Active Concepts
Tomorrow's Vision... *Today!*®

AcquaSeal® Algae
Efficacy Data

Code: 20852
INCI Name: Chlamydomonas Reinhardtii Extract
CAS #: N/A
EINECS #: N/A

Type of Study	Results
Cellular Proliferation Assay	**AcquaSeal® Algae** was shown to have a positive effect on fibroblast growth rate when compared to the positive control.
Cellular Renewal Assay	**AcquaSeal® Algae** significantly reduced the number of days of skin cell turnover in both young and old test subjects.
Cellular Hydration Assay	An *in-vitro* study was conducted to determine if **AcquaSeal® Algae** could modify the ability of stratum corneum cells, harvested from test subjects via scalpel biopsy, to hold water. Results indicate that **AcquaSeal® Algae** is capable of significantly increasing water uptake compared to the control.
Immediate and Long Term Effects Assay	Based on the results in these studies, **AcquaSeal® Algae** is capable of exhibiting both short term and long term skin benefits. Short term use of 2.0% **AcquaSeal® Algae** in a base lotion showed a 41.4% increase in hydration, 44.2% decrease in friction, 40.7% increase in skin slip, and 35.0% increase in skin feel. Long term use of 2.0% **AcquaSeal® Algae** in a base lotion over four weeks showed a 26.0% increase in hydration, 44.7% increase in clarity, and 31.2% increase in firmness. These results support using **AcquaSeal® Algae** in formulations for both short term and long term cosmetic benefits.
Reduction of Skin Irritation	After a single application of the base lotion before application of the irritant (preventative effect), skin redness increased from about 16 to 25, an almost 60% increase in skin redness (a* value). In contrast a single application of 2.0% **AcquaSeal® Algae** in base lotion (before induction of irritation) lessened this increase to 19 (a* units) and increase of only 17% compared to 60%. In the second test product was applied twice a day for two weeks as described above for both the base lotion and experimental.

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	In this study, neither product was applied directly before application of the irritant since we were looking for cumulative benefits. As can be seen, the base lotion had no sparing effects as the irritant increased skin redness about the same as in the other test 60%, while and repeated applications of 2.0% **AcquaSeal® Algae** in base lotion reduced significantly skin redness (only a 30% increase was observed, ameliorative effect).
Skin Genomics Assay	Both RxR and PPAR gamma are nuclear regulatory factors and work together in modulating a number of key genes involved in proliferation and inflammation. **AcquaSeal® Algae** increased expression of both PPAR gamma and RxR. Three genes important in the skin inflammatory process were down-regulated; these include NF-κB, TNFα and COX 2 (cyclooxygenase). CD 44 involved in cell adhesion and usually down regulated during differentiation was up-regulated suggestion a shift from differentiation to proliferation.

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Cellular Proliferation Assay

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3604

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In-vitro Keratinocyte Proliferation Assay

Introduction

During aging fibroblast growth (proliferation) rate decreases and this change is implicated in contributing to age related dermal changes. In the skin and in cell culture fibroblast growth is controlled by a host of specific growth factors including fibroblast growth factor and connective tissue growth factor. The purpose of this study was to determine whether or not **AcquaSeal® Algae** is capable of increasing keratinocyte proliferation.

Materials & Methods

In these studies human normal fibroblasts were obtained from an older donor (age 55) and grown in a minimally supplemented medium. Individual test flasks were supplemented with either a positive control FGF (fibroblast growth factor) or the test material **AcquaSeal® Algae** at various concentrations. Growth rates were calculated as the increase in the number of viable cells for a defined period, (twenty-four hours following supplementation), with an image processor based cell counter. Growth of control cultures was normalized to 1.0.

Tissue or cell samples (commercially sourced NHKE, fibroblasts or Skin Ethic synthetic skin model) are removed from the shipping tray and placed into a 6 or 12-well plate containing 2.5 - 5.0 ml of minimal growth medium (37±2°C). They are incubated for at least 24 hours at 37±2°C and 5±1% CO2. After this initial incubation, the growth medium is replaced with 2.5 - 5.0 ml of fresh medium (37±2°C), and 25-50 ul containing, (a) no additional factors as a control, (b) added FGF (Sigma) 1ug/ml (c), Test material (**AcquaSeal® Algae**) at various levels 0.001-0.1 ug/ml of test material or phosphate buffered saline (negative control) and applied directly onto the surface of the tissue. The 6-well plates are then incubated at 37±2°C and 5±1% CO2 for 24 hours. After 24 hours the contents of the individual wells including all tissue are removed, diluted into 5 ml of PBS and placed into glass tubes and cell count is determined with a Countless II FL cell counter.

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Results

As seen in Figure 1 below the positive control, FGF (1ug/ml supplementation), increased proliferation by about 70% after twenty fours. **AcquaSeal® Algae** also showed a positive effect on growth ranging from 13-56% concentration dependent.

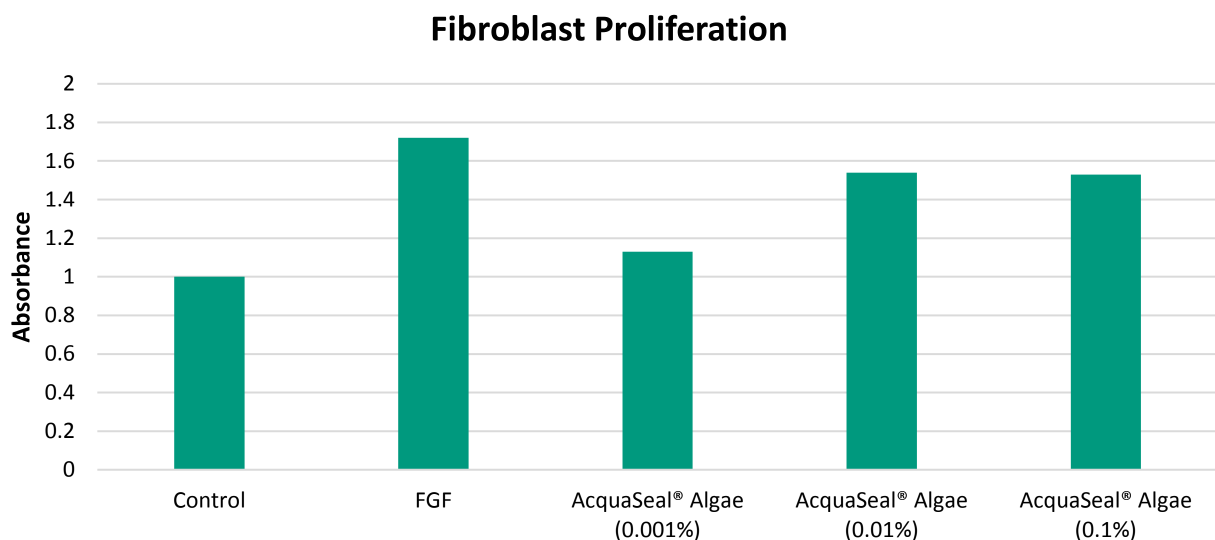

Figure 1. Improvements in fibroblast proliferation.

