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AC CytoSulf PF
Efficacy Data

Code: 20793PF
INCI Name: Plankton Extract
CAS #: 91079-57-1
EINECS #: 293-445-7

Type of Study	Results
Cytostasis Assay	**AC CytoSulf PF** was able to induce cytostasis of HaCaT keratinocytes. By suspending the cell cycle, **AC CytoSulf PF** was able to decrease telomere shortening and prevent mutations to the cellular genome. The results of this assay indicate that **AC CytoSulf PF** has a positive effect on cell cycle progression, perturbing HaCaT keratinocytes primarily in the G2-M phase.
Cellular Viability	**AC CytoSulf PF** was tested to evaluate its effects on the viability of normal human dermal fibroblasts (NDHF). At concentrations of 0.1% and 0.01% **AC CytoSulf PF** nor the preservatives contained therein exhibited any inhibition of cell viability. It can therefore be concluded that at normal use concentrations **AC CytoSulf PF** enhances cellular viability.
High Resolution Ultrasound Skin Imaging	**AC CytoSulf PF** has a strong positive effect on skin's density when used at recommended use levels.
IL-6 ELISA	**AC CytoSulf PF** exhibited anti-inflammatory effects on LPS-treated fibroblasts. This decrease in IL-6 production indicates a reduced inflammatory environment which could decrease the signs of aging and reduce the formation of fine lines and wrinkles. It can therefore be concluded that at normal use concentrations **AC CytoSulf PF** enhances soothing and anti-aging properties.
Moisturization/Hydration Assay	**AC CytoSulf PF** was designed to provide moisturization benefits, however with the present study we can confirm that this ingredient is not only capable of providing protective benefits but also ideal for moisturizing and skin hydrating personal care applications.
Oxygen Radical Absorbance Capacity (ORAC)	**AC CytoSulf PF** exhibited antioxidant activity comparable to 200µM Trolox®. The antioxidant capacity of **AC CytoSulf PF** increased as the concentration increased. As a result we can assure that its ability to minimize oxidative stress is dose dependent. It can therefore be concluded that **AC CytoSulf PF** is capable of providing antioxidant properties.
Transepidermal Water Loss	**AC CytoSulf PF** was designed to provide moisture retention benefits, however with the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing a decrease in transepidermal water loss

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	therefore promoting moisture retention benefits when added to cosmetic applications
Scratch Assay	**AC CytoSulf PF** was able to increase cell migration and close the scratch at a rate comparable to the positive control. The mechanisms of the cells in the in-vitro scratch assay mimic the mechanisms seen in in-vivo wound healing therefore we can be assured that our results are translatable outside the laboratory. With the present study we can be confident that this product has healing abilities and cell proliferation properties.

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Cytostasis Assay Analysis

Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1825

Lot #: NC160218-F

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

Test Performed:
FUCCI Cell Cycle Sensor Assay

Introduction

Sulfur is present in all classes of biomolecules, while disulfide bonds determine the strength and shape of proteins and enzymes. Thus, sulfur is largely responsible for the biological activity of disulfide-containing proteins and enzymes. Select sulfur-rich compounds play essential roles in combating signs and symptoms of aging and these compounds have been shown to have potent chemo preventative effects and anti-inflammatory properties.

Sulfur biology has also been shown to play an important role in cytostasis. Loss of genome maintenance and continual growth and cell turnover contribute to premature aging. Eukaryotic cells are regulated by the cell cycle, which is defined by the major checkpoints G1, S, G2, and M phase. Human skin cells divide daily but this continual division ultimately leads to decay and aging, exhibited as fine lines and wrinkles.

Research has demonstrated the effects of thiol-bearing compounds on antiproliferation, cell cycle arrest, and regulation of cellular signaling changes resulting in increased telomerase inhibition. Sulfur-rich compounds therefore are postulated to have a direct effect on the cell cycle in cultured cell lines, thus playing an essential part in reducing the visible signs of aging. This idea of utilizing sulfur compounds to induce cytostasis can be investigated using a fluorescence-based cell cycle assay.

The FUCCI Cell Cycle Sensor Assay was conducted to assess the ability of **AC CytoSulf PF** to induce cytostasis *in vitro* cultured HaCat Keratinocytes.

- Schiff, J. A., Pathways of assimilatory sulphate reduction in plants and microorganisms. Ciba Foundation Symposium 72, 49-69 (1979)
- Editorial, Biology and Brimstone. Nature Chemical Biology 2(4), 169 (2006)
- Lee et al, Anti-cancer activity of highly purified sulfur in immortalized and malignant human oral keratinocytes. Toxicology in Vitro 22(1), 87-95 (2008)
- Lu, S. C., Regulation of Glutathione Synthesis. Molecular Aspects of Medicine 30(1-2), 42–59 (2009)
- Stewart et al, Cell-cycle dysregulation and anticancer therapy. Trends in Pharmacological Sciences 24(3), 139-145 (2003)

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Assay Principle

The Fluorescence Ubiquitination Cell Cycle Indicator (FUCCI) is a fluorescent protein (FP)-based sensor that employs a red (RFP) and a green (GFP) fluorescent protein fused to different regulators of the cell cycle: Cdt1 and geminin. These two constructs, Cdt1 and geminin, are ubiquitinated by specific ubiquitin E3 ligases targeting them to the proteasome for degradation. The temporal regulation of the activity of these E3 ligases results in the biphasic cycling of geminin and Cdt1 through the cell cycle. In the G1 phase of the cell cycle, geminin is broken down and only Cdt1 tagged with RFP may be visualized, thus identifying cells in the G1 phase with red fluorescent nuclei.

In the S, G2, and M phases, however, Cdt1 is degraded and only geminin tagged with GFP remains, thus identifying cells in these phases with green fluorescent nuclei. During the G1/S transition, as Cdt1 levels decrease and geminin levels increase, both proteins are present in the cells, allowing GFP and RFP fluorescence to be observed—when green and red images are overlaid, the cells appear with yellow fluorescent nuclei. This dynamic color change from red-to-yellow-to-green represents the progression through cell cycle and division and can be measured by mean fluorescence units (MFU).

The Premo™ FUCCI Cell Cycle Sensor combines these Cdt1 and geminin FP constructs with the BacMam gene delivery system. The genetically encoded and pre-packaged reagents enable immediate usage and eliminate the need to purify plasmid or to use lipids, dye-loading chemicals, or other potentially harmful treatments to transduce cells. Cellular transduction is efficient and reproducible in most cell types, including HaCat Keratinocytes, without apparent cytotoxic effects. BacMam 2.0 expands the efficiency and utility of the gene delivery platform as cells can be transduced quantitatively in a simple, one-step process due to inclusion of elements that greatly enhance transduction efficiency and expression levels: a pseudotyped capsid protein for more efficient cell entry and genetic elements (enhanced CMV promoter and Woodchuck Post-Transcriptional Regulatory Element) that boost expression levels.

Premo™ FUCCI Cell Cycle Sensor is designed for live-cell imaging of cell cycle progression and can be used to assess the effect of compounds on the transition of cells through the cell cycle. The workflow is as follows: reagent is added directly to the cells and incubated overnight to allow for the expression of fluorescent proteins. The next day, cell cycle progression in populations of cells can be visualized via traditional fluorescence microscopy.

Materials

A. Kit:	Premo™ FUCCI Cell Cycle Sensor BacMam (Molecular Probes; P36237)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes
D. Cell Line:	HaCat Keratinocytes (AddexBio; T0020001)
E. Media/Buffers:	Basal Medium (Fibrolife; LM-0001), 500µg/mL Human Serum Albumins (Fibrolife; LS-1001), 0.6µM Linoleic Acid (Fibrolife; LS-1001), 0.6µg/mL (Fibrolife; LS-1001), 5ng/mL Fibroblast Growth Factor (Fibrolife; LS-1002), 5mg/mL Epidermal Growth Factor (Fibrolife; LS-1003), 30pg/mL Transforming Growth Factor β-1 (Fibrolife; LS-2003), 7.5mM L-Glutamine (Fibrolife; LS-1006), 1µg/mL Hydrocortisone Hemisuccinate (Fibrolife; LS-1007),

	50µg/mL Ascorbic Acid (Fibrolife; LS-1005), 5µg/mL Insulin (Fibrolife; LS-1004)
F. Culture Plate:	Falcon flat bottom 24-well tissue culture treated plates
G. Reagents:	Phosphate Buffered Saline (PBS)
H. Other:	Sterile disposable pipette tips, 15mL falcon tubes

Methods

HaCat Keratinocytes were seeded into 24-well tissue culture plates and allowed to grow to confluency in complete serum-free media. When confluency was reached, the cells were treated with 0.001% **AC CytoSulf PF** for 24 hours 37°C, 5% CO2, and 95% RH. Cells incubated in complete serum-free media were used as the control.

A cell count was obtained from an untreated seeded well. This cell count was used to calculate the volume of Premo™ geminin-GFP and Premo™ Cdt-1-RFP, provided in the FUCCI Cell Cycle Sensor BacMam kit, required to transfect the keratinocytes. These 2 reagents were combined together and added directly to the **AC CytoSulf PF**-treated and complete serum-free media- treated wells. The cells were incubated for24 hours 37°C, 5% CO2, and 95% RH to allow the transfection to occur.

After 24 hour incubation, the culture media containing the Premo™ geminin-GFP and Premo™ Cdt-1-RFP reagents was removed, the wells were washed with complete serum-free media and fresh complete serum-free media was added. The microplate reader was used to assess the G2-M phase of the cell cycle with 488/555 nm excitation and emission and 510/584 nm excitation and emission for the G1 phase of the cell cycle. PBS was used to assess background fluorescence of the tissue culture plate. The background was subtracted from the fluorescence reading observed from the experimental wells.

Results

The data obtained from this study met criteria for a valid assay and the positive and negative controls performed as anticipated.

Cytostasis in G2-M and G1 Cell Cycle was determined based on fluorescence of the transfected geminin-GFP and Cdt-1-RFP gene.

