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HYALURONIC ACID IN DERMAL REJUVENATION: AN *IN VITRO* STUDY

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The purpose of this paper is to evaluate the role of hyaluronic acid in bio-revitalization by testing several extracellular matrix biological parameters in cultured dermal fibroblasts. To this aim, fibroblastic expressed genes after exposition to three hyaluronic acid medical devices were evaluated. Cells were seeded on a layer of three different medical devices containing 6.2, 10 and 20 mg/ml of hyaluronic acid for 24 h. Real Time Polymerase Chain Reaction was performed to investigate gene expressions. Genes encoding hyaluronic acid synthesis and degradation, Metalloproteinases 2 and 3 and Desmoplakin production as well as GDF6, and IGF1 were activated by hyaluronic acid products. The *in vitro* study showed similar effects on tested genes despite a different concentration of hyaluronic acid contained in the medical devices and the simultaneous presence of other additives. Based on the reported data, gene activations are an aspect of metabolic modulation of signalling pathways rather than the proportional production of a specific connective tissue molecule. Indeed different hyaluronic acid concentration and the presence of other additives did not change the overall effect on the studied genes. We believe that the optimization of extracellular matrix micro-environment, obtained by enhanced structural support with hyaluronic acid, leads to functional and metabolic improvement.

Age-dependent decline of skin seems to be related more to quality of extracellular matrix (ECM) than to chronological age. The accumulation of fragmented collagen in aging impacts on fibroblast function by altering the physical properties of the dermal micro-environment. The ECM usually described as amorphous is instead a highly organized structure connecting the fibroblast cytoskeleton by a complex network (1). Intrinsic and extrinsic skin aging are distinctive events but share some molecular pathways. It is known that ROS (oxygen reactive species) play a major role in both processes (2).

Anti-aging therapies, named bio-stimulation and bio-revitalization, are directed to skin improvement through intra-dermic injection of different drugs in order to improve cellular function directly or via ECM optimization (3).

Hyaluronic acid (HA) is used in different concentrations in bio-revitalization procedures and in different dermal fillers after crosslinking to reduce its hyaluronidase sensitivity. HA is a polymer of dimeric units of *N*-acetyl-glucosamine and

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glucuronic acid synthesized by HA synthetase on the inner surface of plasma membrane, and then secreted in extracellular space (4).

HA degradation occurs by a free radical mechanism or by hyaluronidases (HYAL) with a dynamic turnover rate. The half-life is 3-5 min in the blood, less than a day in the skin and 1-3 week in the cartilage. Among the six human known HYALs, HYAL 1 is the major in serum.

Two main receptors capable of connecting HA to the cell surface are described: the Cluster of Differentiation 44 (CD44), located on the plasma membrane and regulating cell adhesion and migration; and the Receptor for Hyaluronan-Mediated Motility (RHAMM), located in the cytoplasm and responsible for cell growing and migration (5).

The purpose of this study is to investigate the role of HA in bio-revitalization. To this aim, some fibroblastic expressed genes after exposition to three medical devices were evaluated. These products contain different concentrations of HA and are commonly used in aesthetic medicine.

MATERIALS AND METHODS

Primary human dermal fibroblast cell (HFb) culture

Fragments of dermal tissue of healthy volunteers were collected during surgery. The pieces were transferred to 75 cm^2 culture flasks containing DMEM medium (Sigma-Aldrich, Inc., St. Louis, Mo) supplemented with 20% foetal calf serum and antibiotics, i.e. penicillin 100 U/ml and streptomycin 100 µg/ml (Sigma Aldrich, Inc.).

Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Medium was changed the next day and twice a week. After 15 days, the pieces of dermal tissue were removed from the culture flask. Cells were harvested after additional 24 h of incubation.

