

Noninvasive Transcutaneous Vagus Nerve Stimulation Decreases Whole Blood Culture-Derived Cytokines and Chemokines: A Randomized, Blinded, Healthy Control Pilot Trial

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Objectives: The purpose of this study was to test the transcutaneous noninvasive vagus nerve stimulator (nVNS) (gammaCore©) device to determine if it modulates the peripheral immune system, as has been previously published for implanted vagus nerve stimulators.

Materials and Methods: A total of 20 healthy males and females were randomized to receive either nVNS or sham stimulation (SST). All subjects underwent an initial blood draw at 8:00 AM, followed by stimulation with nVNS or SST at 8:30 AM. Stimulation was repeated at 12:00 PM and 6:00 PM. Additional blood samples were withdrawn 90 min and 24 hour after the first stimulation session. After samples were cultured using the Myriad RBM TruCulture (Austin, TX) system (WBCx), levels of cytokines and chemokines were measured by the Luminex assay and statistical analyses within and between groups were performed using the Wilcoxon Signed Ranks Test and Mann-Whitney U with the statistical program R.

Results: A significant percent decrease in the levels of the cytokine interleukin [IL]-1 β , tumor necrosis factor [TNF] levels, and chemokine, interleukin [IL]-8 IL-8, macrophage inflammatory protein [MIP]-1 α , and monocyte chemoattractant protein [MCP]-1 levels was observed in the nVNS group non-lipopolysaccharide (LPS)-stimulated whole blood culture (n-WBCx) at the 24-hour time point ($p < 0.05$). In SST group, there was a significant percent increase in IL-8 at 90 min post-stimulation ($p < 0.05$). At 90 min, the nVNS group had a greater percent decrease in IL-8 concentration ($p < 0.05$) compared to SST group. The nVNS group had a greater percent decrease in cytokines (TNF, IL-1 β) and chemokines (MCP-1 and IL-8) at 24 hour ($p < 0.05$) in comparison to SST. LPS-stimulated whole blood cultures (L-WBCx) did not show a significant decrease in cytokine levels in either the nVNS or SST group across any time points. The nVNS group showed a significant percent increase in LPS-stimulated IL-10 levels at the 24-hour time point in comparison to SST.

Conclusions: nVNS downregulates inflammatory cytokine release suggesting that nVNS may be an effective anti-inflammatory treatment.

Keywords: Cholinergic anti-inflammatory pathway, immunomodulation, neuromodulation, vagus nerve stimulation

Conflict of Interest: Dr. Simon is an employee of electroCore, which manufactures the gammaCore device, and owns stock in the company.

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INTRODUCTION

The vagus nerve (cranial nerve X) is the primary neural component of the parasympathetic nervous system. It is a critical mediator of physiological homeostasis due to its control of heart rate, motility, and secretion of the gastrointestinal tract, pancreatic endocrine and exocrine secretion, hepatic glucose production, and other visceral functions. Importantly vagal activity suppresses innate immune and inflammatory responses to pathogen invasion and tissue injury (1–3). Growing evidence suggests that the innate immune response malfunctions and becomes excessively activated in inflammatory immune-mediated diseases such as rheumatoid arthritis (RA), psoriatic arthritis, and psoriasis (4–6). Furthermore, vagal dysfunction may contribute to the excessive inflammation in RA while vagal nerve stimulation may reduce inflammation in RA (7–9). Recently, hyperinflammatory response has been implicated in the pathophysiology of post-traumatic stress disorder (10,11) and depression (12). Vagal mediated signaling to the adrenal cortex and heart can reduce cortisol secretion and increase heart rate variability supporting a role for vagal mechanisms in stress disorders (13–16).

Pathogen-associated molecules are recognized by sensor/receptors on the nodose ganglion or immune cell surfaces or in intracellular compartments through toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) (17–19), and IL-1 β receptors (20–24). These receptors may individually or collectively activate signal cascades that stimulate release of proinflammatory cytokines and C-reactive protein (CRP) (2,25) (Fig. 1a). Chavan and associates (26) showed that three minutes after intraperitoneal tumor necrosis factor (TNF) injection, mouse vagus nerve firing increased up to approximately 36 Hz from a baseline median firing rate of approximately 7 Hz. Others have shown an increase in vagal afferent firing frequency in response to hepatic IL-1 β administration (27). Efferent vagal action potentials transmit to the celiac mesenteric ganglion and signal post-ganglionic sympathetic splenic nerves to release norepinephrine, however specific mechanisms of this signaling process remain controversial (28–30). Although debated, splenic release of norepinephrine (NE) may activate β adrenergic receptors on splenic T cells that express choline acetyltransferase (ChAT) (31), and synthesize acetylcholine (ACh). ACh then binds to and activates nicotinic acetylcholine receptor subunit $\alpha 7$ ($\alpha 7$ nAChR) that are expressed on the membrane of innate immune cells within the spleen. ChAT + T cells are known to be necessary for $\alpha 7$ nAChR signaling, which inhibits nuclear factor kappa B (NF κ B) activation both directly and indirectly by activating STAT3, thereby decreasing expression of TLR4 and suppressing transcription of inflammatory cytokines (Fig. 1b) (32–38). Nude mice lacking T cells, also lack cholinergic anti-inflammatory capacity, further supporting the hypothesis that ChAT + T cells are pivotal in $\alpha 7$ nAChR signaling (31). Tracey and associates (1–3), describe this vagus nerve, ACh mediated anti-inflammatory pathway, as the cholinergic anti-inflammatory pathway (CAP). Vagal nerve stimulation by CAP decreases leukocyte trafficking through suppression of β -integrin CD11b, thereby decreasing the number of cells which respond to inflammatory chemokine signals (39). More recently, ACh transport through pores on the immune cell membrane directly to mitochondria was demonstrated. ACh activation of $\alpha 7$ nAChR inhibited mitochondrial DNA release, which in turn decreases NACHT, LRR, and PYD domains-containing protein 3 (NLRP3) inflammasome synthesis of cytokines such as IL-1 β and IL-18 (40). Taken together, current preclinical literature supports neuroinflammatory reflexes mediated by vagus nerve signaling result in anti-inflammatory effects thought to be relevant in human models of inflammation.

TNF, IL-6, and lipopolysaccharide (LPS) stimulate the hypothalamic-pituitary-adrenal (HPA) axis, resulting in increased production of cortisol that constrains immune responses to prevent excess inflammation (23,41,42). This stimulation of HPA hormone secretion is due, in part, to afferent vagal fiber activation of the nucleus tractus solitarius [NTS]-to-paraventricular nucleus [PVN] neurocircuit culminating in adrenocorticotrophic hormone (ACTH) and subsequent adrenocortical secretion of cortisol (Fig. 1b). Preclinical studies show that bilateral cervical vagotomy significantly inhibits LPS-induced increase in corticosterone, confirming the importance of the afferent vagal-to-NTS-to-PVN pathway in HPA regulation (33). If stress-induced HPA secretion of glucocorticoids is insufficient to control inflammation, as may occur in inflammatory disorders such as RA, hypersecretion of proinflammatory cytokines from immune cells may abnormally persist (10,43–49).

