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Role of Antioxidants in Dermal Aging: An In Vitro Study by q-RT-PCR

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Abstract
Background Reactive oxygen species production is the final step in skin aging. These unstable molecules can damage and destroy DNA, proteins, and membrane phospholipids. The aim of this study was to test the in vitro effect of an antioxidant precursor, *N*-acetylcysteine (NAC), on human dermal fibroblasts. NAC alone and a solution of NAC and amino acids together, used in aesthetic medicine as intradermal injection treatment, were tested.
Methods The expression levels of some connective related genes (*HAS1*, *HYAL1*, *ELN*, *ELANE*, *DSP*, *GDF6*, and *IGF1*) were analyzed on cultures of dermal fibroblasts using real-time reverse-transcription polymerase chain reaction (real time RT-PCR).
Results All genes were upregulated after 24 h of treatment.
Conclusions An interesting effect of gene induction by administration of NAC and amino acids in vitro was demonstrated. Upregulation of elastin-, hyaluronic acid-, and GDF6-encoding genes supports the evidence of clinical improvement induced by NAC biostimulation in the prevention and correction of skin aging.
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Keywords *N*-Acetylcysteine · Antioxidant · Gene expression · Fibroblasts · Biostimulation

Introduction

Biostimulation is a technique of aesthetic medicine where different injected drugs are used to improve dermal components [1]. These molecules are nucleotides, glucosaminoglycans, and amino acids. The injections are made in the dermis of the face, neck, décolleté, and hands with a 30G needle. The aim of this therapy is to increase the amount of precursor molecules to induce fibroblasts to enhance their activity in terms of replication and secretion of extracellular matrix, without interference with physiological homeostasis [2].

Some authors [3, 4] have pointed out that oxidative stress can be considered the common denominator of both extrinsic and intrinsic aging. Ionizing radiation, environmental pollution, and sun exposure all lead to the first step in the aging pathway: the production of reactive oxygen species (ROS). Intrinsic or chronological aging also contributes, with ROS production as a physiological consequence of oxidative metabolism. In fact, ROS are inevitably created during the process of mitochondrial oxidative energy generation.

Cellular damage occurs when ROS production exceeds the detoxifying ability of antioxidant enzymes, which is

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caused by both decreased performance in scavenging of aged cells [5] and increased ROS production in unfavorable environmental conditions. The presence of impaired electrons leads ROS to react with a great variety of molecules in order to pair their electrons and get to energy stability. DNA, proteins, and membrane phospholipids are the main targets [6]. These reactive species can induce single- and double-strand breaks in nuclear DNA, but it is also well documented that mitochondrial DNA mutations and damage are involved, and in particular the base excision-repair pathway is less efficient in aging [7].

In medical therapy, direct supplementation of antioxidants can interfere with redox equilibrium. In fact, excess antioxidants (ascorbic acid) can produce a paradox pro-oxidant effect [8].

Thus, it is important to act with precursors: *N*-acetylcysteine (NAC) [9] is the precursor of the amino acid cysteine, which is one of the three components of glutathione (gamma-glutamyl-cysteinyl-glycine), the major antioxidant of the body [10] because it works in both intra- and extracellular compartments. NAC is commonly used in acetaminophen overdose and as a mucolytic in pulmonary disease [11], but it has many other benefits: antioxidant, detoxification promoter, hepatic protector, enhancer of nitroglycerine effects, promoter of glutathione synthesis, regulator of lipoprotein and homocysteine levels, and protector against neural cell apoptosis [12].

We performed a series of intradermal injections (0.05–0.1 cc each) of 0.4–0.8 cc of NAC and 3 cc of a solution for parenteral nutrition (Freamine) containing essential and nonessentials amino acids, buffered in sodium bicarbonate (0.5 cc) and lidocaine (0.5 cc). We called this protocol BioNAC and injections were repeated after 14 and 28 days in 20 different points of the face, neck, or hands [2]. Thus the clinical relevance of this study is the evaluation of the effect of these substances in primary cell cultures by using RT-PCR and a panel of specific primers to detect some of their effects on cell metabolism and extracellular matrix components.

