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Comparative in Vitro Study of the Effects Produced by Different Hyaluronic Acids on the Extracellular Matrix and Fibroblasts

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Abstract—*Hyaluronic acid (HA) has emerged as a pivotal molecule in medicine for various medical procedures. Its functionality, applications and the cellular response it triggers vary depending on its molecular weight, presentation and delivery method. The benefits ascribed to hyaluronic acid (HA) in aesthetic medicine have ignited current interest in studying its effects on fibroblasts. This interest stems from its classification as a biostimulant and biorevitaliser. A series of in vitro experiments using adult human dermal fibroblasts (HDFs) as well as a human monocytic cell line (THP-1) were devised to gain further insight into the benefits of the different formulations of commercially available HA products injected into the dermis for the prevention and treatment of skin ageing. The objective of this study was to assess three commercially available hyaluronic acid (HA) products with similar therapeutic aims but varying concentrations and molecular weights. The evaluation aimed to quantify the stimulation of adult HDFs and the inflammatory response in THP-1 monocytes. The results showed that hyaluronic acid of 0.9% concentration and (2700-3400k) Da has shown that it has the ability to biostimulate fibroblasts to secrete elastin, thus improving their morphology, as well as enhancing their regenerative capacity in response to mechanical aggression. On the other hand, it was observed that it also has a greater anti-inflammatory property than HAs of other molecular weights and different concentrations, as evidenced by IL-8 and IL-6 determinations in THP-1 monocyte cultures. In conclusion, the in vitro studies showed that VHMWHA can stimulate adult dermal fibroblasts.*

Keywords— *Very high molecular weight hyaluronic acid, elastin, fibroblast, procollagen type 1, IL-6, IL-8.*

I. INTRODUCTION

Research into the diverse effects of hyaluronic acid (HA) fragments in biomedical and cosmeceutical applications is garnering significant interest in the scientific community, primarily due to the limited literature available on the subject and the benefits of HA[1].

In cosmetic medicine, HA has become the filler of choice due to its ease of use, low immunogenicity, reversibility and longevity [2-3]. There are numerous hyaluronic acid-based injectables on the market that differ mainly in their concentration or molecular weight, among other things [4-7]. These physical-chemical differences result in variations in the rheological properties of the different HAs currently on the market and, therefore, their behavior and the clinical outcomes[8].

The ageing process is multifactorial and manifests through alterations in the different layers of the skin that result in reduced binding of fibroblasts to the extracellular matrix (ECM) and a simultaneous decrease in mechanical forces, dermal thickness and ECM density.

Likewise, the number of fibroblasts diminishes with age, leading to alterations in their biological functions, including a gradual decrease in their ability to produce collagen and elastin[9-10]. Fibroblast ageing affects not only their morphology and collagen and elastin production, it also reduces the expression of genes involved in the production and secretion of other ECM components. Old fibroblasts acquire identity noise as well as adipogenic characteristics, yet concomitantly upregulate the expression of genes involved in inflammation, lipid metabolism, and adipogenesis[11-12]. Clinically, all these changes result in dermal atrophy, wrinkles, and skin fragility, contributing to an aged appearance [11].

HA biorevitalisation is defined as the "enhancement of skin quality through intradermal injection of hyaluronic acid either alone or in combination with other substances" [13]. The same author then defined biostimulation as “the ability to stimulate the anabolic function of dermal fibroblasts” after observing their ability to activate protein synthesis and production of extracellular matrix components, resulting in aesthetic and functional improvement of the skin [14].

Thus, a biostimulant is defined as a substance capable of inducing cellular effects or facilitating temporary tissue regeneration, as well as restoring the mechanical properties of the treated area [15-16].

The concentration of naturally occurring HA in human skin is 500 mg/g, with a predominant molecular weight of approximately 3000 kDa. Its primary functions include homeostasis and tissue organization[17-18].

HA plays a key role in biostimulation in synthetic injectables, as it directly stimulates fibroblasts by enhancing mechanical forces in the ECM[19]. Injection of HA filler into equivalent dermal cultures induces fibroblast elongation and stimulates type 1 collagen synthesis via the TGF-β signaling pathway[20]. This process restores the functional activation of fibroblasts in aged human skin by enhancing ECM structural

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support. This, in turn, further stimulates fibroblast proliferation and vascularization while also increasing epidermal thickness [21-22]. It also plays a role in the regulation of inflammation by binding to membrane receptors such as CD44, CD168 or RHAMM (Receptor for Hyaluronan Mediated Motility). Lastly, it interacts with other components of the ECM, such as proteoglycans [23].

The main objective of this study was to examine the impact of various products primarily composed of hyaluronic acid on dermal fibroblasts *in vitro*, quantify the morphological and physiological changes these fibroblasts undergo when exposed to different molecular weights and/or concentrations of hyaluronic acid, and explore the role of these molecules in the mechanisms of repair and/or regeneration. The secondary objective was to study the inflammatory response in a human monocytic cell line (THP-1) by quantifying IL-8 and IL-6.

II. Materials and Methods

A. Raw Materials

We compared three commercially available products containing hyaluronic acid in different concentrations and molecular weights, with or without cross-linking.

Despite their different compositions, the products share the commonality of yielding similar results when injected into the extracellular matrix of the human dermis for cosmetic medical purposes. Table 1 shows the characteristics of the three products compared in the in vitro study.

TABLE 1. Characterization of the study products. *NS: Not specified by the manufacturer

Product	Type of HA	HA Concentration	Molecular Weight (MW)
PRODUCT 1 (TKN HA3®)	Sodium hyaluronate	0.9%	2700–3400 kDa VHMWHA
PRODUCT 2 (RESTYLANE SKINBOOSTER VITAL®)	Hyaluronic acid, stabilized	2.0%	*NS
PRODUCT 3 (PROFHILO®)	Sodium hyaluronate	3.2%	32 mg LMWHA: 80–100 kDa) + 32 mg HMWHA: 1100–1400 kDa)

B. Experimental systems and culture conditions for in vitro assays

Fibroblast culture

The cells used were adult human dermal fibroblasts (HDF) from Innoprot (Vizcaya, Derio, Spain), specifically the P10858 strain. They were incubated in fibroblast medium (FM) containing basal medium, 2% foetal bovine serum (FBS) from Gibco 10106-169 (Waltham, Massachusetts, USA), 1% fibroblast growth supplement and 1% penicillin/streptomycin solution from Gibco 15140-122 (Waltham, Massachusetts, USA), at 95% humidity and 5% CO_2, in a flask in a temperature-controlled incubator. The seeding density was 5000 cells/cm^2. The fibroblasts were anchored to poly-L-lysine-coated culture vessels to promote cell adhesion.

When the cells reached > 70% but <90% confluence, they were subcultured into new culture flasks. All cell cultures were incubated in standard culture conditions at 37°C, 5% CO_2 and 95% humidity.

