

Enhanced presence of NGF and mast cells number in nasal cavity after autologous stimulation: relation with sensorineural hearing deficit

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Abstract. – OBJECTIVE: Nerve growth factor (NGF) is a neurotrophin which promote and regulate the survival of neurons in the peripheral nervous system. We aimed to evaluate the nasal NGF expressions of mast cells in healthy patients after stimulation with sterilized isotonic solution delivered at high pressure.

PATIENTS AND METHODS: The first part of the study was made with 21 voluntary individuals. The middle third of the inferior turbinate epithelial cells on the right nostril was scraped using a sterile curette and indicated as (pre), than a spray of sterilized isotonic solution at high pressure on the left nostril was delivered and 25 minutes later a similar stimulation was delivered on the same nostril. The stimulation was made with a specific spray. The middle third of the inferior turbinate epithelial cells on the left nostril was scraped using a sterile curette and indicated as (post).

RESULTS: Forced nasal stress induced by local delivery of high pressure physiological solution causes an increase in the number of mast cells and enhances level of NGF in the nasal fluid compared to the control subjects. So based on the first part of our study, since NGF is universally known as effective in protection and repairing of neural cells damage, we started the second part and gave a treatment on the same patients, to increase NGF levels with a six months daily therapy and observed the variations in Sensorineural Hearing Loss (SNHL) and tinnitus intensity from the beginning to the end of the therapy. All patients received sterilized isotonic solution at high pressure (pression emission level: PEL): 7 g/sec for 0.5 sec (emission time: ET) in both nostrils. 25 minutes later a similar stimulation was delivered twice a day. The control group (21 pts) received normal therapy with betahistine dihydrochloride 16 mg twice a day.

CONCLUSIONS: Upon acuphenometry, there was a lower intensity of tinnitus and the improvement was signaled by the patients. Patients with SNHL treated with conventional therapy had a slight worsening, while the patients

treated with our new therapy which increased NGF levels, showed improvement of hearing. This therapy represents a new therapy of SNHL, tinnitus and hearing disorders.

Key Words:

Nasal cavity, Nasal mucosa, Nose, Nerve growth factor, Neurotrophins, Mast cells, Sterilized isotonic solution, Sensorineural hearing loss, Tinnitus.

Introduction

Nerve growth factor (NGF) is the first and best characterized member of a family of neurotrophic factors, produced by neuronal and non-neuronal cells. NGF, released in physiologically significant amounts in the bloodstream, promotes survival peripheral sensory neurons^{1,2}, including those innervating the nasal cavity and auditory cells^{3,4}. NGF signaling is mediated by two distinct receptors: TrkA (a tyrosine kinase receptors), and p75 receptor (a member of the tumor necrosis factor receptor superfamily), while the biological activity on its target cells depends of the ratio of TrkA and p75 present on the surface of NGF-target cells^{5,6}. It has been shown that deficit of NGF release and binding activity can cause neuronal damages, while exogenous NGF administration rescues degenerating peripheral sensory nerve cells⁷ and can stimulate growth guidance of auditory nerve fibers toward their targets within the organ of Corti⁸. Therefore, hearing loss might be the result of lesions of the sensory cells and/or of the neurons of the auditory part of the inner hear⁹. NGF is produced and released by a number of different cells, including olfactory epithelial cells, mucosal cells and mast cells^{10,11}. Other studies have reported that a consistent concentration of NGF expression, in the

nasal mucosa, has been found in mast cells, eosinophil granulocytes, the glandular apparatus and peripheral nerves. Increased expression of NGF may contribute to neural hyperresponsiveness in chronic rhinosinusitis sinus mucosa, particularly those patients with nasal polyps and/or allergies^{12,13}. Mast cells release several biological active mediators¹⁴, including NGF and, in addition to immunological stimuli, these mediators cause immediate vasodilator response characterized by mast cell degranulation¹⁵. Moreover, NGF induces the differentiation of mast cells, supports their survival and function via TrkA receptors that in response to injury contributes to nasal cells activation¹⁶ with other local resident activated cells that accumulate at damaged nerve cells in process of nerve ending damages and repair. Thus, the presence of mast cells and their degranulation has a broad implications for understanding the role in wound healing and peripheral nerve repair in the nasal mucosa and most probably in clinical prospective. We have demonstrated that NGF serum levels is reduced in patients with sensorineural hearing impairment¹⁷ suggesting that low release of NGF by local cells might be critically involved in Sensorineural Hearing Loss (SNHL). To further understand the role and functional significance of NGF release in the nasal cavity, the present study evaluates the effect and presence of mast cells in the nasal mucosa, due to stimulation with sterilized isotonic solution delivered at high pressure.

