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# Endogenous nerve growth factor stimulation: effects on auditory pathway neural cells in a mouse model

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**Abstract. – OBJECTIVE: In the present study, we investigated whether high-pressure hypotonic saline solution (Hphss) affects the basal level of Nerve Growth Factor (NGF) and expression of receptors in the cochlea, bark earing, retina, and visual cortex.**

**MATERIALS AND METHODS: For this study, we used three weeks old female Sprague Dawley (SD) rats (n = 12). Rats were housed in polypropylene cages and were kept under standard conditions (12 h light:12 h dark cycle) with free access to water and food (Purina chow food). A specific dispenser was employed to deliver sterile hypotonic saline at high pressure (pressing emission level (PEL): 7 g/s; emission time (ET): 0.5 s). Rats were divided into two groups: untreated (n = 6) and treated with Hphss (n = 6), three times per day, for 10 consecutive days. Treatment was performed in both nostrils with 50 μl of Hphss using a microsyringe equipped with a plastic tip.**

**RESULTS: We observed a significant enhancement in the level of NGF in the cochlea and bark earing, but not in the retina and visual cortex. This is likely because the nasolacrimal duct pathway does not appear to have an effect on the retina, and the visual cortex appears to be too far from the cribriform plate to be reached by nasal NGF.**

**CONCLUSIONS: This treatment can significantly protect and/or delay degeneration of cochlear auditory NGF-target cells. It is free from side effects and can be used in chronic diseases for as long as needed. It remains to be investigated whether the effects of short-term therapy are long-lasting, or if the treatment must be repeated.**

*Key Words:*
Nerve growth factor (NGF), High-pressure hypotonic saline solution (Hphss), Cochlea, bark earing, Nasal cavity (NC), Olfactory bulb, Brain.

## Introduction

Since its discovery in late 1950, several studies have shown that nerve growth factor (NGF) is produced by a number of neuronal and non-neuronal cells[1]. It acts by promoting outgrowth of sensory and sympathetic neurites[2,3], and by protecting degenerating forebrain cholinergic neurons[4,5]. Moreover, during the last two decades, basal and clinical observations have revealed that NGF can exert a protective action against degeneration of human neuron cells in diabetes[6], pressure ulcer[7], Alzheimer's disease (AD)[8,9], corneal ulcer and retinal cell degeneration[10,11]. These effects are mediated by two distinct receptors, trka$^{NGFR}$ and p75$^{NTR}$. The biological activity of NGF depends on the ratio between the two molecules expressed on the surface of NGF target cells[12-14]. NGF is involved in growth guidance of nerve fibres, including auditory nerve fibres, toward their target tissues[15-21].

In a previous study, we observed that sensorineural hearing loss in humans is related to low levels of serum NGF[22]. We subsequently demonstrated that autologous stimulation, induced by nasal forced stress with an isotonic solution, causes an increase in NGF in the nasal cavity of humans, improving the hearing of patients with sensory neural hearing loss (SNHL) and tinnitus[23]. We, then, demonstrated that nasal administration of high-pressure hypotonic saline solution (Hphss) stimulates the production and release of NGF from cells in the nasal cavity of laboratory mice and it reaches the forebrain[24]. We have also shown that Hphss administration, through olfactory pathways, also induces the release of NGF and up-regulation of NGF recep-

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tors in patients affected by brain tumours, while reducing the number of brain tumour cells by stimulating the release of the anti-tumor protein p73[25]. This is consistent with the hypothesis that enhanced NGF levels, through the up-regulation of p73 protein[26-28], can reduce the number of brain tumour cell proliferation, as suggested by recent findings[29-32]. Overall, the novel findings obtained in our recent studies clearly indicate that nasal Hphss administration can enhance nasal and brain NGF release, affecting cells of the nasal and auditory pathways. However, we have not yet investigated whether Hphss administration alters the basal level of NGF and the expression of NGF-receptors in the cochlea and bark earing, both of which are directly involved in hearing physiopathology.

