

Antiviral effect of high-pressure nasal stimulation

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Abstract. – OBJECTIVE: In a previous study, we reported an increase of nasal nerve growth factor (NGF) in patients treated with high-pressure administration of sterile saline isotonic solution (HPpSIS). Herein we characterized the nasal mucosa in terms of innate immune response and cytokine signature, including antiviral properties. Potential NGF and antiviral benefits of HPpSIS were also discussed.

PATIENTS AND METHODS: Twenty (20) patients (11 males, 9 females; age range 30-75 years old) underwent HPpSIS and nasal samples were collected before and after treatment. Nasal scraping was used for morphological (smears and Quick May-Grunwald Giemsa staining, MGG), biochemical (Histamine, Serotonin; ELISA) and molecular (messenger RNA, mRNA) analyses. Amplification of transcripts specific for Toll-like receptor (TLR) 3 (TLR3), TLR7, TLR9, Interleukin-(IL) 18 (IL18), IL13, IL12, eosinophil-derived neurotoxin (EDN), Eosinophil Cationic Protein (ECP), γ Interferon (γ IFN), tryptase and serotonin was performed using the 2-step real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR). Clinical and laboratory data were analyzed and compared.

RESULTS: The clinical evaluation showed a protective effect of our therapy. Smears showed the presence of leucocytes, eosinophils (EOs) and mast cells (MCs), and increased immunoreactivity for ECP/RNase3 and EDN after HPpSIS. ELISA showed increased levels of Serotonin and EDN associated with unchanged levels of substance P(SP) and histamine. Increased eosinophil-derived neurotoxin eosinophil-derived neurotoxin (EDN) levels were confirmed by *in situ* fluorescent analysis. HPpSIS induced the upregulation of TLR3, TLR7 and TLR9 transcripts, while no changes were observed for Intercellular Adhesion Molecule 1 (ICAM1), IL18, Interleukin-15 (IL15) and IL12 transcripts nor for Interleukin-6 (IL6) and IL13. No changes were also observed for γ IFN and EDN/RNase2 transcripts, while ECP/RNase3 transcripts were significantly upregulated after HPpSIS. Finally, tryptase transcripts were unchanged while serotonin transcripts were significantly increased after HPpSIS.

CONCLUSIONS: The clinical and biomolecular changes observed at the nasal mucosa due to HpSS treatment suggest the activation of an innate surveillance, by means of TLR transcription, and a possible anti-viral response due to EDN upregulation. It remains to be verified if NGF, known to be released locally upon HpSIS treatment, might in part be responsible for this local activation.

Key Words:

Nasal mucosa, Nasal scraping, Anti-viral effects, NGF, Neurotrophins, Eosinophils, Mast cells, Sterilized isotonic solution, Cationic proteins, EDN, ECP.

Introduction

The present study was inspired by our previously published study¹ and the necessity to understand the role and functional significance of nerve growth factor (NGF) in concert with two other molecules released in the nasal cavity: eosinophil-derived neurotoxin (EDN), and eosinophil chemotactic factor (ECF).

The mast cells (MCs)-derived ECF plays an important role in the recruitment of eosinophils (EOs) into the inflammatory regions².

NGF is a neurotrophin promoting and regulating the growth, maintenance, and survival of neurons in the peripheral and central nervous systems³. NGF signaling is mediated by two distinct receptors: the tyrosine kinase trkA receptor (trkA^{NGFR}) and the pan-neurotrophin receptor p75 (p75^{NTR}), a member of the tumor necrosis factor receptor superfamily^{3,4}. The biological activity of NGF depends on the trkA^{NGFR}/p75^{NTR} distribution ratio on the surface of NGF-target cells^{3,4}. Several authors⁵ highlighted the role of NGF in innate immune response, allergic inflammation, and healing of fibrotic processes. NGF and receptors have extended functions and a wide range

of activities at the epithelial level, including nasal and ocular mucosa⁶. As observed for olfactory and ocular mucosa, NGF is produced by different structural cell types, including stromal and epithelial cells and resident immune cells (macrophages, lymphocytes, antigen-presenting cells, EOs and MCs)⁷. EOs granules are released upon cytokine activation during immune defense and contain the EDN/RNase2 and the Eosinophil Cationic Protein (ECP/RNase3). These antimicrobial proteins belong to the ribonuclease A (RNaseA) superfamily and display antiviral activity *in vitro*⁸. In 1997, Domachowske et al described the ability of EDN/RNase2 to function as an effective Antiviral Agent against Respiratory Syncytial Virus (RSV)⁹.

Both EOs and MCs have been described as cells involved in homeostatic function, including developmental biology and innate and adaptive immunity, expanding the well-known role in disease processes, such as infections, allergy/asthma, tumors, as well as brain, ocular, and gut diseases⁸.

Organisms have defense mechanisms for protecting themselves from pathogens. In addition, higher vertebrates depend on both an “adaptive” immune system that targets pathogens with high specificity as well as a broad-action, nonspecific, “innate” immune response that includes the induction of the nonspecific latent ribonuclease (RNaseL). MicroRNAs (miRNAs) are known to regulate gene expression essential for proper development and cell physiology, although the function of the RNA interference (RNAi) pathway in animals remains unclear. These miRNAs are incorporated into microRNA-induced silencing complex (miRISC), which promotes RNA cleavage or translational inhibition upon recognition¹⁰.

RNAi is an evolutionarily conserved mechanism for sequence-specific gene silencing guided by double-stranded RNA¹¹. Among several multiple biological functions, RNases exert a crucial antiviral activity, as reported. Overall, the strategies exerted by RNases against viruses include: i. inhibition of viral replication by its enzymatic activity; ii. regulation of host immune recognition and response; iii. regulation of SGs formation; iv. induction of autophagy; and v. triggering of apoptosis. The EDN/RNase 2 is one of the best studied, which can fight RSV, Para Influenza Viruses (PIV), and human immune deficiency virus (HIV). EDN/RNase2 is essential for antiviral activity but is not unique; eosinophils also possess ECP/RNase3 with anti-RSV activity, although to a much lower extent¹². No synergistic action between EDN and ECP was proven¹².

