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"Bio-revitalizing activity of HA-based MDs on aged dermis spheroids: the role of trehalose in inducing of ECM mechanical boosting by a systemic administration.

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1. Introduction

The aging process is a physiological and degenerative process, as a consequence of cellular senescence of tissues and organs over the time [1]. The chronological aging process induces skin modifications in terms of dermis composition and compactness widely described in dermatological literature. The main skin alterations are related to modification in synthesis and assembly of the crucial components of the extracellular matrix (ECM), the structural support of the dermis compartment [2], [3]. Inside the dermis, fibroblasts play a critical role in ECM assembly and maturation by direct synthesis of key structural component [4], and also by activation of mechanism of remodeling, leading to a modification of dermal mechanical properties [5].

In particular, the dermal ECM actively works as a dynamic reservoir for morphogens, cytokines, and growth factors. These elements are critical in regulating the progressive formation of a specialized and highly complex ECM *in vivo*: collagen and elastic fibers, embedded within an amorphous matrix of glycosaminoglycans (GAGs) represent the main scaffolding of the skin.

In terms of key structural components, dermis is rich in collagen fibers (70-80% of dry wight) providing tensile strength, while the elastic fibre network, making up about 2–4% of the dermal volume, contributes to skin resilience and flexibility [6]. GAGs are essential for retaining water and ensuring tissue hydration [7].

During the aging, these components undergo significant alterations affecting skin strength and elasticity [5], [8], [9]. The dermis experiences changes such as reduced collagen synthesis and decreased solubility, density and thickness of collagen bundles [10], as well as fragmentation and slower degradation of elastic fibers [11], [12]. Several studies suggest that the

gradual dermis degeneration starts around the age of 50, leading to dermal atrophy, characterized by thinner collagen bundles and increased spacing between them [13].

Among all treatments used in aesthetic medicine to counteract the signs of dermis aging, the Medical Devices (MDs) based on hyaluronic acid (HA), such as fillers, are widely used thanks to their ability to restore volume and smooth wrinkles. HA is a natural glycosaminoglycan found in the ECM of the skin, where it plays a crucial role in maintaining hydration and structural integrity [14]. The unique properties of HA, is the high capacity to binding water molecules, making HA (and all medical devices HA-based) an ideal natural molecule for dermal fillers [15]. In addition, HA exhibits no tissue or species specificity, crucial key point to minimize any untoward effect for potential immunologic reactions or transplantation rejection [16].
The biophysical properties of HA fillers, such as molecular weight, concentration, and degree of cross-linking, play a significant role in determining their efficacy; higher molecular weight and greater degree of cross-linking result in more robust fillers that provide greater lift and volume, while lower molecular weight fillers are softer and better suited for fine lines and hydration [17].
In this scenario, innovative in *vitro* models for research and screening are required to deep investigate the pre-clinical efficacy of products and treatments destinated to aesthetic medicine. Micro-physiological systems (MPSs), as spheroids, represent a 3D *in vitro* tissue platform in which cells, in a physiological microenvironment, are able to aggregate together in hanging droplets forming a three-dimensional structure where the *in vivo* spatial and molecular cell-cell-cell-matrix interactions are preserved. This configuration influences cell behavior and the activation of physiological signaling and molecular pathway, triggering the synthesis and deposition of a natural ECM [18], [19]. For these reasons, the *in vitro* tests conducted on spheroids models are more predictive, due to the absence of exogenous scaffolds and to preservation of the physiological orientation and spatial distribution of the cells in the 3D microenvironment, fundamental to ensure a high biomimetic accuracy.

In this study, aged dermis spheroids are applied as advanced 3D tissue model that closely mirror the physiological features of the aged dermis in terms of cells proliferation, differentiation, morphology, genic and proteomic profile [20], [21], as well as cellular responses to external stimuli [22]. These characteristics give 3D spheroids a greater biological relevance and underline their specific potential for advanced applications.

2. Materials and Methods

2.1 Cell source

Primary Human Dermal Fibroblasts (HDF, Zen-Bio, Durham, NC), from the face of a 64-year-old female donor were cultured in 2D for 4 passages. Once reached about 90% of confluence, cells were detached, counted, and seeded to the desired concentration in the hanging drop plates (Akura® PLUS plate) to allow the cellular aggregation (about 10000 cells/spheroids).

