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RESEARCH ARTICLE

Klotho containing serum protects from ultra-violet A induced damage *in vitro*

Elijah Finn[1], Lucia Dussan[1], Scott Rosenthal[1], Peter Snitzer[2], Gail Humble[3], Peter Sykora[4]*

[1]Amelia Technologies, 1121 5th St NW, Washington, DC 20001
[2]Apogee International Inc., Fairfax, VA, 22030
[3]Aesthetic Anti-Aging Clinic, San Francisco, CA, USA
[4]Amelia Technologies LLC, Washington, DC 20001; Department of Biochemistry and Molecular & Cellular Biology, Georgetown University Medical Center, Washington, DC, USA.

*peters@ameliatechnologies.com

ABSTRACT

The skin is the largest organ in the body to come into contact with solar UV radiation. The UV-A (UVA) spectrum makes up over 95% of solar-UV radiation energy reaching the Earth's surface and has been recognized as a significant contributor to skin damage. UVA can penetrate beyond the epidermal skin layer into the dermal layer containing fibroblasts. UVA exposure can induce oxidative DNA damage including 8-oxo-G adducts, as well as single-stranded breaks and apurinic -sites believed to accelerate photo-aging. We investigated the protective impact of klotho protein after UVA exposure. Overexpression of the klotho protein was previously shown to extend lifespan in transgenic mice and give them a youthful appearance compared to litter mates. This rejuvenation impact of klotho was demonstrated to be, in-part, due to the protein inducing protective cellular response pathways including principal antioxidants proteins superoxide dismutase and catalase. Using human fibroblast cells, we measure the ability of klotho to reduce endogenous as well as UVA-associated DNA damage. We report that exposure of fibroblast cells to klotho containing serum reduces the amount of measurable endogenous DNA damage when used at the optimal concentration. Further, using a novel high through-put UVA platform we demonstrate that pre-incubation with 1% klotho containing media significantly reduces the amount of oxidative DNA damage that is induced, post-UVA exposure. Future research will evaluate whether klotho containing media is also protective in other skin cell types and more complex 3D models.

Introduction

Photoaging, as opposed to chronological aging, is the premature aging of the skin due to radiation from the sun or artificial sources such as tanning beds. Ultraviolet A (UVA) radiation has a prominent role in photoaging, it can penetrate deep into the dermis, causing damage to collagen and elastin fibers. This leads to the formation of wrinkles, sagging skin, and the development of age spots. UVA radiation also contributes to the production of reactive oxygen species (ROS) in the skin, leading to oxidative stress and inflammation. Over time, this can result in a loss of skin elasticity and the breakdown of supporting structures, accelerating the aging process. As opposed to the epidermal layer containing primarily keratinocytes, the dermal layer contains fibroblasts that may be more susceptible to UVA induced damage[1,2]. Furthermore, UVA radiation can impair the skin's natural repair mechanisms, making it more vulnerable to environmental damage.

Several compounds have been identified for their ability to protect the skin against UVA damage. These chemicals can be grouped according to the mechanism of action. Zinc oxide and titanium dioxide are physical UVA blockers providing a physical light blocking barrier. In contrast, avobenzone is a chemical blocker of UVA and has been widely used for the previous three decades in the US market. Physical and chemical sunscreen active ingredients stop the UV from penetrating the skin. However, if UVA has penetrated the skin, it is possible to mitigate the extent of induced oxidative damage through the pre-exposure application of antioxidant compounds. Compounds like vitamin C, vitamin E, and various plant-derived antioxidants such as flavonoids and polyphenols help neutralize reactive oxygen species generated by UVA radiation, reducing oxidative stress and related inflammatory response. The final group of UVA protective agents modulate the signaling pathways often associated with metabolism. These agents are often touted as anti-aging compounds and include resveratrol[3], rapamycin[4] and nicotinamide[5]. The common mechanism of action of these metabolic modulators against UVA includes reducing endogenous DNA damage levels and oxidative stress by increasing antioxidant response and activating autophagic pathways. These factors are characteristic of a metabolic switch in the somatotrophic axis[6].

