Cellular Collagen After Light Irradiation

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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 #: 13652

Lot #: 9418748 & N250821C

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 After Light Irradiation

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.

Exposure to different light wavelengths modulates cellular functions such as morphology, migration, or metabolic activity. For example, UV-B light (280-315 nm) and blue light (400-500 nm) stimulate inflammation, reactive oxygen species, DNA mutations, and disruptions in dermal-epidermal junction integrity, which can exacerbate skin wrinkling and aging. Conversely, red light (625-700 nm) and near infrared (IR) light (700-1400 nm) positively impact cells by enhancing mitochondrial function and supporting tissue regeneration which can enhance skin elasticity and reduce wrinkle formation.

Accordingly, a Sirius Red/Fast Green Collagen Assay was conducted to assess the *in vitro* protective effects of **AC LumiVitis** on modulations in collagen synthesis after various light irradiation at time of manufacture and after six months of stability.

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. To understand changes over time, samples from time of manufacture (Lot #: 9418748) and six-month stability (Lot #: N250821C) were tested.

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Materials

- **A. Kit:** Sirius Red/Fast Green Collagen Kit (Chondrex; 9046) *
- **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; Synergy HT Microplate Reader; Accuris UV Transilluminator; elixa LED Blue Light Array; BestQool Portable Red Light Therapy BQ40; Pipettes
- **D. Cell Line:** Normal Human Dermal Fibroblasts (ATCC; PCS-201-012) *
- **E. Media/Buffers:** Fibroblast Basal Medium (ATCC; PCS-201-030) *; Fibroblast Growth Kit (ATCC; PCS-201-041)*: Phosphate Buffered Saline (PBS)
- **F. Culture Plate:** Falcon Flat Bottom 24-Well Tissue Culture Treated Plates
- **G. Software:** Excel Analysis ToolPak (Microsoft)
- **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%, 0.02%, and 0.05% concentrations of **AC LumiVitis** at time of manufacture and after six-month stability were added to CM and incubated at 37°C with fibroblasts. Designated wells on each plate were incubated with CM as Untreated controls with and without light irradiation. Following an 18-hour incubation, the media in all wells was replaced with CM and the tissue culture plates were returned to 37°C for 30 minutes. Next, the fibroblasts were irradiated with UV-B Light or Blue Light or exposed to Red Light or Near Infrared (IR) Light at the dosages outlined in Table 1. After irradiation/exposure, the plates were incubated at 37°C for 24 hours, after which the Sirius Red/Fast Green assay was performed.

Table 1. Light Wavelengths and Dosages

Light Source	Wavelength (nm)	Dose (J/cm^2)
UV-B Light	302	0.076
Blue Light	470	480
Red Light	660	33.6
Near IR Light	850	33.6

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 water. 1 mL of extraction solution was added for color extraction. The optical density was read at 540 nm and 605 nm on a Synergy HT Microplate Reader.

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Three separate experiments were performed for each light source with conditions in duplicate and average values were recorded. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. Collagen protein concentrations of fibroblasts were determined by calculations based on the optical density measurements and expressed in µg. Collagen concentrations were calculated with the following formula:

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

Results

The data obtained met criteria for a valid assay as the controls performed as anticipated. UV-B Light and Blue Light irradiation elicited reductions in collagen levels. Conversely, fibroblasts treated with **AC LumiVitis** attenuated UV-B Light and Blue Light induced reductions in collagen compared to untreated fibroblasts with and without irradiation. Importantly, **AC LumiVitis** did not exhibit any reductions in protection against UV-B Light or Blue Light irradiation after six months of stability.

Exposing fibroblasts to Red Light and Near IR Light elevated collagen levels. Moreover, **AC LumiVitis** demonstrated enhanced collagen levels when exposed to Red Light and Near Infrared Light compared to untreated fibroblasts with and without light exposure. After six-month stability, **AC LumiVitis** promoted collagen levels when exposed to Red Light and Near Infrared Light.