The distinction between young and old HDFs is based on the number of passages in culture. HDFs that are considered young are those in the range of 1 to 15 passages. When this number of passages is exceeded, the metabolic activity of the cells and their cycle begins to slow down, and they are considered as "aged".

Monocyte culture

THP-1, a human monocyte cell line (Ref. TIB-202™, ATCC, University Boulevard, Manassas, Virginia, USA) was used to study the HA-mediated immune response.

The cells were incubated in RPMI complete medium (product code R0883, MERK KGaA, Darmstadt, Germany), 25 mM HEPES (MERK KGaA, Darmstadt, Germany), and Gibco 10% FBS (product code 10106-169, Waltham, Massachusetts, USA), with 50 mM beta-mercaptoethanol (MERK KGaA, Darmstadt, Germany), according to the supplier's guidelines. They were routinely cultured in suspension in cell culture flasks. When the cells reached > 80% confluence, they were subcultured into new culture flasks. All cell cultures were incubated in standard culture conditions (37°C, 5% CO_2 and 95% humidity).

Cytotoxicity assay

To study the cytotoxicity of products 1, 2 and 3, an MTT cell viability assay was performed on HDF and THP-1 cells.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with CAS No. 298-93-1, SIGMA M2128 (MERK KGaA, Darmstadt, Germany), a yellow tetrazole which is reduced to purple formazan in live cells, was used to assess cell metabolic activity and viability.

The cells were previously cultured in 96-well plates, treated with 8 different concentrations of study products 1, 2 and 3 and incubated for 24 hours at 37°C, with 5% CO_2.

Meanwhile, 8 concentrations of sodium dodecyl sulphate (SDS) from SIGMA (product code L6026, MERK KGaA, Darmstadt, Germany) were tested as a positive control to validate the assay. After 24 hours of incubation with the study products and the positive control, the cells were washed with PBS (disodium phosphate [Na_2HPO_4], SIGMA, product code S7907, MERK KGaA, Darmstadt, Germany) and stained with MTT solution. They were then incubated at 37°C for 2 hours. After incubation, the staining medium was removed, and 100 µL dimethyl sulfoxide (DMSO) from PANREAC APPLICHEM (product code 161954, MERK KGaA, Darmstadt, Germany) were added to each well to solubilize the coloured precipitate. Absorbance was measured at a wavelength of 540 nm using the Thermo Scientific™ VARIOSKAN LUX™ spectrophotometer plate reader (Thermo Fisher Scientific S.L., Alcobendas, Madrid, Spain). The percentage of cell viability was calculated relative to the negative control, where the cells were not exposed to any product:

$$\% \text{ cell viability} = AbT/AbC \times 100$$

AbT = absorbance at 540 nm after 24 hours of treatment

AbT = absorbance at 540 nm after 24 hours without treatment (negative control)

Proliferation assay

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To investigate the proliferative effects of the study products, an MTT proliferation assay was conducted on both young and aged HDF.

MTT is a water-soluble yellow tetrazolium salt that is reduced to purple formazan in living cells. This process provides an assessment of cellular metabolic activity and, consequently, cell viability. Cells cultured in 96-well plates were treated with 4 concentrations of the study products and incubated for 24 hours at 37°C with 5% CO_2 in serum-free medium. As a positive control (C+), the cells were incubated in complete medium. After 72 hours of incubation, they were washed with PBS, stained with MTT solution and incubated at 37°C for 2 hours. After incubation, the staining medium was removed, and 100 µL of DMSO added to each well to solubilise the coloured precipitate. Absorbance was measured at 540 nm on the Thermo Scientific™ VARIOSKAN LUX™ spectrophotometer plate reader. The percentage of cell proliferation was calculated relative to the negative (C−) control, where the cells were not exposed to any product:

$$\% \text{ cell viability} = AbT/AbC \times 100$$

AbT = absorbance at 540 nm after 24 hours of treatment
AbT = absorbance at 540 nm after 24 hours without treatment (negative control)

Production of procollagen type 1 assay

Procollagen type 1 production was studied in an *in vitro* assay in which HDFs were cultured in the presence or absence of the study products. Procollagen type 1 production was quantified using the Human Pro-Collagen I alpha 1 DuoSet ELISA Kit (R&D product code DY6220, Bio-Techne® Minneapolis, MN, USA). The cells were cultured in 96-well plates in serum-free medium and treated with a 0.2% v/v concentration of the study products for 72 hours. Untreated cells were used as negative controls (C−) and cells incubated in complete medium as positive controls (C+). After incubation, the supernatant from each well was collected and used to quantify procollagen type 1 production using the VARIOSKAN LUXTM from Thermo Scientific™.

Total collagen production assay

Total collagen production was studied in an in vitro assay in which HDFs were cultured in the presence or absence of the study products. Total collagen production was assessed by colorimetry. The cells were cultured in 12-well plates in serum-free medium and treated with a 0.2% v/v concentration of the study products for 72 hours.

Untreated cells were used as negative controls (C−) and cells incubated in complete medium as positive controls (C+). After incubation, the supernatant was collected from each well and used to quantify total collagen production by colorimetry with the Thermo Scientific™ VARIOSKAN LUX™, using the Sircol Collagen Assay Kit (product code S1000, BIOCOLOR, Co Antrim, United Kingdom).

Elastin production assay

Elastin production was studied in an *in vitro* assay in which HDFs were cultured in the presence or absence of the study products. Elastin production was measured by ELISA. The cells were cultured in 96-well plates in complete medium and treated with a 0.2% v/v concentration of the study products for 24 hours. Untreated cells were used as negative controls (C−) After incubation, the supernatant from each well was collected and used to quantify procollagen type 1 production using a Human Elastin ELISA Kit by ABCAM (product code ab239433, ABCAM, Cambridge, United Kingdom).

Wound healing assay (scratch assay)

Wound healing was studied in an in vitro assay in which HDFs were cultured in the presence or absence of the study products. Wound healing activity is assessed based on the proliferative activity and migratory capacity of the cells. The cells were seeded on both sides of inserts placed in the culture wells and incubated in complete medium at 37°C and 5% CO_2. After the cells reached confluence, typically around 24 hours later, the inserts were removed, and identical scratches made in the cell monolayer in each well by scraping. The wells were then washed with 1 mL PBS after which 1 mL of treatment was added. The scratches were photographed at 24/48/72 hours of incubation. The wound surface area was calculated using ImageJ software. The percentage of wound healing was calculated relative to the negative (C−) control which used untreated cells. The treated cells and the negative control cells were cultured in basal medium. As a positive control (C+), cells were cultured in complete medium or in serum-free medium with hEGF 20 ng/mL.

$$\% \text{ wound closure} = 100 - (AT/AC \times 100)$$

AT = wound surface area after treatment
AC = wound surface area of negative control (cells growing in basal medium)

