Reactive Oxygen Species Scavenging Assay

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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 #: 13337

Lot #: N250520B

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

Test Performed:
Reactive Oxygen Species Scavenging Assay

Introduction

Low levels of intracellular oxidative stress are produced during normal physiological functions. However, UV irradiation, pollutants, foreign substances, and aging elicit unrestricted increases in reactive oxygen species (ROS). These deregulated augmentations in oxidative stress lead to an acceleration of DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation. Moreover, when intrinsic antioxidant capacities are reduced, such as during aging, an imbalance between pro- and anti-oxidant systems further accentuates these hallmarks of cellular aging.

Accordingly, a ROS Scavenging Assay was conducted to assess the *in vitro* effect of **AC ExoRoot** to scavenge unnecessary oxidative stress 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. Attenuating excessive ROS preserves cellular homeostasis and blunts intrinsic and extrinsic age-related declines in hair health.

Assay Principle

Two cell-permeant dyes, CellROX™ Orange Reagent and Hoechst, were utilized in conjunction to provide a specific and quantitative method for determining ROS levels. CellROX™ Orange Reagent fluoresces brightly when bound to ROS indicating oxidative stress, and Hoechst fluoresces when bound to nuclear DNA to indicate cellular nuclei. By displaying the relative fluorescent units (RFU) from the CellROX™ Orange Reagent (ROS Signal) as a function of Hoechst (Nuclear Signal), ROS can be quantified and normalized at the cellular level. To elicit supraphysiological mitochondrial- and non-mitochondrial-derived levels of oxidative stress, the cells were exposed to Antimycin A, a complex III inhibitor of the mitochondrial electron transport chain.

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Materials

A. Kit:	CellROX™ Orange Reagent (ThermoFisher Scientific, C10443)*
B. Incubation Conditions:	37°C, 5% CO_2, and 95% relative humidity (RH)
C. Equipment:	Forma humidified incubator; ESCO biosafety laminar flow hood; Light microscope; Synergy HT Microplate Reader; Pipettes
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)*; Ethanol; Phosphate Buffered Saline (PBS)
F. Reagents:	Hoechst 33342 (ThermoFisher Scientific, 62249)*; Antimycin A (Sigma Aldrich, A8674)*; Minoxidil (Millipore Sigma)
G. Culture Plate:	96 Well Black Side/Clear Bottom Tissue Culture Treated Microplates
H. Software:	Excel Analysis Toolpak (Microsoft)
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 96-well tissue culture microplate and grown to 80%-90% confluency in complete media (CM). 0.01%, 0.02%, 0.05%, and 0.1% concentrations of **AC ExoRoot** in CM were added to cells and placed at 37°C. 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 Complete Media. Control wells were incubated with CM only and all conditions were tested in duplicate. Following an 18-hour incubation, the media in all wells was removed and cells were washed once with PBS. Hoechst and CellROX™ Orange were diluted in CM and added to all wells at final concentrations of 20 µM and 5 µM, respectively. Following a 30-minute incubation at 37°C, the Hoechst and CellROX™ Orange solution was removed, and cells were washed once with PBS. Next, 200 pM of Antimycin A (AntA), initially dissolved in ethanol and further diluted in CM, was added to all wells, except control wells that received CM. Following another 30-minute incubation at 37°C, the AntA and CM was removed, CM was added to all wells, and fluorescence measurements were taken with the following wavelengths (excitation / emission): Hoechst (361 nm / 486 nm) and CellROX™ Orange (545 nm / 565 nm).

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 $p \leq 0.05$. To account for differences in cell counts, ROS levels are expressed as the ROS Signal (CellROX™ Orange) divided by the Nuclear Signal (Hoechst), as calculated by the following equation:

$$ROS\ Levels = \frac{ROS\ Signal}{Nuclear\ Signal}$$

Percent change is expressed relative to AntA and calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{RFU_{Sample} - RFU_{AntA}}{RFU_{AntA}} \times 100$$

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Results

The data obtained from this study met criteria for a valid assay and the positive control performed as anticipated. Compared to untreated cells, AntA (200 pM) increased ROS levels. Dermal papilla cells treated with minoxidil, *Chlorella vulgaris* Extract, **AC ExoRoot**, and **AC ExoRoot** + minoxidil all exhibited reductions in oxidative stress levels compared to cells exposed to AntA.

