

Nasal HPpSIS administration enhances NGF and tumor suppressor protein, p73 in human brain cancer tissues: preliminary data

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Abstract. – **OBJECTIVE:** Nerve Growth Factor (NGF) is a neurotrophic factor known to play a critical role in growth, survival, differentiation and neuroprotection of peripheral sensory and sympathetic neurons, as well as brain neurons. We have recently reported that nasal administration of high-pressure isotonic physiological saline solution (HPpSIS) enhances the level of NGF and the expression of NGF receptors in neurons of the olfactory bulbs and fore-brain cholinergic neurons of laboratory animals. In the present study, we sought to determine whether the same treatment affects the levels of NGF within the brain tumor tissue.

PATIENTS AND METHODS: This study was conducted on eight adult patients, 4 males and 4 females with malignant anterior cranial fossa tumor. Before surgery, four subjects, two males and two females received nasal administration of HPpSIS for ten consecutive days.

RESULTS: The levels of NGF in surgical removed peripheral tumor brain samples of patients treated with nasal HPpSIS administration are more elevated compared to the levels of NGF in peripheral brain tissues of HPpSIS untreated patients.

CONCLUSIONS: We observed that nasal administration of HPpSIS enhances not only the basal brain NGF levels and the expression of NGF receptors but also the tumor suppressor protein p73. The possible functional significance of these observations will be described and discussed.

Key Words:

NGF, HPpSIS, Tumor suppressor protein p73.

of neurotrophic factors, known to play a critical role in growth, survival, differentiation and protection of degenerating neurons (of peripheral sympathetic and sensory nervous system¹⁻² and of brain neurons³⁻⁴ that are involved in memory and learning abilities such as Alzheimer's diseases (AD)^{5,6}. Moreover, in the last two decades, some other studies have shown that NGF is produced by and acts upon a variety of non-neuronal cell types, such as epithelial and immune cells and cancer cells that possess the properties of producing and releasing NGF^{7,8}. Cancer cells produce and release NGF and/or express NGF receptors, though their role signaling in tumor growth, differentiation and proliferation have not been definitively established. Arrighi et al⁹ proposed that a dysregulation of NGF signal transduction was found in some human tumors^{9,10-11}. NGF receptors (NGFRs) are found in numerous immature cell populations in the central nervous system of rats, felines, and primates, but its role in the tumor growth is less clear.

NGF administration is associated to an increased biological activity of NGF-target cells and not related with to induction of cancer cell proliferation^{7,12}.

Vinorea et al¹³ have demonstrated the expression of NGF and NGF-receptors in neural crest-derived cells as well as in tumor cell lines from neuroblastoma, lymphoma, glioma, medulloblastoma adrenal tumors and melanoma, suggesting that NGF administration could lead to cell differentiation and improved prognosis.

We have recently reported that high-pressure administration of sterile saline isotonic solution (HPpSIS), as a nasal spray in patients, improves sensorineural hearing loss (SNHL), and reduces tinnitus symptoms. This effect is associated to

Introduction

Nerve growth factor (NGF) is the first discovered and best characterized member of a family

enhanced release of nerve growth factor (NGF) into the nasal cavity and increase number of NGF-producing cells¹⁴. In a subsequent study, we reported that the same treatment for ten consecutive days reproduced a similar effect in rodents. The result showed that not only HPpSIS administration reproduced the effect observed in human volunteers, but also enhances the level of NGF and expression of NGF-receptors in neurons of the olfactory bulbs and brain¹⁵.

In the present study we investigated whether nasal HPpSIS administration alters the presence of NGF and NGF-receptors in the human brain with a tumor. It was found that nasal administration of HPpSIS enhances not only the expression of NGF receptors and the tumor suppressor protein, p73, but also the basal brain NGF levels. The findings suggest that the NGF released in the nose as the result of HPpSIS treatment goes through the blood brain barrier and promotes up-regulation of NGF-receptors and p73 protein leading to cancer cell differentiation, rather than proliferation. The possible concurrent release of NGF by brain tumor cells is to be investigated. The detailed results and the possible functional significance of these findings will be described and discussed.