Anti-inflammatory activity assay

The anti-inflammatory activity of the study products was investigated using an in vitro assay in which THP-1 cells were exposed to an inflammatory stimulus consisting of E. coli lipopolysaccharide (LPS) obtained from SIGMA, (product code L6529, MERK KGaA, Darmstadt, Germany). The anti-inflammatory effect of the study products was measured by quantifying the secretion of IL-8 and IL-6. This was achieved using the Invitrogen Human IL-8 and Human IL-6 ELISA Kits (product codes KHC0081 and KHC0061 respectively, from Proteintech®, Planegg-Martinsried, Germany). Cells were cultured in 96-well plates and incubated with 0.31 µg/mL PMA to differentiate monocytes and adhere to the plate. The following day, the cells were treated with LPS (10 µg/mL) for 24 hours, using the study products at a concentration of 0.2% v/v as a control. Untreated cells (no product or LPS) were used as a negative control (C−). Cells treated with LPS alone were used as a positive control (C+). After incubation, the supernatant was collected from each well to quantify IL-8 and IL-6 production using the Thermo Scientific™ VARIOSKAN LUXTM system.

Scanning electron microscopy (SEM)

The effect of the study products on the morphology of adult human dermal fibroblasts (HDF) was investigated using scanning electron microscopy with ZEISS EVO 50 (ZEISS, Jena, Germany). The cells were cultured in plates of 24 wells each containing a coverslip and then incubated for 24 hours, using the 0.2% v/v concentration of the study products as the control. Untreated cells were used as negative controls (C−). After incubation, the cells were washed with PBS and fixed in

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2% glutaraldehyde in Sorensen's buffer for 3 hours at 4°C. They were then dehydrated using a series of increasing concentrations of ethanol, starting with 30% ethanol and ending with 100% ethanol. The cells were maintained at each concentration for 10 min. The cells were then exposed to hexamethyldisilazane (product code 804324, MERK KGaA, Darmstadt, Germany) for 10 minutes and allowed to dry overnight. Finally, the cells on the glass coverslips were coated with gold/palladium and visualised by SEM. Images were taken at different magnifications (500X–2000X). The median cell elongation was determined using ImageJ, a public domain program developed by Wayne Rasband (NIH), to measure the maximum length of each cell in three randomly selected 500X fields.

Confocal microscopy for elastin detection

Elastin production was visualised via confocal microscopy using the AXIO OBSERVER Z1M (ZEISS, Jena, Germany) in an *in vitro* assay where HDFs were cultured with or without the study products. The cells were cultured in complete medium 12-well plates for microscopy applications and treated with a 0.2% v/v concentration of the study products for 24 hours. Untreated cells were used as negative controls (C−) After incubation, the cells were fixed in 4% paraformaldehyde (product code 252549, Merck KGaA, Darmstadt, Germany) and permeabilised in 0.1% Triton X-100 (product code 108603.1000, MERK KGaA, Darmstadt, Germany). They were then double stained using MemBrite Fix 660/680 (product code 30098-T, Biotium, San Francisco, USA) and Alexa Fluor 488 elastin antibody (E-11) (product code sc-166543, Santa Cruz Biotechnology, Heidelberg, Germany), and visualised by confocal microscopy. The images were taken using a 40X lens.

C. Statistical analysis

The objective parameters shown in the graphs were reported as the mean ± standard error of the mean (SEM), while the parameters compared to the negative control (C−) or positive control (C+) were reported as percentages. Data distribution normality was evaluated via the Shapiro-Wilk test, revealing normal distribution across all study objective variables. The difference between each product and the negative and positive controls (C− and C+) was analysed using the two-tailed Student's *t*-test for independent samples. Statistical significance was defined as $p < 0.05$. SPSS version 2.0 (IBM, Madrid, Spain) was used for statistical analysis.

III. RESULTS

First, an MTT cytotoxicity study was conducted to determine the optimal concentrations of the products for use with human dermal fibroblasts (HDF) and a human monocyte cell line (THP-1).

The following efficacy studies were then conducted:

- Proliferation assay
- Procollagen type 1 and total collagen production assay
- Elastin production assay
- Wound healing assay (scratch assay)
- Anti-inflammatory assay with IL-6 and IL-8
- Scanning electron microscopy (SEM) for morphological study
- Confocal microscopy for elastin detection

Cytotoxicity assay

An *in vitro* MTT viability assay was used to determine cytotoxicity of the study products on HDFs and THP-1 cells.

Eight different concentrations of each product were tested (Product 1: 0.450, 0.225, 0.113, 0.056, 0.028, 0.014, 0.007, 0.004 v/v; Product 2: 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 v/v; Product 3: 1.0667, 0.3556, 0.1185, 0.0395, 0.0132, 0.0044, 0.0015, 0.0005 v/v).

The concentrations were adjusted based on the hyaluronic acid (HA) content of each product to ensure uniformity across all samples.

The results are shown in the following figures 1 and 2.