Patients and Methods

The First Part of the Study

The study was made with 21 patients with bilateral tinnitus and SNHL (7 male, 14 female, mean age 35 years, age range 19-55 years) in two different sets of test of 7 (A) and 14 (B) subjects. The Ethical Committee of the hospital approved the study and informed consent for tissue analysis was obtained from all the patients. Patients with nasal polyposis, chronic rhinosinusitis, ongoing pregnancy, smokers, and nosebleeds, patients who already underwent nasal surgery, patients with marked septal deviation and or turbinates hypertrophy, patients immunocompromised, and patients with bronchial asthma or chronic obstructive pulmonary disease (COPD), patients who had used antibiotics in the previous 30 days, patients that chronically use immuno-

suppressive corticosteroid were excluded. All patients underwent medical history collection, ear, nose and throat examination, pure tone audiometry and tympanometry. Endoscopic nasal examination was carried out with 0.4 mm rigid endoscope (Arlington Scientific, Inc., Springville, UT, USA). The inferior turbinate epithelial cells on the right nostril was scraped from the medial aspect of the middle third of the inferior turbinate using a sterile disposable Rhino-probe mucosal curette (Arlington Scientific, Inc., Springville, UT, USA) and used as control (pre). Soon afterwards, the left nostril was treated with sterilized isotonic solution at high pressure: 7 g/sec for 0.5 sec with a specific spray. 25 minutes later a similar stimulation was delivered on the same nostril. The nostril was, then, scraped from the medial aspect of the middle third of the inferior turbinate and scraped cells removed and indicated as (post).

The samples collected were, then, centrifuged for 5 min at speed of 2000 rpm, supernatant removed and immediately stored at -70°C for NGF determination; scraped cells used were cytospunned and used for histological and NGF-positive immunocytochemical identification.

NGF Levels Determination

The levels of NGF were measured by a highly sensitive two-site immunoenzymatic assay. Briefly, polystyrene 96-well microtube immunoplates (Nunc) were coated with affinity purified, polyclonal goat anti-NGF antibody, diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA), in order to evaluate the non-specific signal. Following overnight incubation at room temperature and 2 h incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% BSA), plates were washed three times with Tris-HCl, pH 7.4 50 mM, NaCl 200 mM, 0.5% gelatin, 0.1% Triton X-100. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA and 0.5% gelatin), distributed among the wells and left to stand at room temperature overnight. The levels of NGF were measured with a highly sensitive two-site ELISA, (Cat. Nr.G7631, Promega Madison, WI, USA), following the instructions provided by the manu-

facturer. The amount of NGF was determined from the regression line for the NGF standard (ranging from 7.8 to 500 pg/ml of purified mouse NGF). Assays were performed in duplicate and the data are expressed as pg of NGF/mg of total tissue proteins¹⁸.

Histological Studies

Cytospun cells collected from the nasal cavities of pre and post-treated subjects were fixed in 4% paraformaldehyde in phosphate buffer, 0.1M, pH, 7.4 for 5 minutes; then, washed in the same buffer and stained either with toluidine blue to identify mast cells for their metachromatic characteristics^{19,20}. The number of mast cells were, then, counted in 8 comparable microscope fields (magnification 40×) (n=6) of pre and post collected samples and were counted and compared. Asterisk indicates significant differences between groups (**p* < 0.05).

Immunocytochemical Study

Samples of the mucosa, smeared on a glass slide, were fixed as above, in 4% paraformaldehyde in phosphate buffer, 0.1 mole, pH, 7.3 for 5 minutes and after a brief wash in phosphate buffer and immunostained for localization of NGF or NGF receptors. Briefly, cells were first incubated in PBS containing 10% of horse or goat serum for 1 hour, and then left overnight at 4°C with monoclonal anti-NGF antibodies²¹; then, exposed to biotinylated IgG 1:300 (anti-IgG and avidin-conjugated horseradish peroxidase complex) were purchased by Vector Laboratories (Burlingame, CA, USA) with 2% of goat or horse serum, depending on the animal in which

the secondary antibody was produced, for 2 hours at room temperature. Then, immunoperoxidase staining was performed using an ABC (1:100) (Avidin-Biotin complex solution, Vectastain Elite Kit, Vector Laboratories, CA, USA) for 2 hours at room temperature. Sections incubated identically with normal IgG were used as negative controls. Immunostained signals were visualized by DAB (3,3'-diaminobenzidine), and visualized using a Zeiss Axiophot microscope equipped with a 40× objective. The number of NGF immunostained cells in 6 comparable microscope fields (magnification 40×) of different (n=8) pre and post-collected samples were counted and compared. The data obtained are expressed as the number NGF-positive mast cells. Asterisk indicates significant differences between groups (**p* < 0.05).

Statistical Analysis

The statistical analysis of the data was performed using StatView II program for Macintosh (Abacus Concepts Inc., USA) considering the experimental conditions as main factor. Analysis of variance was performed using Tukey-Kramer test. Significance values were taken as those with *p* values of *p* < 0.05. Image analyses were evaluated by one-way ANOVA. A *p*-value less than 0.05 were considered significant.

