

Tradename: AC WonderShroom

Code: 20129

CAS #: 84650-60-2 & 999999-99-4 (or) 68917-13-5 & 999999-99-4 (or) 68917-13-5 & N/A (or) 9057-02-7

Test Request Form #: 12017

Lot #: N240425D

Sponsor: Active Concepts, LLC; 107 Technology Drive Lincolnton, NC 28092

Study Director: Daniel Shill

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Test Performed:

Oxidative Phosphorylation (Mitochondrial Membrane Potential) Assay

Introduction

Cellular metabolism can occur with (aerobic) or without (anaerobic) oxygen to generate ATP (adenosine triphosphate), the end molecular byproduct of metabolism. The stored energy within ATP is harvested and utilized by cells to maintain homeostasis as it is required for cellular migration and proliferation, molecular transportation across membranes, and the biosynthesis of cellular components. Aerobic metabolism, also known as oxidative phosphorylation, occurs within the mitochondria, generates a large amount of ATP, and is the primary source of cellular energy transduction. One driving force of oxidative phosphorylation is the mitochondrial membrane potential ($\Delta\Psi_m$), which represents the transmembrane potential of hydrogen ions. Maintaining $\Delta\Psi_m$ is necessary as the proton flux from cytosol to matrix is harnessed to generate ATP. This electrochemical gradient is not only utilized for ATP synthesis but is also an indicator of mitochondrial function and health as prolonged depressions in $\Delta\Psi_m$ reduce cell viability. Specifically, unregulated mitochondrial membrane potentials produce excessive amounts of oxidative stress which lead to an acceleration of DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation.

Accordingly, a Mitochondrial Membrane Potential Assay was conducted to assess the *in vitro* effect of **AC WonderShroom** to stimulate oxidative phosphorylation in dermal fibroblasts. L-theanine, a standardized component in **AC WonderShroom**, was also tested to demonstrate the superior nature of **AC WonderShroom** compared to L-theanine alone. Activating this biological process maintains cellular homeostasis and vitality.

Assay Principle

The Mitochondrial Membrane Potential Assay is based on the detection of the $\Delta\Psi_m$ in cells. The lipophilic and cationic JC-10 dye selectively enters mitochondria and concentrates in the mitochondrial matrix where it fluoresces red. The JC-10 dye reversibly changes color from green to orange as membrane potentials increase due to shifts in emitted light from 520 nm to 570 nm, expressed as modifications in the JC-10 from monomeric to J-aggregate, respectively. As $\Delta\Psi_m$ increases, ATP synthesis also increases, thus $\Delta\Psi_m$ can be utilized as a surrogate measurement of oxidative phosphorylation.

Materials

A. Kit:	JC-10 Mitochondrial Membrane Potential Assay Kit (Abcam; ab112134)*
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 Neonatal Human Dermal Fibroblasts (ATCC; PCS-201-010)*
E. Media/Buffers:	Fibroblast Basal Medium (PCS-201-030)*; Fibroblast Growth Kit (PCS-201-041)*; Ethanol
F. Reagents:	100x JC-10 Dye Solution (ab112134)*; Assay Buffer A (ab112134)*; Assay Buffer B (ab112134)*; Phosphate Buffered Saline (PBS); Antimycin A (Sigma Aldrich, A8674)*
G. Culture Plate:	96 Well Black Side/Clear Bottom Tissue Culture Treated Microplates
H. Software:	Excel Analysis ToolPack (Microsoft)
I. Other:	Sterile disposable pipette tips

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

Methods

Human neonatal dermal fibroblasts were seeded into a 96-well tissue culture microplate and grew to 80%-90% confluency in Complete Media. Complete Media and 0.01%, 0.05%, and 0.1% concentrations of **AC WonderShroom** in Complete Media were added to cells and placed at 37°C. Additionally, a solution of 0.0025% L-theanine was prepared in CM and placed on cells. This concentration is equivalent to the maximum standardized amount of L-theanine in 0.1% **AC WonderShroom**. Following a 24-hour incubation, the media in all wells was removed and cells were washed once with PBS. Next, 200 pM of Antimycin A, initially dissolved in ethanol and further diluted in Complete Media, was added to all wells, except control and treated wells which received Complete Media. Following a 30-minute incubation at 37°C, the media in all wells was removed and cells were washed once with PBS. Next, 50 µL of the JC-10 Dye Solution was added to all wells and the entire plate was incubated at 37°C for 30 minutes. Lastly, 50 µL of Assay Buffer B was added to all wells and fluorescence measurements were taken at Excitation/Emission wavelengths of 490/525 nm and 540/590 nm.

Regarding experimental controls, Complete Media alone was regarded as a baseline value and considered untreated, and Antimycin A (a known a complex III inhibitor of the mitochondrial electron transport chain) was utilized as a negative control to reduce oxidative phosphorylation.

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 $p \leq 0.05$. A ratio analysis of the fluorescent intensities (525/590) was performed. The $\Delta\Psi_m$ data is expressed as a percent of untreated fibroblasts (Complete Media) and calculated by the following equation:

$$\Delta\Psi_m (\% \text{ of Untreated}) = \frac{525/590 \text{ Ratio}_{\text{Sample}}}{525/590 \text{ Ratio}_{\text{Untreated}}} \times 100$$

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. Compared to untreated fibroblasts (Complete Media), Antimycin A (200 pM) reduced mitochondrial membrane potential. Fibroblasts treated with 0.0025% L-theanine did not experience changes in mitochondrial membrane potential. Fibroblasts treated with **AC WonderShroom** at 0.01%, 0.05%, and 0.1% demonstrated increased $\Delta\Psi_m$ compared to untreated fibroblasts.

