Low-level tragus stimulation improves autoantibody-induced hyperadrenergic postural tachycardia syndrome in rabbits

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BACKGROUND Recent studies have demonstrated that antiadrenergic autoantibodies are involved in the pathophysiology of postural orthostatic tachycardia syndrome (POTS).

OBJECTIVE The purpose of this study was to test the hypothesis that transcutaneous low-level tragus stimulation (LLTS) ameliorates autoantibody-induced autonomic dysfunction and inflammation in a rabbit model of autoimmune POTS.

METHODS Six New Zealand white rabbits were co-immunized with peptides from the α1-adrenergic and β1-adrenergic receptors to produce sympathomimetic antibodies. The tilt test was performed on conscious rabbits before immunization, 6 weeks after immunization, and 10 weeks after immunization with 4-week daily LLTS treatment. Each rabbit served as its own control.

RESULTS An enhanced postural heart rate increase in the absence of significant change in blood pressure was observed in immunized rabbits, confirming our previous report. Power spectral analysis of heart rate variability during the tilt test showed a predominance of sympathetic over parasympathetic activity in immunized rabbits as reflected by markedly increased low-frequency power, decreased high-frequency power, and increased low-to-high-frequency ratio. Serum inflammatory cytokines were also significantly increased in immunized rabbits. LLTS suppressed the postural tachycardia, improved the sympathovagal balance with increased acetylcholine secretion, and attenuated the inflammatory cytokine expression. Antibody production and activity were confirmed with in vitro assays, and no antibody suppression by LLTS was found in this short-term study.

CONCLUSION LLTS improves cardiac autonomic imbalance and inflammation in a rabbit model of autoantibody-induced hyperadrenergic POTS, suggesting that LLTS may be used as a novel neuromodulation therapy for POTS.

KEYWORDS Autoantibody; Autonomic nervous system; Heart rate variability; Inflammatory cytokines; Low-level transcutaneous vagus nerve stimulation; Postural orthostatic tachycardia syndrome

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Introduction

Postural orthostatic tachycardia syndrome (POTS) is a heterogeneous autonomic disorder characterized by excessive orthostatic tachycardia in the absence of orthostatic hypotension.[1] Multiple pathophysiologic mechanisms have been proposed to explain POTS, including autonomic neuropathy, hypovolemia, impaired vasoconstriction, and hyperadrenergic state.[2,3] In recent years, autoimmunity has drawn attention as a potential cause of POTS in some patients.[4,5] We and others have identified the presence of antiadrenergic autoantibodies in a subgroup of POTS patients.[6–8] These autoantibodies act both as direct agonists and more importantly as allosteric modulators to alter receptor function. We have demonstrated that autoantibodies to the α1-adrenergic receptor (α1AR) and β1-adrenergic receptor (β1AR) from POTS patients exerted an inhibitory and facilitatory allosteric effect respectively on the orthosteric ligand responses in vitro.[6,7] Induction of these antibodies with similar functional properties produced a hyperadrenergic POTS phenotype in a rabbit autoimmune model,[9] suggesting that the copresence of the 2 sympathomimetic antibodies is capable of causing an excessive increase in adrenergic activity frequently associated with POTS.

Vagus nerve stimulation is an emerging therapeutic modality for cardiovascular disease, such as heart failure, hypertension, and atrial fibrillation.[10,11] Low-level tragus stimulation (LLTS), a noninvasive approach to stimulate the auricular branch of the vagus nerve, has shown promise in improving cardiovascular autonomic function as well as suppressing inflammation in both animal and human studies.[12,13] In a recent report, we showed that LLTS significantly attenuated muscarinic autoantibody-mediated

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KEY FINDINGS

- Adrenergic autoantibodies caused sympathetic overdrive and increased inflammatory cytokines in a rabbit model of autoimmune postural orthostatic tachycardia syndrome.
- Neuromodulation with low-level tragus stimulation suppressed autoantibody-induced postural tachycardia, improved sympathovagal balance, and decreased inflammatory cytokines.
- These data suggest that noninvasive low-level tragus stimulation may offer a therapeutic advantage in the treatment of postural orthostatic tachycardia syndrome, especially the hyperadrenergic subtype.

cardiovagal dysfunction and inflammation in a new rabbit model of autoimmune POTS.[14] The purpose of the present study was to investigate whether the same LLTS treatment could alter sympathovagal imbalance and postural tachycardia in an established animal model of adrenergic autoantibody-induced POTS.

