Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES

Avantaggiato A.[1], Girardi A[2], Palmieri A.[2], Pascali M.[3], Carinci F[1]

[1]*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;*

[2]*Department of Experimental, Diagnostic and Specialty Medicine, University di Bologna, Bologna, Italy;*

[3]*Plastic Surgeon, Rome, Italy.*

Word count: 2655

Key words: Bio-revitalization, Mesotherapy, Hyaluronic acid, Skin aging, Elastin.

Running title: Bio-revitalizing injective products

Corresponding author:

Palmieri Annalisa, Ph.D.
Department of Experimental, Diagnostic
and Specialty Medicine
University of Bologna,
Via Belmeloro, 8
40126 Bologna
Italy
Phone: +39 0532455582
Fax:: +39 0532455582
annalisa.palmieri@unibo.it

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Abstract

Bio-revitalization is a commonly used technique in aesthetic medicine to improve skin quality and appearance by intra dermal injection of hyaluronic acid (HA) containing compounds.

The present research compares different HA containing injectable as regard their effects on cultured skin fibroblasts over time (24, 48, 72 hours) by using RT-PCR and a panel of genes involved in dermal integrity.

Human dermal fibroblasts were seeded on a layer of five different commercial medical devices containing 6.2, 10, 10bis, 13 and 20 % respectively of HAyaluronic acid alone or associated to other molecules. One of these (10bis) is not specifically intended for the skin but for visco-supplementation in osteoarthritis, whereas the last (20 % in HA concentration) is the unique to contain a reticulation agent. The products differ not only in the HA concentrations but in the content and quality of other ingredients, moreover one of these products contains cross-linked HA. Differences between among medical devices were found. In particular HA concentration seems to be inversely correlated to the Elastin genes activation. As regard to Neutrophil Elastase gene, the two medical devices with the higher concentration in HA displayed the greater effect. Genes encoding for the HA synthase 1, Hyaluronidase 1 and Desmoplakin are enhanced but the HA content of the different products seems to be not directly related to genic activation so the explanation for the differences must be researched in further elements that are distinctive of the specific device.

For the physician it is important to choose among drugs or medical devices which ones to use and in what protocols. The present study performed a comparison that can be useful to better address the skin improvement therapies in aging and in its prevention.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Introduction

The perception of age, health, and beauty seems to be neural encoded and adults and children within and across cultures show high rates of agreement in judgments of facial attractiveness. Skin surface topography and skin coloration affect the perception of age, health, and attractiveness in both men and women.[1]

Bio-revitalization is a commonly used technique in aesthetic medicine to improve skin quality and appearance by intra dermal injection of hyaluronic acid (HA) containing compounds.[2] Many medical devices have been commercialized to this aim with different HA contents and several researches support their use in facial aging. In fact the age dependent decline of skin seems to be mainly related to the quality of the dermal extra cellular matrix (ECM): the highly organized structure connecting fibroblast by a complex network.[3]

HA is a high molecular weight (10^5–10^7 Da) linear non-sulfated glycosaminoglycan (GAG) composed of repeating disaccharides (β-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamide). HA is a polyanion that can self-associate and that can also bind to water molecules giving it a stiff, viscous quality similar to gelatine; HA is responsible for providing the viscoelasticity of some biological fluids and controlling tissue hydration and water transport. HA is omnipresent in the human body occurring in almost all biological fluids and tissues, although the highest amounts of HA are found in the extracellular matrix of soft connective tissues, in the synovial fluid, in the vitreous humour of the eye, and in the hyaline cartilage.[4] HA is synthesized by HA synthase on the inner surface of plasma membrane, and then secreted in the extracellular space.[5] HA degradation occurs by hyaluronidases (HYAL) with an half-life of 3-5 min in the blood, less than a day in the skin and 1-3 weeks in the cartilage. Among the six human known HYALs, HYAL 1 is the major in serum. Two main receptors capable to connect HA to cell surface are described. The Cluster of Differentiation 44 (CD44), located on the plasma membrane and regulating cell adhesion and migration, and