Patients and Methods

Chemicals

A specific high-pressure dispenser with a sterile isotonic solution (pressure emission level: PEL: 7 g/sec for time ET: 0.5 sec emission). Primary antibodies: anti-trkA^{NGFR} (sc-118; Santa Cruz Biotech, Santa Cruz, CA, USA), anti-p75^{NTR} (sc-6188; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p73 1:1000 (Neomarkers, CA, USA) and anti-GAPDH (sc-365062; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Secondary antibodies: Vector anti-rabbit and Vector anti-mouse as well as horseradish peroxidase-conjugated anti-rabbit/mouse specie-specific secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Patients characteristics and Treatment

This study was conducted on eight adult patients, 4 males and 4 females, hospitalized at the Department of Neurooncology of Ancona Hospital with malignant anterior cranial fossa tumor. The age of patients ranged from 40 to 70 years, the mean age 57. Before surgical interven-

tion, four subjects, two males and two females received nasal administration of HPpSIS for ten consecutive days. Four other patients, two males and two females, were left untreated and served as control group. The day after last treatment, the tumor mass was surgically removed in all subjects by the same Neurosurgeon for studying the pathological characteristics of the neoplasm. Small tissue samples at least 1 cm away from the tumor were collected as usually, to verify the radicality of tumor excision. These samples were also used for our structural, biochemical and molecular analyses, related to the expression of NGF and NGF expression and the tumor suppressor protein p73. We planned to investigate the modifications of samples sufficiently away from the tumor limits, to avoid the inflammation present in the tumor site.

The HPpSIS therapy showed in our case series no side effects in more than 21 patients treated for 48 months for sensorineural hearing loss and tinnitus. Furthermore, in our present patients with malignant anterior brain tumor, the treatment was certainly useful for cleaning purpose of the nose cavities, which communicates with anterior cranial fossa through the cribriform plate. This evidence allowed us to forecast a better healing in the anterior cranial fossa, thanks to the reduction of surgical field contamination risks and consequently led us to make the present therapy according to the fundamental ethic requirements.

NGF Assay

For NGF determination, brain tissues were homogenized with ultra-sonication in RIPA buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS and 0.5% DOC; Sodium deoxycholate; 1 mM PMSF; 1 µg/mL leupeptin; AppliChem, Darmstadt, Germany) and centrifuged at 13000 rpm (4°C) for 20 min. The supernatant was collected for NGF analysis (Emax Immunoassay System ELISA kit; Promega Corp., Madison, WI, USA). The assay sensitivity was 3 pg/mL and the NGF recovery ranged from 80% to 90%. The Optical Density (OD) was measured at 575 nm using an ELISA reader (Multiskan EX, Thermo Scientific, Madison, WI, USA) and both standard and sample values were corrected by taking into consideration the non-specific binding. The assays were performed in duplicate and data (pg/µg of total proteins) are shown as fold increase in NGF-treated with respect to untreated one. For normalization, total

proteins were determined by using a spectrophotometer and a DC protein assay reagent (Biorad Laboratories, Hercules, CA, USA).

Western Blotting Analysis

Brain samples were homogenated in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% Triton X-100; 0.1% SDS; 0.5% DOC (Sodium deoxycholate); 1 mM PMSF; 1 µg/ml leupeptin), centrifuged at 4°C for 20 min at 13000 rpm, then supernatant was stored at -20°C. Samples (30 µg of total protein) were dissolved in loading buffer (0.1 M Tris-HCl buffer, pH 6.8, containing 0.2 M dithiothreitol, DTT, 4% sodium-dodecyl-phosphate, SDS, 20% glycerol, and 0.1% bromophenol blue), separated by 8% or 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to PVDF membrane overnight. The membranes were incubated for 1 hr. at room temperature with blocking buffer constituted by non-fat dry milk in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween-20). Membranes were washed three times for 10 minutes each at room temperature in TBS-T followed by incubation at 4°C with primary antibodies overnight: polyclonal rabbit anti-TrkA 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-p75 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-p73 1:1000 (Neomarkers, Fremont, CA, USA). Membranes were washed three times for 10 minutes each at room temperature in TBS-T and incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit IgG 1:4000 or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature. The blots were developed with an ECL chemiluminescent horseradish peroxidase (HRP) substrate as the chromophore (Millipore, Billerica, MA, USA). The public Image J Software was used to evaluate band density, which was expressed as arbitrary units of grey level. The Image J program determines the optical density of the bands using a grey scale shareholding operation. The optical density of polyclonal rabbit anti-GAPDH 1:4000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) bands was used as a normalizing factor. For each gel blot, the normalized values were then expressed as a percentage of relative normalized controls and used for statistical evaluation. Statistical evaluations were performed using the GraphPad Prism package for

Windows and data expressed as means ± SEM of four different samples.

