

## Biostimulation and biorevitalization: effects on human skin fibroblasts

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### Abstract

#### Introduction

Aesthetic medicine uses many injective techniques; biostimulation (BS) and biorevitalization (BR) are among these. The term BS indicates stimulation of the anabolic functions of dermal fibroblasts such as replication, protein synthesis and production of extracellular matrix components (ECM). BR instead uses the same injective technique but different medical devices. It is a direct supplementation of hyaluronic acid (HA) alone or added to other molecules (i.e. vitamins). This study discusses BS and BR and their effects on human skin fibroblasts.

#### Materials and methods

In order to verify the different metabolic effects of BS and BR fibroblast cell cultures, RNA extraction, cDNA synthesis and PCR were performed.

#### Results

BS and BR produce different metabolic effects in fibroblast cell cultures, thus showing that they are different therapies. For example, neutrophil elastase is activated by BS and to a lesser extent by BR, whereas hyaluronan synthase 1 is activated to a higher extent by BR using the medical device with the lowest HA content. Neutrophil elastase, responsible for the degradation of

one of the fibrillar components of ECM, is activated to a lesser extent by BS.

#### Conclusion

Further experiments using more time points (i.e. not only 24 h of cell cultures but also 12, 48 and 72 h) are necessary to give additional insights on fibroblast behaviour after BS and BR. A better comprehension of fibroblast biology will result in a proper clinical application of BS and BR.

#### Introduction

The term biostimulation (BS) indicates stimulation of the anabolic functions of dermal fibroblasts such as replication, protein synthesis and production of extracellular matrix components (ECM). BS can be induced using chemical or physical devices. Two protocols are used to obtain chemical BS<sup>1</sup>:

- Polydeoxyribonucleotide (PDRN) plus glucosamine sulphate (Gluc), which are delivered with multiple intradermal injections (0.05–0.1 cc each) of a solution of 5,623 mg (3 ml) of PDRN plus 400 mg (3 ml) of Gluc, 1 ml of lidocaine and 0.5–1 ml of sodium bicarbonate, to repeat every 7, 14, 21 and 28 days.
- N-acetylcysteine (NAC) and amino acids (Aa), altogether named Bio-NAC, which are delivered with multiple intradermal injections of a solution of Aa 8.5% (3 ml), NAC (0.4–0.8 cc), 1 ml of lidocaine and 0.5–1 ml of sodium bicarbonate, to repeat every 15 and 30 days.

The drugs used in PDRN plus Gluc have a common anti-inflammatory function. In fact, PDRN is indicated in wound healing, and its function

is mediated by adenosine A2 receptors. Gluc is classified among anti-inflammatory non-steroid drugs. The association of PDRN with Gluc is supported by the fact that wound healing is an essential homeostatic mechanism that depends on a series of overlapping phases: inflammation, angiogenesis, formation of new tissue and reorganization.

Bio-NAC has the aim to improve protein synthesis and simultaneously to give a precursor of glutathione (i.e. NAC), because it is the major anti-oxidant mechanism of our body.

Biorevitalization (BR) instead uses the same injective technique, but different medical devices. It is a direct supplementation of hyaluronic acid (HA) alone or added to other molecules (i.e. vitamins).

Since BS and BR have positive effects on dermal fibroblasts in different ways, an experimental study on fibroblasts cell culture is performed in order to get a new insight as regard differences in ECM synthesis and degradation as well as in metabolic signalling.

#### Materials and methods

This work conforms to the values laid down by the Declaration of Helsinki (1964). The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. All subjects gave full informed consent to participate in this study.

#### 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<sup>2</sup> culture flasks containing

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DMEM medium (Sigma-Aldrich, Inc., [www.sigmaaldrich.com](#)).

Cells were incubated in a humidified atmosphere of 5% CO<sub>2</sub> in DMEM medium.

Medium was changed the next day and twice a week. After 15 days, the cells were harvested and lysed for RNA extraction.