Methods

This study protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunization, LLTS, and tilt testing

Six New Zealand white rabbits were immunized and boosted at 2 and 4 weeks with peptides derived from α1AR and β1AR to produce sympathomimetic antibodies as previously described.[9] LLTS (20 Hz frequency, 0.2 ms pulse duration, 2 mA amplitude) was delivered to the bilateral tragus over the auricular concha region with 2 magnet electrodes connected to a TENS device (InTENSity Twin Stim; TENSPros, St Louis, MO) as described.[14] The stimulation, starting at week 6, was administered to conscious rabbits placed in a restrainer for 30 minutes daily for 4 weeks.

The tilt test was performed on conscious rabbits as previously described.[9,14] Briefly, the rabbit was placed in a restrainer secured to a tilt platform. A 3-lead electrocardiogram and an arterial catheter connected to a pressure transducer were applied to measure heart rate (HR) and intra-arterial blood pressure (BP) at horizontal and 60° head-up tilt positions. The HR and BP values before and after tilting were recorded and analyzed using the PowerLab data acquisition system and LabChart data analysis software (AD Instruments, Colorado Springs, CO). The tilt test was performed before immunization, 6 weeks after immunization, and 10 weeks after immunization with 4-week daily LLTS treatment (Figure 1). Each rabbit served as its own control. Pre- and postimmune sera were collected from all animals for immunoassays and bioassays.

HR variability

Cardiac autonomic activity was evaluated by HR variability (HRV) power spectral analysis in the frequency domain using an autoregressive algorithm.[15] A 5-minute electrocardiography segment obtained at horizontal and tilt positions was used for HRV analysis, including low-frequency (LF) power (0.04–0.15 Hz), high-frequency (HF) power (0.15–0.4 Hz), and LF-to-HF ratio. These values were measured and calculated as previously described.[14]

Enzyme-linked immunosorbent assay and cell-based antibody bioassays

Antibodies produced in the rabbit sera were detected by enzyme-linked immunosorbent assay (ELISA) as described.[16] Briefly, ELISA plates were coated with the α1AR or β1AR immunogenic peptides at 10 μg/mL. Sera were diluted 1:10,000, and goat anti-rabbit IgG conjugated with alkaline phosphatase and its substrate paranitrophenyl phosphate 104 were used to detect antibody binding. The optical density values were read at 405 nm at 60 minutes.

Rabbit antibody activity was assessed by serum-induced α1AR and β1AR activation in the GeneBLAzer FRET-based β-lactamase reporter assay (Invitrogen, Waltham, MA) as described.[9] Briefly, α1AR- or β1AR-NFAT-bla CHO-K1 cells were plated and incubated with rabbit sera (1:50), followed by incubation with the β-lactamase substrate CCF4-AM. Data were read and calculated as the ratio of the emissions 460 (blue) and 530 (green) nm after subtraction of the background values and expressed as fold increase over buffer baseline.

Serum acetylcholine and inflammatory cytokine assays

The levels of acetylcholine and inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and interferon γ (IFN-γ) in rabbit sera were measured using ELISA kits from LifeSpan Biosciences (Seattle, WA) and MyBioSource (San Diego, CA), respectively, according to the manufacturers' instructions.

Statistics

Data are expressed as mean ± SD. Comparison between 2 groups was performed by Student's *t* test. One-way analysis of variance followed by Newman-Keuls post hoc test was used for multiple group comparisons. Statistical significance was set at $P < .05$.

Results

Cardiovascular and HRV responses to tilting

The HR, BP, and HRV parameters during the tilt test were measured before immunization, 6 weeks after immunization, and 10 weeks after immunization with 4-week LLTS treatment. The pretilt HR was comparable with no significant differences across the 3 time periods, suggesting that immunization or LLTS had no significant impact on resting baseline HR. In contrast, tilting resulted in a significantly