Statistical Analysis

Data from Western blot, ELISA and image analysis were evaluated using GraphPad Prism 5 software and statistical analysis was conducted using one-way ANOVA or t-test Student. Means from independent experiments were then expressed as means ± SD. For all statistical analysis, p-values < 0.05 or less was considered statistically significant. All experiments were performed in duplicate and provided as mean ± SEM in the graphics. Unpaired Student t-test analysis was performed using GraphPad Prism 5 software package for Windows. A p-value ≤ 0.05 was considered significant. The REST/ANOVA-coupled analysis was carried out for molecular comparisons.

Histological Studies

Tumor samples were fixed immediately after surgical operation and tissues fixed in 4% paraformaldehyde at +4°C for five days and cryopreserved in 10% buffered sucrose, according to a standard procedure. Samples were then sectioned at 10 µm thickness cryostat (Leica CM1850 UV, Leica Microsystems, Wetzlar, Germany) and processed for histological analysis after stained with Hematoxylin and Eosin (H&E).

Immunohistochemical Analysis

For immune-histochemical analyses, coded brain sections were immunostained against TrkA and p75 NGF-receptors and against the tumor suppressor protein p73. Briefly, sections were first incubated in PBS containing 10% horse or goat serum for 1 hour, and then left overnight at 4°C with mouse monoclonal anti-p75NTR 1:400 (sc-56331, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-TrkA 1:400 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p73 1:400 (Neomarkers, Fremont, CA, USA). Sections were then exposed to biotinylated rabbit anti-goat IgG Antibody 1:500 (Vector Laboratories, Burlingame, CA, USA) for 2 hours at room temperature, and the immunoperoxidase staining was performed using an ABC reagent (Avidin-Biotin Complex solution, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA). The sections incubated with normal IgG were used as non-specific staining controls. Immunostained signals were then visualized with 3,3'-diaminobenzidine (DAB) Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame,

CA, USA). Immunostained cells sections were visualized under a Zeiss Standard microscope (Jena, Germany) and cell count was carried out using the image processing and analysis program.

Results

Morphological Observations

Sections of H&E stained peripheral tumor tissues of patients untreated with HPpSIS showed the presence of numerous of pleomorphic and densely stained dead cells invading normal

nerve cells (1A), while a stained section of HPpSIS treated brain show the presence of some normal morphological characteristics of neurons (Figure 1B, arrows). Densely stained cells and dead cells nearly absent. Moreover, some cells with large vacuoles, resembling adipocytes were present in untreated HPpSIS but not in treated HPpSIS tissues.

Brain NGF Levels

As indicate in Figure 1C, the levels of NGF in surgical removed peripheral tumor brain samples of patients treated with nasal HPpSIS adminis-

