stepped up from 0.25% retinol serum to 0.5% retinol serum every night at week 4. The Test Moisturizer (Lipid Infused Cream) was placed

	on top of the retinol serum at each application. The Test Moisturizer was also used in the morning. Cell 2: 15 subjects stepped up from 0.025% Retin-A cream to 0.05% Retin-A cream every night at week 4. CeraVe Moisturizing Cream was placed on top of the Retin-A cream at each application. The CeraVe cream was also applied in the morning. New compliance diaries were dispensed. A compliance text was sent at the second week of stepping up to a higher strength in Study Period 2 to ask subjects to contact the research center if they were experiencing any difficulties with the study products and to encourage subjects to be consistent with treatments and continue to maintain daily diaries. Subjects were asked to return to the research center at week 8 (visit 3) bringing their daily diaries and their test products. Subjects returned to the research center at week 8 (visit 3). The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Subjects completed a self assessment questionnaire (SAQ). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR 4.3 camera system. Diaries were checked for compliance and collected. Subjects underwent TEWL measurements from the left face as allocated in the facial diagram. The subjects were provided new study product and new compliance diaries by the study coordinator, who maintained the blind. The study product was allocated as noted below: Cell 1: 30 subjects stepped up to 1% retinol every night at week 8. The Test Moisturizer was placed on top of the retinol serum at each application. The Test Moisturizer was also used in the morning. Cell 2: 15 subjects stepped up to 0.1% Retin-A cream every night at week 8. CeraVe cream was placed on top of the Retin-A cream at each application. CeraVe cream was also used in the morning. Subjects were asked to return to the research center at week 12 (visit 4) bringing their daily diaries and all their test products. Subjects returned to the research center at week 12 (visit 4). The

	dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Subjects completed a self assessment questionnaire (SAQ). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR 4.3 camera system. TEWL measurements were obtained from the left face. Diaries were checked for compliance and collected. All study products were collected. Skin biopsies were obtained from 6 subjects in cell 1 and 4 subjects in cell 2. Subjects were released from their study participation. *(There is an allowance for an unscheduled visit at any time, if necessary.)*
Study Population:	Photoaged females ages 35-65, Fitzpatrick skin types I-IV, with moderate wrinkling (Glogau III), who are not habitual retinoid users
Number of Patients:	45 subjects (30 subjects in the treatment cell 1 with retinol serum, 15 subjects in cell 2 with Retin-A)
Inclusion Criteria:	The following represented the inclusion criteria: 1. Subject with moderate facial photoaging and free of any facial skin disease. 2. Female subject age 35-65 years. 3. Subject with Fitzpatrick skin types I-IV. 4. Subject agrees not to introduce any new colored cosmetics (lipsticks, eye shadows, facial foundations, blush, powder). Subjects must use the provided cleanser, and sunscreen for the duration of the study. No other products can be applied to the face for the duration of the study. 5. Subject willing to forego waxing or use of depilatories on face for study duration 6. Subjects willing to forego all topical treatments and moisturizers for 24 hours prior to baseline evaluation 7. Willing to avoid excessive solar or UV exposure including: minimizing direct sun exposure and completely avoiding tanning beds. 8. Subject has signed an Informed Consent Form in compliance with 21CFR Part 50: "Protection of Human Subjects." And photography release. 9. Subject is dependable and able to follow directions and is willing to comply with the schedule of visits. 10. Subject is in generally good physical and mental health. 11. Female subjects determined to be of child-bearing potential must agree to practice a medically acceptable form of birth control during the study.
Exclusion	The following represented the exclusion criteria:

Criteria:	1. Subject who is pregnant, planning a pregnancy, or nursing as determined by querying the subject. 2. Subject has a history of acute or chronic dermatologic, medical, physical conditions, which would preclude application of the test material and/or could influence the outcome of the study. 3. Subject who has had cosmetic surgery of the face within the last 12 months. 4. Subject who has used facial retinoids within the last 3 months. 5. Subject who has used facial alpha-hydroxy acids within the last 1 month. 6. Subjects who might need to use any other facial products for medical or other reasons, such as acne medication, steroids. 7. Subjects with known retinoid sensitivity / reactivity 8. Subjects with clinically diagnosed or self-diagnosed sensitive skin. 9. Subjects with excessive facial hair
Endpoints:	<u>Tolerability Endpoint:</u> The tolerability endpoint was the investigator-assessed absence of serious adverse events from the facial study products at any time during the 12-week study. <u>Primary Efficacy :</u> The efficacy endpoint was the investigator assessed improvements in facial skin appearance after 12 weeks of study product use as compared to baseline and between treatment arms. <u>Secondary Efficacy Endpoint:</u> The efficacy endpoint was the subject assessed improvements in facial skin appearance after 12 weeks of study product use as compared to baseline and between treatment arms.
Measures:	<u>Dermatologist Investigator assessed efficacy parameters</u>: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia, crepey cheek skin texture. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). In addition, at week 12, the dermatologist investigator assessed overall clinical improvement from base line, using a 5-point scale (1= much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The investigator referred to baseline images (front, right and left side images) of the subjects in completing this evaluation. <u>Dermatologist Investigator assessed tolerability parameters</u>: itching, stinging

	burning, redness, swelling. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). Subject assessed efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, uneven skin tone, crepey cheek skin texture. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). In addition, at week 12, subjects assessed overall clinical improvement from base line, using a 5-point scale (1=much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The subjects referred to their baseline images (front, right and left side images) in completing this evaluation. Subject assessed tolerability parameters: itching, stinging burning, redness, swelling. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). At post-baseline time points, subjects reported the degree of any of these symptoms they have experienced since the previous time point. Photography: Photographs were taken at each time point with visible light of the central, right, and left face with a Canfield VISIA CR 4.3 camera system. Photographs were performed at the following time points: baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). Transepidermal Water Loss (TEWL): TEWL readings were taken from the left face at baseline (visit 1), week 8 (visit 3), and week 12 (visit 4). Skin Biopsy: Skin biopsies were obtained from 6 subjects in cell 1 and 4 subjects in cell 2. The 2mm skin biopsies were taken from the fold anterior to the right ear at baseline (visit 1) and from the fold anterior to the left ear at week 12 (visit 4). Histologic evaluation for the following attributes: 1. Plumping of the epidermis (and compaction of the SC) 2. Increase in healthy collagen 3. Increased GAG's 3. Decrease in melanin 4. Improved vascularity and DEJ (rete ridges) 5. Less elastosis These analyses were conducted on formalin fixed specimens utilizing H&E, elastic stains, and GAG stains.

2. STUDY VISIT SCHEDULE

Procedures	Visit 1	Text	Visit 2	Visit 3	Visit 4
	BL	Week 2	Week 4	Week 8	Week 12
Informed Consent Procedure	X				
Inclusion/Exclusion Criteria	X				
Brief Medical History and Concomitant Medications Review	X		X	X	X
Investigator Clinical Grading for Efficacy and Tolerability	X		X	X	X
Subject Clinical Grading for Efficacy and Tolerability	X		X	X	X
Compliance Text		X			
Product Dispensing	X		X	X	
Facial Digital Photography (right, left, center VISIA CR 4.3)	X		X	X	X
Transepidermal Water Loss (TEWL)	X		X		X
Skin Biopsies	X				X
Subject Diary Assessment and Compliance Check			X	X	X
Subject Product Accountability and Study Completion					X

3. INTRODUCTION

Aging of the skin can be classified into two components: intrinsic and extrinsic aging. As the names imply, intrinsic aging is due to genetically controlled senescence and extrinsic aging is due to environmental factors superimposed on intrinsic aging. Environmental factors known to accelerate extrinsic aging are sun exposure and cigarette smoking. Cutaneous aging due to sun exposure is known as photoaging.

Youthful skin is characterized by its unblemished, evenly pigmented, smooth, pink appearance. This is in contrast to intrinsically aged skin, which is thin, inelastic and finely wrinkled with deepening of facial expression lines. These changes are evident histologically as a thinned epidermis and dermis with flattening of the rete pegs at the dermo epidermal junction. Extrinsically aged, sun-exposed skin appears clinically as blemished, thickened, yellowed, lax, rough, and leathery. These changes may begin as early as the second or third decade.

The best-studied ingredient category for anti-aging benefits is the retinoids. Retinoids include naturally occurring derivatives, such as retinol, and engineered retinoids, such as tretinoin. Tretinoin is a prescription retinoid with established improvement in skin thickness with an accompanying reduction in fine lines, however, it is irritating, and many consumers cannot tolerate daily use. This has led to the development of OTC retinol formulations that provide many of the same benefits. The goal of this research was to investigate the tolerability and efficacy of a novel OTC retinol formulation.

4. STUDY OBJECTIVES

Objective 1: To investigate the efficacy, tolerability, and consumer acceptance of anti-aging retinol serums in 3 strengths (0.25%, 0.5%, and 1.0%) in combination with a barrier lipid treatment for 12 weeks.

Objective 2: To compare the efficacy tolerability and consumer acceptance of anti-aging retinol serums in 3 strengths (0.25%, 0.5%, and 1.0%) used with a Test Moisturizer, versus an escalating dose of Retin-A cream (0.025%, 0.05% and 0.1%) used with CeraVe cream.

5. STUDY DESIGN OVERVIEW

45 photoaged females ages 35-65, Fitzpatrick skin types I-IV, with moderate wrinkling (Glogau III), who are not habitual retinoid users were enrolled in this double-blind controlled retinoid study. Subjects who signed consent and met all inclusion criteria and none of the exclusion criteria were enrolled at the baseline visit (visit 1). Subjects were asked to continue their self-selected colored cosmetics unchanged throughout the 12-week study. Subjects used the provided gentle cleanser and sunscreen along with the study products. No other skin care products were used.

The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance (rosy glow), lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe). In addition, the dermatologist investigator and the subjects assessed overall clinical improvement from base line at week 12, using a 5-point scale (1= much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The investigator and subjects referred to baseline images (front, right and left side images) of the subjects in completing this evaluation.

Tolerability was assessed in terms of the following parameters: itching, stinging, burning, redness, swelling. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR4.3 camera system. Transepidermal Water Loss (TEWL) measurements were taken from the left face. Skin biopsies were obtained from 6 subjects in cell 1 and 4 subjects in cell 2.

Subjects were randomized by the study coordinator to one of two balanced groups, including the distribution of male subjects between the two groups. The study coordinator maintained the blind. The assignments were allocated as follows for study period 1:
Cell 1: 30 subjects applied 0.25% retinol serum for 4 weeks. The 0.25% retinol serum was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. The Test Moisturizer (Lipid Infused Cream) were placed on top of the retinol serum at each application. The Test Moisturizer was also applied in the morning.

Cell 2: 15 subjects applied 0.025% Retin-A cream for 4 weeks. The 0.025% Retin-A cream was used twice weekly for the week 1, then every other night for week 2, then every night for weeks 3 and 4. CeraVe Moisturizing cream was placed on top of the Retin-A cream at each application. The CeraVe cream was also applied in the morning.

Subjects were provided with a daily compliance diary and asked to return to the research center at week 4, bringing diary and the test products.

A compliance text was sent at week 2 to ask subjects to contact the research center if they are experiencing any difficulties with the study products and to encourage subjects to be consistent with treatments and continue to maintain daily diaries.

Subjects returned to the research center at week 4 (visit 2). The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Subjects completed a self assessment questionnaire (SAQ). Photographs were taken with visible

light of the central, right, and left face with a Canfield VISIA CR 4.3 camera system. Diaries were checked for compliance and collected. The test products were visually inspected for compliance and returned to the subjects.

The subjects were provided new study product by the study coordinator, who maintained the blind. The study product was assigned as follows for study period 2:

Cell 1: 30 subjects stepped up from 0.25% retinol serum to 0.5% retinol serum every night at week 4. The Test Moisturizer (Lipid Infused Cream) was placed on top of the retinol serum at each application. The Test Moisturizer was also used in the morning.

Cell 2: 15 subjects stepped up from 0.025% Retin-A cream to 0.05% Retin-A cream every night at week 4. CeraVe Moisturizing Cream was placed on top of the Retin-A cream at each application. The CeraVe cream was also applied in the morning.

New compliance diaries were dispensed. A compliance text was sent at the second week of stepping up to a higher strength in Study Period 2 to ask subjects to contact the research center if they are experiencing any difficulties with the study products and to encourage subjects to be consistent with treatments and continue to maintain daily diaries. Subjects were asked to return to the research center at week 8 (visit 3) bringing their daily diaries and their test products.

Subjects returned to the research center at week 8 (visit 3). The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Subjects completed a self assessment questionnaire (SAQ). Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR4.3 camera system. Diaries were checked for compliance. Subjects underwent TEWL measurements from the left face as allocated in the facial diagram.

The subjects were provided new study product and new compliance diaries by the study coordinator, who maintained the blind. The study product was allocated as noted below:
Cell 1: 30 subjects stepped up to 1% retinol every night at week 8. The Test Moisturizer was placed on top of the retinol serum at each application. The Test Moisturizer was also used in the morning.

Cell 2: 15 subjects stepped up to 0.1% Retin-A cream every night at week 8. CeraVe cream was placed on top of the Retin-A cream at each application. CeraVe cream was also used in the morning.

Subjects were asked to return to the research center at week 12 (visit 4) bringing their daily diaries and all their test products.

Subjects returned to the research center at week 12 (visit 4). The dermatologist investigator and subjects assessed the following facial efficacy parameters: Overall severity of photodamage, and individual photodamage parameters, dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia (uneven skin tone), and crepey cheek skin texture. In addition, the dermatologist investigator and the subjects assessed overall clinical improvement from base line at week 12, using a 5-point scale (1= much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The investigator and subjects referred to baseline images (front, right and left side images) of the subjects in completing this evaluation.

The dermatologist investigator and the subjects assessed tolerability in terms of the following parameters: itching, stinging burning, redness, swelling. Photographs were taken with visible light of the central, right, and left face with a Canfield VISIA CR4.3 camera system. Subjects answered a self assessment questionnaire (SAQ). TEWL measurements were obtained from the left face. Diaries were checked for compliance and collected. All study products was collected. Skin biopsies were obtained from 6 subjects in cell 1 and 4 subjects in cell 2. Subjects were released from their study participation.

6. STUDY POPULATION

6.1 NUMBER OF SUBJECTS

45 subjects (30 subjects in the treatment cell 1 with retinol serum, 15 subjects in cell 2 with Retin-A)

6.2 POPULATION DESCRIPTION

Photoaged females ages 35-65, Fitzpatrick skin types I-IV, with moderate wrinkling (Glogau III), who were not habitual retinoid users.

6.3 INCLUSION CRITERIA

The following items represented the inclusion criteria:

1. Subject with moderate facial photoaging and free of any facial skin disease.
2. Female subject age 35-65 years.
3. Subject with Fitzpatrick skin types I-IV.
4. Subject agrees not to introduce any new colored cosmetics (lipsticks, eye shadows, facial foundations, blush, powder). Subjects must use the provided cleanser, and sunscreen for the duration of the study. No other products can be applied to the face for the duration of the study.
5. Subject willing to forego waxing or use of depilatories on face for study duration
6. Subjects willing to forego all topical treatments and moisturizers for 24 hours prior to baseline evaluation
7. Willing to avoid excessive solar or UV exposure including: minimizing direct sun exposure and completely avoiding tanning beds.
8. Subject has signed an Informed Consent Form in compliance with 21CFR Part 50: "Protection of Human Subjects." And photography release.

9. Subject is dependable and able to follow directions and is willing to comply with the schedule of visits.
10. Subject is in generally good physical and mental health.
11. Female subjects determined to be of child-bearing potential must agree to practice a medically acceptable form of birth control during the study.

6.4 EXCLUSION CRITERIA

The following items represented the exclusion criteria:

1. Subject who is pregnant, planning a pregnancy, or nursing as determined by querying the subject.
2. Subject has a history of acute or chronic dermatologic, medical, physical conditions, which would preclude application of the test material and/or could influence the outcome of the study.
3. Subject who has had cosmetic surgery of the face within the last 12 months.
4. Subject who has used facial retinoids within the last 3 months.
5. Subject who has used facial alpha-hydroxy acids within the last 1 month.
6. Subjects who might need to use any other facial products for medical or other reasons, such as acne medication, steroids.
7. Subjects with known retinoid sensitivity / reactivity.
8. Subjects with clinically diagnosed or self-diagnosed sensitive skin.
9. Subjects with excessive facial hair.

7. RESULTS

The results are presented in the attached Excel data tables.
Table 1: Retinol Experience
Table 2: Investigator Efficacy
Table 3: Investigator Overall Efficacy
Table 4: Investigator Tolerability
Table 5: Subject Efficacy
Table 6: Subject Overall Efficacy
Table 7: Subject Tolerability
Table 8: Subject Retinol Assessment
Table 9: Retinoid Marketing
Table 10: Moisturizer Assessment
Table 11: Transepidermal Water Loss (TEWL)
Table 12: Retinoid Treatment Assessment

All statistically significant points are bolder for easier recognition. A lower number was indicative of a superior rating for all questionnaires and assessments procured during the study. The data was analyzed using several different comparisons. A longitudinal intragroup analysis was used to determine what was happening in each study arm at each time point. A direct comparison or sequential analysis was done to look at the exact ordinal rating values over time. Finally, a difference from baseline analysis was done where all ordinal values were subtracted from baseline to examine change over time. The direct comparison and difference from baseline are intergroup analytical techniques.

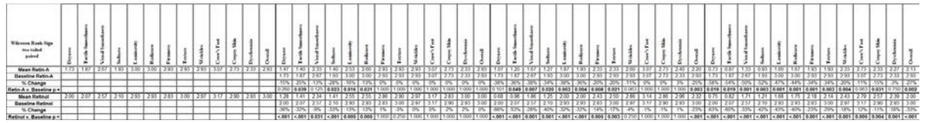
8. DISCUSSION

The results are discussed separately for each collected data set.

Table 1: Retinol Experience
The subjects using the retinoid and ancillary skin care products were asked to rate their experience at the end of the 12 week study. A lower score was indicative of a superior performance. The lipid infused moisturizer used in the retinol group was rated statistically superior to the CeraVe used in the tretinoin group (p=0.038). Subjects in both groups reported improvements in self confidence, beautiful skin, and empowerment. Both products were on average highly recommended by the subjects. The summary chart is presented below:

Experience						
	Week 12					
Mann-Whitney two tailed paired	**Good Skin**	**Self Confidence**	**Beautiful Skin**	**Empowered**	**Rate Moisturizer**	**Recommend**
Mean Retin-A	0.47	1.20	1.33	1.47	0.73	1.33
Mean Retinol	0.61	0.93	1.18	1.18	0.25	0.86
Retin-A v. Retinol p =	0.893	0.273	0.525	0.398	**0.038**	0.057
Z-score =	0.13	1.10	0.64	0.85	2.07	1.90

Table 2: Investigator Efficacy
The investigator assessed efficacy in terms of dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia, crepey cheek skin texture, and overall photodamage. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). There were no statistically significant parameters at any time point between the two groups indicating parity in terms of investigator assessed efficacy for the retinol and tretinoin. The longitudinal intragroup assessment is presented below to examine what happened over time for each group.