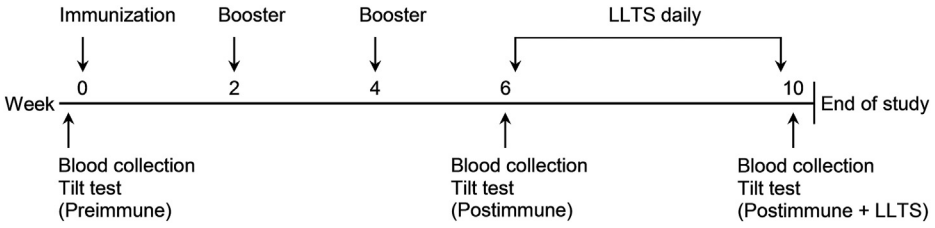

Figure 1 Protocol depiction of the procedures performed on the rabbit. LLTS = low-level tragus stimulation.

greater percent increase in HR at postimmunization compared with preimmunization (33.8 ± 5.0% vs 23.3 ± 5.6%; $P < .05$), which is consistent with our previous findings in this rabbit model of autoimmune POTS.[9] This enhanced postural HR increase was suppressed by LLTS (33.8 ± 5.0% vs 19.1 ± 3.4%; $P < .01$) (Figure 2A). No significant differences in BP response to tilting were observed between the 3 time periods (Figure 2B).

Cardiac autonomic activity was assessed by HRV power spectral analysis. No significant differences were found for the HRV parameters obtained at the 3 study periods before tilting (Figure 3A). The HRV response to tilting, however, showed distinct differences. At postimmunization, tilting caused a greater increase in the LF power, a greater decrease in the HF power, and consequently, a greater increase in the LF-to-HF ratio compared with preimmunization (LF power: preimmune 0.27 ± 0.05 normalized units [n.u.] vs postimmune 0.50 ± 0.06 n.u.; $P < .05$; HF power: preimmune 0.50 ± 0.06 vs postimmune 0.26 ± 0.07 n.u.; $P < .01$; LF-to-HF ratio: preimmune 0.54 ± 0.11 vs postimmune 1.93 ± 0.12; $P < .01$) (Figure 3B), all of which indicate a predominance of sympathetic over parasympathetic activity.[15] These altered changes in the HRV response to tilting were diminished by LLTS (LF power: 0.37 ± 0.07 n.u.; $P < .05$ vs postimmune; HF power: 0.40 ± 0.06 n.u.; $P < .05$ vs postimmune; LF-to-HF ratio: 0.93 ± 0.09; $P < .01$ vs postimmune) (Figure 3B), suggesting an improved sympathovagal balance following LLTS treatment.

Antibody production and activity

Sera collected at the 3 time periods were tested by ELISA and cell-based bioassay to confirm production of functionally active antibodies after immunization and to examine the effect of LLTS on antibody suppression. All immunized rabbits had significantly elevated antibody ELISA optical density values for both α1AR and β1AR compared with preimmunization (anti-α1AR: 1.003 ± 0.242 vs 0.142 ± 0.005; $P < .01$; anti-β1AR: 1.001 ± 0.178 vs 0.029 ± 0.003; $P < .01$) (Figures 4A and 4B). A similar increase in serum antibody activity was observed in immunized animals. Compared with preimmune sera, postimmune sera induced significant activation of α1AR and β1AR (anti-α1AR: 2.79 ± 0.34- vs 2.13 ± 0.42-fold increase over baseline; $P < .01$; anti-β1AR: 2.44 ± 0.60- vs 1.88 ± 0.37-fold increase over baseline; $P < .05$) (Figures 4C and 4D). After LLTS treatment, there were no significant changes in the mean ELISA values (anti-α1AR: 0.806 ± 0.068; $P = .28$ vs postimmune; anti-β1AR: 0.858 ± 0.175; $P = .45$ vs postimmune) (Figures 4A and 4B) and antibody activity (anti-α1AR: 2.71 ± 0.39-fold increase over baseline; $P = .14$ vs postimmune; anti-β1AR: 2.30 ± 0.57-fold increase over baseline; $P = .23$ vs postimmune), suggesting that short-term LLTS had no significant impact on suppressing antibody production and activity.