Recently, we have demonstrated the antiviral activity against RSV of RNase3 expressed in macrophages. Besides, a comparative transcriptome analysis indicates that the protein’s antiviral properties are associated with its catalytic activity¹². The higher antiviral activity of EDN/RNase2 with respect to ECP/RNase3 has been attributed to the presence of a specific region in the former at the C-terminal loop¹².

Several Nasal and oral anti-viral sprays have been launched to allow protection against viruses. Most anti-viral sprays create a protective film on the mucous membrane of the upper respiratory tract to neutralize viruses and prevent multiplication¹³.

We recently reported that the high-pressure administration of sterile saline isotonic solution (HPpSIS) improved sensorineural hearing loss (SNHL) and reduced tinnitus symptoms⁷. This effect was associated with an enhanced release of NGF into the nasal cavity and increased number of NGF-producing cells. In a subsequent study, we reported that a long-lasting treatment for 10 consecutive days reproduced a similar effect in rodents, while enhancing the NGF levels and the expression of NGF-receptors in neurons of the olfactory bulbs and brain⁷.

Herein, we assessed whether HPpSIS could be associated with a change of a specific cellular distribution at the nasal epithelium and if antiviral products from EOs granules (ECP, ECN, EDN, cytokines and soluble mediators) could be related to this effect. Therefore, proinflammatory cytokines and specifically EDN/RNase2 and ECP/RNase3 were analyzed.

Patients and Methods

Reagents

All kits were purchased as specified in the text. Sterile RNase-free plasticware, molecular and analytical-grade reagents were from Starlab (Ahrensburg, Germany) and ICN (Costa Mesa, CA, USA), Sigma-Aldrich (Milan, Italy). Ultrapure RNase-free MilliQ-grade water was provided daily (Direct Q5 apparatus; Millipore, Vimodrone, Milan, Italy) for biochemical analyses and pretreated with Diethyl pyrocarbonate (DEPC) and autoclaved for molecular analyses.

Ethical Issues and Study Population

The Ethical Committee of the hospital (Department of Otolaryngology, Campus Bio-Medico University, School of Medicine, Rome, Italy)

approved the experimental study that was conducted in line with the principles of the Declaration of Helsinki. A written informed consent was obtained by patients adhering to the study. The study was performed with 20 patients (11 males, 9 females; age range 30-75 years old).

The following patients were excluded from the study: patients with nasal polyposis, chronic rhinosinusitis, ongoing pregnancy, smokers, and nosebleeds, patients who already underwent nasal surgery, patients with marked septal deviation, patients immunocompromised, patients with bronchial asthma or chronic obstructive pulmonary disease (COPD), patients who had used antibiotics in the previous 30 days, patients that chronically use immunosuppressive corticosteroid were excluded from this study. All patients underwent medical history collection, ear, nose, and throat examination, pure tone audiometry, and tympanometry. The endoscopic nasal examination was carried out with 0.4 mm rigid endoscope before nasal scraping.

Treatments and Nasal Scraping

Nasal scrapings were performed pre/post HPpSIS therapy by using a nasal swab. Swabs' material was directly smeared onto glass-slide (BDH) providing 3 glass slides per patient. Smears were quickly cytofixed and preserved at 4°C, according to the standard procedure (Bio-fix spray; Bio-Optica, Milan, Italy). The residual material was extracted by rinsing the swab in lysis buffer containing protease inhibitors (50 mM tris-HCl, pH 7.5; 150 mM NaCl; 5 mM Ethylenediamine tetra-acetic acid, EDTA; 1% Triton X-100; 0.1% SDS; 1 mM phenylmethylsulfonyl fluoride, PMSF, and 1x protease inhibitors; Pierce Biotechnology; ThermoFisher Scientific, Waltham, MA, USA). Extracted materials were quickly frozen at -20°C.

Basal Staining and Light Microscopy Acquisitions

Slides were rehydrated and stained according to the quick-differential staining May-Grunwald Giemsa (MGG) procedure or alternatively with Toluidine blue stain for identifying mast cell metachromasia, according to standard procedures¹⁴. Images were evaluated at light transmission by using a direct light microscope (E400 Eclipse; Nikon, Tokyo, Japan) connected to a digital camera (Axiocam 208 color; Carl Zeiss, Jena, Germany). Digital images were provided using the NiS acquisition software (Nikon).

Observations and digital acquisitions were performed on the same day of staining to avoid artifacts due to long-term storage.

Immunofluorescence and Epifluorescent Acquisitions (EDN and ECP)