The aim of the present study is to address if endogenous stimulation of NGF by Hphss administration could regulate levels of NGF and expression of NGF receptors in cochlea, bark earing, retina, and visual cortex.

## Materials and Methods

### Drugs and Devices

A specific dispenser was employed to deliver sterile hypotonic saline at high pressure (pressing emission level (PEL): 7 g/s; emission time (ET): 0.5 s).

### Chemicals

The primary antibodies anti-trka$^{NGFR}$ (sc-118; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p75$^{NTR}$ (sc-58567; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (sc-365062; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

### Animals

For this study, we used three weeks old female Sprague Dawley (SD) rats (n = 12). Rats were housed in polypropylene cages and were kept under standard conditions (12 h light:12 h dark cycle) with free access to water and food (Purina chow food). Housing, care, and experimental animal procedures were in accordance with the guidelines indicated and approved by the Italian National Research Council (CNR). This research conformed to the Intramural Committee and Institutional Guidelines and was performed in accordance with national and international laws (EEC council directive 86/609, OJ L 358, 1, December 12, 1987). All efforts were made to reduce the number of animals and to minimize animal suffering. Rats were divided into two groups: untreated (n = 6) and treated with Hphss (n = 6), three times per day, for 10 consecutive days. Treatment was performed in both nostrils with 50 ul of Hphss using a microsyringe equipped with a plastic tip.

### Tissue Dissection

At the end of the treatments, rats were euthanized with an overdose of carbon dioxide vapor, and the cochlea, bark earing, retina, and visual cortex were dissected out. The presence of NGF and NGF-receptors was measured and used for biochemical and molecular analyses.

### NGF Assay

To determine the level of NGF, tissue samples were homogenized by ultra-sonication 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% sodium deoxycholate; 1 mM PMSF; 1 µg/ml leupeptin), centrifuged at 4°C for 20 min at 13,000 rpm, and then the supernatant was stored at -20°C. NGF levels were measured using a highly sensitive two-site ELISA (Cat. No. G7631, Promega, Madison, WI, USA), following the protocol provided by the manufacturer. Each test was performed in duplicate, and the data are expressed as pg of NGF/µg of total tissue proteins, as previously described[22-24].

### Western Blotting Analysis

Brain tissues were homogenized with ultrasonication 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% sodium deoxycholate; 1 mM PMSF; 1 µg/ml leupeptin), centrifuged at 4°C for 20 min at 13,000 rpm, then the supernatant was storage 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% SDS, 20% glycerol and 0.1% bromophenol blue), separated by 8% or 12% SDS-PAGE, and electrophoretically transferred to PVDF (Bio-Rad, Hercules, CA, USA) membrane overnight. The membranes were incubated for 1 h at room temperature with blocking buffer, comprised of 5% BSA (for Trka$^{NGFR}$) or non-fat dry milk (for p75$^{NTR}$, GAPDH) in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl and 0.1% Tween-20). Membranes were washed three times for 10 min each at room temperature in TBS-T, followed

by incubation at 4°C with primary antibodies overnight: polyclonal rabbit anti-trka$^{NGFR}$ 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-p75$^{NTR}$ 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed three times for 10 min each at room temperature in TBS-T, and incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG 1:4000 (Cell Signaling Technology, Danvers, MA, USA) or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, MA, USA). The blots were developed with an ECL chemiluminescent horseradish peroxidase (HRP) substrate as the chromophore (Millipore, Billerica, MA, USA). 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) band was used as a normalizing factor. For each gel blot, the normalized values were then expressed as a percentage of relative normalized controls and were used for statistical evaluation. At least eight corneas from different rats in each experimental group were used, and experiments were performed in triplicate. Statistical evaluations were performed using the GraphPad Prism package for Windows, and data were expressed as means ± SEM.

### Statistical Analysis

Statistical analysis was performed using the Students's *t*-test. For all statistical analyses, $p < 0.05$ was considered statistically significant.