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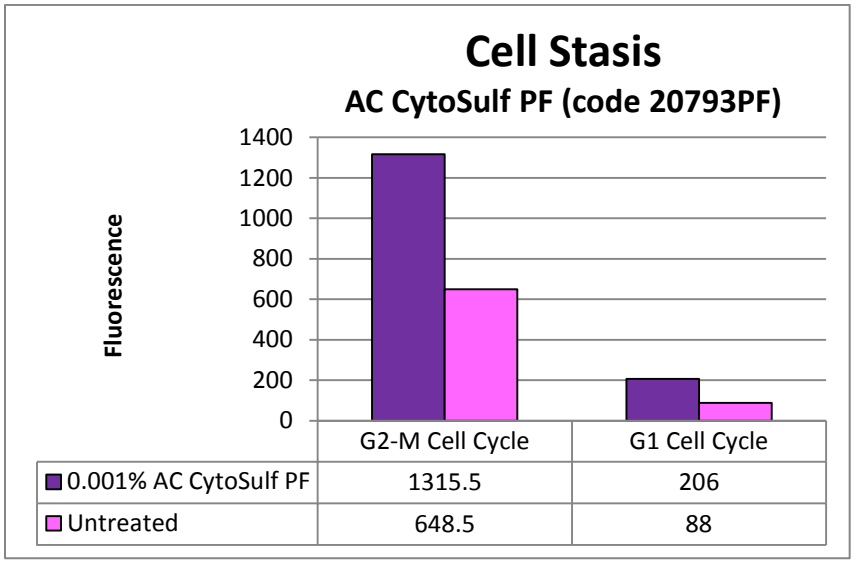

Figure 1: Cytostasis in G2-M and G1 Cell Cycle

Discussion

As shown in figure 1, **AC CytoSulf PF (code 20793PF)** was able to induce cytostasis of HaCaT keratinocytes. By suspending the cell cycle, **AC CytoSulf PF** was able to decrease telomere shortening and prevent mutations to the cellular genome.

AC CytoSulf PF was able to primarily arrest cells in the G2/M phase (1315.5 MFU) followed by the G1 phase (206 MFU), as shown by increased fluorescence (MFU) from the transduced fluorophore-containing gene constructs (GFP and RFP), as seen in Figure 1. These results are compared to the untreated control which showed little to no effect on cell cycle progression.

The results of this assay indicate that **AC CytoSulf PF** has a positive effect on cell cycle progression, perturbing HaCaT keratinocytes primarily in the G2-M phase.

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Cellular Viability Assay Analysis

Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1824

Lot #: NC160218-F

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

Test Performed:
Cellular Viability Assay

Introduction

The cellular viability assay is useful for quantitatively measuring cell-mediated cytotoxicity, cell proliferation and mitochondrial metabolic activity. Increased metabolism in a cell indicates ample cellular respiration and adenosine triphosphate (ATP) production. ATP is the molecular energy of cells and is required in basic cell function and signal transduction. A decrease in ATP levels indicates cytotoxicity and decreased cell function while an increase in ATP levels indicates healthy cells.

The cellular viability assay was conducted to assess the ability of **AC CytoSulf PF** to increase cellular metabolic activity in cultured dermal fibroblasts.

Assay Principle

The assay utilizes a nonfluorescent dye, resazurin, which is converted to a fluorescent dye, resorufin, in response to chemical reduction of growth medium from cell growth and by respiring mitochondria. Healthy cells that are in a proliferative state will be able to easily convert resazurin into resorufin without harming the cells. This method is a more sensitive assay than other commonly used mitochondrial reductase dyes such as MTT. An increase in the signal generated by resazurin-conversion is indicative of a proliferative cellular state.

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Materials

A. Kit:	PrestoBlue™ Cell Viability Reagent (Invitrogen, A13261)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Pipettes
D. Cell Line:	Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers:	Basal Medium (Fibrolife; LM-0001), 500µg/mL Human Serum Albumins (Fibrolife; LS-1001), 0.6µM Linoleic Acid (Fibrolife; LS-1001), 0.6µg/mL (Fibrolife; LS-1001), 5ng/mL Fibroblast Growth Factor (Fibrolife; LS-1002), 5mg/mL Epidermal Growth Factor (Fibrolife; LS-1003), 30pg/mL Transforming Growth Factor β-1 (Fibrolife; LS-2003), 7.5mM L-Glutamine (Fibrolife; LS-1006), 1µg/mL Hydrocortisone Hemisuccinate (Fibrolife; LS-1007), 50µg/mL Ascorbic Acid (Fibrolife; LS-1005), 5µg/mL Insulin (Fibrolife; LS-1004)
F. Culture Plate:	Falcon flat bottom 96-well tissue culture treated plates
G. Reagents:	PrestoBlue™ reagent (10X)
H. Other:	Sterile disposable pipette tips

Methods

Human dermal fibroblasts were seeded into 96-well tissue culture plates and allowed to grow to confluency in complete serum-free media. A 10-fold serial dilution was performed resulting in **AC CytoSulf PF** concentrations of 0.1% and 0.01% in complete serum-free media and incubated with fibroblasts for 24 hours.

Ten microliters of viability reagent was added to 90µL of cell culture media in culture wells and a fluorometric measurement was taken at 560nm for excitation and 590nm for emission.

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Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated.

AC CytoSulf PF did not exhibit negative effects on cell metabolism.

Cellular metabolism results are shown as mean fluorescence units (MFU) and expressed as percentage change, calculated by the below equation:

$$Percent\ (\%)Change = \frac{MFU_{Control} - MFU_{Sample}}{MFU_{Control}} \times 100$$

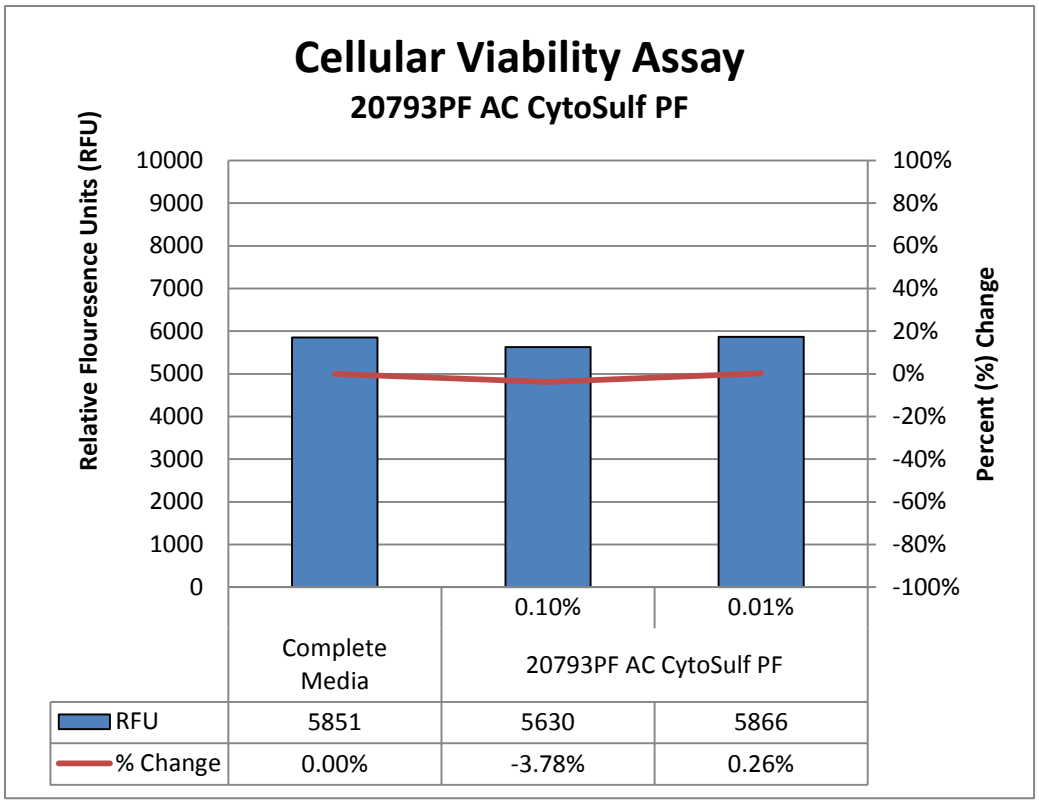

Figure 1: Cellular Metabolism of **AC CytoSulf PF-**treated fibroblasts

Discussion

In this study, **AC CytoSulf PF** (code 20793PF) was tested to evaluate its effects on the viability of normal human dermal fibroblasts (NDHF). At concentrations of 0.1% and 0.01% **AC CytoSulf** PF nor the preservatives contained therein exhibited any inhibition of cell viability. It can therefore be concluded that at normal use concentrations **AC CytoSulf PF** enhances cellular viability.

High Resolution Ultrasound Skin Imaging Assay

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Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1810

Lot #: NC160317-J

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

Test Performed:
High Resolution Ultrasound Skin-Imaging Assay

Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the effect on skin density of **AC CytoSulf PF**. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly improving skin density compared to the control.

Materials

A. Equipment: DermaLab Skin Combo (Ultrasound Probe)

Methods

Ultrasound skin imaging is based on measuring the acoustic response after an acoustic pulse is sent into the skin. The energy of the acoustic pulse is low and will not affect the skin in any way. When the acoustic pulse is emitted and hits different areas of the skin, part of the pulse will be reflected and part will be transmitted further into the skin. The reflected signal travels back and is picked up by the ultrasound transducer. After processing the signal, a cross-sectional image appears on the screen. This image represents an intensity, or amplitude, analysis of the signals.

The intensity of the signals that are received refer to a color scale. Dark colors represent areas of the skin with low reflection. This means that there are no changes or very small changes in density between the structures in the skin. Bright colors represent areas with strong reflections, signifying substantial changes in density between structures.

10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. The DermaLab ultrasound probe was used to determine the skin density of the subject's volar forearms. Baseline elasticity readings were taken on day one of the study.

Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for 4 weeks. The test material consisted of 2.0% **AC CytoSulf PF** in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

Results

AC CytoSulf PF showed improvements in skin density at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

Ultrasound		T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Panelist 1	Experimental	55	60	71	73	75
	Base Lotion	53	66	63	65	67
	Untreated	52	55	53	58	60
Panelist 2	Experimental	55	62	69	72	78
	Base Lotion	39	41	42	45	47
	Untreated	35	36	38	41	45
Panelist 3	Experimental	47	52	55	56	59
	Base Lotion	52	55	56	58	59
	Untreated	42	45	48	51	55
Panelist 4	Experimental	51	57	60	65	68
	Base Lotion	52	59	68	69	72
	Untreated	48	51	55	56	60
Panelist 5	Experimental	51	55	58	61	65
	Base Lotion	45	57	59	63	65
	Untreated	40	42	45	47	44
Panelist 6	Experimental	60	62	63	65	67
	Base Lotion	56	68	69	71	73
	Untreated	45	48	49	51	52
Panelist 7	Experimental	65	68	70	75	77
	Base Lotion	60	59	57	58	63
	Untreated	71	64	73	57	67
Panelist 8	Experimental	30	38	40	40	36
	Base Lotion	35	36	42	45	45
	Untreated	35	33	41	45	34
Panelist 9	Experimental	31	33	65	65	67
	Base Lotion	43	45	63	65	67
	Untreated	35	36	32	38	41
Panelist 10	Experimental	55	58	61	62	65
	Base Lotion	50	52	55	53	54
	Untreated	50	56	60	65	62
Number of Panelists		10	10	10	10	10

Chart 1. Panelist Moisturization Measurements

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Averages	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Experimental (2.0% AC CytoSulf PF in Base Lotion)	50	54.5	61.2	63.4	65.7
Base Lotion	48.5	53.8	57.4	59.2	61.2
Untreated	45.3	46.6	49.4	50.9	52

Chart 2. Average Increase in Skin Density per Individual Test Site

Percent (%) Change	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Base Lotion vs. Untreated	7.06%	15.45%	16.19%	16.31%	17.69%
Experimental (2.0% AC CytoSulf PF in Base Lotion) vs. Untreated	10.38%	16.95%	23.89%	24.56%	26.35%
Experimental (2.0% AC CytoSulf PF in Base Lotion) vs. Base Lotion	3.09%	1.30%	6.62%	7.09%	7.35%

Chart 3. Comparison of Skin Density Changes between Two Test Sites

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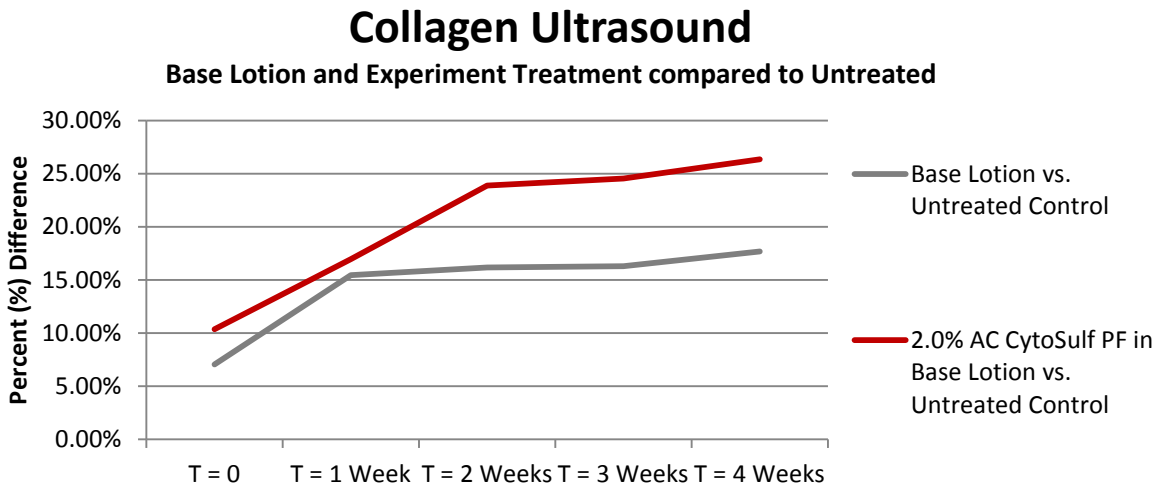

Figure 1. Ultrasound Results Comparing Test Sites to Untreated Control

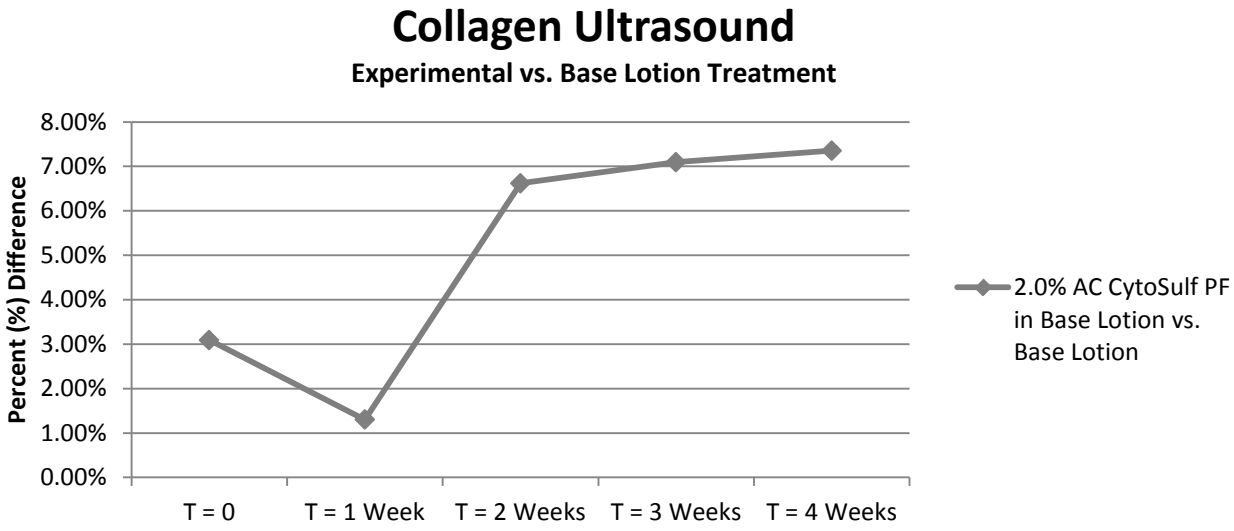

Figure 2. Ultrasound Results Comparing the Difference between the Test Site and the Control Site.

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Discussion

As evidenced in a 4 week efficacy study of **AC CytoSulf PF** on skin, skin density was improved by 16.95% after one week and by 26.35% after 4 weeks when compared to the untreated control. When compared to the base cream **AC CytoSulf PF** improved skin density during each week of the trial, working 6.62% better than the base lotion after two weeks and 7.09% better than the base lotion after four weeks. Results indicate that **AC CytoSulf PF** is capable of improving skin density when compared to both the untreated control as well as the base lotion.

AC CytoSulf PF has a strong positive effect on skin's density when used at recommended use levels.

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IL-6 ELISA Analysis

Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1822

Lot #: NC160218-F

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

Test Performed:
Interleukin (IL)-6 Enzyme-Linked Immunosorbent Assay (ELISA)

Introduction

Interleukin-6 is a proinflammatory cytokine known to play an active role in inflammation, immunology, bone metabolism, reproduction, arthritis, neoplasia, and aging. IL-6 signals through the nuclear factor-kappa B (NF-κB) pathway that results in the transcription of inflammatory mediators, including matrix metalloproteinase-1 (MMP-1). MMP's are responsible for breaking down the extracellular matrix and collagen in the skin leading to wrinkles, fine lines, and loss of skin elasticity. Reducing the level of IL-6 and other inflammatory mediators is believed to slow down degradation of the skin matrix and, possibly, stimulate its replenishment.

Interleukin-6 ELISA was conducted to assess the changes in IL-6 levels in **AC CytoSulf PF**-treated *in vitro* cultured human dermal fibroblasts.

Assay Principle

This ELISA utilizes a colorimetric reaction employing antibodies with antigen specificity to human IL-6. Monoclonal antibodies specific for IL-6 epitopes are coated on a microtiter plate. In positive samples, IL-6 will bind to these antibodies and are tagged a second time with another IL-6-specific antibody labeled with horseradish peroxidase (HRP). The addition of the chromagen solution, containing 3,3',5,5'-tetramethylbenzidine, provides the colorimetric reaction with HRP that is quantitated through optical density (OD) readings on a microplate spectrometer. The standard curve provides a reference from the OD readings for the amount of collagen in each sample.

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Materials

A. Kit:	IL-6 ELISA Kit (Biosource; KAC1261)
B. Incubation Conditions:	37°C at 5% CO_2 and 95% relative humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Microplate Reader; Pipettes
D. Cell Line:	Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
E. Media/Buffers:	Basal Medium (Fibrolife; LM-0001), 500µg/mL Human Serum Albumins (Fibrolife; LS-1001), 0.6µM Linoleic Acid (Fibrolife; LS-1001), 0.6µg/mL (Fibrolife; LS-1001), 5ng/mL Fibroblast Growth Factor (Fibrolife; LS-1002), 5mg/mL Epidermal Growth Factor (Fibrolife; LS-1003), 30pg/mL Transforming Growth Factor β-1 (Fibrolife; LS-2003), 7.5mM L-Glutamine (Fibrolife; LS-1006), 1µg/mL Hydrocortisone Hemisuccinate (Fibrolife; LS-1007), 50µg/mL Ascorbic Acid (Fibrolife; LS-1005), 5µg/mL Insulin (Fibrolife; LS-1004)
F. Culture Plate:	Falcon flat bottom 12-well tissue culture treated plates
G. Reagents:	Lipopolysaccharide (LPS) (1µg/mL); Dexamethasone (10µM)
H. Other:	Sterile disposable pipette tips; wash bottles

Methods

Human dermal fibroblasts were seeded into 12-well tissue culture plates and allowed to grow to confluency in complete serum-free media. 1%, 0.1%, 0.01% concentrations of **AC CytoSulf PF** were added to complete serum-free media containing 1µg/mL LPS and incubated with fibroblasts for 72 hours. Complete serum-free media containing 1µg/mL LPS was used to create an inflammatory environment and dexamethasone (DEX) in the presence of LPS was used as a positive control to quell inflammation.