Smears selected for immunofluorescent analysis were postfixed in cold-buffered 0.05% formaldehyde. After a brief equilibration in Phosphate Buffered Saline (PBS: 20mM PB and 0.9 % saline; pH 7.5) the slides were subjected to the following quick steps: i. antigen retrieval (1 min: 0.05% trypsin-EDTA solution); ii. quenching (5 min; Avidin/Biotin Blocking kit, SP-2001; Vector Laboratories, Burlingame, CA, USA) and iii. blocking/permeabilization (2 min: 1% Bovine Serum Albumin, BSA; 5 min: 0.5% Triton X-100 in PBS) to reduce background and allow better immunofluorescent staining. The slides were probed with monoclonal antibodies specific for EDN (ab103428; anti-EDN3 antibody, ab197374; Abcam, Waltham, MA, USA) and ECP (ab207429, Recombinant Anti-Ribonuclease 3/ECP antibody, EPR20357, Abcam, Waltham, MA, USA). Specific labeling of first antibodies was made using the cyanine2 (Cy2)/green and cyanine5 (Cy5)/blue conjugated species-specific antibodies (1:150-1:300; donkey; Jackson ImmunoResearch, Europe Ltd, Suffolk, UK). The nuclear counterstaining was performed with propidium iodide (PI; Molecular Probes, Eugene, OR, USA). Negative controls (omission of primary antibodies) were used for background calibration. Epifluorescence acquisitions were also carried out with a direct Ni-E200 Eclipse direct microscope equipped with UV lamp, digital camera (Axiocam 208 color) with the following objectives: $\times 10$ /dry 0.75 dic m/n2, $\times 20$ /0.45NA, $\times 40$ /0.60 N and finally $\times 60$ /1.4 oil/immersion (Nikon, Tokyo, Japan). Images were acquired using the ZEN 3.1 (free available blue edition) acquisition software (Carl Zeiss, Jena, Germany). Representative digital images (single acquisitions) were selected and provided as 8-tiff format to be assembled by Adobe Photoshop 7.0 software (Abacus Concepts, Irvine, CA, USA). No changes were carried during panel assembling with respect to original acquisitions. To provide quantitative results, positive cells were quantified in selected cell areas (n=3 per smear/sample). ImageJ software (free, open-source software ImageJ, Fiji distribution) was used for fluorescent Integrated Density (IntDen) quantifications of stained areas (1000x1000 square).

Histamine and Serotonin Quantifications (ELISA)

Protein extracts from nasal scraping swabs were centrifuged at 15000 rpm/15 min to remove debris and clear supernatants were 1:2 diluted with loading buffer for ELISA provided by the kits: Histamine competitive assay kit (ab213975; sensitivity: 30 pg/ml; range 98 - 2500 pg/ml; Abcam, Waltham, MA, USA) and Serotonin ELISA kits (ADI-900-175; Enzo Life Sciences; Ann Arbor, MI, USA and ab133053, Abcam, Waltham, MA, USA; kit IBL). Samples and standards were loaded on 96-well pre-coated plates and the entire procedure of analysis was carried out according to the manufacturer's instructions with minor modifications. Standard ranges were 0.49-500 ng/ml (0.3 ng/ml detection limit) for Histamine and 0.49-500 ng/ml (0.293 ng/ml detection limit) for Serotonin. Absorbance (OD) values were recorded after plate reading at λ 450 nm (corrected to λ 570 nm) in a plate reader platform (Sunrise; Tecan Group Ltd., Männedorf, Switzerland). Normalization for total protein was carried out before assay using the nanodrop spectrophotometer (Nanodrop). The averaged coefficients of variation (CV) were respectively <10% (intra-assay) and <12% (inter-assay).

Cytokine and Soluble Mediator Transcripts Evaluation by Real-Time RT-PCR

Transcription analysis was carried out using the two-step relative real-time PCR performed in an EcoIllumina platform (Illumina Inc., San Diego, CA, USA). Expression profiles were set for human targets Toll-like receptor 3 (TLR3), Toll-like receptor 7 (TLR7), Toll-like receptor 9 (TLR9), Intercellular Adhesion Molecule 1 (ICAM1), Interleukin-18 (IL18), γ Interferon (γ IFN), Interleukin-5 (IL5), Interleukin-6 (IL6), Interleukin-13 (IL13), Interleukin-12 (IL12), EDN, ECP, tryptase and serotonin (**Supplementary Table I**). All primers were designed from the mRNA complete sequences downloaded from NCBI nucleotide and primer3 open source software, using specific parameters (T annealing range 58-65°C; GC% range between 45-55%; primer size 19-21mer). Briefly, complementary DNAs (cDNAs) were produced by using the Reverse Transcription Kit in the presence of random primers and dNTPs (GoScript™ Reverse Transcriptase kit; Promega Italia, Milan, Italy), and the specific amplifications were carried out using the SYBR green master mix (Applied Biosystems, Foster City, CA, USA). Relative transcript

expressions were provided according to the Δ Cq and $\Delta\Delta$ Cq analysis (REST analysis and R-studio script). Referring genes were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal subunit, working at the specific annealing temperatures. Negative/positive controls and single-mode melting curves were used to confirm the specificity of the amplification.

Statistical Analysis

All results are presented as mean \pm standard error of mean (SEM). Statistical significance was assessed with paired samples *t*-test using the R commands (R-studio package). Correlations were assessed by using the Pearson or Spearman correlation of R package, depending on normality check results (Kolmogorov-Smirnov test and the Shapiro-Wilk test). Panels were assembled with Prism5 (GraphPad Software, San Diego, CA, USA).

Results

The results show that forced nasal stress induced by local delivery of HPpSIS can cause an increase in the number of mast cells and the level of EDN/RNase2 and ECP/RNase3 transcripts and their related proteins in the nasal mucosa (scraping) compared to untreated control subjects.

Clinical Evaluation

HPpSIS induced a specific infiltrate in nasal scraping

A representative nasal scraping from control subject is reported in Figure 1A. A recruitment of granulocytes was observed in nasal scraping before (Figure 1B) and after HPpSIS treatment (Figure 1C). In Figure 1C, some granulocytes resemble neutrophils and eosinophils are indicated by red circle. Nasal epithelial cells are visible, and the PAS immunoreactivity was not significantly increased.