### Cell culture

a layer of:

- PDRN 5,625 mg (Placentex integro, Mastelli, Sanremo, Italy) and PDRN 5,625 mg (Placentex integro, Mastelli, Sanremo, Italy) and Milan, Italy).
- NAC (Almus s.r.l., Pomezia, Rome, Italy) and electrolytes were used for intravenous administration. It contains a series of essential amino acids (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, valine) and non-essential amino acids (alanine, asparagine, aspartic acid, serine and cysteine) (Freamine, Almus s.r.l., Pomezia, Rome, Italy) at the concentration of 20 mg/ml as used the *in vivo* study.

A layer of three medical devices with the following characteristics:

- and vitamins (Skink0 E Viscoderm Ibsa//Revitacare, Saint Ouen d'Aumone, France),
- hyaluronic acid (Newest Mastelli, Sanremo, Italy),
- hyaluronic acid (Restylane® (Lundbeck, Sweden).

A set of untreated cells were used as control. The cells were maintained in DMEM medium.

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nized and lysed for RNA extraction.

### RNA processing and real-time PCR

cell lysate using the TaqMan Gene Expression Assay (Applied Biosystems, Austin, TX), following the manufacturer's instructions.

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

systems, Foster City, CA), and the

contamination of reagents. PCR was

and are listed in Table 1.

evaluated as fold changes relative to the control using the  $2^{-\Delta\Delta Ct}$  calculation method. Forward

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### Results

for elastin synthesis) is activated by

BS and less by BR (Figure 1). On the other hand, hyaluronan synthase 1

the medical device with the lowest

Figure 2 shows the effects on growth differentiation factor 6 (GDF6), insulin-like growth factor

GDF6 is more activated by the treatment with NAC/Aa and PDRN/Gluc. IGF1 is strong, inhibited by PDRN/Gluc and very lightly stimulated in the other cases. DSP is always stimulated.

Figure 3 shows the effects of activation given by all the treatments, whether PDRN/Gluc or NAC/Aa. PDRN/Gluc less activated by PDRN/Gluc.

ments enhanced the activity of MMP; only MMP13 is inhibited by PDRN/Gluc and only lightly activated by NAC/Aa.

### Discussion

in wound healing for the ability of nucleotides, in micromolar concentration and intracellular cAMP by increasing the extracellular concentration of adenosine<sup>2</sup>. Adenosine is generated from ATP catabolism and is a

There are at least four different

They are members of the

The increase of deoxyribonucleotides and deoxyribonucleosides is effect is mediated by the activation

Table 1 Primer sequences for SYBR® Green assay		
Gene symbol	Gene name	Primer sequence (5' > 3')
DSP	<i>Homo sapiens</i> desmoplakin	F-ATGACCTGAGGAGAGGACGAA R-AGGCTCTCTTTCTGTACCAC
ELN	<i>Homo sapiens</i> elastin	F-CTAAATACGGTGCTGCTGGC R-CATGGGATGGGGTTACAAAG
HAS1	<i>Homo sapiens</i> hyaluronan synthase 1	F-CTCGGAGATTCCGTGGACTA R-CGCTGATGCAGGATACACAG
GDF6	<i>Homo sapiens</i> growth differentiation factor 6	F-CCCCACGAGTACATGCTGTC R-GAGCATGGACATCAAACAA
IGF1	<i>Homo sapiens</i> insulin-like growth factor 1	F-GCGCAATGGAATAAAGTCCT R-ACAGCGCCAGGTAGAAGAGA
ELANE	<i>Homo sapiens</i> elastase, neutrophil expressed	F-CTACGACCCCGTAACTTGCT R-CCTCACGAGAGTGCAGACGTT
HYAL1	<i>Homo sapiens</i> hyaluronoglucosaminidase 1	F-ACAGATGTATGTGCAACACCG R-AAGGGCCCCAGTGTAGTGTC
MMP13	<i>Homo sapiens</i> matrix metalloproteinase 13	F-AGTTCGGCCACTCCTTAGGT R-TGGTAATGGCATCAAGGGAT
MMP2	<i>Homo sapiens</i> matrix metalloproteinase 2	F-TACGATGGAGGCGCTAATGG R-CGCATGGTCTCGATGGTATT
MMP3	<i>Homo sapiens</i> matrix metalloproteinase 3	F-TTCCCAAGCAAATAGCTGAA R-AGTTCCTTGAGTGTGACTCG
TFRC	<i>Homo sapiens</i> transferrin receptor protein 1	F-CGCTGGTCAATTCTGTGATTA R-GCATTCCCGAAATCTGTTGT