Similarly, the klotho family of protein has also previously been shown to have broad anti-aging properties. There are two main forms of the klotho protein: α-klotho and β-klotho. α-klotho is primarily expressed in the kidneys, parathyroid glands, and choroid plexus of the brain, while β-klotho is more widely expressed in tissues such as the liver, pancreas, and adipose tissue. The α-protein has been associated with several important functions including influencing insulin sensitivity and protecting against oxidative stress. Research has also suggested that klotho may have protective effects against age-related conditions such as cardiovascular disease, chronic kidney disease, and neurodegenerative disorders. How klotho suppresses aging is currently under investigation, however it appears to be implicated in the somatotrophic axis modulating the activity of insulin and insulin-like growth factor (IGF-1) signaling[7]. In previous studies mice that had a KO or reduced expression of α-klotho had elevated levels of oxidative DNA damage markers (8-OHdG), lipid oxidation and apoptosis[8]. Of particular interest for skin research, mice that overexpress α-

klotho protein had a more youthful appearance and extended lifespan[9,10]. Given the mechanistic similarity of klotho with other UVA protective anti-aging compounds we asked whether α-klotho could also mitigate UVA induced DNA damage in human fibroblast cells. To this end, we partially purified human klotho protein, serum and exposed the human fibroblast cells to the enriched media before measuring the genotoxic impact on endogenous and UVA induced DNA damage levels. We report klotho protein supplement in media was able to protect the cells against UVA induced damage.

Materials and Methods

Cell culture

Immortalized Normal Human Dermal Fibroblasts (NHDF) were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Evansville, IN), supplemented with 10% Fetal Bovine Serum (FBS, Peak Serum, Wellington, CO) and 1% Pen Strep (Gibco). Cells were cultured at 37ºC in a 5% CO_2 incubator.

Klotho containing media.

The preparation of α-klotho containing serum has been previously described in[11]. In brief, the klotho ORF was inserted into a commercial lenti-viral plasmid (pLenti-P2A-Puro, Origene, Rockville, MD). In preparation for transfection, human primary Mesenchymal Stem Cells (hMSC): (ATCC, Manassas, VA) were grown in MSC low serum basal media and plated in 6 well plates at 5 x 10^5 cells/1.5 mL media per well. Viromer RED and buffer solution, a polymer-based transfection reagent. (Origene) was used to transfect the cells. Next, 200 µl of the plasmid and Viromer RED complex was directly added to the hMSC cells. The cells were incubated at 37°C in a 5% CO^2 incubator for 1-2 days. Prior to the antibiotic addition the media was gently removed from each well and then 2mL fresh media and antibiotic solution were added to a final Puromycin concentration of 0.5 µg/mL. Cells were further incubated for 2 weeks, and fresh media with puromycin was replaced once every 5-7 days. The cells were then distributed to 10 cm tissue culture dishes and over the next 2-3 weeks, the concentration of puromycin was gradually increased to 1 µg/mL final concentration. During this selection process, 2 mL fresh media and antibiotic solution was replenished once every 5-7 days. Then, single cell colonies were picked up by glass cloning ring method. The colonies were maintained at 1 µg/ml of puromycin selection media, in 24-well plates. The cell culture supernatant was collected after 10-12 days of cell growth and screened for klotho protein expression by quantitative ELISA method following the manufacturer's protocol. The cell culture supernatant wells that indicated the presence of klotho protein were identified as klotho producing hMSC clones and were maintained in puromycin selection media (1 µg/ml) and regularly screened for klotho protein expression in the cell culture supernatant with each passage. These cells (hMSC-klotho) were then scaled up for bulk production of klotho media.

Cellular proliferation assay.

Normal Human Dermal Fibroblasts (NHDFs) were seeded in 96-well plates at 10^4 cells/well and were left overnight in a 37°C incubator prior to klotho treatment and UVA exposure. Cells were then exposed to 0-50% klotho enriched media, 0-237 kJ/m^2 UVA, as well as klotho enriched media followed by UVA exposure. Plates were incubated for 24 hours

following each treatment before viability was measured using DAPI stain after cells had been fixed with 4% paraformaldehyde and permeated with 0.2% Triton. Plates were incubated for 5 minutes at 4°C and cell count was taken using the BioTek Cytation 7 cell imaging multimode reader (Agilent, Wilmington, DE).

Assessment of endogenous DNA damage using the alkaline comet assay.