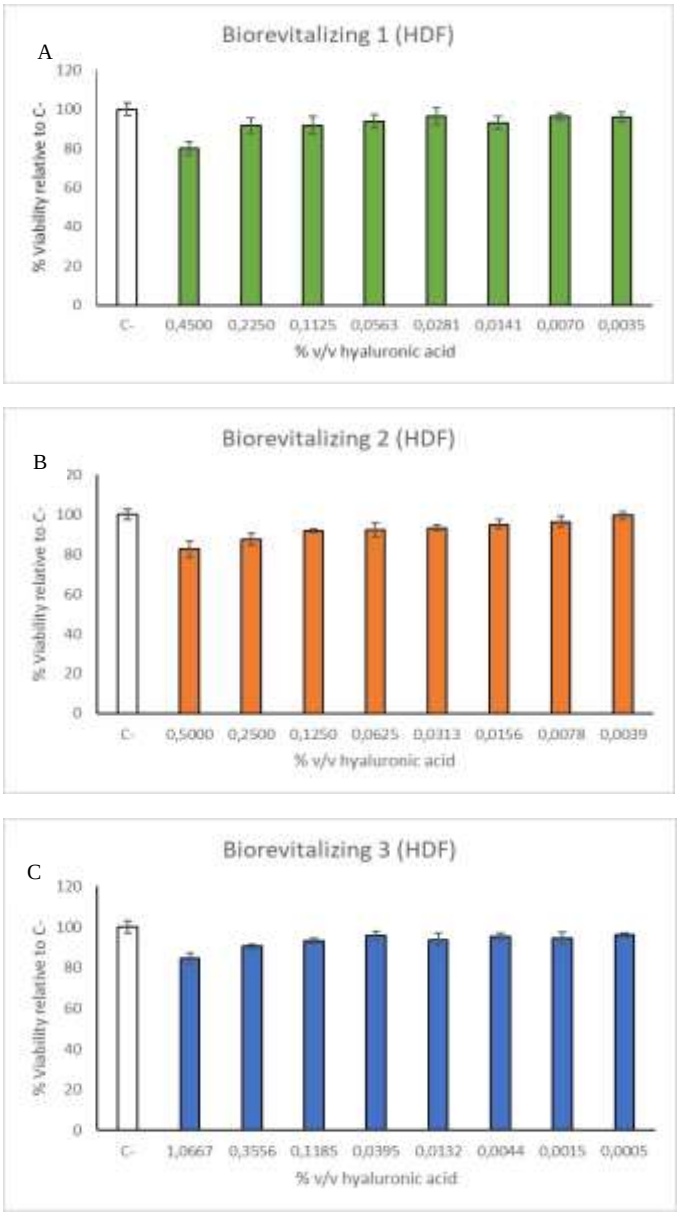

Fig 1. The percentage of cell viability after 24 hours of exposure to 'biorevitalising: a) product 1, b) product 2 and c) product 3. They were measured using the MTT assay with HDFs. The bars represent the average of

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six technical replicates and the error bars the standard deviation. Negative control (C−): untreated cells. Concentration values are expressed as % v/v.

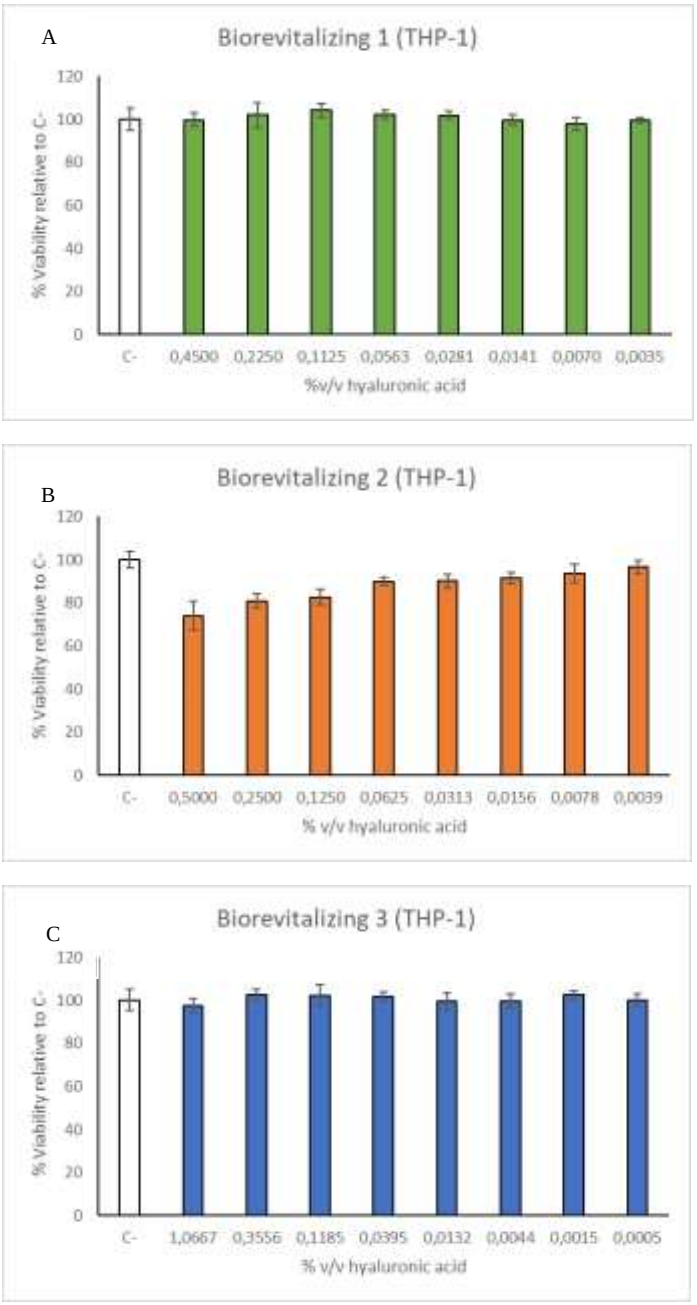

Fig. 2. The percentage of cell viability after 24 hours of exposure to 'biorevitalising: a) product 1 b) product 2, and c) product as measured using the MTT assay with the THP-1 cell line. The bars represent the average of six technical replicates and the error bars the standard deviation. Negative control (C−): untreated cells. Concentration values are expressed as % v/v.

Proliferation assay

The proliferative activity of the study products was evaluated in an in vitro proliferation assay on young and aged HDFs. Taking into account the results of the cytotoxicity assay. The results obtained for the maximum concentration of 0.2% v/v are shown in figures 3.

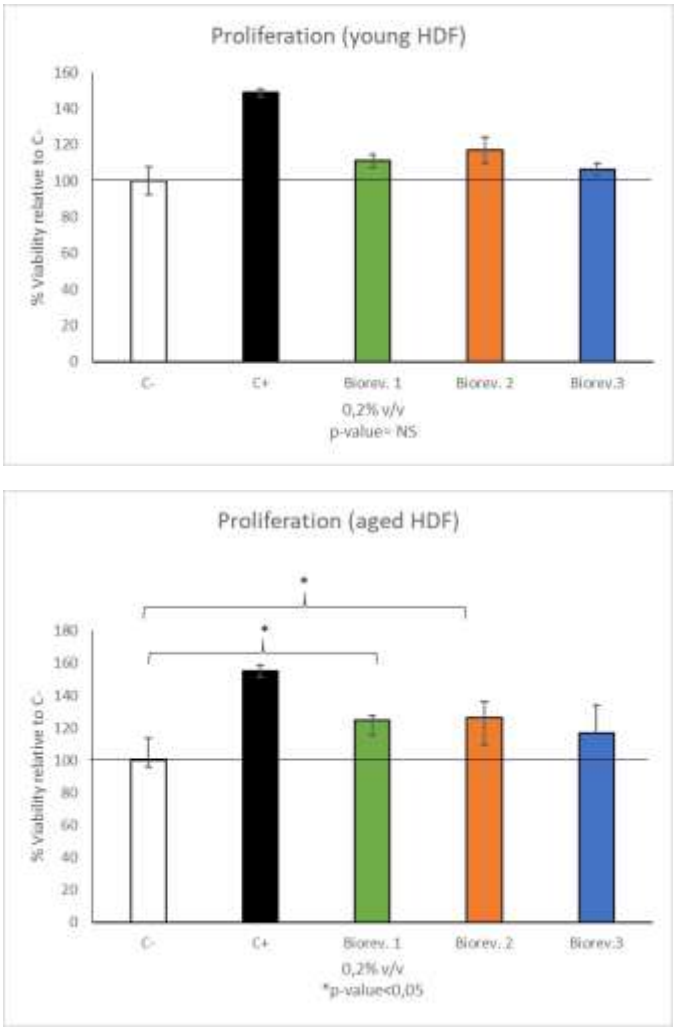

Fig. 3. The percentage of cell proliferation after 72 hours of exposure to the products was measured using the MTT assay with: a) young HDFs b) aged HDFs. The bars represent the average of six technical replicates and the error bars the standard deviation. Negative control (C−): untreated cells in serum-free medium. Positive control (C+): untreated cells in complete medium. Concentration values are expressed as % v/v.