Levels of serum acetylcholine and inflammatory cytokines

Serum concentrations of acetylcholine and inflammatory cytokines TNF-α, IL-1β, and IFN-γ in the rabbit were measured using the ELISA kits to examine the impact of functional adrenergic antibodies and LLTS on the inflammatory reflex. No significant change in acetylcholine level was found after immunization (38.4 ± 2.7 pg/mL vs preimmune 37.9 ± 1.9 pg/mL; $P = .54$). LLTS, on the other hand, produced an increase in serum acetylcholine (44.7 ± 4.6 pg/mL; $P < .05$ vs pre/postimmune) (Figure 5). There was a significant rise in the levels of all 3 measured inflammatory cytokines at postimmunization compared with the preimmune levels (TNF-α: 29.7 ± 5.5 pg/mL vs 7.5 ± 1.4 pg/mL; $P < .01$; IL-1β: 20.9 ± 4.0 pg/mL vs 6.6 ± 1.4 pg/mL; $P < .01$; IFN-γ: 12.9 ± 2.2 pg/mL vs 4.7 ± 1.0 pg/mL; $P < .01$). These increases were markedly suppressed by LLTS (TNF-α: 12.2 ± 2.1 pg/mL; IL-1β: 11.7 ± 2.2 pg/mL; IFN-γ: 8.2 ± 1.3 pg/mL; $P < .01$ vs postimmune) (Figure 6). These data indicate an increased inflammatory response associated with autoantibody-induced sympathetic overactivity, which can be attenuated by LLTS-stimulated acetylcholine release.

Discussion

Hyperadrenergic POTS is a subtype of POTS characterized by sympathetic overactivity with elevated upright plasma catecholamine levels.[2] Our findings of differential antiadrenergic autoantibodies in POTS might help explain the sympathetic overdrive in POTS patients who harbor these autoantibodies.[6,7] In contrast to natural physiological ligands, these autoantibodies do not induce significant receptor desensitization that would otherwise mitigate their activity. This lack of desensitization has been recognized as a characteristic feature of functional autoantibodies targeting G protein–coupled receptors.[17,18] The allosteric inhibitory effect of α1AR antibodies on norepinephrine action is expected to cause an impaired pressor response, which then leads to a

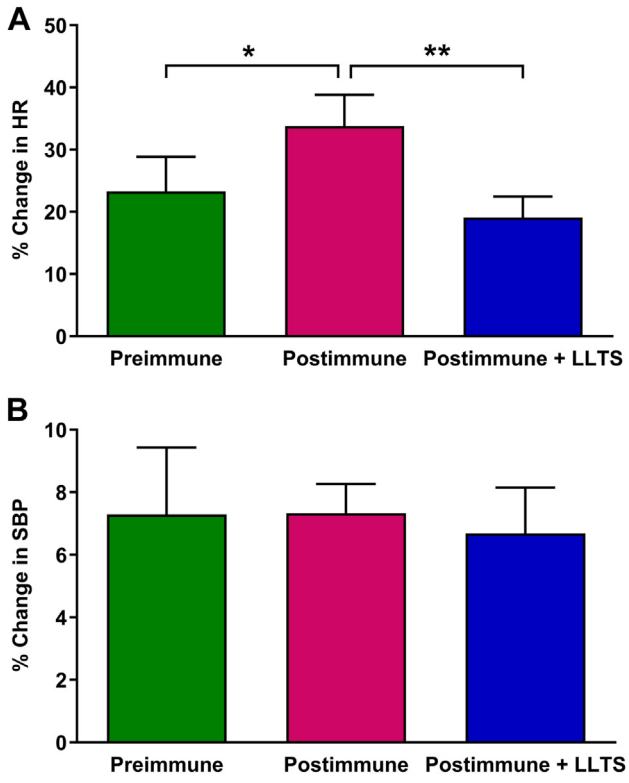

Figure 2 Heart rate (HR) and blood pressure responses to tilting in the rabbit. **A:** There was a greater percent increase in HR upon tilting at postimmunization compared with preimmunization. Low-level tragus stimulation (LLTS) effectively suppressed this enhanced postural HR response. **B:** No significant change in systolic blood pressure (SBP) upon tilting was found before and after immunization and subsequent LLTS treatment. $*P < .05$, $**P < .01$. n = 6.

