Endothelial Permeability Assay

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Tradename: AC ExoVitalize

Code: 60193

CAS #: 7732-18-5 & 8016-20-4 & 90244-99-8 & 123465-35-0 (or) 8002-43-5

Test Request Form #: 10894

Lot #: N230627A

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

Test Performed:
Endothelial Permeability Assay

Introduction

Dark circles and discoloration under the eye, which can be caused by factors such as stress, lack of sleep, or dehydration, are associated with fatigue and a less youthful appearance. Undereye discoloration is a result of poor vascular integrity in the vessels lying close to the epidermis, facilitating the appearance of blue and purple hues of the dark circles. On a cellular level, endothelial function modulates vascular integrity. In particular, endothelial cell permeability plays a fundamental role in the formation of dark circles as increased permeability leads to a pooling of deoxygenated blood under the eye, resulting in discoloration and the appearance of aging.

The relationship between dermal fibroblasts and dermal endothelial cells is vital to maintaining vascular integrity. Specifically, fibroblasts synthesize the extracellular matrix, which provides an anchor point for endothelial cells and greatly reduces endothelial permeability. In response to endogenous and exogenous stimuli, fibroblasts release signaling molecules that promote extracellular matrix synthesis and endothelial cell function. Therefore, vascular integrity and undereye discoloration can be improved by augmenting the fibroblast-released beneficial molecules, resulting in a more youthful appearance.

Accordingly, an Endothelial Permeability Assay was conducted to assess the *in vitro* effect of **AC ExoVitalize** to reduce endothelial cell permeability via fibroblast-released signaling molecules.

Assay Principle

A multicellular approach followed by a colorimetric reaction was utilized to determine relative endothelial permeability. Human dermal fibroblasts are treated with various conditions and the signaling molecules (conditioned media) are collected. The conditioned media is then applied to a monolayer of human dermal microvascular endothelial cells with and without a known inflammatory agent, LPS. Finally, a colorimetric assay is utilized to determine endothelial permeability by observing the quantity of horseradish peroxidase that passes through the endothelial cell monolayer. Conditions that improve endothelial permeability will demonstrate lower absorbance values relative to the complete media + LPS treated monolayer as a result of less horseradish peroxidase passing through the endothelial cells.

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Materials

A. Incubation Conditions: 37°C, 5% CO_2, and 95% relative humidity

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)*; Normal Human Dermal Microvascular Endothelial Cells (ATCC, PCS-110-010)*

D. Media/Buffers: Fibroblast Basal Medium (PCS-201-030)*; Fibroblast Growth Kit (PCS-201-041)*; Vascular Cell Basal Medium (ATCC, PCS-100-030)*; Microvascular Endothelial Cell Growth Kit (ATCC, PCS-110-041)*

E. Reagents: Lipopolysaccharide (LPS) (1 µg/mL) (Fisher, 00-4976-03)*; Streptavidin-Horseradish Peroxidase (HRP) (3 µg/mL) (Biolegends, 405210)*; 3, 3', 5, 5' tetramethyl benzidine (TMB) Substrate Set (BioLegends, 421101)*; Stop Solution (BioLegends, 423001)*

F. Culture Plates: 24 Well Tissue Culture Treated Plates*, 6.5 mm Transwell inserts with 0.4 µm Pore Polycarbonate Membrane in 24 Well Plate (Corning, 3413)*, 96 Well Microplate*

G. Software: Excel Analysis ToolPak (Microsoft)

H. Other: Sterile disposable pipette tips; Sterile forceps

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

Methods

Human dermal fibroblasts were seeded into a 24-well tissue culture microplate and grown to 80%-90% confluency in fibroblast complete media. 0.5% and 1.0% concentrations of **AC ExoVitalize** were added to complete media. Complete media alone was employed as an experimental control. Testing conditions were added to fibroblasts and incubated at 37°C. Following a 48-hour incubation, the fibroblast conditioned media in each well was collected and frozen at -80°C.

Subsequently, human dermal microvascular endothelial cells (DMVECs) were seeded onto the membrane of each 6.5 mm transwell insert and grown to 100% confluency. Confluency was monitored by moving the inserts to empty wells for 5 minutes and evaluating if complete media passed through the cell monolayer and insert. If the well below was dry, cells were considered 100% confluent. A 1 µg/mL LPS + fibroblast conditioned media solution was prepared from the frozen fibroblast media treatments and diluted LPS stock solution. The endothelial monolayers were treated with the LPS + fibroblast conditioned media solutions, while the wells below contained endothelial complete media. LPS is utilized to create an inflammatory environment and fibroblast conditioned media alone was used as an Untreated Control. Following a 24-hour incubation at 37°C, treatment media was removed, and inserts were placed in a new 24-well plate containing fresh media.