## Results

### The Effect of Hphss Treatment on NGF

As shown in Figure 1, nasal administration of Hphss in adult laboratory rats resulted in an in-

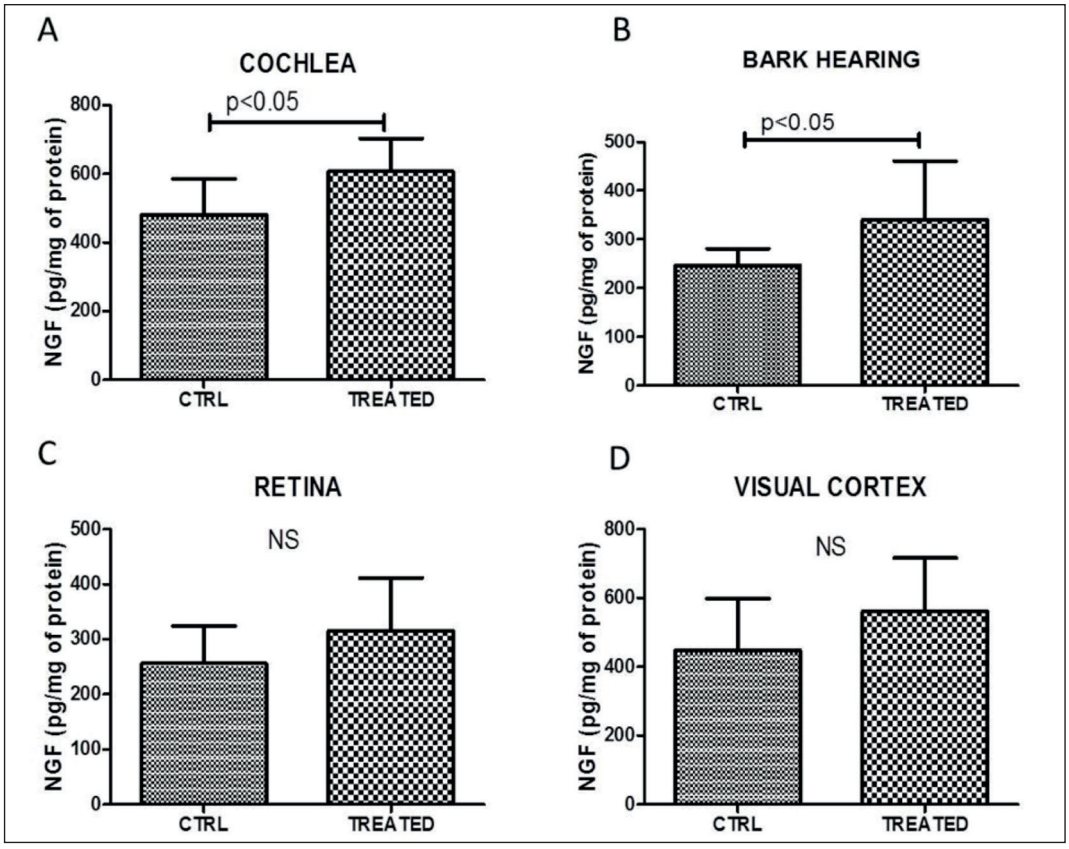


**Figure 1.** Levels of NGF in the cochlea ***(A)***, visual cortex ***(B)***, bark earing ***(C)*** and retina (D) in rats treated for seven consecutive days with Hphss (TREATED) and in untreated rats (CTRL). Significant (*$p < 0.05$) increases in NGF levels were observed in the cochlea (A) and bark earing ***(C)***, but not in the visual cortex ***(B)*** or retina (D), after Hphss treatment, as compared to untreated rats. The results are obtained by ELISA test.

crease in NGF protein in the cochlea (A) and bark earing (C); the results are obtained by ELISA test. These increases were statistically significant ($p < 0.05$). In the visual cortex and retina, there were no statistically significant increases.