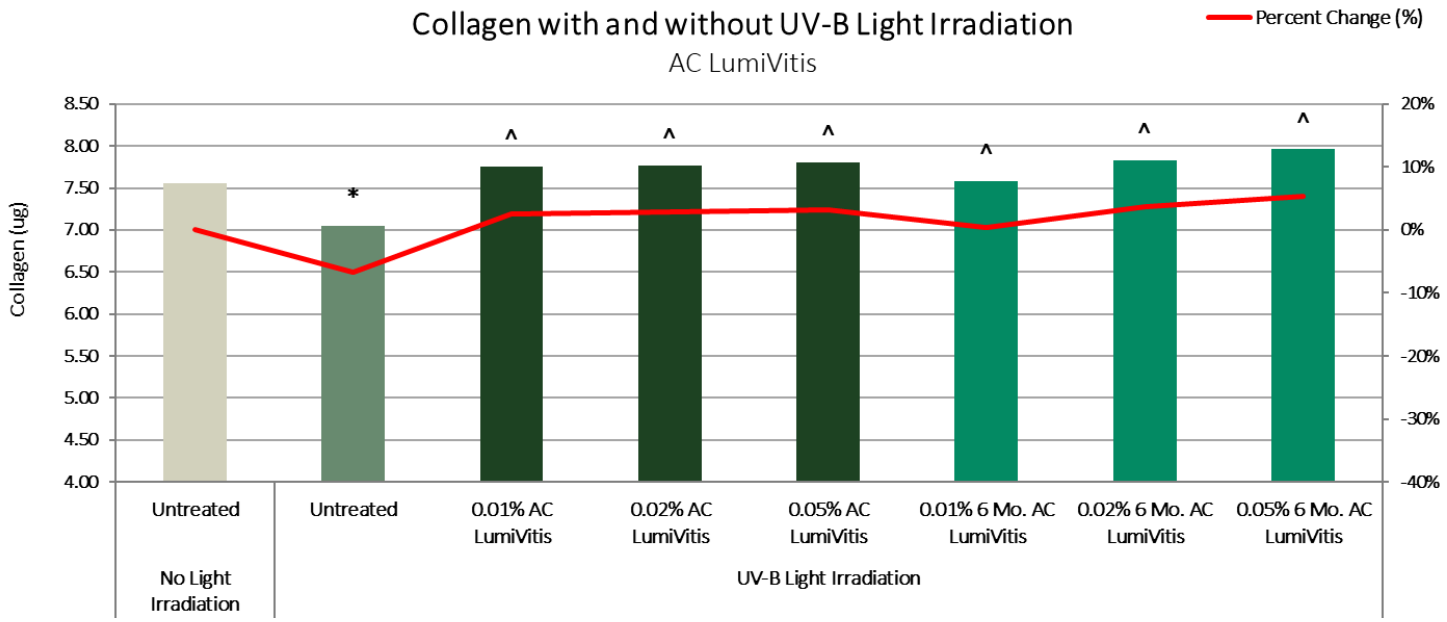

Figure 1. The effect of UV-B Light Irradiation on Collagen. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with irradiation. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

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Table 2. Results from one-way ANOVA Statistical Analysis of Collagen Levels after UV-B Light Irradiation Compared to Untreated Fibroblasts with and without Irradiation. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with irradiation.

	Untreated + Irradiated	0.01% AC LumiVitis	0.02% AC LumiVitis	0.05% AC LumiVitis	0.01% 6 Mo. AC LumiVitis	0.02% 6 Mo. AC LumiVitis	0.05% 6 Mo. AC LumiVitis
Untreated	0.048*	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Untreated + Irradiated	-----	0.001^	< 0.001^	< 0.001^	< 0.022^	<0.001^	< 0.001^

Table 3. Results from one-way ANOVA Statistical Analysis of Collagen Levels after UV-B Light Irradiation Compared to **AC LumiVitis** at time of manufacture. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

	0.01% **6 Mo. AC LumiVitis**	0.02% **6 Mo. AC LumiVitis**	0.05% **6 Mo. AC LumiVitis**
P-value	> 0.05	> 0.05	> 0.05