Similar to *in vivo* pathogen-mediated activation, electrical stimulation of the vagus results in a decrement of multiple inflammatory cytokines (e.g., TNF, IL-6, IL-8) (33,50). Mixed results have been reported for the anti-inflammatory cytokine IL-10 (33,50,51). Thus, vagus nerve stimulation is emerging as a potential treatment for human inflammatory diseases (16,50,52–54).

Preclinical bilateral stimulation of the vagus with distal anesthetic blockade (with presumably afferent-only signaling) exerts anti-inflammatory effects (50), which may result in part from corticosteroids secretion. Interestingly, anti-inflammatory effects seen during bilateral cervical vagotomy followed by afferent only vagal stimulation are dependent on an intact splenic nerve. It is known that the vagal-to-NTS-to-PVN pathway also sends efferent fibers to the Locus Coeruleus (LC) (vagal-to-NTS-to-PVN-to-LC pathway) and then to the sympathetic trunk via the intermediolateral cell column (54). Sympathetic trunk-derived splanchnic nerves signals to the celiac ganglion and then via splenic nerve to the spleen release norepinephrine known to activate the aforementioned CAP, and immune cell β adrenergic receptors that inhibit cytokine production through suppressing NF κ B (23). Anti-inflammatory effects shown with bilateral cervical vagotomy and afferent only vagal nerve stimulation depend on an intact splenic nerve suggest that the vagal-to-NTS-to-PVN-to-LC pathway is a critical component of the inflammatory reflex (55).

Extrapolating from these preclinical and emerging clinical studies, we hypothesize that nVNS using the gammaCore device at 25 Hz may encode specific vagal nerve signaling in afferent-to-efferent and efferent pathways that may result in alteration of peripheral inflammation in healthy control subjects.

MATERIALS AND METHODS

Study Subjects

Twenty subjects were recruited through the Clinical and Translation Research Institute (CTRI) at the University of California, San Diego Health System and randomly assigned to receive either nVNS or SST. All subjects were between the ages of 18 and 61 years, were deemed healthy by medical and physical examination, and did not take any chronic medications. There were 10 active and 10 sham subjects, with an equal ratio of male to females 6:4. Subjects were instructed to refrain from taking any over-the-counter analgesics or anti-inflammatory medications (e.g., nonsteroidal anti-inflammatory drugs and acetaminophen), tobacco products, or alcoholic beverages for one week prior to the study visit. Subjects with prior surgery or abnormal anatomy at the gammaCore[®] treatment site, history of neurologic disease (including transient ischemic attack, seizures, and syncope), and history of cardiovascular disease were excluded from

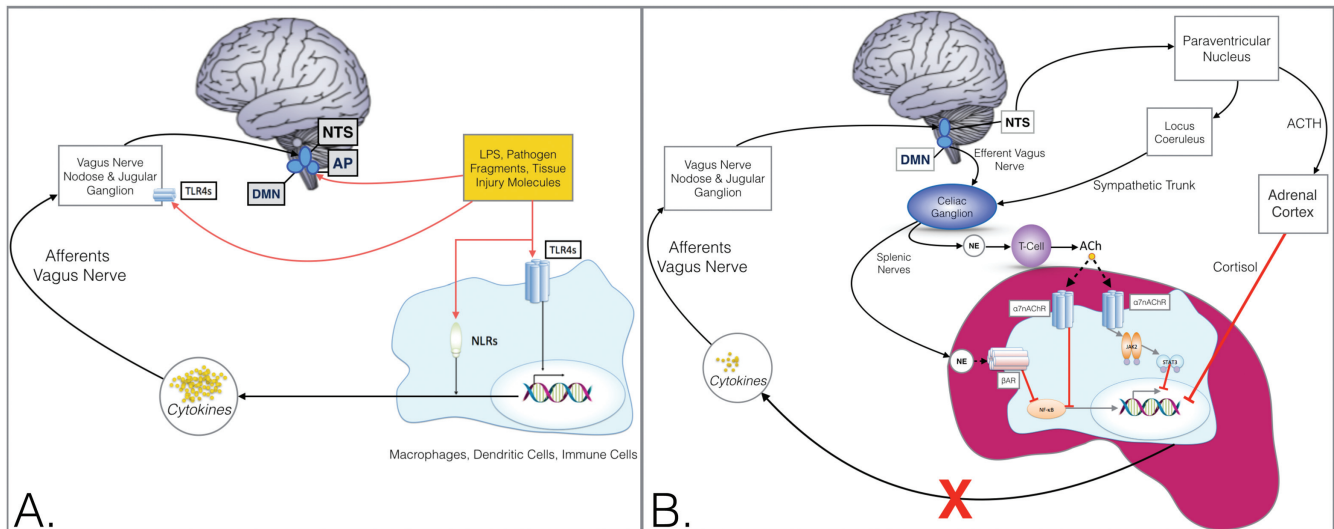


Figure 1. a. Immune responses are activated by pathogen-associated molecular patterns that can be recognized by sensor receptors on the nodose ganglion, immune cell surface or in intracellular compartments through both Toll-like receptors (TLRs), nucleotide binding oligomerization domain-like receptors (NLRs), which activate signal cascades that result in release of proinflammatory cytokines. Pro-inflammatory cytokines may activate vagal afferent fibers in the periphery and within vagal paraganglia cells, as well as act on the area postrema (AP). b. Activated vagal afferents transmit from NTS to the DMN, to efferent vagal fibers that transmit to Celiac Ganglion then to splenic nerve resulting in ChAT + T cell release of the neurotransmitter acetylcholine (ACh), which interacts with innate immune cells that express the nicotinic acetylcholine receptor subunit $\alpha 7$ ($\alpha 7$ nAChR). Activated $\alpha 7$ nAChR inhibits nuclear factor kappa B (NF- κ B) activation both directly and indirectly via Signal Transducer and Activator of Transcription 3 (gene) (STAT3), resulting in decreased expression of TLR4, and suppresses transcription of inflammatory cytokines (red X). Cytokines activate the HPA axis via TLR4 receptors and presumed IL-6 receptors found on vagal paraganglia cells and the Area Postrema (AP), resulting in increased production of cortisol that protects against excess inflammation. Vagal-to-NTS-to-PVN pathway also sends efferent fibers to the Locus Coeruleus (vagal-to-NTS-to-PVN pathway) via the sympathetic trunk, and splanchnic nerve signal to the celiac ganglion and then to splenic nerve to activate β adrenergic receptors (β AR) and the cholinergic anti-inflammatory pathway (CAP). NE = Norepinephrine; NTS = Nucleus Tractus Solitarius; DMN = Dorsal Motor Nucleus; AP = Area Postrema; ACTH = Adrenocorticotrophic hormone; ACh = Acetylcholine.

the study. The Institutional Review Board at the University of California, San Diego Health Systems approved the protocol. Informed consent was obtained for all study procedures.