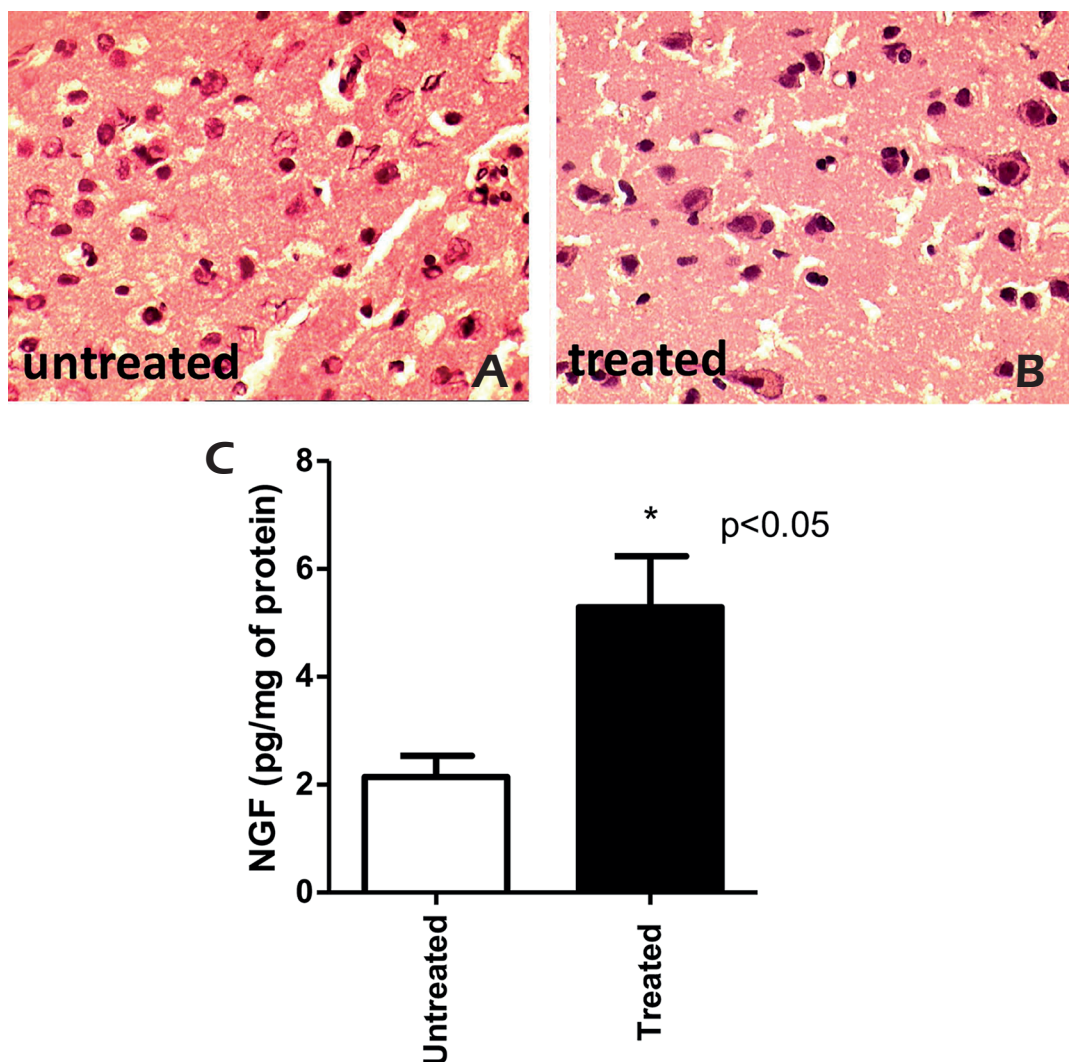


Figure 1. *A-B*, H&E stained sections (*A,B*) showing brain tumor cells of untreated (*A*) and treated samples (*B*) with nasal administration of HpPSIS. Note more numerous pleomorphic tumor cells invading brain tissues in untreated HPpSIS tissues, compared to neurons like-cells present in HPpSIS treated tissues. Magnification, X 300. *C*, Levels of NGF in the brain tumor tissues of untreated and HPpSIS treated patients. Note the enhanced NGF level in HPpSIS treated compared to untreated brain samples. The differences are statistically significant (* $p < 0.05$).

tration is more elevated compared to the levels of NGF in peripheral brain tissues of HPpSIS untreated patients. The difference of NGF presence between these two tissues resulted statistically significant, $p < 0.01$. This observation suggests that nasal administration of HPpSIS induces NGF release, acting most probably also on neoplastic cells.

NGF Receptor Expression

Because the biological activity of NGF is regulated by two NGF-receptors, the low-affinity NGF-receptor (p75) and the high-affinity NGF receptor, TrkA. To measure the expression of NGF receptors, we used the western blotting analysis. As shown in Figure 2, the results showed that the expression p75 receptors 2A,B and to a lesser extend of the TrkA receptors, Figure 2C,D are more expressed in tissues that received the administration of HPpSIS, as compared to the untreated brain tissues. Figure 2B and 2D reports the results of single samples and the differences between HPpSIS treated and untreated tissues.

Immunohistochemistry

To explore differences in NGF-receptor expression between the HPpSIS-treated and the untreated sample, coded brain sections were immunostained against the two NGF-receptors. As illustrated in Figure 2E, F and 2G, H, stained sections showed that the number of both p75 and TrkA immunostained cells are more numerous in tissue samples that received HPpSIS administration, compared to untreated samples.