Biorevitaliser 1 showed a proliferative activity of about 11.09% at the 0.2% v/v concentration tested in young HDFs and about 24.60% in aged HDFs. Biorevitaliser 2 showed a slightly higher proliferative activity of about 17.19% at the 0.2%/v concentration in young HDFs, and about 26.61% in aged HDFs. Biorevitaliser 3 also exhibits proliferative effects on young HDFs, with an increase of approximately 6.55% observed at a concentration of 0.2%. This proliferative activity further increases to 16.87% in aged HDFs.The results showed no significant difference between the negative control (C−) and the three biorevitalising products in the proliferation assay in young fibroblasts, with respective *p*-values of $p = 0.186$, $p = 0.542$, and $p = 0.701$. In contrast, for aged fibroblasts, Biorevitaliser 1 achieved a significance level of $p = 0.008$, while Biorevitaliser 2 showed a significance of $p = 0.005$. However, Biorevitaliser 3 did not reach significance, with a p-value of $p = 0.360$, compared to the negative control (C−).

Procollagen type 1 production assay

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The stimulation of procollagen type 1 production by the study products was assessed through an in vitro assay conducted in HDFs. The results obtained for the maximum concentration of 0.2% v/v are shown in figure 4.

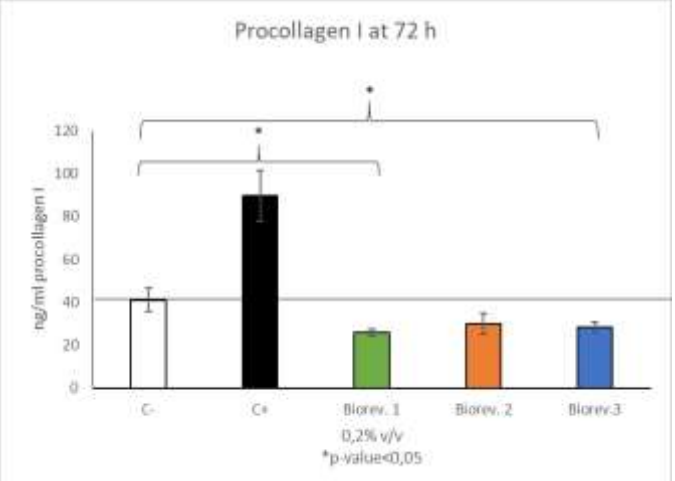

Figure 4. Procollagen type 1 production was measured in HDFs after 72 hours of exposure to the 0.2% concentration of the study products. The bars represent the average of four technical replicates and the error bars represent the standard deviation. Negative control (C−): untreated cells in serum-free medium. Positive control (C+): untreated cells in complete medium. Concentration values are expressed as % v/v.

None of the products under study exhibited an increase in procollagen type 1 production compared to the negative control (C−), which, in fact, was decreased for all three products compared to the negative control (C−). This decrease was significant for Biorevitalisers 1 and 3, with *p*-values of 0.023 and 0.008 respectively, compared to the negative control (C−). On the other hand, Biorevitaliser 2 had a p = 0.109 with respect to the negative control (C−).

Total collagen production assay

The stimulation of total collagen production by the study products was evaluated by an in vitro assay performed on HDFs.

The results obtained for the maximum concentration of 0.2% v/v are shown in figure 5.

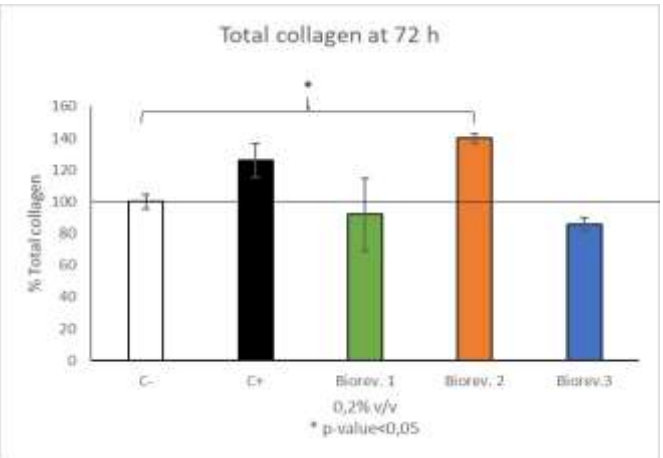

Fig. 5. Total collagen production was measured in HDFs after 72 hours to the 0.2% concentration of the study products. The bars represent the average of three technical replicates and the error bars represent the standard deviation. Negative control (C−): untreated cells in serum-free medium. Positive control (C+): untreated cells in complete medium. Concentration values are expressed as % v/v.

Biorevitaliser 2 was associated with an increase in total collagen production of 39.85% compared to the negative control (C−), while Biorevitalisers 1 and 3 were associated with a decrease in total collagen of 7.97% and 14.11%, respectively. The increase in total collagen production triggered by Biorevitaliser 2 was statistically significant compared to the negative control (C−), with a *p*-value of 0.028. Biorevitalisers 1 and 3 showed no significant increase of total collagen production compared to the negative control (C−), with the obtained p-values being 0.120 and 0.116, respectively.

Elastin production assay

The stimulation of elastin production by the study products was assessed through an *in vitro* assay conducted in HDFs. The results obtained for the maximum concentration of 0.2% v/v are shown in figure 6.

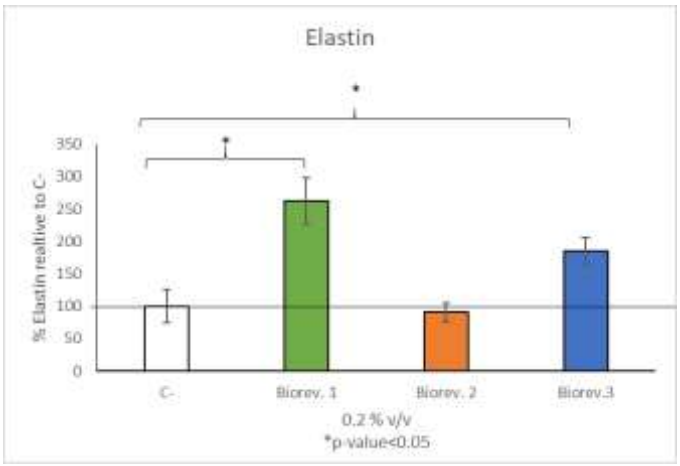

Fig. 6. Elastin production in HDFs after 24 hours of exposure to the study products. The bars represent the average of four technical replicates and the error bars represent the standard deviation. Negative control (C−): untreated cells. Concentration values are expressed as % v/v.