Cell cultures

Human dermal fibroblasts at the second passage were seeded on a layer of three different medical devices:

1 - solution of 6.2 mg/ml of non-reticulated HA of biotechnological origin with a buffered medium containing ammonium molybdate, ammonium metavanadate, calcium chloride, iron sulphate, potassium chloride, copper sulphate, magnesium chloride, manganese sulphate, sodium acetate, sodium hydrogen carbonate, sodium chloride, sodium hydrogen phosphate, sodium metasilicate, sodium selenite, nichel chloride, tin chloride, zinc sulphate, alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glicine, histidine, isoleucine, leucine, lysine, methionine, phenilalanine, proline, serine, threonine, triptophan, tyrosine, valine, adenine (Vit. B4), biotin (Vit. B8), calcium pantothenate (Vit. B5), choline chloride, folic acid (Vit. B9), inositol (Vit. B7), nicotinamide (Vit. B3), pyridoxine (Vit. B6), riboflavin (Vit. B2), thiamine (Vit. B1), vitamin B12, deoxythymidine, glucose, putrescine, sodium pyruvate, lipoic acid, (Viscoderm Skinkò E Ibsa// Revitacare, Saint Ouen d'Aumone, France);

2 - HA sodium salt 10 mg/ml with a buffered medium containing polynucleotides 10 mg/ml, mannitol, sodium phosphate monobasic dihydrate, sodium phosphate dibasic dehydrate (Newest Mastelli, Sanremo, Italy);

3 - Stabilized HA gel 20 mg/ml in saline solution phosphate buffered (Restylane® Vital, Uppsala, Sweden).

A set of untreated cells was used as control. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. After the end of the exposure time, cells were trypsinized and lysed for RNA extraction.

RNA processing and Real Time Polymerase Chain Reaction

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMan Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX), following the manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse-transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc.).

Finally, the cDNA was amplified by Real Time Polymerase Chain Reaction (PCR). The amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and the specific assay was designed for the investigated genes. SYBER assay reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems). Each reaction contained 10 µl 2´ Power SYBR® Green PCR Master Mix (Applied Biosystems), 400 nM concentration of each primer and cDNA.

All experiments performed included non-template controls to exclude contamination of reagents. PCR was performed with two biological replicates.

Expression was quantified using Real Time PCR. The gene expression levels were normalized to the expression of the housekeeping gene *Homo sapiens* transferrin receptor protein 1 (TFRC). Quantification was made with the delta/delta calculation method. Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems), and are listed in Table I.

RESULTS

Fig. 1A shows the effects of the three different

Table I. *Primers sequences for SYBR® Green assay.*

Gene symbol	Gene name	Primer sequence (5'>3')
HAS1	Homo sapiens hyaluronan synthase 1	F-CTCGGAGATTCGGTGGACTA R-CGCTGATGCAGGATACACAG
HYAL1	Homo sapiens hyaluronoglucosaminidase 1	F-ACAGATGTATGTGCAACACCG R-AAGGGCCCCAGTGTAGTGTC
MMP2	Homo sapiens matrix metallopeptidase 2	F- TACGATGGAGGCGCTAATGG R- CGCATGGTCTCGATGGTATT
MMP13	Homo sapiens matrix metallopeptidase 3	F- TTTCCCAAGCAAATAGCTGAA R- AGTTCCCTTGAGTGTGACTCG
GDF6	Homo sapiens growth differentiation factor 6	F-CCCCACGAGTACATGCTGTC R-GAGCATGGACACATCAAACAA
IGF1	Homo sapiens insulin-like growth factor 1	F-GCGCAATGGAATAAAGTCCT R-ACAGCGCCAGGTAGAAGAGA
DSP	Homo sapiens desmoplakin	F-ATGACCTGAGGAGAGGACGAA R-AGGCTCTCTCTTTCCTGTACCAC
TFRC	Homo sapiens transferrin receptor protein 1	F-CGCTGGTCAGTTCGTGATTA R-GCATTCCCGAAATCTGTTGT

medical devices on the primer HAS1 and HYAL1: there is an up-regulation of both genes.

In Fig. 1B, HYAL1, MMP2, MMP 3 (i.e. genes encoding for ECM catabolic enzymes) are compared. All are activated.

In Fig. 1C, the different activation can be appreciated of GDF6, IGF1, DSP metabolic genes.