HPpSIS induced a specific immunoreactivity for EDN, ECP and ECF in nasal scrapings

Immunoreactivity for EDN and ECP was observed in nasal scrapings before and after HPpSIS treatment (respectively left to right panels: EDN Figure 2A and ECF Figure 2B). The epifluorescent acquisition of nasal scrapings immunoprobed simultaneously for EDN (blue) and ECF (green) showed immunoreactivities after HPpSIS treatment (Figure 2C). Specific

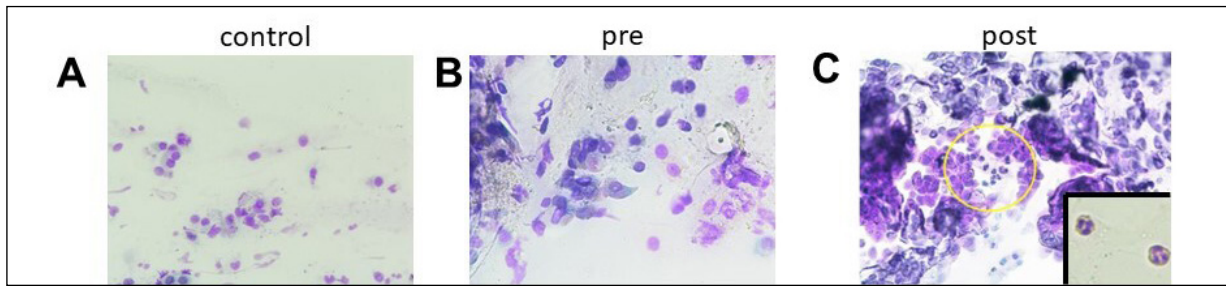


Figure 1. Cytostaining of nasal smears. Microscopical Evaluation of Nasal Cytosmears showing a cellular pattern characterized by neutrophils and monocytes, with rare goblet cells and mucus filaments. **A**, control; **B**, pre HPPpSIS; **C**, post HPPpSIS. MGG staining. In **C**, note the presence of immune cells as framed by red circle. HPPpSIS, high-pressure administration of sterile saline isotonic solution; Magnification x-200.

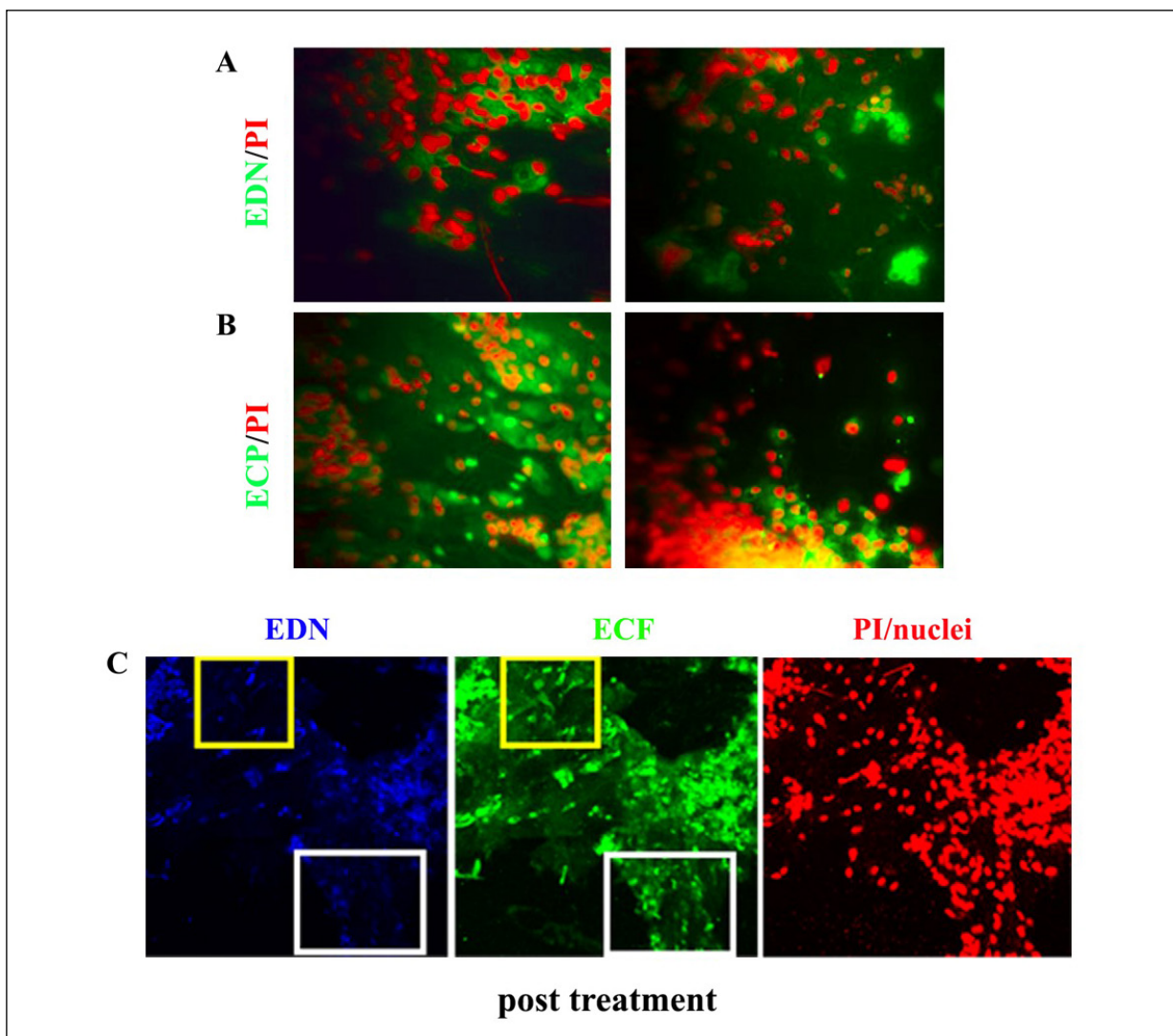


Figure 2. Immunofluorescence of EDN/RNase3 and ECP/RNase2. Epifluorescent acquisitions of Nasal Cytosmears showing a cellular pattern characterized by neutrophils and monocytes, with rare goblet cells and mucus filaments. Panels, with images from pre HPPpSIS and post HPPpSIS cytosmears: panel **A**, EDN immunoreactive cells (green); **B**, ECP immunoreactive cells (green) over red nuclei (Propidium Iodide). In panel **C**, note the distribution of EDN and ECF immunoreactivity (left, EDN/blue; middle, ECF/green; right, nuclei/red) in post HPPpSIS cytosmears. HPPpSIS, high-pressure administration of sterile saline isotonic solution; Magnification x-400.

areas with EDN and ECF co-expression were not observed (Figure 2C).

