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HYALURONIC ACID: THE USE OF ITS PRECURSOR IN SKIN BIO-STIMULATION

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Bio-stimulation is an injective therapy aimed to boost the anabolic functions of dermal fibroblasts to obtain skin improvement. It can be achieved with multiple intradermal injections (0.05–0.1 ml each) of a solution of 400 mg (3 ml) of injectable glucosamine sulphate, plus 5.623 mg (3 ml) of polideoxyribonucleotide, 1 ml of lidocaine and 0.5–1 ml of sodium bicarbonate, to repeat every 7, 14, 21, and 28 days. The administration of glucosamine sulphate to skin fibroblasts is believed to lead to its incorporation in glycosaminoglycans, and thereby to the stimulation of extracellular matrix synthesis, whereas polideoxyribonucleotide possesses anti-inflammatory and regenerative capability. This study aims to elucidate the *in-vitro* effects of this treatment by studying what happens to several genes related to connective tissue integrity. Human dermal fibroblasts were seeded in a culture medium enriched with either two drugs alone or combined: glucosamine sulphate and/or polideoxyribonucleotide. After the end of the exposure time of 24 h, 48 h, and 72 h, the cells were trypsinized and lysed for RNA extraction. Reverse transcription to cDNA was performed directly from cultured cell lysate. Finally, the cDNA was amplified by real-time PCR and a panel of genes involved in dermal integrity was tested. Gene expression of Hyaluronan synthase 1 (HAS1), Elastine (ELN), Insulin like growth factor 1 (IGF1), Growth differentiation factor 6 (GDF6) and of a series of catabolic enzymes, such as Metalloproteases (MMP) 2, 3 and 13, the neutrophyl expressed Elastase (ELANE) and the Hyaluronidase 1 (HYAL1) were tested after 24, 48 and 72 hours of exposure to glucosamine sulphate and polideoxyribonucleotide alone or combined. All the tested genes but one were up-regulated. A negative synergism on several enzymes (particularly appreciable for Insulin-like growth factor 1 and metalloprotease 13) was observed when the two drugs were delivered together. Glucosamine sulphate acts not only as building block in the biosynthesis of glycosaminoglycan chains, but also as a booster of hyaluronan synthase 1. The association of glucosamine sulphate and polideoxyribonucleotide, used in bio-stimulation therapy protocol, has a negative synergism on catabolic genes in dermal fibroblast cultures. The present observations produce further insight into the effects of glucosamine sulphate in the biosynthesis of glycosaminoglycan chains.

Key words: glucosamine sulphate, polideoxyribonucleotide, extracellular matrix, hyaluronic acid

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The progressive atrophy process is the main factor in determining facial aging (1) and this combines perfectly with both the phenotypic manifestations of aging (2) and the histological findings (3).

The first years of the 2000s were dominated by studies on collagen, the major structural protein of the connective tissue (4), which is present in the dermis as fibrillar component of the extra cellular matrix (ECM). In this regard, it has been shown that fibroblasts do not lie inert in the ECM, but connect their cytoskeleton to the collagen fibers to create a three-dimensional network that realizes the consistency of the dermal connective (5). In this view, the glycosaminoglycans of the matrix were progressively studied because the space among cellular and fibrillar components of the connective tissue is embedded by these molecules and by the water they can hold.

In clinical use, the lower immunogenicity of hyaluronic acid (HA) compared to collagen gave rise to its progressive substitution in volume restoration as filler (after cross-linking to reduce its hyaluronidases sensitivity) and in anti-aging therapies, such as the bio-revitalization (where HA is often mixed to aminoacids and vitamins), or bio-stimulation (where HA is virtually absent). These treatments are directed to skin improvement through intra-dermal injection of different drugs in order to promote ECM optimization with regard to its non-fibrillar component (6). HA is a polymer of dimeric units of *N*-acetyl-glucosamine and glucuronic acid synthesized by HA synthase on the inner surface of plasma membrane, and then secreted in the extracellular space (7).

Glucosamine can be extracted and stabilized by chemical modification and is used as a drug for the treatment of osteoarthritis (OA) in Europe, to promote cartilage and joint health, and it is sold over the counter as a dietary supplement in the USA (8).