Results

As reported in Figure 1A-B, the nasal NGF level of the two groups of subjects examined is higher in post subjects (post), compared to con-

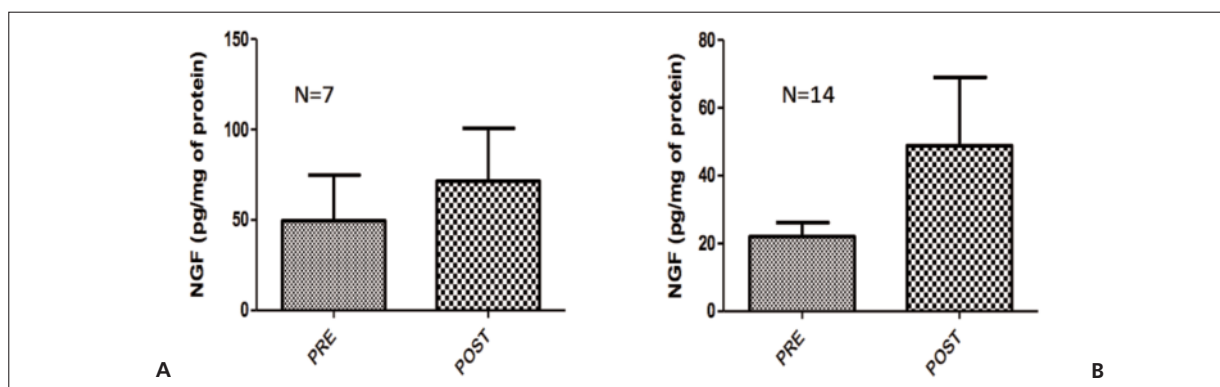


Figure 1. A-B. Levels of NGF in fluid of nasal cavities collected from two different sets of test of 7 and 14 subjects, pre-treated (*pre*) and post-treatment (*post*) as described under subjects and methods. As shown in figures, the concentration of NGF, expressed as pg/mg of protein, is less expressed in the nasal cavities of subjected pre treated, compared to post-treated (*post*) subjects. The differences are statistically significant (*p* < 0,01).

trols (pre) subjects. Thus, the NGF released after high pressure saline administration is markedly enhanced in the nostril of post-treated subjects and the differences are statistically significant. Thus, the treatment stimulates the release of NGF from cells distributed in the nasal cavity. Since mast cells and eosinophils are known to be able to synthesize and release NGF²², we performed histological and immunohistochemical analysis of cells collected from the nasal cavities from pre and post-treated subjects.

As shown in Figure 2A-D, cells collected from the nasal cavities of pre- and post-treated subjects stained positively with toluidine blue and exhibit the metachromatic characteristics typical of mast cells (*arrows*). Moreover, mast cells present in samples collected from post-treated subjects of both groups (Figure 2B-D) are more numerous compared to those in the nasal cavities of pre-treated subjects (Figure 2A-C). As shown in Figure 2E, F, eosinophil (*arrows*) have also been found in the nasal cavity of pre (Figure 2A) and post (Figure 2F). However, these cells are significantly less numerous compared to mast cells, suggesting that the NGF released in the nasal cavity comes from mast cells.

Next, we quantified the number of mast cells present in pre and post treated subjects.

As reported in Figure 3A-B, the number of mast cells of both experiment groups reported in Figure 1A-B are markedly more numerous in post compared to pre-treated subjects. These differences are statistically significant ($p < 0.05$). To further explore whether the mast cells present in nasal fluids produce and release NGF, these cells were immunostained against NGF.

As illustrated in Figure 4A-C the mast cells are markedly immunostained with NGF antibody. This observation is consistent with the hypothesis that the mast cells collected from the nasal cavity produce and release NGF and those post-treated subjects express more NGF than pre-treated subjects.

The Second Part of the Study

In the first part of the study we observed an increase of NGF levels in patients treated with nasal spray forced stimulation. NGF is universally known to have a protective and repairing role on neural cells. So based on the first part of our study, we started the second part and gave a treatment on the same patients, to increase NGF lev-

els with a six months daily therapy and observed the variations in SNHL and tinnitus intensity from the beginning to the end of the therapy.

All patients received sterilized isotonic solution at high pressure (pression emission level: PEL): 7 g/sec for 0.5 sec (emission time: ET) on both nostrils. 25 minutes later a similar stimulation was delivered twice a day. The control group (21 pts; 10 female, 11 male, mean age 42, range 18-75 yrs) received normal therapy with betahistine dihydrochloride 16 mg twice a day.

The patients were asked to mark the severity of their symptoms on a validated Tinnitus Handicap Inventory Questionnaire (THI). They were evaluated at the beginning and 6 months after the treatment.

Upon acuphenometry, the average tinnitus pitch before therapy was 40 dB intensity, while after six months of therapy it was 25 dB intensity, demonstrating a lower intensity of tinnitus; this improvement was signaled by the patients. The patients treated with conventional therapy had no variations, as evident by acuphenometry and patients report (Figure 5).

The patients with SNHL showed average improvement of hearing of 10 db after 6 months of therapy than the control group. The patients treated with conventional therapy had a slight worsening, as referred by audiometry and patients reports (Figures 6 and 7).

Results

The mean THI score reported by patients at 6 month was significantly lower than control group (3.36 ± 1.89 vs 6.95 ± 1.52 ; $p < 0.05$) showing a significant improvement of intensity and tinnitus tolerability.