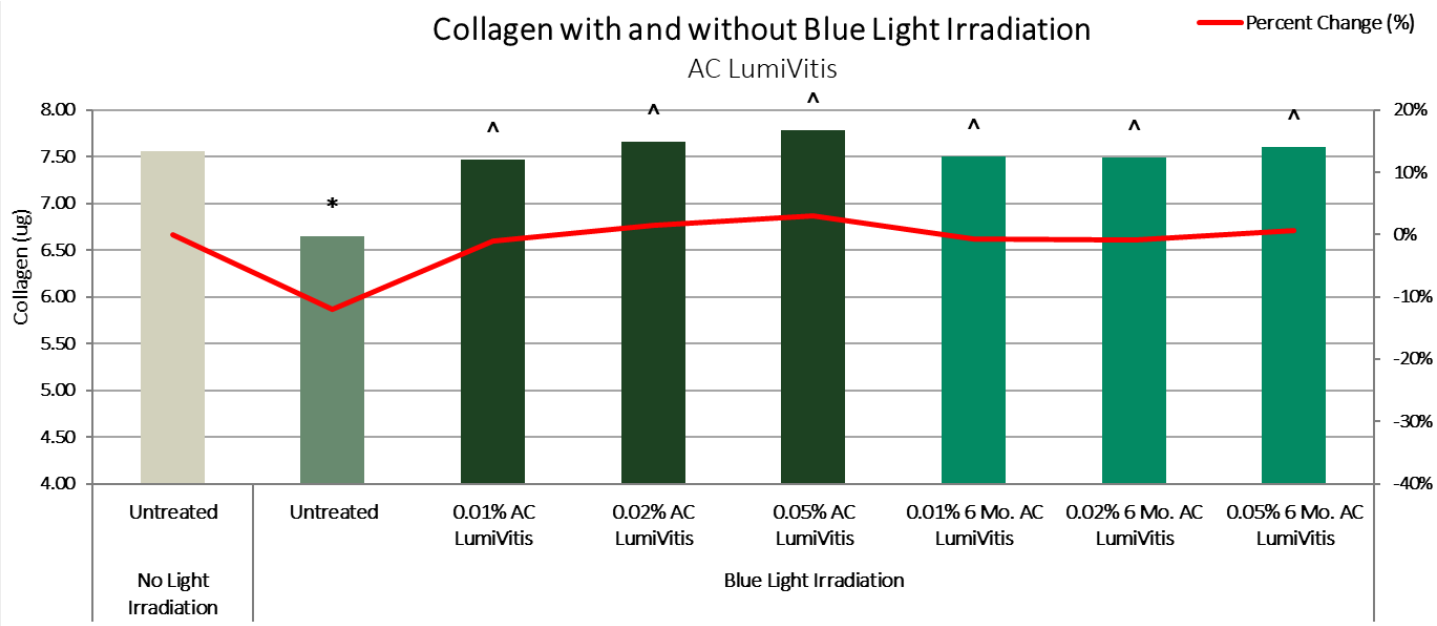

Figure 2. The effect of Blue Light Irradiation on Collagen. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with irradiation. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

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Table 4. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Blue Light Irradiation Compared to Untreated Fibroblasts with and without Irradiation. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with irradiation.

	Untreated + Irradiated	0.01% AC LumiVitis	0.02% AC LumiVitis	0.05% AC LumiVitis	0.01% 6 Mo. AC LumiVitis	0.02% 6 Mo. AC LumiVitis	0.05% 6 Mo. AC LumiVitis
Untreated	0.035*	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Untreated + Irradiated	-----	0.003^	0.004^	0.020^	0.001^	0.002^	0.012^

Table 5. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Blue Light Irradiation Compared to **AC LumiVitis** at time of manufacture. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

	0.01% **6 Mo. AC LumiVitis**	0.02% **6 Mo. AC LumiVitis**	0.05% **6 Mo. AC LumiVitis**
P-value	> 0.05	> 0.05	> 0.05