After 4 weeks of use, there was statistically significant improvement in tactile smoothness, softness, luminosity, and radiance for both groups, but the significance was higher for the retinol group correlating with better improvement. In addition, there was statistically significant improvement in visual smoothness with the retinol (p=0.031), however no significance was seen with the tretinoin. Improvement continued into week 8 with both groups showing statistical

significance in tactile smoothness, visual smoothness, softness, luminosity, radiance, firmness, skin texture, and overall photoaging appearance. There was a highly statistically significant improvement noted in skin dryness ($p<0.001$) with the retinol that was not seen with the tretinoin. After 12 weeks of use, both products demonstrated improvement parity, although the statistical significance continued to be higher for the retinol group. It is important to remember that twice as many subjects used the retinol as tretinoin.

Table 3: Investigator Overall Efficacy
At week 12, the investigator assessed the overall improvement in facial appearance using the following scale: 1= much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse. There was no statistically significant difference between the improvement noted with the retinol and tretinoin, however, the retinol rating was lower at 0.96 than the tretinoin at 1.07 on average. Both ratings rounded to an assessment of much improved for facial appearance. The summary table is presented below:

	Week 12
Mann-Whitney two tailed paired	**Rating**
Mean Retin-A	1.07
Mean Retinol	0.96
Retin-A v. Retinol p =	0.788
Z-score =	0.27

Table 4: Investigator Tolerability
The investigator assessed tolerability by querying the subjects on itching, stinging, and burning while observing directly redness and swelling. There were no statistically significant differences between the retinol and the tretinoin in any of the tolerability criteria.

Table 5: Subject Efficacy
The subjects assessed their skin in terms of dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, uneven skin tone, crepey cheek skin texture, and overall photodamage assessment. All assessments were made on a 5-point ordinal scale (0=none, 1=minimal, 2=mild, 3=moderate, 4=severe) at baseline (visit 1), week 4 (visit 2), and week 8 (visit 3), and week 12 (visit 4). There were no statistically significant differences between the groups at any time except for better visual smoothness seen with the retinol at week 8 ($p=0.045$). The subjects assessed parity between the retinol and tretinoin. The direct ordinal comparison chart is presented below:

It is also worthwhile to examine the intragroup longitudinal assessment. Both the retinol and tretinoin groups experienced improvement, however, there was higher statistical significance at all time points with the retinol as compared to the tretinoin. It is important to remember that twice as many subjects used retinol and tretinoin. There was earlier statistically significant improvement in subject assessed visual smoothness ($p=0.003$), softness ($p=0.006$), crow's feet ($p=0.001$), dyschromia ($p=0.004$), and overall photoaged appearance ($p=0.031$) with the retinol at week 4. Softness continued to be significant for the retinol group at week 8 ($p<0.001$) and not for the tretinoin group. Both groups demonstrated statistical significance for most parameters at week 12. The intragroup longitudinal analysis summary table is presented below:

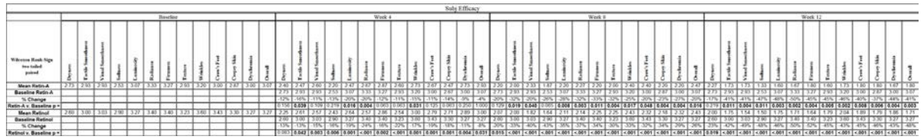


Table 6: Subject Overall Efficacy
After 12 weeks of use, the subjects were asked to assess their overall clinical improvement from baseline, using a 5-point scale (1=much improved, 2=moderately improved, 3=slightly improved, 4=no change, 5=worse). The subjects referred to their baseline images to make the assessment. No statistical difference was noted between the two groups. Both retinol and tretinoin subjects on average felt their facial skin was much improved. The summary chart is presented below:

Subj Overall	
	Week 12
Mann-Whitney two tailed paired	**Rating**
Mean Retin-A	1.27
Mean Retinol	1.18
Retin-A v. Retinol p =	0.697
Z-score =	0.39

Table 7: Subject Tolerability
The subjects rated tolerability in terms of itching, stinging, burning, redness, and swelling with a lower rating indicating less tolerability issues. There was only one statistically significant

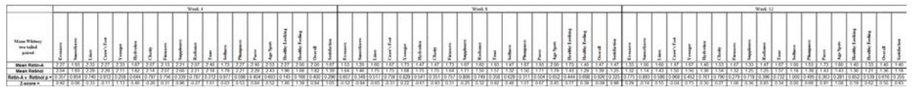
difference between the retinol and tretinoin in terms of itching at week 4. Less itching was experienced by the retinol group (p=0.010). The summary chart is presented below:

Subj Toler																				
	Baseline					Week 4					Week 8					Week 12				
Mann-Whitney two tailed paired	Itching	Stinging	Burning	Redness	Swelling	Itching	Stinging	Burning	Redness	Swelling	Itching	Stinging	Burning	Redness	Swelling	Itching	Stinging	Burning	Redness	Swelling
Mean Retin-A	0.00	0.00	0.00	0.00	0.00	0.40	0.67	0.67	0.73	0.07	0.67	0.93	0.80	1.00	0.13	1.27	1.67	1.67	1.40	0.40
Mean Retinol	0.03	0.00	0.00	0.10	0.00	0.04	0.46	0.50	0.46	0.07	0.50	0.96	1.00	1.04	0.25	0.89	1.14	1.29	1.25	0.50
Retin-A v. Retinol p =	0.579	1.000	1.000	0.579	1.000	**0.010**	0.303	0.403	0.252	0.713	0.791	0.808	0.716	0.582	0.499	0.386	0.346	0.563	0.979	0.611
Z-score =	-0.56	0.00	0.00	-0.56	0.00	2.57	1.03	0.84	1.15	0.37	0.27	0.24	-0.36	-0.55	-0.68	0.87	0.94	0.58	-0.03	-0.51

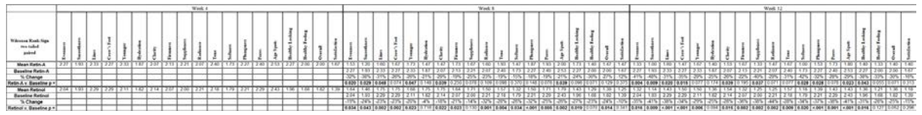
Table 8: Subject Retinol Assessment
At the end of the study, subjects were asked to rated their retinoid experience. The subjects rated the retinal slightly better than the tretinoin, however the difference was not statistically significant. The summary table is presented below comparing the raw ordinal average values for each group.

Product Overall	
	Week 12
Mann-Whitney two tailed paired	Rating
Mean Retin-A	2.07
Mean Retinol	1.89
Retin-A v. Retinol p =	0.556
Z-score =	0.59

Table 9: Retinoid Marketing
The subjects rated a variety of marketing questions. A lower score was indicative of a superior rating. There was no statistically significant difference between the retinol and tretinoin in terms of marketing preference. The direct ordinal comparison is presented below with the mean responses for each group:



The longitudinal comparison is presented below examining the change in means over time within each group. The means represent the overall group assessment and the changes to the responses represent change from baseline in the assessment.



Overall the subjects felt both products performed well after 8 and 12 weeks of product use. The marketing data may be analyzed further at a later date based on the need to support specific claims.

Table 10: Moisturizer Assessment
The subjects were asked to rate their moisturizer at week 4 and week 8. A lower number was indicative of a superior rating. The lipid infused moisturizer used with the retinol product was consistently rated higher than the CeraVe moisturizer used with the tretinoin product. At week 4, there was a statistically significant preference for the retinol lipid infused moisturizer, in terms of being non-sticky (p=0.026), absorption (p=0.034), feel (p=0.023), and experience (p=0.036). The lipid infused moisturizer would be purchased and recommended more by the users than the CeraVe. These preferences persisted into week 8 in terms of being non-sticky (p<0.001), absorption (p=0.010), and feeling good (p=0.028). The subjects would buy (p=0.020) and recommend (p=0.029) the lipid infused moisturizer over the CeraVe at week 8. Thus, the lipid infused moisturizer was statistically better than CeraVe in many attributes. The summary table is presented below:

	Week 4										Week 8									
Mann-Whitney two tailed paired	Easy	Non-Stinging	Non-Sticky	Absorbs	Moisturizes	Tolerated	Feel	Experience	Buy	Like	Recommend	Easy	Non-Stinging	Non-Sticky	Absorbs	Moisturizes	Tolerated	Feel	Experience	Buy	Like	Recommend
Mean Retin-A	0.07	0.40	0.60	0.33	0.27	0.27	0.60	0.47	0.80	0.67	0.67	0.13	0.60	0.93	0.40	0.13	0.33	0.67	0.47	0.73	0.60	0.60
Mean Retinol	0.00	0.11	0.11	0.07	0.07	0.14	0.11	0.11	0.14	0.07	0.07	0.00	0.29	0.07	0.04	0.07	0.14	0.07	0.14	0.11	0.07	0.04
Retin-A v. Retinol p =	0.283	0.711	**0.026**	**0.034**	0.112	0.935	**0.023**	**0.036**	**0.001**	**0.003**	**0.003**	0.086	0.205	**<.001**	**0.010**	0.537	0.590	**0.028**	0.304	**0.020**	0.073	**0.029**
Z-score =	1.07	0.37	2.22	2.12	1.59	0.08	2.27	2.10	3.22	2.99	2.99	1.72	1.27	3.39	2.57	0.62	0.54	2.19	1.03	2.32	1.79	2.18

Table 11: Transepidermal Water Loss (TEWL)
TEWL measurements were taken at baseline, week 8, and week 12. A higher number is indicative of greater barrier damage. While TEWL increased with both the retinol and tretinoin as expected, no statistically significant difference was observed between the 2 groups. The TEWL measurements as compared to baseline summary table is presented below:

TEWL			
T-test two tailed paired	Baseline	Week 8	Week 12
Mean Retin-A	8.94	15.95	18.19
Mean Retinol	9.10	17.34	17.08
Retin-A v. Retinol p =	0.805	0.337	0.604

While both products induced barrier damage, the longitudinal intragroup comparison showed less barrier damage at week 12 with the retinol than the tretinoin. At week 12, there was a 104% increase in TEWL with the tretinoin and an 88% increase with the retinol. While this is not statistically significant, it does show less barrier damage at week 12 with the retinol.

TEWL			
T-test two tailed paired	**Baseline**	**Week 8**	**Week 12**
Mean Retin-A	8.94	15.95	18.19
Baseline Retin-A		8.94	8.94
% Change		79%	104%
Retin-A v. Baseline p =		**<.001**	**<.001**
Mean Retinol	9.10	17.34	17.08
Baseline Retinol		9.10	9.10
% Change		90%	88%
Retinol v. Baseline p =		**<.001**	**<.001**

Table 12: Retinoid Treatment Assessment

The subjects were asked to assess the retinoid treatment based on a variety of parameters. There was a statistically significant preference at week 4 for the purchase of the retinol product over the tretinoin (p=0.010). No other statistically significant differences were noted in the intergroup analysis. Since a lower number is indicative of a superior score, it can be seen that for both products the subject perception was excellent. Additional analysis of this data may be necessary based on the need for claim substantiation. The summary table is presented below:

Treatment																															
	Week 4											Week 8										Week 12									
Mann Whitney two tailed paired	**Easy**	**Non Stinging**	**Non Sticky**	**Absorbs**	**Moisturizes**	**Educated**	**Feel**	**Experience**	**Buy**	**Like**	**Recommend**	**Easy**	**Non Stinging**	**Non Sticky**	**Absorbs**	**Moisturizes**	**Educated**	**Feel**	**Experience**	**Buy**	**Like**	**Recommend**	**Easy**	**Non Stinging**	**Non Sticky**	**Absorbs**	**Moisturizes**	**Educated**	**Feel**	**Experience**	**Buy**	**Like**	**Recommend**
Mean Retin-A	0.27	0.80	0.40	0.27	0.73	0.40	0.73	0.40	1.00	0.87	0.80	0.07	1.07	0.73	0.20	1.07	0.53	0.53	0.73	1.00	0.93	0.80	0.47	1.47	0.67	0.40	0.87	1.20	1.00	1.13	1.33	1.13	1.00
Mean Retinol	0.14	0.71	0.36	0.39	0.39	0.50	0.36	0.36	0.43	0.46	0.43	0.14	1.07	0.39	0.25	0.71	0.64	0.46	0.61	0.57	0.54	0.50	0.21	1.68	0.32	0.21	0.93	1.54	1.11	0.96	0.89	0.89	0.89
Retin-A v. Retinol p =	0.339	0.690	0.682	0.517	0.145	0.810	0.188	1.000	**0.010**	0.062	0.055	0.669	0.945	0.372	0.720	0.208	0.756	0.686	0.756	0.210	0.183	0.326	0.176	0.632	0.064	0.208	0.956	0.386	1.000	0.637	0.196	0.379	0.527
Z-score =	0.96	0.40	0.41	-0.65	1.46	-0.24	1.32	0.00	2.57	1.87	1.92	-0.43	-0.07	0.89	-0.36	1.26	-0.31	0.40	0.31	1.25	1.33	0.98	1.35	-0.48	1.85	1.26	0.06	-0.87	0.00	0.47	1.29	0.88	0.63

9. SUMMARY

9.1 PRODUCT TOLERABILITY ENDPOINT

The tolerability endpoint was the investigator-assessed absence of serious adverse events from the facial study products at any time during the 12-week study. The tolerability endpoint was met.

9.2 SAFETY ENDPOINT

The safety endpoint was the absence of significant adverse reactions. The safety endpoint was met.

9.3 EFFICACY ENDPOINTS

9.3.1 PRIMARY EFFICACY ENDPOINT:

The primary efficacy endpoint was the investigator assessed improvements in overall facial skin appearance (to include all attributes evaluated) after 12 weeks of study product use as compared to baseline and between treatment arms. The primary efficacy endpoint was met for all attributes to include: dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia, crepey cheek skin texture, and overall photodamage.

9.3.2 SECONDARY EFFICACY ENDPOINT:

The secondary efficacy endpoint was the subject assessed improvements in overall facial skin appearance (to include all attributes evaluated) after 12 weeks of study product use as compared to baseline and between treatment arms. The secondary efficacy endpoint was met for all attributes to include: dryness, lack of tactile smoothness, lack of visual smoothness, lack of softness, lack of luminosity, lack of radiance, lack of firmness, poor skin texture, fine facial wrinkles, crow's feet lateral eye wrinkles, dyschromia, crepey cheek skin texture, and overall photodamage.

Enhanced Post-Procedure Healing And Reduced Discomfort With Use Of A Sterile Treatment-Serum Infused Biocellulose Mask.

Michael H. Gold, MD, Gold Skin Care Center, Nashville, TN.

ABSTRACT

Background. Dermatologic procedures may be accompanied by slow healing and post-treatment discomfort. A novel biocellulose mask is designed to relieve post-procedure discomfort, improve rates of healing, and reduce the appearance of redness for 1 week or longer.

Objectives. To evaluate the efficacy and safety of a biocellulose mask to accelerate healing, enhance improvement, and reduce discomfort following a RF/microneedling procedure of the face.

Methods. Ten healthy females aged 35 to 60 years, Fitzpatrick skin type II (n=7) and III (n=3), and mild to moderate wrinkles (Glogau grade II[n=8] or III [n=2]) enrolled in the open-label, single-site pilot study. Subjects were treated once with 2 passes of a microneedle radiofrequency (RF) device (EndyMed PRO™, Intensif Handpiece, EndyMed Medical, Cesarea, Israel) Treatment was immediately followed by application of the biocellulose mask to the entire face for 15 to 20 minutes. Subjects were given an additional six masks for daily home use and asked to return to the office 3 and 7 days later for evaluation of efficacy and safety. Skin responses were tracked by photography of subjects' faces immediately post procedure (pre-and post-mask application), and on days 3 and 7. Clinical grading was performed on days 1, 3 and 7.

Results. Subjects achieved statistically significant improvement in skin radiance, smoothness, texture, and dryness after a single RF/microneedling treatment followed by daily usage of the biocellulose mask for 1 week. Skin tone evenness, red/blotchiness, and overall appearance were trending toward significant improvement by Day 7. Adverse events were not observed in any subject. Seventy percent of subjects would recommend use of the mask after RF/micro needling treatment.

Conclusion. The results demonstrate the effectiveness of the biocellulose mask in soothing skin and accelerating its healing post a RF/microneedling procedure. The mask may be used directly on compromised skin immediately post microneedling, without objective or subjective irritation. Improvement and conditioning of the facial skin using the mask daily for one week after a treatment has been shown.

INTRODUCTION

Dermatologic procedures are often associated acute erythema, edema, post-procedure discomfort and slow healing. An unbranded, sterile, serum-infused, biocellulose mask (Topix Pharmaceuticals, Inc., West Babylon, NY) has been developed to relieve patient discomfort, while accelerating rates of healing, and minimizing inflammation post dermatologic procedures.

Components of the mask include short and long molecular weight hyaluronic acids, matrix repair peptides, green tea polyphenols, resveratrol and other antioxidants. The short chain fractions of HA readily penetrate the skin to deeply moisturize and prevent transepidermal water loss. The high molecular weight HA fractions retain moisture on the surface. The anti-inflammatory, anti-oxidant blend of green tea polyphenols, resveratrol, caffeine, ectoin and ergothioneine promote recovery and support healing. The biocellulose substrate saturated with the serum, comfortably adheres to the face, enveloping post-procedure skin with deep and sustained hydration and healing agents, while providing cooling and soothing comfort.

A non-insulated microneedle radiofrequency (RF) device (EndyMed PRO™, Intensif Handpiece, EndyMed Medical, Cesarea, Israel) has been shown to create microzones of coagulation in the papillary and reticular dermis with minimal epidermal damage, and bulk volumetric heating (Harth 2013, Harth 2014). This technology has shown efficacy in the treatment of acne scars (Harth 2014) and, in combination with non-ablative RF skin tightening and ablative RF fractional skin resurfacing, in full-face skin rejuvenation (Kaplan 2016). RF microneedling can be used on all skin types without concerns over dyschromia

RF microneedling for aesthetics is rapidly increasing in popularity and is a useful model to evaluate the safety and efficacy of the post-procedure treatment mask. The present study assesses the healing benefit and comfort enhancement of the aforementioned biocellulose mask immediately after a single RF/microneedling treatment and for the following week.

