Sirius Red/Fast Green Collagen Analysis

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Tradename: AC ExoEternol

Code: 60200

CAS #: 7732-18-5 & 68333-16-4 (or) 92128-79-5 & 90082-61-4 (or) 68132-21-8 & 123465-35-0 (or) 8002-43-5 & 68333-16-4 (or) 1686112-36-6 (or) 9015-54-7

Test Request Form #: 12955

Lot #: N250701O

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Daniel Shill*
Principal Investigator: *Hannah Stade*

Test Performed:
Sirius Red/Fast Green Collagen Assay

Introduction

Collagen is the main protein of connective tissues, such as skin, bone, tendon and ligament, and the most abundant protein in mammals. Specifically, it accounts for nearly 25% to 35% of the total human protein content. Collagen is a long, fibrous protein that forms bundles called fibers giving structure and support to cells and tissues. Collagen has great tensile strength and is responsible for skin's elasticity, therefore its degradation leads to wrinkles that accompany aging.

Accordingly, a Sirius Red/Fast Green Collagen Assay was conducted to assess the *in vitro* effect of **AC ExoEternol** to trigger collagen synthesis in dermal fibroblasts.

Assay Principle

Sirius Red is a unique dye that binds specifically to the helical structure of types I through V collagen, while Fast Green binds to non-collagenous proteins. These two dyes work in conjunction to provide a semi-quantitative method of determining amounts of collagen and non-collagenous proteins in a sample. After staining samples, the dyes are easily extracted and have optical density (OD) absorptions at 540 nm (Sirius Red) and 605 nm (Fast Green). Collagen concentrations are calculated through equations with OD values.

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Materials

A. Kit:	Sirius Red/Fast Green Collagen Kit (Chondrex; 9046)*
B. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Synergy HT Microplate reader; Pipettes; Light microscope
D. Cell Line:	Normal Human Dermal Fibroblasts (ATCC; PCS-201-012)*
E. Media/Buffers:	Fibroblast Basal Medium (PCS-201-030)*; Fibroblast Growth Kit (PCS-201-041)*; Phosphate Buffered Saline (PBS)
F. Reagents:	Sirius Red/Fast Green dye solution (Chondrex; 9046)*; Extraction solution (Chondrex; 9046)*; Ascorbic Acid-2-Glucose (AA2G) (100 µM or 34 µg/mL); Insulin Growth Factor-1 (IGF-1) (6.5 nM or 50 ng/mL); Glacial Acetic Acid; Ethanol
G. Culture Plate:	Flat Bottom 24-Well Tissue Culture Treated Plates
H. Software:	Excel Analysis ToolPak (Microsoft)
I. Other:	Sterile disposable pipette tips

*Or suitable alternatives, subject to change without notice based off vendor availability

Methods

Human dermal fibroblasts were seeded into 24-well tissue culture plates and allowed to grow to confluency in Complete Media. 0.01% and 0.05% concentrations of **AC ExoEternol** were diluted in serum-free Complete Media and incubated with fibroblasts for 24 hours. Ascorbic Acid-2-Glucose (AA2G) (100 µM or 34 µg/mL) and Insulin Growth Factor-1 (IGF-1) (6.5 nM or 50 ng/mL) were diluted in serum-free Complete Media and utilized as positive controls.

Media was removed from wells containing adherent fibroblasts and the cells were washed with PBS. 500 µL of a cooled 95% ethanol/5% glacial acetic acid solution was added to the wells and incubated for 10 minutes at room temperature, followed by a PBS wash. 200 µL of the Sirius Red/Fast Green dye solution was added to the fixed cell layer and incubated at room temperature for 30 minutes. The dye solution was removed from the cell layer and washed with PBS. 1 mL of extraction solution was added for color extraction and optical density (OD) was read at 540 nm and 605 nm on a Synergy HT Microplate Reader.

Three separate experiments were performed with conditions in duplicate and average values were recorded. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. The collagen and non-collagen protein concentrations of fibroblasts were determined by the following calculations and expressed in µg:

$$Collagen\ (\mu g) = \frac{OD_{540} - (OD_{605} \times 0.291)}{0.0378}$$

$$Non\ Collagen\ Protein\ (\mu g) = \frac{OD_{605}}{0.00204}$$

Percent change is expressed relative to untreated fibroblasts (CM) and calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{Collagen_{Sample} - Collagen_{Complete\ Media}}{Collagen_{Complete\ Media}} \times 100$$

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Results

The data obtained from this study met criteria for a valid assay and the positive controls performed as anticipated. Compared to untreated fibroblasts, AA2G (100 µM or 34 µg/mL) and IGF-1 (6.5 nM or 50 ng/mL) increased collagen concentrations. Fibroblasts treated with **AC ExoEternol** at 0.01% and 0.05% demonstrated increased collagen synthesis compared to untreated fibroblasts.