Fig. 1D gives a global view. It is noteworthy that there is a common trend with regard to relative activation of all genes by the 3 different medical devices.

DISCUSSION

HA degradation and synthesis

HA, the major component of ECM, is the main component of the 3 investigated medical devices and its effects is studied in fibroblast culture especially with regard to its own synthesis and degradation.

HAS1, a gene responsible of HA production, is up-regulated especially with lower HA concentration (i.e. 6.2 mg/ml). This is probably due to the differences among the tested medical devices

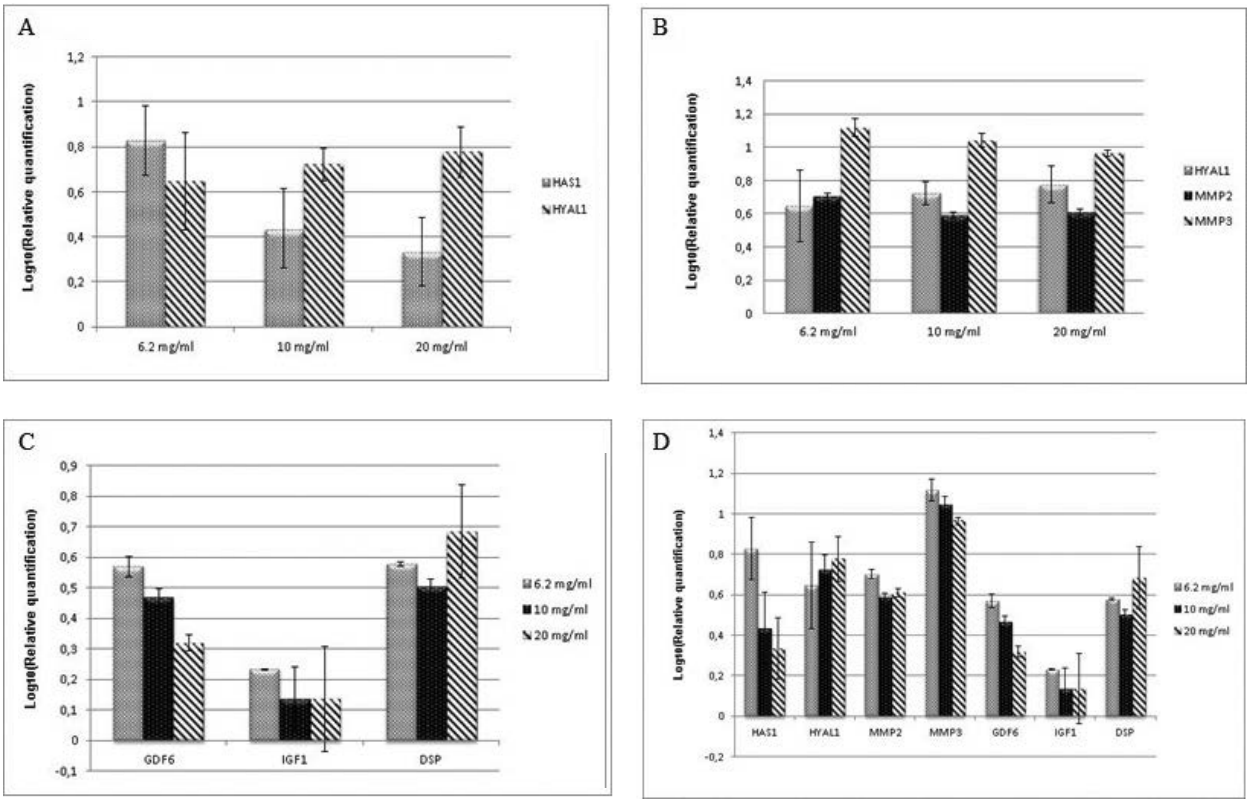

Fig. 1. ***A)*** *Effects of the three different medical devices on the HAS1 and HYAL1 gene expression.* ***B)*** *Comparison of the expression among HYAL1, MMP2 and MMP3 genes.* ***C)*** *Activation of GDF6, IGF1, DSP metabolic genes after treatment.* ***D)*** *Global view of the gene activation after treatment.*