Biorevitaliser 1 was associated with the highest increase in elastin production compared to the negative control (C−), reaching a value of 161.92% at the highest concentration tested. With a significance of $p = 0.005$, compared to the negative control (C−).

Biorevitaliser 2 was associated with a non-significant decrease of 9.13% in elastin production compared to the negative control, with a *p*-value of 0.666.

Biorevitaliser 3 was associated with an 84.67% increase in elastin production compared to the negative control, achieving statistical significance with a p-value of 0.016.

Confocal microscopy for elastin detection

Elastin production in HDFs was visualised by confocal microscopy after exposure of the cells to the products under study. Each product was used at a concentration of 0.2% v/v. The results are shown in Figure 7. Biorevitaliser 1 and 3 were associated with greater elastin production than the negative control (C−). Biorevitaliser 2 was not found to increase elastin

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production. These findings are in line with the elastin secretion values measured by ELISA.

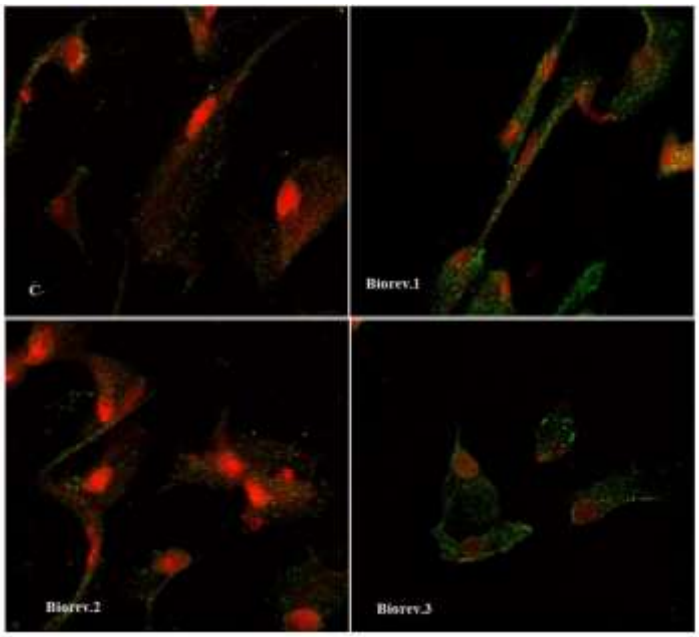

Fig. 7. Confocal microscopy images taken after 24 hours of exposure of HDFs to the study products. The concentration of hyaluronic acid was 0.2% in all products used. Negative control (C−): untreated cells. The images were taken using a 40X lens.

Wound healing assay (scratch assay)

The wound healing activity of the study products was assessed through an *in vitro* assay conducted in HDFs. The results obtained for the maximum concentration of 0.2% v/v are shown in figure 8.

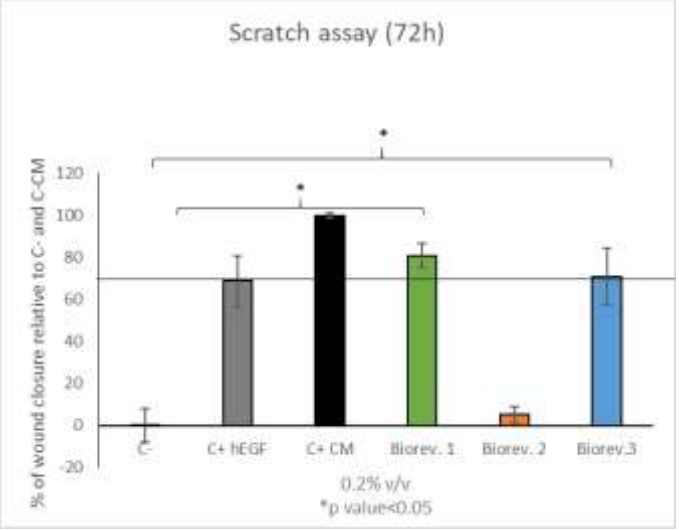

Fig. 8. Results of the scratch assay after 72 hours of exposure of the HDFs to different concentrations of the study products. The bars represent the average of three technical replicates and the error bars represent the standard deviation. Negative control (C−): untreated cells in serum-free medium. Positive control 1 (C+ hEGF): cells in serum-free medium with hEGF 20 ng/mL. Positive control 2 (C+ CM): untreated cells in complete medium. Values are expressed as a % of the surface area.

Microscopy was performed at 72 hours on the different samples to measure the non-healed area for each product. The results are shown in figure 9.

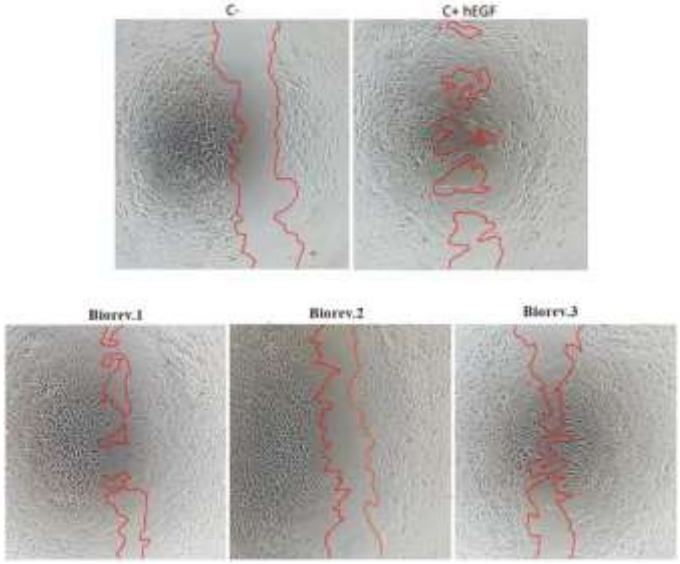

Figure 9. Representative margins of the scratch assay after 72 hours of exposure of the HDFs to different concentrations of the study products. Negative control (C−): untreated cells in serum-free medium. Positive control (C+ hEGF): cells in serum-free medium with hEGF 20 ng/mL. The images were taken using a 4X microscope lens.

At 72 hours after scratching, Biorevitaliser 1 exhibited the highest wound healing activity compared to Biorevitalisers 2 and 3, mimicking the effect of hEGF (C+). Biorevitaliser 1 repaired 80.85% of the wound compared to the negative control (C−), with a significance of $p = 0.007$; Biorevitaliser 2 exhibited only 5.27% repair, showing no wound healing activity ($p = 0.506$). Biorevitaliser 3 demonstrated a wound healing activity of 70.92% ($p = 0.015$) compared to the negative control (C−).