Intervention

Subjects were randomly assigned to receive either SST or nVNS. The nVNS device produced a low-voltage electrical signal (5-kHz sine wave series that occurred for 1 ms and repeated every 40 ms [25 Hz]). Upon application of the device to the neck, it delivered a maximum of 24 V and 60 mA output current while allowing stimulation amplitude adjustment by the user. Two stainless steel contact surfaces and conductive gel applied by the user enabled delivery of stimulations. In both sham and nVNS treatment paradigms, the principle investigator instructed the subject to place the device just under the angle of the mandible, lateral to the trachea and medial to the sternocleidomastoid. In both the nVNS and SST device, there was an initial 30-sec ramp up period, resulting in a total of 90 sec of actual stimulation per two-minute stimulation session. The SST device was identical in appearance, and carried out the same period of stimulation but differed in stimulation parameters with a slowly varying DC signal (.1 Hz) with maximum range of ± 14 V that produced a slight skin tingling sensation. Voltages for sham and active nVNS device were recorded for each stimulation session (Table 1).

Research Design

Subjects were required to be present at the CTRL research facility for two visits during two consecutive days. Subjects underwent an initial blood draw at 8:00 AM, followed 30 min later by stimulation with either nVNS or SST. Stimulation for either case lasted for two minutes, on first the right and then the left side. Left side stimulation was carried out five minutes after right side stimulation. Blood was

then drawn at 10:30 AM, 90 min after the 8:30 AM stimulation. The subject was instructed to deliver the stimulation sequence (once per side) at noon and again at 6:00 PM the same day and return to the CTRL research facility the following morning. On the second day, site staff ascertained any changes to subject's medical history or adverse events, electrocardiographic findings, and vital signs and performed a physical examination. A final third blood draw occurred at 8:00 AM on the second day. The primary endpoints of this study were detection of percent change in cytokines measured with or without additive LPS in whole blood that was cultured for 24 hours (within each group, [nVNS or SST], and between groups).

Serum Collection

Blood was drawn using TruCulture RBM system. Blood samples were obtained at CTRL in a sterile manner, and two 1-mL aliquots (one tube without LPS and one tube with LPS added) of whole blood were cultured for 24 hours at 37°C then stored at -80°C . Cytokine and chemokines were collected using a human cytokine multiplex immunoassay (Myriad Rules-Based Medicine Inc., Austin, TX). LPS tubes had 0.1 $\mu\text{g/mL}$ or 100 ng/mL of LPS in each 1 mL whole blood cultured tube. The multiplex microbead assay is based on Luminex technology and measures proteins similarly to standard sandwich enzyme-linked immunosorbent assay, with comparable sensitivity and range. The lower limit of quantitation (LLOQ) for the cytokines were:

- MCP1: 87 pg/mL
- MIP-1 α : 28.0 pg/mL
- MIP-1 β : 60.0 pg/mL
- TNF: 15.0 pg/mL
- IL-1 β : 6.6 pg/mL
- IL-6: 11.0 pg/mL
- IL-10: 4.9 pg/mL

Table 1. Study Subject Characteristics ($p < .05$ Level of Significance; Two Sample t Test for All Comparisons Except for Race for Which Fishers Exact Test Was Used)

	Sham	Standard Deviation	nVNS	Standard Deviation	Statistical Methods
	Mean		Mean		P
Age (years)	36.6	14.0	35.8	12.9	0.75
Sex (M/F)	6/5		7/4		
Race					0.08
Asian	1		5		
Black	2		0		
White	8		6		
Body mass index	26.1	6.5	25.1	4.65	0.91
Blood pressure first visit (mm Hg)	116.5/72.4	10.7/7.7	117.1/69.2	11.1/9.6	0.77/0.41
Heart rate first visit (beats/min)	65.5	6.9	62.6	10.4	0.45
Blood pressure second visit (mm Hg)	116.5/72.0	11.6/11.1	119.2/70.9	10.0/8.5	0.55/0.80
Heart rate second visit (beats/min)	67.9	8.6	64.9	10.7	0.47
Adverse events	4		5		
Headache	1		2		
Pain at site during stimulation	2		2		
Teeth sensitive with stimulation	1		0		
Neck strain	0		1		
Excluded subjects	1		1		
Vagus nerve ultrasound depth (right/left cm)	1.38/1.24	0.26/0.23	1.27/1.24	0.20/0.26	0.28/0.90
Morning voltage (right/left V)	11/12.86	4.12/4.58	22.59/19.54	2.83/5.17	<.005/<.005
Noon voltage (right/left V)	13.09/12.04	2.38/3.40	21.4/21.5	4.23/4.21	<.005/<.005
Evening voltage (right/left V)	13.09/12.22	2.38/2.59	21.3/21.09	4.97/5.35	<.005/<.005

The LLOQ is the lowest concentration of an analyte in a sample that can be reliably detected and at which the total error meets the laboratory's requirements for accuracy (56). TruCulture tubes were sent Myriad RBM, where multianalyte profiling assays were performed using human inflammation MAP-A and MAP-B technology.

Statistical Methods

We compared independent samples with the Mann-Whitney U test and paired samples with the Wilcoxon Signed-rank test. All calculations were performed using the open source statistical programming language R (www.r-project.org).

RESULTS

Subjects

Subject groups were similar in age, sex, body mass index, heart rate, and blood pressure (Table 1). The average age of the cohort was 36 ± 14 years for the sham group and 35.8 ± 14.5 years for the treated group. The study population was composed of 65% Caucasian, 30% Asian, and 5% African American individuals. Adverse events were minor during stimulation and equal across both groups, with headache and pain during stimulation noted most commonly (Table 1). One male subject who reported muscle strain and persistent pain at the stimulation site was excluded on the second visit day. A second female subject was excluded due to difficulty with blood draw. After subject exclusion, the male to female ratio was 6:4