The Suppressor Protein P73

We next investigated the effect of HPpSIS on the expression of p73. The result of western blotting determination is reported in Figure 3. It was found that p73 expression in brain tumor tissues is enhanced after treatment with HPpSIS as compared to the expression of untreated samples; Figure 3A as indicated also by comparing single values, see Figure 3B. However, to search additional indication about these differences, sections of HPpSIS-treated and untreated brain samples, were immunostained against p73 antibodies. As illustrated in Figure 3C,D, the numbers of p73-immunopositive cells in HPpSIS treated sections are more numerous than p73-immunopositive cells in HPpSIS untreated brain tissues. The quantitative differences of p75, TrkA ip73 immunostained cells are summarized in Table I. Why the high- and low-affinity NGF-receptors are differently

expressed is not known, most probably these differences are related to the different receptors present at the surface of brain cancer cells and to the number of target tissues within the cell population samples, the different roles of the two NGF receptors on cell death and survival and stage of proliferation and/or differentiation^{16,17,10}.

Discussion

The present study was designed to address the following two questions: first, whether HPpSIS nasal administration enhances the level of brain NGF and the expression of NGF-receptor in human peripheral brain tumor tissues and second: whether the HPpSIS administration exerts any effect on brain cancer cell proliferation and differentiation. The rationale behind these two experimental approaches is based on previous and recent observations indicating that cancer cells can express NGF-receptors and exposure to NGF can promote cell differentiation, rather than cell proliferation, not only in isolated cells, but also *in vivo*^{9,16,18,19,10,20-21}.

As far as concern HPpSIS, we have demonstrated that forced nasal administration of HPpSIS in voluntary individuals stimulates the presence of immune cells into the nasal cavity and enhances the release of NGF and NGF-receptor expression, leading to the improvement of SNHL and reducing the tinnitus symptoms¹. Moreover, further investigations revealed also that in laboratory animals HPpSIS treatment enhances the basal presence of NGF and NGF-receptors expression in the olfactory bulbs and brain¹⁵. However, the functional significance of the enhanced brain NGF is not clearly known, though it is known that under normal conditions NGF is produced and released from brain cells localized in the frontal cortex and hippocampus and acts on the survival and neuroprotective support of forebrain cholinergic neurons in mechanism of learning ability and memory deficits in rodents and humans with AD³⁻⁶. It is, therefore, possible that the enhanced NGF released as the result of HPpSIS treatment, may also stimulate the NGF brain cells production.

Regarding the second question, our study revealed that nasal HPpSIS administration not only enhances the expression of NGF and NGF-receptors brain tumor tissue but also the expression of tumor suppressor protein p73. Thus, the concomitant up-regulation of these two neuroprotective