HPpSIS increased the release of Serotonin and EDN proteins in nasal scraping

High serotonin (5HT) content was quantified in nasal scraping after HPpSIS, as compared to pretreatment (untreated samples) (Figure 3A). EDN and ECP transcript expression was not significantly affected (Figure 3B-C). While SP-mRNA was not affected by treatment (Figure 3D), the molecular analysis showed a trend to a decrease for Tryptase (Figure 3E) and a significant increase in VIP-mRNA expression after HPpSIS (Figure 3F).

HPpSIS treatment was associated with a specific cytokine transcription

The analysis of chemokines and cytokines showed no significant changes for ICAM1, IL6 and IL5 (Figure 4A-C). No changes were observed for IL18, IL13 and IL12 (Figure 4D-F). Transcript expression was also found increased for γ IFN and ECP, while no changes were observed for EDN after nasal stimulation (Figure

5A-C). On the other side, a trend to an increase of transcripts' expression was detected for TLR3 (Figure 5D), TLR7 (Figure 5E) and TLR9 (Figure 5F) after HPpSIS.

Discussion

Our findings suggest that the long-lasting nasal spray forced stimulation (HPpSIS) treatment can induce the upregulation of innate surveillance at the nasal mucosa, by means of a trend to an increase of TLR transcription associated with an anti-viral pathway expression (EDN/RNase3 and γ IFN upregulation). Serotonin (5HT) increase was confirmed, while SP, as well as tryptase, was not involved in this HPpSIS treatment. It remains to verify if NGF, known to be released locally after HPpSIS treatment, might be responsible, at least in part, for this specific local activation.

In previous studies, we reported that this nasal forced stress with isotonic solution can cause an increase of NGF and serotonin into the nasal cavity in humans and that the autologous NGF stimulation might be associated with some innate

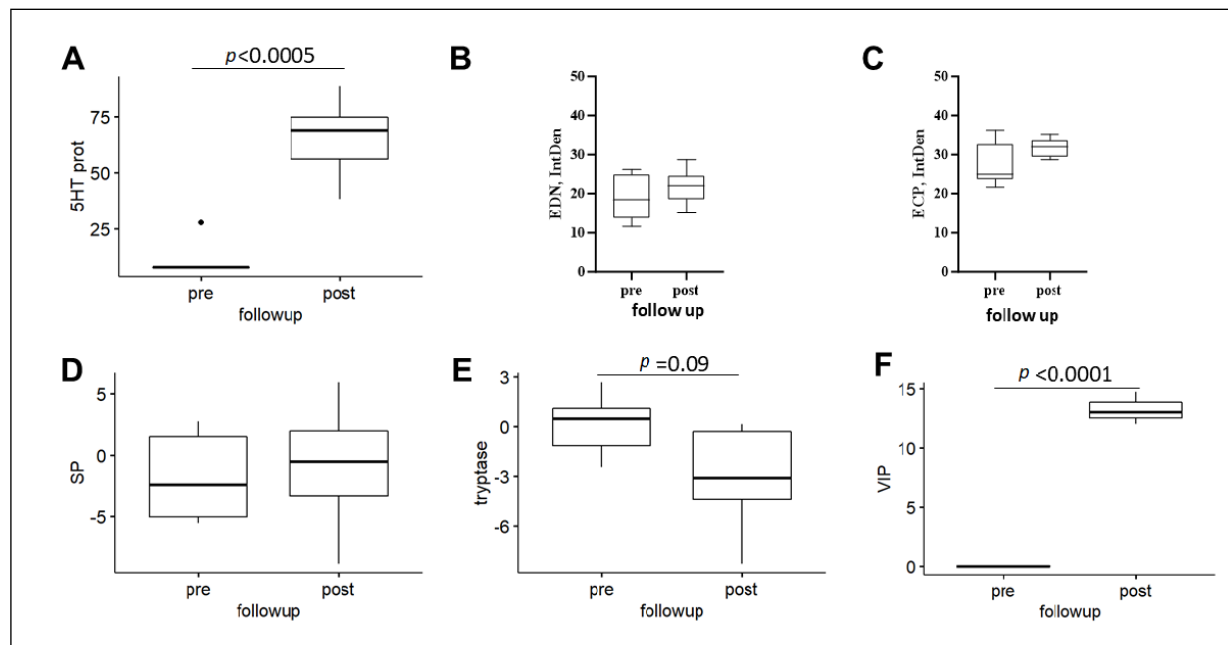


Figure 3. 5HT, EDN/ECP, SP, Tryptase and VIP expression. Biomolecular analysis carried out in parallel on total protein (5HT, EDN/ECP) and total RNA (SP, Tryptase, VIP) samples from pre and post HPpSIS treatment. Comparisons were carried out vs. control nasal scrapings. Note the increased expression of 5HT protein (A) $p < 0.0005$ and unchanged EDN/RNase2 and ECP/RNase3 immunoreactivities after HPpSIS (B, C) $p < 0.08$). The transcripts' analysis showed a significant increase of VIPmRNA (F) $p < 0.0005$ and a trend to a decrease for Tryptase mRNA (E) $p = 0.09$. SP-mRNA was not affected by treatment (D). REST analysis on Cq values produced by amplification carried out (Illumina platform). Pre HPpSIS and Post HPpSIS target expression were calculated with respect to controls, herein referred as 1, according to a standard procedure. Data are 2log expression and bars show standard deviation.

