LETTER TO THE EDITOR

Photo-crosslinking of hyaluronic acid with low-level laser therapy device: a new combined and innovative approach

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To the Editor,

Skin aging is a naturally occurring process characterized by degenerative changes in skin appearance and functionality; both intrinsic and environmental-linked aging factors are involved and, more recently, specific microbiome signatures have been associated with skin aging (1). One of the most common changes characterizing the skin aging process is the significant reduction of the epidermal hyaluronic acid (HA) (2). In recent years, HA has been used as a filler to partially revert wrinkling and to give the skin a "younger" look (3, 4). The use of cross-linked HA has been linked to several side-effects (5). In this sense, the use of light as a catalyst for the cross-linking of HA without incurring chemical substances such as methacrylate could represent a valid and safer alternative. Another increasingly popular approach for treating skin aging is low-level laser therapy (LLLT) (6). The use of LLLT allows to overcome the limitations of other commonly used skin-rejuvenations interventions and is also safer and more effective, as well as requiring minimum postoperative care. Ip and collaborators (7) reported on the usefulness of the combined use of HA injection and LLLT in the prolongation of the longevity of degenerative knee joints.

In the present work, we combined LLLT and a medium-size HA, used as bio-revitalizing agent, to achieve its photo-crosslinking and demonstrate its efficacy *in vitro*. A biorevitalizing gel with HA (Giuliani SpA) was used for photo-crosslinking and *in vitro* and *in vivo* tests. The product consists of medium-chain (1.0-1.5x106 Da) HA, obtained from *Streptococcus equi* bacteria, formulated to a concentration of 20mg/mL in a physiologic buffer. A medium-chain HA from Sigma Aldrich (Milan, Italy) was used for *in vitro* assays.

Cross-linking of HA in 3D skin models

3D Phenion® full-thickness skin models were purchased from Henkel (Phenion® FT; Düsseldorf, Germany) and cultured in 3.5 cm Petri dishes filled with 4 mL pre-warmed air-liquid-interface (ALI) medium, provided by the manufacturer. The medium was refreshed once after an initial overnight equilibration period. Tissues were subjected to experiments after the overnight equilibration at 37°C and 5% CO_2. 0.1 mL of HA was injected into the tissues. A 4 Lights laser (Monodermà Tech, Giuliani SpA, Milan, Italy) was used for tissue irradiation. The laser allows the emission of three different wavelengths, 450nm, 650nm, and 1064nm, simultaneously. Total irradiated energy was 2400 mj/min. After HA injection, the tissues were exposed for 1 min to the emission of the three different wavelengths. Tissues not injected with HA and not

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exposed to laser treatment were used as negative control. Immediately after the 24-h exposure period, 4 biopsy punches (2 mm) were collected and maintained at -80°C until further analysis.

Tandem mass spectrometry (MS/MS)

SANIST platform was used for Tandem Mass Spectrometry analysis as described by Albini and coll. (8) with minor modification. Briefly, prior to LC/MS analysis, samples were treated with a triple extraction procedure using three specific solvents with different polarities (H2O, CH3OH, and CH3CN). This procedure makes it possible to maximize the extraction efficiency of both modified and non-modified hyaluronic acid.

Before mass spectrometric analysis, the samples were separated by liquid chromatography by Ultimate 3000 UPLC (Thermofisher, San Jose, CA, USA) using a Phenomenex reverse-phase C-18 chromatographic column (50 x 2.1 mm; particle size, 5 µm; pore size, 100 Å; San Jose, USA). The mobile phases were: A) 0.2% (v/v) HCOOH(aq) and B) CH3CN. The composition of the elution gradient was 2% (v/v) B between 0 and 2 min; 2 to 30% B between 2 and 7 min; 30 to 80% between 7 and 9 min; 80% B between 9 and 12 min; 80 to 2% B between 12 and 12.1 min; and the column was equilibrated with 2% B between 12.1 and 17 min. The eluent flow was 0.25 mL/min. The injection volume was 15 µL. A mass spectrometer operating in SACI/ESI condition and positive ion mode was employed. Following ion, the source parameters used were: ESI capillary voltage 2750 V, drying gas: 12 L/min, nebulizer gas: 60 psi, and temperature: 350°C. The data were acquired in data-dependent scan mode (auto-fragmentation).