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

the Receptor for Hyaluronan-Mediated Motility (RHAMM), located in the cytoplasm and responsible for cell growing and migration.[6]

For the characteristics of consistency, biocompatibility, and hydrophilicity, HA is an excellent moisturizer in cosmetic dermatology and skin-care products.[7] Its unique viscoelasticity and limited immunogenicity permit its use in several biomedical applications including osteoarthritis treatment, eye surgery, wound regeneration. In addition, HA has currently been explored as a drug delivery agent.[8]

The administration of drugs percutaneously is possible for molecules of low molecular weight, often associated with volatile excipients that may reduce skin hydration. Similarly the electroporation only works for small and polar molecules. The intra-dermal injections (i.e. mesotherapy) instead, allow the overcoming of the epidermal barrier, and the administration of high molecular weight polymers directly in the dermis. This is the reason to investigate in the field of mesotherapy because this injective technique can directly affect the ECM content of a tissue.

Aim of the present research is to compare different HA containing compounds as regard their effects on cultured skin fibroblasts over time by using RT-PCR and a panel of genes involved in dermal integrity. It must to be outlined that this study compares products that differ not only in the HA concentrations but in the content and quality of other ingredients, moreover one of these products contains cross-linked HA. So the observed effects can be due to adjunctive factors and not only to HA concentration. On the other hand the comparison was designed among bio-revitalizing agents that are pre-assembled by manufacturers. The aim is to give adjunctive elements for therapy because it is easy and safer for the practitioner to use commercial medical devices and comparison can be more effective. The only device intended for intra-articular injection was included in the study to assess the effect of HA concentration by comparison with a substance where HA content was the same. In the last case the

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

manufacturer associated HA to nucleotides, that usually are employed in bio-stimulation therapy[2], to the aim to enhance A2-purinic receptors and salvage pathway.

Materials and methods

Primary human dermal fibroblast cell (HFb) culture

Fragments of dermal tissue of healthy volunteers (females aged 55 as average) 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 five different medical devices:

1 – A bio-revitalization solution consisting 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

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

(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 – A product for dermal bio-revitalization consisting of HA sodium salt 10 mg/ml with a buffered medium containing Polynucleotides 10 mg/ml, Mannitol, Sodium phosphate monobasic dihydrate, Sodium phosphate dibasic dihydrate (Newest Mastelli, Sanremo, Italy).

3 – A product used for intra-articular visco-supplementation not intended for dermal rejuvenation containing HA sodium salt 10%, sodium chloride, monobasic dihydrate sodium phosphate, bibasic dodecahydrate sodium phosphate (Hyalgan FIDIA Farmaceutici S.p.A. - Abano Terme, Padua, Italy)

4 – A dermal revitalization medical device containing HA 13,5 mg, mannitol 9 mg, phosphate buffer pH 7,2 (Juvéderm® Hydrate™ Allergan, inc. Irvine, California).

5 – A reticulated product for dermal revitalization, consisting of stabilized HA gel 20 mg/ml in saline solution phosphate buffered (Restylane® Vital, Uppsala, Sweden).

A set of untreated cells were 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, that was 24h, 48h, and 72h, cells were trypsinized and lysed for RNA extraction.

RNA processing and real-time PCR

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.).

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Finally, the cDNA was amplified by real-time PCR. The amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 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 included non-template controls to exclude contamination of reagents. PCR was performed with two biological replicates.

Expression was quantified using real-time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene Homo sapiens transferrin receptor protein 1 (TFRC). Quantification was done 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 1.

Results

Fig.1 reports data about the gene encoding for Elastin, one of the fibrillar component of dermal matrix. HA concentration seems to be inversely correlated to the Elastin genes activation. Elastin encoding genes are progressively (at 24, 48 and 72 h) activated by the product with the lower HA concentration (6.2 mg/ml).

Fig. 2 evidences the activation of Gene encoding for Neutrophil Elastase (ELANE) is showed in Fig. 2 where the two medical devices with the higher concentration in HA (13 and 20 mg/ml), displayed the greater effect. At 24 hours, however the product where HA concentration was 13mg/ml gave rise to the highester result. degree of genic activation.