The administration of glucosamine sulphate (Gluc) to skin fibroblasts is believed to lead to its incorporation in glycosaminoglycans, and thereby to the stimulation of ECM synthesis as a building block without direct use of HA. The advantages are the more physiological approach in order to respect the homeostatic mechanisms of cell biology and the cost that is less expensive for injective glucosamine than for an HA-containing product. The limit is that HA synthesis occurs inside the cells, thus healthy fibroblasts are required, consequently, the potential of bio-stimulation (BS) is better in younger and less photo-damaged skins.

The term BS indicates stimulation of the anabolic functions of dermal fibroblasts such as replication, protein synthesis and production of ECM. It can be achieved with multiple intra-dermal injections (0.05–0.1 ml each) of a solution of 400 mg (3 ml) of injectable Gluc, plus 5.623 mg (3 ml) of PDRN, 1 ml of lidocaine and 0.5–1 ml of sodium bicarbonate, to repeat every 7, 14, 21 and 28 days (9). The second component of this protocol is PDRN, a mixture of deoxyribonucleotide polymers with chain lengths ranging from 50 bp to 2,000 bp obtained from trout sperm by an extraction process. PDRN acts through stimulation of the A2A purinic receptors. Adenosine is a purine nucleoside that is released from a variety of cells in response to several types of stress. It has been suggested that adenosine regulates inflammation via interaction with one or more of its 4 known receptors (A1, A2A, A2B, and A3). Stimulation of adenosine A2 and A3 receptors has been shown to alter the cytokine network by decreasing inflammatory cytokine secretion by macrophages *in vitro* (10).

In a previous paper (6), the effects of this therapy was discussed by analyzing through RT-PCR several genes of cultured human dermal fibroblasts. Those genes are involved in dermal integrity. The aim of the present research is to observe these effects over time, not only after 24 hours but also at 48 and 72 hours after cell stimulation.

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, USA) supplemented with 20% fetal calf serum and antibiotics, i.e. penicillin 100 U/ml and streptomycin 100 µg/ml (Sigma-Aldrich, Inc., St. Louis, Mo, USA). Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Medium was changed the next day and twice a week thereafter. After 15 days, the pieces of dermal tissue were removed from the culture flasks. Cells were harvested after additional 24 h of incubation.

Cell cultures

Human dermal fibroblasts at the second passage were

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

Gene symbol	Gene name	Primer sequence (5'>3')
HAS1	Homo sapiens hyaluronan synthase 1	F-CTCGGAGATTCGGTGGACTA R-CGCTGATGCAGGATACACAG
ELN	Homo sapiens elastin	F- CTAAATACGGTGCTGCTGGC R- CATGGGATGGGGTTACAAAG
IGF1	Homo sapiens insulin-like growth factor 1	F-GCGCAATGGAATAAAGTCCT R-ACAGCGCCAGGTAGAAGAGA
GDF6	Homo sapiens growth differentiation factor 6	F-CCCCACGAGTACATGCTGTC R-GAGCATGGACACATCAAACAA
MMP2	Homo sapiens matrix metalloprotease 2	F- TACGATGGAGGCGCTAATGG R- CGCATGGTCTCGATGGTATT
MMP3	Homo sapiens matrix metalloprotease 3	F-TTTCCCAAGCAAATAGCTGAA R-AGTTCCCTTGAGTGTGACTCG
MMP13	Homo sapiens matrix metalloprotease 13	F-AGTTCGGCCACTCCTTAGGT R-TGGTAATGGCATCAAGGGAT
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

seeded in a culture medium enriched with either two drugs alone or combined: glucosamine sulphate 400 mg (Dona, Rottafarm, Milan, Italy) and polideoxyribonucleotide 5.625 mg (Placentex Integro, Mastelli, Sanremo, Italy). A set of untreated cells was used as control. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. After the end of the exposure time of 24 h, 48 h, and 72 h., respectively, 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, USA), following the manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate was reverse-transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

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, USA), and the specific assay was designed for the investigated genes (Table I). SYBR assay reactions were performed in a 20 µl volume using ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 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 PCR. The gene expression levels were normalized to the expression of the housekeeping gene Homo sapiens transferrin receptor protein 1 (TFRC). Quantification was carried out with the delta/delta calculation method. Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA), and were: Hyaluronic synthase 1 (HAS1) and Elastine (ELN), two proteins involved in connective tissue structure, Insulin like growth factor 1 (IGF1) and Growth differentiation factor 6 (GDF6), two enzymes involved in metabolic regulation and a series of catabolic enzymes as the Metalloproteases (MMP) 2, 3 and 13, the Neutrophyl expressed Elastase (ELANE) and

the Hyaluronidase 1 (HYAL1) after 24, 48 and 72 hours of exposure to Gluc and PDRN alone or combined.