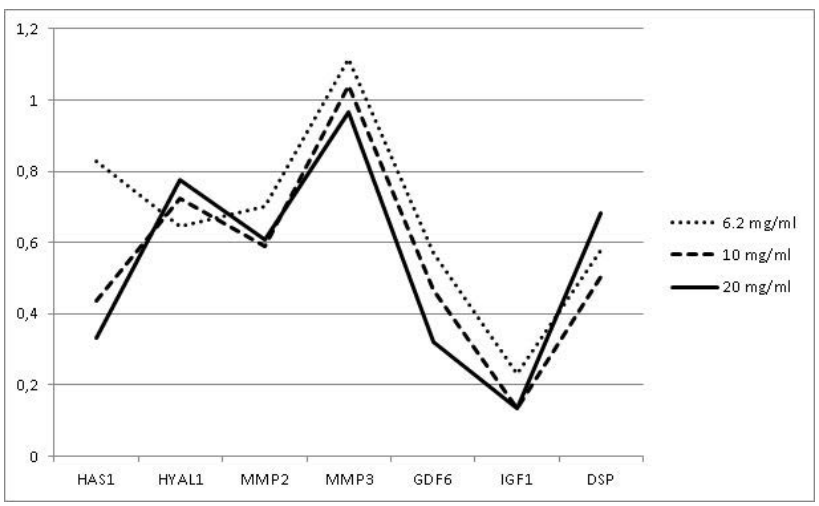

Fig. 2. *Qualitative comparison among the three products used in the experiments to produce bio-revitalization in vitro.*

related to their formulation and enrichment. In fact, the 6.2 mg/ml HA product contains aminoacids and vitamins to improve protein synthesis. The 10 mg/ml HA product, instead, presents the nucleotides in its formulation to obtain an associated bio-stimulating effect.

In addition, HA fragmentation (not declared by productors) is another variable to be considered. In fact, for some Authors it can change HA-receptor interaction (6, 7).

Other Authors observed that HA supplementation to fibroblast cultures results in inhibition of cell proliferation. This inhibition is directly related to HA concentration but it is not dependent on molecular weight. This probably happens because HA is fragmented anywhere after its interaction with membrane receptor CD44 and internalization (8).

Our results demonstrate a strong activation of HYAL1. The product with higher HA concentration exhibited higher HYAL 1 gene expression. This is negative feed-back control and is related to ECM remodeling and tissue reparing. It has been reported that 24 hours after UVB irradiation (i.e. an inflammatory event) there is a major increase of HAS1 compared to HYAL1, both detected with RT-PCR. However, HA ELISA determination decreased from 3 to 24 hours with accumulation of HA degradation products. Only later there was increased HA synthesis even if the dermal balance remains negative (9).

MMPs related genes

ECM degradation is an important factor in tissue healing. It is under the control of metalloproteinases (MMPs) and their antagonists, i.e. tissue inhibitor of metalloproteinases (TIMPs). Twenty-six human MMPs were identified which were Zn-containing enzymes.

Here, MMP2 and 3 are investigated. Quan et al. demonstrated that transmembrane integrine receptors coupling with actin cytoskeleton of fibroblasts adhere to ECM collagen fibrils. This bridge generates mechanical forces which are essential for fibroblast shape: it is more widespread in young and more collapsed and round in aged dermis. In aging, the collagen fibrils cleavage can alter the biomechanical forces of ECM. This impairs fibroblast attachment determining a reduced production of type I collagen and an up-regulation of MMP activity via TGF beta signaling pathway (1).

Starting from this back-ground, two different MMPs related to fibres and proteoglicans were investigated. MMP2 (or gelatinase type A) is specific catabolic enzyme acting against collagen IV of the basal membrane but also on collagen types I, II and III.