### The Effect of Hphss Treatment on NGF-Receptor Expression

We next performed Western blot analysis to determine the expression of the NGF high-affinity receptor. As reported in Figure 2, the results indicated that Hphss administration also enhanced the expression of $Trka^{NGFR}$ in the cochlea (A), bark earing (B), visual cortex (C) and retina (D). However, the administration of nasal Hphss did not alter the basal expression of the low-affinity NGF receptor, $p75^{NTR}$, as shown in Figure 3.

### Structural Analyses

Histological analysis of all brain regions, as well the cochlea and retina, revealed no structural histological alterations following daily Hphss administration (data not shown).

## Discussion

This work investigated the effect of Hphss administration on the expression of NGF and NGF-receptors in the cochlea, bark earing, visual cortex, and retina. The rationale behind this experimental approach was based on a number of published studies showing that NGF exerts protective effects on forebrain cholinergic degenerating neurons. Our recent findings demonstrated that nasal administration of Hphss

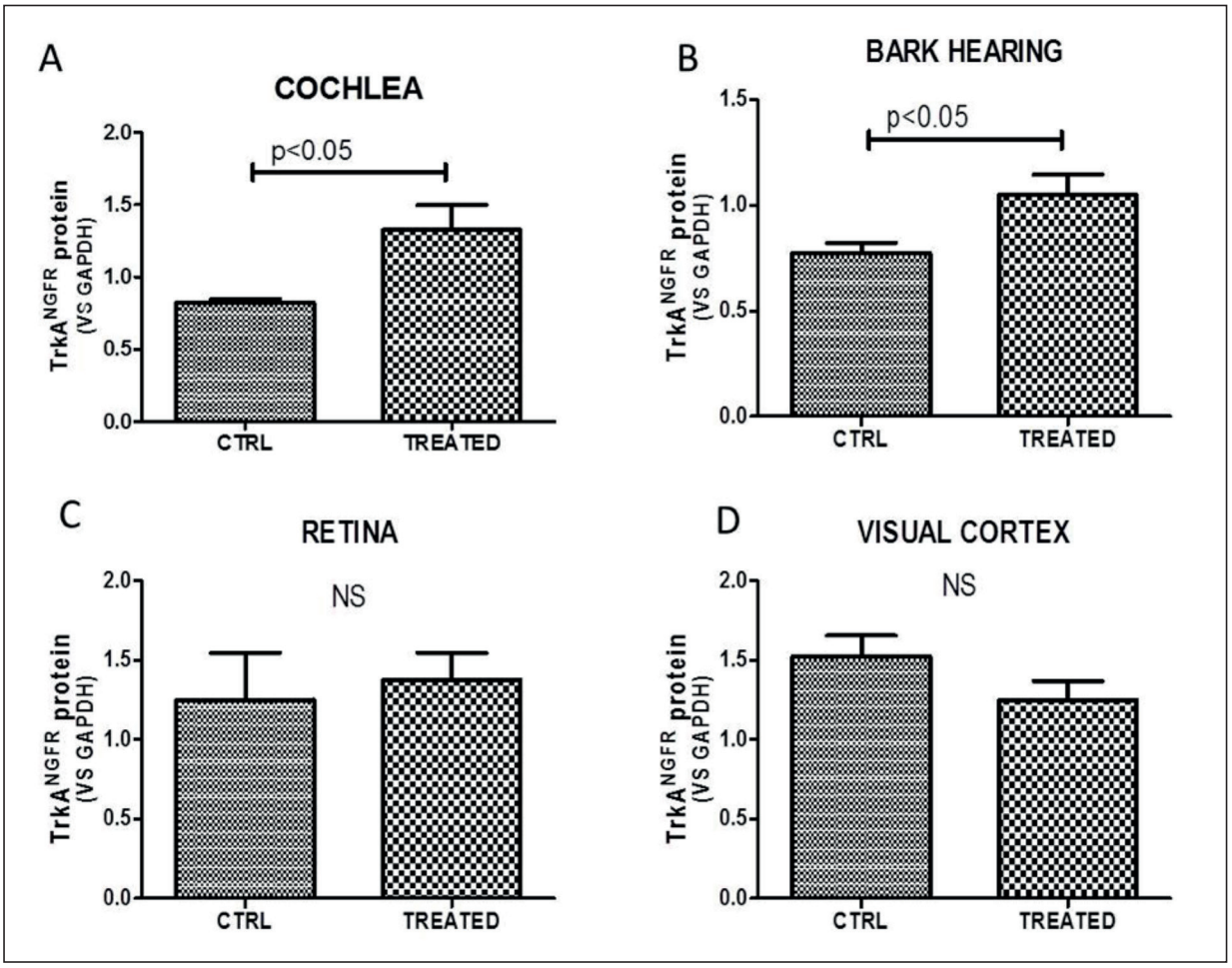


**Figure 2.** Western blot determination of the high-affinity NGF-receptor, $Trka^{NGFR}$, in the cochlea ***(A)***, visual cortex ***(B)***, bark earing ***(C)*** and retina ***(D)*** of rats treated for seven consecutive days with Hphss (TREATED) and untreated rats (CTRL). Significant ($*p < 0.05$) increases in $Trka^{NGFR}$ expression were observed only in the cochlea ***(A)*** and bark earing ***(C)***, after Hphss treatment, as compared to untreated rats.