Discussion

Recent studies have shown that intranasal administration is a potential route for drug delivery to the brain that bypasses the blood brain barrier (BBB) through the cribriform plate in the nasal vault. So it looks feasible a nose to brain pathway for delivery of macromolecules, like NGF^{22,23}. Thus, the olfactory pathway is a safe, non-invasive route to deliver drugs to the brain target cell, including molecules like NGF that might have a potential role for the treatment of neurodegenerative diseases.

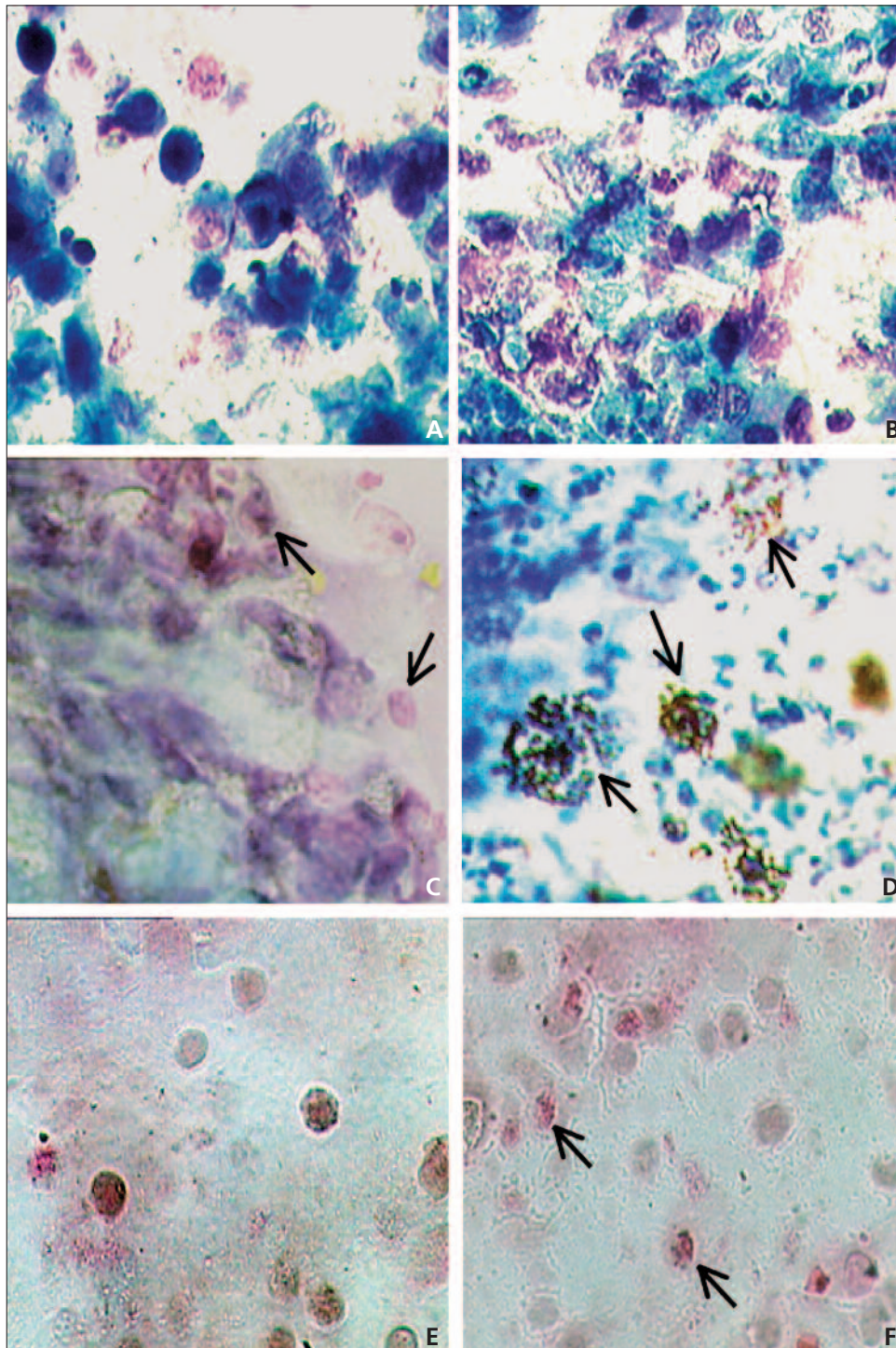


Figure 2. A-F. Representative histological images of cells collected from the nasal cavity of pre-treated (A) and post-treated (B,C) subjects, showing the characteristic of metachromatic staining (arrows) and mast cell degranulation (arrows) in figure D. As shown in figure E, F, the nasal fluid of pre (E) and post (F) treatment contains also eosinophil (arrows), but are less numerous compared to mast cells among the numerous mucosal cells. Tuolidine blue staining, Scale bar: 30 μ m.