Figs. 3 and 4 display The gene activation regarding two of the more involved proteins in HA synthesis and degradation are showed in figs. 3 and 4 respectively. Genes encoding for the

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

HA synthase 1 (HAS1) and Hyaluronidase 1 (HYAL1) are enhanced in every cases but the HA content of the different products seems to be not directly related to genic activation. About HYAL1 the activation is differently distributed over time. The product having the higher HA concentration exhibited the strongest activation at 24 and 48 hours, but at 72 h the activation was similar to that of the product having the lesser HA content. Between the two products containing 10% HA, despite the first contained also nucleotides and mannitol, they had comparable effects, whereas the product with HA concentration of 13%, containing mannitol too, evidenced only a little activation both of HYAL1 and of HAS1.The reason of this behaviour is not clear, maybe that other elements as quality and amount of buffer can affect these results.

Fig. 5 displays the effects on genes encoding for Desmoplakin showing a non-linear correlation between HA concentration and genic activation.

Discussion

Tissue shape, structure, and function require continuous remodelling given by intercellular relationships. Proteoglycans and glycoproteins of cell surface and of ECM play key roles in normal processes such as cell growth, division, differentiation, and migration and in pathophysiological processes such as fibrosis and inflammation. This is the reason to investigate in the field of mesotherapy because it can directly affect the ECM content of a tissue. Mesotherapy has been the subject of many discussions and some studies, that judged it as useless in skin rejuvenation, were much cited in the literature. An interesting study classified as not significant a series of objective improvements demonstrated by the study itself by histological evaluations.[9] The reported not statistical significance can be related more to the very limited sample than to the obtained results. Certainly the improvements can not to be compared to the effects of surgery, as in every medical therapy the results are more

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

progressive and not so immediately evident compared to surgery. This is the reasons because patients self-evaluations are poor after this therapy. Some studies report the death of the cell cultures in vitro by using molecules commonly used in skin revitalization,[10] but they were conducted without comparing the same products at different dilutions. This phenomenon has been also registered in our first experiments and was avoided by a further dosage adjustment of the products. In fact a drug is administered in vivo to a greater tissue extent than the cell culture. Another element of prejudice is that the mesotherapy is often associated with injections of different substances such as multivitamin, homeopathic preparations, nucleotides, antioxidants, hyaluronic acids fragment with different PM. It is obvious that a comparison between among different therapies that share only the injective technique is difficult

The tested products, in the present research, differ in HA content and for the presence of additional molecules that characterizes the formulation of a particular company.

One of the aims of this research was to check if the concentration of HA, per se, could be a significant factor in eliciting the genes encoding for the components of the extracellular matrix (ECM). In this regard differences between medical devices containing the same concentration of HA, without a direct proportion between HA content and gene stimulation, were found.

Elastin encoding genes are progressively (at 24, 48 and 72 h) activated by the product with the lower HA concentration (6.2 mg/ml) but enriched by additional substances, The product containing 20 mg/ml, the only stabilized and thus likely enriched by the not declared reticulation agent 1,4-butanediol diglycidyl ether (BDDE) and consequently having a borderline structure between bio-revitalizing agents and fillers, demonstrated a slow and poor Elastine (ELN) gene activation. If this is an advantage or not must be judged by considering that ELN and Neutrophil Elastase (ELANE) encoding genes were studied included in this

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

study because two elements are distinctive and apparently opposite in skin aging: the loss of elasticity, usually attributed to the intrinsic aging, and the elastosis given by extrinsic processes. ELANE genes were activated by the highest HA concentrations (13 and 20 mg/ml), with an initial stronger effect of the device where HA is only 13%.