To measure the amount of endogenous DNA damage in the cells after klotho serum exposure, the cells were run in an alkaline comet assay[12]. Cells were seeded in 96-well plates at 10^4 cells/well and were left overnight in a 37°C incubator. Cells were then treated with klotho serum for 24-hours at concentrations of 0-4% diluted in culture media. After the treatment, the diluted serum was removed, and cells were gravity loaded into the chip for 15 minutes. After loading, the comet chip was washed with PBS and sealed with 0.8% low melting point agarose (LMPA) before being placed in pH 10 lysis buffer solution for 40 minutes. The comet assay was then transferred to alkaline buffer for 40 minutes, with fresh alkaline buffer being replaced after 20 minutes. The comet assay was run for 50 minutes with a constant setting of 22V. Immediately after electrophoresis the comet slide was neutralized in pH 7.5 400mM Tris-HCl prior to staining with SYBR green diluted in 40mM Tris-HCl. Comet slides were imaged using the BioTek Cytation 7 cell imaging multimode reader and tail DNA was measured using the Trevigen Comet Assay Analysis Software.

Ultra-violet irradiation using the LED UVR DNA damage platform (LUDIS).

The cells were exposed to UVA using the LED UVR DNA damage induction system (LUDIS). The LUDIS is a high throughput system that allows for rapid and precise exposure to UVA (365nm). Using the LUDIS, the effect that UVA has on cells can be measured in a way that is physiologically relevant to everyday exposure to solar radiation. To measure how klotho containing serum effects DNA damage in the cells after UVA exposure, the cells were pretreated with either 0.5% or 1% of the serum for 24 hours prior to UV exposure. With the LUDIS the cells were exposed to exactly 94.8 kJ/m^2 in the 96 well plates.

Statistical analysis:

Statistical evaluation of data was conducted initially using Excel (Microsoft) to annotate and organize raw data that was generated from the cell viability and comet analysis assays. The data was then exported to a dedicated statistical analysis and visualization software (Prism version 10.1.2, Graphpad, Los Angeles, CA). Statistical approaches used in this research include t-test with post-hoc analysis and one-way ANOVA with post-hoc analysis.

Results

NHDF response to klotho containing serum:

We initially determined the toxic impact of the klotho containing serum on the NHDF cells. Cells were grown in the media containing the serum (0.1 – 50% final concentration). A significant ($p<0.0001$, one-way ANOVA) reduction in cell number was measured at only the highest concentration tested (50%) (**Figure 1A**). At serum level (1 – 10%) the klotho containing media was well tolerated. Based on these results, the NHDF cells were exposed to klotho containing serum (0.1 to 5% serum in media) for 24 hours before levels of endogenous

DNA damage were measured using single cell gel electrophoresis[12] (**Figure 1B**). Endogenous levels of DNA damage were significantly ($p<0.0001$) reduced at 0.5 and 1% klotho serum in media however this protective effect was not recapitulated at the highest concentration (5 and 10% serum). We next exposed the NHDF cells to UVA1 (365nm) (**Figure 1C**). The overarching objective of this experiment was to calculate the minimum dose of UVA radiation that induced significant levels of DNA damage. After 94.8 kJ/s/m^2 of radiation using the LUDIS apparatus a significant ($p<0.01$) increase was measured. UVA induced DNA damage plateaued at 189.6 kJ/s/m^2 of irradiation. Cell viability was measured after UVA exposure to ensure that the klothos serum did not sensitize the cells. Durations of exposure up to 237 kJ/s/m^2 (5 mins) did not induce significant cell death. The experiment was then repeated using cells exposed to 1% klotho serum for 24 hours (**Figure 1D**). The combinational treatment did not induce any additional cell death suggesting the klotho was not sensitizing the cells to UVA induced cell-death.

Figure 1:

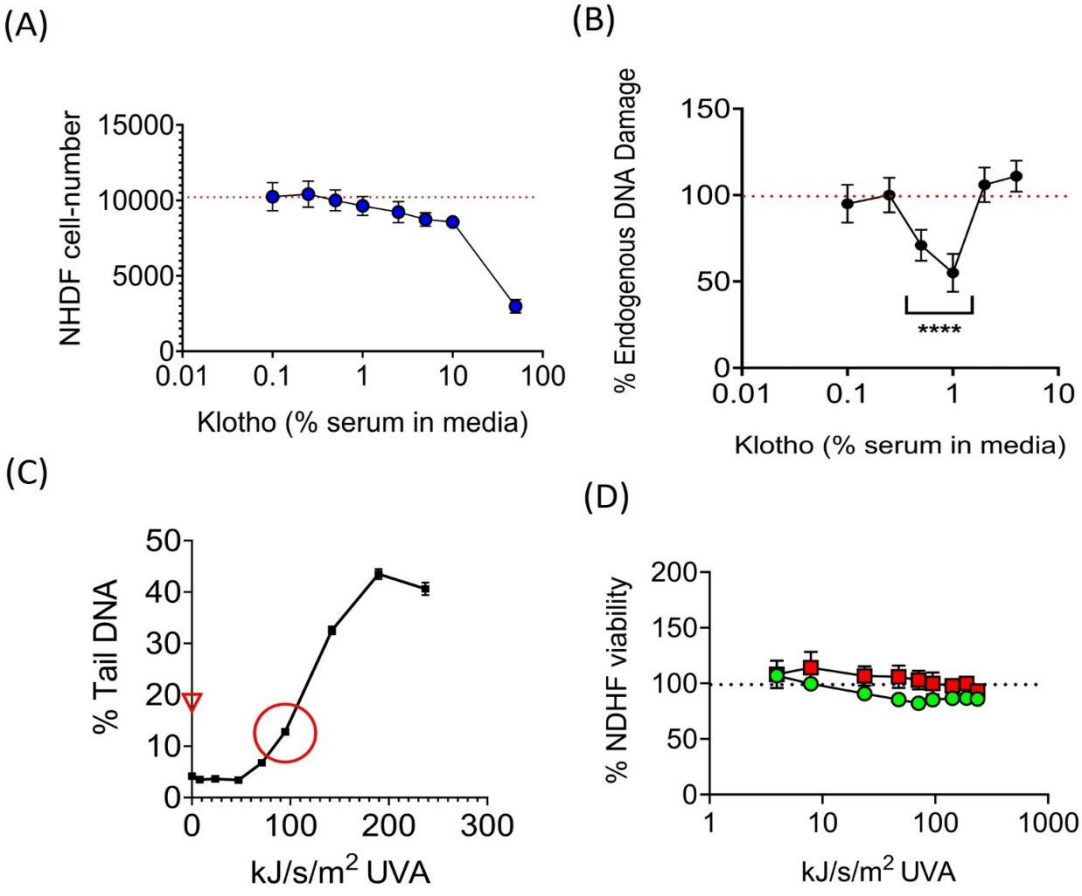

Figure 1: Fibroblast response to UVA and klotho containing serum. **(A)** Human dermal fibroblasts (NHDF) tolerated the klotho containing media up to 10% (v/v). At 50% serum levels there was a significant decrease in viability (****, $p<0.0001$). **(B)** Levels of endogenous DNA damage after 24 hours of serum exposure. The klotho serum reduced endogenous levels of DNA damage at 0.5% ($p< 0.0001$) and 1% ($p< 0.0001$). **(C)** UVA induced DNA damage was measured immediately after exposure. Red circle shows damage measured at 120 sec. used in subsequent experiments. Inverted red triangle is internal positive assay control. **(D)** Klotho does not

sensitize fibroblasts to UVA when irradiating up to 5 min. Green circle- cells pre-exposed to 1% klotho serum + UVA, red square – Only UVA treatment. All error bars show mean± SD of duplicate experiments. Significance was calculated using one way ANOVA with Dunnett's multiple comparisons test comparing to control.

Klothos containing serum reduces the level of UVA induced DNA damage:

Klothos containing serum at 0.5% and 1% (v/v) reduced endogenous levels of DNA damage. We used these concentrations to determine whether the serum was also able to reduce the level of DNA damage induced by UVA. We exposed the cells with or without klotho serum to 94.8 kJ/s/m^2 UVA, the lowest level of UVA that would induce a statistically significant repeatable increase in DNA damage (**Figure 2A**). The sensitivity of the SCGE assay was reduced to enable DNA damage to be measured over a greater range. UVA again induced a significant increase in DNA damage ($p<0.0001$) in all comparison groups. However, only in the cells pre-treated with 1% klotho was there a reduction in the amount of DNA damage after UVA irradiation. To rule out that the difference seen in the klotho 1% cells was due to differences in endogenous DNA damage levels we subtracted the median amount of endogenous DNA damage in each of the three groups (VEH, K 0.5% and K 1%) from the amount of DNA damage induced by the UVA (**Figure 2B**). Using this analytic process derived that K 0.5% did not alter the DNA damage induced by UVA. In contrast, cells incubated in K 1% did significantly ($p<0.01$) decrease the level of UVA induced DNA damage after klotho pre-treatment.

