AnteAGE MDX Exosome Characterization and Functional Testing

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Introduction:

In recent years, the skincare industry has witnessed a remarkable surge in the integration of cutting-edge scientific advancements with beauty and wellness. Among the various breakthroughs, the utilization of exosomes in cosmetics has emerged as a promising trend, revolutionizing the way we approach skincare. Exosomes, once relegated to the realm of cellular communication and biomedical research, have now captured the attention of the beauty industry due to their potential in enhancing skin health and rejuvenation.

Exosomes are small extracellular vesicles that are naturally produced by various cells within the body, acting as essential mediators of intercellular communication. These vesicles play a crucial role in transferring bioactive molecules such as proteins, lipids, and nucleic acids between cells, influencing cellular functions and maintaining tissue homeostasis [1]. Recognizing the inherent regenerative properties of exosomes, researchers and cosmetic manufacturers have sought to harness their potential to transform the cosmetic landscape.

The recent rise of exosomes in cosmetics can be attributed to their unique ability to address fundamental challenges associated with traditional skincare approaches. Unlike conventional skincare products that merely work on the skin's surface, exosomes can penetrate deeply into the skin layers, delivering bioactive compounds directly to target cells [2]. By encapsulating and delivering active ingredients, exosomes offer a sophisticated delivery system that enhances the efficacy and bioavailability of key skincare components. Exosomes have the potential to stimulate various regenerative processes within the skin, promoting cellular repair, collagen production, and overall skin rejuvenation [3, 4]. Their ability to modulate inflammatory responses [5], promote tissue regeneration [6], and regulate cellular processes has garnered immense interest from both scientific researchers and cosmetic formulators alike.

Amidst the excitement surrounding exosomes in cosmetics, it is imperative to emphasize the importance of robust scientific investigation and thorough characterization of these vesicles [7, 8]. Before exosomes are incorporated into cosmetic formulations, it is crucial to conduct comprehensive research to understand their origin, composition, and functional properties. This involves the isolation and purification of exosomes from reliable and verified sources, ensuring their integrity and purity.

Characterization of exosomes involves determining their size, shape, surface markers, and cargo contents. There are scientific guidelines given by the International Society of Extracellular Vesicles (ISEV) on the minimal experimental requirements for characterizing and claiming extracellular vesicles or exosomes [7, 8]. Advanced techniques such as cryo-transmission electron microscopy (Cryo-TEM), nanoparticle tracking analysis (NTA), and flow cytometry are employed to accurately assess these parameters. Additionally, functional testing of exosomes is vital to validate their efficacy and safety in skincare applications. This includes evaluating their ability to deliver active ingredients to target cells and stimulate desired cellular responses.

By adhering to rigorous scientific standards in exosome research, cosmetic manufacturers can confidently develop formulations that are backed by robust scientific evidence. Such an approach ensures consumer safety and promotes transparency within the industry. Furthermore, it enables the development of reliable and effective skincare products that deliver the desired outcomes.

As the field of exosome research progresses, it is essential to establish standardized protocols and guidelines for the characterization and functional testing of exosomes used in cosmetics. This will contribute to the development of best practices and facilitate the evaluation and comparison of different exosome-based formulations. Collaboration between researchers, regulatory bodies, and cosmetic manufacturers is vital in establishing a framework that ensures the quality and consistency of exosome-based cosmetic products.

In recent studies, exosome-based cosmetic formulations have demonstrated remarkable benefits, including improved skin hydration, reduced wrinkles and fine lines, enhanced skin elasticity, and a more radiant complexion [9]. These findings have sparked widespread interest and excitement within the cosmetic industry, leading to an increased focus on incorporating exosomes into skincare products. However, to ensure the safety, efficacy, and reliability of exosome-based cosmetic formulations, it is imperative to prioritize robust scientific research, thorough characterization, and functional testing of these vesicles. By adhering to stringent scientific standards, the skincare industry can confidently harness the potential of exosomes, offering consumers innovative and effective products that enhance skin health and rejuvenation while upholding the principles of scientific rigor and consumer safety. As research continues to uncover the full potential of exosomes, we can anticipate a future where exosome-based skincare approaches revolutionize the way we care for our skin, providing unprecedented results and redefining the concept of beauty and wellness.

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Materials and Methods

Stem Cell Culture

Bone marrow MSCs and Umbilical cord MSCs were cultured in the same way. Cells were cultured in xenofree media. 10 million cells were seeded into a vitronectin (0.25µg/cm^2; ThermoFisher, Waltham, MA) coated 5 layer cell stack and cultured for 72hours. After 72 hours cells were passaged by dissociation with trypsin (TrypLE, Gibco). Viable cell counts were established using trypan blue exclusion and counting on an automated cell counter (Countess 3, Thermo Fisher Scientific; Waltham, MA). 10 million cells were added into vertical wheel bioreactors (PBS bioreactors) containing 6.25g of Corning Synthamax II Microcarriers. After 1 hour to allow cells to attach to microcarriers, the total volume of media was bought up to 500mL in each bioreactor. Cells were cultured for 72 hours before supplementing with a cell growth supplement and returned to culture for a further 48 hours. After 48 hours, media was collected, the microcarriers washed twice with PBS and a particle free chemically defined media added to the microcarrier-cell suspension for 48 hours, resulting in a chemically defined stem cell conditioned media rich in extracellular vesicles. Collected conditioned media was immediately filtered using a 0.2um filter and stored at -80 until purification. Media stored in the -80 freezer was given a shelf life of 6 months.