Changes in interleukin-1 beta, hyaluronan synthetase and metalloproteinase-12 gene expressions

Normal human keratinocyte NCTC 2544 (National Institute on Cancer Research, Italy) were cultured in 5% CO_2, at 37°C on RPMI medium containing 2 mM l-glutamine, 1% of penicillin (100U/ml)/streptomycin (100U/ml) supplemented with 10% Fetal Bovine Serum (FBS) (basal medium). Mouse fibroblasts (Balb 3 T3, clone CCL-163™) were purchased from ATCC Culture Collection (Middlesex, UK), and were cultured under humidified atmosphere (5% CO_2, 37°C), using Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% (w/v) calf bovine serum (CBS), 1% penicillin (10,000 U/mL)/ streptomycin (10,000 U/mL) mixture, and 1% non-essential amino acid solution (NEAA). Both cell types were incubated in 25 cm^2 surface culture flasks at 37°C with 5% CO_2 until ca. 80% of confluence was reached. Cells were then harvested with trypsin/ EDTA and seeded at a density of 1×10^6 cells per well into 12-well plates for qRT-PCR.

Twenty-four hours after seeding on 12-well plates, NCTC2544 and BALB3T3 80% confluent cells were exposed to 1 mM hydrogen peroxide (100 µl/well) for 2 h and 30 min, respectively. After the treatment medium was removed, cells were washed and exposed to 200µg/mL of HA followed by irradiation with the three different wavelengths (450, 650, and 1064nm), simultaneously. RNA for qRT-PCR analysis was extracted 24 h after the treatment. Tri Reagent (Sigma Aldrich) method, as described by Chomczynski and Mackey (9), was used and the cDNA was then synthesized from 2 µg RNA template in a 20 µl reaction volume, using the PrimeScript RT-PCR Kit (Takara, Japan). The cDNA was amplified and detected by the Stratagene Mx3000P Real-Time PCR System (Agilent Technologies Italia S.p.A., Milan, Italy). The amplification of cDNA from NCTC cells was conducted using the following Taqman gene expression assays: Hs01555410_m1 (interleukin-1 beta, IL-1β,) andHs999999 m1 (human glyceraldehyde-3-phosphate dehydrogenase, GAPDH). The following genes were used for BALB3T3 cell: Mm03048195_m1 (hyaluronan synthetase, HAS1), Mm00500554_m1 [metalloproteinase-12 (MMP-12)] and Mm00466519ml (beta-actin, β-actin). GAPDH and β-actin were used as housekeeping genes, respectively. PCR amplifications were carried out in 20 µl of total volume. The mixture of reaction contained 10 µl of 2X Premix Ex Taq (Takara, Japan), 1 µl of 20× TaqMan gene expression assay, 0.4 µl of RoX Reference Dye II (Takara, Japan), 4.6 µl of water, and 4 µl of DNA. PCR conditions were the following: 95°C for 30 s followed by 40 cycles

Table I. *Over-expressed signals (m/z) m/z signals in samples injected with HA and irradiated with the combination of the three wavelengths (Combo-x), compared to the negative control (injected HA/no laser)*

	m/z
Combo-1	768.1016084
Combo-2	631.577331
Combo-3	695.0770477
Combo-4	483.223441
Combo-5	404.986113
Combo-6	203.6698455

of 95°C for 5 s, 60°C for 20 s. PCR reactions were performed in duplicate using an MX3000p PCR machine (Stratagene, La Jolla, CA, USA). Δ cycle threshold was used for the calculation of the relative abundance in the expression of each gene.

RESULTS

The photo-crosslinking of HA was tested on 3D Full Thickness skin models through Tandem Mass Spectrometry Analysis (MS/MS). Samples irradiated with the combination of the three different wavelengths (450-650-1064nm) were compared with the negative control sample (not irradiated). The analyte m/z value, retention time, and their peak chromatographic area are shown in Fig. 1. The following over-expressed signals (m/z) were found in samples injected with HA and irradiated with the combination of the three wavelengths, compared to the negative control (injected HA/no laser): 768.10, 631.58, 695.08, 483.22, 404.99, 203.67. Each over-expressed signal corresponds to a different chemical structure. The identification of each unknown structure was made through a library-search procedure (National Institute for Standard and Technology-NIST). This search is based on the criterion of similarity and allows the identification of the structural features of an unknown compound from its electron-ionization mass spectrum. SANIST software was used for this purpose (8). Following data acquisition, the software automatically proceeds to data elaboration using a proprietary (ISB - Ion Source & Biotechnologies S.r.l., Bresso, Italy) Bayesian elaboration model.