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Cellular Renewal Assay

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Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3605

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
Cellular Renewal

Purpose

To determine the ability of **AcquaSeal® Algae** to modify the rate of skin turnover (i.e. stratum corneum replacement time) versus a non-treated control and a placebo. Skin cell turnover or renewal rates, (transit time for an epidermal basal cell, to mature, and slough off the skin surface) can be easily measured via DHEA or a dansyl chloride staining method.

Materials & Methods

The epidermal cellular renewal rate, or the transit time for an epidermal basal cell, to mature, and slough off the skin surface is known as skin cell renewal and is assessed via a dansyl chloride or DHA staining method. As the skin ages the epidermal turnover time increases from about 15-20 days for a thirty year-old to about 30 days for a 70 year-old. Exfoliants such as AHAs, and retinoids can increase the rate of skin cell renewal and thus improve properties of the skin. Evidence has been published that longer-term increases in cell renewal rates can result in skin rejuvenation.

To assess skin cell renewal rates between eight (8) and twelve (12) subjects were recruited and refrained from using any moisturizer product and any cleansing product on the volar forearm for 5 days prior to and throughout the course of the study. Subjects should have no adverse skin conditions. One group of subjects (8) was of an average age of 53; the other test group (10) was of an average age of 27.

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The full thickness epidermis is stained with a fluorescent dye as follows:
5.0% ultra pure dansyl chloride is milled into petrolatum and applied as a very thin film to standard Band-Aids (2 x 3 cm). Six to eight test sites (3-4 per arm) are marked on the volar forearm and the Band-Aids are firmly and snugly applied to each test site. These must remain undisturbed, without washing on each subject for 24 hours. After 24 hours the Band-Aids are removed, the sites washed to remove residual stain, and staining confirmed by viewing the sites with a long wave UV light source to detect dansyl chloride fluorescence.

To assess product effects on cell renewal rates test products are applied (b.i.d.) in a randomized fashion to the test sites with an untreated site serving as a control. Application is usually twice-a-day unless otherwise indicated. Removal of the stain, reflecting loss of stratum corneum is monitored daily with the UV light source until complete removal (usually 3-4 weeks) of all stain is noted. End points are determined empirically by trained technicians.

Results

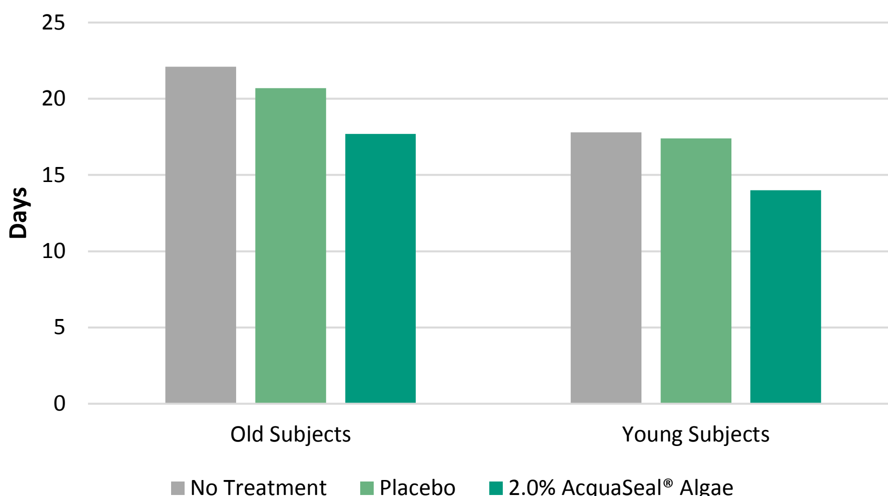

Figure 1. Number of Days for Stain Removal.

Discussion

As can be seen in the above table, treatment with 2.0% **AcquaSeal® Algae** significantly reduced the number of days for removal of fluorescent stain of this test groups (older subjects average age 53) compared to an untreated site. The results indicate that 2.0% **AcquaSeal® Algae** is capable of increasing cellular renewal by 20.0% and 21.0% per age group respectively when compared to the untreated control.

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In-vitro Cellular Hydration Assay

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Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3606

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In-vitro Cellular Hydration Assay

Introduction

An *in-vitro* study was conducted to determine if **AcquaSeal® Algae** could modify the ability of stratum corneum cells, harvested from test subjects via scalpel biopsy, to hold water. Results indicate that **AcquaSeal® Algae** is capable of significantly increasing water uptake compared to the control.

Materials & Methods

Harvesting of Cells

Superficial stratum corneum cells were isolated from the lower leg of 10 test subjects, age 60 or greater via scalpel shave biopsies. All subjects had dry and scaly, whitish looking skin prior to treatment. All treatments were done *in vivo* so the cells were harvested after subjects had been treated to reflect real in use conditions. No treatments were applied after the cells had been isolated.

Samples were taken before treatment started and after two weeks of treatment with 2.0% **AcquaSeal® Algae** twice per day approximately 3mg/ cm2 or a placebo. Sufficient squame samples were taken from each subject (about 50-100 mg) for accurate measurement on a Mettler Microbalance with a sensitivity of 0.01mg. The samples were pooled and thoroughly mixed to factor out differences between subjects, so all starting materials were identical. Samples were visual inspected with a microscope and generally were clumps of perhaps tens to hundreds of cells. Both untreated and treated samples look similar with respect to clump size under the microscope.

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Sample Hydration

Cell samples (about 30-60 mg per sample) were equilibrated at 0% humidity (dry control) for twenty four hours, or soaked in water with mixing to ensure maximal hydration and equilibrated at 100% humidity (fully hydrated state). After equilibration and blotting off excess water, samples were weighed to determine water content. Water content was determined as dry weight subtracted from wet weight at various times.

Two different tests were conducted and treated and untreated samples compared. First, maximal water uptake (the % water cells were able to take up at 100% humidity) was determined. Second, the rate of loss of water when cells were transferred from 100% to 0% humidity was also assessed.