METHODS

Subjects. Ten healthy females aged 35 to 60 years, Fitzpatrick skin type II (n=7) and III (n=3), and mild to moderate wrinkles (Glogau grade II[n=8] or III [n=2]) enrolled in the open-label, single-site pilot study. Inclusion criteria were willingness to (1) use only their current skin care regimen during the study; (2) use no products other than the dispensed skin cleanser, sunscreen, and biocellulose masks; and (3) willingness to wear a hat or apply SPF 30 or greater sunblock if sun exposure is necessary. Grounds for exclusion were pregnancy, breast feeding, plans for pregnancy, or unwillingness to use appropriate methods of birth control; excessive facial exposure to UV radiation (sunlight or artificial); presence or recent history of a facial condition (e.g., moderate to severe acne vulgaris, atopic dermatitis, psoriasis, rosacea, seborrheic dermatitis, excessive facial hair or coloration) which, in the opinion of the principal investigator, might interfere with evaluation of study parameters; presence of implanted metal devices in the treatment area; invasive or non-invasive skin treatments (e.g., hair removal, injectable fillers or toxins) in the target area within the previous 3 months; permanent makeup, tattoos, body piercing, or excessive hair in the treatment area; history of squamous cell carcinoma or melanoma in the treatment area within the previous 5 years; allergy or hypersensitivity to any of the product ingredients; current or recent (past 30 days) participation in another research study; or per the principal investigator's

discretion, any other mental or physical condition that might make it unsafe for the subject to participate in this study. All subjects consented to photography and provided signed informed consent to participate in the study.

Study Design. The protocol is outlined in Table 1. Subjects were screened and qualified subjects were enrolled during Visit 1 (Day 1). Screening and baseline evaluations occurred during Day 1 unless a washout period was required. Subjects were instructed to stop using topical astringents and abrasives (1 week), antibiotics (2 weeks), retinoids (2 weeks), and glycolic or lactic acids (2 weeks) on the face. Photographs (full front, 45° left and right) were obtained with a mounted digital camera (Canfield Scientific, Parsippany, NJ).). For photography, subjects wore no makeup, including foundation, blush, eye shadow, lipstick and mascara. They were required to remove all jewelry, use headbands to keep hair back away from face, and keep their eyes closed for all photographs.

Table 1. Protocol outline

Procedure	Day		
	1	3 (± 1)	7 (± 1)
Investigator global assessments*	x	x	x
Subject tolerability assessments	x	x	x
Glogau grading	x	x	x
RF/Microneedling treatment	x		
Mask Posttreatment for 15-20 minutes	x		
Dispense diary	x		
Reconcile/collect diary		x	x
Concomitant medications query	x	x	x
Adverse event query		x	x
Subject satisfaction questionnaire			x
Clinical photography*	x	x	x

*Before and after treatment.
RF = radiofrequency

Pretreatment. The face of each subject was washed thoroughly with soap and lukewarm water and dried. The target area was photographed and assessed visually to determine initial RF/microneedling treatment parameters. Topical anesthetic was applied and later removed with a gauze pad moistened with 70% medical grade alcohol. Care was taken to remove all anesthetic before RF/microneedling treatment.

Treatment device. The FDA-approved RF/microneedling device uses a consumable sterile tip containing a matrix of tiny RF micro-needle (300-micron diameter) electrodes arranged to deliver RF energy. Using the microprocessor control, the operator can modify the treatment parameters to achieve specific tissue effects directly related to the subject 's skin condition.
Treatment area: Describe how the full face was treated before getting into detail about the test spot, otherwise readers may think only a test spot was treated.

Determining treatment parameters. A test spot was done in the target area of each subject to define the optimal treatment parameters. Low parameters were used initially and adjusted according to observed skin reactions and needs of the subject. For example, if the skin had mild to moderate redness and edema and the subject felt mild to supportable discomfort for a given set of parameters, the operator did not alter the settings. If erythema and edema were absent and the subject felt no discomfort at the

initial settings, the pulse duration was increased by 30 msec or the power was increased by 2 watts. If the jaw, zygomatic arch, or temples were treated and the skin was edematous and the subject felt great discomfort, the pulse duration was reduced by 30 msec or the power was decreased by 2 watts. The recommended initial parameters for each facial area are shown in Table 2.

Table 2: Recommended initial parameters for radiofrequency/microneedling treatment.

Area Of Treatment	Energy (watts)	Needles Depth (mm)	Pulse Duration
Forehead	13	1.2-1.4	110
Periorbital	12	1-1.2	110
Cheeks	14	2.2-2.8	110-140
Perioral	12	1.2-1.5	110

Mask. When the appropriate treatment parameters were determined each subject was treated with 2 passes of the RF/microneedling device over the whole face. Treatment was immediately followed by application of the biocellulose mask to the entire face for 15 to 20 minutes. Subjects were given an additional six masks for daily home use and asked to return to the office on 3 and 7 days later for evaluation and maintain daily treatment diaries. Compliance was evaluated by notes in the diary.

Study objectives. The primary objective was to show the efficacy and safety of using the biocellulose mask to accelerate healing after the RF/microneedling procedure. The secondary objective was to evaluate improvement and conditioning of the facial skin using the mask once daily for one week after a single RF microneedling treatment.

Assessments. Wrinkles were evaluated at each visit using the Glogau scale (Type I, no wrinkles with minimal to no discoloration and no keratoses; Type II, wrinkles in motion, slight lines near the mouth or eyes, no keratoses; Type III, wrinkles at rest, always visible, noticeable discolorations, visible keratoses; Type IV, only wrinkles throughout, makeup appears to cake and crack when applied, gray or yellow discoloration of skin, history of skin cancer). Full-face Investigator Global Assessments (Table 3) were made at each visit, before and after treatment and before and after application of the mask, using a scale of 0 to 4.

Table 3. Global assessments of skin attributes

Skin Attribute	Rating	
	0	4
Radiance	dull	glowing
Tone (evenness)	Even, healthy color	Uneven, discolored
Smoothness	smooth appearance	severe, rough appearance
Texture	smooth, even feeling	rough, uneven feeling
Red/Blotchy	clear	severe redness
Dryness/Flakiness	smooth	rough and dry
Overall Appearance	Healthy, youthful appearance	poor appearance

Full-face investigator objective tolerability was assessed as erythema, edema, dryness, and peeling using the following scale: 0=none, 1=minimal, 2=mild, 3=moderate, and 4=severe.
Full-face subjective tolerability was assessed as stinging, tingling, itching, burning, and itching using the following scale: 0=none, 1=minimal, 2=mild, 3=moderate, 4=severe

Data analysis. The investigators hypothesized that the use of mask immediately after the RF/microneedling procedure would result in (1) improved scores in the Investigator Global Assessments (a measure of efficacy) and (2) acceptable scores in the Investigator Objective and Subjective Tolerability Assessments (indicators of safety). The investigators further hypothesized that facial skin condition would be improved by the healing components of the mask. Since the scales are discrete whole numbers, comparisons with baseline were made using the non-parametric Wilcoxon Signed Rank Test. Corrections for multiple comparisons were not made.

Adverse events. Subjects were asked to record adverse events in their diaries. Severity and relationship to the study treatment were assessed and recorded.

RESULTS

Adverse events were not observed in any subject during the study. Efficacy, tolerability, and subject satisfaction are shown below.

Radiance. The median pretreatment assessment for all skin attributes, including radiance, was 2.0 initially (Table 1). For radiance, a higher score indicated improvement and the highest score for any subject was 3.0 throughout the study. The number of subjects achieving 3.0 at each time point is shown in Figure 1. Median improvement compared to baseline was significant at Day 7 (Table 1) in which 8/10 subjects achieved improvement at 3.0.

Table 1. Investigator global assessments (median [IQR]) with comparisons to baseline

	Day 1			Day 3	Day 7
Skin Attribute	PreT, PreM*	PostT, PreM	PostT, PostM	PostT, PostM	PostT, PostM
Radiance	2.0 (0.2)	2.0 (0.1)	2.0 (0.0)	2.0 (1.0)	3.0 (0.1) (P = 0.0313)
Evenness	2.0 (0.0)	2.0 (1.0)	2.0 (1.0)	2.0 (0.1)	2.0 (1.0)
Smoothness	2.0 (0.0)	3.0 (0.1)	2.0 (0.0)	1.5 (1.0)	1.0 (0.0) (P = 0.0195)
Texture	2.0 (0.0)	3.0 (0.1)	2.0 (0.1)	2.0 (1.1)	1.0 (0.0) (P = 0.0078)
Blotchy, Red	2.0 (0.0)	3.0 (0.0)	2.0 (0.0)	2.0 (1.0)	1.0 (1.0)
Dryness	2.0 (0.0)	2.0 (0.1)	2.0 (1.0)	1.5 (1.0)	1.0 (0.0) (P = 0.0078)
Overall Appearance	2.0 (0.0)	2.5 (1.0)	2.0 (0.0)	2.0 (1.0)	2.0 (1.0)

*Baseline.
IQR = interquartile range; PreT = pretreatment; PreM = premask; PostT = posttreatment; PostM = postmask.

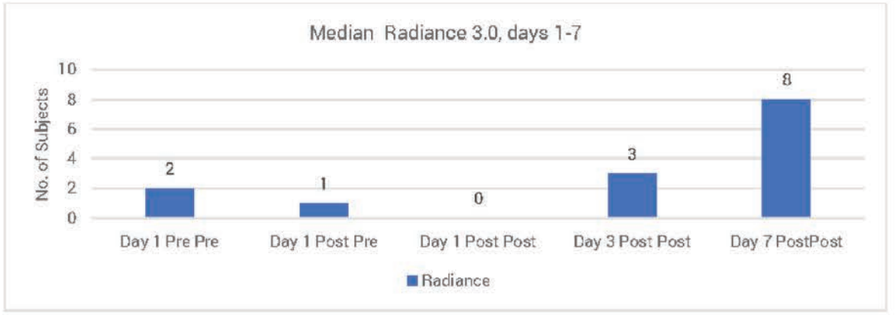


Figure 1. Number of subjects who achieved a score of 3.0 for radiance 1 to 7 days after a single radiofrequency/microneedling treatment and application of the biocellulose mask.

Other Assessments. For the remaining skin attributes, a lower score indicated improvement and the lowest score for any subject was 1.0 throughout the study. The number of subjects achieving 1.0 at each time point is shown in Figure 2 for each attribute. Improvement became apparent for all attributes by day 3. At Day 7, median improvement compared to baseline was significant for smoothness, texture, and dryness (Table 1). Trends (non-significant) toward improvement were evident for evenness (p = 0.1250), red blotchy (p =0.0547), and overall appearance (p = 0.1250) at Day 7. On Day 1 (PosT, PreM), 10 subjects were scored 2 or 3 for evenness, red/blotchiness, and overall appearance. On the third day, 9, 7, and 8 subjects were scored 2 or 3 and 1, 3, and 2 subjects were scored 1, respectively. On the seventh day, 6, 4, and 6 subjects were scored 2 or 3 and 4, 6, and 4 subjects were scored 1, respectively. These data clearly indicate a trend toward improvement for these attributes with daily usage of the mask.

Figure 2. Number of subjects who achieved a score of 1.0 for skin attributes 1 to 7 days after a single radiofrequency/microneedling treatment and application of the biocellulose mask

Tolerability. Objective and subjective tolerability for all subjects during the study are shown graphically in Figures 3 and 4. Erythema (Figure 3) was resolved in all subjects by Day 7 and edema (Figure 3) was absent in all subjects by Day 3.

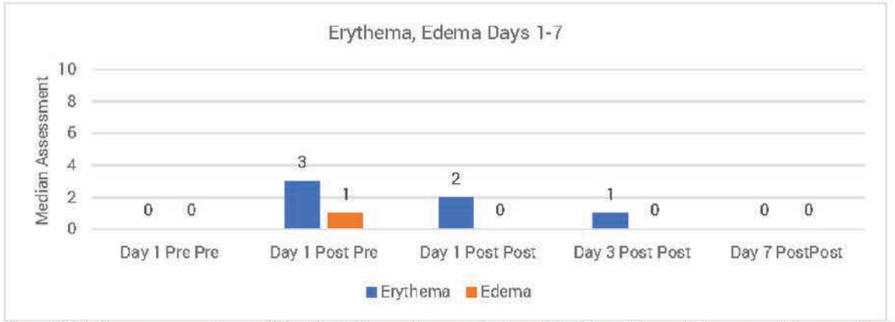


Figure 3. Median assessment score (all subjects) for erythema and edema 1 to 7 days after radiofrequency/microneedling treatment and application of the biocellulose mask

Median tolerability scores for stinging, tingling, and burning during the study are shown in Figure 4. Stinging (Figure 4) was either 1 or 2 at Day 1 (PosT, PreM); either 1 or 0 at Day 1 (PosT, PosM); and 0 in all subjects thereafter. On Day 1 (PosT, PreM), tingling (Figure 4) was either 0 or 1 in 9 subjects and 2 in a single subject. On Day 1 (PosT, PosM), tingling was 0 in 9 subjects and 1 in a single subject. Tingling was 0 in all subjects thereafter. Itching was 0 or 1 on Day 1(PosT, PreM); 0 in all subjects on Day 1 (PosT, PosM); 0 or 1 on Day 3, and 0 in all subjects on Day 7. Burning (Figure 4) was 0 to 2 on Day 1 (PosT, PreM); 0 in 9 subjects on Day 1 (PosT, PosM), and 0 in all subjects for the remainder of the study.

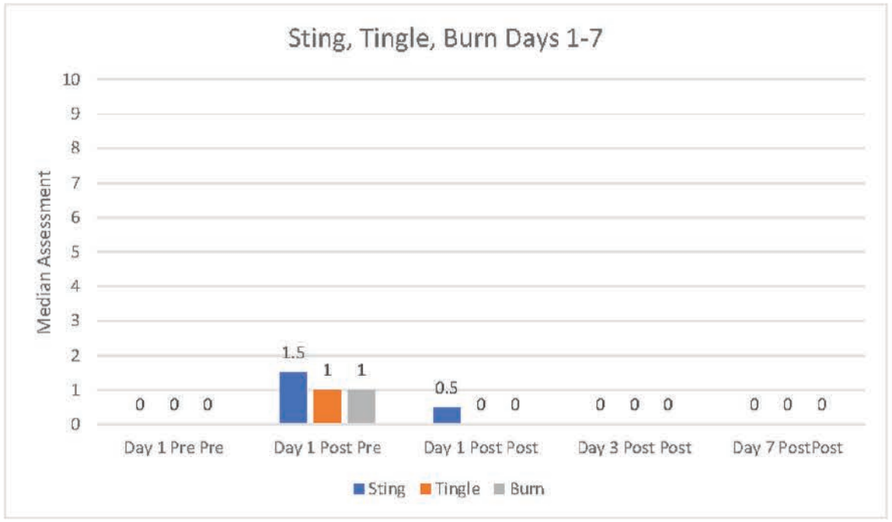


Figure 4. Median assessment score (all subjects) for stinging, tingling, and burning 1 to 7 days after a single radiofrequency/microneedling treatment and application of the biocellulose mask.

Dryness was scored 0 in most subjects at each time point. The remaining subjects were scored 1 (n=1, Day 1 [PosT, PreM]; n=1, Day 3; and n=2, Day 7). Peeling was scored 0 in all subjects at each time point.

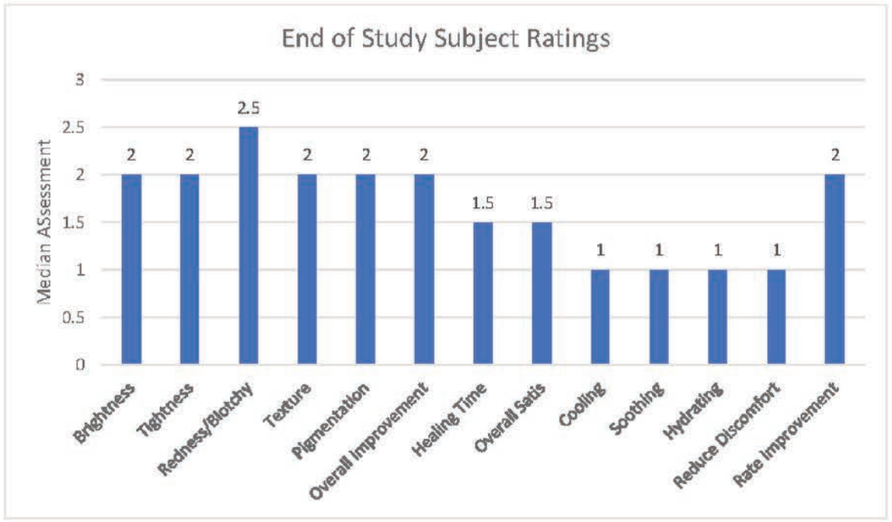
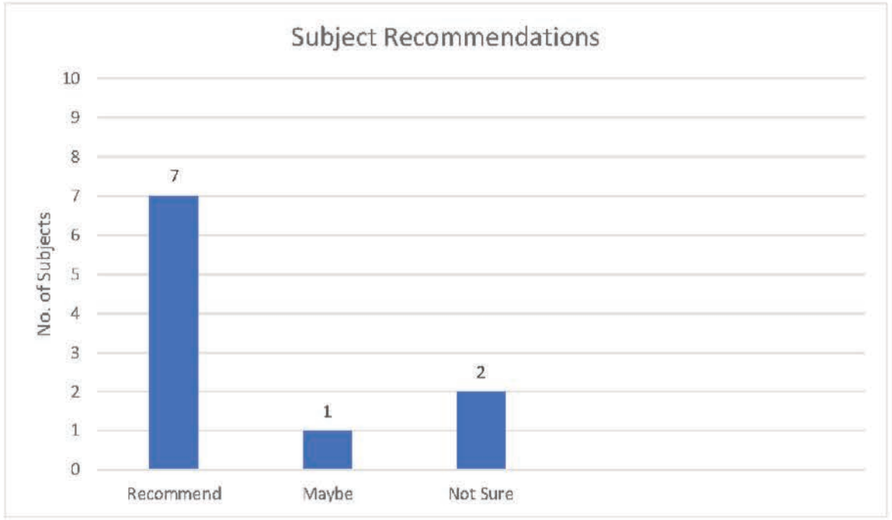


Figure 5. Median assessment score (all subjects) for end-of-study parameters 1 to 7 days after a single radiofrequency/microneedling treatment and application of the biocellulose mask.

Subject Self-Assessments. Subjects were asked to assess the results (0-4 with 4 the most favorable) for the parameters shown in Figure 5. The median value was highest (2.5) for redness/blotchy followed by brightness, tightness, texture, pigmentation, overall improvement, and rate improvement (all 2.0), and then healing time and overall satisfaction (all 1.5) and cooling, soothing, hydrating, and reducing discomfort (all 1.0). The minimum value of all parameters was 1.0 and the maximum values were 4.0 (redness blotchy, hydrating, reducing discomfort, rate improvement), 3.0 (brightness, tightness, texture, pigmentation, overall improvement, healing time, overall satisfaction) and 2.0 (cooling, soothing).



(Figure 6) Seventy percent of subjects Would recommend the treatment with face mask.