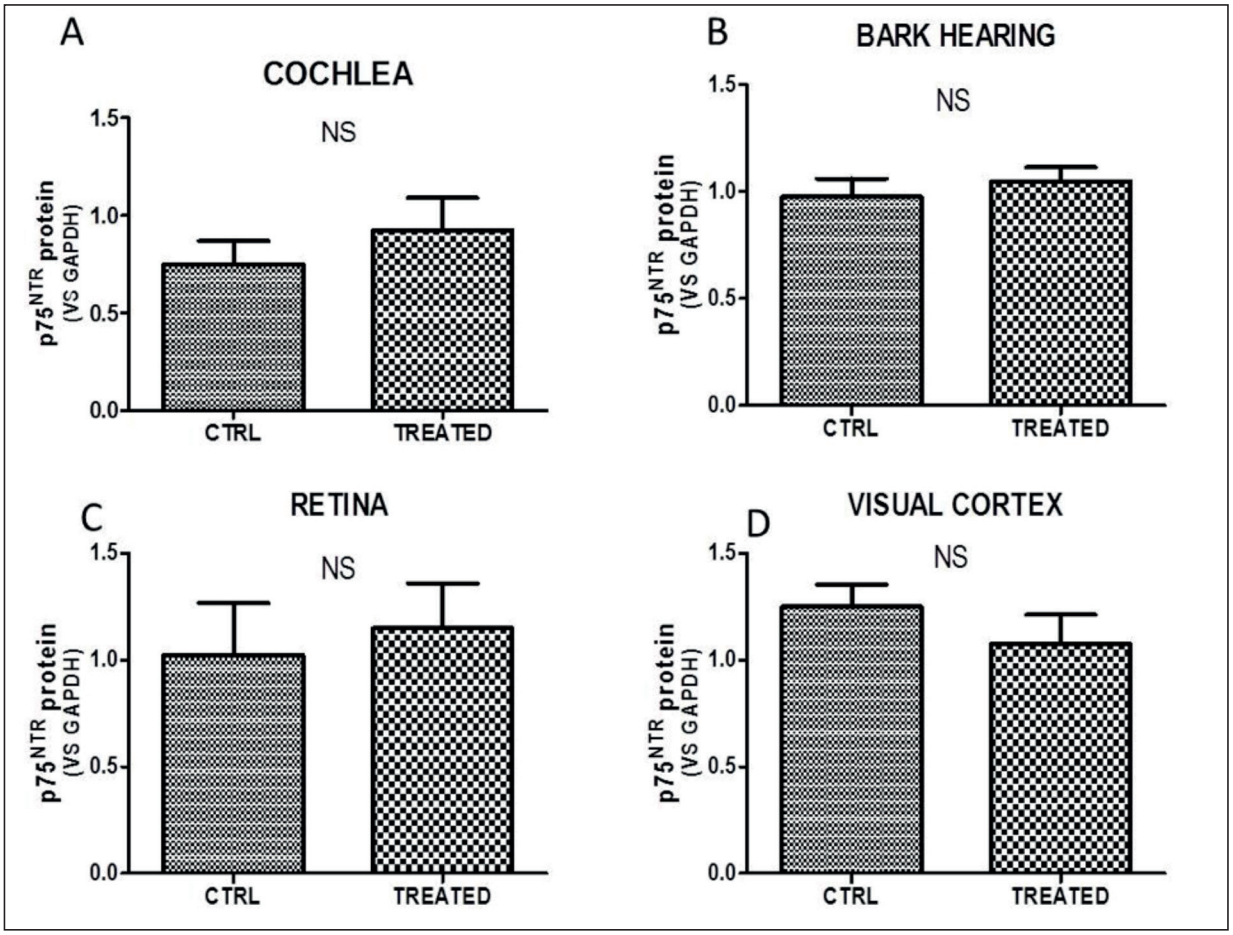


**Figure 3.** Western blot determination of the low-affinity NGF-receptor, $p75^{NTR}$, in the cochlea ***(A)***, visual cortex ***(B)***, bark earing ***(C)*** and retina ***(C)*** of rats treated for seven consecutive days with Hphss (TREATED) and untreated rats (CTRL). Significant (*$p < 0.05$) increases in $p75^{NTR}$ expression were only observed in the cochlea ***(A)*** and bark earing ***(C)*** after Hphss treatment, as compared to untreated rats.

stimulates the basal levels of nasal NGF, and can reach brain neurons via olfactory pathways. However, it was not known whether the released NGF could be transported from the olfactory cavity to the auditory and visual cortex, and the cochlea and retina.

Within the central nervous system, the largest quantity of NGF is produced and released by brain cells localized in the frontal cortex and the hippocampus. The action of NGF in these brain regions is to regulate survival and protect forebrain cholinergic neurons that are known to be involved in functional activities, including learning and memory abilities[1-5]. However, other cells, including immune cells, are able to produce and release NGF[1]. We recently reported, for the first time, that forced nasal administration of Hphss enhances the presence of immune cells in the nasal cavity and the release of NGF and NGF-receptor expression in the olfactory bulbs and the linked brain areas[23,24]. Moreover, we also found that nasal Hphss administration, in patients affected by brain tumours localized in the anterior portion of the cranial fossa, induces a significant increase in NGF and its receptors, as demonstrated by biochemical, molecular and immunochemical analyses[25]. This effect was associated with an increase in NGF and the p73 protein in tumour tissue; the p73 protein is known to down-regulate the proliferation of tumor cells by enhancing cell differentiation[25-32].