2.2 Evaluation of revitalizing effect of filler HA-based

Once aggregated, spheroids were treated with three HA-based MDs, with different compositions (HMW-HA, MMW-HA, and a mixture of MMW+LMW-HA combined with trehalose). A preliminary cytotoxicity assay was performed to define the non-toxic safe dose. The efficacy of the products was evaluated by PICP release (pro-collagen C-terminal type I) by ELISA assay (Cloud Corporation, Katy, TX) after 2 and 7 days of systemic exposure, and by immunofluorescence on collagen type III for signal quantification (on 3D whole mount samples) afer 7 days of treatment.

2.3 Kinetic evaluation of Collagen type I deposition by ELISA assay

ODs related to the biological replicate have been interpolated in the concentration-OD curve of standards (assay range 0.312-20 ng/mL) to calculate the concentration in the samples.
If one sample either treated with negative control or with positive control, is outside the sensitivity range (LOD <0.129 ng/mL), the results have been reported as <0.129 ng/mL.

2.4 Collagen type III evaluation by 3D Whole mount Immunofluorescence

After 7 days of exposure, untreated and/or treated tissues were collected and foxed in 10% formalin buffered solution overnight at 4°C. Pooled tissues (n=10 spheroids/series) were permeabilized with 0.2% Triton X-100 (Merck, Germany) diluted in PBS 1X, and incubated for 4h in blocking solution (5%FBS, 0.2% Triton X-100 diluted in PBS 1X) at RT. 4h after, tissues were incubated with rabbit polyclonal primary antibody anti-human collagen type III (abcam, ab7778, 1:1000) overnight. The day after, tissues were incubated with Alexa Fluor 555 PLUS Donkey-anti-rabbit (Life Technologies, A32794) as secondary antibody and with DAPI for nuclei staining (Merck, MBD0015, 1:3000), overnight. The acquisition was performed by a widefield fluorescence microscope THUNDER Imager Live Cell & 3D Assay DMi8 (Leica Microsystems, Wetzlar, Germany). 6-10 spheroids were acquired for each experimental condition by Z-stack was performed to observe the signal distribution in the whole volume of spheroids. To reduce the fluorescence noise, all acquisitions are elaborated with High Content Imaging and processed with LASX 3.7.5 software (Leica Microsystems, Wetzlar, Germany).
The quantification of expression signals was performed on 2D maximum projection of the whole Z-volume for each spheroid, at 7 days (D7) of exposure/treatments. The Sum of Intensity was considered as parameter to evaluate the intensity, and the amount of signal expression was calculated by normalization to each spheroids area (in μm^2).

3. Results

3.1 Preliminary Cytotoxicity Assay

Figure 1 shows the ATP quantification after 24h of treatment with different concentration of products for dose-finding study, aiming to the evaluation of the highest non-toxic dose applicable (ATP quantification was performed on 6 spheroids/each series).