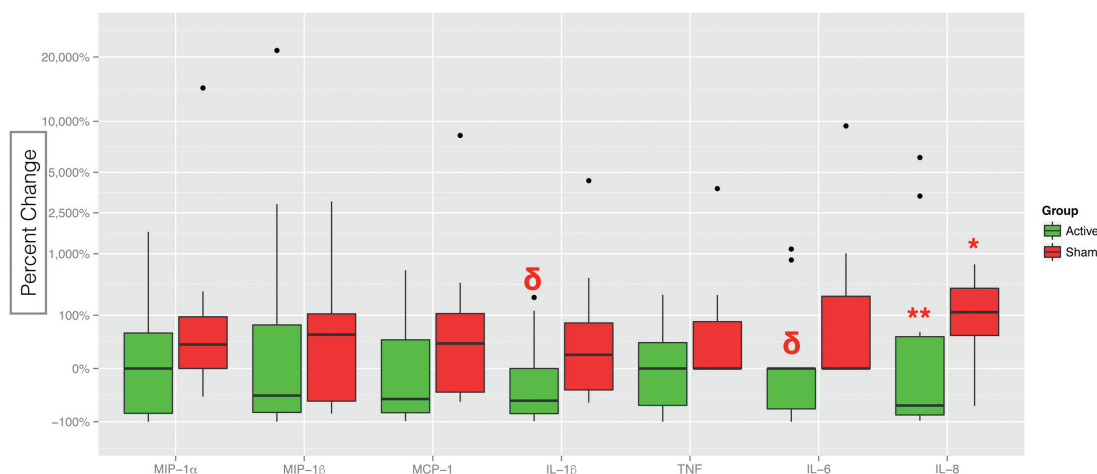


Figure 2. At 90 min, the SST group demonstrated a significant percent increase in IL-8 (* = < 0.05). At 90 min, the nVNS group had a greater percent decrease in IL-8 concentration (** = < 0.05) and a greater percent decrease in IL-1 β and IL-6 approached a significant level ($\delta < 0.06$) when compared to SST.

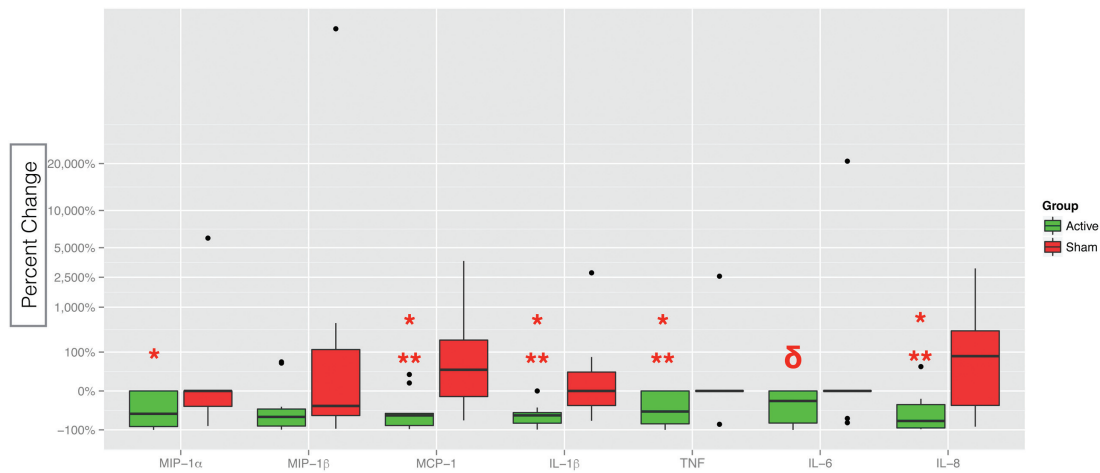


Figure 3. Within the nVNS group, non-LPS-stimulated whole blood culture (n-WBCx) demonstrated a significant percent decrease in cytokine (TNF, IL-1 β) and chemokine (MCP-1 and IL-8) levels at the 24-hour time point (* = < 0.05). In the nVNS group, IL-6 approached a significant decrease at the 24-hour time point as well (δ = < 0.06). Compared with the SST group, the nVNS group had a significantly greater percent decrease in cytokine (IL-1 β , TNF) and chemokine (MCP-1 and IL-8) levels at 24 hours (** = < 0.05).

in both the sham and the nVNS group (Table 1). Vagal nerve depth, as measured by ultrasonography, did not differ significantly between groups and ranged from 1.27 to 1.38 cm for the right side and 1.24 and 1.25 cm for the left side (Table 1).

Cytokine Analyses

Within Group Non-LPS-Stimulated Whole Blood Culture Cytokine Analysis

At 90 min, there was no significant percent decrease compared to baseline in any cytokine or chemokine levels within the SST group in non-LPS-stimulated whole blood culture (n-WBCx) (Fig. 2). The SST group did, however, demonstrate a significant percent increase in the chemokine IL-8 (p < 0.05) at this time. At the 24-hour time point all of the levels in SST subjects were comparable to baseline, while in the nVNS group, n-WBCx demonstrated a significant percent decrease in cytokine (IL-1 β , TNF) and chemokine (MIP-1 α , MCP-1, IL-8) levels (p < 0.05) (Fig. 3). Within the nVNS group, IL-6 levels had a strong tendency to decrease at the 24-hour time point (p < 0.06) (Fig. 3).

Between Group n-WBCx Cytokine Analysis

Raw baseline (prior to stimulation) n-WBCx cytokine and chemokine values did not differ between groups (p > 0.1), except for the cytokine IL-1 β , which was elevated in the nVNS group (p = 0.041) (Supporting Information Table 1). Compared to the SST group, the nVNS group had a greater percent decrease in the chemokine IL-8 concentration (p < 0.05) at 90 min (Fig. 2). At this time cytokines, IL-6 and IL-1 β approached a significant decrease when the nVNS group was compared to SST (p < 0.06). Compared with the SST group, the nVNS group had a significantly greater percent decrease in cytokine (IL-1 β , TNF) and chemokine (MCP-1, IL-8) levels at 24 hours (p < 0.05).

LPS-Stimulated Whole Blood Culture Analysis

LPS-stimulated cytokines and chemokines did not differ significantly in the nVNS vs. SST, (Supporting Information Figs. 1 and 2), except for IL-10, which increased at 24 hours in the nVNS group when compared to the SST group (p < 0.005) (Fig. 4). Raw baseline (prior to stimulation) L-WBCx cytokine and chemokine values did not differ between groups (p > 0.1).

DISCUSSION

In this pilot study, nVNS significantly decreased the release of pro-inflammatory cytokines in whole blood culture when compared to sham stimulation.