In the current report, we found that Hphss significantly enhanced the level of NGF in the cochlea and bark earing, but not in the retina and visual cortex. The nasolacrimal duct pathway does not appear to affect the retina, and the visual cortex appears to be too far from the cribriform plate to be reached by nasal NGF. More-

over, the expression of NGF-receptors, which mediate NGF's biological and functional activities, is enhanced only in the cochlea and bark earing. Characterization of the pharmacokinetics for intranasal delivery of NGF revealed that intranasal NGF rapidly spreads through brain tissue[33,34]. Indeed, published findings indicate that the presence of NGF in the olfactory cells play an essential protective role in regeneration, maintenance, and development in the olfactory system[35] and the olfactory epithelium and sensory cells[36,37]. Therefore, it might be possible for the nasal administration of Hphss to usefully regulate the levels of NGF in the cochlea and bark earing[24]. The present data are consistent with the efficacy of our therapy for the treatment and prevention of sensorineural hearing loss and tinnitus.

## Conclusions

We clearly indicate that Hphss is free from side effects and can be used in chronic diseases for as long as needed. However, whether this treatment can permanently delay degenerative events in the cochlea and bark earing, and whether this effect is long lasting, remains to be investigated.

### Acknowledgements

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### Conflict of Interest

The Authors declare that they have no conflict of interests.

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