SA-β-gal Activity | Cellular Aging Model

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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 #: 13566

Lot #: N250520B

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

Test Performed:
Senescence-Associated β-galactosidase (SA-β-gal) Activity
Cellular Aging Model: Fibroblasts

Introduction

Cellular senescence is a state of permanent cell cycle arrest that accompanies aging and contributes to a decline in normal scalp physiology and hair health. Contrary to apoptosis, defined as programmed cell death resulting in the clearance of damaged cells, senescent cells are stable, viable, and communicate with neighboring cells. Senescence is characterized by changes in cellular morphology, metabolism, signaling pathways, and biochemical profiles that manifest as increased collagen breakdown, reduced hair growth, and thinning hair. SA-β-gal is the gold standard biomarker to identify senescence *in vitro* as the enzyme β-galactosidase explicitly accumulates in the lysosomes of senescent cells.

Accordingly, a cellular aging model was developed to assess the *in vitro* effect of **AC ExoRoot** to reduce SA-β-gal activity in "aged" fibroblasts. 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 cellular senescence could blunt or prevent the age-related decline in scalp physiology and hair health.

Assay Principle

A model of cellular aging was developed by utilizing fibroblasts at different passage numbers (P), which represent the number of times cells have been harvested and reseeded into subsequent cell culture vessels (i.e. the higher the passage number, the more times those cells have been harvested and reseeded). Fibroblasts at low passages (≤ P5) demonstrate morphological, metabolic, and senescent biomarker profiles matching that of young skin *in vivo*. Conversely, fibroblasts at high passages (≥ P8) exhibit the cellular characteristics of older skin *in vivo*.

Hoechst and SPiDER-ßGal dyes were utilized in conjunction to provide a specific and quantitative method for determining cellular senescence. Hoechst, a cell-permeant dye, fluoresces brightly when bound to nuclear DNA to indicate cellular nuclei, whereas SPiDER-ßGal fluoresces when bound to β-galactosidase indicating cellular senescence. SA-β-gal activity is normalized by displaying the relative fluorescent units (RFU) from the SPiDER-ßGal dye (β-galactosidase Signal) as a function of the Hoechst dye (Nuclear Signal) RFU and set relative to "young" fibroblasts to underscore the effect of cellular aging on senescence.

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Materials

A. Kit:	Cellular Senescence Plate Assay Kit – SPiDER-ßGal (Dojindo, SG05)*
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); Minoxidil (Millipore Sigma)
F. Reagents:	Hoechst 33342 (ThermoFisher Scientific, 62249)*; Dimethyl Sulfoxide (DMSO)
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

"Young" and "aged" human dermal fibroblasts from the same lot were seeded into a 96-well tissue culture microplate and grown to 80%-90% confluency in complete media (CM) at 37°C. "Young" fibroblasts were utilized in this assay at passage P4, while "aged" fibroblasts were utilized at passage P8. "Young" and "aged" fibroblasts incubated with CM only are utilized as controls, whereas 0.02% minoxidil in CM and 0.01%, 0.02% and 0.05% concentrations of *Chlorella vulgaris* Extract, **AC ExoRoot**, and **AC ExoRoot** with 0.02% minoxidil in CM were added to "aged" fibroblasts only. Following a 24-hour incubation, the media in all wells was removed and cells were washed once with PBS. The nuclear dye Hoechst was diluted in CM, added to all wells at final concentration of 20 µM, and incubated at 37°C.

Following a 30-minute incubation, the Hoechst dye was removed, all cells were washed once with PBS, CM was added to all wells, and fluorescence measurements were taken to determine the Nuclear Signal (excitation: 361 nm / emission: 486 nm). Subsequently, the CM was removed, all cells were washed once with PBS, and Lysis Buffer was added to each well. After a 10-minute incubation at room temperature, the SPiDER-ßGal dye was added to each well and incubated for 30 minutes at 37°C. The Stop Solution was then added to each well and fluorescence measurements were taken to determine the β-galactosidase Signal (excitation: 535 nm / emission: 580 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, normalized SA-β-gal activity is calculated as the β-galactosidase Signal (SPiDER-ßGal dye) divided by the Nuclear Signal (Hoechst dye), as shown by the following equation:

$$Normalized\ SA\text{-}\beta\text{-}gal\ Activity = \frac{\beta\text{-}galactosidase\ Signal}{Nuclear\ Signal}$$