We have been the first in this study to obtain through a forced nasal stress induced by high pressure local delivery of physiological solution

with a specific spray an increase in the number of mast cells and enhanced level of NGF in the nasal fluid compared to the control subjects. This

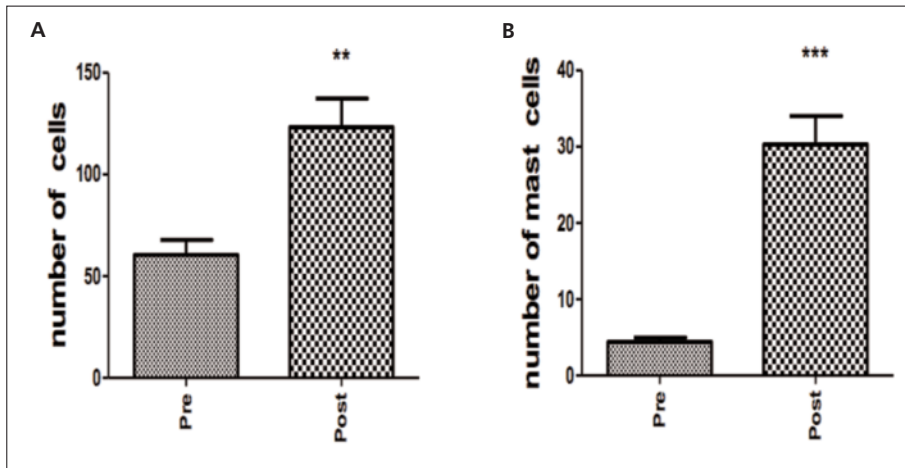


Figure 3. A-B. Mean number of mast cells collected from the nasal cavity of subjects before (pre) and after treatment (post) subjects. Note significant increase of mast cells after nasal spray treatment. The differences are statistically significant ($p < 0.01$).

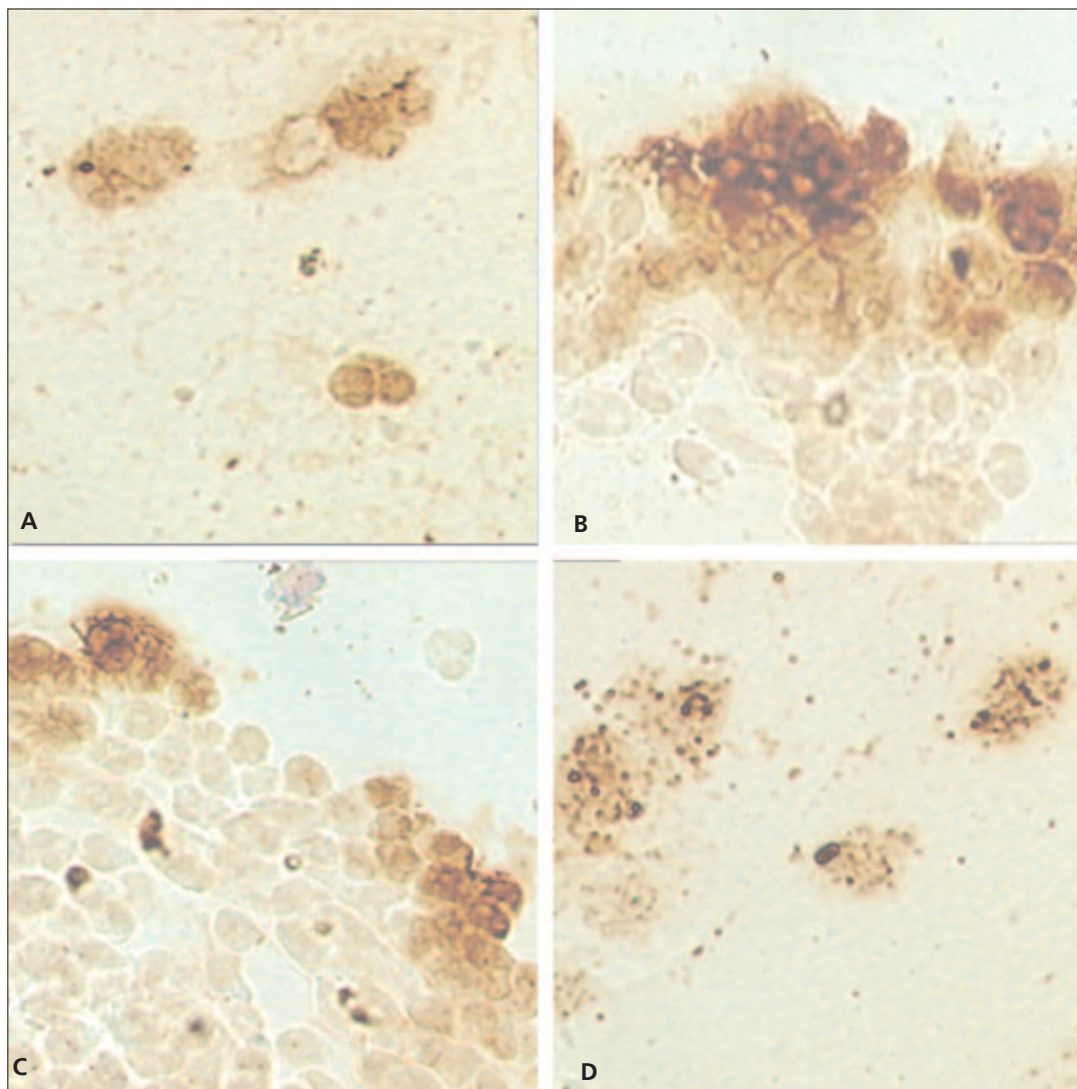
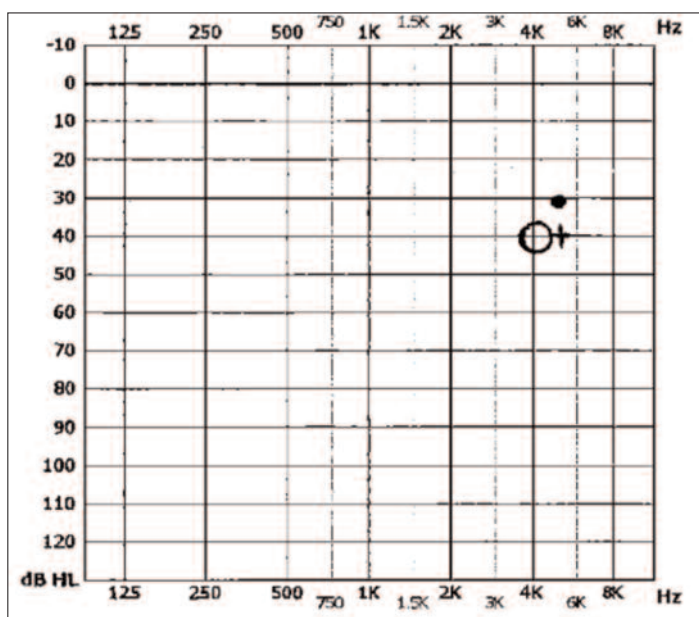