Exosome Isolation

The same process was carried out for either BM or UC exosome isolation. The only difference being the starting cells that produced the conditioned media. All cells were selected from young, healthy and consenting donors.

Tangential Flow Filtration (TFF)

4-10L of particle free chemically defined stem cell conditioned media was thawed in a temperature-controlled environment overnight inside an ISO Certified clean room. Concentration was carried out using a KR2i Tangential Flow Filtration device (Repligen, Compton, CA). TFF columns were pre-washed,

equilibrated and tested for membrane integrity prior to use according to the manufacturers instructions. Following equilibration, conditioned media was concentrated at a consistent trans-membrane pressure, diafiltrated with PBS for a final concentration/diafiltration of 100x.

Size Exclusion Chromatography (SEC)

Size exclusion chromatography was carried out to purify exosomes from other contaminating soluble proteins. 12 fractions were collected and 10µL from each fraction was assayed by direct ELISA for the tetraspannin CD81. Fractions enriched in CD81 (exosome rich fractions) were combined.

Particle concentrations were determined by nanoparticle tracking analysis NTA (See below). BM and UC exosomes were combined (1:1) and diluted with PBS containing 25mM Trehalose (Sigma-Aldrich, St Louis, MO) to give a concentration between $2.5x10^{10}$ – $3.5x10^{10}$p/mL. 500uL of this solution was then added into pre-sterilized 5mL borosilicate glass vials inside the class 5 clean room. Each vial containing 500uL of exosome containing at least $1.5x10^{10}$ particles/exosomes.

Exosome Lyophilization

Exosomes were lyophilized immediately after vial filling using a Labconco Freezezone 6 with stoppering tray dryer. Samples were frozen in the tray dryer on a -40oC shelf for 8 hours. After 8 hours, the vacuum was turned on and samples lyophilized using a per-determined program consisting of a primary, secondary and tertiary drying. Lyophilized vials were stoppered under vacuum, removed from the tray dyer and stored in a temperature-controlled environment.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 9.5.1 for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com). Data was assessed for normality using a Shapiro-Wilk test for normality and if Gaussian distribution was confirmed mean values was performed using one-way analysis of variance (ANOVA) with post hoc Tukey test (equal variances assumed) or a Games-Howell test (unequal variances determined). Statistical significance was assumed at $P<0.05$.

Exosome Characterization

Exosomes were characterized in accordance with MISEV 2014 [7] and MISEV 2018 [8].

Nanoparticle Tracking Analysis (NTA)

NTA was carried out on exosomes using a ZetaView R30 Mono (Particle Metrix) according to the manufactures instructions. The machine was pre calibrated using 100nm polystyrene beads to confirm sensitivity and accuracy. Exosomes were diluted to a concentration the resulted in approximately 100 particles/ field of view. Sensitivity was set at 80 with a shutter setting of 140.

Cryo TEM

Cryogenic Transmission electron microscopy (Cryo-TEM) was carried out through a collaboration with the University of California Irvine. EVs were directly adsorbed onto glow-discharged holey carbon grids (QUANTIFOIL, Großlöbichau, Germany). Grids were then blotted at 95% humidity and rapidly plunged into liquid propane with the aid of a VITROBOT (Maastricht Instruments BV, Maastricht, The Netherlands). Vitrified samples were imaged at liquid nitrogen temperature using a JEM- 2100F Transmission Electron Microscope (JEOL, Tokyo, Japan), equipped with a field emission gun and operated at an acceleration voltage of 200 kV.

Plate Assay

The plate assay was used to determine relative expression of positive exosome markers CD9, CD81, Alix, TSG101 and negative marker Calnexin. 100uL of a solution containing 1x109p/mL was added to a high protein binding 96 well plate overnight at RT. The following day unbound particles were removed by washing 3x using ELISA wash buffer (R&D Systems). Wells that contained targets that were intra-luminal were permeabilized with 0.5% TritonX-100 for 20 minutes. After 20 minutes, wells were washed three times with ELISA wash buffer. Non-specific antibody binding was blocked with 1% (v/v) Reagent Diluent (R&D Systems) for 1 hour at RT. After one hour, primary antibodies (all mouse anti-human): CD9 (R&D Systems), CD 81 (ThermoFisher), Alix (SantaCruz, Santa Cruz, CA), TSG101 (SantaCruz) and Calnexin (SantaCruz) were diluted to 1ug/mL in 1% (v/v) in reagent diluent and incubated at RT for 1 hour. After an hour, wells were washed three times with ELISA wash buffer and anti-mouse-biotin antibody diluted to 1µg/mL in 1% (v/v) Reagent Diluent for one hour at RT. After one hour, wells were washed three times with ELISA wash buffer and 1x streptavidin-HRP (R&D Systems) diluted in 1% (v/v) Reagent Diluent for 20 minutes in the dark.

After 20 minutes, wells were washed three times with ELISA Wash buffer. 100uL of TMB substrate was added to each well for 20 minutes in the dark. After 20 minutes 50uL of 2N H_2SO_4 was added to each well to stop the reaction. Absorbance was measured at 450 and 570nm using a SpectraMax ID5 multimode plate reader.

ExoView

ExoView imaging was carried out through a collaboration with the Extracellular Vesicle Core Facility at the Children's Hospital Los Angeles.