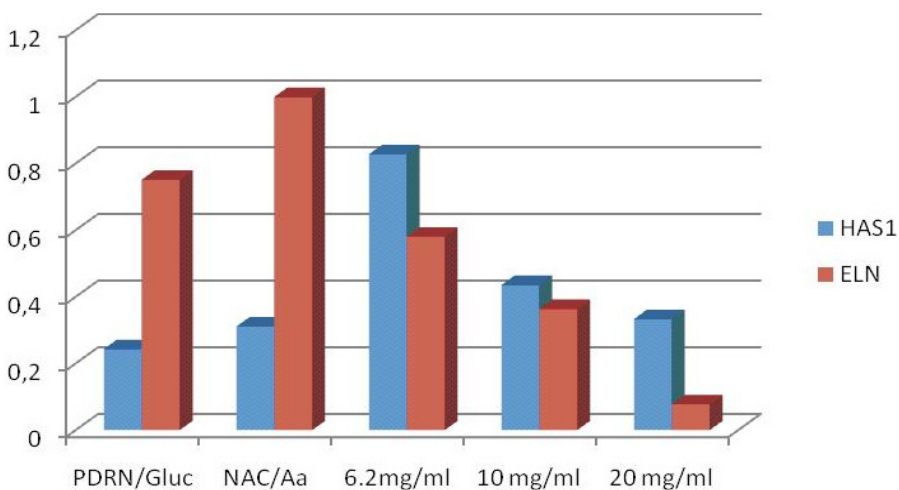


Figure 1: Treatment effects (BR and BS) on neutrophil elastase expression after 24 h of incubation.

A2 subtype<sup>5</sup>. Vascular endothelial growth factor and protein wound content were enhanced after PDRN wound injection in diabetic mice. Furthermore, it demonstrated an

increased wound breaking strength, increase in CD31 and induced transglutaminase II and angiopoietin-1 expression<sup>6</sup>. PDRN is demonstrated to increase nucleic acid biosynthesis

and enhance both cellular replication and protein synthesis. In fact, the nucleotides and nucleosides derived from PDRN degradation can be used as signalling transducers in the extracellular environment or can be internalized. In the intracellular compartment, they can provide purinic and pyrimidinic rings for nucleic acid synthesis via activation of the salvage ways<sup>7</sup> that permits a major speed and an energy spare with respect to the *de novo* metabolic ways.

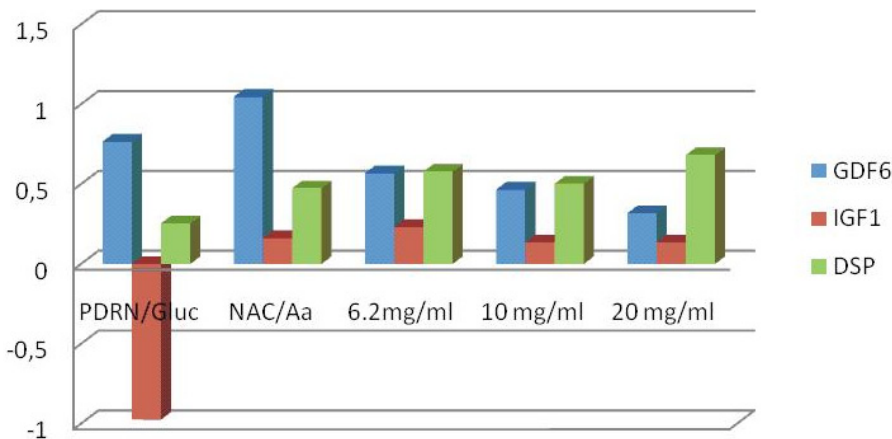
Gluc is classified among anti-inflammatory non-steroid drugs and used in knee arthrosis. Gluc is one of the major precursors in glucosaminoglycans synthesis. The glucosamine and acetic acid together produce *N*-acetylglucosamine, and its polymerization with glucuronic acid gives HA.