Figure 2:

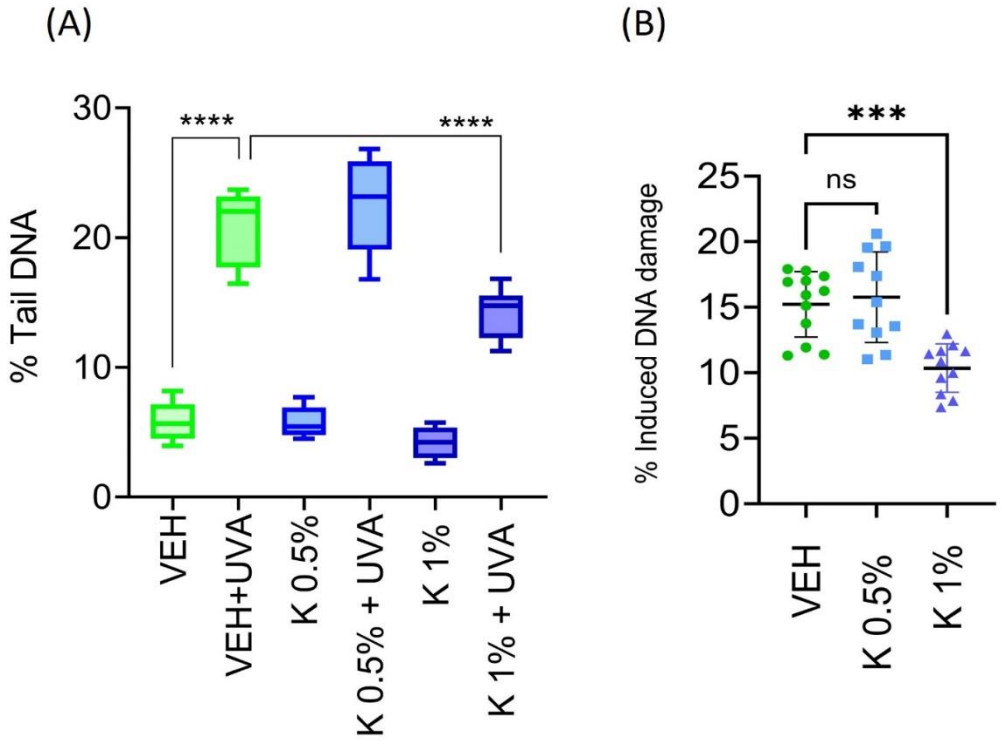

Figure 2: 1% Klothos containing media protects against endogenous and UVA induced DNA damage. (A) DNA damage levels were measured in cells pre-treated with klotho containing media and then exposed to

UVA. Klothos at 1% (v/v) significantly reduced the DNA damage induced by UVA (****, p<0.0001) **(B)** Protective response was not associated with differences in starting endogenous levels but a decrease in induced UVA associated damage (***, p<0.01). n<11 technical replicates from duplicated biological assays. Significance was calculated using one way ANOVA with Dunnett's multiple comparisons test comparing to control.

Discussion

Solar UV radiation interacts with DNA upon exposure to sunlight, inducing DNA damage through distinct mechanisms for both UVA and UVB. UVB radiation primarily induces DNA lesions through direct DNA absorption, resulting in the formation of cyclobutene pyrimidine dimers (CPDs) and pyrimidine photoproducts ((6-4)PP)[11]. In contrast, UVA1 radiation causes DNA damage predominantly through indirect mechanisms, primarily via the generation of reactive oxygen species (ROS)[12]. ROS, like singlet oxygen and superoxide radicals can oxidize DNA bases, leading to the formation of oxidative DNA lesions like 8-oxo-7,8-dihydroguanine (8-oxoG) and DNA strand breaks[13]. It is the accumulation of these oxidative lesions that impair the cellular repair mechanisms, leading to cellular senescence, inflammation, and potential carcinogenesis[14].