Placebo Composition Formulation

Ingredient	%
Phase A	
Deionized Water	62.600%
Magnesium Aluminum Silicate	0.400%
Xanthan Gum	0.150%
Acrylates/C10-30 AlkylAcrylate Crosspolymer	0.750%
Phase B	
Butylene Glycol	4.000%
Disodium EDTA	0.050%
Phase C	
Caprylic/Capric Triglyceride	8.500%
Octyl Palmitate	4.000%
Cetearyl Alcohol	2.000%
PEG-8 Stearate	1.000%
PEG-100 Stearate	0.800%
Phase D	
Triethanolamine 99%	0.100%
Phase E	
Water	14.000%
Phenoxyethanol	0.500%
Potassium Sorbate	0.100%
Methylisothiazolinone	0.050%
Butylene glycol	1.000%

Results

Maximal Water Uptake

Results are as described in the following Table 1. As can be seen below, the cells isolated from subjects treated with 2.0% **AcquaSeal® Algae** *in vivo* for two weeks, had an increased capacity for water uptake compared to the vehicle treated control. While control cells when fully hydrated, adsorbed about 4.7 mg of water, the cells from **AcquaSeal® Algae**-treated subjects absorbed about 80% more water 8.5 mg. This represents a significant increase in water holding capacity.

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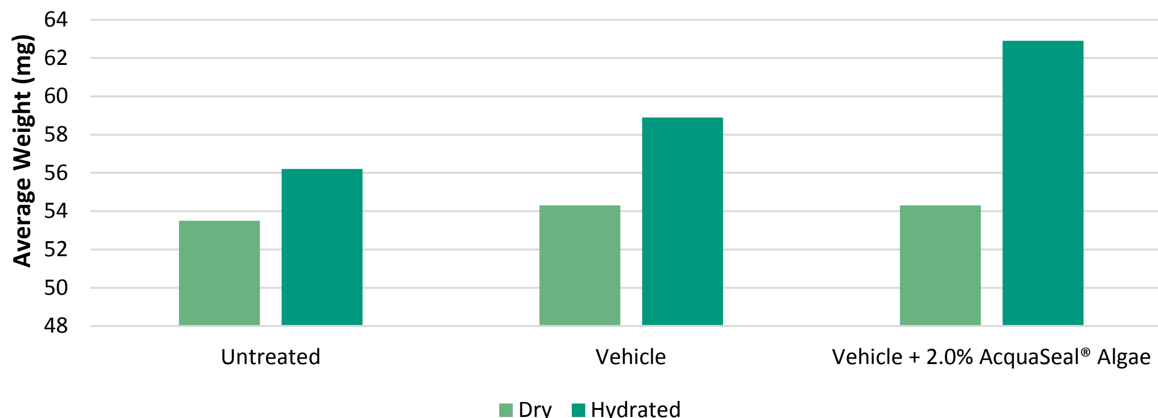

Figure 1. Average weight of skin cells for water holding capacity.

Cellular Water Loss Rates

Afterwards, the fully hydrated cell samples from the above experiment were relocated to an isolated chamber at a humidity of less than 5% and the weight of the samples was monitored over time. The total amount of water adsorbed by the cells and the rate of water loss would be indicative of the ability of the collected cells to maintain moisture. As can be seen below for both the untreated and the vehicle, more than 50% of the gained water was lost within the first 5 minutes. In contrast, the cells from the 2.0% **AcquaSeal® Algae** treated subjects only lost about 20% over that same time period. After two hours (test completion) both untreated and vehicle controls had lost all absorbed water while the 2.0% **AcquaSeal® Algae** samples still retained about 30% of the adsorbed water.

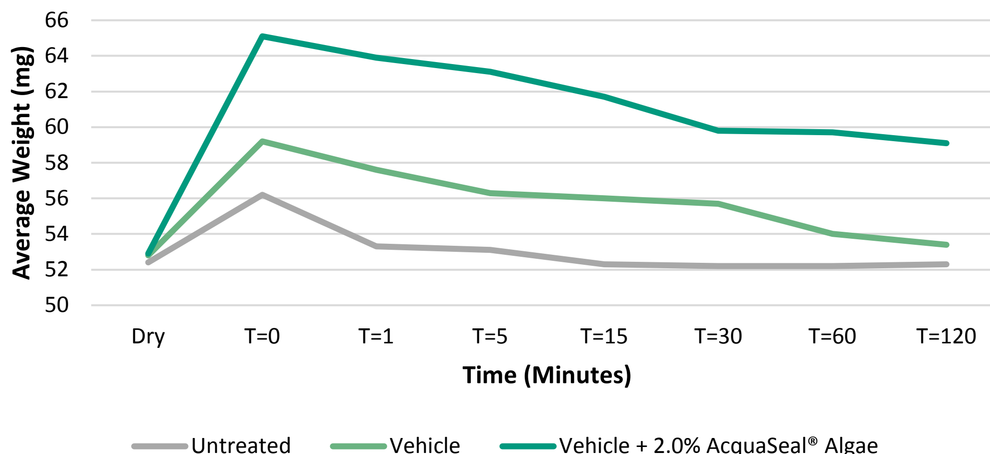

Figure 2. Average weight of skin cells for cellular water loss.

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Discussion

AcquaSeal® Algae modifed the ability of stratum corneum cells to hold water, increasing both the maximal percent adsorbed, and reducing the rate of loss when cells were placed under low humidity conditions compared to untreated and a vehicle control (placebo). Since these tests were conducted on isolated cells and not structured layers of skin, the results reflect in a large part, the actual ability of the cells to hold water as opposed to an intact skin structure. This data indicates that **AcquaSeal® Algae** is capable of significantly increasing water uptake compared to the control.

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In-vivo Immediate and Long Term Effects

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3607

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In-vivo Immediate and Long Term Analysis

Purpose

To determine the ability of **AcquaSeal® Algae** when used at 2.0% in a simple lotion to modify the skin cosmetic properties *in vivo* after a single use and with repeated use. A single use fifteen (15) subject study evaluated the immediate impact of **AcquaSeal® Algae** on skin properties. A fifteen (15) subject study over a four week period evaluated the longer term and cumulative effects of **AcquaSeal® Algae** on skin properties related to cosmetic benefits and skin health.

Materials & Methods

Single Use Study

A single use fifteen (15) subject study evaluated the immediate impact of **AcquaSeal® Algae** on skin properties. **AcquaSeal® Algae** was formulated into a lotion base at 2.0% and applied to the face (one side) of test panelists. To the other side the control lotion was applied. A number of skin parameters were assessed after one hour. Measurements included skin feel, skin friction and skin hydration. Tests were run in the morning and subjects refrained from using any cosmetic product on their faces for at least three days prior to the test. Subjects equilibrated in a controlled environment room for thirty minutes prior to the test.