Anti-inflammatory activity assay

The anti-inflammatory activity of the study products was evaluated in an *in vitro* assay in THP-1 monocytes. The results obtained for the maximum concentration of 0.2% v/v are shown in figures 10 and 11.

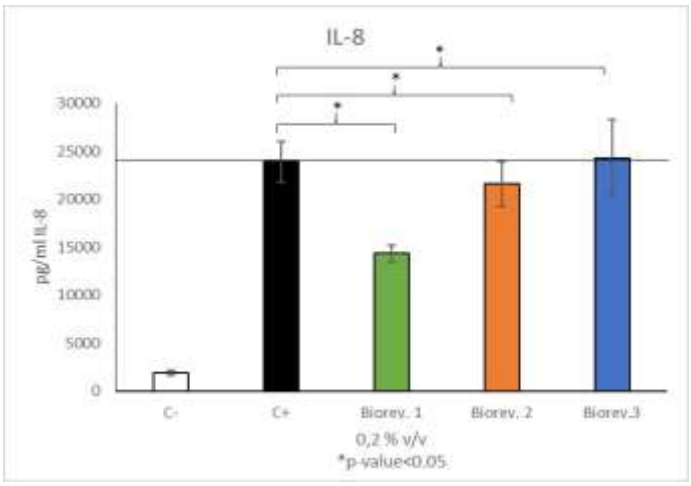

Fig 10. Inflammatory cytokine (IL-8) secretion by THP-1 cells after 24 hours of exposure to LPS and the study products. The bars represent the average of

Maria Llanos Pérez González, Begoña Garcia Diaz, Andrea Palacio Moreno, and Laura Molina Antoli "Comparative in vitro study of the effects produced by different hyaluronic acids on the extracellular matrix and fibroblasts," *International Research Journal of Pharmacy and Medical Sciences (IRJPMS)*, Volume 7, Issue 5, pp. 57-66, 2024.

four technical replicates and the error bars represent the standard deviation. Negative control (C−): cells not exposed to any product or LPS. Positive control (C+): cells exposed to LPS. Concentrations expressed as % v/v.

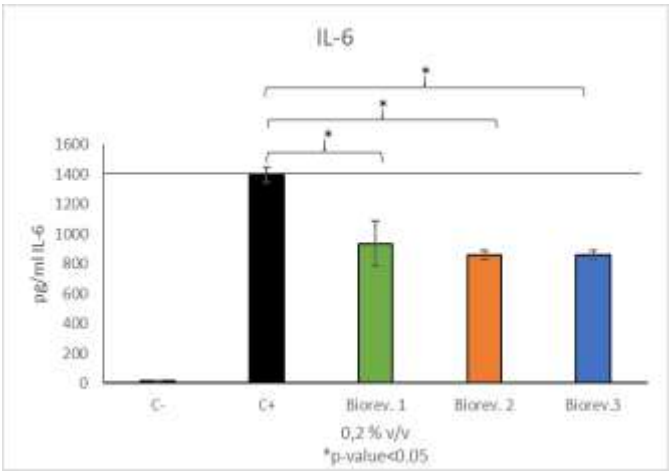

Fig 11. Inflammatory cytokine (IL-6) secretion by THP-1 cells after 24 hours of exposure to LPS and the study products. The bars represent the average of four technical replicates and the error bars represent the standard deviation. Negative control (C−): cells not exposed to any product or LPS. Positive control (C+): cells exposed to LPS. Concentrations expressed as % v/v.

Biorevitaliser 1 exhibited anti-inflammatory activity for IL-8 and IL-6, with reductions of −39.94% and −33.02%, respectively, compared to the positive control (C+), with *p*-values of 0.004 and 0.011. Biorevitaliser 2 exhibited anti-inflammatory activity for IL-8 and IL-6, with reductions of −9.70% and −38.39%, respectively, compared to the positive control (C+), with *p*-values of 0.118 and $1.32 \cdot 10^{-5}$. Biorevitaliser 3 exhibited anti-inflammatory activity for IL-8 and IL-6, with reductions of +1.41% and −38.39%, respectively, compared to the positive control (C+), with *p*-values of 0.80 and 0.006.

Scanning electron microscopy (SEM)

The morphology of HDFs was evaluated by SEM after exposure to the products under study. Each product was used at a concentration of 0.2% v/v. The results are shown in figures 12 and 13.

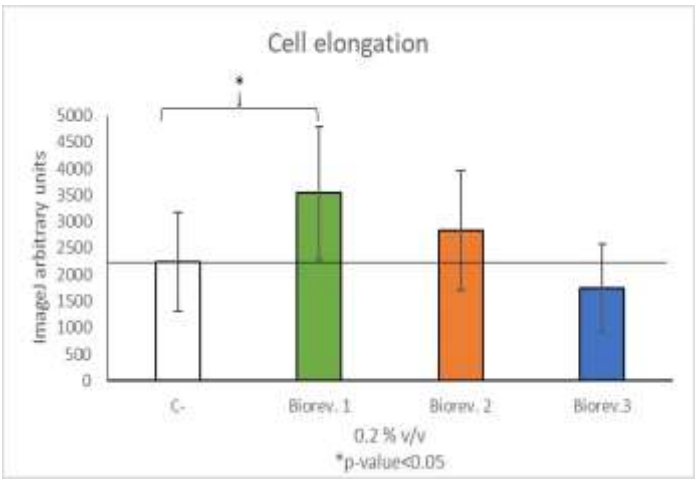

Fig. 12. Cell elongation quantified by ImageJ software in arbitrary units after 24 hours of exposure of the HDFs to the study products. The bars represent the average of at least 16 cells (distributed in three 500X fields) and the error bars represent the standard deviation. Negative control (C−): untreated cells.

Biorevitaliser 1 was visualised by microscopy to induce a more elongated cell shape at the tested concentration compared to the negative control (C−), with an increase of +57.61% and a significance of $p = 0.041$.

Biorevitaliser 2 induced a cell elongation that was +26.63% greater than that of the negative control (C−), with a *p*-value of 0.729.

Biorevitaliser 3 was associated with a −22.47% reduction in elongation compared to the negative control (C−), with a *p*-value of 0.159.