Microarray chips (NanoView Biosciences, Boston, MA, USA Product No.: EV-TETRA-C) were coated with anti-CD9 (HI9a), -CD63 (H5C6), and -CD81 (JS-81) antibodies. Baseline measurements of pre-adhered particles were obtained by pre-scanning the chips according to the manufacturer's instructions. Diluted AnteAGE MDX Exosomes were carefully loaded onto the pre-scanned chips (40 µL) and incubated overnight in a sealed 24-well plate (Greiner Bio-One) at room temperature. The following day, the antibody detection mixture, containing anti-CD9 (CF®-488-labeled), -CD63 (CF®-647-labeled), and -CD81 (CF®-555-labeled) antibodies, diluted 1:500 in blocking buffer, were incubated for 90 minutes at room temperature. Unbound antibodies were washed out with solution A, followed by three wash cycles. Excess detergent was removed with Solution B (NanoView Biosciences) in three wash steps. Finally, the chips were dried by carefully placing them in a Petri dish filled with Milli-Q water, removing them at a 45° angle to allow the surface tension to dry the chips, and draining the remaining water on lint-free Kimtech wipes (Kimberly-Clark, Dallas, TX, USA). The chips were then transferred to the sample stage for fluorescent and interferometric imaging.

Fluorescent labelling and uptake of exosomes

Exosomes were fluorescently labelled using 200µM Memglow-488. Purified exosomes were incubated with dye for 2 hours at room temperature. Unbound dye was removed by purifying 488 Memglow labelled exosomes on a qEV1 size exclusion chromatography column. Unbound dye was retained inside the column and fractions containing fluorescent exosomes collected. Fluorescently labelled exosomes were incubated with dermal fibroblasts and a keratinocyte cell line (HaKaT) for 2 hours in the dark. After 2 hours, cells were washed twice with PBS to remove any fluorescent exosomes that were not taken up by cells. Cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% TritonX-100 for 5 minutes and stained with phalloidin-atto-565 (red) and Hoechst (blue) for 1 hour in the dark. Cells were imaged using a Keyence BZ810 Fluorescent microscope.

Functional Testing

Anti-Inflammatory

Anti-Inflammatory properties of exosomes were determined using a THP-1 based assay. THP-1 cells (ATCC, Manassas, Virginia) were cultured in RPMI (GIBCO) supplemented with 10% (v/v) Heat Inactivated Fetal bovine serum (FBS; Gibco) and 1% (v/v) Antibiotic, Antimycotic (Corning, NY). 6x104 viable THP-1 cells were seeded into each well of a black 96 well plate (Grenier Bio-one, Austria) in RPMI containing 5% FBS and supplemented with phorbol-12-myristate-13 -acetate (PMA) for 24 hours. After 24 hours, media was removed, and test media added. Test media contained 10ng/mL Lipopolysaccharide LPS (Sigma-Aldrich) along with exosomes diluted to $1x10^9$p/mL. Control conditions included, no LPS (untreated), LPS only and LPS + Dexamethasone. After 24 hours of treatment, media from each individual well was collected and stored at -80 prior to ELISA and cells were fixed with 4% paraformaldehyde for immunohistochemistry staining.

Protein concentrations for IL6, IL8, and TNFa, were determined by ELISA. ELISAs were purchased from R&D Systems and carried out according to the manufacturers instructions. Protein concentration was determined based on the absorbance values plotted again a standard curve.

Immunohistochemistry was carried out to determine levels of iNOS in exosome treated cells. Cells were fixed with 4% PFA for 10min. After 10min cells were permeabilized with 0.1% TritonX-100 for five minutes. Non-specific antibody binding was blocked for 1hour with normal goat serum. Mouse Monoclonal primary antibody to iNOS (clone EPR16635; Abcam, Cambridge, UK) was diluted in 0.1% TritonX-100 to a concentration of 2µg/mL and added to cells overnight at 4oC. The following day, cells were washed five times with wash buffer and a goat anti-mouse secondary antibody diluted 1:500 added for two hours at RT. In addition a Phalloydin-595 conjugated antibody (10nM) diluted to 1:1000 and Hoeschest at 2µM was included in this 2 hour incubation. After two hours, cells were washed and imaged using a Keyence-BZ810 fluorescent microscope.

Within each well, four random images were captured. Results display a representative image.

Cell Proliferation

Human Dermal Fibroblasts (PromoCell; Mexico) and Hair Follicle Dermal Papilla Cells (Cell Applications, San Diego, CA) were cultured in RPMI containing 10% (v/v) FBS and 1% (v/v) Anti-Anti. For the proliferation assay, 5x103 cells were seeded into each well of a 96 well plate in normal culture media. After 24 hours, media was removed and replaced with a serum free media to growth arrest cells for 24 hours. After 24 hours, exosome treatments were added. Exosomes were diluted in serum free RPMI media to a concentration of 1x109p/mL and added to cells for 72 hours. After 72 hours, 5uL of Orangu ™ was added to each well for 2 hours. Orangu™ utilizes WST-8 which is metabolized by cells to give an orange-colored formazan dye. The amount of dye formed is directly proportional to the number of living cells. Absorbance at 450nm was determined with a SpectraMax ID5 multimode plate reader. Data is presented as the percentage difference in treated (with exosomes) compared to untreated (no exosome) controls.

In vivo skin application

AnteAGE MDX treatments were carried out and applied as directed by the manufacturer. One vial of AnteAGE MDX exosomes containing 10 billion particles along with a hyaluronic acid diluent vial was bought to RT prior to use. Facial skin was washed with AnteAGE cleanser and thoroughly dried. Skin was numbed for 15min and micro-needled using Exceed Pen (Candela) at a depth ranging 0.5-1.2mm. AnteAGE MDX exosome vial was reconstituted with the supplied hyaluronic acid diluent and applied to the face. Images were taken prior to treatment and at various times post treatment.