Standards were prepared in concentrations ranging from 2476pg/mL to 0pg/mL. 50µL of Solution B was added to wells for standards and assay controls and 50µL of Solution A was added to experiment wells. 100µL of standards, controls, and samples were added to appropriate wells. After a one hour incubation at room temperature and washing, 50µL Solution A and 100µL anti-IL-6 conjugate was added to all wells. Following a one hour incubation and washing, 100 µL chromagen solution was added for the colorimetric reaction. One-hundred µL stop solution was added to stop the reaction after 15 minutes. The optical density was read at 450nm on the Synergy HT Microplate Reader.

A standard curve was created by reducing the data and generating a linear curve fit. The IL-6 concentration of **AC CytoSulf PF** treated-fibroblasts was determined by extrapolation from the standard curve and expressed in pg/mL.

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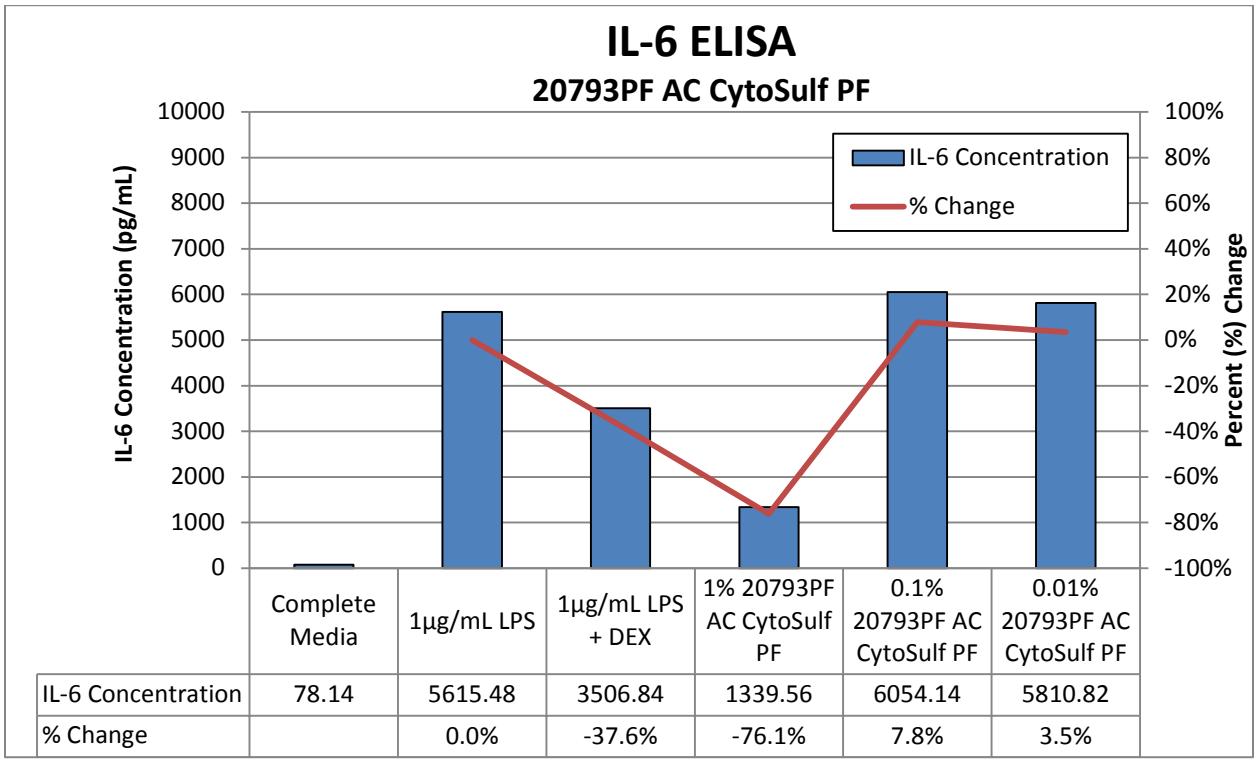
Results

The data obtained from this study met criteria for a valid assay and the positive and negative controls performed as anticipated.

AC CytoSulf PF at a concentration of 1% was able to decrease IL-6 production.

IL-6 production percent decrease is calculated by the following formula:

$$Percent\ (\%)\ Change = \frac{IL\ 6\ Concentration_{Sample} - IL\ 6\ Concentration_{1\mu M/mL\ LPS}}{IL\ 6\ Concentration_{1\mu M/mL\ LPS}} \times 100$$



	Complete Media	1µg/mL LPS	1µg/mL LPS + DEX	1% 20793PF AC CytoSulf PF	0.1% 20793PF AC CytoSulf PF	0.01% 20793PF AC CytoSulf PF
IL-6 Concentration	78.14	5615.48	3506.84	1339.56	6054.14	5810.82
% Change		0.0%	-37.6%	-76.1%	7.8%	3.5%

Figure 1: AC CytoSulf PF-treated fibroblasts IL-6 concentrations and percent change

Discussion

As shown in figure 1, **AC CytoSulf PF** (code 20793PF) exhibited anti-inflammatory effects on LPS-treated fibroblasts. This decrease in IL-6 production indicates a reduced inflammatory environment which could decrease the signs of aging and reduce the formation of fine lines and wrinkles. It can therefore be concluded that at normal use concentrations **AC CytoSulf PF** enhances soothing and anti-aging properties.

Active Concepts

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Moisturization/ Hydration Assay

Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1810

Lot #: NC160317-J

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

Test Performed:
Moisturization/ Hydration Assay

Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the moisturization benefits of **AC CytoSulf PF**. 10 M/F subjects between the ages of 23-45 participated in the study. Results indicate that this material is capable of significantly increasing moisturization compared to the control.

The Moisturization Assay was conducted to assess the moisturizing ability of **AC CytoSulf PF**.

Materials

A. Equipment: DermaLab Skin Combo (Hydration/ Moisture Pin Probe)

Methods

The moisture module provides information about the skin's hydration by measuring the conducting properties of the upper skin layers when subjected to an alternating voltage. The method is referred to as a conductance measurement and the output is presented in the unit of uSiemens (uS). A moisture pin probe is the tool used to gather hydration values.

10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. A Dermalab Corneometer was used to measure the moisture levels on the subject's volar forearms. The Corneometer is an instrument that measures the amount of water within the skin. The presence of moisture in the skin improves conductance therefore results in higher readings than dry skin. Therefore the higher the levels of moisture, the higher the readings from the Corneometer will be. Baseline moisturization readings were taken on day one of the study.

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Following initial measurements, all subjects were asked to apply 2 mg of each test material on their volar forearms. Measurements were taken immediately after application of test materials and then weekly for 4 weeks. The test material consisted of 2.0% **AC CytoSulf PF** in a base lotion.

For added perspective, measurements of an untreated test site and a site treated with a base lotion (Cetaphil Moisturizing for All Skin Types) were recorded.

Results

AC CytoSulf PF showed very high moisturizing capabilities at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

Moisturization		T = 0	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks	T = -24 Hours	T = -1 Week	T = -2 Weeks
Panelist 1	Experimenta	45	100	165	173	188	210	180	93	42
	Base Lotion	32	111	102	118	120	136	114	81	54
	Untreated	83	95	105	122	133	150	100	72	65
Panelist 2	Experimenta	130	130	168	245	256	272	175	152	128
	Base Lotion	128	175	201	235	272	283	120	109	105
	Untreated	106	125	156	180	195	200	125	93	89
Panelist 3	Experimenta	133	165	200	225	245	256	196	135	129
	Base Lotion	102	136	172	188	196	205	145	110	109
	Untreated	95	110	136	156	166	169	120	95	89
Panelist 4	Experimenta	62	136	182	196	210	250	125	88	49
	Base Lotion	79	106	175	172	185	194	85	75	69
	Untreated	61	96	115	145	155	183	82	76	45
Panelist 5	Experimenta	77	113	145	182	196	201	188	45	36
	Base Lotion	54	98	125	145	156	176	90	63	45
	Untreated	74	88	101	110	125	130	86	60	30
Panelist 6	Experimenta	91	115	265	286	296	310	173	93	63
	Base Lotion	79	95	195	215	206	243	125	92	83
	Untreated	39	77	188	190	196	210	86	66	45
Panelist 7	Experimenta	75	114	124	182	245	263	163	110	66
	Base Lotion	92	115	164	190	195	210	96	84	105
	Untreated	93	98	145	155	158	160	136	65	113
Panelist 8	Experimenta	102	125	225	235	286	291	163	98	83
	Base Lotion	110	125	215	230	256	271	115	101	55
	Untreated	85	100	193	200	210	215	105	65	65
Panelist 9	Experimenta	53	169	210	245	285	301	201	85	55
	Base Lotion	51	186	210	263	288	295	163	140	63
	Untreated	63	120	133	139	125	127	115	72	53
Panelist 10	Experimenta	161	253	276	301	315	322	195	93	76
	Base Lotion	127	183	276	287	295	301	193	88	45
	Untreated	126	237	255	263	269	271	180	63	55
Number of Panelists		10	10	10	10	10	10	10	10	10

Chart 1. Panelist Moisturization Measurements

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Averages	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks	T = -24 Hours	T = -1 Week
2.0% AC CytoSulf PF in Base Lotion	142	196	227	252.2	267.6	175.9	99.2
Base Lotion	133	183.5	204.3	216.9	231.4	124.6	94.3
Untreated	114.6	152.7	166	173.2	181.5	135.5	72.7

Chart 2. Average Moisture Increase and Regression Scores of Individual Test Sites

Percent (%) Change	T = 24 Hours	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks	T = -24 Hours	T = -1 Week
Base Lotion vs. Untreated	16.05	20.17	23.07	25.23	27.49	9.779	29.71
2.0% AC CytoSulf PF + Base Lotion vs. Untreated	23.91	28.35	36.74	45.61	47.43	54.97	36.45
2.0% AC CytoSulf PF in Base Lotion vs. Base Lotion	6.766	6.811	11.11	16.27	15.64	41.17	5.196

Chart 3. Comparative Moisture Increase and Regression Scores Between Individual Test Sites