RESULTS

Gene expression of HAS1, ELN, IGF1, GDF6 and of a series of catabolic enzymes, such as MMP2, MMP3 and MMP13, ELANE and HYAL1, were tested after 24, 48 and 72 hours of exposure to Gluc and PDRN alone or combined. All the tested genes but one (IGF1) were up-regulated (Fig. 1). A lesser activation of HAS1 could be observed when PDRN alone was added to culture medium (Fig. 2). Association of PDRN and Gluc acted not only as building block in the biosynthesis of glycosaminoglycan chains, but acted as a booster of HAS1 (Fig. 3). A negative synergism on several enzymes (particularly appreciable for IGF1 and MMP13) was observed when the two drugs were delivered together (Fig. 3).

DISCUSSION

Bio-stimulation is an injective therapy aimed to boost the anabolic functions of dermal fibroblasts to obtain a skin improvement. It can be achieved with multiple intradermal injections of a solution of Glucosamine sulphate (Gluc), plus polideoxyribonucleotide (PDRN). The administration of Gluc to skin fibroblasts is believed to lead to its incorporation in glycosaminoglycans, and thereby to the stimulation of extracellular matrix synthesis, whereas PDRN possesses anti-inflammatory and regenerative capability. This research is aimed to elucidate the *in-vitro* effects of this treatment by studying what happens to several genes related to connective tissue integrity.

HAS1 gene expression is enhanced in Gluc cultured fibroblasts.

HAS1 gene expression was enhanced after Gluc supplementation to cultured fibroblasts (Fig.1). The activation was increasing after 24, 48 and 72 hours. This behavior supports the idea that Gluc is not only important as building block in the biosynthesis of glycosaminoglycan chains, but can act as a booster for HA synthesis and for other cellular products.