200 µL of 3 µg/mL HRP solution was added to each insert and incubated for 15 minutes at 37°C. After incubation, transwell inserts were discarded and 20 µL of the media in the wells was transferred in duplicate to a 96-well plate. 50 µL of TMB substrate solution was added to all wells for the colorimetric reaction. After a 5-minute incubation in the dark, 25 µL of stop solution was added to stop the reaction and optical density (OD) was read at 450 nm. Permeability was normalized to the Untreated Control.

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 $p \leq 0.05$.

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Results

The data obtained from this study met criteria for a valid assay as the negative control performed as anticipated. Compared to Untreated Control, LPS (1 µg/mL) increased permeability of the monolayer. DMVECs exposed to LPS and treated with 0.5% and 1.0% **AC ExoVitalize** demonstrated reductions in endothelial permeability compared to LPS-treated DMVECs.

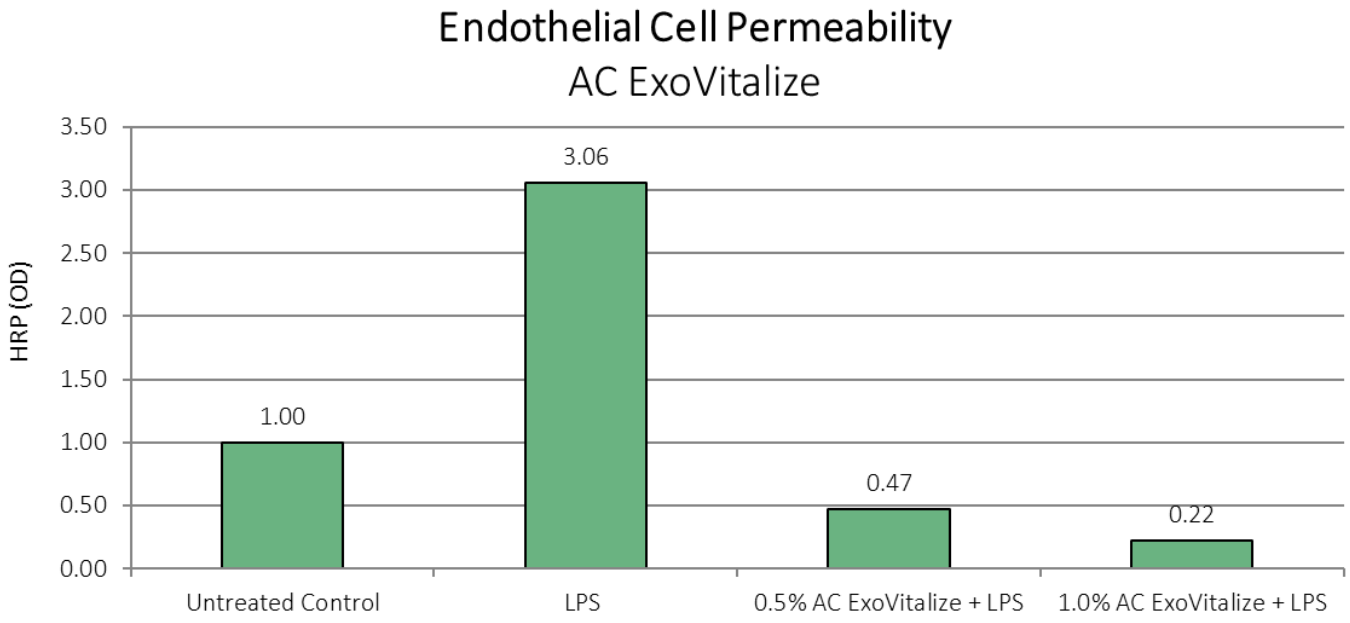

Figure 1. The Effect of **AC ExoVitalize** on Dermal Microvascular Endothelial Cell Permeability Relative to the Untreated Control.

Table 1. P-values from one-way ANOVA Statistical Analysis Compared to LPS-Treated DMVECs

	Untreated Control	0.5% **AC ExoVitalize**	1.0% **AC ExoVitalize**
P-value	0.019	< 0.001	< 0.001

Discussion

The ability of **AC ExoVitalize** to improve endothelial cell permeability via fibroblast-released signaling molecules was assessed. As shown in Figure 1, DMVECs incubated with LPS, a substance known to induce an inflammatory state, exhibited a 206% increase in endothelial permeability compared to the Untreated Control. This data demonstrates permeability of DMVECs is dynamic and can be manipulated with exogenous compounds.

Conversely, DMVECs treated with 0.5% and 1.0% **AC ExoVitalize** demonstrated 85% and 93% reductions in endothelial permeability compared to LPS-treated fibroblasts, respectively. Moreover, 0.5% and 1.0% **AC ExoVitalize** enhanced endothelial cell permeability by 53% and 78% compared to the Untreated Control, respectively. These data demonstrate **AC ExoVitalize** improves endothelial permeability.

Taken together, these data indicate **AC ExoVitalize** augments endothelial cell permeability by beneficially altering the signaling molecules released by fibroblasts. Collectively, **AC ExoVitalize** improves vascular integrity, diminishing the appearance of dark circles and discoloration under the eyes.