Figure 6. Willingness of subjects to recommend the RF/microneedling procedure with face mask to friends and family.

Discussion

There is no set protocol for post procedure in today's world. We have been recommending moist environments for many years – which started with petrolatum and has progressed to include products with various growth factors or silicone-based products to help wounds heal faster than what we have with just plain petrolatum. The advantage of the mask is first – the initial soothing effect on the skin which works faster than most anything I know. And with its novel combination of anti-inflammatory ingredients, it also helps reduce redness very quickly – and was very accepted by everyone that used it.

This is the first study to evaluate the efficacy and safety of a biocellulose mask to accelerate healing, enhance improvement, and reduce discomfort up to 1 week after a single RF/microneedling procedure of the face.

Subjects achieved statistically significant improvement in skin radiance, smoothness, texture, and dryness after a single RF/microneedling treatment and daily usage of the biocellulose mask for 1 week (Table 1). The earliest noticeable improvements were in smoothness and dryness as shown by

the decrease in median scores from 2.0 to 1.5 on Day 3 for both skin attributes. Data for evenness, red/blotchiness, and overall appearance suggested trends toward improvement in these attributes by Day 7.

Adverse events were not observed in any subject. Tolerability was indicated by favorable trends in both objective and subjective parameters. Erythema was resolved in 4 subjects by Day 3 and in all subjects by Day 7 while edema was resolved in 6 subjects on Day 1 (PosM) and in all subjects by Day 3 (Figure 3). Stinging, tingling, and burning were gone by Day 3 (Figure 4) while itching was absent by day 7. Minimal dryness was noted in only a few subjects during the study. Peeling was not observed in any subject during the study.

Seventy percent of subjects would recommend the treatment and mask and the remaining subjects were not sure. No subject stated that he or she would not recommend the mask. Subjects were most satisfied with improvement in skin redness/blotchiness, brightness, tightness, texture, pigmentation, overall improvement, and rate of improvement.

These encouraging results justify additional studies with more subjects, longer duration, and a control group undergoing RF/microneedling treatment without the biocellulose mask.

CONCLUSIONS

The results demonstrate the efficacy and safety of the biocellulose mask to accelerate healing post RF/microneedling. Improvement and conditioning of the facial skin using the mask daily for one week has been shown.

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Enhanced post-procedure healing and reduced discomfort with use of a sterile treatment-serum infused biocellulose mask.

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Green Tea *(Camelia sinensis)* Suppresses B Cell Production of IgE Without Inducing Apoptosis

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Abstract. Green tea *(Camelia sinensis)* is known to possess biological properties that are antioxidative and antimutagenic. Recent studies demonstrated beneficial effects of green tea in inflammatory allergy. However, the effect of green tea on anti-allergic activity/IgE responses in vitro has not been studied. U266 myeloma cells (2 x 10^6/ml), which secrete IgE, were cultured for 0-72 hr with or without green tea extract (1-300 ng/ml), and IgE levels in the supernatants were determined (24-72 hr) by ELISA. The effects of green tea extract on U266 cell numbers, viability, and apoptosis were studied by flow cytometry. High levels of IgE produced by U266 cells were observed at 24, 48, and 72 hr (1.3 ± 0.3 x 10^3, 1.7 ± 0.3 x 10^3, 2.8 ± 0.4 x 10^3 IU/ml, respectively). Addition of green tea extract either as (a) a single dose, or (b) repeated daily doses, suppressed IgE production with increasing suppression over time (up to 90%; $p < 0.05$); the suppression was dose-dependent with the highest concentrations resulting in the greatest suppression. The suppression of IgE production by green tea extract was not mediated by apoptosis or cell death. This study demonstrates that green tea extract has immunoregulatory effects on human IgE responses in vitro.

Keywords: IgE, U266 myeloma cells, B cell, polyphenols, flow cytometry, annexin, propidium iodide

Introduction

Green tea *(Camelia sinensis)* contains a number of bioactive ingredients, including polyphenols such as epigallocatechin gallate (EGCG), caffeine, and vitamins [1-2], which have antioxidative and free-radical scavenging properties [3-6], cancer-preventing actions [7-10], and cause induction of apoptosis of cancer cells [11]. EGCG, the major catechin in green tea, is believed to be the primary source of green tea's beneficial effects [1]. However, the O-methylated derivative of EGCG, (-)-epigallocatechin-3-O-(3-O-methyl)-gallate (EGCG"3Me), which was isolated from oolong tea, is reported to have more inhibitory effects on type I and IV allergies in mice than does EGCG [1]. EGCG"3Me can inhibit histamine release in a human basophilic cell line KU812 [12], as well as suppress the expression of FcεR1 α and γ chain genes [1]. Shiozaki et al [13] found that tea catechins and caffeine have important inhibitory roles in type I allergic reactions in rats.

Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenyl natural product from green tea, modulates the inflammatory allergic reaction and decreases IgE-induced histamine release from mast cells [14]. Gallic acid also has an anti-allergic effect in allergy models in vivo and in vitro [14], and

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might be useful in the treatment of allergic skin reactions [14]. Kim et al [14] reported that gallic acid inhibits mast cell-derived inflammatory allergic reactions by blocking histamine release and pro-inflammatory cytokine expression.

IgE plays a major role in asthma and allergic reactions through its ability to bind to Fc-epsilon RI on mast cells [15]. The effect of green tea on the production of IgE by B cells has yet to be established. In the present study, we assessed the effect of unseparated green tea extract (GTE) on IgE response in vitro. We used unseparated GTE because this likely closely mimics the advantageous effects of green tea, since it includes all of the potentially bioactive ingredients. We also investigated whether the effect of GTE on IgE production in U266 cells was due to apoptosis, or was independent of cell death. The present study demonstrates that green tea extract suppresses in vitro IgE production, suggesting a potential therapeutic application of GTE in IgE mediated inflammatory diseases such as allergies, asthma, and atopic dermatitis.

Materials and Methods

U266 cell culture. Cultures of human B cell myeloma line U266 (ATCC, Rockville, MD) (2 x 10^6/ml), which secretes IgE, were cultured for 0-72 hr in complete medium (RPMI containing heat-inactivated fetal calf serum (FCS) (10%) (Gibco, Grand Island, NY), L-glutamine (2 mM) (Gibco), 2 mercaptoethanol (50 µM) (Sigma, St. Louis, MO), and streptomycin (20 ng/ml) (Lilly, Indianapolis, IN), with or without various concentrations of green tea (1-300 ng/ml). Cells were cultured for 0-72 hr at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell viability was >90%, as judged by trypan blue exclusion. Cell culture supernatants were collected (0, 12, 24, 48, 72 hr) and frozen (-70°C) until assayed.

Fresh green tea extract. Whole green tea *(Camelia sinensis L)* extract was purchased from Topix Pharmaceuticals (West Babylon, NY). As per manufacturer, the composition of green tea extract is 90% polyphenol isolate from whole leaf, containing 80% catechins, of which 70% of the catechin content is EGCG, with no significant lot-to-lot variation. The green tea extract powder was freshly prepared for each experiment by suspension in RPMI media (Gibco) (1 g/100 ml) prewarmed to room temperature and diluted (1-300 ng/ml). Three hundred ng/ml was selected as the maximum concentration of GTE in this study, based on previous studies that found maximum serum levels of 300 ng/ml in human volunteers after multi-gram daily supplements [16-17].

Quantification of IgE production. In vitro quantitative determination of IgE content in cell culture supernatants was performed using a solid-phase sandwich enzyme-linked immunoadsorbent assay (IgE ELISA Test Kit, Bioquant, San Diego, CA). All ELISAs were performed according to the manufacturer's recommended procedure. Specimens were analyzed in triplicate and a standard curve was derived from known concentrations of IgE. Plates were read at 450 nm using an automated microplate reader (Model Elx800; Bio-Tek Instruments, Winooski, VT). Optical densities were converted to IU/ml based on the standard curve.

Flow cytometry. Flow cytometry was performed with an Epics XL/MCL flow cytometer with system II software (Beckman Coulter, Fullerton, CA). The fluidics system and optical alignment were verified daily using flow-check fluorospheres (Beckman Coulter). The gain on the photomultiplier tube detecting fluorescence intensity was adjusted so that 99% of cells with background fluorescence staining were scored between 10^0-10^1 on a 4-decade log scale. Color compensation was performed between channels FL1 to FL2 to exclude the effects of overlap of the fluorescence spectra. Briefly, overlap of FL1 and FL2 was excluded by adjusting the compensation value so that cells that are singly labeled with a FL1 fluorescent antibody will result in positive and negative cell populations that have the same median fluorescence intensity, which is distributed symmetrically around the mean FL2 channel autofluorescence value. Specific fluorescence was reported as the % of cells with relative fluorescence intensity scored above background (isotype control); 50,000 events were counted for all samples.

Apoptosis. Apoptotic cell death was determined using a combination of annexin V (AV) and propidium iodide (PI) labeling and analyzed by flow cytometry. In apoptotic cells, membrane phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the extracellular environment. Annexin V is a 36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS and binds to cells when PS is exposed to the external cellular environment, which occurs when membrane integrity is affected in the early phase of apoptosis [18-20]. PI is a vital dye that binds to DNA, a process implying disrupted cellular membrane and exposed DNA, compatible with late, irreversible cell necrosis, either primary or secondary to late apoptosis.

U266 cells were gently separated from culture tubes with a rubber policeman, washed twice with cold phosphate buffer solution (PBS) (Gibco), and resuspended in binding buffer (0.1 M Hepes, 1.4 M NaCl, 25 mM $CaCl_2$) (BD Biosciences Pharmingen, San Diego, CA). Annexin V-FITC and PI were then added with a volume of 1:20 the volume of cells (typically 5 µl in 100 µl of cells in solution), followed by gentle vortexing, and incubation for 15 min at room temperature (25°C) in the dark. Finally, 400 µl of 1X binding buffer was added to each tube, and cells were analyzed by flow cytometry within 30 min. Apoptosis and necrosis were distinguished by the combination of labeling of annexin V (AV) and propidium iodide (PI) (BD Biosciences Pharmingen), and analyzed using WinMDI software (Vers. 2.8, Scripps Research Institute, San Diego, CA). AV-PI- was defined as viable cells; AV+PI- was defined as early apoptosis; AV-PI+ was defined as early necrosis; AV+PI+ was defined as late stage cell death, either by apoptosis or necrosis.

Absolute cell counts. Absolute cell counts after treatment with GTE were determined by single platform flow cytometric immunophenotyping analysis using flow-count fluorospheres (Beckman Coulter) [21-22]. Briefly, the fluorospheres were transferred to a test tube with an equal volume of cells in solution (100 µl each), followed by vigorous vortexing. After analysis by flow cytometry, the absolute cell count was calculated by the following formula: Absolute Count = [(cells counted) x (fluorospheres added)] / (fluorospheres counted).

Statistics. Data are reported as mean ± SD. Non-parametric statistical techniques were employed; the Kruskal-Wallis one way ANOVA and pair-wise Mann-Whitney U tests were used to compare medians between treatment and control groups. Values (two-tailed) of $p \leq 0.05$ were considered significant.

Results

Effect of green tea on IgE responses induced in vitro. With no GTE treatment, high levels of IgE were first detected in vitro at 12 hr (6.4 ± 5.6 x 10^2 IU/ml) and continued to increased at 24, 48, and 72 hr (1.3 ± 0.3 x 10^3, 1.7 ± 0.3 x 10^3, 2.8 ± 0.4 x 10^3 IU/ml, respectively) (Fig. 1A). We focused on 24, 48, and 72 hr for studies of the effects of in vitro treatment with green tea extract (GTE) (1-300 ng/ml) on suppression of IgE production.

At 24 hr after treatment with 300 ng/ml GTE, there was moderate suppression of IgE production (15.2 ± 1.3%), but no or mild suppression with 1,

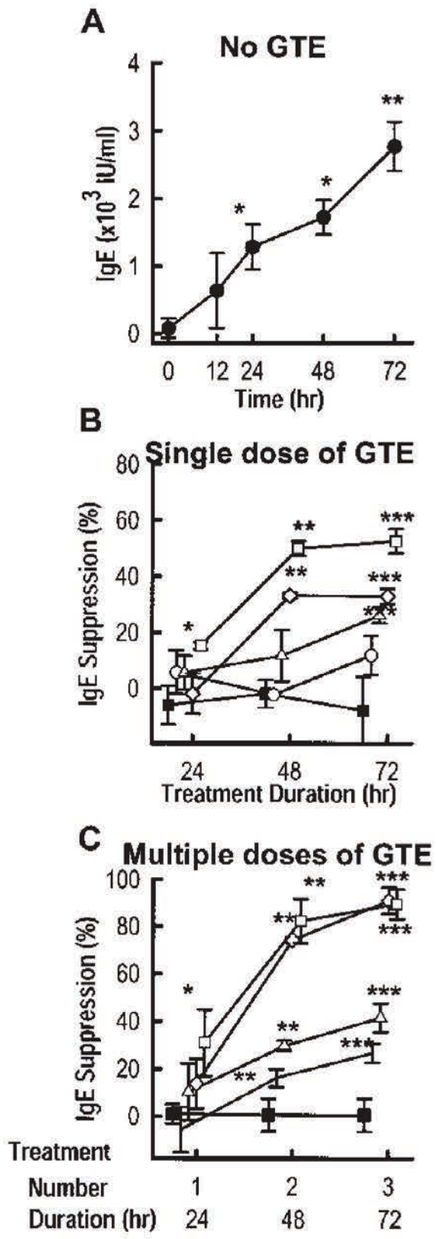

Fig. 1. Green tea extract (GTE) suppresses IgE production by U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Cells received either: (A) no treatment (solid square), (B) a single treatment at day 0, or (C) multiple treatments at days 0, 1, and/or 2 of 1 (open circle), 10 (open triangle), 100 (open diamond), or 300 (open square) ng/ml GTE added in vitro. Cell culture supernatants were collected at 0, 12, 24, 48, or 72 hr. Sandwich ELISA was performed (triplicate). Data are reported as IU of IgE per ml (mean ± SD; n = 4 experiments). Panel A: *, ** = significant difference from 0 hr ($p = 0.04$) and 24 hr ($p = 0.03$), respectively. Panel B: *, **, *** = significant difference from other treatment groups at 24 hr ($p = 0.05$) and 48 hr, and from similar treatment at 24 hr ($p < 0.04$) and 72 hr, and from similar treatment at 24 hr ($p < 0.0001$), respectively. Panel C: *, **, *** = significant difference from other single treatment groups at 48 hr ($p < 0.03$) and 72 hr, and from similar treatment at 48 hr ($p < 0.05$).

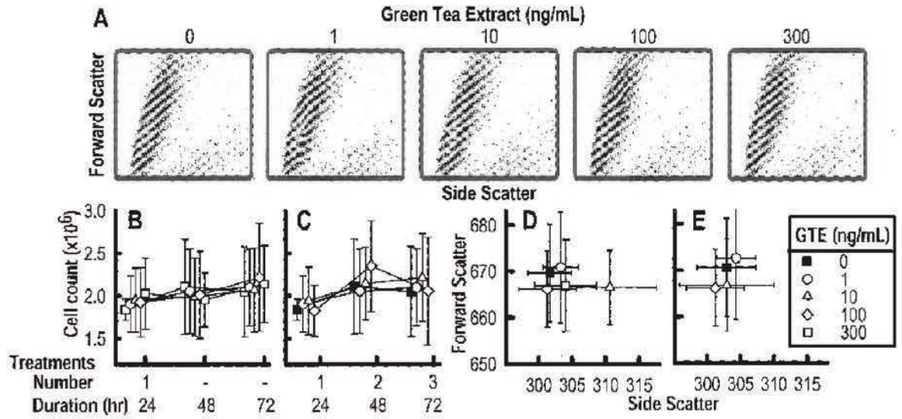

Fig. 2. Green tea extract (GTE) is not toxic to U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Cells received either (A, B, D) a single treatment at day 0, or (C, E) multiple treatments at days 0, 1, and/or 2 of 1, 10, 100, or 300 ng/ml GTE. Panels A, D, and E: forward scatter (FS) and side scatter (SS) parameters are relative measures of cell size and surface irregularity, respectively. Panels B and C: absolute cell counts were determined by flow cytometry using flow-count fluorospheres (50,000 cells counted). Data are expressed as means ± SD (n = 3 experiments).

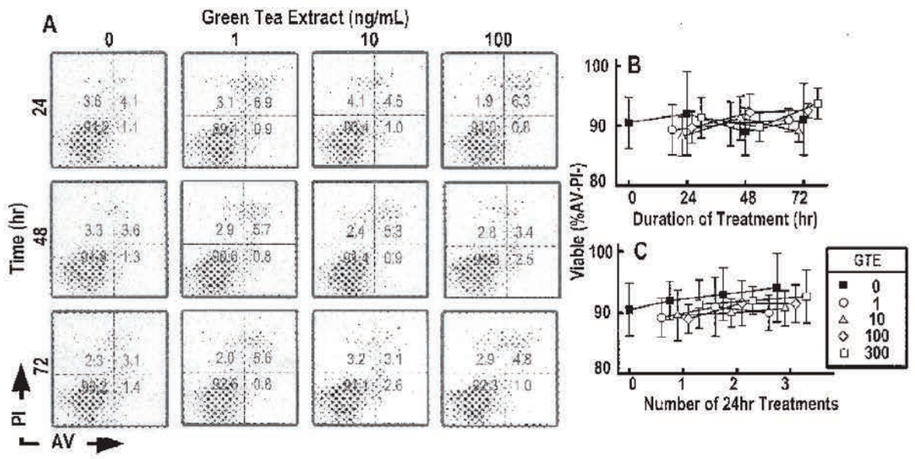

Fig. 3. Green tea extract (GTE) does not induce apoptosis or necrosis of U266 cells. U266 cells were grown in complete RPMI medium to logarithmic growth phase. Panel A, B: At day 0, a single treatment of 1, 10, 100, or 300 ng/ml GTE was added. Panel C: At days 0, 1, and 2, a treatment of 1, 10, 100, or 300 ng/ml GTE was added. Cells were collected at 24, 48, or 72 hr, washed, and stained with annexin V (AV) and propidium iodide (PI). Apoptosis and necrosis were distinguished by flow cytometry (50,000 cells counted) using the combined labeling of AV and PI. AV-PI- was defined as viable cells; AV+PI- was defined as early apoptosis; AV-PI+ was defined as early necrosis; AV+PI+ was defined as late stage cell death, either by apoptosis or necrosis. Panel A: Representative data for a single treatment with 1, 10, and 100 ng/ml. Panels B and C: Data are expressed as % of cells that were AV- and PI- (means ± SD, n = 3 experiments).