Figure 4. A-D. Representative images of NGF-immunostained cells collected from the nasal cavity of control (A) and treated (B-D) subjects. Numerous cells are markedly stained positively for NGF, indicating that they produce and release NGF. Figure D, shows degranulated mast cells. Scale bar: 30 μ m.

Figure 5. 21 People affected by tinnitus treated for 6 months and examined with acuphenometry. o People affected by tinnitus, average 40 db intensity, before treatment; • After therapy PEL 7 g/sec ET 0.5 sec + After conventional therapy.



result was suggested by findings showing that nasal epithelial cells release NGF, and that cells present in the nasal fluid synthesize and release NGF²⁴. It is known that NGF is constitutively expressed in nasal cavities and that epithelial cell, mast cells and eosinophil cells produce and release growth factor¹². A functional role of NGF in the nasal cavity is also suggested by findings

that NGF modulates differentiation, growth, maintenance of nerve cells of the central and peripheral sensory nerve cells^{2,25}, including olfactory sensory neurons. Notably, olfactory neurons are unique in the mammalian nervous system because of their capacity to regenerate in adult animals. In our research immunohistochemical analysis revealed that cells collected from nasal

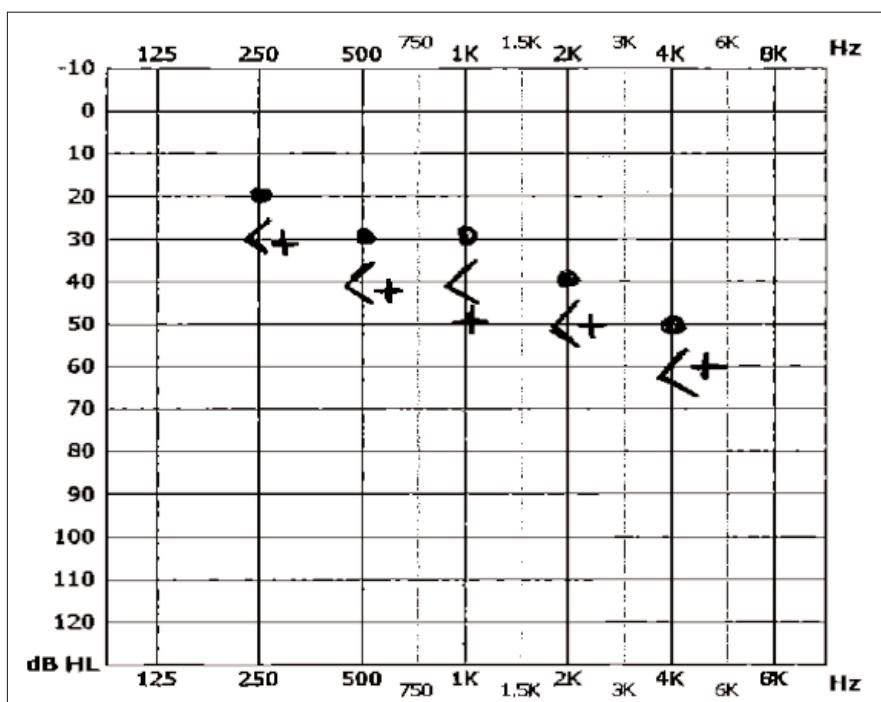


Figure 6. 21 People with SNHL treated for 6 months and examined with bone audiometry (BA). < left ear bone audiometry before treatment; • After Therapy PEL 7 g/sec ET 0.5 sec; + After conventional therapy.