Beyond the potential for carcinogenic initiation, UVA1 plays a major role in photoaging of the skin due to its ability to penetrate the dermis. The dermal layer is made up primarily of fibroblasts cells. The dermis plays a crucial role in the structure and function of the skin. It provides support and nutrition and is involved in the regulation of body temperature and the maintenance of homeostasis[15]. The dermis also contributes to the skin's protective function by providing a barrier against mechanical forces[16]. It is a dynamic and complex structure, with a complex network of extracellular matrix macromolecules that connect the epidermal layer to the dermis, and a distinctive microarchitecture that strengthens dermal-epidermal connectivity[17]. The oxidative damage that UVA1 causes leads to the degradation of the extracellular matrix (ECM) found in the dermis[18]. Constant exposure to UVA1 radiation can lead to the chronic activation of nuclear factor-kappa B (NF-κB), which induces the expression of pro-inflammatory cytokines such as TNF-a. It has been shown that chronic activation of NF-kB can be harmful to the skin, as the inflammatory response can lead to tissue damage and photoaging of the skin, along with its ability to induce the expression of Matrix Metalloproteinases (MMPs), which break down the elastin and collagen fibers of the ECM that are essential for maintaining skin firmness and elasticity[19]. The research conducted in this study uses Normal Human Dermal Fibroblasts (NHDFs) as a model system, physiologically relevant as they reside in the dermis and are more susceptible to UVA induced oxidative stress, in comparison to keratinocytes found in the epidermis[20].

The purpose of this work was to investigate the protective effect that the klotho protein has against solar radiation, specifically targeting its protection against UVA1. To do this it was imperative to establish a physiologically relevant amount of UVA1 exposure to cells, which was achieved by calculating the typical UVA1 irradiance at 37.5W/m^2 [21]. This allowed us to correlate our UVA1 dose into time spent in midday sun exposure on an average day, giving us a range from 0 to 100 minutes of midday sun exposure (0- 237kJ/s/m^2 UVA1).

To combat the effects that UVA has on the skin, alpha-klotho was used in this work. Alpha-klotho is a transmembrane protein expressed in the kidney and brain that has been shown to attenuate oxidative stress by enhancing the expression and activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase[22,23]. The expression of these enzymes are able to reduce the accumulation of ROS and oxidative damage in DNA and cellular components[24,25]. Additionally, alpha-klotho can inhibit the expression of pro-inflammatory cytokines and signaling pathways[26], including nuclear factor-kappa B (NF-κB) and tumor necrosis factor-alpha (TNF-α)[27], which mitigates inflammation-mediated cellular damage and promotes tissue homeostasis. Alpha-klotho possesses β-glucuronidase activity, which cleaves extracellular sugars from glycoproteins, leading to the shedding of the extracellular domain of klotho[28]. Klotho can then act as a circulating hormone or exert paracrine effects on nearby cells. Through its interactions with fibroblast growth factor receptors (FGFRs) and other signaling molecules, klotho can activate intracellular signaling pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt pathway and the forkhead box O (FoxO) transcription factors, which play critical roles in cellular stress response and longevity[29,30].

The Klotho protein has been shown to have a protective effect against UVB radiation and oxidative stress in a variety of cells[31,32,33]. Our goal was to investigate the possible protective effects the protein has against UVA induced damage specifically. We found that NHDFs treated with 0.5% and 1% klotho serum for 24 hours had a significant decrease in endogenous DNA damage levels. We also found that pretreatment with the 1% klotho serum for 24 hours significantly reduce UVA related DNA damage. It was also found that there was no decrease in cellular proliferation with the addition of klotho serum added into culture media at concentrations up to 10%. With the use of the alpha-klotho protein as a pretreatment on NHDF cells we were able to show its protective effects against UVA irradiation, as well as its ability to reduce endogenous DNA damage. Further research will continue to investigate exactly how klotho protects against UVA damage. It can be theorized that since klotho is involved with many different pathways, it is protecting cells on multiple levels. As an antioxidant, it can protect against oxidative stress[34] brought on by UVA radiation. Klotho has also been reported to have anti-apoptotic and anti-senescent properties, reducing caspase 3, caspase 9, and p53 [35]. It also can reduce inflammation by inactivating NF-kB[36], TNF-α[37], and may also play a role in DNA repair due to its regulation of DNA glycosylases, key components of Base Excision Repair (BER)[38,39]. Klotho plays a major role in anti-aging, anti-inflammation, and protection of the skin from external stressors like solar radiation and because of this it would make a great addition to new skincare formulations focused on fighting the effects of sun damage and photoaging.

Conflict of Interest Statement:

Dr. Peter Sykora is CSO and director of Amelia Technologies LLC. Dr. Gail Humble is a CEO of the Aesthetic Anti-Aging Clinic, San Francisco, United States.

Acknowledgement Statement:

ChatGPT was used to originally develop the framework for the introduction, however this has been significantly edited and few of the original AI-derived sentence structures still remain in the document.

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