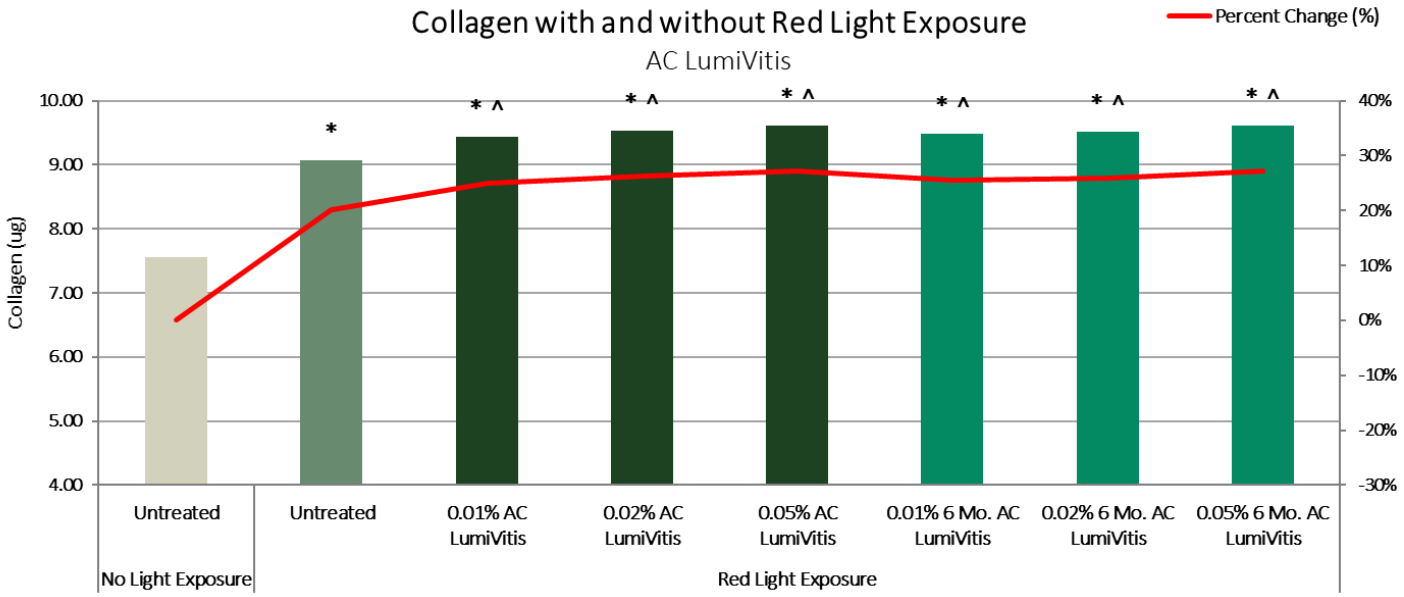

Figure 3. The effect of Red Light Exposure on Collagen. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with exposure. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

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Table 6. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Red Light Exposure Compared to Untreated Fibroblasts with and without Exposure. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with exposure.

	Untreated + Irradiated	0.01% AC LumiVitis	0.02% AC LumiVitis	0.05% AC LumiVitis	0.01% 6 Mo. AC LumiVitis	0.02% 6 Mo. AC LumiVitis	0.05% 6 Mo. AC LumiVitis
Untreated	0.007*	0.001*	0.001*	0.003*	0.001*	0.004*	< 0.001*
Untreated + Exposure	-----	0.022^	0.047^	0.022^	0.025^	0.035^	0.014^

Table 7. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Red Light Exposure Compared to **AC LumiVitis** at time of manufacture. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

	0.01% **6 Mo. AC LumiVitis**	0.02% **6 Mo. AC LumiVitis**	0.05% **6 Mo. AC LumiVitis**
P-value	> 0.05	> 0.05	> 0.05