10, or 100 ng/ml (5.7 ± 7.8%, 5.6 ± 6.1%, and -2.0 ± 7.2%, respectively) (Fig. 1B). At 48 hr after treatment with 100 and 300 ng/ml GTE, there was strong suppression of IgE production (33.1 ± 1.1% and 49.9 ± 2.7%, respectively), but no suppression with 1 and 10 ng/ml GTE (-2.3 ± 0.07% and 11.6 ± 9.2%, respectively) (Fig. 1B). At 72 hr after treatment with 10, 100, and 300 ng/ml GTE, there was strong suppression of IgE production (26.0 ± 2.9%, 32.8 ± 2.8%, and 52.4 ± 4.4%, respectively), but no significant suppression with 1 ng/ml GTE (11.7 ± 6.9%) (Fig. 1B).

To determine the effects of sustained GTE exposure on IgE production by U266 cells, repeated treatments of GTE were added in vitro every 24 hr. As shown in Fig. 1C, repeated GTE dosing caused additional suppression of IgE production by U266 cells compared with single dosing. After 2 doses (48 hr treatment duration), there was even greater suppression of IgE production with 1, 10, 100, and 300 ng/ml GTE than a single dose at 48 hr (1: 16.3 ± 3.7% vs -2.3 ± 0.07%; 10: 29.9 ± 2.2% vs 11.6 ± 9.2%; 100: 74.3 ± 1.2% vs 33.1 ± 1.1%; 300: 82.4 ± 9.3% vs 49.9 ± 2.7%) (Fig. 1C). Similarly, after 3 doses (72 hr treatment duration), there was even greater suppression of IgE production with 1, 10, 100, and 300 ng/ml GTE than with a single dose at 72 hr (1: 26.8 ± 4.1% vs 11.7 ± 6.9%; 10: 41.8 ± 5.9% vs 26.0 ± 2.9%; 100: 91.0 ± 5.6% vs 32.9 ± 2.8%; 300: 89.6 ± 6.3% vs 52.4 ± 4.4%) (Fig. 1C). Further, after 3 doses, there was significantly greater suppression of IgE production with 1, 10, and 100 ng/ml GTE than after 2 doses ($p < 0.04$), but not with 300 ng/ml.

GTE is non-toxic to U266 cells. Since GTE was previously shown to kill cancer cells, including multiple myeloma cells in vitro [23], we investigated whether GTE suppression of IgE was related to cell death. U266 cell morphology was analyzed via forward scatter (FS) and side scatter (SS) parameters of flow cytometry, which are relative measures of cell size and surface irregularity, respectively (representative data, Fig. 2A). A single treatment with any concentration of GTE did not change U266 cell morphology (Figs. 2A, 2D). Similarly, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not change cell morphology (Fig. 2E). In particular, there were no increases in numbers of small dense cells (decreased FS and increased SS, respectively) that would be consistent with the morphological changes of apoptosis [24].

The absolute numbers of U266 cells in culture after 24, 48, and 72 hr without GTE treatment ranged from 1.7 - 2.5 x10^6 cells. A single treatment with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not affect the absolute numbers of cells at 24, 48, or 72 hr (Fig. 2B). Moreover, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not have any effect on the absolute numbers of cells (Fig. 2C).

GTE does not induce apoptosis of U266 cells. To determine whether concentrations of GTE sufficient to suppress IgE production by U266 cells induced apoptosis, we studied U266 apoptosis using co-labeling with annexin V (AV) and propidium iodide (PI) via flow cytometry. AV-PI- cells represent viable cells; AV+PI- cells represent early apoptosis; AV-PI+ cells represent early necrosis; AV+PI+ cells represent either primary necrosis, or secondary necrosis in apoptotic cells (representative data, Fig. 3A). The majority of U266 cells in the untreated group were viable at all time points tested (90.5–92.0%) (Fig. 3A).

A single treatment with any concentration of GTE did not increase the numbers of apoptotic or necrotic cells in vitro at 24, 48, or 72 hr (Fig. 3B). Similarly, multiple treatments at 24 hr intervals (1, 2, or 3) with any concentration of GTE (1, 10, 100, or 300 ng/ml) did not increase the numbers of apoptotic or necrotic cells (Fig. 3C).

Discussion

The present study demonstrates that green tea extract suppresses human IgE production in vitro in a dose-dependent fashion, where the highest concentration of GTE (300 ng/ml) results in the strongest suppression of IgE production. A single treatment with GTE suppresses IgE production as early as 24 hr, with strongest suppression at 72 hr. The continued suppression of IgE production at 72 hr suggests that GTE modifies certain transcription pathways with a sustained effect.

Previous studies showed EGCG, the principal green tea catechin, to be oxidized in cell culture medium with a half-life of only 130 min for HT-29 human colon adenocarcinoma cells [25] and 30 min for esophageal squamous cell carcinoma KYSE 150 cells and epidermoid squamous cell carcinoma A431 cells [26]. These studies suggest that a single dose of EGCG in cell cultures would be almost entirely consumed within 12 hr. Therefore, it seems likely that a single dose of GTE suppresses U266 cell production of IgE, with sustained effect at 48 and 72 hr by propagation of other mediators. In the present study, repeated daily GTE treatments resulted in stronger suppression than a single equivalent dosage. These data suggest that there may be a cumulative stimulatory effect with repeated and dose-dependent efficacy.

The results of this study demonstrate a novel role for GTE in suppressing B cell production of IgE. Previous reports established that green tea polyphenols (eg, epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG)), exhibit anti-mutagenic and anticarcinogenic activity in microbial systems (*Salmonella typhimurium* and *Escherichia coli*), mammalian cell systems, and in vivo [27]. However, the role of GTE in suppression of allergic/IgE responses in vitro has not been studied previously and the mechanism(s) are undefined. The present study in U266 B cells suggests that GTE acts directly on B cells, resulting in suppression of IgE production.

Several studies have tested the effects of natural plant derivatives in suppression of IgE production and treatment of atopic disease, because the plant derivatives are generally safe, affordable, and easily accessible. Kim et al [34] demonstrated suppression of IgE production by *Siegesbeckia glabrescens* whole plants in U266 cells in vitro. Preparations from the kiwifruit, *Actinidia arguta*, induced suppression of in vitro IgE production by U266 cells and in vivo IgE suppression in human subjects [35]. Two catechins isolated from Taiwanese oolong tea, (-)-epigallocatechin 3-O-(3-O-methyl)gallate and (-)-epigallocatechin 3-O-(4-O-methyl)gallate, inhibited type I allergic reactions in mice sensitized with ovalbumin and Freund's incomplete adjuvant [36]. The efficacy of various plant extracts in inhibiting IgE production suggests a general suppressive effect of plant-derived catechins.

In contrast, other investigators have reported that green tea or its components can be deleterious or induce clinical asthma [37-39]. In those studies, the asthma was reported in individuals who worked in green tea factories. In such cases, occupational exposure to components involved in the processing of green tea might conceivably cause hyper-responsiveness to green tea or its components, which would not be found in the general population. Future studies will examine whether individual catechins and other plant extracts cause suppression of IgE production in vivo.

The present study of GTE effects in an IgE producing cell line may have limited physiological relevance and further studies are necessary to test GTE suppression of IgE production in vivo in animals and humans. Nevertheless, the present study demonstrates suppression of IgE responses in vitro by single and repeated treatments with GTE; the suppression appears to be independent of green tea's anti-cancer properties and does not reflect altered cell viability and proliferation.

In this study, GTE did not cause increased apoptosis, necrosis, or changes of cell morphology, suggesting that suppression of IgE production by GTE is not mediated by apoptosis or cell death. In contrast, previous studies demonstrated specific killing of multiple myeloma cells by high doses of EGCG extracted from green tea [23,40]. These differences may be explained by (a) a lower concentration of specific catechins, or (b) the presence of multiple other catechins and bioactive ingredients that may interact with the effects of EGCG. Mechanisms by which tea polyphenols may possibly act include the inactivation of mutagens and carcinogens, modulation of DNA replication or repair, and inhibition of invasion and metastasis of tumor cells [27]. Ahmad et al [41,42] suggested that EGCG-caused cell cycle deregulation and apoptosis of cancer cells may be mediated through NF-kappaB inhibition. Most of these reports require further investigation [27]. Further, the effects of bioactive ingredients of green tea other than EGCG are not entirely known. The health effects associated with consumption of green tea reflect the biologic activities of all its polyphenols, rather than of a single component [43-45].

The mechanism of GTE suppression of IgE production is unknown. It may be that green tea modulates U266 IgE production at the transcriptional or translational level by downregulation of key IgE potentiating genes, such as IL-6, STAT3, TLR-2, or BSAP/Pax5, as we have shown with other anti-inflammatory agents [46,47].

The present study used unseparated green tea extract, whereas other studies examined the cellular effects of separated ingredients from green tea, such as EGCG and ECG [1,6,10,23,40-42,48]. We elected not to use such products as there are numerous bioactive ingredients in green tea that may contribute to its potential therapeutic effects. Moreover, there may be additive or multiplicative interactions between various catechins and other molecules present in green tea. Thus, an unseparated extract is more analogous to the green tea beverages that are ubiquitously consumed in Asia and other parts of the world.

The concentrations of green tea extract used in this study merit discussion. Green tea polyphenols have low bioavailability in serum, with maximum serum levels of approximately 300 ng/ml even after multi-gram daily supplements [16,17]. In the present in vitro study, we selected a range of 1-300 ng/ml concentrations, which is comparable to achievable serum concentrations in vivo. We therefore believe that green tea suppression of IgE production may be achievable in the clinical setting. However, it seems likely that metabolism and binding proteins could account for differential effects of green tea and its bioactive components when ingested in vivo.

In conclusion, GTE suppression of IgE production in vitro is not associated with cell apoptosis or necrosis. The GTE concentrations used in this study are likely to be physiologically achievable, suggesting that GTE suppression of IgE production may also be demonstrated clinically. However, in vivo confirmation of these findings in animals and humans is needed, and the mechanism by which GTE suppresses IgE responses remains to be elucidated. The model system used in the present study may be used to identify the mechanism of green tea suppression of IgE production. Clinical studies of green tea supplements are warranted to test their therapeutic efficacy in allergies, asthma, and atopic dermatitis.

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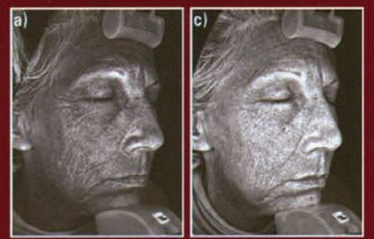

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Dose-dependent Antioxidant Function of Resveratrol Demonstrated Via Modulation of Reactive Oxygen Species in Normal Human Skin Fibroblasts In Vitro

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ABSTRACT

The study of free radicals is particularly relevant in the context of human skin carcinogenesis and photoaging because of their ability to induce DNA mutations and damaging lipid peroxidation byproducts. Therefore, it is important to identify and evaluate agents with the ability to modulate intracellular free radicals. Significant interest exists in evaluating the chemotherapeutic and anti-oxidant properties of resveratrol (trans-3,4',5-trihydroxystilbene). Resveratrol is a phytoalexin, a naturally occurring compound derived from the skin of grapes and other plants. Resveratrol was selected for evaluation because of demonstrated chemopreventive and chemotherapeutic properties in a murine skin cancer model and other human cancer models through a variety of mechanisms. However, the intracellular anti-oxidant properties of resveratrol on free radicals in human skin cells in vitro is not well characterized. The purpose of this research is to investigate the ability of resveratrol to modulate the hydrogen peroxide-induced upregulation of reactive oxygen species (ROS) free radicals in normal human skin fibroblast cells in vitro. Hydrogen peroxide is a well known generator of free radicals that occurs during endogenous and UV-induced oxidation processes in the human skin and was used to upregulate ROS in normal human skin fibroblast cells. Using a flow cytometry-based assay, the results demonstrate highly significant ($P<0.001$) dose-dependent reduction of intracellular hydrogen peroxide-upregulated ROS by resveratrol at 0.01%, 0.001% and 0.0001% concentrations in human skin fibroblasts in vitro.

INTRODUCTION

The study of free radicals is particularly relevant because of their ability to induce DNA mutations and lipid peroxidation byproducts associated with human skin carcinogenesis and photoaging. Therefore, it is important to identify and evaluate agents with the ability to modulate intracellular free radicals. Significant interest exists in evaluating the chemotherapeutic and anti-oxidant properties of resveratrol (trans-3,4',5-trihydroxystilbene).

Resveratrol is a phytoalexin, a naturally occurring molecule that defends plants against infections and nutrient depravation.[1] Resveratrol is commonly found in the skin of grapes and in red wines that include the grapes' skin in the fermentation process.[2] It should be noted that other plants contain resveratrol, including Japanese knotweed (*Polygonum cuspidatum*), a common source for resveratrol included in over-the-counter (OTC) products and supplements.[1] Additional sources of resveratrol include peanuts, grapeseeds, cranberries and blueberries.[1]

Resveratrol gained the attention of the scientific community due to the association with the phenomenon referred to as "the French Paradox." This theory is that resveratrol's antioxidative properties are responsible for increased longevity and improved cardiovascular health in the French population.[2,3] Resveratrol demonstrates positive effects on the cardiovascular system including anti-atherosclerotic properties, protection of intact endothelium, prevention of endothelial ischemic injury and suppression of platelet aggregation.[4] Other positive effects are attributed to resveratrol's ability to promote vasorelaxation and to reduce lipid oxidation.[1] Resveratrol has also been shown to reduce serum concentrations of cholesterol and triglycerides.[1] Many of these effects are related to the antioxidative properties of resveratrol, but resveratrol has other favorable properties.[1]

Despite the apparent interest resveratrol would seemingly generate in the field of dermatology, limited clinical data is available regarding the usefulness of several topical formulations that advertise resveratrol as a component. Furthermore, the bioavailability of orally consumed resveratrol in human skin is unknown. A clinical study by Baxter demonstrated a resveratrol based topical skin cream had greater antioxidative properties than idebenone (a compound that is found in commercially available anti-aging products).[5]

The authors selected resveratrol for evaluation because of the demonstrated chemopreventive and chemotherapeutic proper-

ties in both human and murine skin cancer models. However, the intracellular antioxidative properties of resveratrol on free radicals in human skin cells in vitro are not well characterized. The purpose of this research is to investigate the ability of resveratrol to modulate the hydrogen peroxide-induced upregulation of reactive oxygen species (ROS) free radicals in normal human skin fibroblast cells in vitro.

METHODS

Cell Culture

Normal human skin fibroblast AG13145 primary cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Gibco) in a humidified incubator at 37°C with an atmosphere containing 5% CO_2.

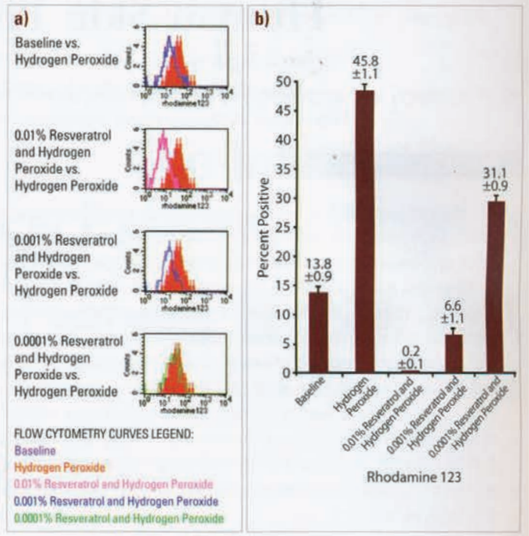
Flow Cytometry Assays of Intracellular Free Radicals

Normal human AG13145 skin fibroblast cells (Coriell, Camden, NJ) were seeded at $2x10^5$ cells per 35 mm tissue culture dishes. Forty-eight hours later the cells were pre-treated for four hours with the resveratrol concentrations 0.01%, 0.001% and 0.0001% (courtesy of Topix Pharmaceuticals). The cellular experimental conditions were then washed with 1x phosphate buffer solution (PBS) (Gibco), intravitally stained with dihydrorhodamine (DHR) (Calbiochem/EMD Biosciences, San Diego, CA), for 30 minutes, and then exposed to 1.2 mM H_2O_2 (Sigma-Aldrich, St. Louis, MO), a well known generator of intracellular ROS in DMEM without FBS for 30 minutes to generate intracellular ROS.[6,7] The cells were then washed with 1x PBS, collected with trypsin (Gibco) and processed for flow cytometry analysis. The ROS were evaluated by flow cytometry analysis immediately with a Becton-Dickinson FACScan flow cytometer at wavelength 490 nm. Statistical analysis of the data was performed using the paired two-tailed Student's *t* test, highly significant at $P<0.05$ and highly significant at $P<0.01$. Data is represented as the means±the standard error of the mean (SEM).

RESULTS

The effect of various concentrations of resveratrol on normal human skin fibroblast cells AG13145 was assessed via a flow cytometry based assay that measured intracellular free radicals using dihydrorhodamine. Dihydrorhodamine when exposed to ROS free radicals undergoes a conformational change to rhodamine 123 and produces a red fluorescence that is quantifiable by flow cytometry. Representative flow cytometry data of experimental conditions performed in triplicate are illustrated in Figure 1a. The baseline amount of endogenous ROS free radicals in the normal human skin fibroblasts assayed is represented by the purple curve in Figure 1a. Exposure to 1.2 mM H_2O_2 caused an upregulation of intracellular ROS indicative of the red solid curve that is shifted to the right (Figure 1a).

FIGURE 1. Dose Response Reduction of Hydrogen Peroxide-upregulated Free Radicals by Resveratrol in Normal Human Skin Fibroblasts. **a)** Flow cytometry data representative of experiments done in triplicate **b)** Statistical analysis of flow cytometry data. All data were highly significant ($P<0.001$) when compared to the hydrogen peroxide data set.



Resveratrol concentrations of 0.01%, 0.001% and 0.0001% demonstrated dose dependent inhibition of the H_2O_2 upregulated ROS free radicals when compared to the population of normal human skin fibroblast cells treated with H_2O_2 to upregulate intracellular ROS free radicals (Figures 1a and 1b). Statistical analysis performed using two-tailed Student's *t* test revealed highly significant reductions of intracellular ROS free radicals ($P<0.001$) for all concentrations of resveratrol when compared to the H_2O_2 upregulated free radical conditions. The 0.01% resveratrol concentration resulted in the greatest reduction of intracellular free radicals of the conditions tested (0.2±0.1) and also reduced intracellular free radicals below endogenous ROS levels. The other two concentrations of resveratrol, 0.001% and 0.0001%, demonstrated a dose dependent reduction on intracellular free radicals (6.6±1.1 and 31.1±0.9, respectively). The endogenous baseline free radicals were also statistically significantly less than the exogenously upregulated free radicals (13.8±0.9).

DISCUSSION

Evidence exists supporting a central role of free radicals in the initiation and promotion of skin cancer. Using a flow cytometry-based assay, the results demonstrate that resveratrol demonstrates a dose dependent modulation of hydrogen peroxide-upregulated free radicals in human fibroblast skin cells in vitro.