Skin hydration was assessed with the DPM value of the Nova Impedance Meter as per manufacturer's instructions. Measurements were made in triplicate and averaged. Skin slip and overall feel was self-assessed on a 0-10 Clinical Scale, subjects being supervised by trained clinicians. Subjects would place a line of card on which a visual linear 0-10 scale had been placed.

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Friction was assessed with a Courage Friction Meter FR 700 as per manual instructions for measuring skin friction using a 16mm head. Data was acquired as AFU and converted with proprietary software to a 1-10 scale wherein 1 was a synthetic skin treated with dimethicone to dramatically reduce slip or friction and 10 was the skin mimic treated with a sucrose/ water solution to maximize resistance. Measurements are essentially arbitrary with respect to specific numbers and all results should be viewed comparatively, i.e. A versus B or before versus after. Skin-feel and skin slip were graded on a 1-10 clinical scale with subjective self-assessment under supervision of trained clinicians.

Long Term (Four Week) Clinical Study

A fifteen (15) subject study over a four week period evaluated the longer term and cumulative effects of **AcquaSeal® Algae** on skin properties related to cosmetic benefits and skin health. After an initial one week dry out phase (no cosmetic product use) subjects (average age 52) applied a 2.0% **AcquaSeal® Algae** lotion to their half of their face twice-a-day for four weeks. The placebo was applied to the other half. Evaluations were made prior to the study start and after four weeks. At the four week evaluation, assessments were made at least six hours after production application, i.e. applications were made first thing in the morning and prior to retiring, thus measurements were taken mid to late afternoon following the previous morning application.

Hydration was assessed with the NOVA meter (DPM value) as per manufacturer's instructions.
All measurements were done in triplicate. Superficial facial lines (SFL's) were assessed (half face) by trained graders using the method of Packman (Packman, E., and Gans, E.H. Topical moisturizers: quantification of the effect on superficial facial lines. Soc. Cos. Chem. 29, 79-90 (1992). In this method a total wrinkle or SFL is determined based upon the summation of the number of wrinkles, fine (1), moderate, (2) and deep (3). The number in parenthesis refers to a multiple weight factor for each wrinkle class.
Clinical grading of overall skin condition was done via expert graders on a 0-10 point scale as above using a visual linear 0-10 scale.

Clarity (luminosity L value) was assessed with the Minolta Chroma Meter (Muizzuddin, N., Marenus, K., Maes, D., and Smith, W. Use of a chromameter in assessing the efficacy of antiirritants and tanning accelerators. J. Soc. Cos. Chem. 1990.).

Skin Firmness was assessed with a Cutometer 575 using the ratio of Ue/Ur as a measure of firmness.

Placebo Composition Formulation

Phase A	
Deionized Water	62.600%
Magnesium Aluminum Silicate	0.400%
Xanthan Gum	0.150%
Acrylates/C10-30 AlkylAcrylate Crosspolymer	0.750%
Phase B	
Butylene Glycol	4.000%
Disodium EDTA	0.050%
Phase C	
Caprylic/Capric Triglyceride	8.500%
Octyl Palmitate	4.000%
Cetearyl Alcohol	2.000%

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PEG-8 Stearate	1.000%
PEG-100 Stearate	0.800%

Phase

Triethanolamine 99%	0.100%

Phase E

Water	14.000%
Phenoxyethanol	0.500%
Potassium Sorbate	0.100%
Methylisothiazolinone	0.050%
Butylene glycol	1.000%

Results

Single Use Study

As shown in Figure 1 below, the placebo did not significantly alter skin hydration or feel or hydration characteristics after one hour. **AcquaSeal® Algae** on the other hand, significantly improved skin hydration, reduced skin friction and provided a positive skin feel.

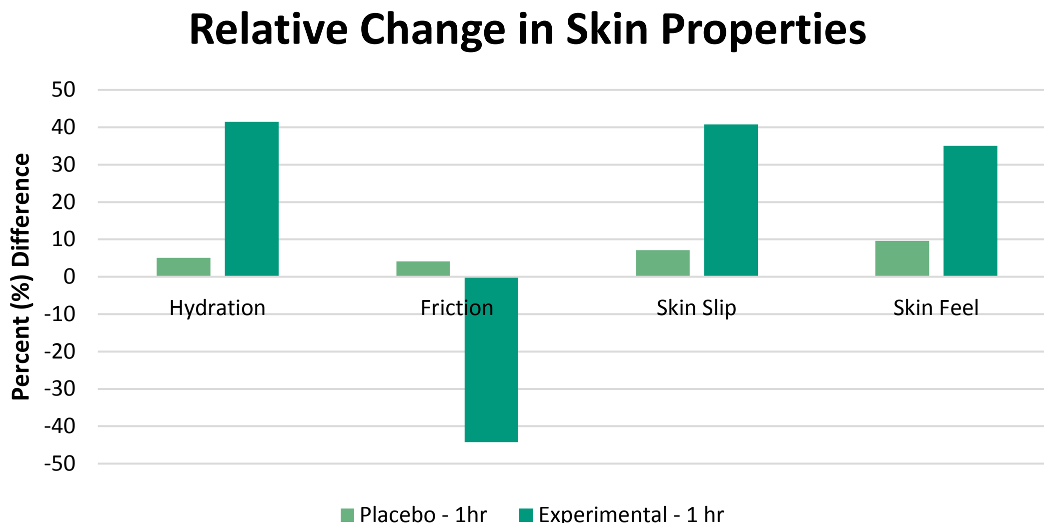

Figure 1. Relative change in skin properties after one hour.

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Long-Term Use-Long Term Skin Changes
Summarized below in Figure 2 are the results after one month product usage; results are expressed as percent change from the pre-treatment assessments, both for the clinical and laboratory evaluations. **AcquaSeal® Algae** produced considerable positive changes in skin quality after both four weeks. Statistically significant improvements in skin hydration, clarity, lines and wrinkles, firmness, flakiness and overall appearance were observed with continuing use of **AcquaSeal® Algae**. The placebo showed no such changes.

