Advanced Glycation End-Products Assay

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Tradename: AC LumiVitis

Code: 21032

CAS #: 8013-01-2 & 85594-37-2 (or) 84929-27-1 & 68333-16-4 (or) 1686112-36-6 (or) 9015-54-7

Test Request Form #: 13655

Lot #: 9418748

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

Test Performed:
Advanced Glycation End-Products (AGEs) Assay

Introduction

Advanced glycation end-products (AGEs) are harmful compounds formed between sugars and proteins resulting in oxidative stress, inflammation, and cellular disfunction. Accumulation of AGEs on collagen immobilizes fibroblasts by enhancing adhesion thereby reducing migration, proliferation, and expression of extracellular matrix proteins. As a result, glycated collagen exhibits slowed wound healing, reduced elasticity, and greater expression of wrinkles, all characteristics of aged skin. Therefore, cosmetic applications aimed at reducing AGEs-induced fibroblast functional impairments will promote healthier and more youthful skin.

Accordingly, a glycated collagen model was developed to assess the *in vitro* ability of **AC LumiVitis** to reduce AGEs-induced fibroblast dysfunction via adherence and migration. Attenuating glycation of collagen could blunt or prevent the age-related decline in skin function and physiology.

Assay Principle

A model of glycated collagen was developed by utilizing the advanced glycation end-products (AGEs) precursor 3-deoxyglucosone (3DG) to understand changes in cell adherence and migration. Human dermal fibroblasts were seeded onto pre-treated collagen-coated wells and cell adherence was evaluated over time. Subsequently, an artificial scratch was created in each well and images were collected to monitor cell migration.

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Materials

A. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
B. Equipment:	Forma humidified incubator, ESCO biosafety laminar flow hood, Synergy HT Microplate reader; Pipettes; Light microscope
C. Cell Line:	Normal Human Dermal Fibroblasts (ATCC; PCS-201-012)*
D. Media/Buffers:	Fibroblast Basal Medium (PCS-201-030)*; Fibroblast Growth Kit (PCS-201-041)*; Phosphate Buffered Saline (PBS)
E. Reagents:	Bovine Collagen Coating Solution (Cell Applications Inc; 125); 3-Deoxyglucosone (Cayman Chemical; 16347); Hoechst 33342 (ThermoFisher Scientific; 62249); Dimethyl Sulfoxide (DMSO)
F. Culture Plate:	24 Well Tissue Culture Treated Microplates
G. Software:	Excel Analysis ToolPak (Microsoft)

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

Methods

Collagen coated 24-well tissue culture microplates were incubated with 0.01%, 0.02%, and 0.05% concentrations of **AC LumiVitis** diluted in Complete Media (CM) containing 1 mM 3-Deoxyglucosone (3DG). 3DG was utilized to create glycated collagen while CM alone was utilized as the untreated collagen control. Following a 24-hour incubation at 37°C, treatments were aspirated and wells were washed three times with PBS.

Next, human dermal fibroblasts were seeded into pre-treated wells in CM and incubated at 37°C. Following a 24-hour incubation, media was removed and cells were briskly rinsed with PBS to remove loose cells. Hoechst was diluted to 20 µM in CM and added to all wells. Following a 30-minute incubation at 37°C, the nuclear dye was removed, all wells were washed once with PBS, CM was added to all wells, and fluorescence measurements were taken to determine the prevalence of cells adhered to collagen via Nuclear Signal (excitation: 361 nm / emission: 486 nm).

Subsequently, scratches were made down the middle of each well in a straight line, generating an *in vitro* "wound" devoid of cells. The rate at which the fibroblasts migrate to fill the area indicates wound healing. All wells were washed with sterile PBS to remove cellular debris caused by the scratch. CM was added to each well and was not changed throughout the duration of the experiment. Images were captured immediately after the scratch and 48 hours afterwards.

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 presence of cells adhered to collagen is relative to the untreated CM control and assessed as follows:

$$Cellular\ Presence\ (\%) = \frac{Nuclear\ Signal_{Sample}}{Nuclear\ Signal_{Untreated}} \times 100$$

Percent scratch closure was calculated for each well by the following equation and is expressed relative to the untreated CM control:

$$Scratch\ Closure\ (\%) = \frac{Final\ Area - Initial\ Area}{Initial\ Area} \times 100$$

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Results

The data obtained met criteria for a valid assay and the control performed as anticipated. Collagen treated with 3DG exhibited increased fibroblast adhesion and reduced migration compared to untreated collagen. Fibroblasts grown on collagen pretreated with **AC LumiVitis** in the presence of 3DG exhibited reduced adhesion and increased migration compared to untreated fibroblasts on glycated collagen.