Several in vitro studies have proposed that resveratrol demonstrates chemoprotective effects in skin cancer, functioning through a variety of anti-carcinogenic mechanisms. Resveratrol plays a protective role in all steps of carcinogenesis (initiation, promotion and progression).[8,9] Mechanisms by which resveratrol may play a role in the chemoprevention of melanoma include acting as a free radicals scavenger, cyclooxygenase inhibitor and apoptosis activator.[10] In normal HaCaT keratinocytes resveratrol prevented ultraviolet (UV) B associated apoptosis, decreased activation of apoptosis associated caspases 3 and 8 and free radical generation.[11] Resveratrol also inhibited UVB-mediated activation of nuclear factor kappa-beta, a role player in cutaneous carcinogenesis, in a dose dependent manner in a normal human epidermal keratinocyte model.[12] Interestingly, high doses of resveratrol caused apoptosis in normal HaCaT keratinocytes severely by propogating DNA breakage associated with oxidative stress and resultant cell death, removing the ability of these cells to undergo carcinogenesis.[13]

In animal models of cutaneous carcinogenesis, resveratrol shows promise as a chemopreventative compound. Topically administered resveratrol has been shown to inhibit skin cancer formation in several mouse models of carcinogenesis. In comparison to other antioxidants, resveratrol when applied topically in a two stage CD-1 mouse skin cancer model using 9,10-dimethyl-1,2-benzanthracence (DMBA) as initiator and phorbol 12-myristate 13-acetate (TPA) as promoter, was an effective chemopreventive agent quantified by inhibition of skin tumor formation.[14] When consumed orally, resveratrol is absorbed more efficiently when compared to other red wine polyphenols catechin and quercetin.14 It is hypothesized that resveratrol may be the most effective bioavailable anti-cancer polyphenol in red wine.[14]

Topical application of resveratrol to hairless mice inhibited UVB induced increase in skin thickness and edema, markers of inflammation.[15] This is significant because human malignancies sometimes arise in sites of chronic inflammation, which suggests that inflammation may be a prerequisite for cancer development in some cases.[16] Another mouse model showed that topical application of resveratrol before or after UVB exposure in SKH-1 mice undergoing a UVB initiation-promotion protocol (UVB twice weekly for 28 weeks) led to highly significant inhibition in tumor incidence, delay in onset of tumorigenesis and increase in apoptosis.[17] Survivin, an apoptosis inhibitor protein that is expressed in squamous cell carcinoma, basal cell carcinoma, hypertrophic actinic keratosis and Bowen's disease, was upregulated at the protein and mRNA levels in UVB-induced tumors.[17] Additionally, the proapoptotic Smac/DIABLO protein was downregulated in these tumors.[17] These responses were attenuated with the application of resveratrol before or after the UVB exposure.[17]

Resveratrol has also been studied in non-cutaneous models. Resveratrol demonstrated potent inhibition of Vascular Endothelial Growth Factor (VEGF) in several in vitro cancer models.[18-20] Another studied mechanism is enhancement of enzymatic processes that decrease susceptibility to carcinogens. This was shown in a cultured mouse hepatoma model, as resveratrol induced quinone reductase activity, a phase 2 drug metabolizing enzyme that detoxifies carcinogens.[8] Upregulation of a carcinogen reducing enzyme may have a central role in prevention of carcinogenesis and explanation of the French Paradox. Resveratrol also inhibited de novo formation of inducible nitric oxide synthase (NOS) in lipopolysaccharide-stimulated mouse macrophages, which is important as aberrant expression inflammatory mediators such as NOS have been reported in a variety of neoplasms and may act as tumor promoters.[16] Additionally, in a prostate cancer cell line, resveratrol inhibited cell growth by selectively targeting the G1-S phase transition, which was associated with increased expression of P21 and decreased expression of cyclin D1 and cyclin dependent kinase 4 proteins, thus arresting cell growth.[21] Resveratrol also has the capability of inducing cancerous cell death. In a human pancreatic cancer in vitro model, resveratrol upregulated cell death, as pancreatic cancer cells exposed to resveratrol histologically demonstrated features of apoptosis including cellular rounding and membrane blebbing.[22]

Resveratrol demonstrates intracellular antioxidative properties in normal human skin fibroblasts in vitro and has significant promise as a chemotherapeutic agent worthy of further evaluation. The authors hypothesize that clinical topical application of resveratrol, alone or in combination with other agents, might have anti-carcinogenic properties, through antioxidant and other mechanisms.

DISCLOSURES

Dr. Neil Brody contributed intellectually to the design of a commercially available topical product that contains resveratrol (distributed by Topix Pharmaceuticals).

Topix kindly provided the resveratrol for use in the assays described in this paper.

None of the authors have any financial conflicts of interest.

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REDUCTION OF FACIAL REDNESS WITH RESVERATROL ADDED TO TOPICAL PRODUCT CONTAINING GREEN TEA POLYPHENOLS AND CAFFEINE

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INTRODUCTION

Facial redness can occur in association with a large number of medical problems. The most common causes of facial redness and rashes include inflammatory dermatoses, infections, and connective tissue disorders. Rosacea is associated with flushing, erythema, telangiectasia, papules, and pustules. Its etiology is unknown. Perioral ocular dermatitis is an erythematous eruption of unknown etiology while atopic dermatitis, an inflammatory skin disease, frequently affects the cheeks in infants and other facial areas in adults. Contact dermatitis, seborrheic dermatitis, psoriasis, cellulitis, discoid lupus erythematosus, dermatomyositis, and impetigo are also associated with facial redness *Tavadia 2003). Chronic sun damage, genetic flusher-blusher, and acne are also frequently encountered.

While redness is the final clinical manifestation, the pathophysiology leading to the redness may be quite varied. We refer to the common denominator in all of these as inflammation, and we now understand many molecules are involved in the inflammatory process. Many of the pathways of inflammation involve reactive oxygen species (ROS). It is probably true quenching ROS, should be considered an anti-inflammatory agent.

Many topical formulations include antioxidants to improve the antioxidant capability of the skin (Berson 2008, Farris 2007, Palmer 2010). An antioxidant that has received considerable attention is resveratrol (3,5,4'-trihydroxystilbene), a polyphenolic phytoalexin found in red wines, colored berries, and peanuts (Baxter 2007).The myriad of clinical benefits of resveratrol led to the hypothesis that the addition of this agent to a topical preparation containing green tea polyphenols and caffeine (both of which protect skin from UV injury [Elmets 2001, Hefferan 2009]) might be an even more effective skin care product. The present study evaluated the ability of a resveratrol-enriched product containing green tea polyphenols and caffeine to reduce facial redness in human skin.

METHODS

Stage 1. In a preliminary split-face study, 16 volunteers applied topical antioxidant product containing green tea polyphenols and caffeine to one side of the face and the same product with resveratrol added to the other side of the face. Product was applied twice daily for 12 weeks. Both products were well tolerated. After 12 weeks subjects with facial redness showed a reduction in redness on the side treated with resveratrol-enriched product (data not shown). These results led to the present study in which subjects presenting with facial redness applied resveratrol-enriched product to the entire face to evaluate the consistency of the apparent reduction in redness.

Stage 2. Subjects (n = 16) presenting with facial redness applied the resveratrol-enriched product twice daily to the entire face. Reduction in redness was evaluated and photographed at 2-week intervals for up to 9 weeks. Photography was obtained by Canfield Visia Software Version 5.2.0 2010-0503a. This unit has a mode that spectrally separates the red portion of the image allowing enhanced ability to see changes in skin redness. Improvement was evaluated by nine trained staff members and 21 house staff residents on a scale of 1 to 9. The baseline score was assigned a value of 5 for each subject. Posttreatment scores lower than 5 denoted redness reduction while scores above 5 indicated an increase in redness. Evaluators compared photographs taken before treatment and at 2-week intervals for up to 9 weeks. All subjects provided signed informed consent to treatment and photography.

RESULTS

All subjects completed the study. Adverse effects were not observed in any subject. Data were analyzed by non-parametric statistics because the 9-point scale is not continuous and scoring data were not normally distributed as shown by the Shapiro-Wilk test.

As shown in Figure 1, the collective data show that median redness scores ranged from 2 to 6 and that most subjects (69%-99%) achieved a redness reduction of at least 1 score level at the end of their treatment period. Redness in the remaining subjects (0%-31%) either did not change (0%-19%) or increased by 1 score value (0%-19%).

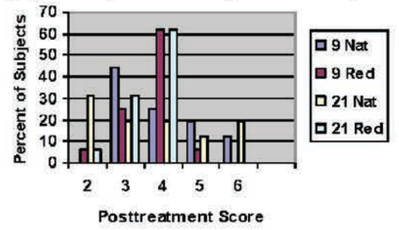

Figure 1. Median posttreatment score vs. percentage of subjects for four sets of data. Baseline score was set at 5 for each subject. Posttreatment scores less than 5 indicated reduced redness while scores greater than 5 denoted increased redness.

Figure 2. Graph of 9 evaluators-natural photo data.
A 75-year-old female (skin type 2) before treatment (left) and 4 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

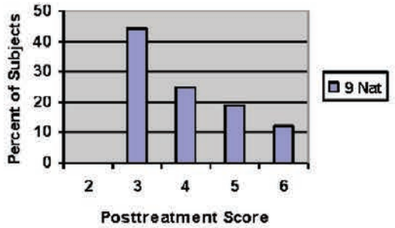

Figure 3. Graph of 9 evaluators-redness photo data.
A 72-year-old male (skin type 2) before treatment (left) and 9 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

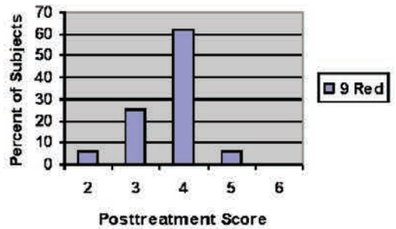

Figure 4. Graph of 21 evaluators-Natural photo data
A 27-year-old female (skin type 1))before treatment (left) and 5 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

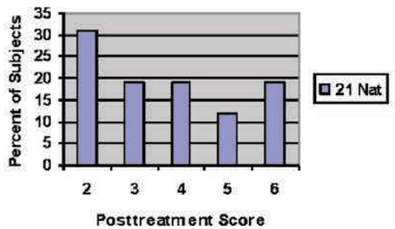

Figure 5. Graph of 21 evaluators- redness photo data.
The possible role of treatment duration on facial redness reduction was also evaluated for each of the four data sets (Table 1). For the 3 to 6-week treatment period the proportions of subjects among the four data sets did not differ significantly by Pearson's chi-square test ($p = 0.1967$). A similar result ($p = 0.1059$) was obtained when the 7 to 9 week treatment period data were compared. These results indicate that for each of the four sets of data, the distribution of subjects among median scores did not differ significantly within each of the two treatment periods.

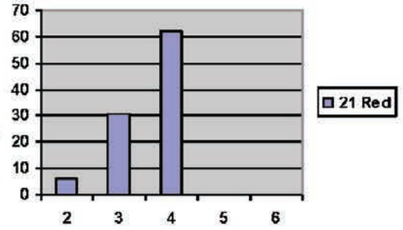

Table 1. Distribution of subjects among median scores for the 3 to 6 and 7 to 9-week treatment periods for each of the four data sets

Median Score	Weeks of Treatment							
	3 to 6				7 to 9			
	9 Nat	9 Red	21 Nat	21 Red	9 Nat	9 Red	21 Nat	21 Red
2	0	1	4	1	0	0	1	0
3	6	4	3	5	1	0	0	0
4	4	7	2	6	0	3	1	4
5	1	0	2	0	2	1	0	0
6	1	0	1	0	1	0	2	0
	$X^2 = 15.88$, df = 12, p = 0.1967 (ns)				$X^2 = 18.33$, df = 12, p = 0.1059 (ns)			

The distributions of subjects among the median scores for the 3 to 6-week treatment period were compared with those for the 7 to 9-week treatment period for each of the four data sets. As shown in Table 2, differences in proportions of subjects for the 3 to 6 and 7 to 9-week treatment durations did not achieve statistical significance for any of the four data sets.

Table 2. Comparisons of distributions of subjects among median scores for the 3 to 6 and 7 to 9-week treatment periods

Median Score	9* Nat		9* Red		21* Nat		21* Red	
	3-6†	7-9†	3-6†	7-9†	3-6†	7-9†	3-6†	7-9†
2	0	0	1	0	4	1	1	0
3	6	1	4	0	3	0	5	0
4	4	0	7	3	2	1	6	4
5	1	2	0	1	2	0	0	0
6	1	1	0	0	1	2	0	0
	P = 0.1573 (ns)		P = 0.1870 (ns)		P = 0.3283 (ns)		P = 0.2019 (ns)	

*No. of evaluators.
†Weeks of treatment.
Nat = natural photos; red = red images.

However, the Table 2 data suggest that 3 to 6 weeks may be sufficient time for most subjects (56%-75%) to achieve a reduction score of 2 to 4. It is not known if the small number of subjects (6%-25%) who achieved a score of 2 to 4 after 7 to 9 weeks of treatment actually achieved these reductions earlier. A few subjects showed no change in redness (0%-12%) or a slight increase in redness (0%-6%) after 3 to 6 weeks while the remaining subjects (0%-19%) failed to achieve a reduction in redness after 7 to 9 weeks of treatment.

DISCUSSION

Overall, the results suggest that the treatment effect (i.e., reduction in facial redness) requires up to 6 weeks of treatment for most subjects. It is possible that subjects achieving redness reduction in 3 to 6 weeks may improve further. However, if redness has not been reduced after 6 weeks of treatment, it is unlikely that further treatment will reduce redness. Clinical examples are presented in Figures 2-4.

Many topical formulations include antioxidants. Common examples include the polyphenols (found in tea), vitamin C, vitamin E, silymarin, and soy isoflavones (Pinnell 2003). Interest in resveratrol became stronger when, in 1997, resveratrol was shown to have cancer chemopreventative effects in tumor initiation, promotion, and progression stages in humans (Jang 1997). Resveratrol has since been shown to reduce intracellular hydrogen peroxide-upregulated ROS in human fibroblasts in vitro (Jagdeo 2010), modulate genetic expression (Baxter 2007), inhibit inflammatory mediators (Baxter 2007), prevent skin cancer (Aziz 2005), exhibit antiproliferative activity in multiple forms of cancer (Athar 20078, Ding 2002), promote apoptosis in tumor cells (Delmas 2003), improve dermal wounds (Khanna 2002, Sen 2002, Khanna 2001), inhibit UVB-induced skin damage (Afaq 2003), and protect against LDL oxidation (Brito 2003). Resveratrol has also been shown to have antifungal and antibacterial properties (Chan 2002) and to reduce levels of ROS in HaCaT keratinocytes exposed to UVA light (Baxter 2007).

Green tea polyphenols (GTPs) are antioxidants shown in mice to protect against skin inflammation and tumorigenesis (Mukhtar 1994, Katiyar 2000) and phototoxicity induced by psoralen plus UV-A radiation (Zhao 1999). GTPs (catechins) include (—) epicatechin, (—) epicatechin-3-gallate, (—) epigallocatechin, and (—) epigallocatechin-3-gallate derivatives. When administered topically in mice, (-)-epigallocatechin-3-gallate protects against photocarcinogenesis (Gensler 1996) and is regarded as the most effective catechin.

Topical caffeine has been shown to protect against UV damage in mice by eliminating UV-damaged keratinocytes (Koo 2007) and subsequently inhibiting skin cancer development. Topical caffeine has also been to inhibit formation of galactose cataracts (Varma 2010) and improve psoriasis vulgaris (Vali 2005).

Exposure of the skin to UV radiation induces inflammatory responses associated with a variety of skin disorders, including cancer. Regarded as early events in tumor promotion, development, or both, inflammatory responses are characterized by erythema, edema, hyperplastic responses, and increases in blood flow blood vessel permeability, and levels of COX-2 and prostaglandin. These responses are also associated with the induction of inflammatory cytokines (tumor necrosis factor-α, IL-6, and IL-1β) (Meeran 2009).

It is useful to summarize mechanisms by which the combination product of the present study reduces facial redness and inflammation. Facial redness may occur in a variety of inflammatory dermatologic disorders and an effective treatment of facial redness without the side effects of steroids would be useful (Oh 2010). Since the molecular targets of each component are not identical, the components may act independently and synergistically to reduce cutaneous inflammation.

Resveratrol
Jang and colleagues (1997) showed that resveratrol inhibited cyclooxygenase (COX)-1 activity, a significant finding because COX catalyzes the production of pro-inflammatory substances (e.g., prostaglandins) that stimulate the growth of tumor cells (Plescia 1975, Goodwin 1984). Subbaramaiah and colleagues (1998) reported that resveratrol inhibited expression of the COX-2 gene by inhibiting the protein kinase C (PKC) signal transduction pathway. This is important because PKC is up-regulated in some types of cancer (O'Brien1989, Gordge 1996, Subbaramaiah 1998). **Dr. Brody: Is VEGF associated with inflammation? I did not find references to support that.**

Green Tea Polyphenols
Influxes of neutrophils and macrophages into a UV-irradiated skin site) are reduced by topical application of GTPs and via green tea in drinking water in mice (Katiyar 2001) and in humans (Katiyar 1999). One study (Elmets 2001) on the anti-inflammatory component of GTPs showed that, after pretreating human skin with green tea extract and then exposing the treated area to solar-simulated light, the green tea extract inhibited UV-induced erythema in a dose-dependent manner, reduced the number of sunburn cells, and protected the epidermal Langerhans cells. Meeran and colleagues (2009) studied the effect of GTP administration (via drinking water) on mice in which skin tumors developed after exposure to UV radiation. The authors observed reductions in inflammation markers COX-2, prostaglandin E_2, proliferating cell nuclear antigen, and cyclin D1). The authors concluded that GTPs prevent photocarcinogenesis and that this is mediated through repair of UV-damaged DNA. Other studies showed that GTPs (1) inhibit ornithine decarboxylase, COX, and lipoxygenase; (2) inhibit release of interleukins 1, 8, 10, and 12; and (3) inhibit UV-induced infiltration of neutrophils and macrophages (Hsu 2005). Green tea extracts have also been shown to inhibit phosphorylation of vascular endothelial growth factor receptors (VEGFR) (Lamy 2002), which play a central role in tumor angiogenesis.

Caffeine
Although caffeine is widely known to reduce facial redness by constricting blood vessels, little data have been published on the effects of topical caffeine on skin (Koo 2007). Topical caffeine inhibits cyclic AMP phosphodiesterase which results in increased levels of cAMP in skin which, in turn, reduces inflammatory reactions (Kaplan 1978, Kerzendorfer 2009). Heffernan and colleagues (2009) recently showed that caffeine protects against UV-induced damage in human keratinocytes. These authors also suggested that caffeine might prevent or reverse UV damage by inhibiting the ataxia-telangiectasia and Rad3-related protein (ATR)-checkpoint kinase 1 (Chk1) pathway. ATR is a protein kinase that senses DNA damage and activates pathways that lead to inhibition of cell cycle progression (Sancar 2004). CHk1 is a kinase involved in cell cycle control. Topical caffeine has also been to inhibit formation of galactose cataracts (Varma 2010) and improve psoriasis vulgaris (Vali 2005).