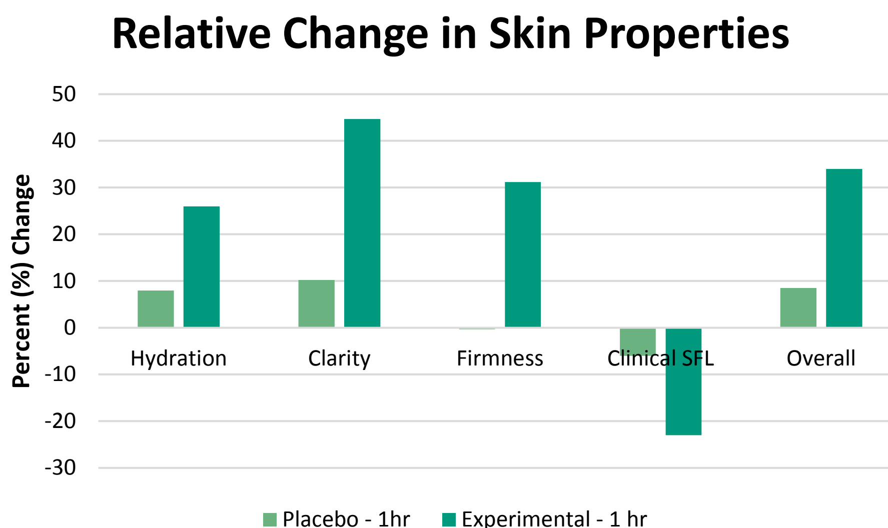

Figure 2. Percent change in skin properties after four weeks.

Discussion

Based on the results in these studies, **AcquaSeal® Algae** is capable of exhibiting both short term and long term skin benefits. Short term use of 2.0% **AcquaSeal® Algae** in a base lotion showed a 41.4% increase in hydration, 44.2% decrease in friction, 40.7% increase in skin slip, and 35.0% increase in skin feel. Long term use of 2.0% **AcquaSeal® Algae** in a base lotion over four weeks showed a 26.0% increase in hydration, 44.7% increase in clarity, and 31.2% increase in firmness. These results support using **AcquaSeal® Algae** in formulations for both short term and long term cosmetic benefits.

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Reduction of Skin Irritation

info@activeconceptsllc.com • Phone: +1-704-276-7100 • Fax: +1-704-276-7101

Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3608

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
In-vivo Reduction and Prevention of Skin Irritation

Introduction

An *in-vivo* Reduction and Prevention of Skin Irritation assay was preformed to determine the ability of **AcquaSeal® Algae** to reduce Balsam of Peru-mediated skin inflammation/irritation.

Materials & Methods

Erythema was induced on the volar forearm of 15 subjects by application of 5.0% Balsam of Peru milled into pure petrolatum and applied to test sites under a semi-occlusive Band-Aid brand bandage (2 X 2 inches) for fifteen to thirty minutes. After removal of the tape the test site was wiped free of residual petrolatum and skin erythema was assessed by determining the a* value of the Minolta Chroma.

For most tests, experimental test materials are applied to several sites, while other sites serve as nontreated controls. Erythema, measured for example with the Minolta Meter is assessed every 15 or 30 minutes over a two hour period after which, the transient erythema induced by Balsam of Peru dissipates. Erythema on the sites treated with anti-irritants is compared with that on the control sites. In general Balsam of Peru treatment will result in an increase in the a* value (red color) by about 10 units or so within a few minutes after removal the bandage.

Two variations a single use test and a multiple use test were conducted. In the single use test immediately prior to application of Balsam of Peru and induction of irritation, sites were treated with 2.0% **AcquaSeal® Algae** in a base lotion or the base lotion alone. In a second longer term test, the forearm was treated for one week (twice a day) with 2.0% **AcquaSeal® Algae** in a base lotion or the base lotion alone. Balsam of Peru induced irritation was evaluated as above.

Evaluations were made at least one hour after the last application of test product. In this way, longer term or cumulative effects are observed rather than immediate effects.

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Results

As seen in Figure 1 below Balsam of Peru increased skin redness (a*) value dramatically of skin treated simply with the control vehicle. In Contrast a single application (before induction of irritation) and repeated applications of 2.0% **AcquaSeal® Algae** in a base lotion reduced significantly skin redness.

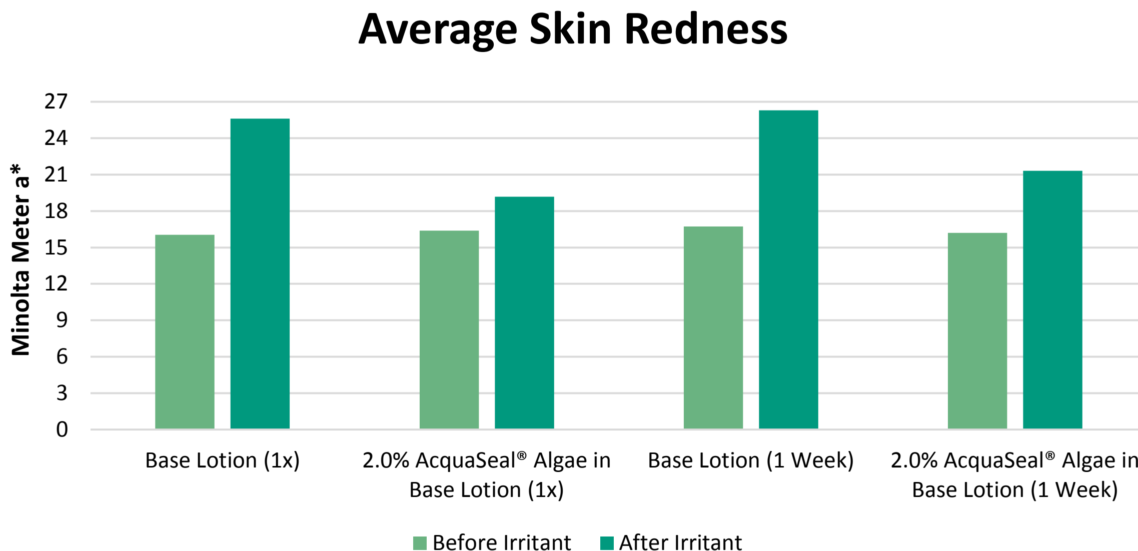

Figure 1. Average skin redness before and after Balsam of Peru application.

Discussion

After a single application of the base lotion before application of the irritant (preventative effect), skin redness increased from about 16 to 25, an almost 60% increase in skin redness (a* value). In contrast a single application of 2.0% **AcquaSeal® Algae** in base lotion (before induction of irritation) lessened this increase to 19 (a* units) and increase of only 17% compared to 60%. In the second test product was applied twice a day for two weeks as described above for both the base lotion and experimental. In this study, neither product was applied directly before application of the irritant since we were looking for cumulative benefits. As can be seen, the base lotion had no sparing effects as the irritant increased skin redness about the same as in the other test 60%, while and repeated applications of 2.0% **AcquaSeal® Algae** in base lotion reduced significantly skin redness (only a 30% increase was observed, ameliorative effect).

