IL-6 ELISA Analysis

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Tradename: AC ExoRoot

Code: 60202

CAS #: 7732-18-5 & 91079-57-1 (or) 223749-83-5 & 123465-35-0 (or) 8002-43-5 & 68333-16-4 (or) 1686112-36-6 (or) 9015-54-7

Test Request Form #: 13008

Lot #: N250520B

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

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 decreased dermal structure and weakened hair follicles. 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.

Accordingly, an interleukin-6 ELISA was conducted to assess the *in vitro* effect of **AC ExoRoot** to elicit changes in IL-6 levels in dermal papilla cells. The key active ingredient in **AC ExoRoot**, *Chlorella vulgaris* Extract, was tested to demonstrate the superior nature of bioauthentic exosomes as a delivery system. Additionally, minoxidil and a blend of **AC ExoRoot** with minoxidil were tested to elucidate any interactive effects.

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 IL-6 in each sample.

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Materials

A. Kit:	Human IL-6 ELISA Kit (Invitrogen; BMS213-2)*
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:	Human Hair Follicle Dermal Papilla Cells (HFDPCs) (Cell Applications Inc; 602K-05a)*
E. Media/Buffers:	Dermal Papilla Growth Media (DPGM) (Cell Applications Inc.; C-26501)*; Collagen Coating Solution (Cell Applications Inc.; 125-100)*; Phosphate Buffered Saline (PBS)
F. Reagents:	Lipopolysaccharide (1 µg/mL); Dexamethasone (10 µM); Minoxidil (Millipore Sigma)
G. Software:	Excel Analysis ToolPak (Microsoft)
H. Culture Plate:	12 Well Tissue Culture Treated Plates
I. Other:	Sterile disposable pipette tips

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

Methods

Human dermal papilla cells were seeded into collagen coated 12-well tissue culture plates and allowed to grow to confluency in complete media (CM). 0.01%, 0.02%, 0.05%, and 0.1% concentrations of **AC ExoRoot** were added to CM containing 1 µg/mL LPS and incubated with cells for 72 hours. LPS is utilized to create an inflammatory environment and dexamethasone (DEX) in the presence of LPS was used as a positive control to quell inflammation. Additionally, a 0.02% solution of minoxidil and 0.01%, 0.02%, 0.05%, and 0.1% solutions of *Chlorella vulgaris* Extract and **AC ExoRoot** with 0.02% minoxidil were prepared in CM containing 1 µg/mL LPS and incubated with cells.

Standards were prepared in concentrations ranging from 0 pg/mL to 200 pg/mL. Prior to samples being added all wells were washed with 1X wash buffer four times. 50 µL of assay buffer was added to the sample and assay control wells. 50 µL of the controls and samples were added to appropriate wells. 100 µL of each standard concentration was added to the appropriate well. 50 µL of Biotin-conjugate were added to all wells. After a two-hour incubation at room temperature and washing, 100 µL Streptavidin-HRP was added to all wells. Following a one-hour incubation and washing, 100 µL of TMB substrate solution was added for the colorimetric reaction. After a 10-minute incubation in the dark, 100 µL of stop solution was added to stop the reaction. The optical density (OD) was read at 450 nm on the Synergy HT Microplate Reader.

Assays were repeated three separate times with each sample run in duplicate. Duplicates for each replicate were averaged, and the average of all three experiments is displayed. Data was analyzed using a one-way ANOVA with statistical significance accepted at $p \leq 0.05$. A standard curve was created by reducing the data and generating a linear curve fit. The IL-6 concentration of **AC ExoRoot** treated-dermal papilla cells was determined by extrapolation from the standard curve and expressed in pg/mL. Percent change is expressed relative to CM + LPS and calculated by the following equation:

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

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. Compared to untreated dermal papilla cells, LPS (1 µg/mL) increased IL-6 production whereas DEX (10 µM) reduced the LPS-induced inflammation. Dermal papilla cells treated with *Chlorella vulgaris* Extract alone did not experience reduced inflammation compared to cells treated with LPS. Comparatively, cells treated with minoxidil, **AC ExoRoot**, and **AC ExoRoot** with minoxidil all exhibited reductions in LPS-induced IL-6 levels.