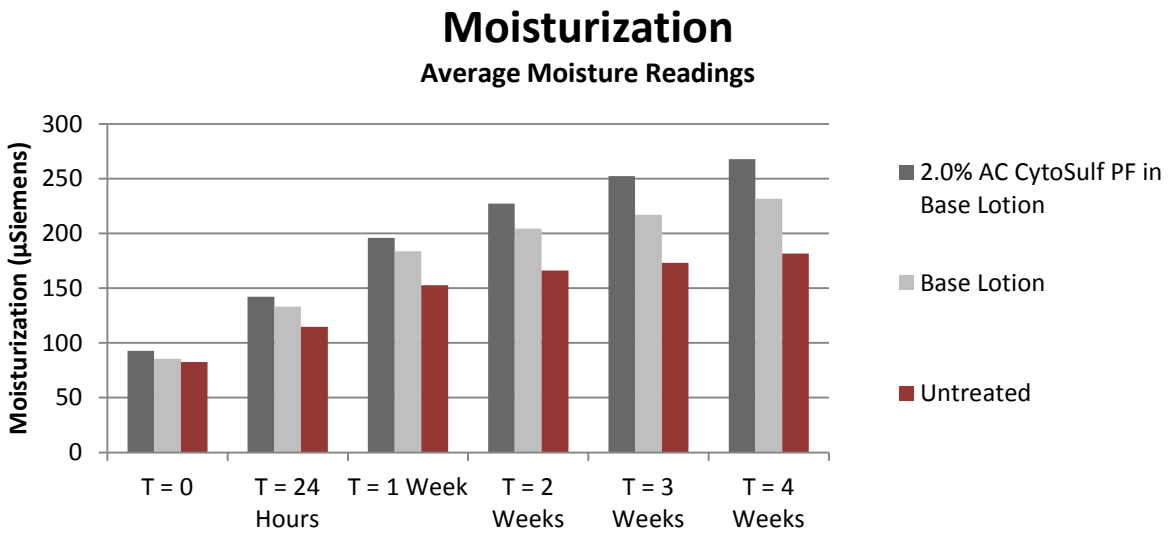

Figure 1. Average increase in moisturization per test site

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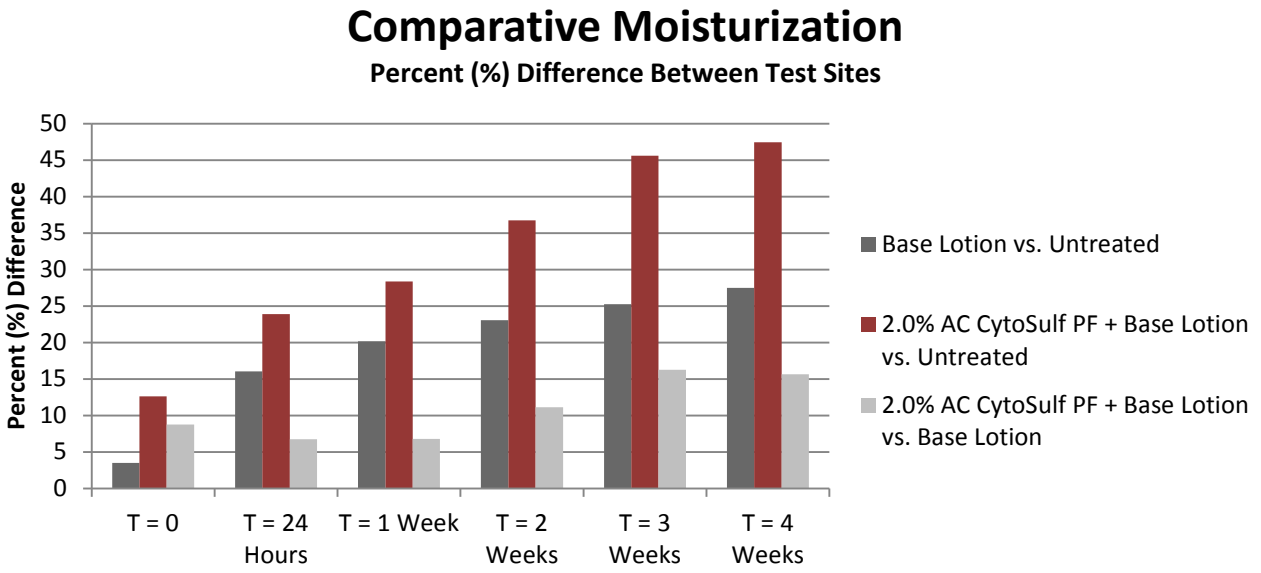

Figure 2. Percent difference in moisturization between two test sites over four weeks

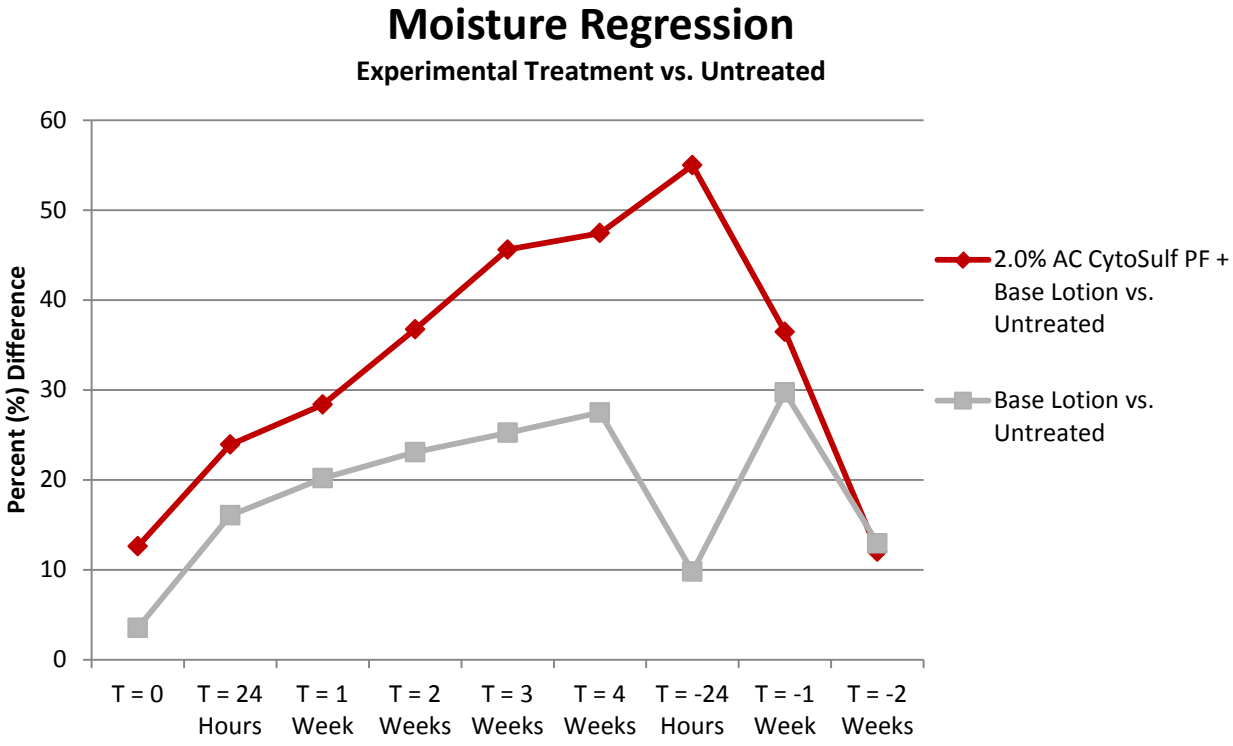

Figure 3. Regression in skin moisturization after application of experimental and base lotion material ceased

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Discussion

As evidenced in a 4 week efficacy study of **AC CytoSulf PF** on skin, moisture levels were improved by 24% after 24 hours and by 47% after 4 weeks when compared to the untreated control. Comparisons of the base lotion to the Experimental Lotion containing 2.0% **AC CytoSulf PF** demonstrate the experimental material moisturized the skin 6.8% better after 24 hours. After four weeks the base lotion containing 2.0% **AC CytoSulf PF** moisturized skin 15% better than the base lotion alone. Results indicate that **AC CytoSulf PF** is capable of increasing moisturization when compared to both the untreated control as well as the base lotion.

Furthermore, when examining the moisture levels on the skin after application of test materials stopped, it was determined that **AC CytoSulf PF** is capable of sustaining increased skin moisturization when compared to the skin site that remained untreated through the duration of the study. After 24 hours, the site testing 2.0% **AC CytoSulf PF + Base Lotion** was approximately 55% more moisturized than the site which did not receive treatment. After one week, the experimental test site was still yielding moisturization results that were 36% higher than the untreated site. Additionally, in comparison to the site tested with the base lotion alone, the site treated with 2.0% **AC CytoSulf PF + Base Lotion** moisturized the skin 41% better after 24 hours after and was still 5.2% more effective in moisturizing the skin when reading were taken one week after the applications of both test materials ceased.

AC CytoSulf PF was designed to provide moisturization benefits, however with the present study we can confirm that this ingredient is not only capable of providing protective benefits but also ideal for moisturizing and skin hydrating personal care applications.

Oxygen Radical Absorbance Capacity (ORAC) Assay

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Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1823

Lot #: NC160218-F

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

Test Performed:
Oxygen Radical Absorbance Capacity (ORAC)

Introduction

Reactive oxygen species (ROS) are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS are detrimental to cellular structures and functional molecules (i.e DNA, proteins, lipids) as they act as strong oxidizing agents or free radicals. The oxygen radical absorbance capacity (ORAC) assay is a standard method used to assess antioxidant capacity of physiological fluids, foods, beverages, and natural products. The assay quantitatively measures a sample's ability to quench free radicals that have the potential to react with and damage cellular components.

Oxygen Radical Absorbance Capacity (ORAC) assay was conducted to assess the antioxidant capacity of **AC CytoSulf PF**.

Assay Principle

This assay is based upon the effect of peroxyl radicals generated from the thermal decomposition of 2, 2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) on the signal intensity from the fluorescent probe, fluorescein, in the presence of an oxygen radical absorbing substance. The degree of change is indicative of the amount of radical damage and the presence of antioxidants results in an inhibition in the free radical damage to the fluorescein. The antioxidant protection of the sample can be calculated by comparing it to a set of known standards. Trolox®, a water soluble vitamin E analog, with known antioxidant capabilities is used in this ORAC assay as the standard for measuring the antioxidant capacity of unknown substances. ORAC values, expressed in µM of Trolox® equivalents (TE), are calculated using the area under the curves (AUC) of the test product, Trolox®, and the control materials. Trolox equivalency is used as the benchmark for antioxidant capacity of mixtures since it is difficult to measure individual components.