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Skin Genomics Assay

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Tradename: AcquaSeal® Algae

Code: 20852

CAS #: N/A

Test Request Form #: 3609

Lot #: NC170831-I

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principle Investigator: *Jennifer Goodman*

Test Performed:
DNA Microarray Analysis

Introduction

Mediation of gene expression was evaluated in cultured keratinocytes or commercial skin equivalents per protocol. About twenty genes, important in a variety of skin functions were evaluated. A number of genes undergoing a significant up or down regulation are highlighted and discussed below. Both the RxR and PPAR gamma gene code for nuclear regulatory factors and work together in modulating a number of other key genes involved in proliferation and inflammation. **AcquaSeal® Algae** increased expression of both PPAR gamma and RxR, indicating that the use of **AcquaSeal® Algae** would likely result in increased epidermal proliferation.

Materials & Methods

DNA Microarray Analysis General Overview

Cultured cells are grown in a 6-well plate until confluent using appropriate culture conditions. Upon reaching confluency, three of the six wells are treated with culture media supplemented with **AcquaSeal® Algae** at a concentration of 2.0% in water. The remaining three wells are treated with culture media alone and act as a control. After applying the test material, the cells are incubated for 24 hours at 37±2°C and 5±1% CO2. At the end of the incubation period, the culture media is removed via aspiration and the cells are washed once with cold phosphate buffered saline ("PBS") using approximately 1 ml per well. After the wash, a trypsin/EDTA solution is added to the wells to detach the cells. Trypsin neutralizing solution is then added to the wells. The treated cells and the untreated cells are pooled into separate 15 ml centrifuge tubes and pelleted by centrifuging at 1000 RPM at 4±2 °C. After removing the supernatant, the pelleted cells are lysed by adding 300 ml of guanidinium thiocyanate lysis solution to each tube and then repeatedly drawing and releasing the solution into the pipette until the cell pellet is dissolved. The cell lysates are stored at -75°C until the RNA extraction process as described below is completed.

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RNA Isolation

RNA isolation is performed using the RNAqueous Kit from Ambion Inc. (Austin, Texas). To the cell lysates or tissue homogenates prepared above, an equal volume of 64% ethanol is added and the tubes are vortexed. Up to 700 ml of the mixture is transferred to a glass fiber filter cartridge, which is loaded into a 1.5 ml collection tube and the cartridge is centrifuged for 1 minute at 14,000 RPM. The flow through is discarded. Any remaining mixture is loaded into the filter cartridge and the centrifugation process is repeated until all of the mixture has been processed. The filter is then washed to remove any residual cellular debris from the RNA bound to the glass fibers by applying 700 ml of a first wash solution (1 time) and 500 ml of a second wash solution (2 times) to the filter cartridge and centrifuging at 14,000 RPM for 1 minute to pass each wash through the cartridge. The flow through is discarded after each wash. After the final wash, one final spin is performed without wash solution to remove any residual wash solution in the filter cartridge. The RNA bound to the glass fibers within the cartridge is then be eluted by applying 30 ml of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, preheated to 70-80°C, herein below “TE buffer”) to the cartridge and centrifuging the cartridge in a new collection tube at 14,000 RPM for one minute. For samples prepared from cell lysates and small tissues, the elution process is repeated with an additional 30 ml of preheated TE buffer. For samples prepared from larger tissues (*i.e.,* full thickness tissues) the elution process is repeated two additional times. After the RNA is eluted, RNA concentration is quantified using a Ribogreen assay. RNA quality is assessed via gel electrophoresis.

RNA Concentration Assay

Ribogreen reagent is provided as a stock solution in DMSO. Prior to use, the reagent is diluted 2000 fold in TE buffer. The RNA assay requires 200 ml of diluted Ribogreen reagent per sample to be tested and 1 ml of the reagent for the standards. Once prepared, the diluted reagent is stored protected from light. A series of RNA standards are prepared by diluting purified ribosomal RNA derived from *E. coli* to the following concentrations: 2 mg/ml, 1 mg/ml, 200 ng/ml, 40 ng/ml and 0 ng/ml (blank). Prior to assaying, the RNA samples prepared above are diluted 1000 fold in TE buffer. For the RNA assay, 100 ml of the diluted samples or standards are transferred to the wells of a black 96-well plate. The samples and standards are assayed in duplicate. After the samples/standards are added to the plate 100ml of diluted Ribogreen assay reagent is added to the wells and the plate is gently mixed and allowed to incubate for 5-10 minutes protected from the light. After this incubation, the plate is read with a fluorometer using an excitation wavelength of 500 nm and an emission wavelength of 525 nm.

RNA Gel Electrophoresis

A 1% RNA gel is prepared by adding 0.3 g agarose to 21.6 ml diethylpyrocarbonate (DEPC) treated water. The agarose is dissolved by boiling the water in a microwave oven. After the solution is cooled to approximately 55°C, 5.4 ml of formaldehyde and 3.0 ml 10xMOPS (0.2 M MOPS [pH 7.0], 20 mM sodium acetate, 10 mM EDTA, made in DEPC H2O and filter sterilized). After mixing, the agarose gel is cast in the horizontal gel apparatus with loading slots placed on the side of the gel closest to the negative terminal. The gel is allowed to set for at least 1 hour at room temperature. While the gel is setting, 175 ml of 1x MOPS is prepared by diluting the 10x stock. After the gel is set, the comb is removed and the buffer chamber of the gel apparatus is filled with 150-175 ml 1x MOPS (enough buffer is added to cover the gel with approximately 3 mm of buffer). The cover is placed on the apparatus, the electrical leads are attached to the power source, and the empty gel is run at 40 V (4 V/cm) for 5-10 minutes.

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While the gel is running, the RNA samples are prepared by transferring approximately 1 mg of each sample RNA to a 600 ml PCR tube. DEPC H2O is used to bring the total volume of all the samples to a common level and then 1-3 volumes of a gel-loading buffer (i.e. 5% glycerol, 1 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 20% formaldehyde, 50% formamide, 10 mg/ml ethidium bromide) are added. The samples are denatured by placing them at 65-70°C for 5-15 minutes and then placed on ice to cool. The samples are then carefully loaded into the lanes (each loading slot can hold 10-15 ml of sample, depending upon the thickness of the gel) and run on the gel at 40 V for 1-3 hours. At the end of the run, the RNA is visualized by placing the gel on a UV transilluminator. An RNA sample is used for subsequent processing if both the 18S and 28S ribosomal bands are clearly visible and there is little or no staining below the 18S band.

mRNA Amplification

mRNA is amplified using the MessageAmp aRNA kit from Ambion Inc.