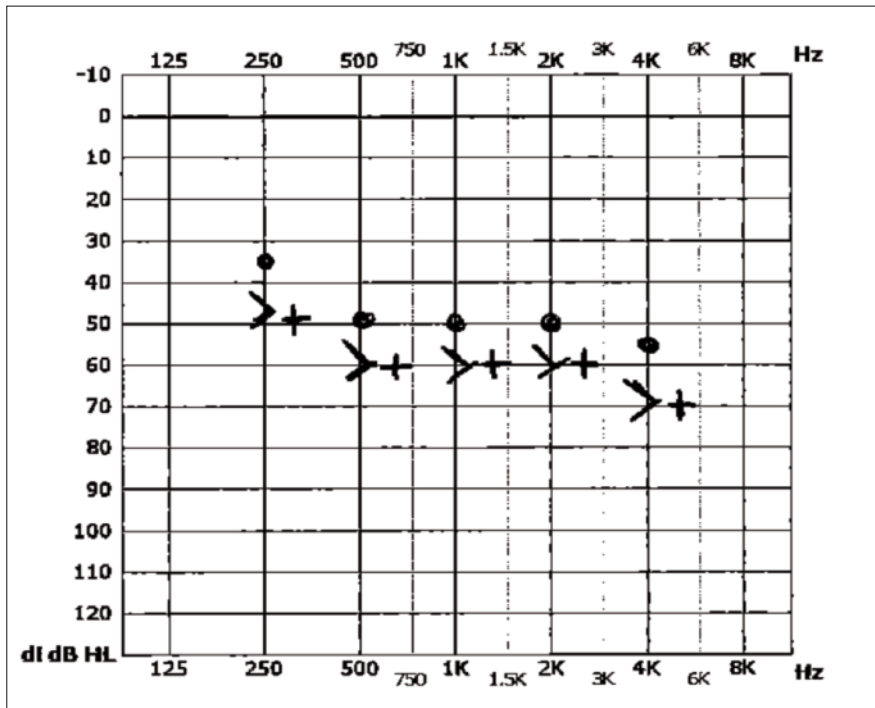


Figure 7. 21 People with SNHL treated for 6 months and examined with bone audiometry (BA). > right ear bone audiometry before treatment; • After therapy PEL 7 g/sec ET 0.5 sec; + After conventional therapy.

lavages, after nasal stress treatment, are markedly immunostained with anti-NGF antibody. Therefore, they release NGF and/or express NGF receptors. These observations are consistent with studies showing that NGF is produced by and acts upon mast cells²⁶, and that NGF is regulated by autocrine mechanisms^{25,27}. Moreover, we observed few epithelial cells and eosinophils positive to NGF mostly in post-treated subjects, suggesting that these cells also release the enhanced levels of NGF in the nasal fluid.

We have previously reported that in patients affected by sensorineural hearing loss (SNHL) the amount of circulating NGF is significantly lower in comparison to that found in age-matched health controls. These observations support the hypothesis that NGF might play a critical role in the pathophysiology of human hearing deficits¹⁷.

This role is also suggested by other findings showing that NGF in the olfactory bulb is involved in the development, maintenance, and regeneration of olfactory receptor cells, in the

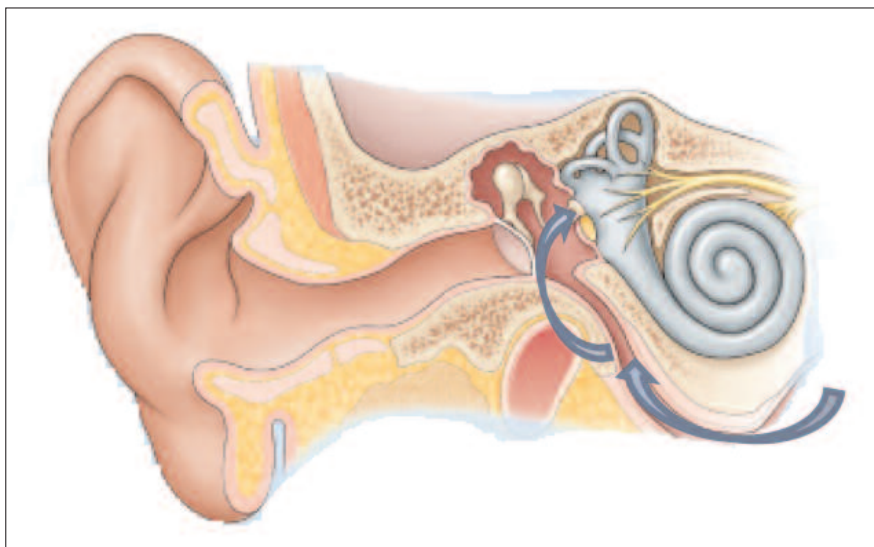


Figure 8. Anatomical way from the nose to the cochlear cells, along the Eustachian tube, the middle ear cavity and through the round window membrane.

maintenance of olfactory nerve cells, and in the differentiation and survival of mature olfactory nerve cell^{6,26}. It is, therefore, possible that the NGF expressed and released in the nasal mucosa might be involved in mechanism for maintaining and/or restoring homeostatic function of nasal tissue also during or after local inflammation and tissue damage.