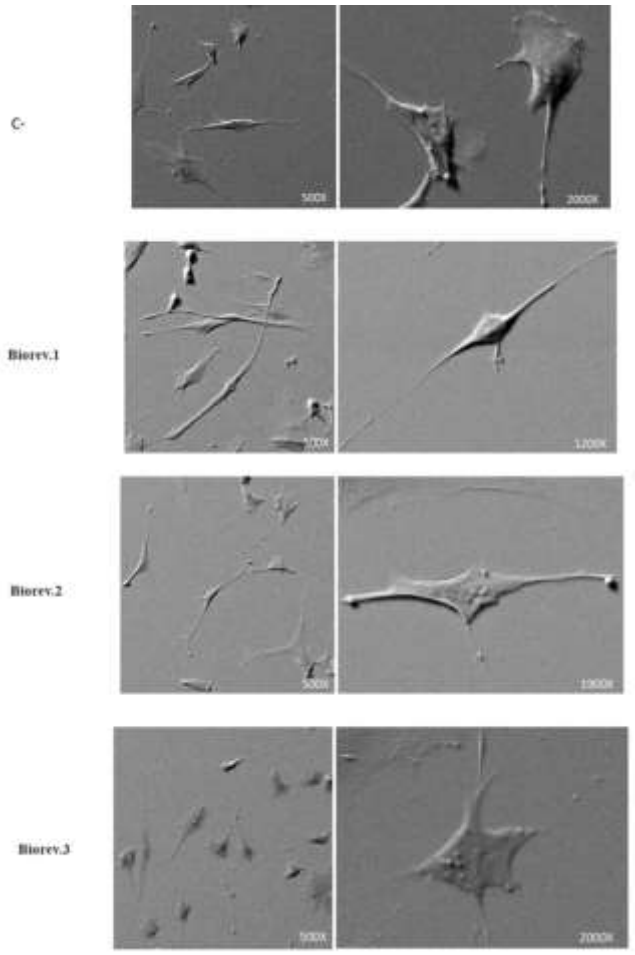

Fig. 13. SEM images taken after 24 hours of exposure of HDFs to the study products. The concentration of hyaluronic acid was 0.2% in all products used. Negative control (C−): untreated cells

IV. Discussion

Hyaluronic acid is one of the most abundant molecules in the skin, where it performs functions related to homeostasis and cellular organisation. Its molecular weight is typically around 3000 kDa [26-27].

This *in vitro* study evaluated three hyaluronic acid-based injectables currently available on the market to determine whether they exhibited bio-stimulation capacity on fibroblasts. We assessed their ability to induce cell proliferation,

production of procollagen type 1, total collagen and elastin, and their regenerative or wound healing capacity. This is because many authors have reported that fibroblasts represent a heterogeneous group of cells showing characteristic phenotypes in various physiological or pathophysiological conditions [33-35]. The results indicate that the very high molecular weight Biorevitaliser 1 exhibited a proliferative activity of 11.09% at the 0.2% v/v concentration in young HDFs and 24.60% in aged HDFs. Similarly, Biorevitaliser 2, molecular weight unknown, showed slightly higher proliferative activity of 17.19% in young HDFs and 26.61% in aged HDFs at the highest concentration tested. In contrast, Biorevitaliser 3, consisting of high and low molecular weight HA, showed a lower proliferative activity of 6.55% in young HDFs and 16.87% in aged HDFs.

Fibroblast proliferation can be stimulated by various mechanisms, including increased mechanical forces and the presence of HA [31-32,36]. Activation of the α1β1, the main membrane-bound collagen-binding receptor, is thought to play a role in the regulation of dermal fibroblast growth and collagen production, acting as a switch that controls the proliferation of these skin cells both *in vivo* and *in vitro* [37]. Other authors also confirm that procollagen type 1 expression is dependent on the TGF-β/CCN2 axis. This expression was found to increase after the addition of HA fillers to similar dermal skin cell cultures, which resulted in the expansion of collagen networks [10,38-39]. None of the products in our study stimulated the production of procollagen type 1 compared to the negative control (C−). Conversely, the highest total collagen production was recorded for Biorevitaliser 2, followed by Biorevitaliser 1. No collagen production was observed for Biorevitaliser 3.

It's important to note that the elongation of fibroblasts is linked to a positive regulation of the TGF-β signalling pathway, with subsequent targets being CTGF/CCN2 and procollagen type 1. Thus, the structural properties of dermal ECM appear to play an important role in modulating fibroblast function during human skin ageing [39]. In human aged skin *in vivo*, dermal fibroblasts express high levels of CCN1, a protein that negatively regulates collagen homeostasis by suppressing collagen synthesis and increasing its degradation [38]. Further research into the role of CCN1 revealed that it alters collagen homeostasis by promoting the expression of specific secreted proteins, including matrix metalloproteinases and pro-inflammatory cytokines. Moreover, it was found that CCN1-induced secretory proteins are elevated in aged human skin. Hence, it has been suggested that CCN1 triggers an "age-associated secretory phenotype" in dermal fibroblasts, contributing to collagen depletion and fragmentation in aged human skin [39]. This study examined microscopic changes in fibroblast morphology in response to HA exposure, revealing that: fibroblasts exposed to Biorevitaliser 1 exhibited a more elongated shape compared to the negative control. Biorevitaliser 2 caused no changes, and Biorevitaliser 3 not only did not elongate the cells, but it also led to a more compact cell pattern.

While there is extensive literature on the stimulation of collagen production in response to HA-based fillers and their interactions with the extracellular matrix [30-36], there is a lack of references regarding the mechanisms of elastin production in response to exposure to dermal fillers, both *in vivo* and *in vitro* [40-42]. This study demonstrated that Biorevitaliser 1, formulated with very high molecular weight hyaluronic acid, increased elastin secretion by 161.92% above the control value. Biorevitaliser 3 also showed an increase to 84.76%. In contrast, Biorevitaliser 2 was associated with a 9.13% reduction in elastin secretion. These results were confirmed by confocal microscopy after exposure of HDFs to the products under study, employing the same quantification method as for the assessment of elastin secretion.

In vivo studies have revealed that the degree of inflammatory response induced varies depending on several factors, including the biological material, dosage, injection site, method and individual patient characteristics [43]. However, these factors are not the sole determinants, as some authors have linked the inflammatory response to fillers with the molecular weight of HA. Ultra-low molecular weights have been documented to stimulate the immune response and promote inflammation [22,24] while high and very high molecular weights tend to inhibit them. The results of this study in monocytes indicate that Biorevitaliser 1 exhibits higher wound healing activity compared to the positive control, whereas Biorevitaliser 2 shows no wound healing activity. Biorevitaliser 3 demonstrated a similar wound healing activity to Biorevitaliser 1.

V. CONCLUSIONS

These *in vitro* assays demonstrated that very high molecular weight hyaluronic acid has the capacity to stimulate adult dermal fibroblasts. It promotes the proliferation of both young and aged fibroblasts, induces morphological changes in aged fibroblasts, giving them an appearance similar to that of young fibroblasts, and causes a significant increase in the secretion of elastin. Furthermore, it promotes wound healing.

However, further *in vivo* studies are necessary to determine whether the response observed *in vitro* aligns with the experience of dermal filler recipients *in vivo*.

Authors Contributions

Every author made an equal contribution to the planning, execution, analysis, and preparation of the research work manuscript.

Conflict of interests

There were no conflicts of interest related to this study.

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