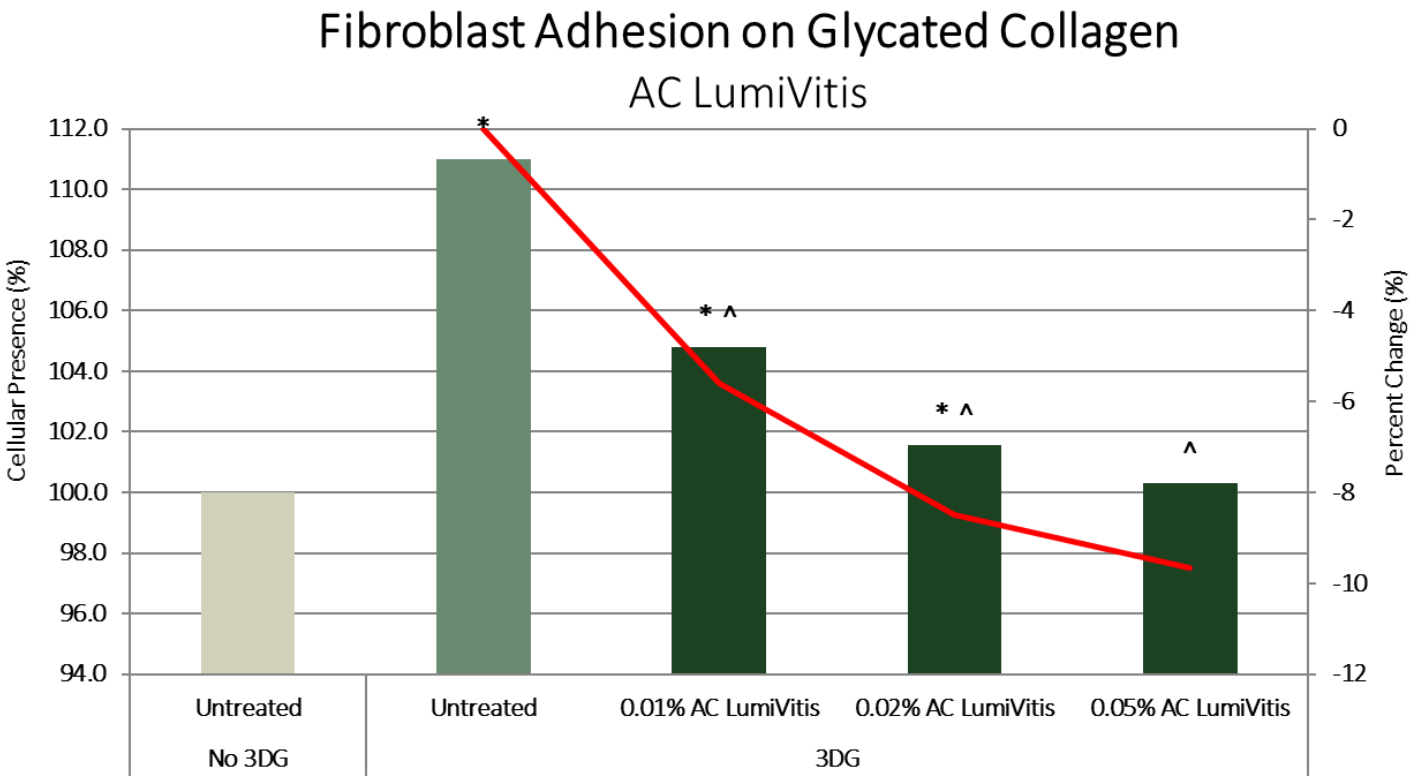

Figure 1. The Effect of **AC LumiVitis** on Fibroblast Adhesion to 3DG-treated Collagen. * indicates significance ($p \leq 0.05$) compared to untreated collagen. ^ indicates significance ($p \leq 0.05$) compared to 3DG-treated collagen.

Table 1. Results from one-way ANOVA Statistical Analysis of Fibroblast Adhesion Compared to Untreated and 3DG-treated Collagen. * indicates significance ($p \leq 0.05$) compared to untreated collagen. ^ indicates significance ($p \leq 0.05$) compared to 3DG-treated collagen.