Some Authors (11) demonstrated that addition

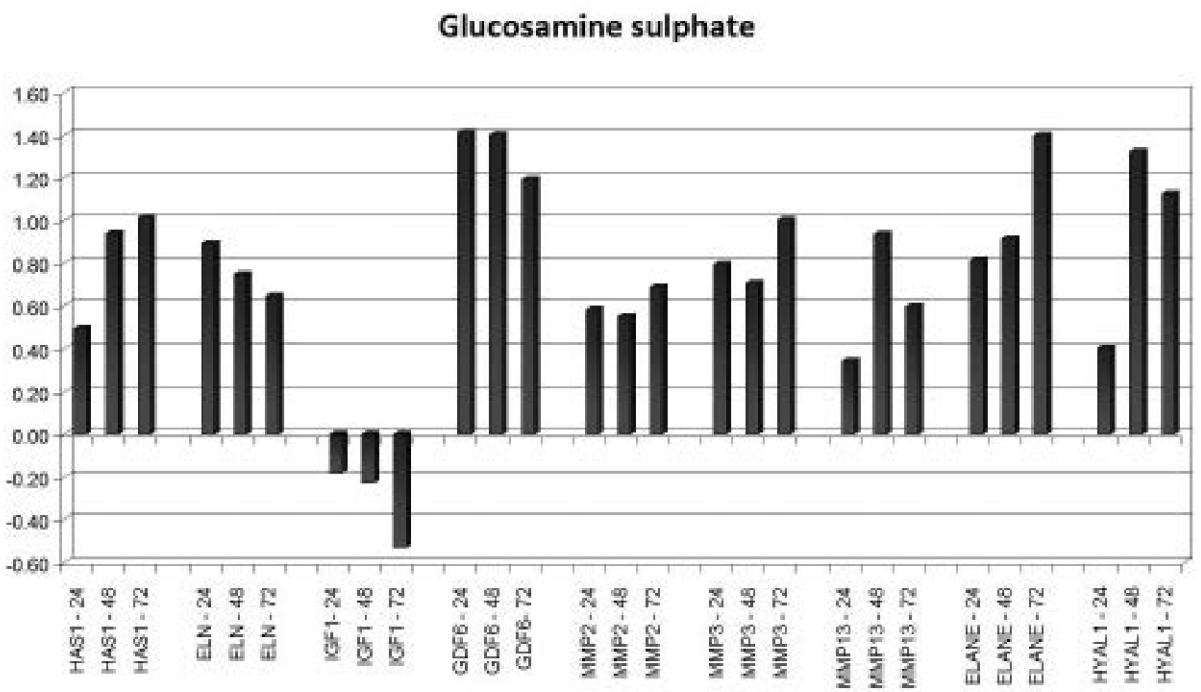

Fig. 1. *Gene activation after administration of glucosamine sulphate; all the tested genes were up regulated except insulin like growth factor 1.*

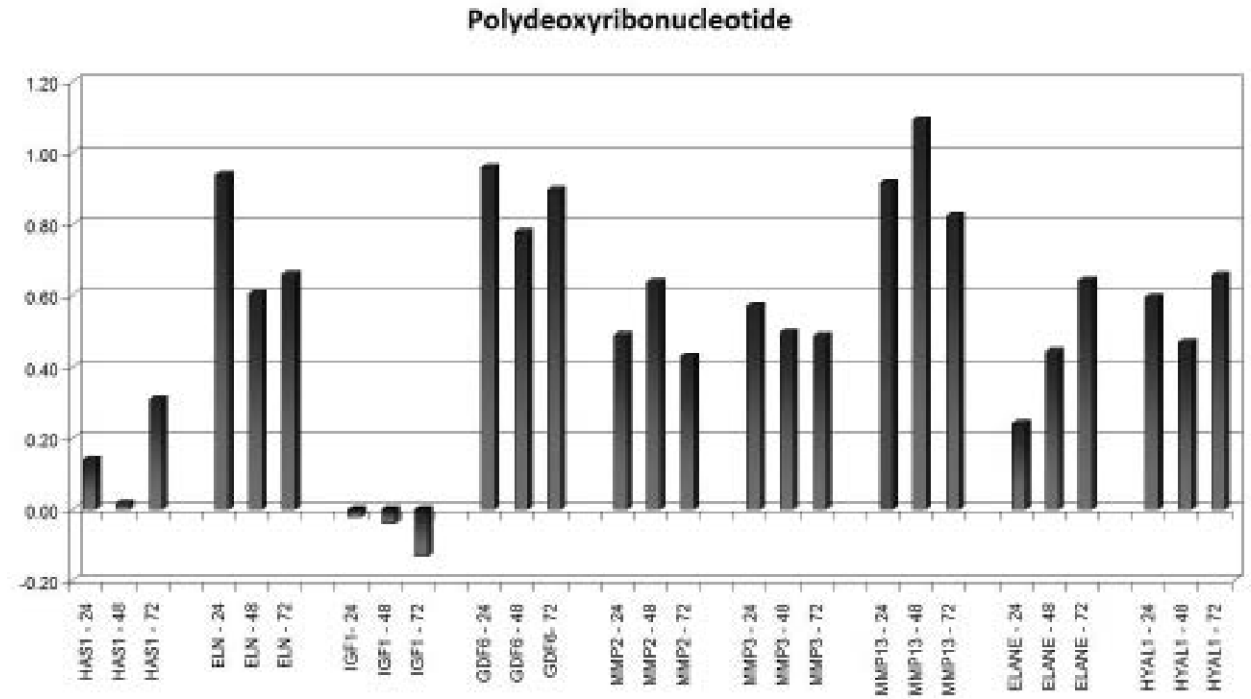

Fig. 2. *Polideoxyribonucleotides are usually associated to Gluc in the bio stimulation protocol. A lower activation can be observed of hyaluronan synthase 1 when polideoxyribonucleotide alone is added to the culture medium.*