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Materials

A. Equipment:	Synergy H1 Microplate reader (BioTek Instuments, Winooski, VT); Gen5 software (BioTek Instuments, Winooski, VT); Pipettes
B. Buffers:	75mM Potassium Phosphate (pH 7.4); Deionized H_2O
C. Reagents:	2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (153mM); 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®); Fluorescein Sodium Salt (4nM)
D. Preparation:	Pre-heat (37°C) Synergy H1 Microplate reader; Prepare Trolox® standards, sample dilutions, fluorescein solution, and AAPH.
E. Microtitre Plates:	Corning 96 Well Black Side/Clear Bottom Microplates

Methods

Solutions of **AC CytoSulf PF** and Trolox® (positive control) were prepared in 75mM potassium phosphate buffer. Materials were prepared at three different concentrations/dilutions. Trolox® was used as a reference for antioxidant capacity and prepared at a concentrations ranging from 12.5µM to 200µM in 75mM potassium phosphate buffer.

For the ORAC assay, 25µL of test material and Trolox® were combined with 150µL of fluorescein in 75mM potassium phosphate buffer and incubated in the Synergy HT Microplate reader at 37˚C for 30 minutes. At the end of the incubation period, 25µL of AAPH were pipetted into each well. Fluorescent measurements were then taken every 2 minutes for approximately 2 hours at an excitation wavelength of 485nm and an emissions wavelength of 520nm.

The AUC and Net AUC values of the standards and samples were determined using Gen5 2.0 Data Reduction Software using the below equations:

$$AUC = 0.5 + \frac{R2}{R1} + \frac{R3}{R1} + \frac{R4}{R1} + \cdots + \frac{Rn}{R1} \rightarrow Where\ R\ is\ fluorescence\ reading$$

$$Net\ AUC = AUC_{sample} - AUC_{blank}$$

The standard curve was obtained by plotting the Net AUC of different Trolox® concentrations against their concentration. ORAC values of samples were then calculated automatically using the Gen5 software to interpolate the sample's Net AUC values against the Trolox® standard curve. ORAC measurements for the test material were expressed in micro moles Trolox® equivalents (µMTE), where 1 ORAC unit is equal to 1 µMTE.

ORAC values are also calculated in Units/milliliter (U/mL). The equation used for the calculation is shown below:

$$ORAC\ (U/mL) = (50 \times Dilution\ Factor) \times \left(\frac{AUC_{Sample} - AUC_{Blank}}{AUC_{Trolox} - AUC_{Blank}}\right)$$

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Results

AC CytoSulf PF began exhibiting antioxidant activity at a 0.05% concentration.

The ORAC value expressed in U/mL for 0.1% **AC CytoSulf PF** is 63558.8.

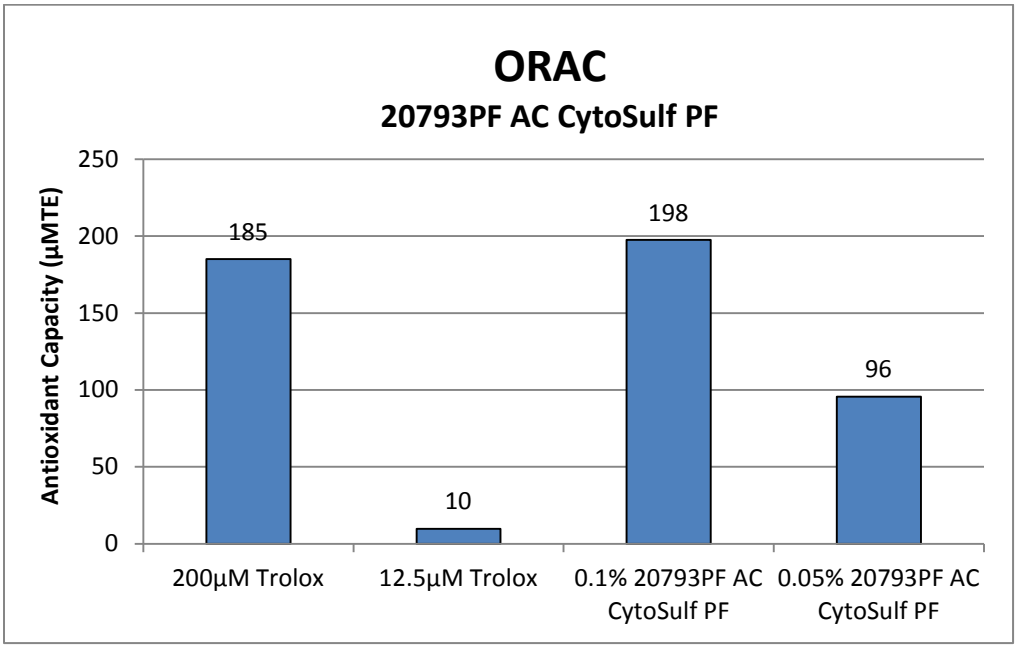

Figure 1: Antioxidant capacities

Discussion

As shown in figure 1, **AC CytoSulf PF** (code 20793PF) exhibited antioxidant activity comparable to 200µM Trolox®. The antioxidant capacity of **AC CytoSulf PF** increased as the concentration increased. As a result we can assure that its ability to minimize oxidative stress is dose dependent. It can therefore be concluded that **AC CytoSulf PF** is capable of providing antioxidant properties.

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Transepidermal Water Loss Assay

Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1810

Lot #: NC160317-J

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

Test Performed:
Transepidermal Water Loss (TEWL) Assay

Introduction

An *in-vivo* study was conducted over a period of four weeks to evaluate the ability of **AC CytoSulf PF** to enhance barrier function through reduction in Transepidermal Water Loss (TEWL). Results indicate that this material is capable of efficiently reducing TEWL which allows moisture retention.

The Transepidermal Water Loss Assay was conducted to assess the moisture retention capabilities of **AC CytoSulf PF.**

Materials

A. Equipment: DermaLab Skin Combo (Transepidermal Water Loss Probe)

Methods

Transepidermal water loss is measured by the DermaLab Combo based on Nilsson's Vapor Pressure Gradient method. This method involves an open chamber with minimal impact on the skin, and therefore, a very low bias. Two temperature and humidity sensor sets are mounted in a chamber at different heights above the surface of the skin. The evaporation rate of the skin follow's Fick's Law of Diffusion:
Rate = P x [c1 – c2] / T
where P=permeability coefficient of membrane, (c1-c2) = concentration gradient, and T=thickness of membrane).

10 volunteers M/F between the ages of 23 and 45 and who were known to be free of any skin pathologies participated in this study. A DermaLab Combo was used to measure TEWL on the subject's volar forearms. The instrument consists of a probe that is based upon the vapor gradient with an open chamber. This open chamber design maintains the free natural evaporation from the skin without interfering with the environment over the measurement area.

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This ensures unbiased and accurate readings. Operation of the water loss module is fully menu drive, allowing for pre-setting and standard deviation or measurement time. Baseline TEWL readings were taken on day one of the study.

Following initial measurements, all subjects were asked to apply 2 milligrams of each test material on their volar forearms. Measurements were taken immediately after application of the test materials and then weekly for four weeks. The test material consisted of 2.0% **AC CytoSulf PF** in a base lotion.

Results

AC CytoSulf PF showed very effective moisture retention capabilities at a 2.0% concentration. Please note, each value is an average of three consecutive readings per test site.

TEWL		T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Panelist 1	Experimental	3.2	1.8	2.5	3	3.5
	Base Lotion	4.5	4.5	5	4.8	4.2
	Untreated	3.8	3.7	2.7	2.5	4.5
Panelist 2	Experimental	4.2	3.1	4.2	3.6	3.2
	Base Lotion	6.3	4.2	4.3	4.3	4
	Untreated	5.8	5.5	5.7	5.5	6.3
Panelist 3	Experimental	11.8	7.1	7.1	7.2	3.2
	Base Lotion	5.5	5.6	3.5	3.8	8
	Untreated	4.2	3.28	3.3	3.2	4.5
Panelist 4	Experimental	3	3.5	1.7	2.3	2.5
	Base Lotion	2.9	3	1.6	2	2.3
	Untreated	3.5	5.1	5.5	5.6	5.8
Panelist 5	Experimental	9.4	2	3.3	2.4	3
	Base Lotion	4.2	3.3	2.4	2.8	2.9
	Untreated	2.7	2.4	3	2.8	3.5
Panelist 6	Experimental	3.9	2.3	4.2	4.5	4.6
	Base Lotion	2.3	2.3	3.7	3.9	4.1
	Untreated	6.3	4.1	6.1	6.1	6.1
Panelist 7	Experimental	6.5	4.9	4.1	3.8	5.5
	Base Lotion	6.3	6.2	3.8	5.1	7.8
	Untreated	5.4	4.9	3.7	3.9	6.3
Panelist 8	Experimental	7.8	7.1	7.5	7.3	4.2
	Base Lotion	8.6	5	5.6	6.1	6.3
	Untreated	8.1	6	6.1	6.3	7.2
Panelist 9	Experimental	9.1	6.4	6.9	7.2	5.2
	Base Lotion	8.1	7.7	8.3	8.5	8.5
	Untreated	4.8	7.3	7.5	8.1	8.3
Panelist 10	Experimental	2.6	5.4	5.5	5.7	5.8
	Base Lotion	3.3	4.4	4.2	4.5	4.8
	Untreated	9.8	7.6	7.5	7.5	7.4
Number of Panelists		10	10	10	10	10

Chart 1. Panelist Moisturization Measurements

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Averages	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Experimental (2.0% AC CytoSulf PF + Base Lotion)	6.15	4.36	4.70	4.70	4.07
Base Lotion	5.20	4.62	4.24	4.58	5.29
Untreated	5.44	4.98	5.11	5.15	5.99