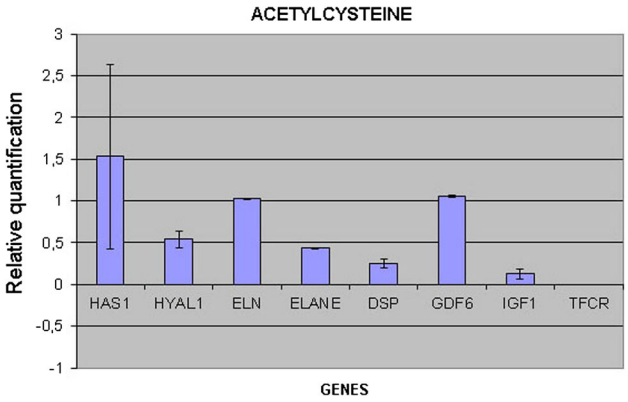

Fig. 1 Human dermal fibroblast gene expression profile after 24 h of treatment with acetylcysteine; SYBR® Green assay. *HAS1* = 1.53, *HYAL1* = 0.54, *ELN* = 1.02, *ELANE* = 0.44, *DSP* = 0.25, *GDF6* = 1.05, *IGF1* = 0.13

Materials and Methods

Primary Human Dermal Fibroblast Cell (HFb) Culture

Dermal tissue fragments were obtained from three women with a mean age of 50 years who had undergone reductive mastoplasty. The pieces were transferred to 75-cm^3 culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, MO, USA) supplemented with 20 % fetal calf serum and antibiotics [penicillin 100 U/ml and streptomycin 100 μg/ml (Sigma Aldrich)]. Cells were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C. The 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 24 days of incubation.

Cell Culture

For the investigation, HFb at the second passage were seeded on a layer of NAC (Almus s.r.l. Pomezia, Rome, Italy) or with Freamine III (Baxter S.p.A., Rome, Italia) at a concentration of 20 mg/ml, or with NAC and Freamine together as in the in vivo protocol. Freamine III is a solution of amino acids (8.5 %) and electrolytes used for parenteral nutrition. It contains a series of essential (isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and nonessential (alanine, arginine, histidine, proline, serine, and cysteine) amino acids and different electrolytes in physiological proportion useful for protein synthesis.

A set of untreated cells were used as controls. 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 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). Finally, the cDNA was amplified by real-time PCR. All amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City,

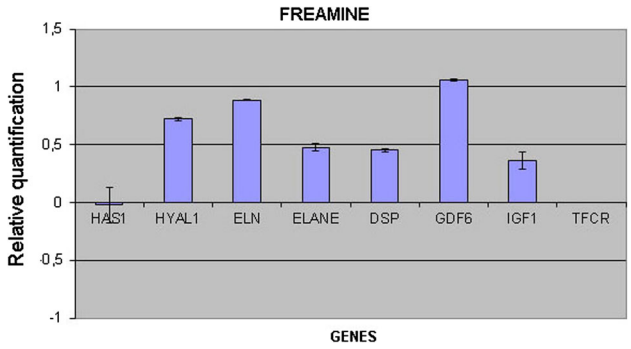

Fig. 2 Human dermal fibroblast gene expression profile after 24 h of treatment with Freamine; SYBR® Green assay. *HAS1* = −0.02, *HYAL1* = 0.72, *ELN* = 0.89, *ELANE* = 0.48, *DSP* = 0.45, *GDF6* = 1.06, *IGF1* = 0.36

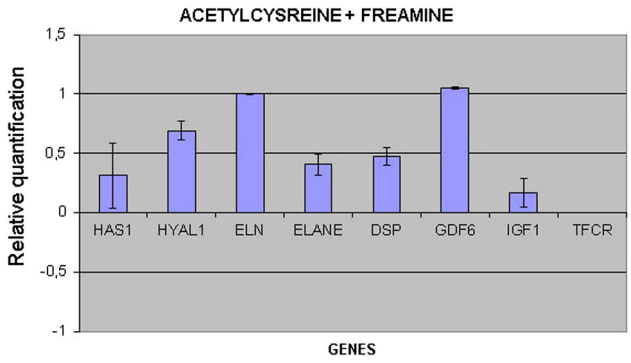

Fig. 3 Human dermal fibroblast gene expression profile after 24 h of treatment with Freamine + acetylcysteine; SYBR® Green assay. *HAS1* = 0.31, *HYAL1* = 0.69, *ELN* = 1.00, *ELANE* = 0.40, *DSP* = 0.47, *GDF6* = 1.05, *IGF1* = 0.16

CA, USA) and the specific assay designed for the investigated genes. SYBR® Green 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, 400 nM concentration of each primer, and cDNA. All experiments were performed including nontemplate controls to exclude reagent contamination. PCRs were performed with two biological replicates.