The reported result on BS shows an opposite activation of genes responsible for the formation and degradation of fibrillar and amorphous components of ECM (Figures 1 and 3). In fact, it showed less activation of HAS1 but double activation of HYAL1, strong activation of ELN and very light activation of ELANE. Thus, BS increases the synthesis of one of the fibrillar components of ECM and can enhance the degradation of amorphous component of ECM.

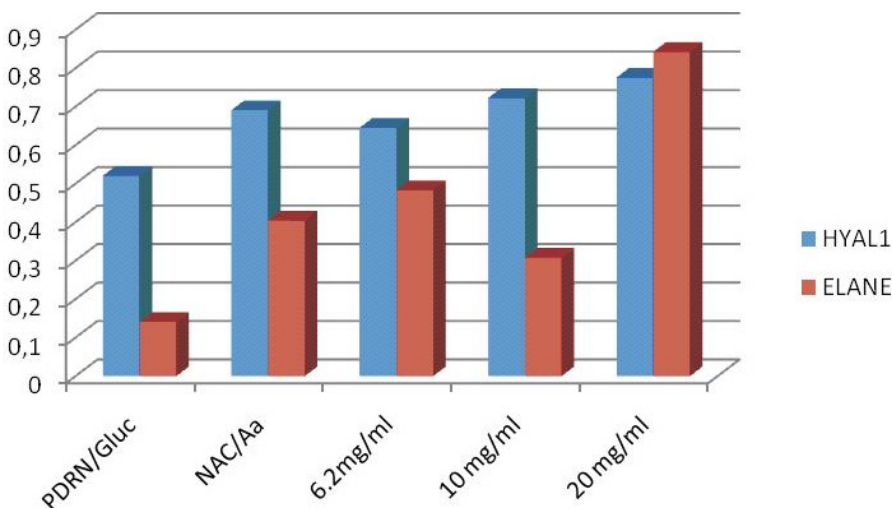
The second BS protocol (NAC/Aa) has the aim to improve protein synthesis and simultaneously administer a precursor of glutathione; it is an indirect way to give an antioxidant without interfering with the homeostatic assessment. *N*-acetylcysteine is the precursor of an amino acid, cysteine, which is one of the three components of glutathione (gamma-glutamyl-cysteine-glycine)<sup>8</sup>. Glutathione is an antioxidant system that works either in intracellular or extracellular compartment. *N*-acetylcysteine is commonly used in acetaminophen poisoning and as a mucolytic<sup>9</sup>, and possesses many

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**Figure 2:** Treatment effects on some genes such as growth differentiation factor 6 (GDF6), insulin-like growth factor 1 (IGF1) and desmoplakin (DSP).



**Figure 3:** Effects of activation on hyaluronidase 1 (HYAL1) by all treatments.

other useful effects. In fact, it is an antioxidant, a hepatic protector, a booster of nitroglycerine, a promoter of glutathione synthesis and a depressor of synthesis of lipoproteins and homocysteine<sup>10</sup>. It can act as a modulator and protect neural cells from apoptosis<sup>11</sup>. The behaviour is very similar to BS performed with PDRN/Gluc, but with NAC/Aa, we did not see any strong inhibition of IGF1. The reason for this inhibition is not clear at the moment, even if some studies on animal models and on centenarians relate the reduction of GH or IGF1 to the length and quality of life<sup>12,13</sup>.