Chart 2. Average Transepidermal Water Loss of Individual Test Sites

Percent (%) Change	T = 0	T = 1 Week	T = 2 Weeks	T = 3 Weeks	T = 4 Weeks
Base Lotion vs. Untreated	-4.41%	-7.38%	-17.03%	-11.07%	-11.69%
Experimental (2.0% AC CytoSulf PF + Base Lotion) vs. Untreated	-13.05%	-12.59%	-8.02%	-8.74%	-32.05%
Experimental (2.0% AC CytoSulf PF + Base Lotion) vs. Base Lotion	-18.27%	-5.63%	-10.85%	-2.62%	-23.06%

Chart 3. Comparative Transepidermal Water Loss Results Between Individual Test Sites

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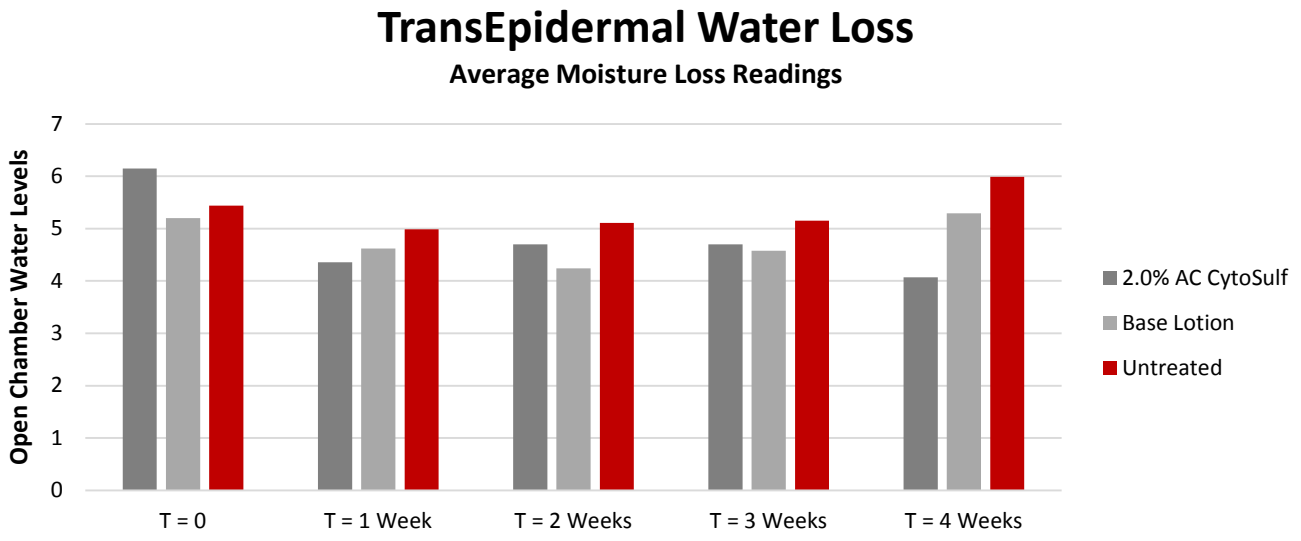

Figure 1. TEWL measurements taken at individual test sites

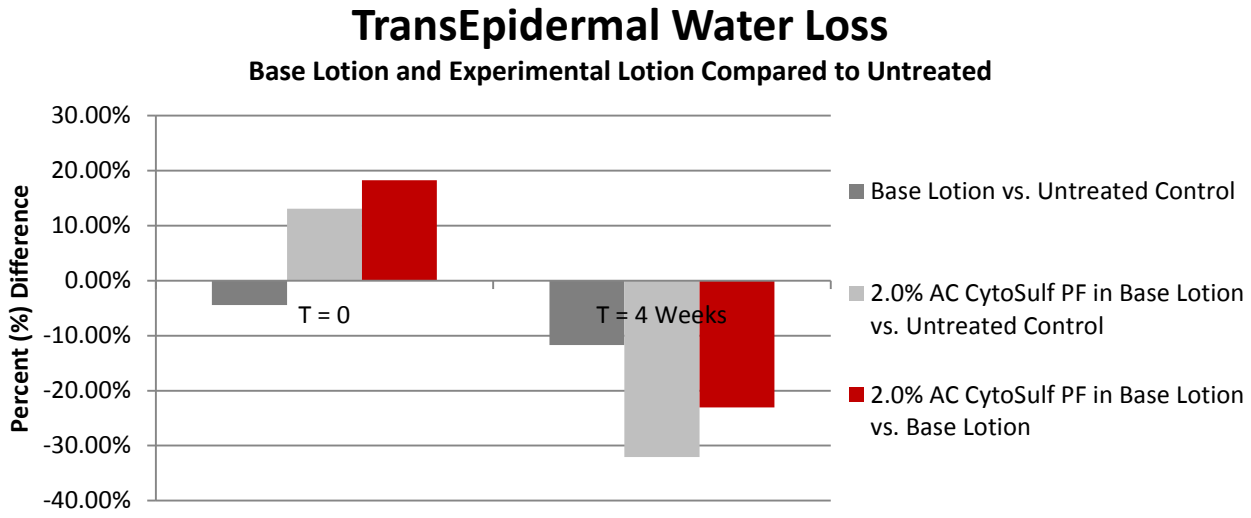

Figure 2. Comparison of percent reduction in water loss over time between two test sites

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Discussion

As evidenced in a four week efficacy study of **AC CytoSulf PF** on the skin, it can be used to effectively reduce transepidermal water loss with better results over time. When compared to the base cream **AC CytoSulf PF** was shown to decrease transepidermal water loss by 23.06% and by 32.05% when compared to the untreated control after four weeks. Results indicate that **AC CytoSulf PF** is capable of reducing TEWL, which allows for moisture retention.

AC CytoSulf PF was designed to provide moisture retention benefits, however with the present study we can confirm that this unique ingredient is not only capable of providing functional benefits but it is also capable of providing a decrease in transepidermal water loss therefore promoting moisture retention benefits when added to cosmetic applications.

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Tradename: AC CytoSulf PF

Code: 20793PF

CAS #: 91079-57-1

Test Request Form #: 1821

Lot #: NC160218-F

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

Test Performed:
Scratch Assay

Introduction

Wounded tissue begins a complex and structured series of events in order to repair the damaged region. Some of these events include upregulation of angiogenic factors causing increased vascularization, increased deposition of extracellular matrix, and increased cell proliferation. The wound healing process begins as cells polarize toward the wound, initiate protrusion, migrate, and close the wound area. These processes reflect the behavior of individual cells as well as the entire tissue complex.

The scratch assay was conducted to assess the wound healing properties of **AC CytoSulf PF-**treated *in vitro* cultured human dermal fibroblasts.

Assay Principle

The *in vitro* scratch assay is a well-known and widely used method to study cell migration and proliferation. This assay is based on the observation that when an artificial gap or scratch is made on a confluent cell monolayer, the cells will migrate towards the opening and close the scratch. The basic steps involve creating a scratch in a cell monolayer and capturing images throughout the healing or cell migration process. Through these images we can quantify the rate of cell migration.

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Materials

A. Incubation Conditions:	37°C at 5% CO_2 and 95% Relative Humidity (RH)
B. Equipment:	Forma Humidified Incubator, ESCO Biosafety Laminar Flow Hood, Inverted Microscope; Camera; Pipettes
C. Cell Line:	Normal Human Dermal Fibroblasts (NHDF) (Lonza; CC-2511)
D. Media/Buffers:	Basal Medium (Fibrolife; LM-0001), 500µg/mL Human Serum Albumins (Fibrolife; LS-1001), 0.6µM Linoleic Acid (Fibrolife; LS-1001), 0.6µg/mL (Fibrolife; LS-1001), 5ng/mL Fibroblast Growth Factor (Fibrolife; LS-1002), 5mg/mL Epidermal Growth Factor (Fibrolife; LS-1003), 30pg/mL Transforming Growth Factor β-1 (Fibrolife; LS-2003), 7.5mM L-Glutamine (Fibrolife; LS-1006), 1µg/mL Hydrocortisone Hemisuccinate (Fibrolife; LS-1007), 50µg/mL Ascorbic Acid (Fibrolife; LS-1005), 5µg/mL Insulin (Fibrolife; LS-1004), Phosphate Buffered Saline (PBS)
E. Reagents:	Epidermal Growth Factor-1 (100ng/mL); Paraformaldehyde (3.7%); Crystal Violet Stain
F. Culture Plate:	Falcon Flat Bottom 6-Well Tissue Culture Treated Plates
G. Other:	Sterile Disposable Pipette Tips; Wash Bottles; 15mL Conical Tubes

Methods

Human dermal fibroblasts were seeded into 6-well tissue culture plates and allowed to grow to confluency in complete serum-free media. A 0.1% concentration of **AC CytoSulf PF** was added to the culture media and incubated with fibroblasts for the extent of the experiment. Epidermal Growth Factor-1 was utilized as the positive control and complete serum-free media was used a negative control.

When cell growth reached confluency scratches were made across the well in a cross or 'X' pattern. The wells were washed with sterile PBS and fresh media containing **AC CytoSulf PF** and the controls were added. Initial images were captured immediately after the scratch took place and every 24-hours afterwards, up to 72-hours. Cells were fixed with 3.7% paraformaldehyde and stained with crystal violet for enhanced microscopy.

ImageJ software was used to analyze the images and calculate the area of the scratch and the closure rate.

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Results

The data obtained from this study met criteria for a valid assay and the positive and negative controls performed as anticipated.

AC CytoSulf PF at a 0.1% concentration was able to increase cell migration and wound healing compared to our negative control.