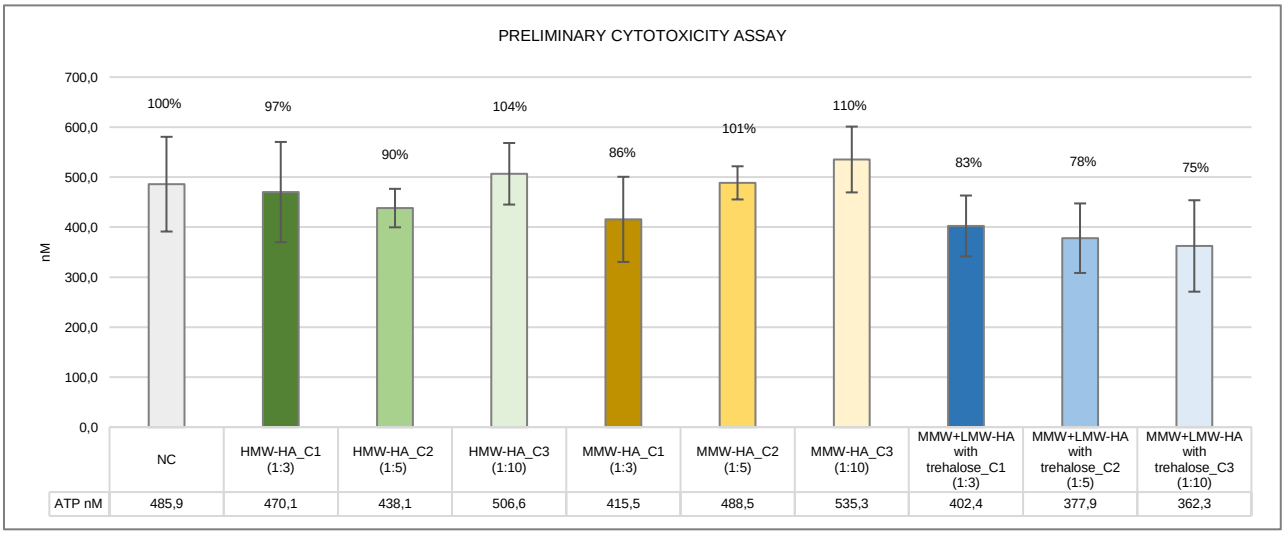

Figure 1. ATP content in nM measured for all series spheroids. Percentage of viability was calculated by normalization to negative control (NC). (n° of replicates= 6 wells/each series).

NC values are defined as homeostatic baseline and defined with 100% of viability. Exposure of spheroids to the 3 products-HA based at 3 concentrations (HMW-HA, MMW-HA and MMW+LMW-HA with trehalose) for 24h did not affect cellular viability, with values of ATP amount close to untreated series (NC), in all series greater than 50% (defined as cutoff value). As a result, the study was conducted on spheroids exposed to the highest concentration (1:3) for all test items.

3.2 ELISA Assay for PICP Release

Procollagen Type I C-Terminal Propeptide (PICP) is a biomarker closely linked to collagen type I production, a key component of the dermis. It reflects the rate of collagen synthesis, making it valuable for monitoring skin health, wound healing, and aging processes [23]. Elevated PICP levels may indicate increased collagen turnover, potentially associated with skin repair or fibrotic disorders [24], [25].

The culture media of all series were collected at D2 and D7, to evaluate a kinetic of PICP release in all treatments compared to unexposed series during this experimental window. In the Figure 2 (A and B) the PICP amount dosed in the culture media of all samples was quantified by ELISA assay. T-test (reported in the Tab. I) was applied as statistical analysis (only statistically significant results are included).