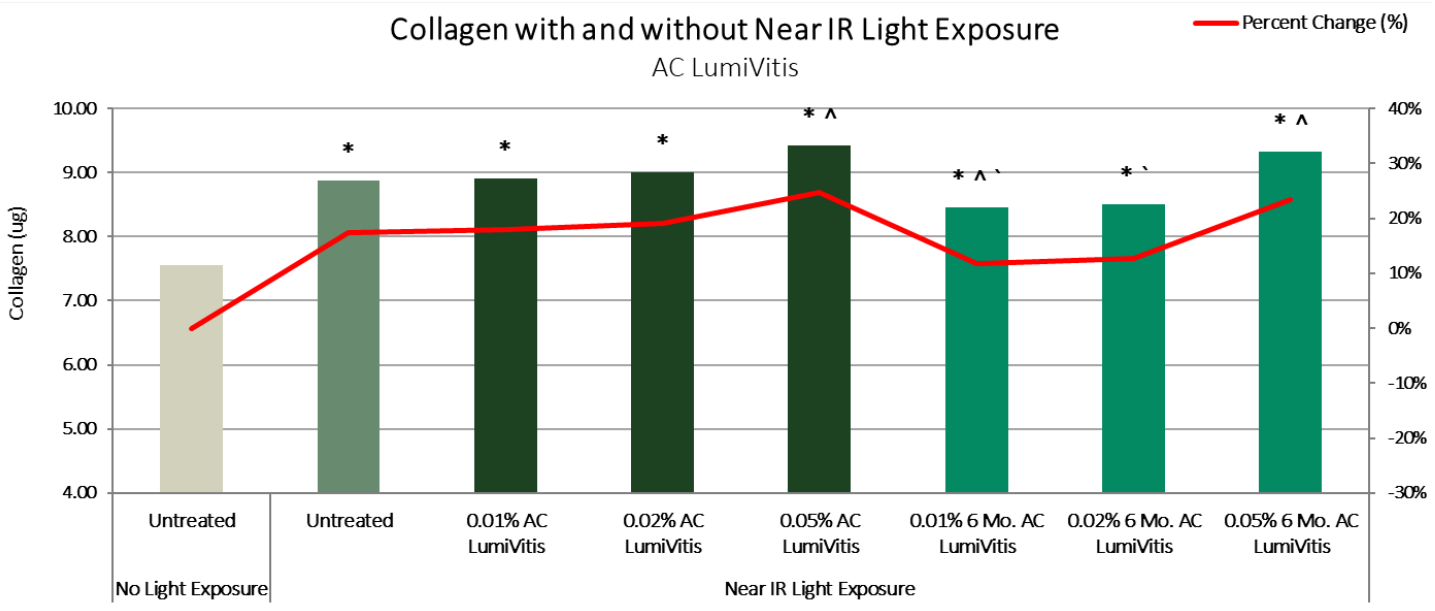

Figure 4. The effect of Near IR Light Exposure on Collagen. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with exposure. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

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Table 8. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Near IR Light Exposure Compared to Untreated Fibroblasts with and without Exposure. * indicates significance ($p \leq 0.05$) compared to untreated fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to untreated fibroblasts with exposure.

	Untreated + Irradiated	0.01% AC LumiVitis	0.02% AC LumiVitis	0.05% AC LumiVitis	0.01% 6 Mo. AC LumiVitis	0.02% 6 Mo. AC LumiVitis	0.05% 6 Mo. AC LumiVitis
Untreated	0.011*	0.011*	0.004*	0.001*	0.009*	0.034*	0.001*
Untreated + Exposure	-----	> 0.05	> 0.05	< 0.001^	< 0.001^	> 0.05	0.017^

Table 9. Results from one-way ANOVA Statistical Analysis of Collagen Levels after Near IR Light Exposure Compared to **AC LumiVitis** at time of manufacture. ` indicates significance ($p \leq 0.05$) between time of manufacture and six-month **AC LumiVitis** batches.