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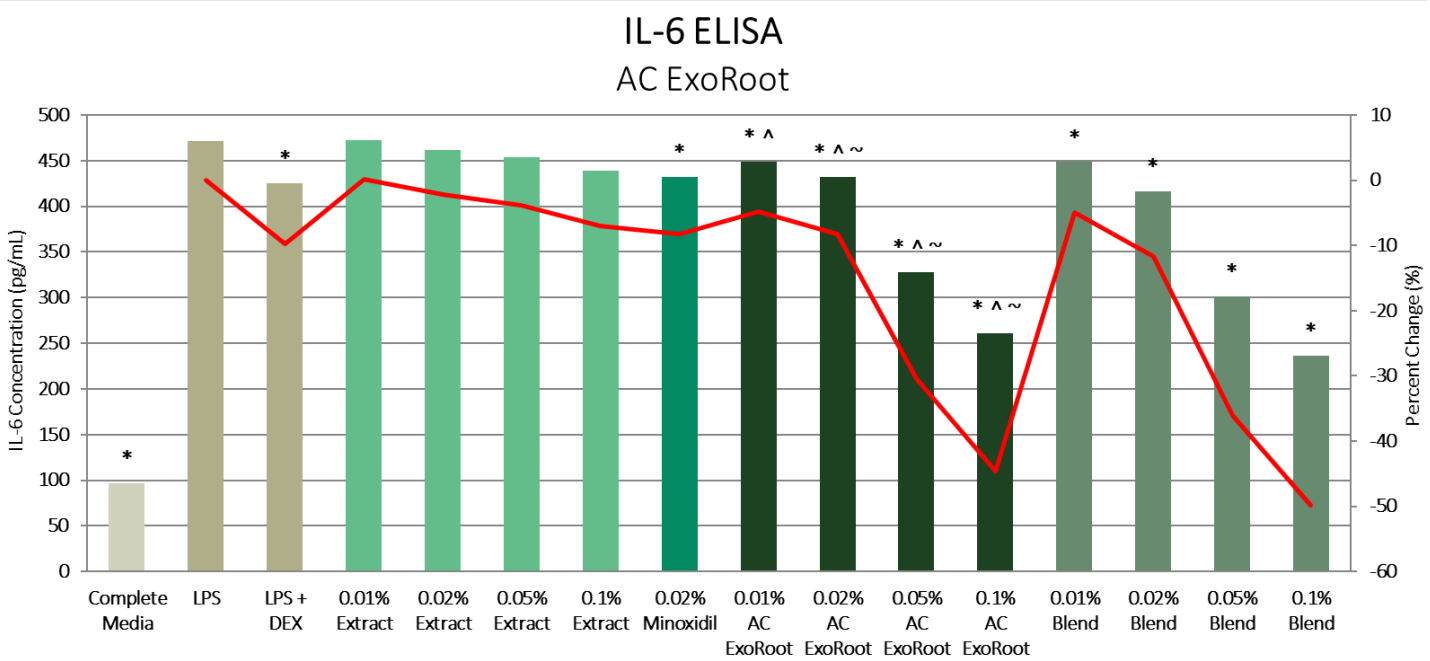

Figure 1. The effect of **AC ExoRoot** on IL-6 concentrations in dermal papilla cells. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to dermal papilla cells incubated with LPS. ^ indicates significance ($p \leq 0.05$) compared to *Chlorella vulgaris* Extract. ~ indicates significance ($p \leq 0.05$) compared to **AC ExoRoot** + 0.02% minoxidil.

Table 1. Results from one-way ANOVA Statistical Analysis Compared to LPS treated Cells. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to dermal papilla cells incubated with LPS.

	DEX	0.01% Extract	0.02% Extract	0.05% Extract	0.1% Extract	0.02% Minoxidil	0.01% **AC ExoRoot**	0.02% **AC ExoRoot**	0.05% **AC ExoRoot**	0.1% **AC ExoRoot**	0.01% Blend	0.02% Blend	0.05% Blend	0.1% Blend
P Value	0.001*	> 0.05	> 0.05	> 0.05	> 0.05	< 0.001*	0.011*	< 0.001*	0.012*	0.007*	0.019*	0.015*	0.010*	0.001*

Table 2. Results from one-way ANOVA statistical analysis between two conditions compared at equivalent use levels. ^ indicates significance ($p \leq 0.05$) compared to *Chlorella vulgaris* Extract. ~ indicates significance ($p \leq 0.05$) compared to **AC ExoRoot** + 0.02% minoxidil.

	0.01% **AC ExoRoot**	0.02% **AC ExoRoot**	0.05% **AC ExoRoot**	0.1% **AC ExoRoot**
Chlorella vulgaris Extract	0.019^	0.038^	0.016^	0.011^
AC ExoRoot + 0.02% minoxidil	> 0.05	0.033~	0.033~	0.018~

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Discussion

As shown in Figure 1, dermal papilla cells incubated with LPS, a substance known to induce an inflammatory state, exhibited a 79% increase in IL-6 levels compared to untreated cells. Conversely, cells exposed to LPS and DEX elicited a 10% significant reduction in IL-6 compared to cells treated with LPS (Table 1). These data demonstrate the inflammatory environment in dermal papilla cells is dynamic and can be manipulated with exogenous compounds.

Similarly, dermal papilla cells treated with **AC ExoRoot** at 0.01% 0.02%, 0.05%, and 0.1% demonstrated 5%, 8%, 30%, and 45% reductions in IL-6 levels compared to LPS-treated cells, respectively (Table 1). Comparatively, *Chlorella vulgaris* Extract at 0.01% 0.02%, 0.05%, and 0.1% only elicited 0%, 2%, 4%, and 7% reductions in IL-6 levels compared to LPS-treated cells, respectively, and was significantly less effective than **AC ExoRoot** highlighting the superior nature of the bioauthentic exosomes as a delivery system (Table 2). These data demonstrate **AC ExoRoot** reduces inflammation in a dose-dependent fashion.

Dermal papilla cells treated with 0.02% minoxidil experienced an 8% reduction in IL-6 levels compared to LPS-treated cells (Figure 1; Table 1). Moreover, cells treated with 0.01% 0.02%, 0.05%, and 0.1% **AC ExoRoot** with 0.02% minoxidil demonstrated 5%, 12%, 36%, and 50% reductions in IL-6 levels compared to LPS-treated cells, respectively. At 0.02% concentration and higher, the blend exhibited a synergistic effect compared to **AC ExoRoot** alone (Table 2). These data indicate **AC ExoRoot** can be used in conjunction with minoxidil to quell inflammation thereby promoting hair growth.

Collectively, a decrease in IL-6 production indicates a reduced inflammatory environment which could diminish the signs of aging and minimize hair thinning and loss. These data indicate **AC ExoRoot** exerts an anti-inflammatory effect, which may help to attenuate characteristics of cellular aging.