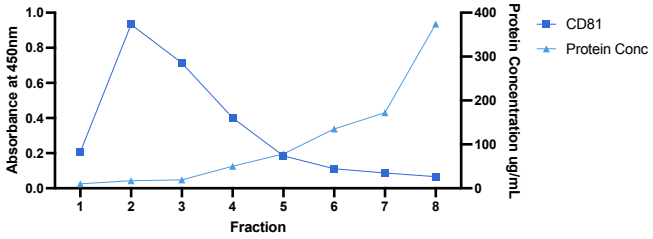
Results

The utilization of exosomes in cosmetics has yielded compelling outcomes, revealing their significant impact on skin health and rejuvenation. We present the results of our characterization and functional testing of MDX exosomes. Through rigorous experimentation and comprehensive analysis, we have uncovered promising findings that not only showcase the potential of exosomes in skincare but also attain to the importance of scientific research for product safety, consistency, and optimal patient outcomes. Our results contribute to the growing body of evidence supporting the incorporation of exosomes into cosmetic products, reinforcing the need for scientific rigor to ensure reliable and effective skincare solutions. These findings hold immense value for the industry, guiding future formulation strategies and setting a benchmark for the responsible development of exosome-based cosmetics.

AnteAGE MDX are characterized as sEVs according to MISEV

SEC separates exosomes and other large particles from the smaller soluble proteins that are also found in CM. Figure 1 demonstrates the protein concentrations in the fractions collected after SEC as well as the relative abundance of the tetraspannin CD81 (indicating exosomes). Figure 1 demonstrates that the exosomes elute and peak in fractions 2-4 where as the proteins are enriched and peak in the following fractions. Combining fractions 2-4 enables the enrichment of exosomes in the final product.

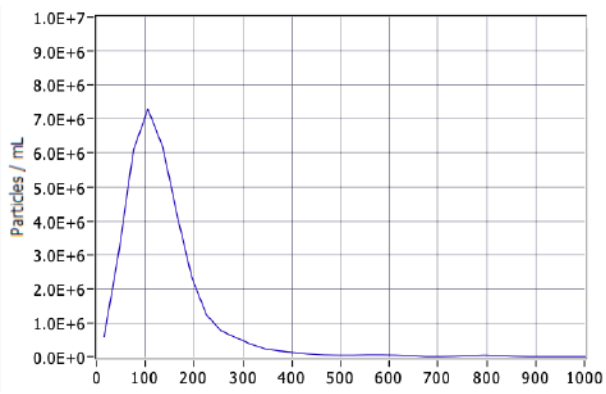
Figure 1: Exosomes elute in fractions 2-4. Size Exclusion Chromatography (SEC) of concentrated conditioned medium separates extracellular vesicles (CD81+) from protein containing fractions. Exosomes are most concentrated in fractions 2, 3 & 4. Fractions 2-4 are combined in the final AnteAGE MDX product, demonstrating a high level of exosomes purity.



The MISEV guidelines enable researchers to specify extracellular vesicles based on some minimal experimental criteria. Here, based upon the experimental results, the AnteAGE MDX exosomes are classified as sEVs. They have a modal particle size of 103nm (Fig. 2A) but NTA measurement.

Figure 2: AnteAGE MDX are characterized as sEVs according to MISEV. Nanoparticle tracking analysis demonstrates a modal particle size of 103nm (A), which was confirmed by Cryo-TEM (B) [Continued on next page].

Fig. 2A



Looking at individual vesicles by Cryo-TEM confirms particles delineated with a double limpid membrane and in the size range for sEVs (Fig. 2B). As well as determining particle size of a heterogeneous population of EVs as well as individual EVs, the MISEV guidelines also require the determination of proteins that are known to be enriched in sEVs. ExoView data demonstrates the presence of the tetraspannins CD9, CD81 and CD63 on the surface of the EVs. MDX exosomes demonstrate 41.67% of particles express all three tetraspannins on their surface (Fig. 2C). 7.57% express CD9 and CD81 and no CD63, 15.38% express CD9 and CD63 and no CD81, 11.38% express CD81 and CD63 and no CD9, and 21.07% express only CD9, 0.53% only CD81 and 7.57% only CD63 (Fig. 2C). As well as demonstrating the presence of the tetraspannins on the surface of the EVs, MISEV also requires the identification of endosomal proteins inside the vesicles as well as demonstrating the negative expression of cell markers such as calnexin. Using a plate based assay, AnteAGE MDX exosomes demonstrate the presence of the endosomal protein Alix and no expression of cell protein calnexin when compared to the IgG control (Fig. 2D). These experiments demonstrate the characteristics of AnteAGE MDX exosomes, and meet the requirements of ISEV. In addition to these experiments, when conducting functional experiments, it is also important to demonstrate the exosomes are being taken up by recipient cells. Without uptake, there would be significantly less function of the exosomes. Fluorescent AnteAGE MDX exosomes (Green) demonstrate uptake into both keratinocytes and dermal fibroblasts. Exosomes demonstrate a peri-nuclear localization and can be observed within the cell cytoplasm (Fig. 2E).

Figure 2: AnteAGE MDX are characterized as sEVs according to MISEV. The tetraspannin co-localization percentages are shown along with the particle counts on each chip and a fluorescent image of MDX Exosomes on the ExvView (B). Internally, exosomes are positive for the endosomal marker Alix and negative for cell marker Calnexin (D). Fluorescently labelled MDX Exosomes (green) are taken up into human skin cells – Fibroblasts (Left) and Keratinocytes (Right). Actin is stained with Phalloidin-565 (red) and the nucleus stained blue (E).