SA-β-gal activity is displayed as relative to the normalized SA-β-gal activity in "young" fibroblasts by the following equation:

$$SA\text{-}\beta\text{-}gal\ Activity = \frac{Normalized\ SA\text{-}\beta\text{-}gal\ Activity_{Sample}}{Normalized\ SA\text{-}\beta\text{-}gal\ Activity_{Young\ Fibroblasts}}$$

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Percent change is calculated from the SA-β-gal activity in "aged" fibroblasts and calculated by the following equation:

$$Percent\ Change\ (\%) = \frac{SA\text{-}\beta\text{-}gal\ Activity_{Sample} - SA\text{-}\beta\text{-}gal\ Activity_{Aged\ Fibroblasts}}{SA\text{-}\beta\text{-}gal\ Activity_{Aged\ Fibroblasts}} \times 100$$

Results

The data obtained from this study met criteria for a valid assay and the controls performed as anticipated. Compared to "aged" fibroblasts, "young" fibroblasts demonstrated lower levels of SA-β-gal activity. "Aged" fibroblasts treated with *Chlorella vulgaris* Extract alone did not exhibit significant reductions in SA- β-gal activity compared to untreated "aged" fibroblasts. Comparatively, "aged" fibroblasts treated with minoxidil, **AC ExoRoot**, and **AC ExoRoot** with minoxidil all exhibited reductions in SA-β-gal activity compared to untreated "aged" fibroblasts.