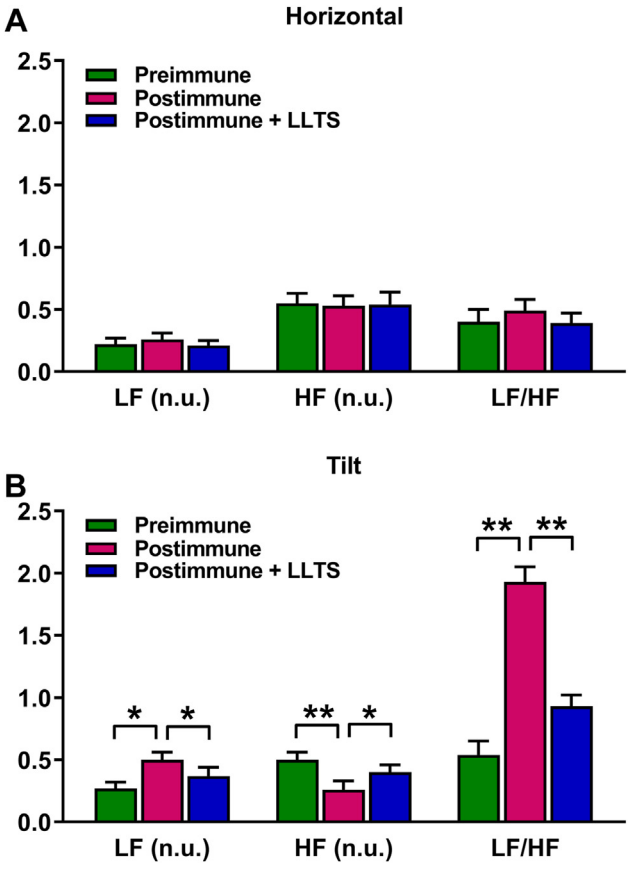

Figure 3 Heart rate variability (HRV) response to tilting in the rabbit. HRV parameters including the low-frequency (LF) and high-frequency (HF) components, both expressed in normalized units (n.u.), and LF-to-HF ratio were measured before and after tilting. **A:** No significant differences in the HRV parameters obtained at preimmunization, postimmunization, and post–low-level tragus stimulation (LLTS) were observed before tilting. **B:** At postimmunization, the rabbits demonstrated higher LF and LF-to-HF ratio and lower HF values upon tilting compared with preimmunization. LLTS diminished these changes. $*P < .05$, $**P < .01$. n = 6.

compensatory increase in the sympathetic output in an effort to normalize vasoconstriction and BP. This increased adrenergic activity along with the facilitatory β1AR antibodies would result in an overstimulation of cardiac β1AR with an enhanced tachycardia. This hypothesis was supported by the observation that α1AR and β1AR co-immunized rabbits exhibited an attenuated BP response and a heightened HR response to α1AR and β1AR agonist infusions, respectively, in an in vivo model of autoimmune POTS.[9]

In the present study, we used HRV analysis to assess cardiac autonomic dysfunction associated with α1AR and β1AR antibodies in immunized rabbits. As previously reported, α1AR and β1AR co-immunization produced an enhanced postural HR increase in the absence of significant BP change in the rabbit, confirming a POTS-like phenotype of this model.[9] No significant changes in the HRV parameters were found at recumbency. However, upon tilting, immunized animals showed an altered sympathovagal balance with sympathetic dominance as indicated by the reduced HF power and increased LF power and LF-to-HF ratio. These findings are consistent with clinical observations that POTS patients present a lower HF power and a higher LF-to-HF ratio than control subjects during head-up tilt.[19,20] Production of receptor-activating antibodies to the α1AR and β1AR in immunized rabbits was confirmed by the in vitro assays.

Despite many years of research, POTS remains difficult to treat. There are no widely accepted treatment algorithms or consensus on the management strategy for different subtypes of POTS.[1,2] Pharmacologic treatment of hyperadrenergic POTS primarily focuses on suppressing sympathetic activity or increasing parasympathetic activity. β-blockers and acetylcholinesterase inhibitor pyridostigmine can help control symptoms in POTS by antagonizing sympathetic overactivity, but their use is limited by inconsistent success and side effects.[2] More recently, transcutaneous vagus nerve stimulation, a noninvasive neuromodulation approach, has gained increased attention as a promising new paradigm for the treatment of POTS.[21] The efficacy of this treatment for POTS is currently under evaluation in several clinical trials (NCT02281097, NCT03124355, NCT04632134, and NCT05043051). With this intervention, we demonstrated that daily LLTS for 4 weeks effectively suppressed postural tachycardia, reduced sympathetic activation, and improved sympathovagal balance in immunized animals. Acetylcholine from vagal stimulation