Fig. 2B

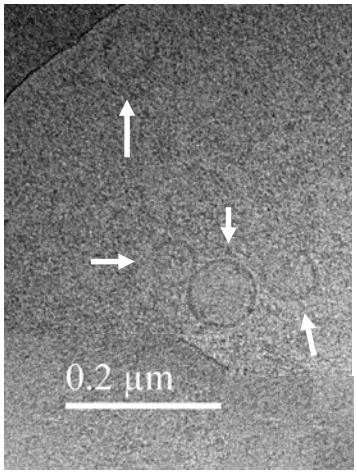
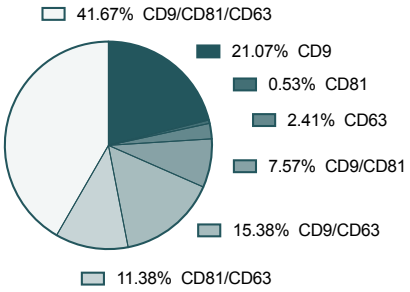
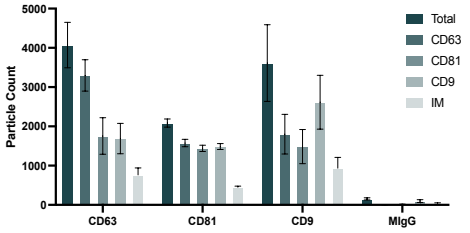


Fig. 2C





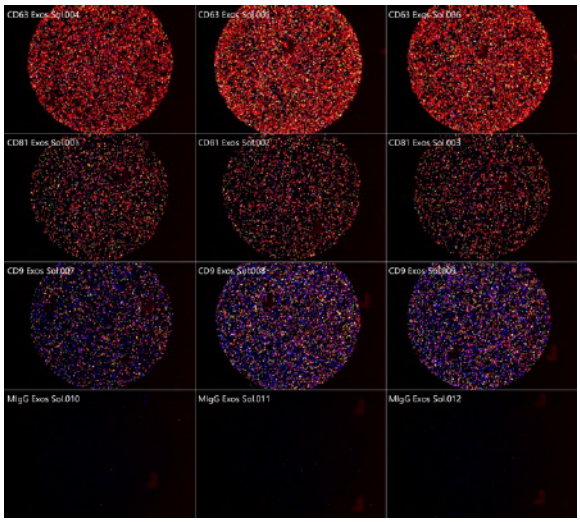


Fig. 2D

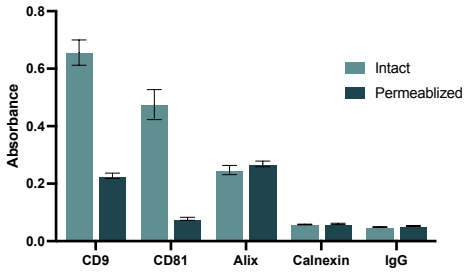
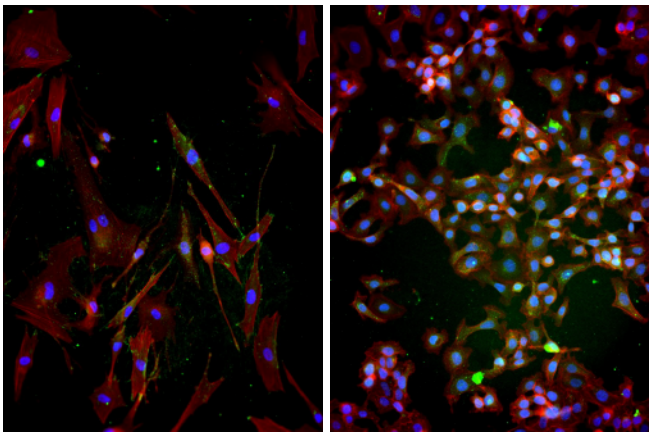


Fig. 2E



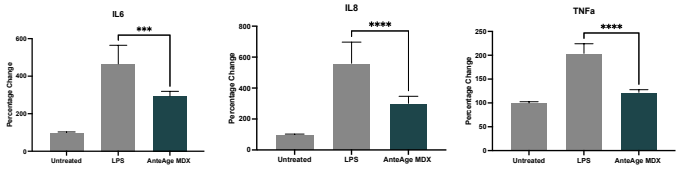
AnteAGE MDX demonstrates anti-inflammatory functions in vitro

It is important to control inflammation within the skin. Chromic inflammation in the skin can lead to accelerated ageing breaking down collagen and elastin leading to the formation of fine lines, wrinkles and sagging skin. In a simple in vitro model of inflammation, exosomes demonstrate anti-inflammatory characteristics. When activated THP-1 cells (macrophages) are exposed to LPS they demonstrate a potent inflammatory response, significantly increasing the expression of inflammatory cytokines, IL6, IL8 and TNFα (Fig. 3A). When cells are also exposed to exosomes the inflammatory response is significantly ($P<0.05$) reduced.

Macrophages can become activated/polarized into either pro-inflammatory, M1, or alternatively activated anti-inflammatory, M2, macrophages. M1 macrophages are routinely characterized by the intracellular expression of iNOS. Figure 2B demonstrates the significant increase in the number of cells expressing iNOS following LPS exposure for 24 hours. Like the cytokine profiles, the addition of exosomes significantly reduced the number of iNOS positive cells (Fig. 3B). Alternatively activated M2 macrophages are characterized by the expression of CD206 on the cell surface. When exposed to LPS, the number of CD206 expressing cells significantly reduced (Fig. 3B), when exposed to exosomes, the number of CD206 expressing cells significantly increased. This data together with the cytokine expression indicates exosomes are capable of polarizing macrophages towards an anti-inflammatory, alternatively activated phenotype.