Data from Tracey (1) indicate that afferent vagal-mediated signals are relayed to the NTS and that the Dorsal Motor Nucleus (DMN) activates vagal efferents targeted to the celiac ganglion, thereby stimulating the CAP (deactivating monocytes) and resulting in decreased release of peripheral cytokines. Vagus nerve stimulation is believed to mediate an anti-inflammatory effect through: 1) induction of the CAP via ChAT +T cells, altered cytokine expression, either through ACh ligand-induced activation of α 7nAChRs on the cell membrane or in mitochondria that modulate the NLRP3 inflammasome and inhibit procaspase-1; 2) activation of the NTS-to-PVN pathways, resulting in ACTH stimulation of adrenocortical release of glucocorticoids; 3) activation of the NTS-to-PVN-to-LC pathway ultimately signaling to splenic nerve resulting in norepinephrine release that can activate the CAP via ChAT +T cells and immune cell β adrenergic receptors that can inhibit cytokine production; and 4) decreased leukocyte trafficking through suppression of β -integrin CD11b, thereby decreasing the number of circulating leukocytes able to mobilize to an inflammatory chemokine signal. Vagus nerve electrical stimulation mediated by these potential pathways has been shown to decrease peripheral inflammation, evidenced by a

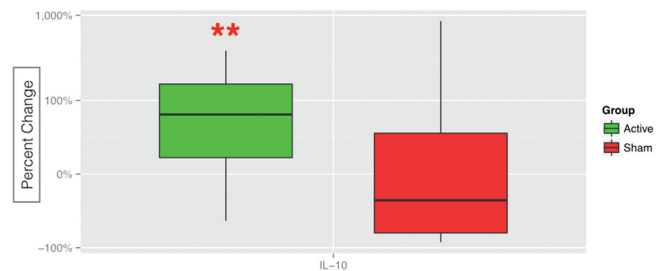


Figure 4. L-WBCx did not show a significant decrease in cytokine levels in either the nVNS or SST groups across any time points. The nVNS group showed a significant percent increase in LPS-stimulated IL-10 levels at 24 hours compared with values in the SST group (* = < 0.05).

reduction in multiple inflammatory cytokines, including TNF, IL-6, and IL-8, and CRP in dogs (57) and humans (52).

The pilot data in our study support the anti-inflammatory effect of nVNS, likely due to activation of the CAP, with a significant percent decrease in IL-8 at 90 min (Fig. 3) and significant percent decreases in cytokine and chemokine levels at the 24-hour time point (Fig. 4).

The SST group did not demonstrate a significant percent decrease in any measured cytokine or chemokine levels at either the 90-min or 24-hour poststimulation n-WBCx. Instead the SST group had an increase in IL-8 at 90 min (Fig. 3).

Cytokine concentrations vary over a 24-hour period with circadian rhythm. The circadian rhythm of inflammatory cytokines is believed to be controlled by central gene expression clocks activated via the suprachiasmatic nucleus and the hypothalamus, which induce entrainment of peripheral clock inflammatory cytokine gene expression as well as release of peripheral cortisol by way of paraventricular nucleus ACTH signaling to the adrenal cortex to release cortisol (58). Beyond cytokine synthesis and release, the number of inflammatory cells also has a circadian rhythm, with monocyte nadir occurring at 8:00 AM and acrophase (peak cells per milliliter) at 11:00 PM (59). Inflammatory disease states such as RA, bronchial asthma, allergic rhinitis, ankylosing spondylitis, and polymyalgia rheumatica demonstrate early morning increases in symptom severity. In RA, bronchial asthma, and polymyalgia rheumatica symptom, severity has been correlated with a clear early morning circadian increase in inflammatory cytokines (14). Sham stimulation in our study resulted in a significant percent increase in IL-8 when comparing the 8:00 AM and 10:30 AM draws, coinciding with likely circadian rhythmicity of this cytokine. In contrast, the nVNS group showed a percent decrease in all cytokines, although it was not statistically significant at the 10:30 AM draw. The three stimulation sessions in nVNS group resulted in a significant percent drop in MIP-1 α , MCP-1, TNF, IL-1 β , and IL-8 at the 24-hour time point. The 24-hour collection point controlled for individual differences due to circadian alterations in cytokine expression because blood draws were recorded at the exact same time each day (8:00 AM). It is possible that if a larger number of study subjects were enrolled there may have been a 90-min decrease in cytokines in the nVNS group.

This cohort of healthy volunteers did not show any reduction in LPS-stimulated whole blood cultures (L-WBCx), although this has been reported in preclinical literature (33,50). The primary difference between these preclinical studies and the current study are use of surgically implanted vagus nerve stimulators and *in vivo* administration of LPS. *In vivo* LPS administration results in the recruitment and activation of circulating monocytes that may subsequently undergo modulation when circulated through the spleen. The healthy control cohort in our study did not demonstrate signs of illness and, therefore, were unlikely to have increased recruitment and mobilization of immune cells that decreased the measured effect of LPS in culture. Peak cytokine levels are dependent on culture time when stimulated with LPS, which may not have been captured in a 24-hour culture. The only cytokine that increased in the L-WBCx was IL-10 in the nVNS group. IL-10 acts on immune cells, including monocytes/macrophages at IL-10R1, and IL-10R2 through STAT3, resulting in decreased synthesis of cytokines such as TNF, IL-1 β , IL-6, and the chemokine IL-8 (60–62). Taken together, IL-10 has the potential to inhibit inflammatory cytokine and chemokine synthesis and release. nVNS treatment greater than one day (as in the current study) may result in further IL-10 anti-inflammatory effects in LPS mediated culture.

Limitations

Because this pilot study involved only ten subjects per group, the small sample size may not adequately represent a larger population, which could skew the results. Nonetheless, these positive findings in such a small cohort of healthy control subjects were robust and significant warranting further investigation on peripheral immune system effects of transcutaneous nVNS in larger cohort of healthy control subjects as well as in subjects with inflammatory immune-mediated diseases. In our future research, we plan to quantify mRNA transcript levels as careful RNA sequencing and analysis may provide important insight into nVNS regulation of immune gene expression. Although the target of this stimulator is the vagal nerve, cutaneous afferents to the skin and innervation of the underlying tissue may have been stimulated as well. Further studies that block cutaneous afferents and or underlying tissue may elucidate target specific mechanisms of transcutaneous cervical stimulation as delivered by the gammaCore device.

CONCLUSION

For the first time to our knowledge, we show transcutaneous nVNS modulates human inflammatory cytokines and chemokines, as measured by WBCx. The significant decrement in cytokines and chemokines seen in this study supports the concept that such stimulation activates cholinergic-mediated anti-inflammatory responses the vagal NTS-PVN-HPA and NTS-PVN-LC pathway. Other as yet undiscovered reflex pathways might also contribute. Future studies will focus on nVNS anti-inflammatory mechanisms in immune-mediated inflammatory diseases that may eventually lead to a viable nVNS anti-inflammatory therapy.

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Authorship Statements

Dr. Lerman designed and wrote the study. Dr. Lerman and Katie Lam carried out the study. Drs. Lerman, Baker, Hauger, Davis, Huang, Sorkin and Simon prepared the manuscript. James Proudfoot carried out the statistical analysis of all data. All authors had complete access to the study.