BR uses the same injective technique, but the drugs are medical devices with different types and concentrations of HA. HA is a high-molecular mass polysaccharide of the extracellular matrix especially of soft connective tissues. It is synthesized in the plasma membrane of fibroblasts by addition of sugars to the reducing end, whereas the non-reducing end protrudes into the pericellular space. It is a polymer of dimeric units of *N*-acetylglucosamine and glucuronic acid<sup>14</sup>. Among its important biological functions, there are the modulation of cellular proliferation, migration

and differentiation, regulation of the extracellular water content and protein homeostasis. HA is largely used in aesthetic medicine for its hydration capability, but its role is not only a filler. In fact, there are two different receptors: the cluster of differentiation 44 (CD44) located on the plasma membrane and the receptor for hyaluronan-mediated motility located in the cytoplasm. The individualization of these receptors attributes to the role HA has in cellular motility, proliferation and angiogenesis<sup>15</sup>. For some authors, the receptor interaction is different on the basis of HA fragmentation<sup>16,17</sup>, but others have observed that hyaluronan supplementation to fibroblast culture results in inhibition of cell proliferation, and this is positively related to the concentration of HA but not related to molecular weight, probably because after interaction with receptor CD44 and internalization, HA is always fragmented<sup>18</sup>.

In these experiments, HA is present either as a delivered substance in BR or as a primer in the quality of HAS1 and HYAL1. It can be observed that the medical device with the lowest concentration of HA produced the major stimulation of HAS1 (Figure 1). Moreover, there are evidences of stimulation of HYAL1 with all the tested products.

ECM degradation is an important factor in tissue repair. It is regulated by MMPs and by tissue inhibitors of metalloproteinases. In man, there are 24 different MMPs.

In our experiment, MMP3 is particularly activated by BR procedures, MMP2 is activated in a uniform way in all the cases and MMP13 is strongly inhibited by PDRN/Gluc and only very lightly activated by NAC/Aa (Figure 2).

### Conclusion

BS and BR produce different metabolic effects in 24 h fibroblast cell cultures, thus showing that they are

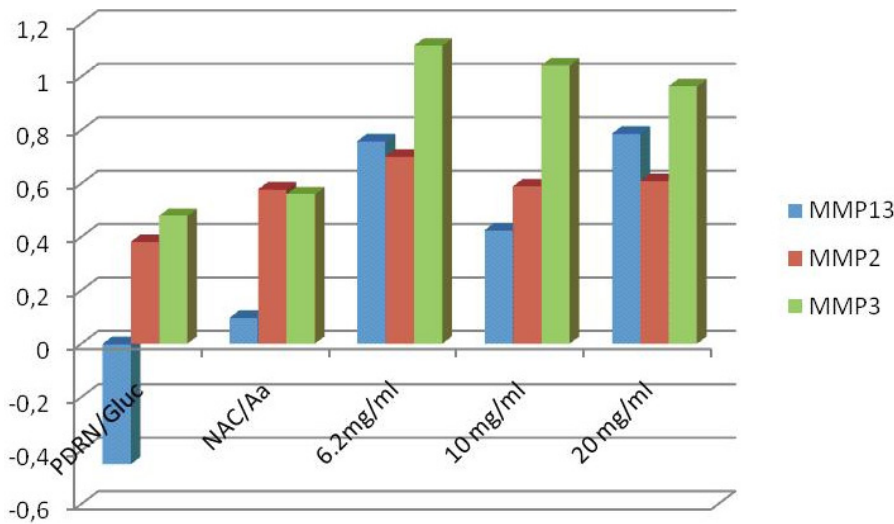


Figure 4: Treatment effects on expression of metalloproteinases (MMP).

different therapies. Additional experiments using more time points (i.e. not only 24 h of cell cultures, but also 12, 48 and 72 h) are necessary to give additional insights as regard early stages of fibroblast response to BS and BR.

A better comprehension of fibroblast biology will result in a proper clinical application of BS and BR.

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