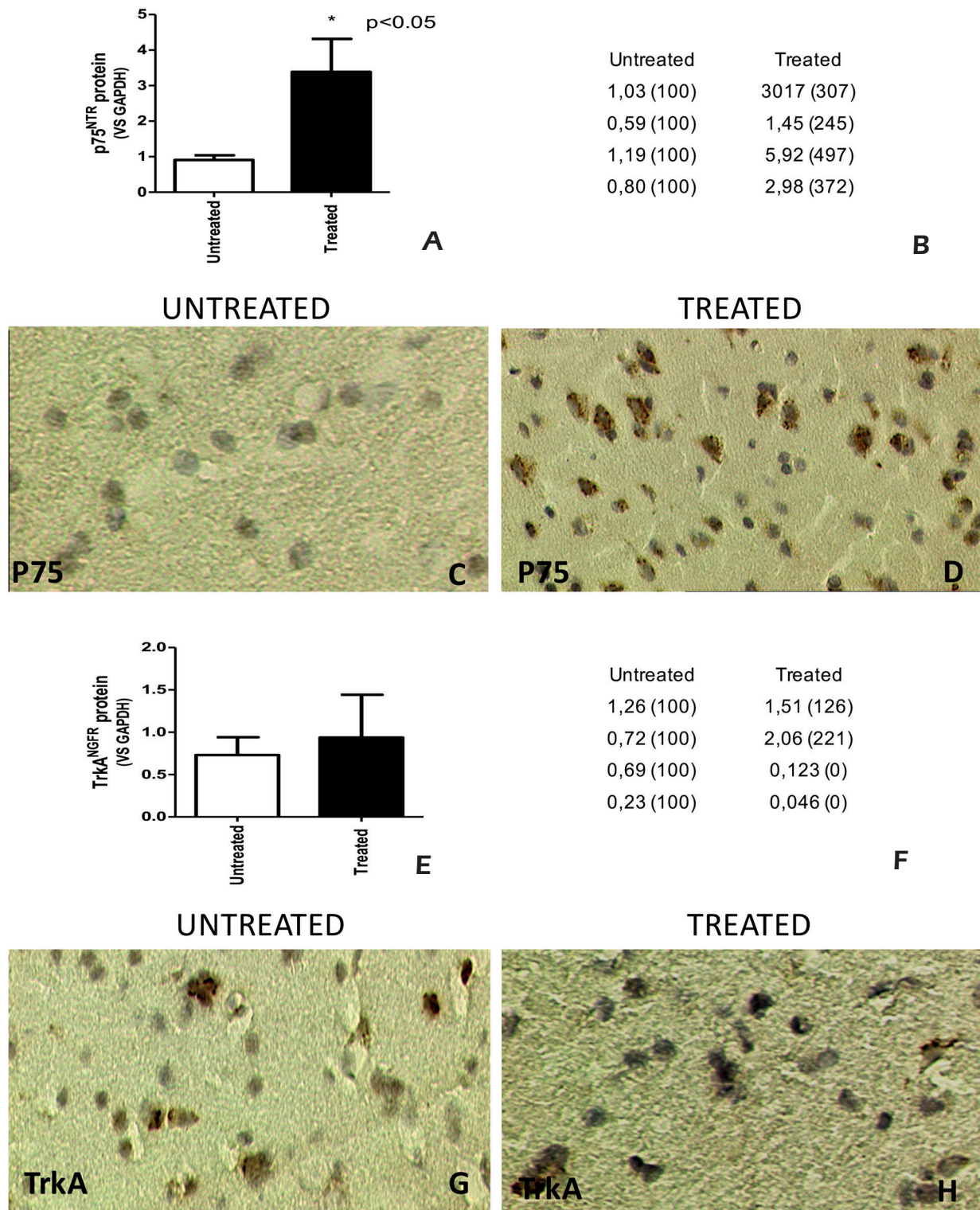


Figure 2. *C-D*, Western blotting determination showing the effect of nasal HPpSIS administration on the expression of low-affinity NGF-receptors p75 (*A,B*) and the immunostained brain tissues of untreated (*C*) and of nasal HPpSIS of low-affinity NGF receptor, p75 expression (*D*). *E-F*, Western blotting determination showing the effect of nasal HPpSIS administration on the expression of high-affinity NGF-receptors p75 (*E,F*) and the immunostained brain tissues of untreated (*G*) and of nasal treated HPpSIS brain tissues of high NGF-affinity NGF receptor, TrkA (*F*). Note that only the increase p75 resulted statistically significant ($p < 0.01$), while the increase of TrkA and is evident only in few single tissue samples.