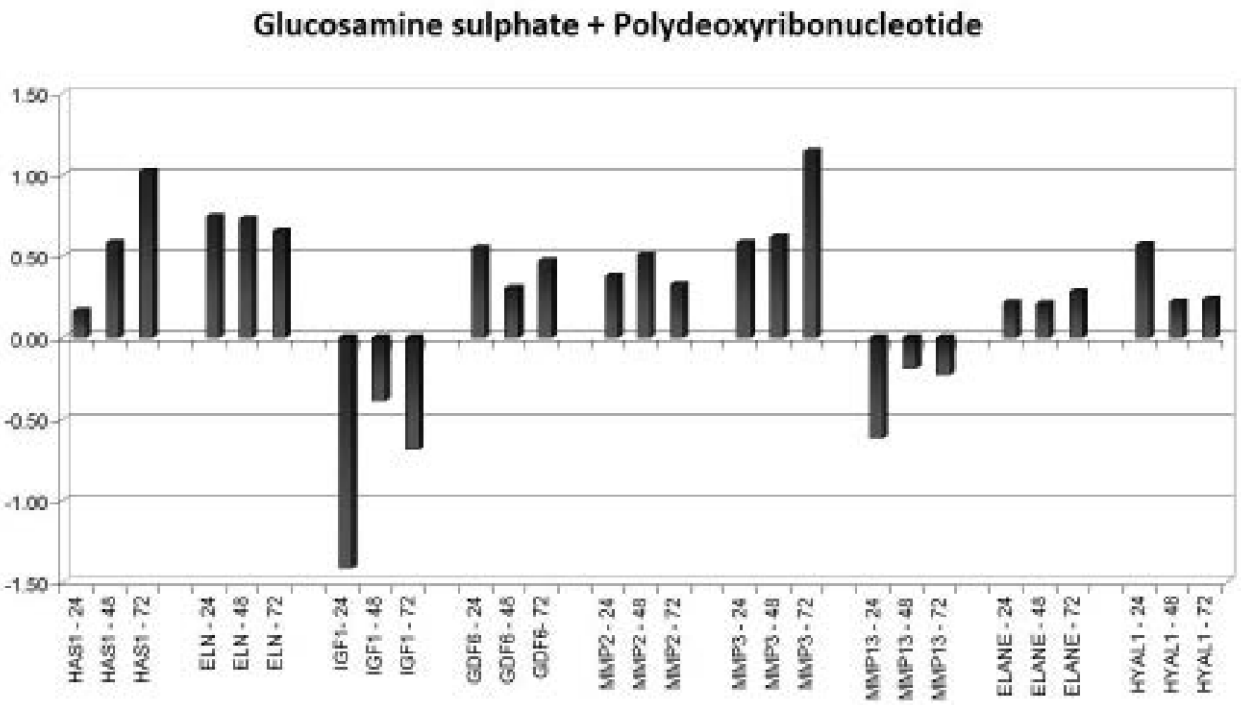

Fig. 3. *Effect of the association of the two substances with a negative synergism on several enzymes, particularly appreciable for insulin like growth factor 1 and metalloprotease 13.*

of glucosamine hydrocloride or glucose did not significantly alter HAS gene expression on cultured chondrocytes. Since they inferred that the registered increased amount of HA in the culture medium might be explained by the fact that addition of glucosamine hydrocloride simply implements the building blocks that are required for the HA synthesis.

In the present study, instead, an activation of HAS1 gene was demonstrated but the increasing effect over time was obtained by using injectable Gluc, and thus a different molecule. This drug has shown efficacy and safety in clinical trials in knee osteoarthritis (OA) when orally administered at a dose of 1,500 mg once a day. In addition to the clinical data, its use is supported by a distinct pharmacologic profile and well-described pharmacokinetics (12).

Gluc produces anabolic and catabolic gene activation.

Gluc has been studied in OA where it has been demonstrated to reduce prostaglandin E2 production, to interfere with nuclear factor kappa B DNA binding in chondrocytes and synovial cells, and to be associated with decreased expression of interleukin-1b. Long-term oral administration of Gluc reduces the destruction of cartilage and the up-regulation of MMP-3 mRNA in a model of spontaneous OA. It was suggested that since glucosamine inhibits both anabolic and catabolic genes, the therapeutic effects might be due to anti-catabolic rather than to anabolic activities (13). The present study on human dermal fibroblasts confirms what was previously described in chondrocytes, demonstrating an enhancement in both anabolic and catabolic gene activation. Chondrocyte and fibroblast can share some mechanisms associated to senescence. Several factors (cell apoptosis, oxidative stress, decreased responsiveness to growth factors, telomere erosion, glycation of ECM macromolecules) contribute to the modification of ECM production in a quantitative and qualitative manner and, as a consequence, this may lead to decreased tissue repair (14).

