IL-6 ELISA Analysis

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Tradename: AC Melatonin Liposome SF

Code: 61012

CAS #: 7732-18-5 & 123465-35-0 & 73-31-4

Test Request Form #: 5982

Lot #: N200114B

Sponsor: *Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092*
Study Director: *Maureen Danaher*
Principal Investigator: *Michael Hovis*

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 wrinkles, fine lines, and loss of skin elasticity. 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 Melatonin Liposome SF** to elicit changes in IL-6 levels in dermal fibroblasts. Additionally, Non-Encapsulated Melatonin was tested to demonstrate the superior nature of the liposome system.

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:	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:	Lipopolysaccharide (LPS) (1 µg/mL); Dexamethasone (DEX) (10 µM)
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 fibroblasts were seeded into 12-well tissue culture plates and allowed to grow to confluency in complete media (CM). 0.01%, 0.1%, and 1.0% concentrations of **AC Melatonin Liposome SF** were added to CM containing 1 µg/mL LPS and incubated with fibroblasts 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 4.0% solution of Non-Encapsulated Melatonin was prepared and further diluted to 0.01%, 0.1%, and 1.0% in CM. This concentration of melatonin is equivalent to the amount present in **AC Melatonin Liposome SF**.

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 Melatonin Liposome SF** treated-fibroblasts 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 fibroblasts, LPS (1 µg/mL) increased IL-6 production whereas DEX (10 µM) reduced the LPS-induced inflammation. Fibroblasts treated with 0.01%, 0.1%, and 1.0% Non-Encapsulated Melatonin also had slightly reduced IL-6 levels. Fibroblasts treated with **AC Melatonin Liposome SF** at 0.01%, 0.1%, and 1.0% demonstrated a reduction in LPS-induced IL-6 levels.

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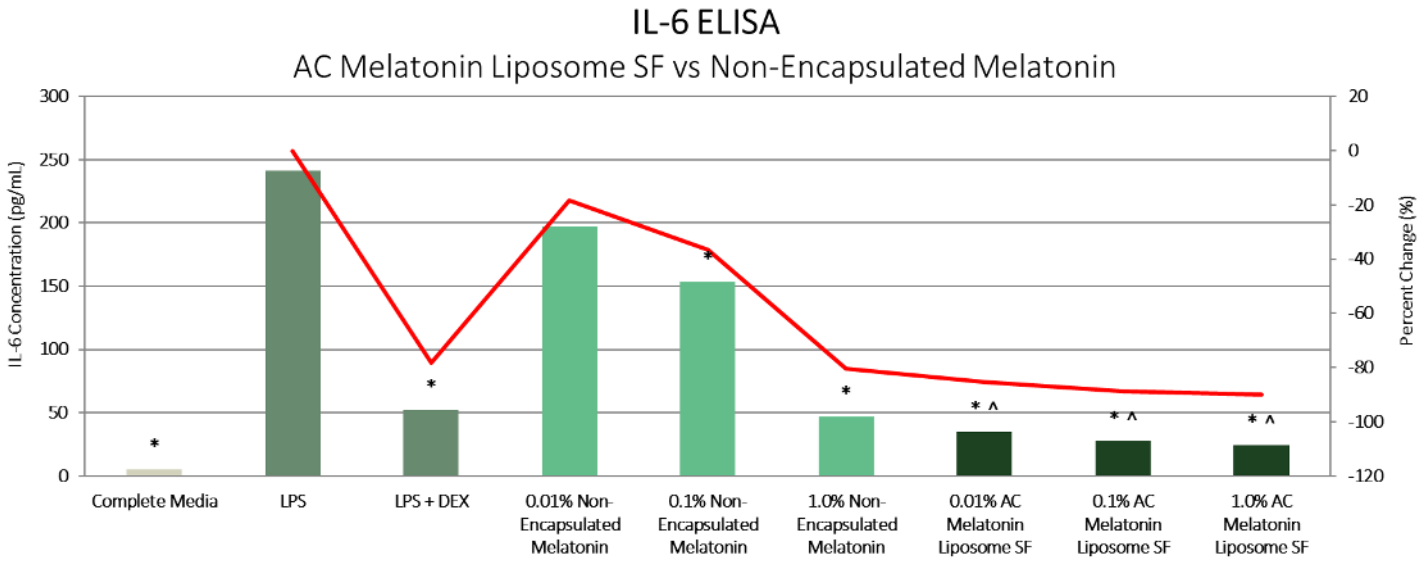

Figure 1. The effect of **AC Melatonin Liposome SF** on IL-6 concentrations in fibroblasts. * indicates significance ($p \leq 0.05$) compared to fibroblasts incubated with LPS. ^ indicates significance ($p \leq 0.05$) compared to fibroblasts incubated with Non-Encapsulated Melatonin at the equivalent concentration.

Table 1. Results from one-way ANOVA Statistical Analysis Compared to Fibroblasts Incubated with LPS. * indicates significance ($p \leq 0.05$) compared to fibroblasts incubated with LPS.

	DEX	0.01% **Non-Encapsulated Melatonin**	0.1% **Non-Encapsulated Melatonin**	1.0% **Non-Encapsulated Melatonin**	0.01% **AC Melatonin Liposome SF**	0.1% **AC Melatonin Liposome SF**	1.0% **AC Melatonin Liposome SF**
P-Value	0.001*	> 0.05	0.043*	0.001*	< 0.001*	< 0.001*	< 0.001*

Table 2. Results from one-way ANOVA Statistical Analysis Compared to Fibroblasts Incubated with Non-Encapsulated Melatonin. ^ indicates significance ($p \leq 0.05$) compared to fibroblasts incubated with Non-Encapsulated Melatonin.

	0.01% **AC Melatonin Liposome SF** vs Non-Encapsulated Melatonin	0.1% **AC Melatonin Liposome SF** vs Non-Encapsulated Melatonin	1.0% **AC Melatonin Liposome SF** vs Non-Encapsulated Melatonin
P-Value	< 0.001^	< 0.001^	0.001^

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Discussion

As shown in Figure 1, fibroblasts incubated with LPS, a substance known to induce an inflammatory state, exhibited a 98% increase in IL-6 levels compared to untreated fibroblasts. Conversely, fibroblasts exposed to LPS and DEX elicited a 78% significant reduction in IL-6 compared to fibroblasts treated with LPS (Table 1). These data demonstrate the inflammatory environment in fibroblasts is dynamic and can be manipulated with exogenous compounds.

Similarly, fibroblasts treated with AC Melatonin Liposome SF at 0.01%, 0.1%, and 1.0% demonstrated 85%, 89%, and 90% reductions in IL-6 levels compared to LPS-treated fibroblasts, respectively (Table 1). Comparatively, the 0.01%, 0.1%, and 1.0% Non-Encapsulated Melatonin only decreased IL-6 levels by 18%, 36%, and 81%, respectively, compared to LPS-treated fibroblasts and was significantly less effective than AC Melatonin Liposome SF highlighting the superior nature of the liposome system (Table 2). These data demonstrate AC Melatonin Liposome SF reduces inflammation.

Collectively, a decrease in IL-6 production indicates a reduced inflammatory environment which could diminish the signs of aging and minimize the formation of fine lines and wrinkles. These data indicate AC Melatonin Liposome SF exerts an anti-inflammatory effect, which may help to attenuate characteristics of cellular aging.