Sirius Red/Fast Green Collagen Analysis

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Tradename: AC ExoRestore

Code: 60195

CAS #: 7732-18-5 & 93333-80-3 & 123465-35-0 (or) 8002-43-5

Test Request Form #: 10228

Lot #: N230919F

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Drumwright*
Principle Investigator: *Hannah Duckett*

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 and, 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 ExoRestore** to trigger collagen synthesis in dermal fibroblasts. The key active ingredient in **AC ExoRestore**, *Momordica charantia* (Bitter Melon) Fruit Extract, was tested to demonstrate the superior nature of bioauthentic exosomes as a delivery system.

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 (RH)
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. 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 (CM). 0.01% and 0.1% concentrations of **AC ExoRestore** were diluted in serum-free CM and incubated with fibroblasts for 24 hours. AA2G (100 µM or 34 µg/mL) and IGF-1 (6.5 nM or 50 ng/mL) were diluted in serum-free CM and utilized as positive controls. Additionally, solutions of 0.002% and 0.02% *Momordica charantia* (Bitter Melon) Fruit Extract was prepared in CM. These concentrations of *Momordica charantia* (Bitter Melon) Fruit Extract are equivalent to the amount present in 0.01% and 0.1% **AC ExoRestore**, respectively.

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. 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.

The collagen and non-collagen protein concentrations of fibroblasts were determined by the following calculations and expressed in µg:

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

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

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 the *Momordica charantia* (Bitter Melon) Fruit Extract solutions demonstrated increased collagen to a similar degree. Similarly, fibroblasts treated with **AC ExoRestore** at 0.01% and 0.1% demonstrated increased collagen synthesis compared to untreated fibroblasts.

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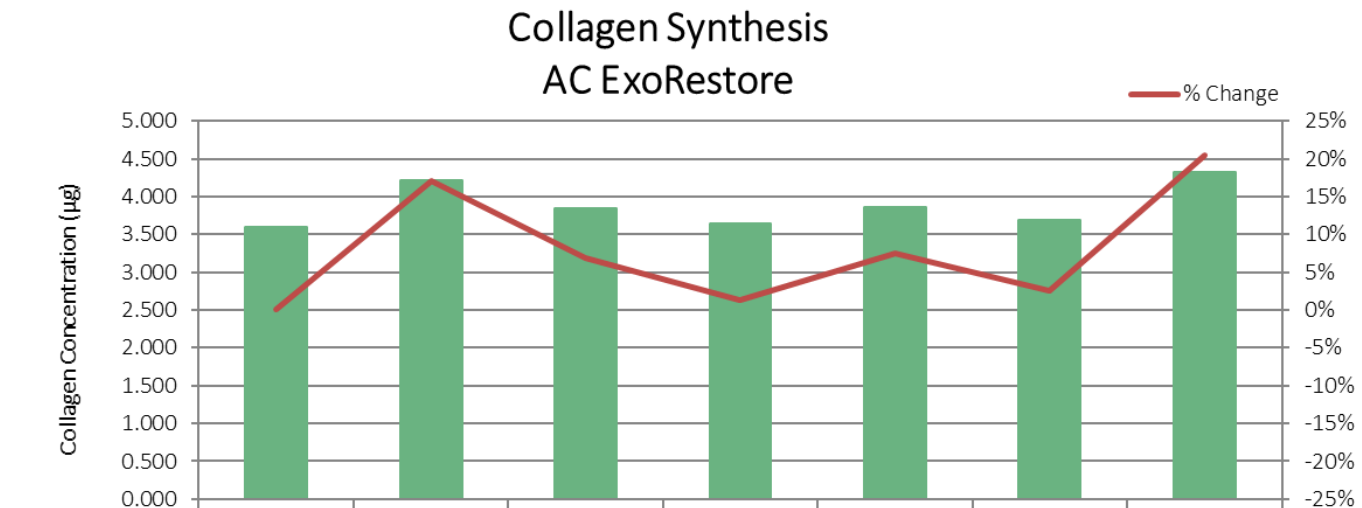

	Complete Media	AA2G	IGF-1	0.002% Momordica charantia Fruit Extract	0.02% Momordica charantia Fruit Extract	0.01% AC ExoRestore	0.1% AC ExoRestore
Collagen (ug)	3.594	4.210	3.842	3.641	3.862	3.684	4.329
% Change	0%	17%	7%	1%	7%	3%	20%

Figure 1. The effect of AA2G (100 µM or 34 µg/mL), IGF-1 (6.5 nM or 50 ng/mL), *Momordica charantia* Fruit Extract, and **AC ExoRestore** on collagen concentrations in dermal fibroblasts. Please note that 0.002% and 0.02% *Momordica charantia* (Bitter Melon) Fruit Extract are equivalent to 0.01% and 0.1% **AC ExoRestore**, respectively.

Discussion

As shown in Figure 1, fibroblasts incubated with Ascorbic Acid-2-Glucose (AA2G) and Insulin Growth Factor-1 (IGF-1), both known to stimulate collagen synthesis, exhibited increases in collagen synthesis of 17% and 7%, respectively. These data demonstrate collagen in fibroblasts is dynamic and can be manipulated with exogenous compounds.

Fibroblasts treated with **AC ExoRestore** at 0.01% and 0.1% demonstrated potent increases in collagen synthesis of 3% and 20%, respectively, compared to untreated fibroblasts. Comparatively, the 0.002% and 0.02% *Momordica charantia* (Bitter Melon) Fruit Extract treatments (equivalent to 0.01% and 0.1% **AC ExoRestore**, respectively) only increased collagen by 1% and 7%, respectively, highlighting the superior nature of bioauthentic exosomes as a delivery system. Overall, these data demonstrate **AC ExoRestore** 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 ExoRestore** stimulates collagen synthesis which may assist cosmetic applications designed to enhance collagen deposition and provide a younger and healthier dermal complexion.