Cellular Detoxification Assay

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Tradename: AC Det'Ox Hair

Code: 21030

CAS #: 8013-01-2 & 68333-16-4 (or) 92128-79-5

Test Request Form #: 13095

Lot #: N250115A

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

Test Performed:
Cellular Detoxification / Autophagy Detection Assay

Introduction
Autophagy is the self-identification and delivery of damaged cellular proteins and organelles, intracellular microbes, and toxins to lysosomal vacuoles for breakdown. The byproducts of this process are either excreted from the cell, utilized for energy production, or to support catabolic pathways. This removal of cellular damage regulates cell survival and function, as a reduction in autophagy (i.e. an accumulation of cellular damage) increases inflammation and reactive oxygen species, perturbs metabolism, and promotes senescence, all of which disrupt hair health at cellular level.

An Autophagy Detection Assay was conducted to assess the *in vitro* effect of **AC Det'Ox Hair** to restore normal autophagy in hair follicle dermal papilla cells in the presence of heavy metals. Exposure to heavy metals disrupts this biological detoxification process that maintains cellular homeostasis and accelerates the age-related decline in hair follicle cell function.

Assay Principle
The Green Detection Reagent, supplied in Abcam's Autophagy Detection Kit, fluoresces when bound to autophagic-lysosomal vacuoles produced during autophagy to indicate autophagic activity, while the Nuclear Stain fluoresces when bound to nuclear DNA to indicate cellular nuclei. These two dyes work in conjunction to provide a specific and quantitative method for determining autophagic activity. By displaying the Green Detection Reagent fluorescent signal as a function of the Nuclear Stain fluorescent signal, autophagic activity at the cellular level can be quantified and normalized.

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Materials

A. Kit:	Autophagy Detection Kit (Abcam; ab139484)*
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:	Human Hair Follicle Dermal Papilla Cells (HFDPCs) (Cell Applications Inc; 602K-05a)*
E. Media/Buffers:	Complete Follicle Dermal Papilla Cell Growth Medium (Cell Applications Inc.; C-26501)*; Collagen Coating Solution (Cell Applications Inc.; 125-100)*; Dimethyl Sulfoxide (DMSO); 10x Assay Buffer (ab139484)*; Deionized water
F. Reagents:	Rapamycin (ab139484)*; Chloroquine (ab139484)*; Nuclear Stain (ab139484)*; Green Detection Reagent (ab139484)*
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 Hair Follicle Dermal Papilla Cells (HFDPCs) were seeded into a collagen coated 96-well tissue culture microplate and grew to 80%-90% confluency in Complete Media. 0.01%, 0.02%, and 0.04% concentrations of **AC Det'Ox Hair** were added to Complete Media with and without the presence of Heavy Metals (Table 1), while Rapamycin and Chloroquine were initially reconstituted in DMSO and deionized water, respectively, and further diluted with just Complete Media. As experimental controls, Chloroquine at 40 µM was utilized as a negative control to inhibit autophagic-lysosomal vacuole development, and Rapamycin at 10 µM was utilized as a positive control to augment autophagic-lysosomal vacuole development. All conditions, tested in duplicate, were added to HFDPCs and incubated at 37°C. Following an 18-hour incubation, the media in all wells was removed and cells were washed twice with 1x Assay Buffer. The Nuclear Stain and Green Detection Reagent were diluted in Complete Media and added to all wells. After a 30-minute incubation at 37°C, the Nuclear Stain and Green Detection Reagent were removed from all wells, and cells were washed with 1x Assay Buffer. Next, fluorescence measurements were taken with the following wavelengths (excitation / emission): Nuclear Stain (350 nm / 461 nm) and Green Detection Reagent (463 nm / 534 nm).

Table 1. Heavy Metal Elements and Concentrations.

Element	Concentration (ppm)
Lead	0.1
Cadmium	0.05
Manganese	3.0
Chromium	1.0
Nickel	1.0
Zinc	50
Copper	13
Fluoride	40
Selenium	0.5
Thallium	0.02
Calcium	500

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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, Cellular Detoxification values are expressed as the Autophagic-Lysosomal Vacuoles Signal (Green Detection Reagent) divided by the Nuclear Signal (Nuclear Stain), as calculated by the following equation:

$$Cellular\ Detoxification = \frac{Autophagic/Lysosomal\ Vacuoles\ Signal}{\text{Nuclear Signal}}$$

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

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

Results

The data obtained met criteria for a valid assay as the positive control, negative control, and Heavy Metals performed as anticipated. Compared to untreated HFDPCs, Chloroquine (40 µM) reduced autophagic-lysosomal vacuoles whereas Rapamycin (10 µM) and exposure to Heavy Metals increased autophagic-lysosomal vacuoles. HFDPCs exposed to Heavy Metals and treated with AC Det'Ox Hair at 0.01%, 0.02%, and 0.04% normalized autophagic activity compared to untreated HFDPCs.