	3DG	0.01% **AC LumiVitis**	0.02% **AC LumiVitis**	0.05% **AC LumiVitis**
Untreated	< 0.001*	0.002*	0.025*	> 0.05
3DG	-----	0.032^	0.001^	0.021^

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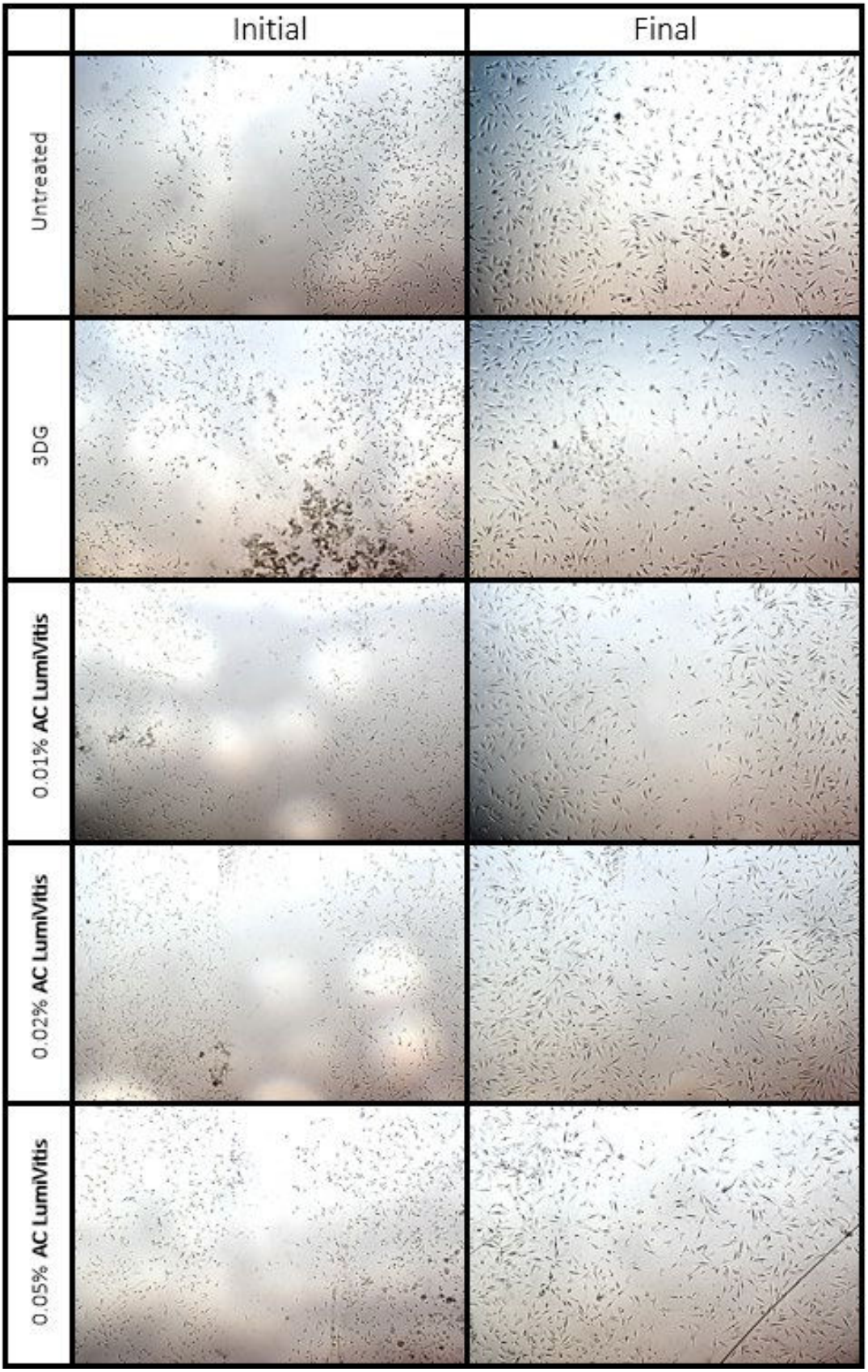

Figure 2. Representative Images of Fibroblast Migration Over Time.