Figure 3: AnteAGE MDX demonstrate anti-inflammatory functions in vitro. Using a simple in vitro anti-inflammatory assay, LPS Stimulated THP1 cells demonstrate a potent inflammatory response that is inhibited with the addition of AnteAGE MDX Exosomes (A). In the same assay, THP1 treated cells were stained for iNOS (Green, left panel; M1 marker) and CD206 (Green, right panel, M2 Marker) (B). Cells were also stained for actin (red) and the nucleus (blue).

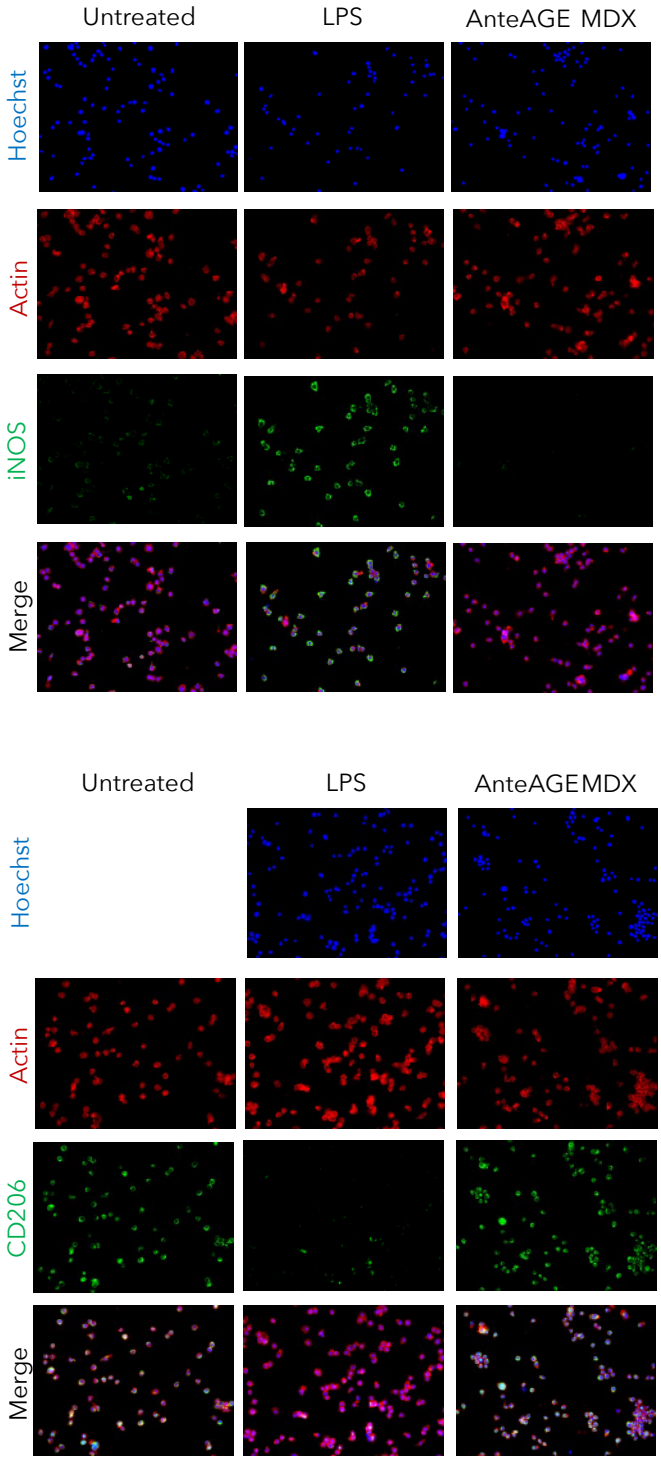
Fig. 3A



AnteAGE MDX demonstrates proliferative capabilities among skin cells

Within the skin fibroblasts are the primary cell type responsible for collagen synthesis, giving the skin structure; providing firmness and elasticity. Modulating the rates of fibroblast proliferation will therefore have direct influence on the structure and

Fig. 3B



visible age of the skin. After serum starving dermal fibroblasts to inhibit their proliferation, the addition of exosomes was sufficient to significantly ($P<0.05$) increase the rate of fibroblast proliferation (Fig. 4A). Exosomes were also capable of significantly ($P<0.05$) increasing the rate of hair follicle dermal papilla cell proliferation (Fig. 4B). Being able to significantly increase the rate of proliferation in hair follicle dermal papilla cells gives promising utility for the use of exosomes in hair regeneration.

Figure 4: AnteAGE MDX demonstrate proliferative capabilities among skin cells. AnteAGE MDX Exosomes stimulated both dermal fibroblast and Hair Follicle Dermal Papilla Cell proliferation after 72 hours of exosome exposure.