Oxidative Phosphorylation

AC WonderShroom

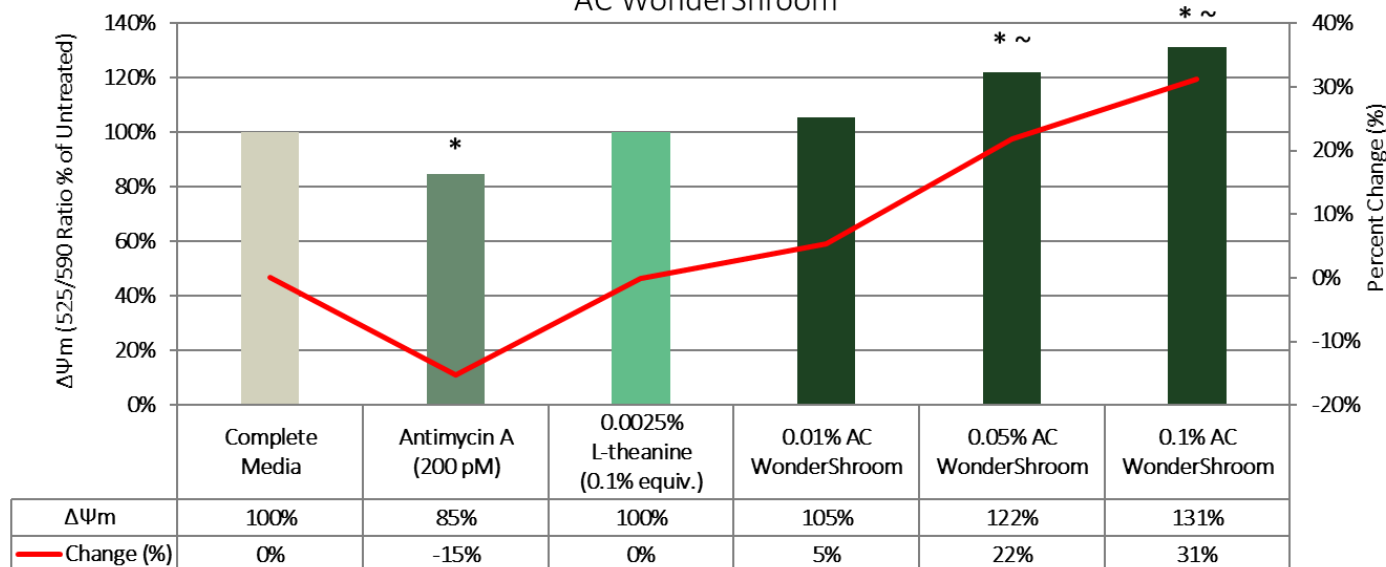


Figure 1. The Effect of AC WonderShroom on $\Delta\Psi_m$ in Dermal Fibroblasts. * indicates significance ($p < 0.05$) compared to Complete Media. ~ indicates significance ($p < 0.05$) compared to L-theanine. 0.0025% L-theanine is equivalent to the maximum standardized amount of L-theanine in 0.1% AC WonderShroom.

Table 1. Results from one-way ANOVA Statistical Analysis of $\Delta\Psi_m$. Results represent p-values between two conditions compared. * indicates significance ($p < 0.05$) compared to complete media. ~ indicates significance ($p < 0.05$) compared to L-theanine.

	Antimycin A	0.0025% L-theanine	0.01% AC WonderShroom	0.05% AC WonderShroom	0.1% AC WonderShroom
Complete Media	0.044*	> 0.05	> 0.05	0.031*	0.009*
0.0025% L-theanine	0.020~	-----	> 0.05	0.040~	0.018~

Discussion

As shown in Figure 1, fibroblasts incubated with Antimycin A, a known oxidative phosphorylation disruptor, exhibited a significant 15% reduction in $\Delta\Psi_m$ compared to untreated fibroblasts (Table 1). These data demonstrate $\Delta\Psi_m$ in fibroblasts is dynamic and oxidative phosphorylation can be manipulated with exogenous compounds.

Conversely, fibroblasts treated with **AC WonderShroom** at 0.01%, 0.05%, and 0.1% demonstrated significant 5%, 22%, and 31% increases in $\Delta\Psi_m$ compared to untreated fibroblasts, respectively (Figure 1; Table 1). Comparatively, L-theanine did not change $\Delta\Psi_m$ compared to untreated fibroblasts and was significantly less effective than the equivalent 0.1% **AC WonderShroom**. These data demonstrate **AC WonderShroom** increases $\Delta\Psi_m$ and oxidative phosphorylation in fibroblasts and highlight the superior nature of the product compared to the actives alone.

In summary, oxidative phosphorylation is a biological process that generates ATP to assist in cellular migration and proliferation, molecular transportation across membranes, and the biosynthesis of cellular components. A necessary component of oxidative phosphorylation is the maintenance of $\Delta\Psi_m$ given prolonged reductions in this electrochemical gradient are indicative of poor mitochondrial health. Increasing oxidative phosphorylation not only maintains $\Delta\Psi_m$, but also reduces the formation of reactive oxygen species which augments the ability of dermal cells to reduce characteristics of cellular aging (e.g. DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation). Collectively, these data indicate **AC WonderShroom** stimulates oxidative phosphorylation, demonstrated by increases in mitochondrial membrane potential, which assists in the maintenance of cellular homeostasis, vitality, mitochondrial function, and attenuate the physical signs of cellular aging.