The presence of over-expressed signals (m/z) (Table I) in irradiated samples and the analysis of similarity confirmed the photo-crosslinking of HA. The analysis also revealed that the photo-crosslinking of HA takes place on the glycosidic bonds present in free linear polymer or fragment of

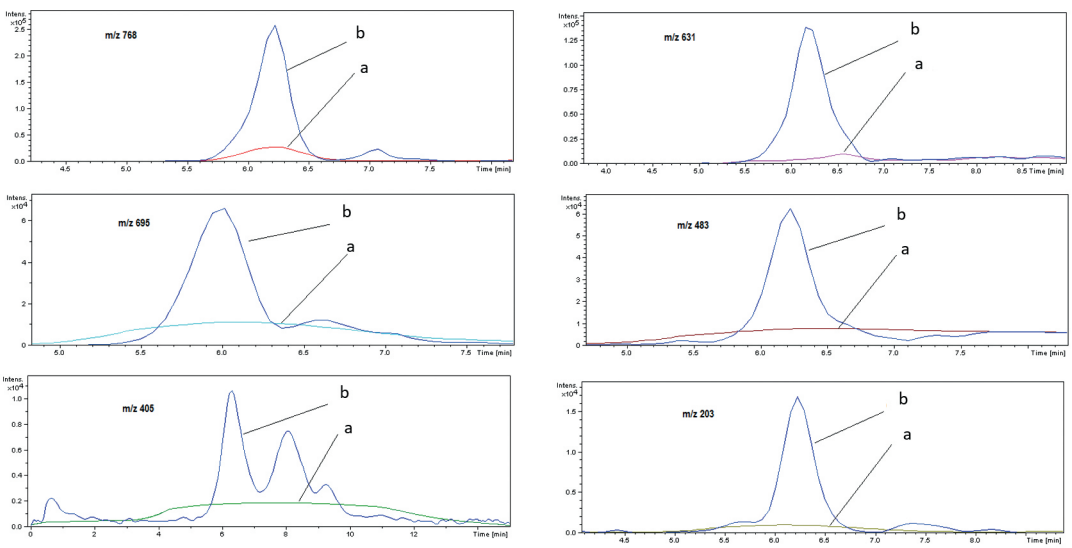

Fig. 1. *Representative full scan (base peak) extracted ion 'SANIST' mass chromatograms.* ***a)*** *HA injected Full-thickness 3D model.* ***b)*** *HA injected and LLLT irradiated (450,650 and 1064nm, combined) Full-thickness 3D model.*

HA and other species inside the tissues and is able to create such bonds. The comparative analysis (expressed in counts/s) carried out on the average of the abundance of ions collected in the samples shows a higher Area (count/s) in the sample irradiated compared with negative control, and confirms the photo-crosslinking of injected HA.

In vitro activity of photo-crosslinked hyaluronic acid

In the present work, linear HA was combined with LLLT (450-650-1064nm, combined). After the injection of linear HA, LLLT was applied by means of a 3-wavelengths LLLT device. This leads to a rearrangement of linear HA in a self-aggregate natural cross-linked system. The intra- and inter-chain interactions via glycosidic bonds are responsible for the formation of a temporary network structure also capable of incorporating free circulating HA. Coupling linear HA to LLLT results in a volumizing and filling effect of the skin without incurring the use of chemical substances. We evaluated the anti-aging efficacy of the combined treatment with HA and LLLT by means of qRT-PCR, examining gene expression profiles associated with skin aging on human keratinocytes NCTC 2544 and murine fibroblast BALB3T3.

Even though the injection of the medium-size HA alone did not reduce the expression of *IL-1β* gene in human keratinocytes NCTC2544, compared to negative control (Fig. 2a) the combined use of LLLT laser (3 combined wavelengths, 450/650 e 1064nm) produced a significant ($p<0.05$) decrease of *IL-1β* gene (Fig. 2a). *HAS-1* gene expression was significantly ($p<0.05$) decreased after the combined treatment of HA and LLLT devices (Fig. 2b). The exposition of fibroblast BALB3T3 to the combination of 3 wavelengths of LLLT device after HA injection produced a significant ($p<0.05$) decrease of *MMP-12* compared to the sample injected with HA alone and negative control (Fig. 2c).