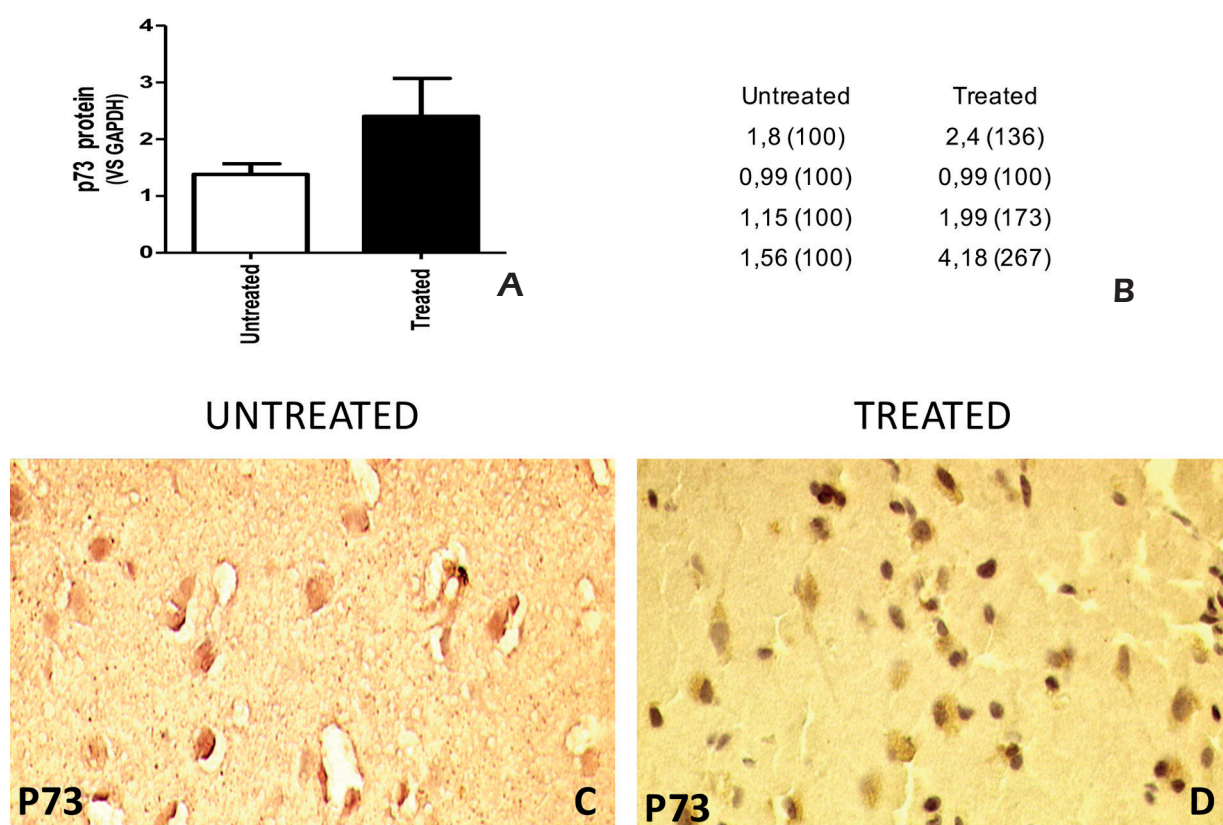


Figure 3. A-D. Western blotting determination showing the effect of nasal HPpSIS administration on the expression of p73 protein (**A,B**) and the immunostained brain tissues of untreated (**C**) and of nasal treated HPpSIS samples. Note the enhanced expression of the anti-tumor expression of p73 protein following nasal HPpSIS administration as compared to untreated samples. Magnification: x 125.

molecules by tumor cells suggests that NGF and p73 might be involved in promoting cancer cell differentiation rather than cancer proliferation. This hypothesis is suggested by a number of other previous and recent experimental indicating that exposure of NGF to cancer promotes cell differentiation, rather than proliferation, and that NGF by itself is unable to induce and/or to generate cancer cell proliferation in normal cells

within and outside the central nervous systems^{7,8} and that under normal conditions, physiological amounts of NGF is produced and secreted by several cells and tissues and released the human bloodstream¹².

The hypothesis of the anti-proliferative action of NGF on cancer is also suggested by evidence indicating this effect is regulated by MafK pathways as reported and that NGF and MafK are

Table I. Quantitative determination of p75, TrkA, and p73 immunostained peripheral tumor brain samples of Patients untreated and treated with nasal HPpSIS. The results reported in the table indicate that HPpSIS administration enhances significantly the number of immunostained brain cells, compared to the number of immunostained cells of untreated samples.

	I	I	III	IV	V	VI	VII	VIII
P75	20 ± 2	18 ± 3	19 ± 2	16 ± 2	43 ± 3	40 ± 3	35 ± 3	41 ± 3
TrkA	17 ± 3	14 ± 2	15 ± 2	15 ± 1	41 ± 3	38 ± 3	37 ± 3	40 ± 3
P73	24 ± 2	19 ± 2	16 ± 1	24 ± 2	38 ± 3	32 ± 3	42 ± 4	30 ± 3

Number of immunopositive cells in 40 square millimeters in four different areas of each tissue samples. Values are means of ± SEM. Control sample I, II, III, IV; HPpSIS treated V, VI, VII, VI.

both expressed in developing hippocampal neurons, both involved in the regulation of neuronal cell differentiation.

Despite the numerous studies regarding the effect of NGF on brain neurons under normal and degenerative events^{21,4}, not much is known about the role of NGF on brain tumor cells. Our present observations suggest that the concomitant presence of NGF and tumor repressor protein has a protective role in reducing cancer cells proliferation. As for the differences in the number of NGF-expressing receptors in peripheral cancer tissues samples, see table, it should be taken into due consideration that all brain cancer cells might not be a homogenous cancer cell population and/or not all be equally responsive to NGF. Certainly, a better understanding of the effect of HPpSIS and the action of NGF will depend on a careful optimization of the experimental approach and a larger number of individuals with a brain tumor. Even though not all brain cancer cells will stop to proliferate and start to differentiate, it might be possible that longer HPpSIS treatment and further release of NGF and p73 might produce better results might be worth of further investigation also in the tumor cells. The present experimental approach might offer a novel additional strategy, jointly with actual therapies, to reduce cancer cell proliferation and further studies are ongoing.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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