Percent scratch closure and migration rate are expressed by the following formula:

$$\frac{Scratch\ Area\ _{t=x}\ - Scratch\ Area\ _{t=0}}{Scratch\ Area\ _{t=0}} \times 100 = \ \%\ Scratch\ Closure$$

$$\frac{Change\ in\ Area\ of\ Scratch\ (nm^2)}{Migration\ Time\ _{t=x}} = Migration\ Rate$$

$$Where\ x = time\ (hours)\ post\ scratch$$

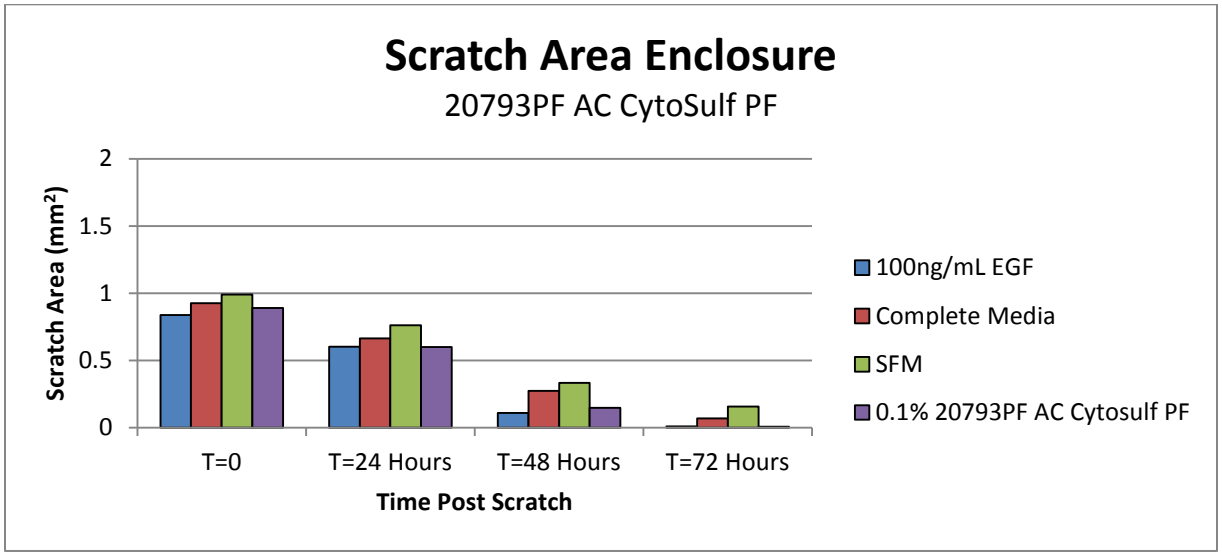

Figure 1: Area of scratch

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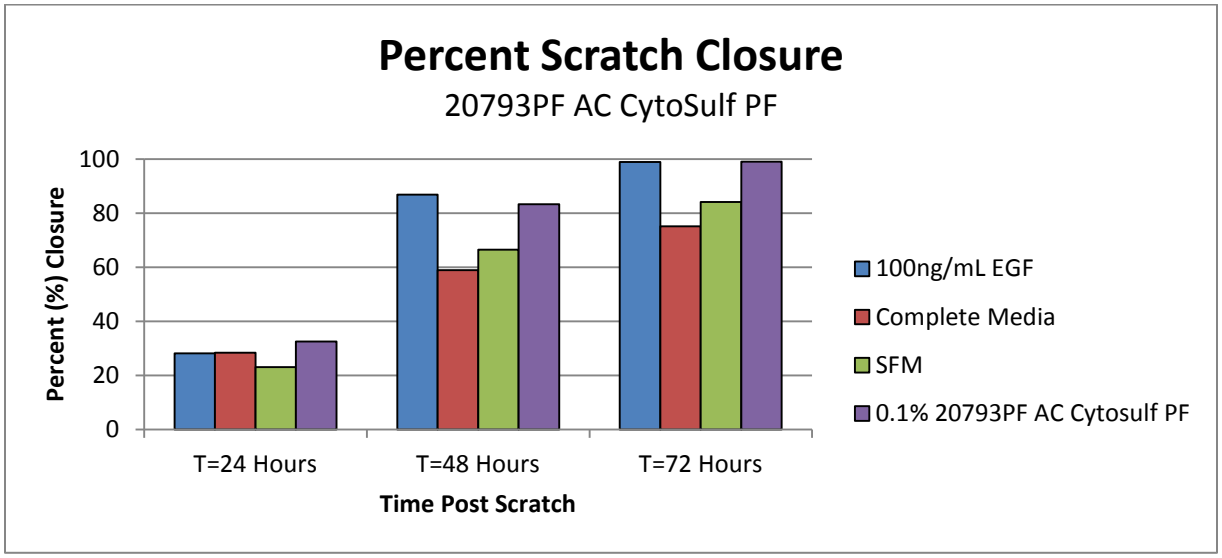

Figure 2: Percent scratch closure

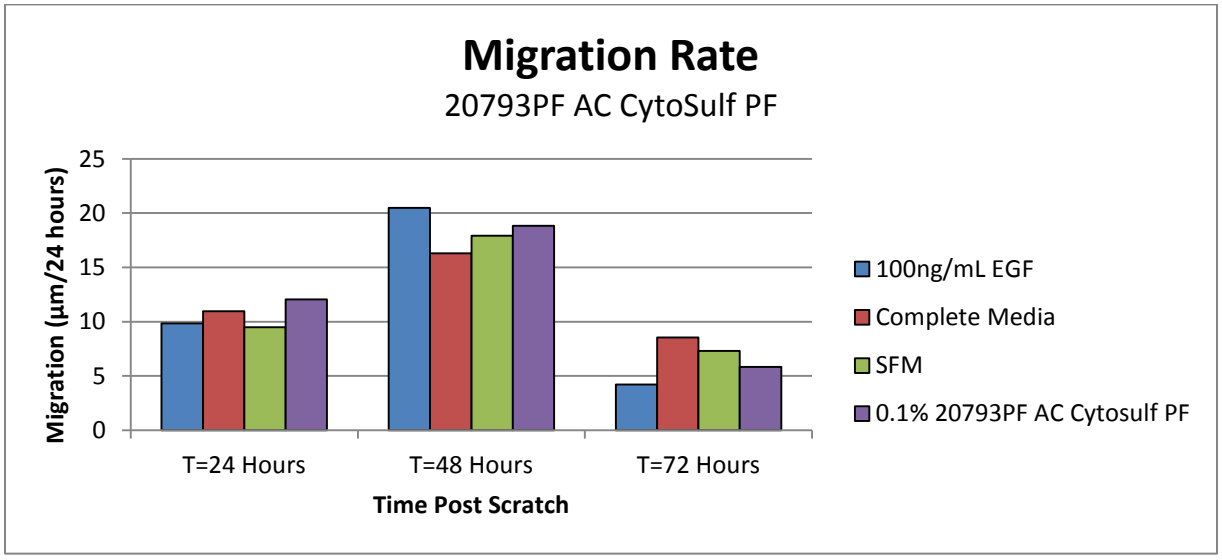

Figure 3: Cell migration rate

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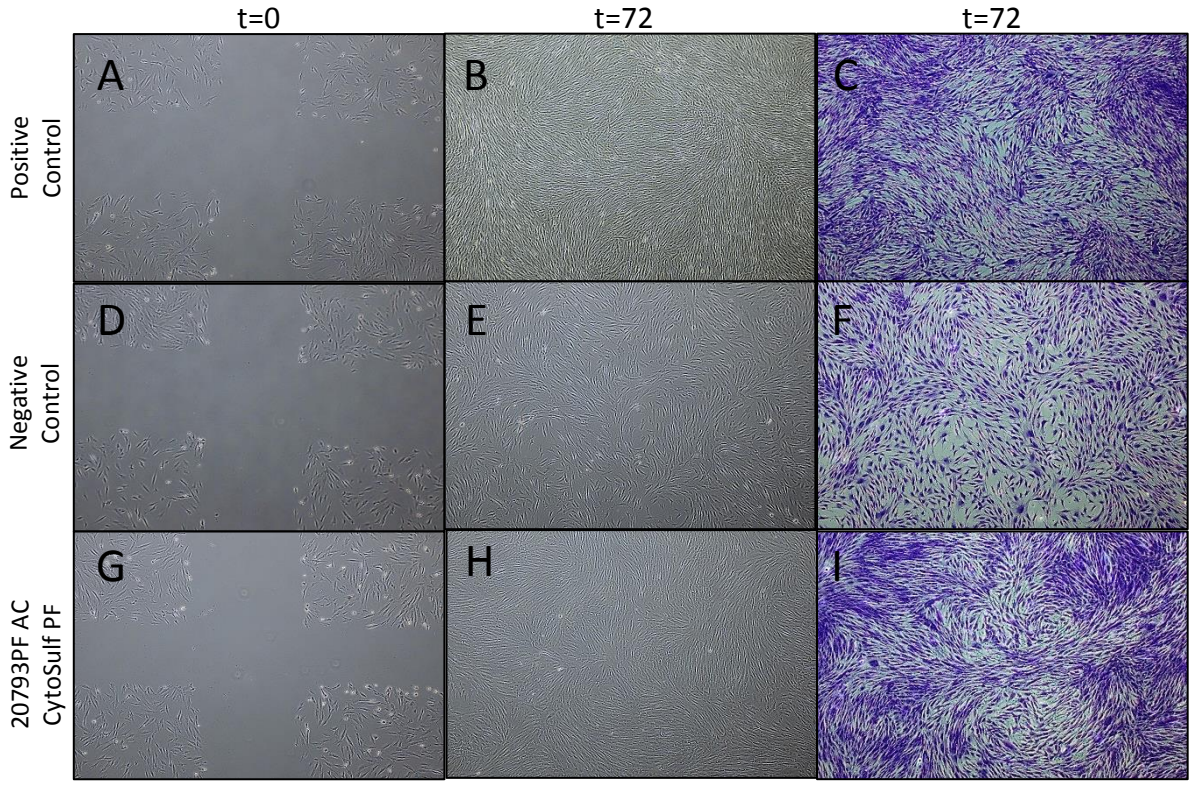

Figure 4: Images at t=0 hours (A, D, G) and t=72 hours (B, E, H) for **AC CytoSulf PF**, positive control (EGF-1), and negative control (SFM). At experiment completion (t=72 hours), cells were fixed in paraformaldehyde and stained with crystal violet (C, F, I).

Discussion

AC CytoSulf PF (code 20793PF) was able to increase cell migration and close the scratch at a rate comparable to the positive control. The mechanisms of the cells in the *in vitro* scratch assay mimic the mechanisms seen in *in vivo* wound healing therefore we can be assured that our results are translatable outside the laboratory. With the present study we can be confident that this product has healing abilities and cell proliferation properties.