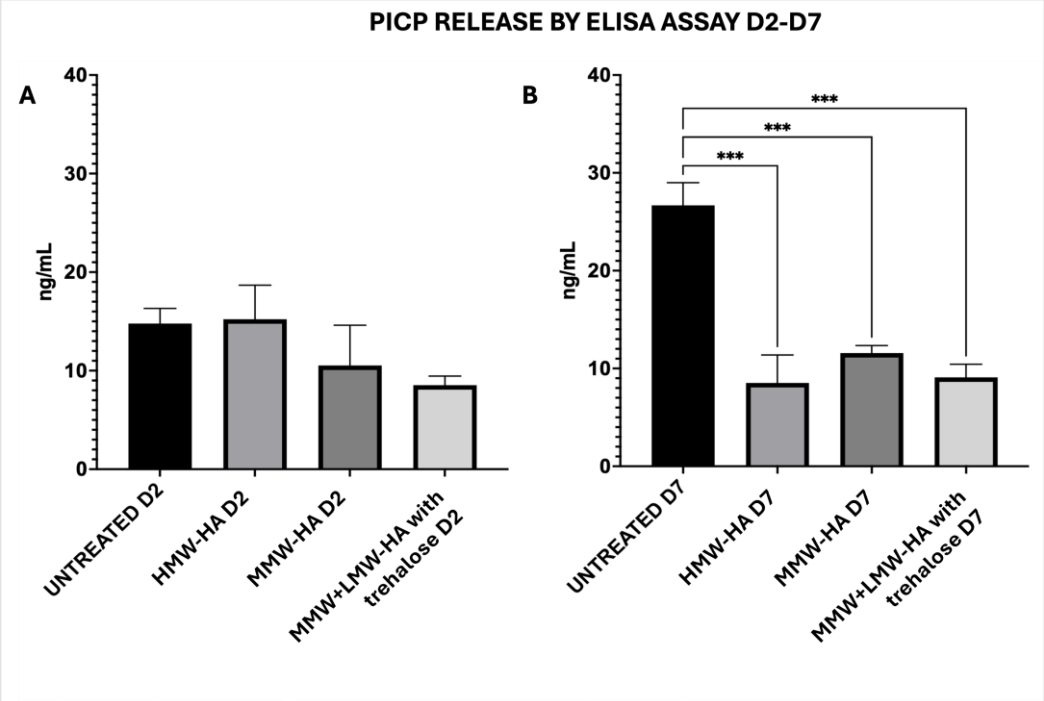

Figure 2. PICP amount dosed by ELISA assay. Pro-collagen C-terminal type I release was measured (ng/mL) for all series spheroids after 2 (A) and 7 days (B). (n° of biological replicates= 3 of 30 pooled /each series) (***p<0.001).

SERIES	T-test statistical analysis between D2-D7
UNTREATED D7 vs D2	** p<0.002

Table 1. PICP ELISA assay at D2-D7- T-Test statistical analysis.

In untreated basal conditions, physiologically PICP release increase during the time of culture, showing a dynamic and physiological evolution of 3D dermis spheroids: the quantification of the pro-collagen C-terminal type I is directly related to the metabolic process of collagen type I synthesis and deposition.

At D2 all treatments did not visibly affect PICP production and, as consequence, the mature collagen type I assembly and deposition: all observed differences between them are not statistically significant and the amount was comparable to untreated series.

At D7 of exposure, the effect of all HA-based fillers tested did not show differences if compared to untreated series at 7 days of culture.

These results suggested that the systemic administration of all injectable fillers did not show a direct efficacy on collagen I synthesis, suggesting a possible different MoA: during 7 days of treatment, in all exposed series is visible possible negative feedback associated to collagen I production and metabolism.

3.3 Histological Analysis for Collagen III and signal Quantification

Collagen type III is a key component of the dermis, providing elasticity and flexibility to the skin. It forms a network of fine fibers that interweaves with collagen type I, contributing to the skin's overall structure and integrity [26].

Figure 3 shows the most representative focal planes of 3D whole mount immunolabelled samples, and, in the Figure 4 the quantification of signal expression for Col III after 7 days (D7) of treatment (quantification was performed on a mean of n= 10 replicates/each series) is reported.