Fig. 4A

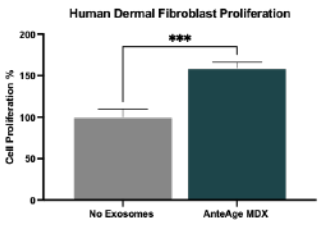
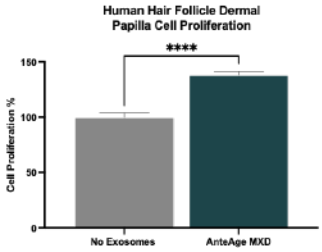


Fig. 4B



AnteAGE MDX demonstrates long term stability

The ability to store exosomes safely and conveniently for the practitioner is critical for the production of a well accepted and integrated product. Storing exosomes with a short shelf life at hard to maintain temperatures such as -80oC is not very user friendly. Being able to store an exosome product at a user friendly and convenient temperature such as 4oC is beneficial for everyone. To be able to increase stability over a long period at these temperatures, water needs to be removed from the exosome solution. This can be achieved by lyophilization or freeze drying. Lyophilization can have detrimental effects on the stability and viability of exosomes if not done properly [10]. The use of a cryoprotectant is essential for maintaining exosome structure and integrity and therefore in vivo function. Exosomes are lyophilized with the cryoprotectant trehalose. After lyophilization and storage for 1 month at 4oC exosome particle concentration reduced by 20% however, particle concentration was measured to be 1.19x1010p/mL (11.9Billion; Fig. 5A). After 6 & 9 months of storage, the percentage reduction of exosome particles was consistent at around 20% reduction compared to the freshly isolated exosomes (Fig. 4A) but again, the total number of particles was still above the 10 billion particles / vial. Importantly, after lyophilization exosomes retained their structure with an intact lipid bilayer as observed using Cryo-TEM (Fig. 5B&C).

Figure 5: AnteAGE MDX demonstrate long term stability. AnteAGE MDX Exosomes retain their stability following lyophilization as demonstrated with their paricle concentration and total number of particles per vial (A). Cryo-TEM also confirms the maintenance of an intact lipid bilayer following lyophilization (C) when compared to freshly isolated exosomes (B). The high background observed after lyophilzation is due to the increase in salt concentration when resuspending in a small volume of particle free water for Cryo-TEM.

Fig. 5A

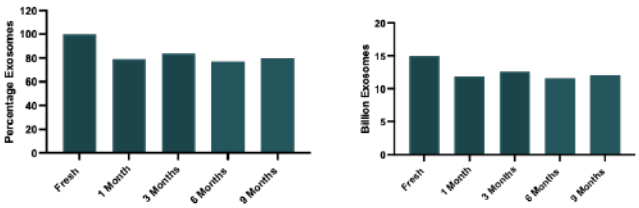


Fig. 5B

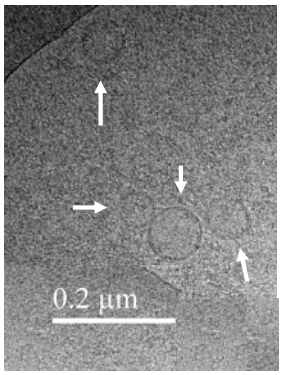
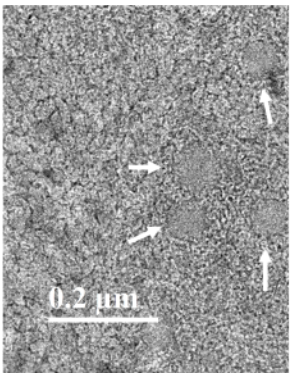


Fig. 5C



AnteAGE MDX visibly improves skin health

The application of MDX exosomes to the skin following microneedling visibly improved skin quality (Fig. 6 A&B). One exosome treatment demonstrates significant reduction in fine lines and wrinkles as well as improving skin tone (Fig. 6A). Two MDX exosome microneedling treatments demonstrated significant improvement in skin tone and color, fine lines and wrinkles as well as reducing redness and acne (Fig. 6B). Overall, the application of AnteAGE MDX exosomes significantly improved the quality and youthfulness of the skin.

Figure 6: AnteAGE MDX visibly improves skin health. The addition of AnteAGE MDX exosomes significantly reduces redness, uneven skin tone and fine lines and wrinkles. These beneficial responses can be observed at 3 weeks post treatment (A) and at 2.5 months post treatment (B).

Fig. 6A

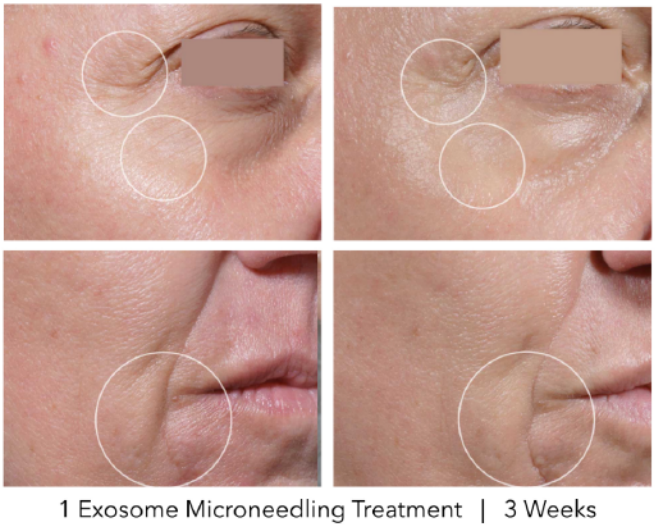
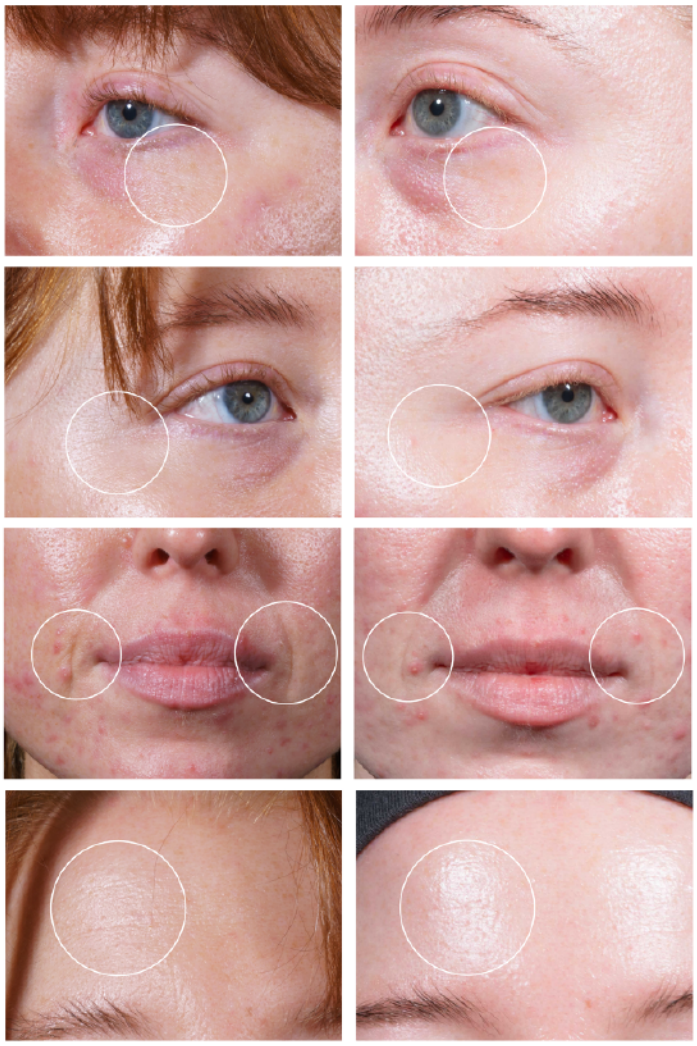