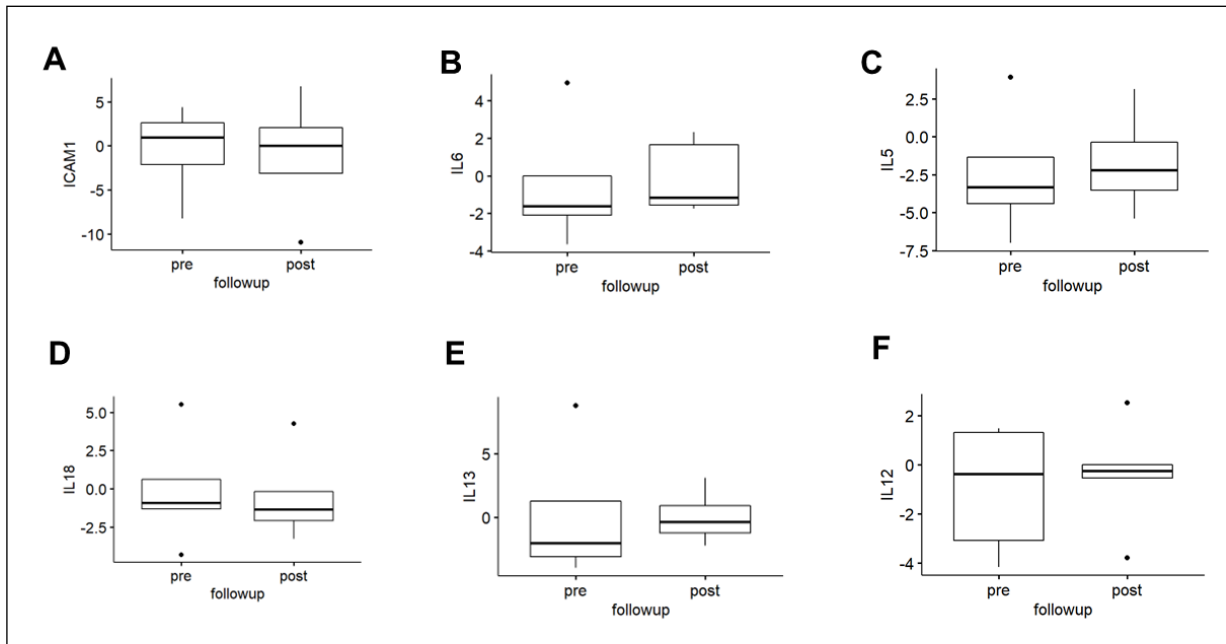


Figure 4. A, ICAM1, B, IL6, C, IL5, D, IL18, E, IL13 and F, IL12 transcripts' expression. Molecular analysis carried out in parallel on total RNA samples extracted from pre and post HPPSIS, as compared to control expression. REST analysis on Cq values produced by amplification (Illumina). Control is referred to 1, according to a standard procedure. Data are 2log expression. Bars are provided with standard deviation.

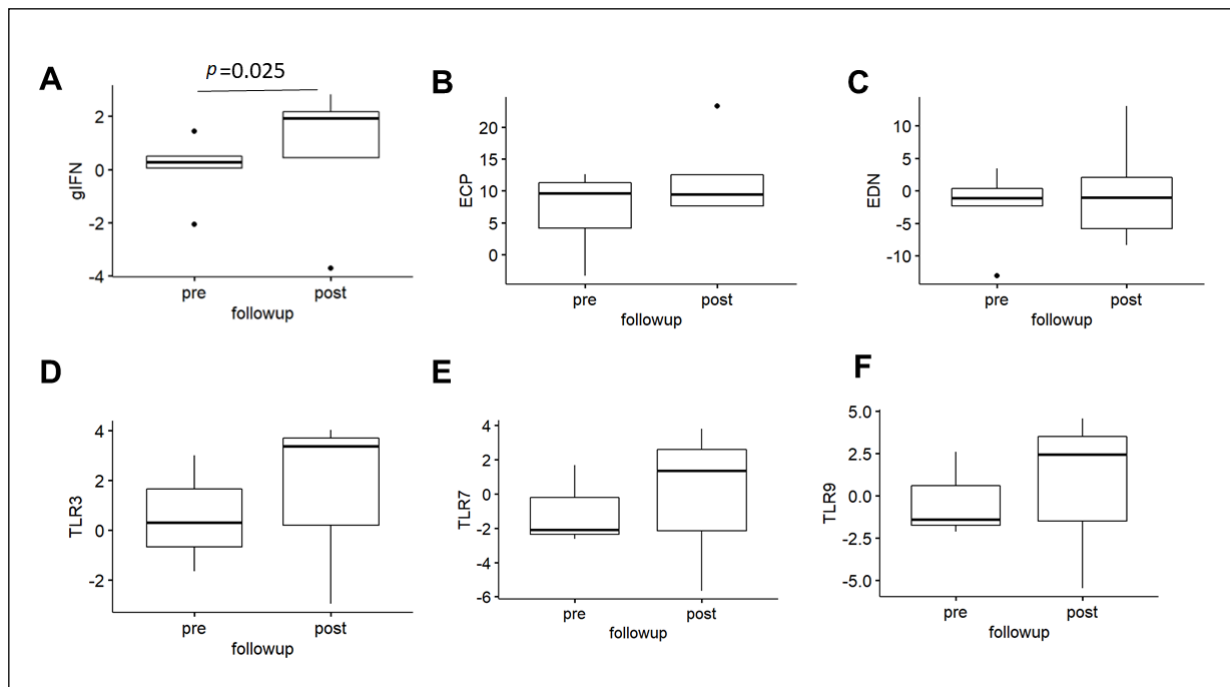


Figure 5. γ IFN, ECP, EDN, TLR3, TLR7 and TLR9 transcripts' expression. Molecular analysis carried out in parallel on total RNA samples extracted from pre and post HPPSIS, as compared to control expression. Note the increased expression of γ IFN IFN, ECP (A,B) and a trend to an increase was detected for all TLR at follow-up (post HPPSIS) (D, E, F). No change was observed for EDN (C). REST analysis on Cq values produced by amplification carried out in Illumina platform. Control is referred to 1, according to a standard procedure. Data are 2log expression. Bars are provided with standard deviation.

immune changes at mucosal levels¹. Since we observed a reduction in the respiratory symptoms of our patients, we decided to conduct a study to prove the presence of antiviral molecules in the nasal cavity⁷. This hypothesis is supported by observations that forced nasal administration of HPpSIS in voluntary individuals, stimulated the presence of immune cells into the nasal cavity, and enhanced the expression/release of NGF and NGF-receptor locally, leading to the improvement of SNHL and reducing the tinnitus symptoms¹. Since we previously reported an increased NGF expression in nasal scraping from patients treated with HPpSIS in concomitance with a reduction in episodes of influenza, we started to think about the anti-inflammatory role of NGF throughout the modulation of local innate response¹⁵.