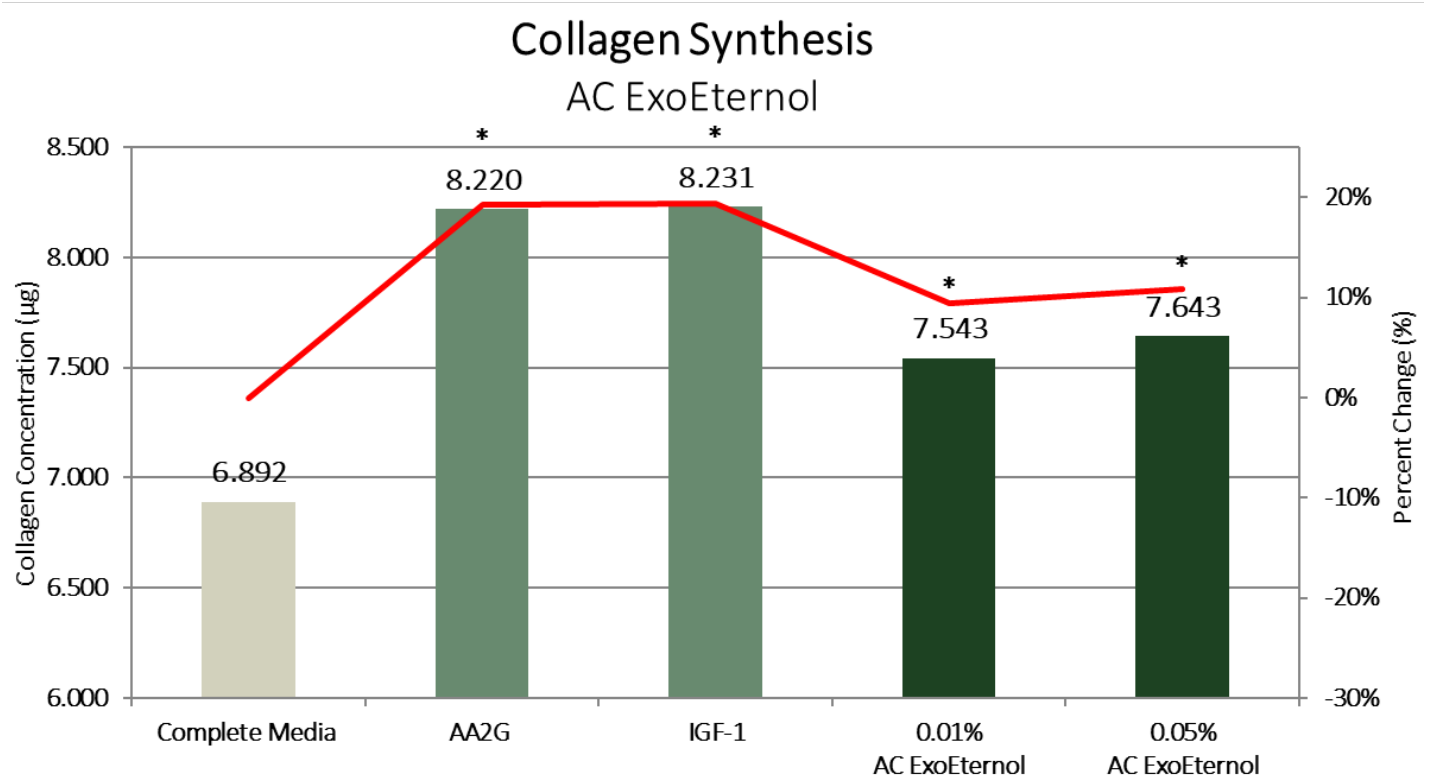

Figure 1. The effect of AA2G (100 µM or 34 µg/mL), IGF-1 (6.5 nM or 50 ng/mL), and **AC ExoEternol** on collagen concentrations in dermal fibroblasts. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts.

Table 1. Results from one-way ANOVA Statistical Analysis Compared to Complete Media. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts.

	0.01% **AC ExoEternol**	0.05% **AC ExoEternol**
P-value	0.012*	0.029*

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Discussion

As shown in Figure 1, fibroblasts incubated with AA2G and IGF-1, both known to stimulate collagen synthesis, exhibited increases in collagen synthesis of 19% and 19% compared to untreated fibroblasts, respectively. These data demonstrate collagen in fibroblasts is dynamic and can be manipulated with exogenous compounds.

Similarly, fibroblasts treated with AC ExoEternol at 0.01% and 0.05% demonstrated potent increases in collagen synthesis of 9% and 11%, respectively, compared to untreated fibroblasts. Overall, these data demonstrate AC ExoEternol activates collagen synthesis in dermal fibroblasts.

Collectively, increases in collagen production may lead to improvements in dermal-epidermal junction integrity as well as an improved scaffolding matrix. These data indicate AC ExoEternol stimulates collagen synthesis which may assist cosmetic applications designed to enhance collagen deposition and provide a younger and healthier dermal complexion.