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REFERENCES

- Tracey KJ. Reflex control of immunity. *Nat Rev Immunol* 2009;9:418–428.
- Tracey KJ. The inflammatory reflex. *Nature* 2002;420:853–859.
- Andersson U, Tracey KJ. Reflex principles of immunological homeostasis. *Annu Rev Immunol* 2012;30:313–335.
- Gierut A, Perlman H, Pope RM. Innate immunity and rheumatoid arthritis. *Rheum Dis Clin North Am* 2010;36:271–296.
- Maciejewska Rodrigues H, Jüngel A, Gay RE, Gay S. Innate immunity, epigenetics and autoimmunity in rheumatoid arthritis. *Mol Immunol* 2009;47:12–18.
- Bachelez H. Immunopathogenesis of psoriasis: recent insights on the role of adaptive and innate immunity. *J Autoimmun* 2005;25(Suppl.):69–73.
- Bruchfeld A, Goldstein RS, Chavan S, et al. Whole blood cytokine attenuation by cholinergic agonists ex vivo and relationship to vagus nerve activity in rheumatoid arthritis. *J Intern Med* 2010;268:94–101.
- Koopman FA, Schuurman PR, Vervordeldondk MJ, et al. Vagus nerve stimulation: a new bioelectronics approach to treat rheumatoid arthritis? *Best Pract Res Clin Rheumatol* 2014;28:625–635.
- Levine YA, Koopman FA, Faltys M, et al. Neurostimulation of the cholinergic anti-inflammatory pathway ameliorates disease in rat collagen-induced arthritis. *PLoS One* 2014;9:e104530.
- Baker DG, Nievergelt CM, O'Connor DT. Biomarkers of PTSD: neuropeptides and immune signaling. *Neuropharmacology* 2012;62:663–673.
- Breen MS, Maihofer AX, Glatt SJ, et al. Gene networks specific for innate immunity define post-traumatic stress disorder. *Mol Psychiatry* 2015;20:1538–1545.
- Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat Rev Neurosci* 2010;9:46–56.
- Straub RH, Paimela L, Peltomaa R, Schölermerich J, Leirisalo-Repo M. Inadequately low serum levels of steroid hormones in relation to IL-6 and tumor necrosis factor in untreated patients with early rheumatoid arthritis and reactive arthritis. *Arthritis Rheum* 2002;46:654–662.
- Straub R. Origin of typical disease sequelae: circadian rhythms of symptoms. *The origin of chronic inflammatory systemic diseases and their sequelae*. Waltham, MA: Elsevier Inc., 2015:225–227.
- Crofford LJ, Kalogeras KT, Mastorakos G, et al. Circadian relationships between interleukin (IL)–6 and hypothalamic-pituitary-adrenal axis hormones: failure of IL-6 to cause sustained hypercortisolism in patients with early untreated rheumatoid arthritis. *J Clin Endocrinol Metab* 1997;82:1279–1283.
- Koopman FA, Stoof SP, Straub RH, Van Maanen MA, Vervordeldondk MJ, Tak PP. Restoring the balance of the autonomic nervous system as an innovative approach to the treatment of rheumatoid arthritis. *Mol Med* 2011;17:937–948.
- Baccala R, Gonzalez-Quintanilla R, Lawson BR, et al. Sensors of the innate immune system: their mode of action. *Nat Rev Rheumatol* 2009;5:448–456.
- Chen GY, Nuñez G. Sterile inflammation: sensing and reacting to damage. *Nat Rev Immunol* 2010;10:826–837.
- Andersson U, Tracey KJ. HMGB1 is a therapeutic target for sterile inflammation and infection. *Annu Rev Immunol* 2011;29:139–162.
- Goehler LE, Gaykema RP, Hansen MK, Anderson K, Maier SF, Watkins LR. Vagal immune-to-brain communication: a visceral chemosensory pathway. *Auton Neurosci* 2000;85:49–59.
- Goehler LE, Gaykema RP, Nguyen KT, et al. Interleukin-1beta in immune cells of the abdominal vagus nerve: a link between the immune and nervous systems? *J Neurosci* 1999;19:2799–2806.
- Ek M, Kurosawa M, Lundeberg T, Ericsson A. Activation of vagal afferents after intravenous injection of interleukin-1β: role of endogenous prostaglandins. *J Neurosci* 1998;18:9471–9479.
- Sternberg EM. Neural regulation of innate immunity: a coordinated nonspecific host response to pathogens. *Nat Rev Immunol* 2006;6:318–328.
- Watkins LR, Maier SF. Implications of immune-to-brain communication for sickness and pain. *Proc Natl Acad Sci USA* 1999;96:7710–7713.
- Medzhitov R. Inflammation 2010: new adventures of an old flame. *Cell* 2010;140:771–776.
- Chavan SS, Robbiati S, Steinberg BE, et al. Tumor necrosis factor induces specific neurograms in the sensory vagus nerve [abstract]. Presented at Society of Neuroscience Neuroscience 2014. Washington, DC, 2015. <http://www.abstractsonline.com/Plan/ViewAbstract.aspx?mId=3527&sKey=7ce6178f-43ee-41f3-8eda-f0dbce9a-faa1&ckey=135933ff-fb59-4c3e-b1bc-25d1d597f8f7&mKey=54c85d94-6d69-4b09-afaa-502c0e680ca7>
- Nijima A. The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat. *J Auton Nerv Syst* 1996;61:287–291.
- Martelli D, McKinley MJ, McAllen RM. The cholinergic anti-inflammatory pathway: a critical review. *Auton Neurosci* 2014;182:65–69. doi: 10.1016/j.autneu.2013.12.007.
- Bratton BO, Martelli D, McKinley MJ, Trevaks D, Anderson CR, McAllen RM. Neural regulation of inflammation: no neural connection from the vagus to splenic sympathetic neurons. *Exp Physiol* 2012;97(11):1180–1185. doi: 10.1113/expphysiol.2011.061531.
- Vida G, Peña G, Deitch EA, Ulloa L. A7-cholinergic receptor mediates vagal induction of splenic norepinephrine. *J Immunol* 2011;186(7):4340–4346. doi: 10.4049/jimmunol.1003722.
- Rosas-Ballina M, Olofsson PS, Ochani M, et al. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science* 2011;334(6052):98–101. doi: 10.1126/science.1209985.