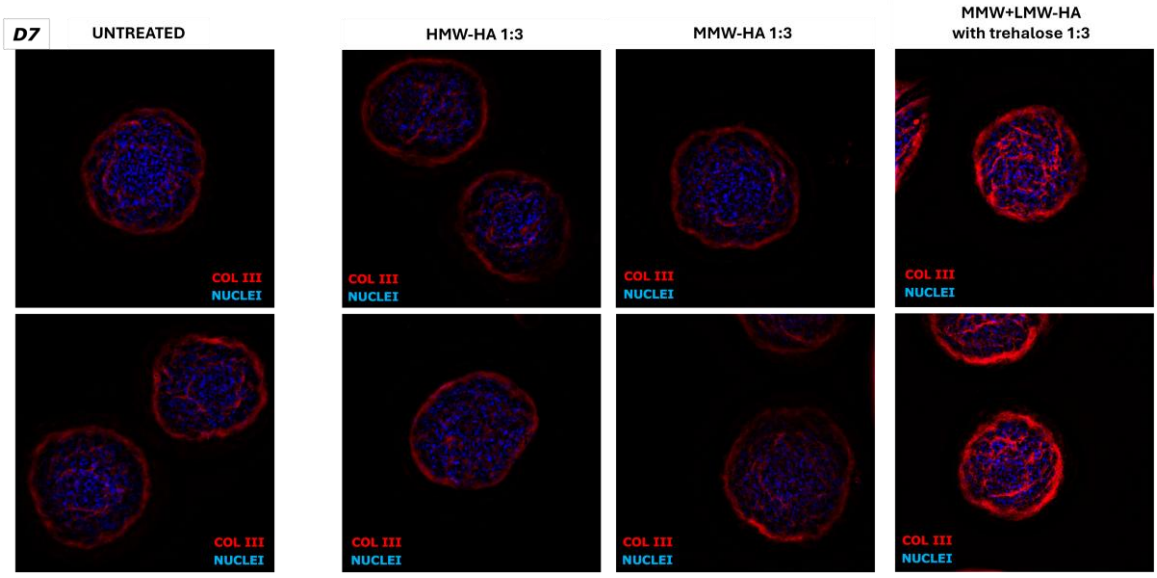

Figure 3. Collagen type III IF on 3D Whole mount samples. Most representative focal planes were showed for each series (collagen III in red, nuclei in blue). 20X Mag.

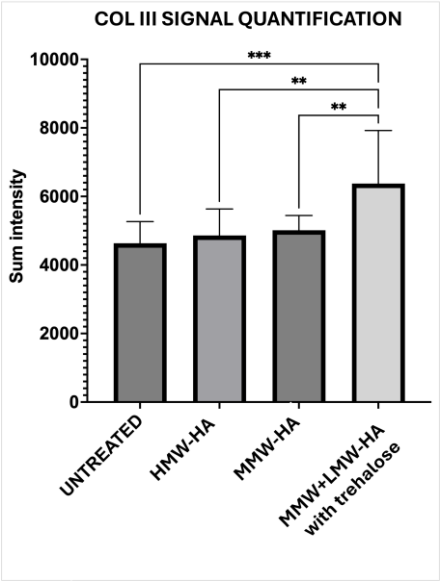

Figure 7. Quantification of Col III signal expression after 7 days of treatments. The analysis is performed on maximum projection images derived from Z-stacks of 3D whole mount samples (*** p< 0.001, **p=0.001 vs. HMW-HA and **p=0.002 vs MMW-HA, respectively).

At 7 days of treatment, a positive effect on Col III was observed after exposure to the mixture MMW+LMW-HA with trehalose: a visible increase of collagen III expression was observed if compared to untreated unexposed series (*** p< 0.001) and to all other fillers tested (**p=0.001 vs. HMW-HA and **p=0.002 vs MMW-HA). The synergic action of HA at MMW (1200-1500 kDa) and HA at LMW (200-400 kDa) in combination with trehalose, showed a direct modulation on collagen III synthesis contributing to give a boost to ECM composition, structure and stiffness, suggesting that the systemic administration could globally stimulate dermis fibroblasts metabolism in stromal improvement and remodeling.
On the contrary, MMW-HA and HMW-HA based fillers did not affect significantly the collagen expression, after 7 days of exposure.

4. Discussion

Dermis spheroids model represents an innovative MPS (Micro Physiological System) able to mimic the native dermis features, preserving the phenotype of cell source during extended long-term culture. The model was demonstrated to be a relevant biological testing platform to predict human skin response for anti-aging and photo-protective cosmetics ingredients [20], [21], [27], [28]. The main limitation related to the administration could affect the testing platform choice in order to maximize the products efficacy in a defined exerimental window. In particular, the administration by injection (as required for a filler) allows forcing the products to interact with the dermis compartment because they are put in mechanical contact with the stroma (by direct injection). In the case of systemic administration, the products are diluted in the culture media in a defined dose and the efficacy depends on their composition and to their ability to biologically interact with the most exposed cells, by absorption and metabolization at the cytoplasmic level, exerting a biological effect [29].
All the products HA-based did not show a direct effect on the metabolic synthesis collagen I (monitored by PICP release), suggesting possible negative feedback especially after long term exposure (D7). In particular, a similar behaviour could be seen also on collagen III synthesis for HMW-HA based and MMW-HA based MDs: they did not show a visible impact on Col III modulation (in terms of fibers configuration and signal expression) at 7 days of systemic exposure. In particular, untreated series showed a dynamic behaviuor of aged dermis spheroids, in terms of PICP release: the amount increased during the culture time reaching highest values after 7 days, suggesting a dynamic and physiological evolution of dermis ECM development in terms of structural collagen I deposition.
A basal expression of Col III was observed inside the spheroid’s architecture as red thin fibers confirming a physiological assembly that mirrors the native dermis architecture. The exposure to HMW-HA based filler showed no differences observed in terms of PICP release at D2 of exposure if compared to untreated series, and the same level was maintained after 7 days, suggesting a late effect in reducing collagen I enzymatic synthesis, due to a possible negative feedback. No modulation of collagen III expression was observed by IF on whole mount samples confirming a different possible activity and MoA in the defined experimental window.