Therefore, we characterized the i. nasal scrapings at the cytological level; ii. the expression of some neuromediators (SP, VIP, Serotonin) and verified iii. the expression of innate immune response (chemokines, cytokines, ECP/RNase3 and EDN/RNase2) and finally iv. we assessed the eventual release of tryptase and other toxic related products.

In our nasal smears, the presence of neutrophils and some EOs was observed upon HPpSIS. This expression was also associated with an increased immunoreactivity for EDN and ECP, and biochemical analysis corroborated quantitatively the EDN expression. Of interest, no specific upregulation of SP was observed, while an increased expression of Serotonin was quantified. As known, the healthy nasal mucosa is composed of numerous ciliated cells that characterize the pseudo-stratified epithelium and few neutrophils¹⁶. Long-lasting exposure to the allergen is often associated with a minimal “flogosis”, characterized by a persistent infiltration of neutrophils and few eosinophils (EOs)¹². The degranulation with the release of preformed mediators is often associated with tissue degradation in a vicious route that is characterized by the recall of EOs and MCs in areas of interest. EOs are innate immune cells infiltrating the mucous membranes, as demonstrated in the inflammatory (antibacterial/antiviral), allergic response and in the case of tumors. A crosstalk between EOs and MCs modulates the inflammatory response and drives tissue repair and homeostasis if required¹⁷. This crosstalk highlights the role of MCs as “gatekeepers” in mucosal tissue homeostasis¹⁶.

In 1986, Gleich et al¹⁸ confirmed the ribonuclease activity of EDN/RNase2 and ECP/

RNase3. Moreover, extracellular EDN is used as an indicator of eosinophil activation and degranulation *in vitro*¹⁹.

Herein, we used the molecular approach to verify the transcription of the indicators of inflammation²⁰⁻²¹⁻²², fibrosis and innate response upon treatment. Increased EDN levels were corroborated by *in situ* fluorescent analysis. HPpSIS induced the upregulation of TLR3, TLR7 and TLR9 transcripts, while no changes were observed for ICAM1, IL18, IL15 and IL12 transcripts nor for IL6 and IL13. This is an interesting finding as it would suggest that HPpSIS might contribute to increasing the innate immune surveillance. No changes were also observed for γ IFN and EDN/RNase2 transcripts, while ECP/RNase3 transcripts were significantly upregulated after HPpSIS. Finally, tryptase transcripts were unchanged, while serotonin transcripts were significantly increased after HPpSIS²³.

The searching of new specific therapeutic targets for antiviral defense represents an emerging field and several peptides have been prospected as promising antiviral drugs because of their high efficacy and low toxic side effects²⁴. In this context, VIP represents a prospective antiviral peptide as described in HIV, vesicular stomatitis virus (VSV), RSV, Zika virus (ZIKV) and cytomegalovirus (CMV) and even Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infections²³. Since viral attacks can also trigger severe complications due to inflammatory and immune responses hyper-activation, the potent anti-inflammatory and immunoregulatory properties of VIP might avoid overactive immune signaling and tissue degradation. VIP signatures HPpSIS-induced might provide a background for “modulating” the immune response upon viral attack and for protecting the mucosa²⁵.

Limitations

Some limitations can be identified from this biomolecular study: i. the small study population; ii. the unique time point for detections (pre/post-treatment).

Conclusions

To summarize, HPpSIS treatment triggered the expression of ECP/RNase3 and EDN/RNase2, as confirmed by immunofluorescent and ELISA

analyses. EOs and MCs are known to release several preformed mediators involved in type-I response, some enzymes (histaminase, arylsulfatase) and their granules contain several cytotoxic proteins, including EDN, ECF and myelin basic protein (MBP)¹⁸. EDN/RNase2 and ECP/RNase3 are released from eosinophil granules when cells are activated by cytokines and other proinflammatory mediators. EOs' activation leads to the release of various cytotoxic proteins, some with antiviral properties, including EDN, ECF, and MBP. It has been reported that local cell activation (structural and immune cells) can lead to granule protein release in the extracellular microenvironment. *In vitro* studies described that the extracellular EDN quantification was a useful tool to evaluate the eosinophil activation and degranulation, as observed in tryptase and histamine release by mast cells^{9-12,16}. Notably, EOs and MCs activations are also associated with the release of various growth factors, including NGF.

Taken together, the present experimental approach might offer a novel additional strategy, jointly with actual therapies, to reduce viral infection and further studies are ongoing.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Approval

The Ethical Committee of Campus Bio-Medico University approved the study that was conducted in line with the principles of the Helsinki Declaration (63/18 OSS).