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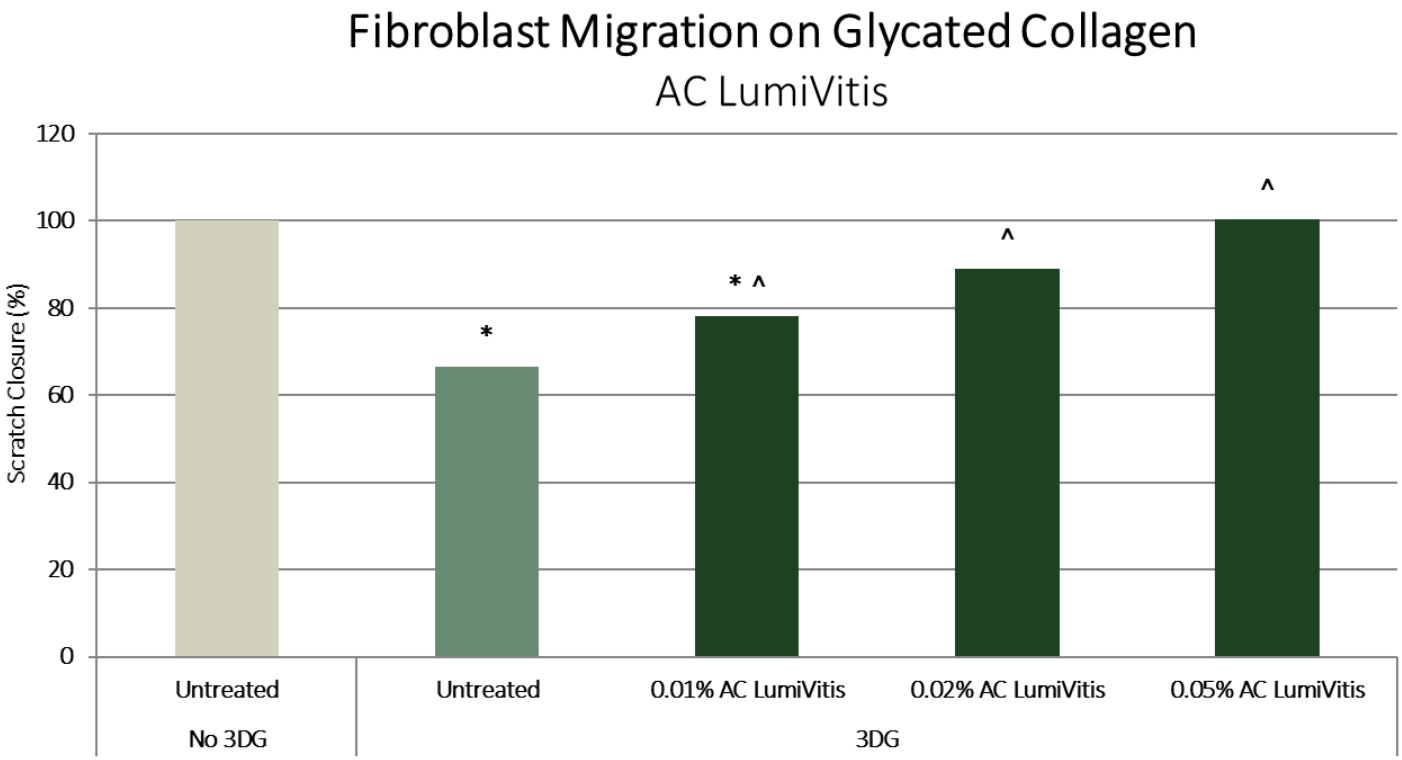

Figure 3. The Effect of **AC LumiVitis** on Fibroblast Migration Across 3DG-treated Collagen. * indicates significance ($p \leq 0.05$) compared to untreated collagen. ^ indicates significance ($p \leq 0.05$) compared to 3DG-treated collagen.

Table 2. Results from one-way ANOVA Statistical Analysis of Fibroblast Migration Compared to Untreated and 3DG-treated Collagen. * indicates significance ($p \leq 0.05$) compared to untreated collagen. ^ indicates significance ($p \leq 0.05$) compared to 3DG-treated collagen.

	3DG	0.01% **AC LumiVitis**	0.02% **AC LumiVitis**	0.05% **AC LumiVitis**
Untreated	0.004*	0.007*	> 0.05	> 0.05
3DG	-----	0.012^	0.019^	0.001^

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Discussion

As shown in Figure 1, fibroblasts grown on 3DG glycated collagen demonstrated 11% higher cell adherence than untreated collagen. These data demonstrate fibroblast adherence increases with AGEs. Alternatively, collagen treated with 3DG in the presence of **AC LumiVitis** at 0.01%, 0.02%, and 0.05% elicited 6%, 9%, and 10% reductions in cell adherence compared to glycated collagen, respectively (Table 1). Moreover, at 0.05% **AC LumiVitis** exhibited adherence comparable to untreated collagen (Table 1). These data demonstrate **AC LumiVitis** has anti-glycation capabilities demonstrated by a reduction in fibroblast adherence.

Fibroblasts on collagen treated with 3DG demonstrated a reduction in migration by a scratch closure of 66% compared to untreated collagen. These data demonstrate fibroblast migration reduces with AGEs. Alternatively, fibroblasts on collagen co-incubated with 3DG and **AC LumiVitis** at 0.01%, 0.02%, and 0.05% exhibited wound closures of 78%, 89%, and 100% compared to untreated collagen, respectively (Table 2). These data demonstrate **AC LumiVitis** has anti-glycation capabilities demonstrated by a restoration of fibroblast migration.

Collectively, glycated collagen increases fibroblast adhesion and inhibits migration, attributes typically observed in aged cells. These data indicate **AC LumiVitis** reduces AGEs induced fibroblast dysfunction which may help to attenuate characteristics of cellular aging by promoting skin elasticity and wound healing.