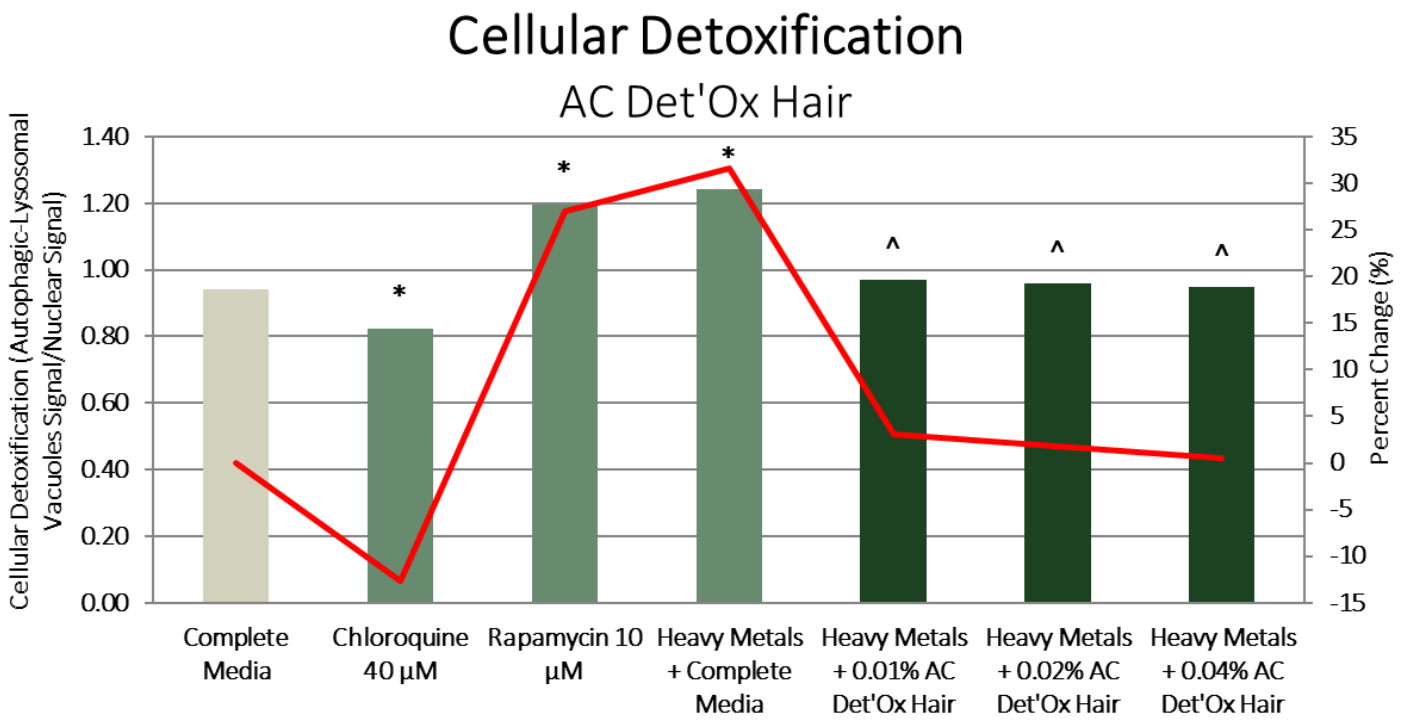

Figure 1. The Effect of AC Det'Ox Hair on Hair Follicle Dermal Papilla Cells Cellular Detoxification With and Without Heavy Metals Exposure. * indicates significance ($p \leq 0.05$) compared to Complete Media. ^ indicates significance ($p \leq 0.05$) compared to Heavy Metals.

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Table 2. P-values from one-way ANOVA Statistical Analysis between the two conditions compared. * indicates significance ($p \leq 0.05$) compared to Complete Media. ^ indicates significance ($p \leq 0.05$) compared to Heavy Metals.

	0.01% AC Det'Ox Hair	0.02% AC Det'Ox Hair	0.04% AC Det'Ox Hair
Complete Media	> 0.05	> 0.05	> 0.05
Heavy Metals	< 0.05^	< 0.05^	< 0.05^

Discussion

As shown in Figure 1, HFDPCs incubated with Chloroquine, a known autophagy inhibitor, exhibited a 13% reduction in autophagic-lysosomal vacuoles compared to untreated HFDPCs. Conversely, HFDPCs exposed to Rapamycin elicited a 27% increase in autophagic-lysosomal vacuoles compared to untreated HFDPCs. Similarly, HFDPCs exposed to Heavy Metals demonstrated a 32% increase in autophagic activity compared to untreated HFDPCs. These data demonstrate cellular detoxification in HFDPCs is dynamic and can be manipulated with exogenous compounds.

Conversely, HFDPCs treated with Heavy Metals and AC Det'Ox Hair at 0.01%, 0.02%, and 0.04% demonstrated 22%, 23%, and 24% reductions in autophagic-lysosomal vacuoles compared to HFDPCs treated with Heavy Metals, respectively. Importantly, HFDPCs treated with Heavy Metals and AC Det'Ox Hair restored autophagic activity similar to the level of untreated HFDPCs. These data demonstrate AC Det'Ox Hair protects HFDPCs against Heavy Metal-induced increases in cellular detoxification.

Collectively, autophagy is a biological process that identifies toxins within the cell and removes or recycles damaged cellular components to maintain homeostasis. These data indicate AC Det'Ox Hair protects this cellular detoxification process against Heavy Metal-induced autophagic perturbations, which may help to attenuate characteristics of cellular aging.