MMP3 (or stromelysine) degrades specifically the non-fibrillar part of ECM. Specifically, it has a catabolic action against proteoglycans, fibronectin, laminin type IV collagen but not on type I collagen. Thus MMP3 is for some aspects similar to hyaluronidases. Our results support this idea: in Fig. 1B both enzymes exhibited an up-regulation. This effect was more marked for stromelysine and was independent from the HA concentration (10).

In these experiments, gene expression of MMPs were always enhanced by all three medical devices. MMP2 activated collagen catabolism, therefore it participates in tissue remodeling. In addition, MMP2 possess an anti-inflammatory role through monocyte chermoattractant protein 3. Moreover, TGFbeta1 results activating on gelatinases and inhibiting on the other MMPs (11).

Thus interaction of MMPs and cytokines can be either enhancing or inhibiting, and this represent a modulatory role on inflammatory response of tissues (12).

Moreover, cellular response to growth signals and oxidative stress are also mediated by MAP kinases. Induction of transcription factor c-Jun, via stress activated protein kinase (MAPK), promotes expression of MMPs 1, 3, 9 and prevention of the expression of procollagen I (13).

Metabolic signalling related genes

GDF 6 is the growth differentiation factor 6, a member of transforming growth factor beta (TGF beta) family. It is important in embryo development and growth, and in dermal youth maintenance. GDF6 is also involved in the patterning of the epidermis. It induces tendon and cartilage formation. This phenomenon leads to collagen and proteoglycan synthesis, similarly to what happens in other connective tissues. The inhibition of procollagen gene expression after UV irradiation depends upon blocking of TGF beta type II receptor (14).

Chang et al. demonstrated that GDF6 induces epidermis and inhibits neural tissue in dissociated cells by binding to the neural inductor noggin. The Authors underline that GDF6 can synergize with BMP in embryo to regulate patterning of the ectoderm (15).

In these experiments, GDF6 gene expression was inversely up-regulated in respect to HA concentration (Fig. 1C).

Insulin-like growth factors (IGF) or somatomedins are a family of peptides which play important roles in mammalian growth and development. IGF1 mediates many of the effects of growth hormone by stimulating the incorporation of sulphate into cartilage. Human IGF1 is a single chain of 70 amino acids that displays homology to proinsulin. In our study it is lightly stimulated by all the products (16).

DSP encodes desmoplakin, an important protein in cell-cell junction maintenance and thus in promoting epidermal integrity. Its importance in the dermal compartment is outlined by different papers where the presence was demonstrated of DSP in cell, derived not only from ectoderm but also is from mesenchima such as cardiac, corneal fibroblasts and odontoblasts (17-19). In this study, DSP encoding gene is strongly activated in all samples (Fig. 1C and 1D) and this finding supports the idea of ECM optimization performed by bio-revitalization procedures.

Fig. 1D shows a quantitative comparison among gene expressions. The strongest effect on MMP3, and the relatively homogeneous behaviour of gene activation with the different products except for HAS1 can be noted.

Fig. 2 shows a qualitative comparison among the three products used in these experiments to produce bio-revitalization *in vitro*. It is evident that bio-revitalization with different medical devices and with different HA concentrations can interact with structural and regulative pattern of dermis despite the differences in HAS1 expression gene.

In conclusion, the *in vitro* testing of the three bio-revitalization commercial products determined an up-regulation of genes responsible for HA synthesis and degradation, for Metalloproteinases 2 and 3 activation, for Desmoplakin production and for metabolic signalling (GDF 6, IGF1).

Bio-revitalization procedures have a similar effect despite different HA concentrations and the concomitant presence of other molecules (i.e. aminoacids, vitamins, polynucleotides) which are different in quality and amount. Only HAS1 gene shows a different modulation by using the 3 products.

Further investigations are needed to understand whether this behaviour differs after different time points, whether the proteins are really present and whether others components in these medical devices can interfere. This gene activation can represent more an aspect of metabolic modulation of signalling pathways than a real and proportional production of connective tissue related molecules. This is the most interesting aspect of these therapies. Optimization of micro-environment i.e. form amelioration, obtained by enhanced structural support, seems to lead to a functional improvement and thus it contributes to anti-age therapies.

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