The encouraging results of the present study justify additional experiments with similar subjects treated for periods exceeding 9 weeks to determine the duration of improvement and the requirements for maintaining reduction in redness. Limitations of the study include the small number of subjects, the variable durations of treatment., and the absence of a green tea-caffeine control.

CONCLUSION
The skin product combination of resveratrol, green tea polyphenols, and caffeine reduces facial redness in most patients after 3 to 6 weeks of continuous treatment and may provide further improvement with additional treatment.

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Bibliography available upon request

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REDUCTION OF FACIAL REDNESS WITH RESVERATROL ADDED TO TOPICAL PRODUCT CONTAINING GREEN TEA POLYPHENOLS AND CAFFEINE

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Natasha Phrsai BS, and Neil Brody MD PhD

J Drugs Dermatol. 2012;12(7):770-774.

Reduction of Facial Redness With Resveratrol Added to Topical Product Containing Green Tea Polyphenols and Caffeine

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ABSTRACT

Background/Objective: Many topical formulations include antioxidants to improve the antioxidant capability of the skin. This study evaluated the ability of a unique combination of antioxidants including resveratrol, green tea polyphenols, and caffeine to reduce facial redness.
Methods: Subjects (n=16) presenting with facial redness applied the resveratrol-enriched product twice daily to the entire face. Reduction in redness was evaluated by trained staff members and dermatology house staff officers. Evaluators compared clinical photographs and spectrally enhanced images taken before treatment and at 2-week intervals for up to 12 weeks.
Results: 16 of 16 clinical images showed improvement and 13 of 16 spectrally enhanced images were improved. Reduction in facial redness continued to evolve over the duration of the study period but was generally detectable by 6 weeks of treatment. Adverse effects were not observed in any subject.
Conclusion: The skin product combination of resveratrol, green tea polyphenols, and caffeine safely reduces facial redness in most patients by 6 weeks of continuous treatment and may provide further improvement with additional treatment.

J Drugs Dermatol. 2013;12(7):770-774.

INTRODUCTION

Facial redness can occur in association with a large number of medical problems. The most common causes of facial redness include inflammatory dermatoses, such as rosacea, perioral oral/ocular dermatitis, contact, seborrheic and atopic dermatoses and chronic sun damage. While redness is the final clinical manifestation, the biologic pathway leading to the redness may be quite varied. We refer to the common denominator in all of these as inflammation, and we now understand many molecules are involved in the inflammatory process. Many of the pathways of inflammation involve reactive oxygen species (ROS). Therefore one may conclude that molecules quenching ROS should be considered anti-inflammatory agents.

There are a number of topical formulations that include antioxidants to improve the antioxidant capability of the skin.[1,2,3,4] Two antioxidants, green tea polyphenols and caffeine, have been shown in the laboratory[5,6,7] to be very effective and have been used in a commercially available product that has been well tolerated. A third compound that has received considerable attention is resveratrol (3,5,4'-trihydroxystilbene), a polyphenolic phytoalexin found in red wines, colored berries, and peanuts.[8] Resveratrol has also been shown in the laboratory[9] to be a potent antioxidant. The myriad of clinical benefits of resveratrol led to the hypothesis that the addition of this agent to a topical preparation containing green tea polyphenols and caffeine (both of which protect skin from UV injury[10,11]) might create an even more effective skin care product. The present study demonstrates that this combination of GTP, caffeine, and resveratrol reduces facial redness.

METHODS

Stage 1

In a preliminary split-face study, volunteers applied topical antioxidant product containing green tea polyphenols and caffeine to one side of the face and the same product with resveratrol added to the other side of the face. Product was applied twice daily for 8-12 weeks. Both products were well tolerated. Facial redness was reduced on the side treated with resveratrol-enriched product (data not shown). These results led to the present study in which subjects presenting with facial redness applied resveratrol-enriched product to the entire face to evaluate the consistency of the clinically apparent reduction in redness.

Stage 2

Subjects (n = 16) presenting with facial redness applied the resveratrol-enriched product twice daily to the entire face. Reduction in redness was evaluated and photographed at 2-week intervals for up to 12 weeks. Photography was obtained by Canfield Visia Software Version 5.2.0 2010-0503a. This unit has a mode that spectrally separates the red portion of the image allowing enhanced ability to see changes in skin redness. Improvement was evaluated by nine trained staff members and 21 dermatology residents on a scale of 1 to 9. The baseline score was assigned a value of 5 for each subject. Post treatment scores lower than 5 denoted redness reduction while scores above 5 indicated an increase in redness. Evaluators compared photographs taken before treatment and at 2-week intervals for up to12 weeks. All subjects provided signed informed consent to treatment and photography.

FIGURE 1. Clinicians (red) and trained observers (blue) independently rated clinical images of the same 16 patients. There was 100% agreement between the two groups of observers. All 16 patients exhibited signs of reduced redness after 6 weeks of treatment, based on clinical images. Note: All lines above baseline (level 5) denote improvement.

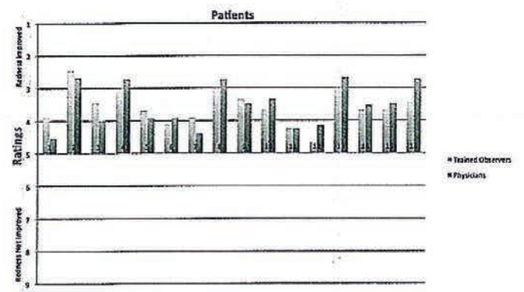
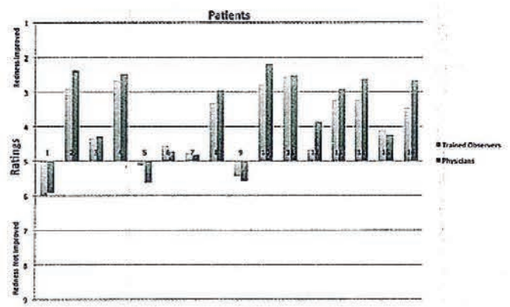


FIGURE 2. Clinicians (red) and trained observers (blue) independently rated computer-generated images of the same 16 patients. There was 100% agreement between the two groups of observers. Thirteen out of 16 patients showed reduced redness following 6 weeks of treatment, based on computer-generated images. Patients 1, 5, and 9 did not show signs of reduced redness based on computer-generated images. Note: All lines above baseline (level 5) denote improvement.



RESULTS

All subjects completed the study. Adverse effects were not observed in any subject. Two sets of images were evaluated. One set were clinical photos, the other spectrally enhanced red images that were computer generated by the Canfield software. The sets of observers evaluated the before and after images independently. During their evaluations there was no inter-observer discussion. There was 100% agreement between the groups as to which patients improved and which did not. Improvement once attained was sustained throughout the course of the study. The data suggest that 3 to 6 weeks may be sufficient time for most subjects to achieve a reduction score of 2 to 4. (Note added in proof: most of the patients have now been informally followed for more than 1 year and have maintained their improvement.)

DISCUSSION

As has been said, now that we know it works in fact, how does it work in theory? The product that reduced facial redness is a combination of a number of products produced by mother nature that each has individual histories of providing benefits by association with epidemiologic data. Uniquely these products are associated with a plethora of benefits but a noticeable absence of deleterious effects. Scientists, including our own group, have studied these molecules in test tube and animal models and drawn lots of conclusions about the nature of their activities. Discussed here are some of our favorites, but do they answer the question of how this product produces its admirable effects? Historically the first product that we evaluated clinically contained just green tea polyphenols. Note that this is was a concentrated assortment of all the molecules available from gentle extraction and concentration of the green tea leaf, intentionally not focusing on any single component as the epidemiology of benefits is for green tea and not individual components. This product was cosmetically accepted and conceptually was a potent topical antioxidant. Clinical observation suggested it had a calming effect on the users' skin, including those with inflammatory dermatoses, suggesting its anti-inflammatory nature. Our laboratory found that the addition of caffeine increased the antioxidant capacity of green tea polyphenols in a test tube model using human fibroblasts. Caffeine had already been administered topically and was being redeemed as systemically beneficial and therefore might add to the antioxidant qualities of our existing product. Back in the lab, resveratrol had proven to have antioxidant capacities and some other unique activities.[12] Guided by this laboratory data and an excellent epidemiology story, we added resveratrol producing a unique product in which all of these components were present in high concentrations in a stabilizing base. Each translation from bench top to commercial product has a period in which the older product is compared to the new for cosmetic acceptability. Everyone liked the qualities of the new product and we noticed that in those individuals with facial redness, the side treated with the resveratrol addition had reduced redness. Translational work is thought of as from the bench to the bedside but clinical observation yields facts and what remains is why.

A suggestion for the mechanism of action of this combination product centers on the concept that individual cells in an environment, in this case the skin, produce molecules that influence all the surrounding tissues and on some level the host.[13] This is now a more accepted doctrine in the cancer literature and recently in the aging literature.[14] The data presented here on redness in the skin may be explained by having the mixture of active principles in this product change how individual cells

FIGURE 3. A 35-year-old male (skin type 2) before treatment (left) and 9 weeks after treatment with resveratrol-enriched product (right). Clinical image. Redness reduction was scored at 3.

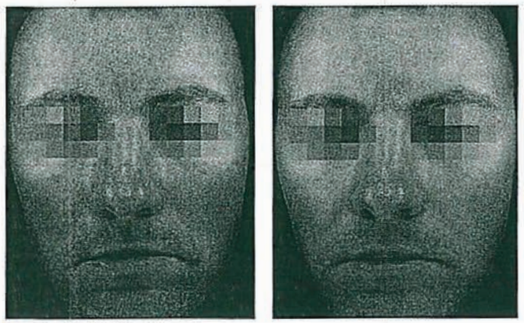


FIGURE 4. A 35-year-old female (skin type 2))before treatment (left) and 9 weeks after treatment with resveratrol-enriched product (right). Spectrally enhanced image. Redness reduction was scored at 3.

view injury and therefore the array of molecules they produce or induce their neighbors to produce. The injured cell has at least four alternatives: complete repair, autophagy, apoptosis or necrosis. Each of these pathways has a consequence to the surrounding tissue. An example of complete repair may be the excision-repair proteins for damaged DNA that may be going on continuously without upsetting the cellular environment. Additional stress to the cell is a reactive oxygen species (ROS) assault, which could be quenched by the cell's store of antioxidants or by the exogenous antioxidants applied to the skin or consumed. This simplistic explanation needs to be expanded. Cell necrosis is the most inflammatory path for the injured cell with release of all the contents initiating myriad inflammatory pathways and immune activation. This kind of pathway probably accounts for the exacerbation of lupus after acute UV damage. Apoptosis or programmed cell death is the least inflammatory method of removing non-repairable cells as represented by the sunburn cell. The combination of resveratrol, green tea pholyphenols, and caffeine in our product proved effective in reducing redness. Each of these three compounds yields an acclamatory effect on individual cells in the surrounding environment, which may account for the observed benefits.

Green tea polyphenols (GTPs) are antioxidants shown in mice to protect against skin inflammation, associated tumorigenesis[15,16] and phototoxicity induced by psoralen plus UV-A radiation.[17] The polyphenol portion of green tea (the catechins) includes epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate derivatives. When only the catechin portion of green tea is administered topically in mice, epigallocatechin-3-gallate (EGCG) protects best in a photocarcinogenesis model.[18] Because of this and similar models, EGCG is regarded as the most effective catechin.[19] It is important to remember that the epidemiology is for green tea and not any individual molecules. Surrogates are valid for their models. They are used for their expediency. Intentionally, the studied product uses all of the molecules in green tea leaves that would be present in the beverage on which the epidemiology is based.

Another approach to discerning mechanisms by which the combination product of the present study reduces facial redness involves pathways of inflammation. Facial redness may occur in a variety of inflammatory dermatologic disorders.[20] Since the molecular targets of each component are not identical, the components may act independently or synergistically to reduce cutaneous inflammation. All three of the components in our product have been shown to improve or protect against UV-induced skin damage. Exposure of the skin to UV radiation induces formation of ROS, which leads to inflammatory responses associated with a variety of skin disorders, including cancer. Inflammatory responses are characterized by erythema, edema, hyperplastic responses, and increases in blood vessel permeability. Both topical GTP-application and GTPs in drinking water reduce inflammation.[21] One study[22] on the anti-inflammatory component of GTPs showed that, after pretreating human skin with green tea extract and then exposing the treated area to solar-simulated light, the green tea extract inhibited UV-induced erythema in a dose-dependent manner, reduced the number of sunburn cells, and protected the epidermal Langerhans cells. Resveratrol, as a natural polyphenol, is also a pigment. This property allows it to absorb UV radiation, and when applied topically, it can reduce the penetration of UV radiation into the skin.[23] In this way, topical resveratrol acts as a natural sunscreen and reduces the inflammation and oxidative damage associated with UV exposure. Furthermore, pre-treatment of keratinocytes with resveratrol increases cell survival after these cells have been exposed to UV radiation.[24] This is also associated with a reduction in the production of ROS, and subsequent anti-inflammatory effects. Green tea phenols add to this anti-inflammatory effect. GTPs can inhibit the UV-induced infiltration of neutrophils and macrophages.[25] In our product, this effectiveness is further supplemented by caffeine. Topical caffeine has been shown to protect against UV damage in mice by eliminating UV-damaged keratinocytes,[26] and subsequently inhibiting skin

cancer development. The topical application of caffeine to human skin provides protection from UV light via DNA repair mechanisms.[27] Caffeine has been shown to prevent or reverse UV damage by inhibiting the ataxia-telangiectasia and Rad3-related protein (ATR)-checkpoint kinase 1 (Chk1) pathway[28] involved in cell cycle control.[29] By inhibiting the ATR-Chk1 pathway, caffeine prevents tumor growth and promotes apoptosis. Lastly, should UV-damage occur, topical caffeine can eliminate UV-damaged keratinocytes[30] and subsequently inhibit skin cancer development. While this discussion frequently uses UV light as the inducer of ROS, it has been shown that both visible light and infrared also induce ROS.[31]

Our product provides a "second line" of defense in that its components also directly inhibit various inflammatory pathways. The cyclooxygenase (COX)-2 and lipoxygenase (LOX) pathways catalyze the production of pro-inflammatory substances, including prostaglandins and leukotrienes.[32,33] Various biochemical pathways are also associated with the induction of inflammatory cytokines (tumor necrosis factor-α, IL-6, and IL-1β) that stimulate the growth of tumor cells.[34] Resveratrol works to diminish inflammation by stopping COX-2 activity,[35] likely by inhibition of the protein kinase C (PKC) signal transduction suppressing COX-2 expression.[36] This finding is important because PKC is up regulated in some types of cancer.[37,38,39] Green tea phenols (GTPs) have also been shown to have an effect on the COX pathway. GTPs in drinking water reduced inflammation markers COX-2, prostaglandin E2, proliferating cell nuclear antigen, and cyclin D1 in mice with skin damage that developed after exposure to UV radiation.[40] Other studies showed that GTPs: (1) inhibit ornithine decarboxylase, COX, and LOX; and (2) inhibit release of interleukins 1, 8, 10, and 12,[41] which are all pro-inflammatory molecules. The third compound in our product, caffeine, takes yet another approach in countering inflammation. Topical caffeine inhibits cyclic AMP phosphodiesterase, which results in increased levels of cAMP in skin, which, in turn, reduces inflammatory reactions.[42,43]

Lastly, it is important to consider that an increase in cutaneous blood supply would carve a convenient pathway for inflammatory markers to reach the skin. Angiogenesis is defined as the production of new blood vessels and/or altered permeability of existing blood vessels. A key element that stimulates angiogenesis is vascular endothelial growth factor (VEGF). Resveratrol, GTPs, and caffeine down regulate angiogenesis. A study by Pietrasik and colleagues[44] demonstrated that resveratrol modulates normal somatic cells, leading to a decrease of the angiogenic activity of endothelial cells. Mesothelial cells treated with resveratrol created an angiogenesis-suppressive milieu, reflected by the inhibited proliferation and migration of endothelial cells. This suppressive effect continued even after the cells were removed from resveratrol exposure. Endothelial cells treated directly with resveratrol also showed anti-angiogenic activity. The anti-angiogenic effect of resveratrol may be associated with its activation of glycogen-synthase kinase 3b (GSK3b), which results in decreased production of VEGF via down-regulation of b-catenin.[45] GTPs play an anti-angiogenic role by inhibiting phosphorylation of VEGF receptors[46] required for VEGF binding. Meanwhile, pretreatment of cells with caffeine significantly reduces adenosine-induced VEGF promoter activity and VEGF and IL-8 expression.[47] The anti-angiogenic effects of all three compounds in our product may directly reduce redness.

CONCLUSION

The skin product's unique combination of resveratrol, green tea polyphenols, and caffeine reduces facial redness in most patients after 3 to 6 weeks of continuous treatment and may provide further improvement with additional treatment.

DISCLOSURE

The study was initiated and funded by one of the authors (N.I.B.). That author contributed to the conceptualization and design of the product but holds no patents and does not benefit from its sale. This product is commercialized as Replenix Power of Three by Topix Pharmaceuticals.

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MODIFICATION OF GENE EXPRESSION USING TOPICAL ANTIOXIDANTS: A PROOF OF CONCEPT STUDY

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INTRODUCTION Non-melanoma skin cancer is the most common malignancy in the United States. The number of skin cancers continues to rise and is a significant cause of morbidity and mortality (1). The role of solar ultraviolet light (UV) in the pathogenesis of human skin carcinogenesis, has been clearly demonstrated (2). UV radiation is known to generate hydrogen peroxide (H2O2) and other reactive oxygen species (ROS) free radicals (3). Oxidative stress due to free radicals, reactive aldehydes and other oxidation byproducts is a critical component of carcinogenesis and photoaging through the generation of gene mutations and post-translational modifications of essential cancerrelated proteins. These changes alter cellular pathways such as apoptosis, DNA repair, cell cycle regulation and growth (4,5,6). Cells altered by ROS can alter the funtional capacity of their organs even without becoming malignant. Therefore, it is critical to develop and evaluate helpful topical anti-oxidant agents to counteract oxidative stress and prevent skin cancer and aging. In this proof of concept study, through the use of genetic microarrays, we aim to demonstrate that the topical application of antioxidants (GTP, caffeine and resveratrol) effectively modifies genetic expression.