The results permit to infer that bio-revitalization could reduce the elastotic component in aged skin by elastase activation that is related to the HA concentration, being higher at concentration more of 13 mg/ml, while the product with 20 mg/ml of HA did not perform a further activation. It must to be outlined that the term "elastase" is used to describe an enzyme capable of releasing soluble peptides from insoluble elastin, which was the first substrate employed for characterizing ELANE activities, but does not necessarily imply that its activities are always physiologically related to digestion of this target alone. In fact this serine protease is involved in pathogen destruction, in the regulation of pro-inflammatory processes and in a variety of inflammatory human conditions.[11]

In this view it will be relevant interesting to study the real protein content and not only the genes activation and this will be the goal of a further evaluation and, to compare the clinical results more specifically on elastotic photo damaged skins. On this topics an interesting study[12] was conducted on aging hands demonstrating a good improvement by combining filler and bio-revitalization and, despite lack of comparative elements about products, clinical results confirm our experimental findings. This element supports the argument that, among injective treatments, the bio-revitalization, carried out each time that non reticulated HA is directly injected, is a procedure lying, theoretically, between the bio-stimulation (obtained by injecting drugs precursors of the elements of the dermal matrix or of intracellular constituents) and the use of fillers. Thus it is inappropriate the term bio-rejuvenation, often indifferently used, because this is a therapy that involves an enrichment in ECM content and a certain degree of camouflage and thus is a palliative care, a revitalization properly and not a

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

rejuvenation. A real cell activation is performed by bio-stimulation that instead is really a drug dependent effort to enhance cell function.

In a recent study[2] the different devices have been studied in cultured fibroblasts after 24 hours of exposure, the objective is now to observe the effect over time, by considering what happens also after 48 and 72 hours. In fact, the direct administration of a substance already present in the body could lead to inhibition of its synthesis or to an increase of its catabolism for an homeostatic mechanism. For this reason it is relevant particularly interesting to observe what happens to the genes involved in synthesis and catabolism of HA when HA content is artificially enhanced. There is apparently no relationship between HA content and HAS1 activation during the first 24 hours, but at 72 hours there is an HAS1 gene activation increase less or more pronounced, but always present. Interestling The two products containing also mannitol displayed a reduced effect

About Hyaluronidase 1 the activation is differently distributed over time. The product having the higher HA concentration exhibited the strongest activation at 24 and 48 hours, but at 72 h the level was similar to that of the product having the lesser HA content. Between the two products containing HA at 10%, despite the first contained also nucleotides and mannitol, they had comparable effects, whereas the product with HA concentration of 13%, containing mannitol too, evidenced only a little activation both of HYAL1 and of HAS1.The reason of this behavior is not clear, maybe that other elements as quality and amount of buffer can affect these results. In addition HA fragmentation (not declared by productors) for some Authors can change HA-receptor interaction.[13,14] Others Authors instead observed that the inhibition of cell proliferation following an HA supplementation to fibroblast cultures is directly related to HA concentration but it is not depending on molecular weight. In fact, HA is anywhere fragmented after its interaction with membrane receptor CD44 and internalization.[15]

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

A recent study on wound healing evidenced how native HA (molecular weight 1000-1400 kDa) is not the best biopolymer to stimulate wound healing and cell proliferation, whereas medium molecular wheight HA (100-300 kDa) promoted wound closure and cell proliferation This occurred without CD44 receptor involvement probably because HA binding to CD44 leads to its redistribution into lipid rafts, with multiple signal molecules cascades activation. Among the receptors present in lipid raft, the slow activation of P2X7 purinergic receptor increases mitochondrial calcium and promotes cell proliferation, whereas a further activation results in cytolysis. HA with low molecular weight (less than 160kDa) instead has been shown to have a strong antimicrobial activity.[16]

A further element to investigate is the inflammation that these multiple injection can produce. It has been reported that 24 hours after UVB irradiation (i.e. an inflammatory event) there is a major increase of HAS1 than 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 dermal balance remains negative.[17]

DSP encodes desmoplakin, a protein related to cell-cell junction maintenance and thus important in promoting epidermal integrity. Several researches evidenced Its importance in the dermal compartment too, in fact the presence of DSP is demonstrated also in cell derived from mesenchima such as cardiac[18], corneal fibroblasts[19] and odontoblasts.[20]