Gene expression was quantified using real-time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene *TFRC*. We evaluated the expression as fold changes relative to the expression of the untreated HFb. Quantification was done using the delta/delta calculation method [13]. The data were then processed using the base 10 logarithm and reported as relative quantification in Figs. 1, 2 and 3. Forward and reverse primers for the selected genes were designed using Primer Express software (Applied Biosystems) and are listed in Table 1.

Results

The gene expression levels of several skin growth-, health-, and elasticity-related genes (*HAS1*, *HYAL1*, *ELN*, *ELANE*, *DSP*, *GDF6*, and *IGF1*) of treated cells were upregulated after 24 h of treatment compared with untreated fibroblasts. Figure 1 shows the upregulation of these genes for NAC treatment alone, Fig. 2 reports the result of Freamine treatment alone, and Fig. 3 reports the combination of NAC plus Freamine treatment.

The genes encoding hyaluronic synthase were highly stimulated after incubation with NAC, whereas the amino acid solution had only a poor effect. Hyaluronidase was quite stimulated by NAC but more so by Freamine treatment. NAC and Freamine are responsible for elastin and elastase (in lesser amounts) gene activation. Desmoplakin was stimulated by the NAC and amino acid solution. The *GDF6*, as a growth and differentiation index, was strongly activated by NAC and amino acids, whereas the *IGF1* was only lightly stimulated by Freamine and very poorly stimulated by NAC.

Discussion

The SH groups of NAC are essential against reactive oxygen species. NAC is a powerful scavenger of hypochlorous acid and is capable of reducing hydroxyl radicals and hydrogen peroxide [14]. In cellular cultures, there is evidence that NAC increases the intracellular concentration of glutathione (GSH) by enhancing the uptake of cystine by the medium and its reduction to cysteine [15]. Although intracellular thiols are major redox buffers, their role in maintaining redox homeostasis is not completely understood, particularly during aging. NAC treatment has a role not only in GSH activity, but in increased catalase and mitochondrial manganese superoxide dismutase activities [16]. NAC has an immunologic modulator role and not an indiscriminate stimulating effect. It has a role in increasing or decreasing immune functions depending on the cell state [17].

Hyaluronic acid (HA) is synthesized from the gene *HAS1* [18], which seems to be overexpressed in this study. *HAS1* upregulation could lead to higher HA production and, consequently, it can be expected to have a volume restoration effect. In fact, HA is widely used in aesthetic medicine for its invaluable water-holding skill, a fundamental requirement in giving the skin shape, elasticity, and moisture.

Intracellular degradation of HA occurs after endocytosis followed by digestion by hyaluronidases [19], a family of endoglucosaminidases. *HYAL1* is the gene that encodes hyaluronoglucosaminidase 1, the major hyaluronidase in

Table 1 Primer 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
GDF6	*Homo sapiens* growth differentiation factor 6	F-CCCCACGAGTACATGCTGTC
		R-GAGCATGGACACATCAAACAA
IGF1	*Homo sapiens* insulin-like growth factor 1	F-GCGCAATGGAATAAAGTCCT
		R-ACAGCGCCAGGTAGAAGAGA
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

human plasma. In this study, *HYAL1* expression increased after incubation with cultured fibroblasts. The implication of this enzyme's induction in a tissue and, in particular, the importance of catabolic enzymes in skin needs more investigation. We suppose that in tissue, physiology remodeling has the same importance of production as a single matrix component.