The treatment with a MMW-HA filler led to a similar trend to HMW-HA observed both for PICP release during the time and for collagen III expression, suggesting that both formulations showed a similar behavior during 7 days of exposure.
The mixture MMW+LMW-HA combined with trehalose showed no direct and no significant modulation of PICP release on treated derms spheroids early at 2 days of exposure, with similar quantified amount close to the other series.
At 7 days, a global PICP reduction was observed, suggesting a similar MoA on collagen I metabolism.
On the contrary, a visible and significant increase of collagen III deposition and expression was visible, suggesting a direct positive stimulation of dermis fibroblasts metabolism in increasing stiffness and structural key protein of ECM.
Unlike them, the synergic mixture of MMW+LMW-HA with trehalose showed a positive effect on Coll III, with a visible increase of protein expression, suggesting a direct mechanical activity, boosting of ECM stiffness and structural assembly due to the dermis stimulation. The combined activity of two different HA molecules in the presence of the trehalose, enhanced the product efficacy even after systemic administration with a direct interaction with the dermis stroma and a mechanical stimulation of the cells to produce *de novo* structural proteins of the ECM [30], [31]. In particular, the trehalose works as a stabilizer, contributing to the long-term maintenance of HA molecules within the intracellular space [32], [33] by two different mechanisms:
i) By establishing an adequate steric hindrance against the enzyme hyaluronidase (confirmed by IR analysis), by interacting with HA at the -NH groups.
ii) By increasing in the hydration sphere of the HA molecules (expansion of the coordination sphere of water molecules), as showed by calorimetric data (TGA and DSC): in this way the glycosidic bond became even more inaccessible to the enzyme hyaluronidase, allowing a long stability of the molecule during the time.

These data fully support the protective action of trehalose towards hyaluronic acid, increasing its resistance to degradation by the specific enzyme hyaluronidase and, consequently, its bioavailability.
Globally, the positive mechanical boosting of ECM induced by exposure to a selected formulation of HA molecules combined with trealose, in Col III synthesis could induce an improvement of compactness and mass density of dermis stroma, suggesting a modulation also on mechanical and viscoelastic properties, that could be further investigated by advanced techniques based on nanoindentation.

5. Conclusion

The dermis spheroid model proved to be a robust and innovative Micro Physiological System (MPS), able of replicating aged dermal features while preserving cellular phenotype during long-term culture. Its predictive reliability makes it a valuable tool for testing the efficacy of anti-aging cosmetic ingredients, such as injectable fillers HA-based. However, the method of administration of such medical devices, such as injectable fillers, plays a crucial role in

determining their biological impact. While systemic delivery showed limited effects on collagen I and III synthesis, particularly for HMW-HA and MMW-HA formulations, the combination of MMW+LMW-HA with trehalose demonstrated an increase in collagen III deposition. This suggests a distinct mechanism of action, likely due to enhanced mechanical stimulation and ECM remodeling.

Importantly, the synergistic formulation enriched with trehalose not only improved the stability and bioavailability of HA by protecting it from enzymatic degradation, but also enhanced its biological performance. These effects were observed even under conditions mimicking systemic exposure, using advaced 3D dermis spheroids models that faithfully replicate the structural and functional features of aged human skin. These models provide a robust and physiologically relevant platform for the pre-clinical assessment of dermo-cosmetic treatments.

Overall, these findings highlight the potential of combining different HA molecular weights with trehalose to optimize dermal regeneration and ECM reorganization, offering promising insights for the development of next-generation cosmetic and dermatological solutions.

6 References

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