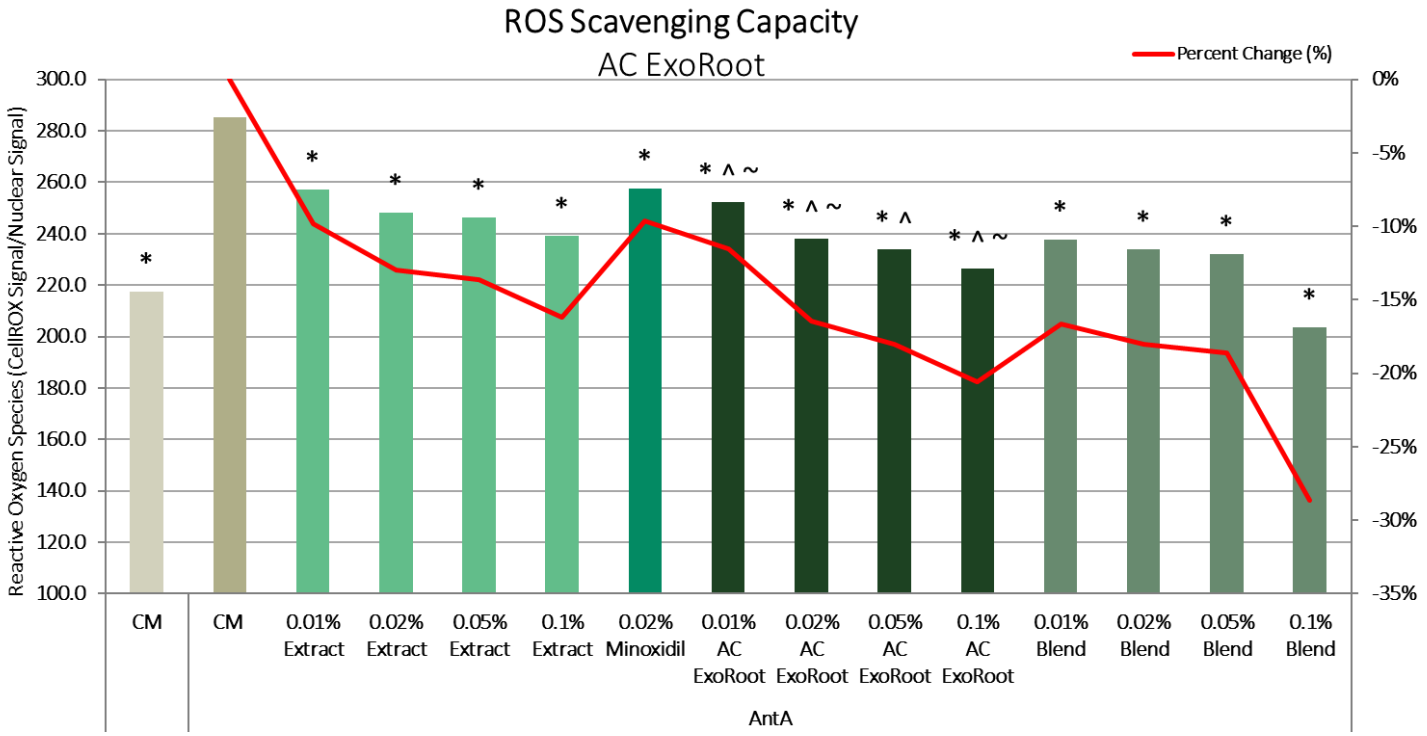

Figure 1. The effect of **AC ExoRoot** on ROS scavenging. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to AntA treated cells. ^ indicates significance ($p \leq 0.05$) compared to *Chlorella vulgaris* Extract. ~ indicates significance ($p \leq 0.05$) compared to **AC ExoRoot** + 0.02% minoxidil.

Table 1. P-values from one-way ANOVA Statistical Analysis compared to AntA treated cells. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to AntA treated cells.

	CM	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*	0.023*	0.032*	0.012*	0.008*	0.030*	0.019*	0.008*	0.007*	0.004*	0.024*	0.018*	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	0.006^	0.016^	0.027^	0.026^
AC ExoRoot + 0.02% Minoxidil	0.018~	0.046~	> 0.05	0.010~

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Discussion

As shown in Figure 1, dermal papilla cells incubated with AntA, a known inducer of oxidative stress, elicited a 31% increase in ROS levels, compared to untreated cells. These data demonstrate the supraphysiologic level of ROS induced by AntA and the magnitude of ROS in dermal papilla cells is dynamic.

Conversely, dermal papilla cells treated with **AC ExoRoot** at 0.01%, 0.02%, 0.05%, and 0.1% demonstrated significant 12%, 16%, 18%, and 21% reductions in ROS levels compared to cells treated with AntA, respectively (Table 1). Similarly, cells treated with *Chlorella vulgaris* Extract at 0.01%, 0.02%, 0.05%, and 0.1% demonstrated significant 10%, 13%, 14%, and 16% reductions in ROS levels compared to cells treated with AntA, respectively (Table 1). However, at all concentrations **AC ExoRoot** demonstrated significantly lower ROS levels compared to *Chlorella vulgaris* Extract highlighting the superior nature of the bioauthentic exosomes as a delivery system (Table 2). These data demonstrate **AC ExoRoot** attenuates excessive oxidative stress in a dose-dependent fashion.

Similarly, cells treated with 0.02% minoxidil exhibited 10% reduction in ROS levels compared to cells treated with AntA (Figure 1; Table 1). Moreover, cells treated with 0.01%, 0.02%, 0.05% and 0.1% **AC ExoRoot** with 0.02% minoxidil exhibited 17%, 18%, 19%, and 29% reductions in ROS levels compared to AntA treated cells, respectively. At 0.01%, 0.02% and 0.1%, the blend exhibited a significant synergistic effect compared to **AC ExoRoot** alone (Table 2). These data indicate **AC ExoRoot** can be used in conjunction with minoxidil to reduce ROS and protect hair follicles.

Collectively, intrinsic and extrinsic factors perturb hair homeostasis by stimulating abundant levels of ROS that amplify DNA mutation, cellular senescence, advanced glycation end products, protein oxidation, and collagen degradation. These data indicate **AC ExoRoot** scavenges unnecessary ROS, which may help to attenuate characteristics of cellular aging.