The increase of elastin RNA is of paramount importance because elastin is responsible for dermal elasticity and flexibility due to its role in forming elastic fibers. Moreover, it has a known role in damaged skin repair and regeneration. Elastic fibers are formed by two components: a more abundant amorphous component (elastin) and a microfibrillar component. Elastin is composed largely of glycine, proline, and other hydrophobic residues and contains multiple lysine-derived crosslinks such as desmosines, which link the individual polypeptide chains into a rubber-like network [20]. Elastin is initially synthesized as a soluble polypeptide, then the single molecules are aligned on a scaffold of microfibrils composed of fibrillin. This alignment is stabilized by the formation of intermolecular crosslinks known as desmosines, which contribute to the insolubility of elastin [21]. In our study, both NAC and Freamine increased the activity of *ELN*, the gene that encodes elastin, maybe for the largest amount of amino acid precursors for protein synthesis and for optimization of cellular function.

Neutrophil elastase is a serine protease of neutrophil and monocyte granules. Its key physiologic role is in innate host immunity, but it can also participate in tissue remodeling and local inflammatory responses [22]. The expression level of *ELANE*, which encodes neutrophil elastase, increased consistently in all experiments. The fibroblast source of *ELANE* might be a significant fraction of the total protein expression in the tissue, along with white blood cell production. Further experiments are required to better clarify this behavior.

DSP encodes desmoplakin, an important protein in cell–cell junction maintenance and thus in promoting epidermal integrity. We can easily see that an undamaged tissue prevents cells from further environmental damage (e.g., UV radiation) and bacterial infections, and that fibroblasts, like other mesenchymal cell phenotypes, may be altered by the dysregulation of DSP. Freamine and, to a lesser amount, NAC enhanced *DSP* in cultured fibroblasts. The reason for this activation is under examination but some authors have pointed out the importance of desmoplakin in cells derived from the mesenchyme such as cardiac, corneal fibroblasts, and odontoblasts [23–25].

GDF6 is growth differentiation factor 6, a member of the transforming growth factor β superfamily. It is known that GDF6 is a cellular growth and differentiation protein, also involved in the patterning of the epidermis. It induces tendon and cartilage formation in a process that involves extracellular matrix reconstitution. This phenomenon leads to collagen and proteoglycan synthesis, similar to what happens in other connective tissues such as skin. GDF6 plays an important role in embryo development and growth and in dermal maintenance. UV irradiation inhibits procollagen gene expression by blocking TGFβ type II receptor [26]. Chang et al. [27] demonstrated that GDF6 induces epidermis and inhibits neural tissue in dissociated cells by binding to noggin, a neural inductor. They showed that GDF6 can work in synergy with BMP in the embryo to regulate patterning of the ectoderm. Additional data were produced by Banka et al. [28]. In our experiments,

expression of *GDF6* was upregulated by NAC and by Freamine.

Insulin-like growth factors or somatomedins are a family of peptides that play important roles in mammalian growth and development. IGF1 mediates many of the growth-promoting effects of growth hormones by stimulating the incorporation of sulfate into cartilage. Human IGF1 is a single chain of 70 amino acids that displays homology to proinsulin. In our study, it was only slightly stimulated by NAC and a little more by Freamine.

Conclusions

The aging process in skin depends upon the overlapping of intrinsic factors, called chrono-aging, and extrinsic or environmental factors, called photo-aging. These two elements simultaneously produce wrinkles, laxity, and atrophy of the skin via ROS production. In this experimental study the induction of extracellular genes by NAC and Freamine in vitro was demonstrated. This might occur because NAC is a modulator of glutathione synthesis. High stimulation of genes encoding hyaluronic acid, elastin, and GDF6 after incubation of dermal fibroblasts with NAC and amino acid solution was seen. These findings support the clinical evidence seen with the use of these antioxidants through injection intradermic therapy for skin-aging prevention and amelioration.

The apparently contradictory effect of stimulation of synthesis and degradation of elastin and hyaluronic acid is probably related to the remodeling of the extracellular matrix that occurs with this therapy. However, our study was performed in vitro and an increased number of subjects could give more reliable results. Further investigations are needed to clarify the relationships between gene induction, related protein, and ROS level inside tissues.

Conflict of interest The authors have no conflicts of interest to disclose.

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