In this study DSP encoding gene is always enhanced. The least effect was given by the product containing less HA, but for the others, there is no apparent relationship to HA content. (Fig.5) However this element represents a further explanation of the clinical improvement. A possible explanation of DSP activation in bio-revitalization is the assessment of a three-dimensional network foe fibroblast and keratinocytes communications.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Conclusions

The commercial offer in bio-revitalizing product is intriguing and the use of preassembled medical devices is very comfortable, but medical therapy must be a function of the diagnosis, as every medical act, and not targeted only to a camouflage, so for the physician is important to choose among drugs or medical devices which ones to use and in what protocols. The present study performs a comparison that can be useful to better address the skin improvement therapies in aging and in its prevention.

Author Disclosure Statement

“No competing financial interests exist."

This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
Rejuvenation Research

References

1. Fink, B & Prager, M. The effect of incobotulinumtoxin a and dermal filler treatment on perception of age, health, and attractiveness of female faces. J Clin Aesthet Dermatol. 2014, 7:36-40.
2. Avantaggiato, A, Palmieri, A, Carinci, F, Pasin, M & Bertuzzi, GL. Biostimulation and biorevitalization: effects on human skin fibroblasts. Annals of Oral & Maxillofacial Surgery. 2013, 1:11.
3. Quan, T, Wang, F, Shao, Y, Rittie, L, Xia, W, Orringer, JS, Voorhees, JJ & Fisher, GJ. Enhancing structural support of the dermal microenvironment activates fibroblasts, endothelial cells, and keratinocytes in aged human skin in vivo. J Invest Dermatol. 2013, 133:658-67.
4. Fakhari, A & Berkland, C. Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. Acta Biomater. 2013, 9:7081-92.
5. Laurent, TC, Laurent, UB & Fraser, JR. The structure and function of hyaluronan: An overview. Immunol Cell Biol. 1996, 74:A1-7.
6. Papakonstantinou, E, Roth, M & Karakiulakis, G. Hyaluronic acid: A key molecule in skin aging. Dermatoendocrinol. 2012, 4:253-8.
7. Jegasothy, SM, Zabolotniaia, V & Bielfeldt, S. Efficacy of a New Topical Nano-hyaluronic Acid in Humans. J Clin Aesthet Dermatol. 2014, 7:27-9.
8. Burdick, JA & Prestwich, GD. Hyaluronic acid hydrogels for biomedical applications. Adv Mater. 2011, 23:H41-56.
9. El-Domyati, M, El-Ammawi, TS, Moawad, O, El-Fakahany, H, Medhat, W, Mahoney, MG & Uitto, J. Efficacy of mesotherapy in facial rejuvenation: a histological and immunohistochemical evaluation. Int J Dermatol. 2012, 51:913-9.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

10. Jager, C, Brenner, C, Habicht, J & Wallich, R. Bioactive reagents used in mesotherapy for skin rejuvenation in vivo induce diverse physiological processes in human skin fibroblasts in vitro- a pilot study. Exp Dermatol. 2012, 21:72-5.

11. Korkmaz, B, Horwitz, MS, Jenne, DE & Gauthier, F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev. 2010, 62:726-59.

12. Dallara, JM. A prospective, noninterventional study of the treatment of the aging hand with Juvederm Ultra(R) 3 and Juvederm (R) Hydrate. Aesthetic Plast Surg. 2012, 36:949-54.

13. Yoneda, M, Shimizu, S, Nishi, Y, Yamagata, M, Suzuki, S & Kimata, K. Hyaluronic acid-dependent change in the extracellular matrix of mouse dermal fibroblasts that is conducive to cell proliferation. J Cell Sci. 1988, 90 (Pt 2):275-86.

14. Wang, YZ, Cao, ML, Liu, YW, He, YQ, Yang, CX & Gao, F. CD44 mediates oligosaccharides of hyaluronan-induced proliferation, tube formation and signal transduction in endothelial cells. Exp Biol Med (Maywood). 2011, 236:84-90.