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Authors' Contributions

All authors contributed to the study conception and design. The first conceptualization was performed by Fabrizio Salvinelli and Fabio Greco. Material preparation, data collection and analysis were performed by Bijorn Balzamino, Alessandra Micera and Valeria Frari. The first draft of the manuscript was written by Francesca Bonifacio, Giulia

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References

- 1) Salvinelli F, Frari V, Rocco ML, d'Eramo A, Salvinelli E, Salvinelli B, Aloe L. High-pressure physiological saline isotonic solution administration enhances brain NGF and NGF-receptors expression. *Eur Rev Med Pharmacol Sci* 2015; 19: 3822-3832.
- 2) Ishimitsu T, Torisu M. The role of eosinophil chemotactic factor in localized tissue eosinophilia by parasitic infection. *Nihon Rinsho Jpn J Clin Med* 1993; 51: 675-680.
- 3) Micera A, Lambiase A, Stampachiacchiere B, Bonini S, Bonini S, Levi-Schaffer F. Nerve growth factor and tissue repair remodeling: trkA(NGFR) and p75(NTR), two receptors one fate. *Cytokine Growth Factor Rev* 2007; 18: 245-256.
- 4) Aloe L, Rocco ML, Stigliano E, Angelini F, Iacangelo M, Frari V, Salvinelli F. Nasal HPpSIS administration enhances NGF and tumor suppressor protein, p73 in human brain cancer tissues: preliminary data. *Eur Rev Med Pharmacol Sci* 2017; 21: 618-626.
- 5) Lambiase A, Micera A, Sgrulletta R, Bonini S, Bonini S. Nerve growth factor and the immune system: old and new concepts in the cross-talk between immune and resident cells during pathophysiological conditions. *Curr Opin Allergy Clin Immunol* 2004; 4: 425-430.
- 6) Micera A, Jirsova K, Esposito G, Balzamino BO, Di Zazzo A, Bonini S. Mast Cells Populate the Corneoscleral Limbus: New Insights for Our Understanding of Limbal Microenvironment. *Invest Ophthalmol Vis Sci* 2020; 61: 43.
- 7) Salvinelli F, Frari V, Capuano F, Salvinelli B, Rocco ML, Aloe L. Endogenous nerve growth factor stimulation: effects on auditory pathway neural cells in a mouse model. *Eur Rev Med Pharmacol Sci* 2018; 22: 7013-7019.
- 8) Yang D, Rosenberg HF, Chen Q, Dyer KD, Kurosaka K, Oppenheim JJ. Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells. *Blood* 2003; 102: 3396-3403.
- 9) Domachowske JB, Dyer KD, Bonville CA, Rosenberg HF. Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective an-

- tiviral agent against respiratory syncytial virus. *J Infect Dis* 1998; 177: 1458-1464.
- 10) Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. *Nature* 2004; 431: 343-349.
 - 11) Ungheri D, Verini MA, Ganceff L, Mazue G, Carminati P. Antiviral activity of nerve growth factor in vitro. *Drugs Exp Clin Res* 1993; 19: 151-157.
 - 12) Ying S, Meng Q, Zeibecoglou K, Robinson DS, Macfarlane A, Humbert M, Kay AB. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. *J Immunol Baltim Md* 1950 1999; 163: 6321-6329.
 - 13) Lin HR, Ganem D. Viral microRNA target allows insight into the role of translation in governing microRNA target accessibility. *Proc Natl Acad Sci U S A* 2011; 108: 5148-5153.
 - 14) Atiakshin D, Samoilova V, Buchwalow I, Boecker W, Tiemann M. Characterization of mast cell populations using different methods for their identification. *Histochem Cell Biol* 2017; 147: 683-694.
 - 15) Nagineni CN, William A, Cherukuri A, Samuel W, Hooks JJ, Detrick B. Inflammatory cytokines regulate secretion of VEGF and chemokines by human conjunctival fibroblasts: Role in dysfunctional tear syndrome. *Cytokine* 2016; 78: 16-19.
 - 16) Garaci E, Caroleo MC, Aloe L, Aquaro S, Piacentini M, Costa N, Amendola A, Micera A, Calìò R, Perno CF, Levi-Montalcini R. Nerve growth factor is an autocrine factor essential for the survival of macrophages infected with HIV. *Proc Natl Acad Sci U S A* 1999; 96: 14013-14018.
 - 17) Kabata H, Artis D. Neuro-immune crosstalk and allergic inflammation. *J Clin Invest* 2019; 129: 1475-1482.
 - 18) Levi-Schaffer F, Micera A, Zamir E, Mechoulam H, Puxeddu I, Piliponsky AM, Aloe L, Pe'er J. Nerve growth factor and eosinophils in inflamed juvenile conjunctival nevus. *Invest Ophthalmol Vis Sci* 2002; 43: 1850-1856.
 - 19) Choi Y, Le Pham D, Lee DH, Lee SH, Kim SH, Park HS. Biological function of eosinophil extracellular traps in patients with severe eosinophilic asthma. *Exp Mol Med* 2018; 50: 1-8.
 - 20) Crick F. Central Dogma of Molecular Biology. *Nature* 1970; 227: 561-563.
 - 21) Massingale ML, Li X, Vallabhajosyula M, Chen D, Wei Y, Asbell PA. Analysis of inflammatory cytokines in the tears of dry eye patients. *Cornea* 2009; 28: 1023-1027.
 - 22) Usai C, Gibbons JM, Pade C, Li W, Jacobs SRM, McKnight A, Kennedy PTF, Gill US. The β -NGF/TrkA Signalling Pathway Is Associated With the Production of Anti-Nucleoprotein IgG in Convalescent COVID-19. *Front Immunol* 2022; 12.
 - 23) Salvinelli F, Frari V, Bonifacio F, Cavicchioni G, Greco F, Micera A. Enhanced presence of sero to in nasal cavity after autologous stimulation. *Eur Rev Med Pharmacol Sci* 2022; 26: 2970-2974..
 - 24) Gröger M, Bernt A, Wolf M, Mack B, Pfrogner E, Becker S, Kramer MF. Eosinophils and mast cells: a comparison of nasal mucosa histology and cytology to markers in nasal discharge in patients with chronic sino-nasal diseases. *Eur Arch Otorhinolaryngol* 2013; 270: 2667-2676.
 - 25) Zhu Y. SARS-CoV-2: phylogenetic status, mutations and therapeutic research based on spike protein. *Eur Rev Med Pharmacol Sci* 2021; 25: 5843-5852.