In a recent paper (15), the Authors studied whether the concentration of HA in several bio-revitalizing medical devices could represent a significant factor in eliciting the gene encoding for some components of the ECM. Among these, the elastine (ELN) encoding gene displayed a progressively (at 24, 48 and 72 h) more relevant activation using a medical device with a low HA concentration (6.2 mg/ml) but enriched by aminoacids and vitamins. Instead, the product containing a higher (20 mg/ml) HA concentration exhibited a slow and poor ELN gene activation. In contrast, the neutrophil expressed elastase (ELANE) gene was more activated by the products displaying the highest HA concentrations (13 and 20 mg/ml). Noticeably, in the presented experiments Gluc produces activation both of ELN and ELANE genes, thus we hypothesize that this can be due to the progressive transformation of Gluc in HA. Because of the decrease of Gluc and of the consequent increase of HA over time, the gene activation of ELN decreases while ELANE expression increases. This can be a further element supporting the view that the added Gluc really becomes HA.

PDRN enhances the tested genes in lesser amount compared to Gluc

Here the role of PDRN (Fig. 2) was studied. All the tested genes but one were enhanced, the main difference is that HAS1 is virtually unaffected, whereas all the catabolic genes were activated. A possible explanation is an enhancement in cellular metabolism. Further researches are needed to better understand whether it is the cause or consequence of cellular replication.

It is known that PDRN acts as mitogen for fibroblasts, endothelial cells, and pre-adipocytes, working with different growth factors. PDRN is used in plastic and dermatologic surgery for its regenerative properties in ischemic skin flaps and its role has been demonstrated in the clinical improvement after local sub-dermal administration, focusing on its anti-inflammatory and positive regenerative effects (16).

PDRN treatment significantly ameliorated clinical signs of arthritis, improved histologic damage, reduced the cartilage expression and circulating levels of high mobility group box chromosomal protein 1, tumor necrosis factor beta, and interleukin-6 and -10 expression. The concomitant administration of a PDRN antagonist ablated the PDRN-induced protective effect in experimental arthritis. PDRN reduced cytokine production from stimulated human chondrocytes (10).

A recent study confirmed the positive effects of PDRN on the survival of random pattern skin flaps

in rats. The underlying mechanism is likely related to the activation of the PDRN-adenosine A2A receptor-VEGF pathway (17).

Gluc and PDRN exhibit negative synergism on many connective tissue-related genes

In a previous study (18), it was demonstrated that MMP13 and IGF1 gene expression in fibroblast cultures are strongly inhibited after 24 h of incubation with the association of Gluc and PDRN, whereas Gluc and PDRN alone produced a modest inhibition of IGF1 and activation of MMP13. Moreover, ELANE gene was highly over-expressed after incubation with Gluc, less over-expressed with PDRN, but there was a negative synergistic effect with the association that could be useful to reduce ELANE activation given by Gluc alone. The present series of experiments was carried out after 24, 48 and 72 hours of exposure to Gluc and PDRN together, and the negative synergism on all the studied catabolic genes including ELANE was confirmed (Fig. 3).

The relationship between IGF1 and TGF beta has been studied in OA where an increased cartilage inorganic phosphate generation occurs in response to TGF beta and is normally antagonized by IGF1 (19). GDF6 is related to TGF beta super-family and its activation is less pronounced when Gluc is associated to PDRN, and this is further antagonized by the negative synergism observed for IGF1 in the present research.

Bio-stimulation is a therapy aimed to improve the skin appearance by means of cell activation. In this context, the tested drugs seem to produce a tissue repair effect with a further advantage when they are administered simultaneously. In fact, Gluc and PDRN given together produced a negative synergism on all the tested catabolic genes. This can be relevant in anti-aging therapies where the cellular metabolism can be slow and fibroblasts are less represented than in young patients. Thus, it is important to preserve all the components of the extracellular matrix. Moreover, Gluc acts not only as building block in the biosynthesis of glycosaminoglycan chains, but as a booster of HAS1 and this is a further explanation of the hydration improvement observed after the injections.

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