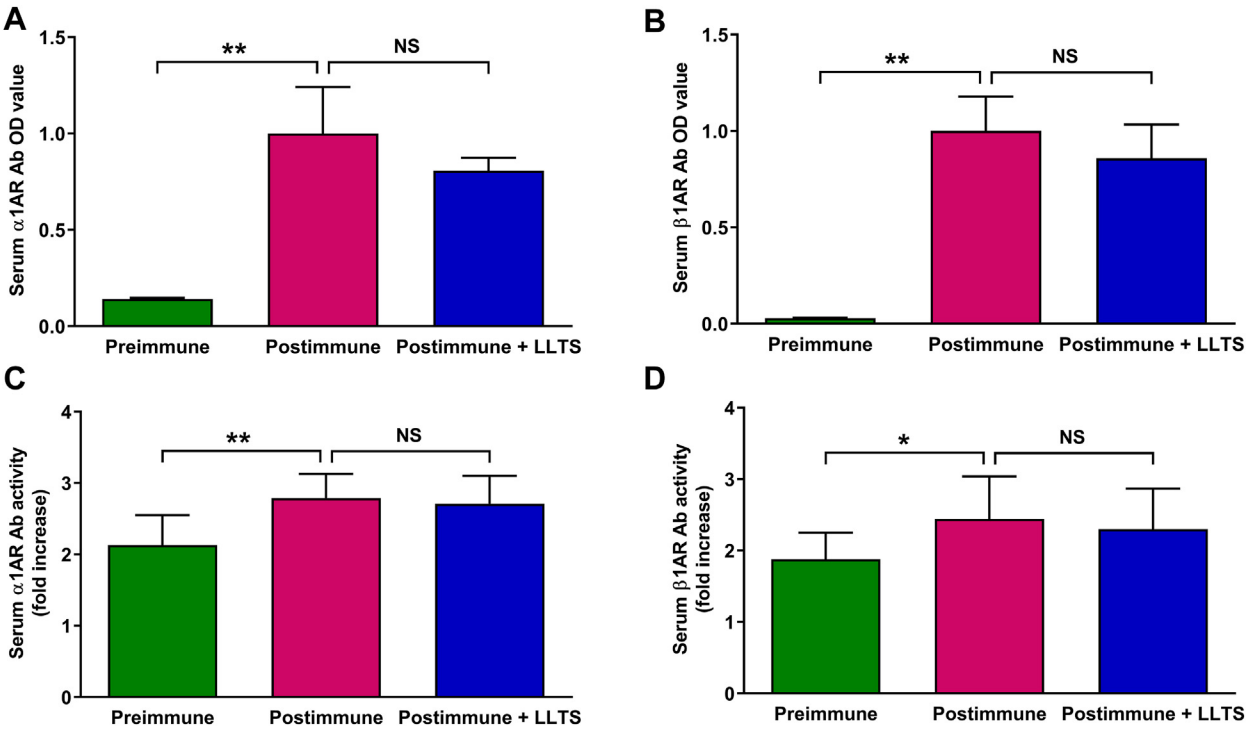

Figure 4 Production of active antibodies to the α1-adrenergic receptor (α1AR) and β1-adrenergic receptor (β1AR) in the rabbit. High antibody levels to both α1AR (**A**) and β1AR (**B**) were detected in the postimmune sera by enzyme-linked immunosorbent assay. Functional activity of serum antibodies, expressed as fold increase over buffer baseline, was confirmed by postimmune serum-induced activation of α1AR **(C)** and β1AR **(D)** in cell-based bioassays. There were no significant changes in the antibody expression and activity levels after low-level tragus stimulation (LLTS). $*P < .05$, $**P < .01$. n = 6. Ab, antibody; NS = not significant; OD = optical density.

has been shown to produce cardioprotective effects in animal studies.[22,23] The increased acetylcholine levels after LLTS observed in this rabbit model suggest that LLTS improves the POTS phenotypes similarly by increasing acetylcholine secretion from cholinergic neurons.