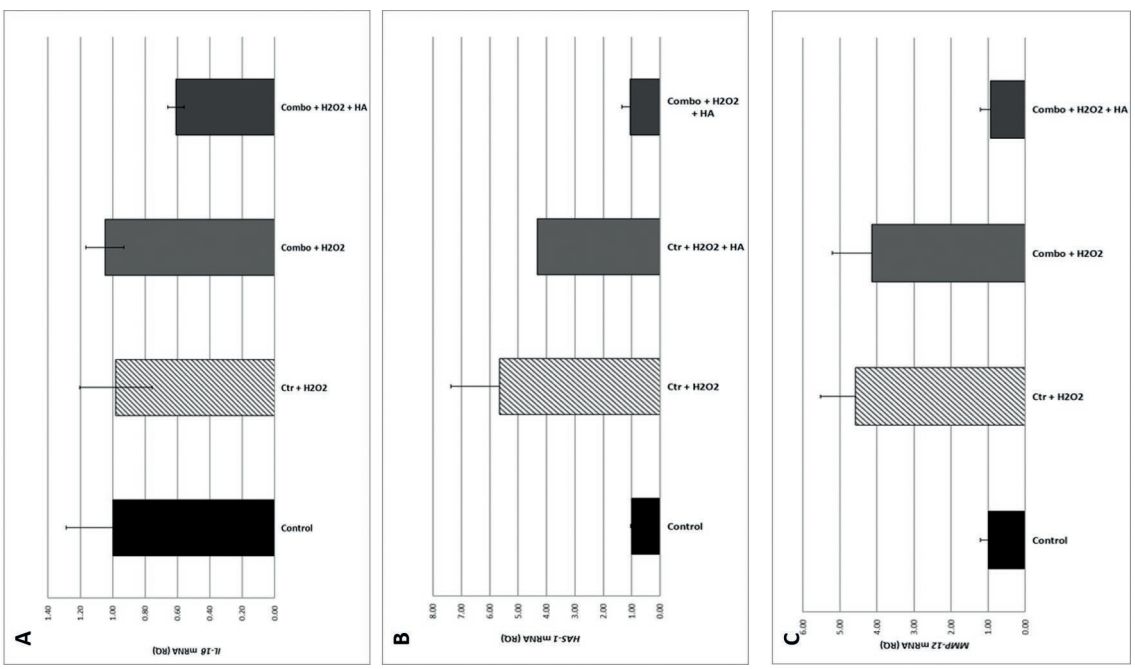

Fig. 2. *Expression of the interleukin 1 beta (IL-1β) (**a**), hyaluronan synthetase 1 (HAS-1) (**b**), and matrix metalloproteinase 12 (MMP-12) (**c**) genes as determined by RT-PCR. NCTC or BALB3T3 cells were treated with: medium alone (control); medium and 1mM hydrogen peroxide (Ctr+H2O2); LLLT laser (3 combined wavelengths, 450/650 e 1064nm) and 1mM hydrogen peroxide (Combo+H2O2); LLLT laser (3 combined wavelengths, 450/650 e 1064nm), hyaluronic acid and 1mM hydrogen peroxide (Combo+H2O2+HA). Analyses were carried out after incubation at 37°C for 24 h, under 5% CO2. Data are the means±SD of three separate experiments performed in triplicate. Statistical differences between mean values were determined with Tukey's HSD test. The asterisk indicates a significant difference (P < 0.05) to the control.*

DISCUSSION

Photo-crosslinking is a chemical reaction that takes advantage of some specific wavelengths to catalyze a chemical reaction. In the present work, we tested the hypothesis that the polymerization of HA could be obtained intradermally via LLLT irradiation.

As a complex process, skin aging is regulated both from endogenous and exogenous factors (1). This leads to progressive changes in pathways related to energy metabolism, inflammation, epidermal barrier and oxidative stress (10). Nowadays, the use of biorevitalizing agents such as HA has acquired importance due to its great versatility and efficacy (3, 4). The main limitation of HA, used in its linear form (not cross-linked), is its rapid enzymatic degradation, and for this reason, HA is often subjected to cross-linking by means of chemical substances (mainly Butanediol diglycidyl ether, divinyl sulfone and methacrylate). Moreover, the use of LLLT represents a useful tool to reverse dermal and epidermal signs of aging (6).

The aging process of the skin is characterized by a chronic progressive increase of the proinflammatory response of the cells (11). "Inflamm-aging" is the term coined in 2000 to described this process and while the causes are not completely understood, the involvement of a dysregulation of the inflammatory cytokine network has been reported (11), including the stimulation of NF-κB and the IL-1β-mediated inflammatory cascade (11). The aging process is also reported to increase the expression of hyaluronidase 1 and 2 and metalloproteinases (2, 12). An increased level of HAS genes means more availability of HA, even in the photo-crosslinked form, and improvement of the skin rejuvenation process. Other proteases strictly involved during the aging process of the skin are metalloproteinases that can enzymatically accelerate the aging process from decades to hours (12). MMPs produced by different skin cell types have different functions and roles during aging. In particular, MMP-12 secreted by macrophages and fibroblasts is responsible for elastin degradation.

The present findings on the effect of LLLT-photocrosslinked HA on *IL-1β, HAS1, MMP-12* genes corroborate the usefulness of LLLT for reducing inflammation in many skin conditions, including skin aging, and suggest a role of photo-crosslinked HA in reducing inflammatory cascade caused by the aging process.

In conclusion, in the present work, we confirmed by MS/MS the cross-linking of HA by LLLT. Data from *in vitro* assays on human keratinocytes and murine fibroblasts also reported that the ability of photo-crosslinked HA effectively counteracts skin aging by reducing inflamm-aging, HA, elastin, and collagen degradation. Future clinical work should be aimed at investigating the *in vivo* safety and efficacy of LLLT photo-crosslinked hyaluronic acid.

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