First Strand cDNA Synthesis: To start the first strand synthesis, 5 mg of total RNA for each sample are added to 600 ml PCR tubes and the total volume of liquid in the tube is adjusted to 12 ml with DEPC H2O. To each tube, 1 ml of T7 Oligo(dT) primer is added and the tube is incubated at 70±2°C for 10 minutes to denature the RNA and is then placed on ice to allow the primer to anneal to the poly A ends of the mRNA. After cooling, 2 ml of 10 x first strand buffer, 1 ml of RNAse inhibitor and 4 ml of dNTP mix is added to each tube, and the tube is placed at 42°C. As soon as the tube is heated, 1 ml of reverse transcriptase is added and the tubes are returned to 42±2°C for 2 hours. At the end of the two hours, the tubes are briefly centrifuged to collect all of the fluid at the bottom of the tube and then placed on ice.

Second Strand Synthesis and cDNA Purification: For the synthesis of the second strand of cDNA the following ingredients are added sequentially to the tubes: 63 ml DEPC H2O, 10 ml 10 x second strand buffer, 4 ml dNTP mix, 2 ml DNA Polymerase and 1 ml of RNAse H. The tube is mixed and then incubated at 16±2°C for 2 hours. Towards the end of the 2 hour incubation, a sufficient quantity of DEPC H2O is warmed to 50±2°C, and a cDNA purification filter cartridge is equilibrated with 50 ml of cDNA binding buffer (one cartridge per sample) for at least 5 minutes. After the samples are finished incubating, 250 ml of cDNA binding buffer are added to each tube and thoroughly mixed. The contents of the PCR tube are then transferred to the cDNA purification filter cartridge. The cartridge is then placed in a collection tube and centrifuged at 10,000 RPM for 1 minute. The flow-through is discarded and 650 ml of cDNA wash solution is added to the cartridge. The cartridge is centrifuged again, the flow-through is discarded, and is then centrifuged one additional time to ensure that the wash buffer has been completely emptied from the filter. The cDNA is eluted by applying 10 ml of preheated DEPC H2O to the filter and centrifuging the filter in a new collection tube at 10,000 RPM for one minute. This elution is performed one additional time to give a total volume of 16-18 ml of cDNA solution.

In Vitro Transcription to Synthesize aRNA and aRNA Purification

The in vitro transcription begins by adding the following to the cDNA solution: 4 ml each of T7 ATP solution, T7 CTP solution, T7 GTP solution, T7 UTP solution, 4ml of 10xReaction buffer, and 4 ml of T7 enzyme mix. The tube is mixed and then incubated at 37±2°C for 6-14 hours. Towards the end of the incubation, a sufficient volume of Elution Solution is warmed to 50-60°C and an aRNA filter cartridge is equilibrated with 100 ml of aRNA binding buffer for at least 5 minutes. At the end of the incubation period, 350 ml of aRNA binding buffer is added to the sample tubes and thoroughly mixed. An additional 250 ml of absolute ethanol is also added to each tube. The mixture is then transferred to an aRNA filter cartridge; the cartridge is then inserted into a collection tube and centrifuged at 10,000 RPM for 1 minute. The flow-through is discarded and 650 ml of aRNA wash buffer is added to the cartridge followed by centrifuging at 10,000 RPM for one minute.

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After discarding the flow through, the cartridge is spun one final time to remove all traces of the wash buffer. The cartridge is then transferred to a new collection tube. 25 ml of pre-warmed Elution Solution is added to the cartridge. The cartridge is incubated for 2 minutes at room temperature and then aRNA is eluted by centrifuging for 1 minute at 10,000 RPM. This elution is performed one additional time to give a total volume of 45-50 ml of aRNA solution. The final concentration of the aRNA is determined by the Ribogreen assay described above. In addition, the quality of the aRNA is checked via gel electrophoresis as described above. An aRNA sample is used for subsequent processing if a broad band of RNA is observed.

Labeling and Purification of aRNA

aRNA is labeled with fluorescent dyes using the PerkinElmer ASAP RNA Labeling Kit. Two tubes are prepared for the labeling process – for the untreated sample Cy3 labeling (green), and for the treated sample Cy5 labeling (red). To the Cy3 tube add 2 mg of aRNA prepared from the untreated/control sample and add enough DEPC H2O to bring the total volume up to 4 ml. To the Cy5 tube add 2 mg of aRNA prepared from the sample treated with the test material and add enough DEPC H2O to bring the total volume up to 4 ml. To both tubes, add 5 ml of ASAP labeling buffer and 1 ml of the specific dye for the tube (Cy3 or Cy5). Incubate the tubes for 15 minutes at 85±2°C. At the end of the 15 minutes, place the tubes on ice to cool and then add 2.5 ml of ASAP stop solution to each tube. The above proportions are sufficient for analyzing one microarray chip. If more chips are to be used then the labeling is increased proportionately.

To purify the labeled aRNA, a micronon YM-30 filter column is inserted into a collection tube and filled with 400 ml of TE buffer. The Cy3 and Cy5 probes are combined (12.5 ml of each) and then added to the micron filter and thoroughly mixed with the TE buffer. The filter is centrifuged at 12,000 RPM for 8 minutes and the flow through is discarded. The column is washed twice with 400 ml of TE buffer, discarding the flow through each time. After the final wash, the filter column is inverted, placed into a new collection tube and centrifuged at 12,000 RPM for 2 minutes to collect the probe (the probe is concentrated in a volume of 2-30 ml of residual TE buffer).