	0.01% 6 Mo. AC LumiVitis	0.02% 6 Mo. AC LumiVitis	0.05% 6 Mo. AC LumiVitis
P-value	0.011`	0.022`	> 0.05

Discussion

As shown in Figure 1, UV-B Light irradiation produced a 6.6% reduction in collagen synthesis compared to untreated fibroblasts, demonstrating the negative effects of UV-B Light on collagen production. Alternatively, fibroblasts irradiated with UV-B Light and treated with **AC LumiVitis** at 0.01%, 0.02%, and 0.05% exhibited increases in collagen of 9.9%, 10%, and 11%, respectively, compared to untreated fibroblasts with irradiation and slightly boosted collagen by 2.6%, 2.8%, and 3.3%, respectively, compared to untreated fibroblasts (Table 2). After six months, the protective effects of **AC LumiVitis** did not significantly differ than time of manufacture with increases in collagen of 7.5%, 11%, and 13%, at 0.01%, 0.02%, and 0.05%, respectively, compared to untreated fibroblasts with irradiation (Table 3). This data indicates **AC LumiVitis** protects collagen synthesis against the deleterious impacts of UV-B Light irradiation and maintains efficacy over time.

Similarly, Blue Light irradiation produced a 12% reduction in collagen synthesis compared to untreated fibroblasts, demonstrating the negative effects of Blue Light on collagen production (Figure 2). Alternatively, fibroblasts irradiated with Blue Light and treated with **AC LumiVitis** at 0.01%, 0.02%, and 0.05% exhibited increases in collagen of 12%, 15%, and 17%, respectively, compared to untreated fibroblasts with irradiation and did not further enhance collagen compared to untreated fibroblasts (Table 4). After six months, the protective effects of **AC LumiVitis** did not significantly differ than time of manufacture with increases in collagen of 13%, 13%, and 14%, at 0.01%, 0.02%, and 0.05%, respectively, compared to untreated fibroblasts with irradiation (Table 5). This data indicates **AC LumiVitis** protects collagen synthesis against the adverse impacts of Blue Light irradiation and maintains efficacy over time.

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Red Light exposure enhanced collagen synthesis by 20% compared to untreated fibroblasts, demonstrating the positive effects of Red Light on collagen production (Figure 3). Fibroblasts exposed to Red Light and treated with **AC LumiVitis** at 0.01%, 0.02%, and 0.05% exhibited increases in collagen of 25%, 26%, and 27%, respectively, compared to untreated fibroblasts and significantly boosted the benefits of Red Light exposure by 3.9%, 5.0%, and 5.9%, respectively, compared to untreated fibroblasts with light exposure (Table 6). After six months, the synergistic effects of **AC LumiVitis** did not significantly differ than time of manufacture with increases in collagen of 26%, 26%, and 27%, at 0.01%, 0.02%, and 0.05%, respectively, compared to untreated fibroblasts (Table 7). This data indicates **AC LumiVitis** amplifies the benefits of Red Light exposure with respect to collagen synthesis and maintains efficacy over time.

Finally, Near IR Light exposure enhanced collagen synthesis by 17% compared to untreated fibroblasts, demonstrating the positive effects of Near IR Light on collagen production (Figure 4). Fibroblasts exposed to Near IR Light and treated with **AC LumiVitis** at 0.01%, 0.02%, and 0.05% exhibited increases in collagen of 18%, 19%, and 25%, respectively, compared to untreated fibroblasts and significantly boosted the benefits of the Near IR Light by 6.2% at 0.05% concentration, respectively, compared to untreated fibroblasts with light exposure (Table 8). After six months, the synergistic effects of **AC LumiVitis** did not differ from time of manufacture with increases in collagen of 12%, 13%, and 23%, at 0.01%, 0.02%, and 0.05%, respectively, compared to untreated fibroblasts (Table 9). This data indicates **AC LumiVitis** ameliorates the benefits of Near IR Light exposure with respect to collagen synthesis.

Collectively, these data demonstrate that **AC LumiVitis** attenuates UV-B Light and Blue Light induced disruptions in collagen synthesis while complementing Red Light and Near IR Light augmentations in collagen production. In summary, utilizing **AC LumiVitis** may lead to improvement in the dermal-epidermal junction integrity, as well as an improved scaffolding matrix, helping prevent visible signs of aging.