15. Croce, MA, Boraldi, F, Quaglino, D, Tiozzo, R & Pasquali-Ronchetti, I. Hyaluronan uptake by adult human skin fibroblasts in vitro. Eur J Histochem. 2003, 47:63-73.

16. Ghazi, K, Deng-Pichon, U, Warnet, JM & Rat, P. Hyaluronan fragments improve wound healing on in vitro cutaneous model through P2X7 purinoreceptor basal activation: role of molecular weight. PLoS One. 2012, 7:e48351.

17. Averbeck, M, Gebhardt, CA, Voigt, S, Beilharz, S, Anderegg, U, Termeer, CC, Sleeman, JP & Simon, JC. Differential regulation of hyaluronan metabolism in the epidermal and dermal compartments of human skin by UVB irradiation. J Invest Dermatol. 2007, 127:687-97.

18. Bowers, SL, McFadden, WA, Borg, TK & Baudino, TA. Desmoplakin is important for proper cardiac cell-cell interactions. Microsc Microanal. 2012, 18:107-14.

19. Vanderburg, CR & Hay, ED. E-cadherin transforms embryonic corneal fibroblasts to stratified epithelium with desmosomes. Acta Anat (Basel). 1996, 157:87-104.

20. Sawa, Y, Kuroshima, S, Yamaoka, Y & Yoshida, S. Intracellular distribution of desmoplakin in human odontoblasts. J Histochem Cytochem. 2005, 53:1099-108.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Figure legends

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

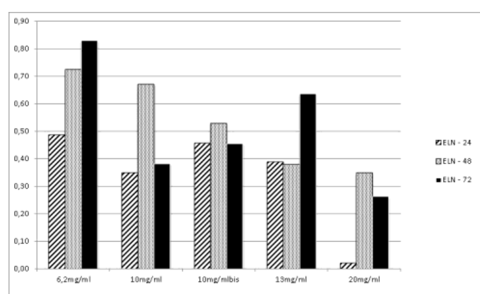

Fig.1: Elastin gene expression in Human Dermal Fibroblasts treated with products containing different HA concentration, at 24h, 48 h and 72 h of exposure. The product containing less HA with amioacids and vitamins exhibited the greater effect mainly after 72 hours, whereas the product where HA is more concentrated gave rise only to a poor gene stimulation. Noticeably the last is a bio-revitalizing device containing stabilized HA thus differing for a cross-linking that classify it among the NASHA (non animal stabilized HA) compounds. The two products with 10 % HA content differ for the presence of nucleotides in the first and this seems to not affect elastin gene expression. The product containing 13 % HA produces a delayed enhancement in elastine gene activation.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

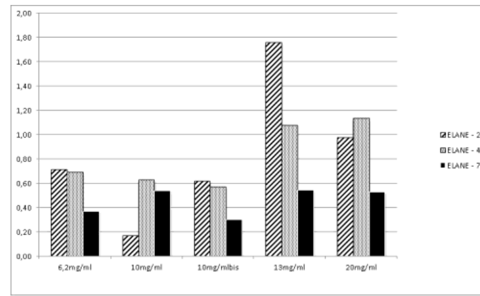

Fig. 2: Neutrophyl expressed elastase gene in Human Dermal Fibroblasts treated with different HA concentration, after 24h, 48 h and 72 h of exposure. The first three compounds exhibit differences in activation timing. The product with 13% HA and 9% mannitol produced a great activation of the gene expression of this serine prothease after the first 24 h, thereafter the result is similar to that obtained by the 20% HA product.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