Microarray Hybridization and Washing

For hybridization, 45 ml of 10x control target RNA (supplied with Agilent Technologies In Situ Hybridization Kit) is mixed with 160 ml of DEPC water and 9 ml of 25x Agilent Fragmentation Buffer. This mixture is incubated at 60°C for approximately 30 minutes in a hybridization oven. At the end of the incubation, 225 ml of Agilent Hybridization Buffer is added along with the fluorescent aRNA probes prepared above. The mixture is then incubated at 70°C for 5-10 minutes in a water bath. During this incubation period, an Agilent SUREHYB hybridization chamber is prepared by inserting a glass gasket slide into the bottom half of the chamber. At the end of the incubation, the hybridization mixture (approximately 450 ml) is applied to the glass gasket slide and an Agilent Human 1A Oligo Microarray Chip is placed face down on top of the gasket such that the hybridization solution is sandwiched between the glass gasket slide and the microarray face of the chip. The top half of the chamber is attached and the connecting thumbscrew tightened. After verifying that there is good bubble formation in the chamber, it is placed into the hybridization oven for approximately 17 hours (60°C and rotating at 4 RPM). At the end of the hybridization period, the microarray/glass gasket is removed from the SUREHYB chamber and placed in 50 ml of a first wash solution (room temperature, 6x SSC, 0.005% Triton X-102). After the gasket has fallen away from the microarray, the array is transferred to 300 ml of fresh wash solution 1 on a magnetic stir plate. The array is washed while the solution is mixed at medium speed for 10 minutes and is then transferred to 300 ml of wash solution 2 (0.1x SSX, 0.005% Triton X-102, 4°C) for 5 minutes. After the final wash, the array is centrifuged at 500 RPM for 5 minutes until dry.

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Microarray Scanning and Analysis

The microarrays are scanned with an Axon GenePix 4100A Scanner with the scanning resolution set to 10 mm and analyzed with GenePix Pro software. During the initial scan the PMT gains for the scanner are adjusted such that the Cy5/Cy3 image count ratios are between 0.88 and 1.12.

To derive the standard curve for the Ribogreen assay, the relative fluorescent units versus the known RNA concentrations in mg/ml for the standards is plotted and subjected to regression analysis to establish the line that best fits these data points. Mean RFU values for the test materials and untreated samples are then used to estimate the amount of RNA present in each sample.

The level of gene expression is related to the fluorescence intensity of the probed gene marker on the microarray. Fluorescence measurements between the Cy3 and Cy5 probes are normalized. The total fluorescent signal for both dyes is normalized with a correction factor such that the ratio of total intensities for both dyes equal to one. Control genes are used as reference standards.

Criteria for evaluating changes in gene expression are known to those of ordinary skill in the art and include the following: First, the ratio of Cy3/Cy5 (untreated/treated) fluorescence intensity is greater than 1.5 or less than 0.66. (This relates to a change in gene expression of at least +/-30%.) Second, the fluorescence intensity of the gene marker is greater than the background intensity. Third, the gene feature is clearly marked specifically by the aRNA probes and is not due to non-specific fluorescence. The first two criteria are filtered via computer analysis. The last criterion requires visual inspection of the array.

Ratios of greater than about 1.3 are interpreted to indicate that a gene is upregulated by the treatment, whereas ratios of less than about 0.7 are interpreted to indicate a down regulated gene. Thus, a ratio of 1.3, where the treated value is 130% of the untreated value, indicates a 30% increase in gene expression. Similarly, a ratio of 0.7, means that the treated value was 70% of the untreated value. This indicates a 30% decrease in gene expression.

Results

As shown in Figure 1, three genes important in the skin inflammatory process were down-regulated; these include NF-κB, TNFα and COX 2 (cyclooxygenase). CD 44 involved in cell adhesion and usually down regulated during differentiation was up-regulated suggestion a shift from differentiation to proliferation. All data presented herein was statistically significant.

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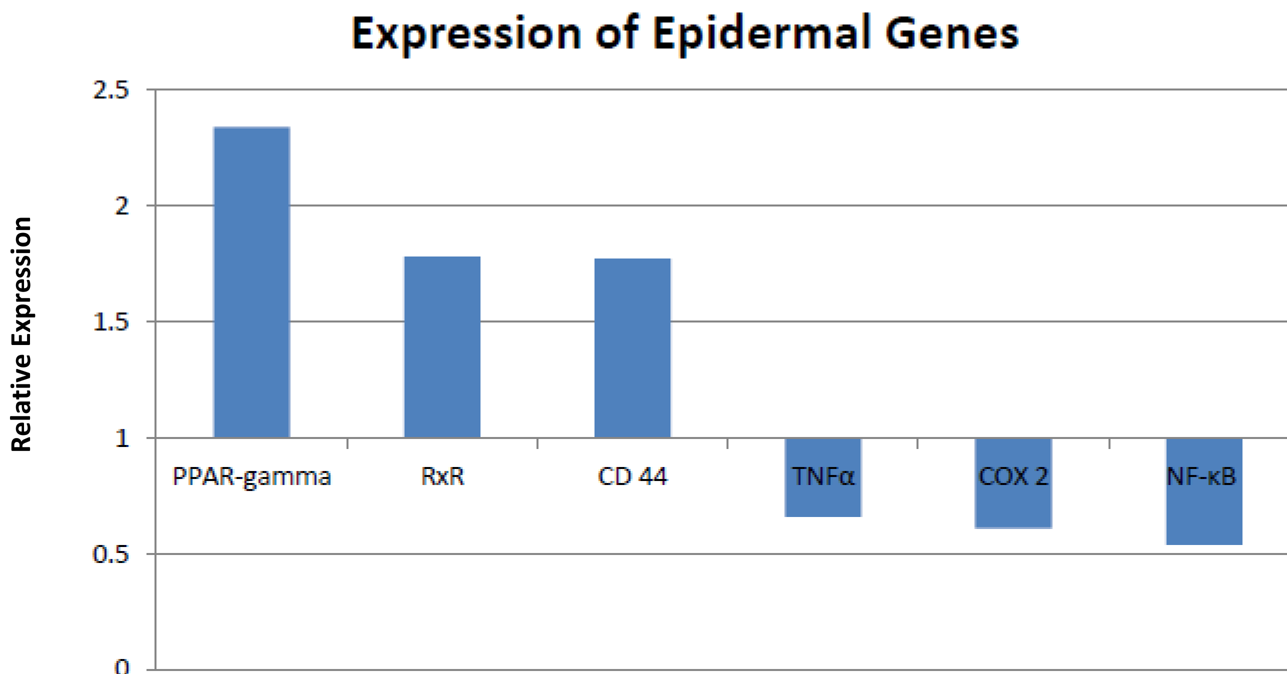

Figure 1. Expression of epidermal genes.

Discussion

Mediation of gene expression was evaluated in cultured keratinocytes as per commercial protocols as referenced above. Twenty genes, important in a variety of skin functions were evaluated. Both RxR and PPAR gamma are nuclear regulatory factors and work together in modulating a number of key genes involved in proliferation and inflammation. **AcquaSeal® Algae** increased expression of both PPAR gamma and RxR. Three genes important in the skin inflammatory process were down-regulated; these include NF-κB, TNFα and COX 2 (cyclooxygenase). CD 44 involved in cell adhesion and usually down regulated during differentiation was up-regulated suggestion a shift from differentiation to proliferation.

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