- Wang H, Yu M, Ochani M, et al. Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. *Nature* 2003;421:384–388.
- Wang H, Liao H, Ochani M, et al. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. *Nat Med* 2004;10:1216–1221.
- Borovikova LV, Ivanova S, Zhang M, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;405:458–462.
- Saeed RW, Varma S, Peng-Nemeroff T, et al. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. *J Exp Med* 2005;201:1113–1123.
- Parrish WR, Rosas-Ballina M, Gallowitsch-Puerta M, et al. Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling. *Mol Med* 2008;14:567–574.
- Pavlov VA, Ochani M, Yang LH, et al. Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis. *Crit Care Med* 2007;35:1139–1144.
- Hamano R, Takahashi HK, Iwagaki H, Yoshino T, Nishibori M, Tanaka N. Stimulation of alpha7 nicotinic acetylcholine receptor inhibits CD14 and the toll-like receptor 4 expression in human monocytes. *Shock* 2006;26:358–364.
- Huston JM, Rosas-Ballina M, Xue X, et al. Cholinergic neural signals to the spleen down-regulate leukocyte trafficking via CD11b. *J Immunol* 2009;183:552–559. doi: 10.4049/jimmunol.0802684.
- Lu B, Kwan K, Levine YA, et al. Alpha7 nicotinic acetylcholine receptor signaling inhibits inflammasome activation by preventing mitochondrial DNA release. *Mol Med* 2014;20:350–358.
- Fernandez R, Acuña-Castillo C. Neural reflex control of inflammation during sepsis syndromes. In: Azevedo L, ed. *Sepsis - an ongoing and significant challenge*. 2012: 133–156. <http://www.intechopen.com/books/sepsis-an-ongoing-and-significant-challenge/neural-reflex-control-of-inflammation-during-sepsis-syndromes>
- Silverman MN, Pearce BD, Biron CA, Miller AH. Immune modulation of the hypothalamic-pituitary-adrenal (HPA) axis during viral infection. *Viral Immunol* 2005; 18:41–78.
- Elenkov IJ, Tezzoni DG, Daly A, Harris AG, Chrousos GP. Cytokine dysregulation, inflammation and well-being. *Neuroimmunomodulation* 2005;12:255–269.
- McEwen BS. Physiology and neurobiology of stress and adaptation: central role of the brain. *Physiol Rev* 2007;87:873–904.
- Raison CL, Miller AH. When not enough is too much: the role of insufficient glucocorticoid signaling in the pathophysiology of stress-related disorders. *Am J Psychiatry* 2003;160:1554–1565.
- Buske-Kirschbaum A. Cortisol responses to stress in allergic children: interaction with the immune response. *Neuroimmunomodulation* 2009;16:325–332.
- Chrousos GP, Kino T. Glucocorticoid signaling in the cell. Expanding clinical implications to complex human behavioral and somatic disorders. *Ann N Y Acad Sci* 2009; 1179:153–166.
- Crofford LJ, Kalogeras KT, Mastorakos G, et al. Circadian relationships between interleukin (IL)–6 and hypothalamic-pituitary-adrenal axis hormones: failure of IL-6 to cause sustained hypercortisolism in patients with early untreated rheumatoid arthritis. *J Clin Endocrinol Metab* 1997;82:1279–1283.
- Pfiffis KN, Papadimitriou A, Nicolaidou P, Chrousos GP. Dysregulation of the stress response in asthmatic children. *Allergy* 2009;64:18–31.
- Olofsson PS. A stimulating concept: bioelectronic medicine in inflammatory disease. *Bioelectron Med* 2014;1:30–33.
- Zhao YX, He W, Jing XH, et al. Transcutaneous auricular vagus nerve stimulation protects endotoxemic rat from lipopolysaccharide-induced inflammation. *Evid Based Complement Alternat Med* 2012;2012:627023. doi: 10.1155/2012/627023.
- Koopman FA, Miljko S, Grazio S, et al. Pilot study of stimulation of the cholinergic anti-inflammatory pathway with an implantable vagus nerve stimulation device in patients with rheumatoid arthritis [abstract]. *Arthritis Rheum* 2012;64(Suppl. 10):451.
- Zitnik RJ. Treatment of chronic inflammatory diseases with implantable medical devices. *Cleve Clin J Med* 2011;78(Suppl. 1):S30–S34.
- Pavlov VA, Wang H, Czura CJ, Friedman SG, Tracey KJ. The cholinergic anti-inflammatory pathway: a missing link in neuroimmunomodulation. *Mol Med* 2003;9: 125–134.
- Rosas-Ballina M, Ochani M, Parrish WR. Splenic nerve is required for cholinergic anti-inflammatory pathway control of TNF in endotoxemia. *Proc Natl Acad Sci USA* 2008; 105:11008–11013.
- Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale discovery, for human cytokine profiling. *J Immunol Methods* 2009;340:55–64.
- Zhang Y, Popovic ZB, Bibevski S, et al. Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model. *Circ Heart Fail* 2009;2:692–699.
- Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. *Nat Rev Immunol* 2013;13:190–198.
- Haus E. Chronobiology of the immune system. In: Committee on Military Nutrition Research, Food and Nutrition Board, Institute of Medicine, ed. *Military strategies for sustainment of nutrition and immune function in the field*. Washington, DC: National Academy Press, 1999:437–496. http://books.google.com/books?id=V_ZJkM024ooC&pgis=1
- Sabat R, Grütz G, Warszawska K, et al. Biology of interleukin-10. *Cytokine Growth Factor Rev* 2010;21:331–344.
- Murray PJ. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. *Proc Natl Acad Sci USA* 2005;102: 8686–8691. doi: 10.1073/pnas.0500419102.
- de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE. Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 1991;174:1209–1220.