METHODS This is an open-label, prospective study with two human volunteers. A topical preparation containing green tea polyphenols, caffeine and resveratrol was applied BID to the right forearm and two different vehicles serving as control were applied to the left forearm x 15 days. On day 15, 3 separate sites, each corresponding to a different topical application site (see figure 1) were irradiated with 50-70 mJ/cm2 of narrow band UVB. An hour later 6 punch biopsies were performed in a manner to only harvest epidermis and as little reticular dermis as

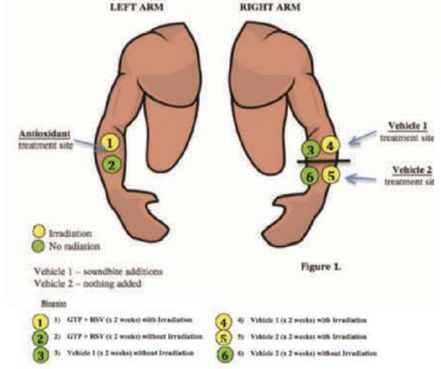

Figure 1.

RESULTS

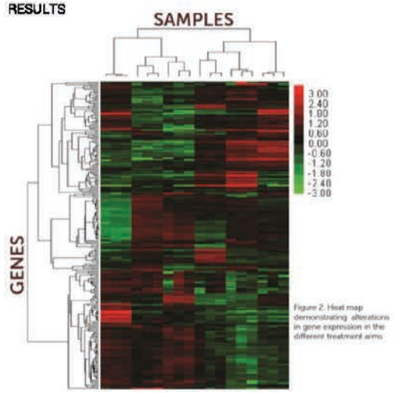

Figure 2. Heat map demonstrating alterations in gene expression in the different treatment arms

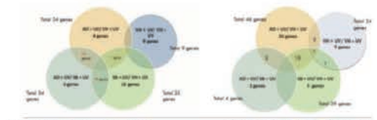

Figure 3a. Venn Diagram illustrating the number of genes changed using the different topical applications with and without UVB irradiation in patient number 1. AO – antioxidant, VH – vehicle 1, SB – Vehicle 2

Figure 3b. Venn Diagram illustrating the number of genes changed using the different topical applications with and without UVB irradiation in patient number 2. AO – antioxidant, VH – vehicle 1, SB – Vehicle 2

DISCUSSION Two very important facts are demonstrated in this study. First, there's always some question about whether or not many of the marketed cosmoceuticals have any effects. The major tool in most studies compares before and after photographs that in the best scenarios are subjective. Shown here is the up and down regulation of many genes objectifying the penetration and functional alteration of keratinocyte gene activity by the tested products. Second, the methods codified in this study can be a powerful tool to study which genes are turned on/off in response to applied products. The power of this methodology is barely hinted at here. Without the application of a topical agent but exposure to a known, frequented agent, UVB, it may be possible to asses the individuals genomic sensitivity to a known age inducer/carcinogen. The gene alteration patterns may be helpful in illuminating why different individuals have different responses to exogenous environmental stimuli. Dietary alterations may be shown to change these patterns in an individual. The power of this methodology seems outstanding.

FUTURE DIRECTION

This study only shows that the particular compilation of chemicals used in these formulations can alter the activity of genes in two volunteers. Much work needs to be done first to demonstrate that these gene alterations are reproducible both in the same patient and additional patients. Cells are continuously responding to their environment (including neighboring cells) and the rate of response is much faster than previously accepted. There are exciting possibilities for the field of dermatology now that we have shown that it is possible to make products that change the direction of cellulargene activity with topical application. Our grandmothers taught us that "one rotten apple spoils the barrel". There are exciting publications in the last several years that show that " bad cells in an organ system" diminish the function of that organ. The ability to control the damage caused by ROS in the skin may be analogous in that getting rid of or preventing environmental stimuli like UV light from injuring some of the cells in the skin will improve its function with age.

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Reduction of Facial Redness with Resveratrol Added to Topical Product Containing Green Tea Polyphenols and Caffeine

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Introduction

Facial redness can occur in association with a large number of medical problems. The most common causes of facial redness and rashes include inflammatory dermatoses, infections, and connective tissue disorders. Rosacea is associated with flushing, erythema, telangiectasia, papules, and pustules. Its etiology is unknown. Perioral ocular dermatitis is an erythematous eruption of unknown etiology while atopic dermatitis, an inflammatory skin disease, frequently affects the cheeks in infants and other facial areas in adults. Contact dermatitis, seborrheic dermatitis, psoriasis, cellulitis, discoid lupus erythematosus, dermatomyositis, and impetigo are also associated with facial redness *Tavadia 2003). Chronic sun damage, genetic flusher-blusher, and acne are also frequently encountered.

While redness is the final clinical manifestation, the pathophysiology leading to the redness may be quite varied. We refer to the common denominator in all of these as inflammation, and we now understand many molecules are involved in the inflammatory process. Many of the pathways of inflammation involve reactive oxygen species (ROS). It is probably true quenching ROS, should be considered an anti-inflammatory agent.

Many topical formulations include antioxidants to improve the antioxidant capability of the skin (Berson 2008, Farris 2007, Palmer 2010). An antioxidant that has received considerable attention is resveratrol (3,5,4'-trihydroxystilbene), a polyphenolic phytoalexin found in red wines, colored berries, and peanuts (Baxter 2007).The myriad of clinical benefits of resveratrol led to the hypothesis that the addition of this agent to a topical preparation containing green tea polyphenols and caffeine (both of which protect skin from UV injury [Elmets 2001, Heffernan 2009]) might be an even more effective skin care product. The present study evaluated the ability of a resveratrol-enriched product containing green tea polyphenols and caffeine to reduce facial redness in human skin.

Methods

Stage 1. In a preliminary split-face study, 16 volunteers applied topical antioxidant product containing green tea polyphenols and caffeine to one side of the face and the same product with resveratrol added to the other side of the face. Product was applied twice daily for 12 weeks. Both products were well tolerated. After 12 weeks subjects with facial redness showed a reduction in redness on the side treated with resveratrol-enriched product (data not shown). These results led to the present study in which subjects presenting with facial redness applied resveratrol-enriched product to the entire face to evaluate the consistency of the apparent reduction in redness.

Stage 2. Subjects (n = 16) presenting with facial redness applied the resveratrol-enriched product twice daily to the entire face. Reduction in redness was evaluated and photographed at 2-week intervals for up to 9 weeks. Photography was obtained by Canfield Visia Software Version 5.2.0 2010-0503a. This unit has a mode that spectrally separates the red portion of the image allowing enhanced ability to see changes in skin redness. Improvement was evaluated by nine trained staff members and 21 house staff residents on a scale of 1 to 9. The baseline score was assigned a value of 5 for each subject. Posttreatment scores lower than 5 denoted redness reduction while scores above 5 indicated an increase in redness. Evaluators compared photographs taken before treatment and at 2-week intervals for up to 9 weeks. All subjects provided signed informed consent to treatment and photography.

Results

All subjects completed the study. Adverse effects were not observed in any subject. Data were analyzed by non-parametric statistics because the 9-point scale is not continuous and scoring data were not normally distributed as shown by the Shapiro-Wilk test.

As shown in Figure 1, the collective data show that median redness scores ranged from 2 to 6 and that most subjects (69%-99%) achieved a redness reduction of at least 1 score level at the end of their treatment period. Redness in the remaining subjects (0%-31%) either did not change (0%-19%) or increased by 1 score value (0%-19%).

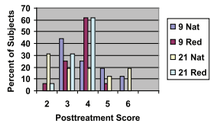

Figure 1. Median posttreatment score vs. percentage of subjects for four sets of data. Baseline score was set at 5 for each subject. Posttreatment scores less than 5 indicated reduced redness while scores greater than 5 denoted increased redness.

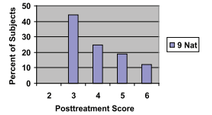

Figure 2. Graph of 9 evaluators-natural photo data.

A 75-year-old female (skin type 2) before treatment (left) and 4 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

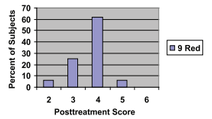

Figure 3. Graph of 9 evaluators-redness photo data.

A 72-year-old male (skin type 2) before treatment (left) and 9 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

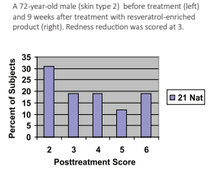

Figure 4. Graph of 21 evaluators-Natural photo data

A 27-year-old female (skin type 1))before treatment (left) and 5 weeks after treatment with resveratrol-enriched product (right). Redness reduction was scored at 3.

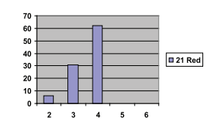

Figure 5. Graph of 21 evaluators- redness photo data.

The possible role of treatment duration on facial redness reduction was also evaluated for each of the four data sets (Table 1). For the 3 to 6-week treatment period the proportions of subjects among the four data sets did not differ significantly by Pearson's chi-square test (p = 0.1967). A similar result (p = 0.1059) was obtained when the 7 to 9 week treatment period data were compared. These results indicate that for each of the four sets of data, the distribution of subjects among median scores did not differ significantly within each of the two treatment periods.

Table 1. Distribution of subjects among median scores for the 3 to 6 and 7 to 9-week treatment periods for each of the four data sets

Median Score	Weeks of Treatment							
	3 to 6				7 to 9			
	9 Nat	9 Red	21 Nat	21 Red	9 Nat	9 Red	21 Nat	21 Red
2	0	1	4	1	0	0	1	0
3	6	4	3	5	1	0	0	0
4	4	7	2	6	0	3	1	4
5	1	0	2	0	2	1	0	0
6	1	0	1	0	1	0	2	0
	X^2 = 15.88, df = 12, p = 0.1967 (ns)				X^2 = 18.33, df = 12, p = 0.1059 (ns)			

The distributions of subjects among the median scores for the 3 to 6-week treatment period were compared with those for the 7 to 9-week treatment period for each of the four data sets. As shown in Table 2, differences in proportions of subjects for the 3 to 6 and 7 to 9-week treatment durations did not achieve statistical significance for any of the four data sets.

Table 2. Comparisons of distributions of subjects among median scores for the 3 to 6 and 7 to 9-week treatment periods

Median Score	9* Nat		9* Red		21* Nat		21* Red	
	3-6†	7-9†	3-6†	7-9†	3-6†	7-9†	3-6†	7-9†
2	0	0	1	0	4	1	1	0
3	6	1	4	0	3	0	5	0
4	4	0	7	3	2	1	6	4
5	1	2	0	1	2	0	0	0
6	1	1	0	0	1	2	0	0
	P = 0.1573 (ns)		P = 0.1870 (ns)		P = 0.3283 (ns)		P = 0.2019 (ns)	

*No. of evaluators.
† Weeks of treatment.
Nat = natural photos; red = red images.

Discussion

Overall, the results suggest that the treatment effect (i.e., reduction in facial redness) requires up to 6 weeks of treatment for most subjects. It is possible that subjects achieving redness reduction in 3 to 6 weeks may improve further. However, if redness has not been reduced after 6 weeks of treatment, it is unlikely that further treatment will reduce redness. Clinical examples are presented in Figures 2-4.

Many topical formulations include antioxidants. Common examples include the polyphenols (found in tea), vitamin C, vitamin E, silymarin, and soy isoflavones (Pinnell 2003). Interest in resveratrol became stronger when, in 1997, resveratrol was shown to have cancer chemopreventative effects in tumor initiation, promotion, and progression stages in humans (Jang 1997). Resveratrol has since been shown to reduce intracellular hydrogen peroxide-upregulated ROS in human fibroblasts in vitro (Jagdeo 2010), modulate genetic expression (Baxter 2007), inhibit inflammatory mediators (Baxter 2007), prevent skin cancer (Aziz 2005), exhibit antiproliferative activity in multiple forms of cancer (Athar 20078, Ding 2002), promote apoptosis in tumor cells (Delmas 2003), improve dermal wounds (Khanna 2002, Sen 2002, Khanna 2001), inhibit UVB-induced skin damage (Afaq 2003), and protect against LDL oxidation (Brito 2003). Resveratrol has also been shown to have antifungal and antibacterial properties (Chan 2002) and to reduce levels of ROS in HaCaT keratinocytes exposed to UVA light (Baxter 2007).

Green tea polyphenols (GTPs) are antioxidants shown in mice to protect against skin inflammation and tumorigenesis (Mukhtar 1994, Katiyar 2000) and phototoxicity induced by psoralen plus UV-A radiation (Zhao 1999). GTPs (catechins) include (—) epicatechin, (—) epicatechin-3-gallate, (—) epigallocatechin, and (—) epigallocatechin-3-gallate derivatives. When administered topically in mice, (-)-epigallocatechin-3-gallate protects against photocarcinogenesis (Gensler 1996) and is regarded as the most effective catechin.

Topical caffeine has been shown to protect against UV damage in mice by eliminating UV-damaged keratinocytes (Koo 2007) and subsequently inhibiting skin cancer development. Topical caffeine has also been to inhibit formation of galactose cataracts (Varma 2010) and improve psoriasis vulgaris (Vali 2005).

Exposure of the skin to UV radiation induces inflammatory responses associated with a variety of skin disorders, including cancer. Regarded as early events in tumor promotion, development, or both, inflammatory responses are characterized by erythema, edema, hyperplastic responses, and increases in blood flow blood vessel permeability, and levels of COX-2 and prostaglandin. These responses are also associated with the induction of inflammatory cytokines (tumor necrosis factor-α, IL-6, and IL-1β) (Meeran 2009).

It is useful to summarize mechanisms by which the combination product of the present study reduces facial redness and inflammation. Facial redness may occur in a variety of inflammatory dermatologic disorders and an effective treatment of facial redness without the side effects of steroids would be useful (Oh 2010). Since the molecular targets of each component are not identical, the components may act independently and synergistically to reduce cutaneous inflammation.

Conclusion

The skin product combination of resveratrol, green tea polyphenols, and caffeine reduces facial redness in most patients after 3 to 6 weeks of continuous treatment and may provide further improvement with additional treatment.

Topical Green Tea Polyphenols and Caffeine as a Treatment for Acne Vulgaris

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Introduction

Acne vulgaris is the most prevalent skin disorder in the US with 40-50 million individuals affected. It affects 85% of adolescents, from ages 12-24, and can continue on into adulthood, with 20% of men and 35% of women still affected into their 30s.[1] Although acne is not life threatening it causes significant psychosocial morbidity and has physical sequelae including lifelong scarring.[2]Acne is a complex interaction of the immune system recognition of *Propionibacterium acnes* based on an individuals genetics played out in the follicular apparatus. *P. acnes* is a key player in the pathogenesis of acne. These Gram-positive, anaerobic/microaerophilic rods are found within sebaceous follicles. They produce porphyrins that fluoresce under Wood's lamps illumination.[1]

Each of the popular acne treatments is a target of attack in the literature and lay press for assorted reasons. Isotreinoin, one of the most effective treatments has been attacked because of sides effects, including liver enzyme abnormalities, dyslipidemia and teratogenicity, and its use is limited in many countries.[3] Oral and topical antibiotics, while effective for inflammatory lesions are accused of leading to bacterial resistance.[4] Topical retinoids, the current first-line treatment for acne, cause irritation and burning on initiation.[5] As a result, there exists a need for new drugs with fewer side effects without diminishing efficacy. Green tea polyphenol (GTP) has gained interest in recent years because of its potent antimicrobial and anti-inflammatory activities.[6] Evidence has shown that GTP has antibacterial effects on P. acnes, as well as sebosupressive, apoptotic, and anti-inflammatory effects on sebocytes.[7] Caffeine is another well-known anti-inflammatory ingredient. Together, GTP and caffeine synergistically protect cells from oxidative challenge.

Methods

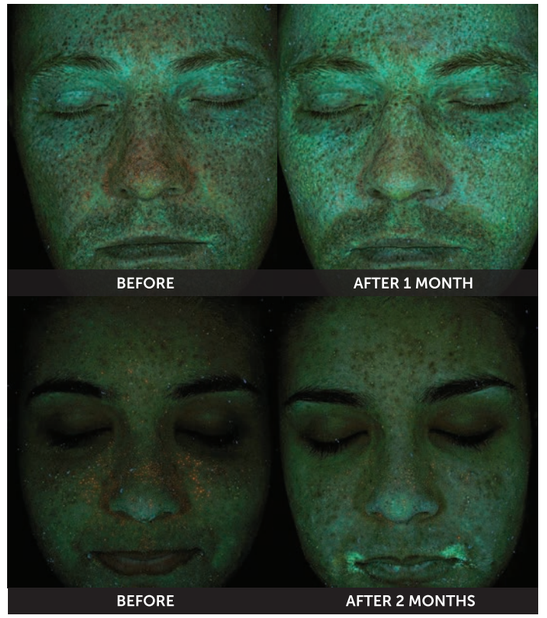
We identified a group of 13 patients from our private practice database from 2008 to 2016 who had the requisite images during 6 months of treatment. We analyzed the images of the patients (ages 18 to 40) with mild to moderate acne vulgaris to evaluate the efficacy of a commercially available topical product containing 90% GTP and caffeine USP. Subjects were photographed under fluorescing conditions using Canfield imaging equipment before treatment and at monthly follow-ups. 13 blinded pairs of pre and post-treatment photographs were shown to 28 dermatology residents who served as evaluators. Evaluators were asked to select which photo in a pair had the fewest number of orange spots. The evaluators were not told the orange spots were of porphyrin fluorescence. Intraclass correlation coefficient (ICC) was used to measure the inter-rater reliability of evaluators of the photograph pairs.

Results

- The evaluators found a decrease in orange spots corresponding to *P. acnes* fluorescence in 12 out of 13 pairs of images.
- There was a single set of photos in which the majority of evaluators were not able to distinguish the post-treatment image.
- The average measures ICC amongst evaluators was 0.934 (95% CI 0.868 to 0.976)
- The decrease in fluorescence in *P. acnes* was seen as early as 1 month and continued for the 6 months that the patients were followed.
- There was subjective improvement in the appearance of acne and treatment was well tolerated with no adverse outcomes. (Data presented elsewhere)

Discussion

- The GTP and caffeine topical treatment decreased the porphyrin levels, which indicated a decrease or change in metabolic function of *P. acnes*. We did not evaluate by colony counts that the *P. acnes* was reduced but there was correspondence between the decrease in porphyrin levels and the clinical improvement in acne.
- Our initial use of this commercially available antioxidant in our acne patients was to the mitigate the irritant effects of tretinoin. This product contains all of the naturally occurring portions of green tea which includes molecules that historically were known to have antibacterial properties that include *P. acnes*.



Limitations

- Small sample size
- We did not quantitate bacteriologically a reduction in *P. acnes*.

Conclusion

GTP and caffeine are a novel treatment that obviates most of the reasons that other standard acne medications are attacked in literature. The formulations either cream or serum does not irritate the skin and may decrease the irritation of retinoids.

Topical GTP and caffeine were found to be a viable non-antibiotic and non-irritating addition to our armamentarium of acne treatments.

Disclosures

The commercially available product was generously donated by Topix Pharmaceuticals, Inc. It is marketed as Replenix CF and available in cream and serum formulations. Dr. Brody was involved in the conceptualization and design of the product.

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