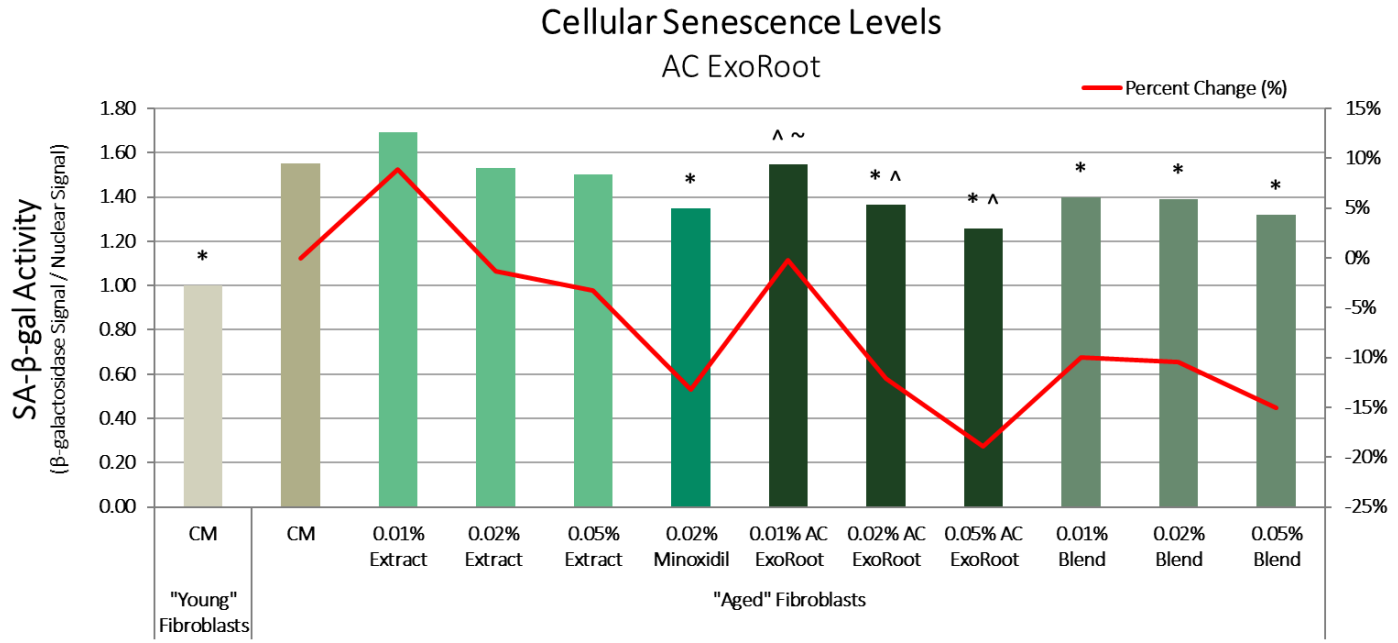

Figure 1. The effect of **AC ExoRoot** on cellular senescence levels in "aged" fibroblasts. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to "aged" fibroblasts. ^ indicates significance ($p \leq 0.05$) compared to *Chlorella 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 "aged" fibroblasts. Extract: *Chlorella vulgaris* Extract. Blend: % **AC ExoRoot** + 0.02% minoxidil. * indicates significance ($p \leq 0.05$) compared to "aged" fibroblasts.

	"Young" Fibroblasts	"Aged" Fibroblasts									
		0.01% Extract	0.02% Extract	0.05% Extract	0.02% Minoxidil	0.01% **AC ExoRoot**	0.02% **AC ExoRoot**	0.05% **AC ExoRoot**	0.01% Blend	0.02% Blend	0.05% Blend
P Value	< 0.001*	> 0.05	> 0.05	> 0.05	0.015*	> 0.05	0.045*	0.021*	0.005*	0.029*	0.033*

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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**
Chlorella vulgaris Extract	0.036^	0.036^	0.043^
AC ExoRoot + 0.02% Minoxidil	0.012~	> 0.05	> 0.05

Discussion

As shown in Figure 1, "aged" fibroblasts demonstrated 55% higher levels of SA-β-gal activity compared to "young" fibroblasts. These data demonstrate "young" fibroblasts exhibit reduced levels of cellular senescence compared to "aged" fibroblasts.

"Aged" fibroblasts treated with **AC ExoRoot** at 0.02% and 0.05% elicited significant 12% and 19% reductions in SA-β-gal activity compared to untreated "aged" fibroblasts, respectively (Figure 1; Table 1). Comparatively, *Chlorella vulgaris* Extract at 0.02% and 0.05% only elicited reductions of 1% and 3% in SA-β-gal activity compared to untreated "aged" fibroblasts, respectively, and was significantly less effective than **AC ExoRoot** highlighting the superior nature of the bioauthentic exosomes as a delivery system (Table 2). Both *Chlorella vulgaris* Extract and **AC ExoRoot** at 0.01% did not elicit reductions in SA-b-gal activity. These data demonstrate **AC ExoRoot** attenuates cellular senescence in "aged" fibroblasts in a dose-dependent fashion.

Similarly, "aged" fibroblasts treated with 0.02% minoxidil exhibited a 13% reduction in SA-β-gal activity compared to untreated "aged" fibroblasts (Figure 1; Table 1). Moreover, "aged" cells treated with 0.01%, 0.02%, and 0.05% **AC ExoRoot** with 0.02% minoxidil exhibited 10%, 10%, and 15% reductions in SA-β-gal, respectively, compared to untreated "aged" fibroblasts. At 0.01% the blend exhibited an additive effect compared to **AC ExoRoot** alone, but there were no significant benefits or drawbacks observed at 0.02% and 0.05% (Table 2). These data indicate **AC ExoRoot** can be used in conjunction with minoxidil without negatively impacting **AC ExoRoot**'s anti-aging properties.

Senescence is a hallmark characteristic of cellular aging that contributes to the age-associated modifications of scalp function and composition *in vivo*, such as loss of scalp barrier function, increased hair thinning, and reduction in hair growth. Specifically, cellular senescence is associated with increased inflammation, oxidative stress, and impaired collagen homeostasis. Collectively, these data indicate that **AC ExoRoot** reduces cellular senescence and may attenuate or reverse the alterations in scalp physiology that occur during aging.