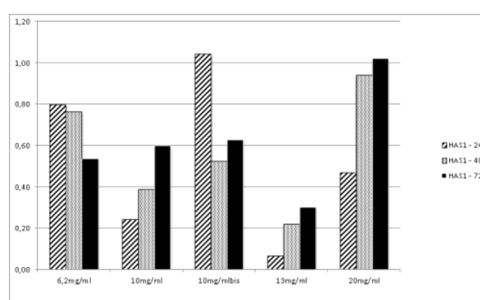

Fig. 3: HA synthase 1 gene expression in Human Dermal Fibroblasts treated with different medical devices containing various HA concentration, at 24h, 48 h and 72 h of exposure. The second and fourth compounds containing mannitol in their formulation gave rise to similar effect. The lowest HA concentration produced an early activation in HA synthase 1 gene that is more pronounced in the visco-supplementation device where aminoacids and vitamins are absent (third product). The last column series where HA is more concentrated and cross-linked exhibits a slower but progressive activation probably because cross-linking will affect sustainability of the product and may modulate enzyme expression.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

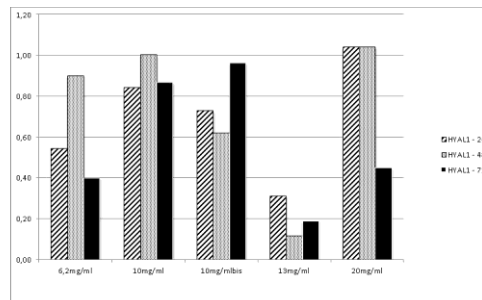

Fig. 4: Hyaluronidase 1 (HYAL1) gene expression in Human Dermal Fibroblasts treated with different HA concentration, at 24h, 48 h and 72 h of exposure. The less and more concentrated HA products share similar effects except for a greater activation of HYAL1 by the second in the first 24 h that could be explained as homeostatic answer. Among the other products no relationship with HA content and gene activation is appreciable, so the explanation for the differences must be researched in other elements that are distinctive of the specific device. The least HYAL1 gene activation was performed by the 13% HA 9% mannitol product.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

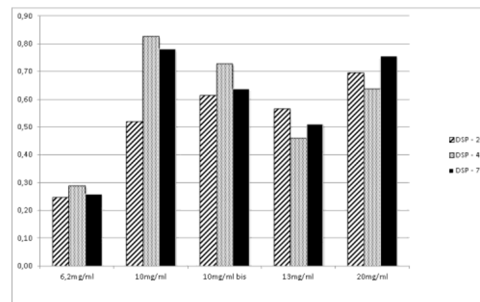

Fig. 5: Desmoplakin gene expression in Human Dermal Fibroblasts treated with different HA concentration, at 24h, 48 h and 72 h of exposure. By comparing the two products with 6.2 and 13 % in HA content, DSP activation is double in the second and is further increased with the device containing 20% HA. The products containing 10% HA are more difficult to compare giving that the nucleotides and mannitol included in the formulation of the first product can affect the DSP gene activation.

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.

Tab.1- Primers sequences for SYBR® Green assay

Gene symbol	Gene name	Primer sequence (5'>3')
DSP	Homo sapiens desmoplakin	F- ATGACCTGAGGAGAGGACGAA R- AGGCTCTCTCTTTCCTGTACCAC
ELN	Homo sapiens elastin	F- CTAAATACGGTGCTGCTGGC R- CATGGGATGGGGTTACAAAG
HAS1	Homo sapiens hyaluronan synthase 1	F- CTCGGAGATTCGGTGGACTA R- CGCTGATGCAGGATACACAG
ELANE	Homo sapiens elastase, neutrophil expressed	F- CTACGACCCCGTAAACTTGCT R- CCTCACGAGAGTGCAGACGTT
HYAL1	Homo sapiens hyaluronoglucosaminidase 1	F- ACAGATGTATGTGCAACACCG R- AAGGGCCCCAGTGTAGTGTC
TFRC	Homo sapiens transferrin receptor protein 1	F- CGCTGGTCAGTTCGTGATTA R- GCATTCCCGAAATCTGTTGT

Rejuvenation Research
COMPARISON AMONG BIO-REVITALIZING INJECTIVE PRODUCTS: A STUDY ON SKIN FIBROBLAST CULTURES (doi: 10.1089/rej.2014.1654)
This article has been peer-reviewed and accepted for publication, but has yet to undergo copyediting and proof correction. The final published version may differ from this proof.