COMMENTS

The purpose of this pilot study was to determine if VNS modulates the peripheral immune system (β and $\text{TNF}\alpha$) in whole blood culture when compared to sham stimulation. These data have potential therapeutic implications in the domain of inflammatory disorders based on the anti-inflammatory properties of the VN.

Indeed, the VN activates the hypothalamic-pituitary-adrenal axis through the activation of vagal afferents by peripheral pro-inflammatory cytokines that convey the peripheral immune information to the nucleus tractus solitarius (NTS) in the brainstem thus activating noradrenergic neurons in the NTS that project to the paraventricular nucleus of the hypothalamus around corticotrophin-releasing (CRF) neurons. The release of CRF will then activate the release of ACTH by the hypothalamus then stimulating the release of glucocorticoids by the adrenal glands [1]. It is termed the neuro-endocrine-immune axis. More recently, an anti-inflammatory role of vagal efferents has been described by the group of KJ Tracey. Using a model of endotoxic shock in rats [2], they showed that the release of $\text{TNF}\alpha$ was increased both in the blood and in the liver and this $\text{TNF}\alpha$ level was increased in vagotomized animals. In contrast, when performing VNS of the distal end cut of the VN, activating for sure vagal efferents, they dampened the release of $\text{TNF}\alpha$. They argue for an inflammatory reflex as represented by a vago-vagal reflex, called the cholinergic anti-inflammatory pathway [see for review Ref. 3]. They showed that this anti-inflammatory reflex was mediated through the binding of acetylcholine (ACh), the neuromediator of the VN, with $\alpha 7$ -nicotinic ACh receptors ($\alpha 7\text{nAChR}$) of macrophages since this effect was blunted in $\alpha 7$ knockout animals [3]. This group also argues for an interaction of the VN with the sympathetic splenic nerve that innervates the spleen. Activation of the VN was supposed to inhibit the release of $\text{TNF}\alpha$ by spleen macrophages where norepinephrine released at the distal end of the splenic nerve was able to act on $\beta 2$ adrenergic receptor of spleen lymphocytes to release ACh then activating $\alpha 7\text{nAChR}$ of spleen macrophages to inhibit the release of $\text{TNF}\alpha$ [3]. This anti-inflammatory spleen pathway has been revisited recently by Martelli et al. [4]. Another pathway could be the activation of sympathetic nerves by the central autonomic nervous system through the integration of peripheral inflammatory signals in the brain by vagal afferents then stimulating descending pathways that activate the spinal pre-ganglionic sympathetic neurons [5]. Thus the VN has an anti- $\text{TNF}\alpha$ effect and its activation by VNS, originally used to treat drug-resistant epilepsy through stimulation of vagal afferents, could be used in $\text{TNF}\alpha$ -mediated diseases such as inflammatory bowel diseases (IBD; Crohn's disease and ulcerative colitis), rheumatoid arthritis, psoriasis as well as other inflammatory conditions such as pancreatitis and postoperative ileus [6]. Most of these diseases are characterized by an imbalance of the autonomic nervous system as represented by a blunted vagal tone in IBD [7]. Thus VNS, by restoring a normal vagal tone, could be an interesting tool in the armamentarium to treat such diseases. In particular, VNS could be an alternative non-drug therapy to classical anti- $\text{TNF}\alpha$ drugs. Invasive VNS used in the treatment of drug resistant epilepsy is performed through a spiral electrode wrapped around the left cervical VN, because the right VN innervates the sinoatrial node (involved in the pace-maker function of the heart), then tunneled subcutaneously to and connected with a pulse generator located in the left chest wall [6]. The implantation (~1h duration) is performed under general anesthesia generally by neurosurgeons familiar with epilepsy. The VNS device is manufactured by Cyberonics (Houston, TX, USA). Using this device and based on preliminary data in animals with colitis [8], we reported the first case of VNS in a patient with Crohn's disease [9], and we have very recently shown that VNS could be of interest in mild to moderate Crohn's disease [10]. Non-invasive VNS eliminat-

ing the need for surgical implantation and thus improving the safety and tolerability of VNS is of interest. The cymba concha of the external ear is innervated exclusively by the auricular branch of the VN that projects to the NTS. Transcutaneous auricular VNS (ta-VNS), by activating the inflammatory reflex could be used in the management of inflammatory disorders. Ta-VNS has been shown to protect endotoxemic rat from lipopolysaccharide-induced inflammation [11]. Ta-VNS using the device NEMOS (Cerbomed, Erlangen, Germany) has been used in the management of refractory epilepsy [12]. GammaCore (electroCore LLC, Basking Ridge, NJ, USA) is another non-invasive VNS that uses proprietary electrical signals to treat primary headache [13]. It consists of a portable stimulator with a battery, signal-generating and -amplifying electronics and a digital control user interface that controls signal amplitude. Two stainless steel round discs function as skin contact surfaces that deliver a proprietary, low-voltage electrical signal to the cervical VN. Such a device could be used in the treatment of inflammatory disorders.

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REFERENCES

1. Fleshner M, Goehler LE, Hermann J, Relton JK, Maier SF, Watkins LR. Interleukin-1 beta induced corticosterone elevation and hypothalamic NE depletion is vagally mediated. *Brain Res Bull* 1995, 37:605–10.
2. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000, 405:458–62.
3. Pavlov V, Tracey KJ. Neural circuitry and immunity. *Immunol Rev* 2015, 63:38–57.
4. Martelli D, McKinley MJ, McAllen RM. The cholinergic anti-inflammatory pathway: a critical review. *Auton Neurosci* 2014, 182:65–9.
5. Saper CB. The central autonomic nervous system: conscious visceral perception and autonomic pattern generation. *Annual Review of Neuroscience* 2002, 25:433–69.
6. Bonaz B, Picq C, Sinniger V, Mayol JF, Clarençon D. Vagus nerve stimulation: from epilepsy to the cholinergic anti-inflammatory pathway. *Neurogastroenterol Motil* 2013, 25:208–21.
7. Pellissier S, Dantzer C, Mondillon L, Trocme C, Gauchez AS, Ducros V, Mathieu N, Toussaint B, Fournier A, Canini F, Bonaz B. Relationship between vagal tone, cortisol, $\text{TNF}\alpha$, epinephrine and negative affects in Crohn's disease and irritable bowel syndrome. *PLoS One* 2014, 9:e105328.
8. Meregnani J, Clarençon D, Vivier M, Peinnequin A, Mouret C, Sinniger V, Picq C, Job A, Canini F, Jacquier-Sarlin M, Bonaz B. Anti-inflammatory effect of vagus nerve stimulation in a rat model of inflammatory bowel disease. *Auton Neurosci* 2011, 160:82–9.
9. Clarençon D, Pellissier S, Sinniger V, Kibleur A, Hoffman D, Vercueil L, David O, Bonaz B. Long term effects of low frequency (10 hz) vagus nerve stimulation on EEG and heart rate variability in Crohn's disease: a case report. *Brain Stimul* 2014, 7:914–6.
10. Bonaz B, Sinniger V, Hoffmann D, Clarençon D, Mathieu N, Dantzer C, Vercueil L, Picq C, Trocme C, Faure P, Cracowski JL, Pellissier S. Chronic vagus nerve stimulation in Crohn's disease: a 6-month follow-up pilot study. *Neurogastroenterol Motil* 2016 (in press).
11. Zhao YX, He W, Jing XH, Liu JL, Rong PJ, Ben H, Liu K, Zhu B. Transcutaneous auricular vagus nerve stimulation protects endotoxemic rat from lipopolysaccharide-induced inflammation. *Evid Based Complement Alternat Med* 2012, 2012:627023.

12. Rong P, Liu A, Zhang J, Wang Y, He W, Yang A, Li L, Ben H, Li L, Liu H, Wu P, Liu R, Zhao Y, Zhang J, Huang F, Li X, Zhu B. Transcutaneous vagus nerve stimulation for refractory epilepsy: a randomized controlled trial. *Clin Sci (Lond)*. 2014 Apr 1. [Epub ahead of print].
13. Nesbitt AD, Marin JC, Tompkins E, Rutledge MH, Goadsby PJ. Initial use of a novel noninvasive vagus nerve stimulator for cluster headache treatment. *Neurology* 2015, 84:1249–53.

Comments not included in the Early View version of this paper.