Mast cells are involved in inflammatory and hypersensitivity reaction²⁸, and occur in many peripheral tissue, in perivascular regions in close apposition to innervating sensory or autonomic nerves²⁹ and also within the peripheral and central nervous systems. Secretory products of activated mast cells can stimulate or facilitate axon reflex, there by inducing positive feedback loop³⁰. Activated mast cells also secrete a wide range of pluripotent cytokines and other inflammatory mediators²⁷ and may, thus, act as bidirectional carrier of information between the nervous and immune systems. Given the potential involvement of mast cells and NGF in neuroimmune interactions and the close microanatomical associations between mast cells and sensory or autonomic fibers in several tissues, mast cells, in fact, produce NGF. A more complete understanding of their local stimulatory and inhibitory regulation might open avenues to the management of inflammatory disease states, including those of autoimmune origin. The dual nature of the mechanism of action of NGF provides some explanation as to its potency in altering mast cell population and underlines its possible *in vivo* role at sites of tissue injury where mast cells hyperplasia is often observed.

The mechanisms(s) involved in the regulation and/or releasing the NGF in the nasal cavity is not known. It has been demonstrated that stress can stimulated the release of NGF from NGF-producing cells^{31,32}.

We found NGF elevated in post-treated subjects; mast cells in the nasal fluid were positive to NGF demonstrating for the first time that NGF produced by mast cells can be increased stimulating a local nasal inflammation through a new therapy based on a saline solution delivered at high pressure.

It is, therefore, possible that stress induced by forced intranasal administration of physiological solution could be used to enhance the constitutive amount of NGF, to reinforce and protecting the damaged sensory nerve endings in SNHL and tinnitus. This hypothesis is supported by our previous observations showing that patients with

sensorineural hearing impairment have reduced levels of circulating NGF¹⁷ and that NGF has been shown to play a critical trophic role on ear innervation. The clinical and audiometric improvement in patients with SNHL and tinnitus treated with our new therapy, which increase NGF levels, represent a clinical confirm to this hypothesis.

NGF participates in the pathophysiology of hearing, most probably to stimulate neuritis outgrowth from hearing neural sensory neurons and may have a crucial role in the auditory pathway, promoting the survival and preventing the degeneration of sensory neural cells. Moreover, since NGF plays a critical role on peripheral sensory nerve cells, including the hearing cells of the inner hear, it is possible that the recruitment of mast cells and release of NGF in the nasal cavity might positively modulate hearing deficits in sensorineural hearing loss by reducing and/or protecting the sensory deficit.

Conclusions

We presume that the improvement in SNHL and in tinnitus in our patients could be related to the penetration of increased NGF from nasal mucosa through eustachian tube up to the middle ear and through the round window membrane up to the cochlear auditory cells (Figures 8 and 9).

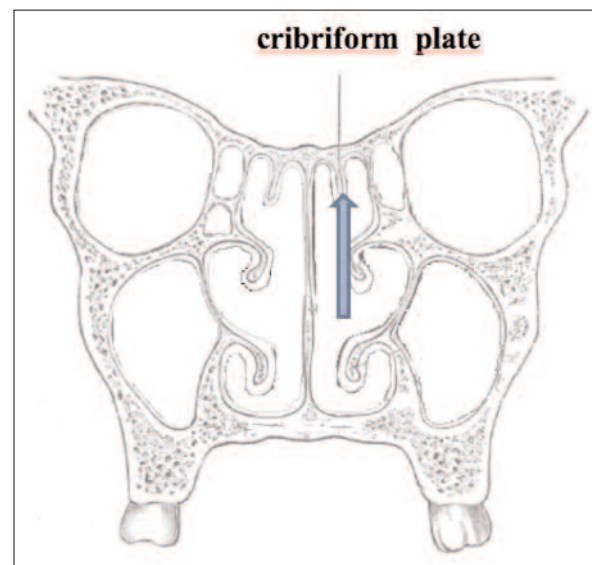


Figure 9. Anatomical way from the nose to the brain, along the cribriform plate.

Another way of NGF penetration is well known, through the cribriform lamina up to the endocranial space. We are trying with ongoing experiments on animal models to better understand whether the neurotrophins, individually or in combination, are effective *in vivo* in preventing the degeneration and promoting neuronal hearing cell repair. Our data support the hypothesis that NGF and the presence of mast cells and their degranulation have a broad implications for understanding the role in wound healing in the nasal mucosa and peripheral neural cells repair. We first discovered a therapy able to stimulate and enhance NGF production in nasal mucosa. The novelty is based on the particular physical parameters in delivering physiologic solution. The physiologic solution showed to give no side effects in a six months daily therapy.

The clinical and audiometric improvement in patients with SNHL and tinnitus treated with this new therapy represent a clinical confirm to our hypothesis and a new therapy of SNHL, tinnitus and hearing disorders. We are increasing the number of patients in a new study aiming to better understand the critical role of NGF not only in the therapy of SNHL, tinnitus and hearing disorders but also in neurological diseases.

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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