Fig. 6B



2 Exosome Microneedling Treatments | 2.5 Months

Discussion

This data demonstrating the characteristics, as well as in vitro and in vivo function of exosomes, provides valuable insights into the potential of exosomes in skincare and emphasizes the importance of robust scientific research, characterization, and functional testing of these vesicles. The integration of exosomes in cosmetics has gained significant interest due to their ability to enhance skin health and rejuvenation. By encapsulating and delivering bioactive compounds, exosomes offer a sophisticated delivery system that can penetrate deep into the skin layers, promoting cellular repair, collagen production, and overall skin rejuvenation [2, 9].

To ensure the safety, efficacy, and reliability of exosome-based cosmetic formulations, it is crucial to prioritize robust scientific research, thorough characterization, and functional testing of these vesicles. Rigorous scientific investigation involves comprehensive research on the origin, composition, and functional properties of exosomes. Isolation and purification of exosomes from reliable and verified sources are essential to ensure their integrity and purity. The International Society of Extracellular Vesicles (ISEV) provides guidelines on the minimal experimental requirements for characterizing and claiming extracellular vesicles or exosomes [7, 8, 11].

Characterization of exosomes involves determining their size, shape, surface markers, and cargo contents. Techniques such as cryo-transmission electron microscopy (Cryo-TEM), nanoparticle tracking analysis (NTA), and ELISA are employed for accurate assessment [7, 8, 12]. Cryo-TEM allows visualization of exosomes at high resolution, providing insights into their morphology and structural details. NTA enables quantification of exosomes and determination of their size distribution. ELISA helps identify specific surface markers present on exosomes, contributing to their characterization. The AnteAGE MDX exosomes fit all criteria for characterization as sEVs (exosomes) set out by ISEV.

Functional testing of exosomes is crucial to validate their efficacy and safety in skincare applications. This includes evaluating their ability to deliver active ingredients to target cells and stimulate desired cellular responses. For instance, fluorescent labeling and uptake studies can assess the internalization of exosomes by skin cells, such as dermal fibroblasts and keratinocytes [13, 14]. Furthermore, functional assays can be conducted to evaluate the anti-inflammatory properties of exosomes using cell-based models, such as THP-1 cells, and measuring the expression of inflammatory markers like IL-6, IL-8, and TNF-α. Stem cell exosomes have previously demonstrated [15-17].

By adhering to stringent scientific standards, the skincare industry can confidently harness the potential of exosomes and offer consumers innovative and effective products that enhance skin health and rejuvenation while upholding the principles of scientific rigor and consumer safety. Standardized protocols and guidelines for the characterization and functional testing of exosomes used in cosmetics should be established to facilitate the evaluation and comparison of different exosome-based formulations [18]. Collaboration between researchers, regulatory bodies, and cosmetic manufacturers is essential in developing a

framework that ensures the quality and consistency of exosome-based cosmetic products.

Recent studies have demonstrated the benefits of exosome-based cosmetic formulations, including improved skin hydration, reduced wrinkles and fine lines, enhanced skin elasticity, and a more radiant complexion [9, 19]. These findings have sparked widespread interest within the cosmetic industry, leading to increased focus on incorporating exosomes into skincare products. However, it is crucial to note that scientific research and comprehensive characterization of exosomes are imperative for the safety, efficacy, and reliability of these formulations.

In conclusion, exosomes have emerged as promising tools in the skincare industry due to their unique ability to penetrate deep into the skin layers and deliver bioactive compounds to target cells. AnteAGE MDX exosomes are fully characterized as sEVs and demonstrate potent in vitro and in vivo function in multiple model systems. However, the robust scientific research behind the AnteAGE MDX product including: comprehensive characterization, and functional testing are only a minimal experimental requirement that other exosome cosmetic providers should also be providing. This first AnteAGE MDX exosome product can help redefine the concept of beauty and wellness through the utilization of exosomes.

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