There is some evidence supporting an inflammatory component associated with increased sympathetic activity in POTS.[24] Elevated inflammatory biomarkers have been found in POTS patients with adrenergic autoantibodies,[25] suggesting that chronic autoimmune activation and inflammation may contribute to a hyperadrenergic state. In line with these clinical findings, a significant increase in inflammatory cytokines including TNF-α, IL-1β, and IFN-γ was observed in this rabbit model of autoimmune POTS. It is well known that adrenergic receptors are also expressed in both innate and adaptive immune cells, and adrenergic activation can increase production of proinflammatory cytokines.[26] The presence of functional antiadrenergic autoantibodies in POTS would be expected to interact with the immune and inflammatory pathways. β1AR autoantibodies from patients with cardiomyopathy or heart failure have been shown to induce T cell proliferation and increased release of proinflammatory cytokines such as TNF-α from macrophages.[27,28] Moreover, α1AR autoantibodies from patients with hypertension activate the transcription factor nuclear factor-kappa B that may increase inflammatory responses.[29] These autoantibodies therefore would be involved not only in regulation of chronotropic and contractile responses, but also in gene regulation of the immune and inflammatory responses, which may play an additional role in disease pathogenesis.

Vagal activation has been known to suppress proinflammatory cytokine production through cholinergic stimulation of the α7 nicotinic acetylcholine receptor of the immune cells in spleen and other organs.[30] The discovery that vagus nerve stimulation attenuates systemic inflammation has led to extensive investigations of the cholinergic anti-inflammatory pathway.[31] Many studies support a positive effect of transcutaneous auricular vagus nerve stimulation in treating autoimmune and inflammatory conditions.[32,33] Moreover, clinical studies on cardiovascular diseases, such as atrial fibrillation and heart failure, also provided evidence for both anti-inflammatory and antiarrhythmic effects of LLTS.[34–36] In this study, we observed a similar decrease in inflammatory cytokines in immunized animals with LLTS treatment. As previously reported in a similar study,[14] there was no change in antibody levels after LLTS treatment due to the short duration of the study. Further clinical and experimental studies are warranted to assess the long-term effect of LLTS on suppressing autoimmunity and inflammation and ameliorating autonomic dysfunction in POTS.

Clinical implications

POTS is a heterogeneous disease, with multiple pathophysiologic mechanisms.[2,37] Management of POTS remains challenging, and pharmacologic treatment is often limited by lack

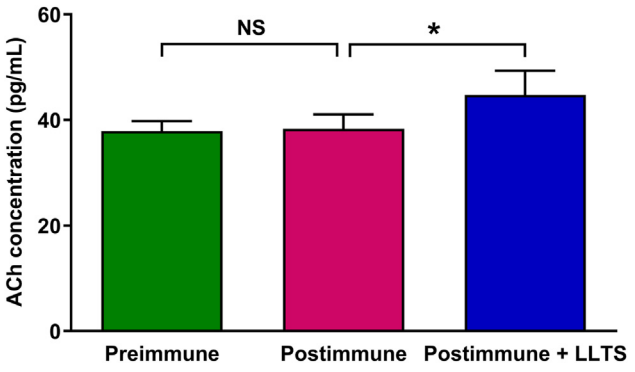

Figure 5 Acetylcholine (ACh) levels in the rabbit sera measured by enzyme-linked immunosorbent assay. No significant change in the serum ACh concentration was found after immunization. However, low-level tragus stimulation (LLTS) caused an increase in ACh production. $*P < .05$. n = 6. NS = not significant.

of efficacy or side effects.[37] Our data support the concept of autoantibody-induced hyperadrenergic state and inflammation in POTS, lending credence to the notion of individualization of therapies for each patient.[2] Importantly, these data suggest that the use of LLTS is a safe, effective, and noninvasive intervention for the treatment of POTS. LLTS has the potential to make a significant impact in light of the limited success of the currently available treatment options, especially those with the hyperadrenergic phenotype. Clinical trials testing this notion are ongoing (NCT02281097, NCT03124355, NCT04632134, and NCT05043051).

Limitations

There are several limitations to the present study, including a small sample size, short study duration, and lack of sham intervention. While these limitations should be acknowledged, it is important to note that this work was intended to provide a prototypical animal model of autoimmune hyperadrenergic POTS and evaluate the therapeutic potential of LLTS for the treatment of POTS. Our data suggest that with the noninvasive nature and ability to suppress sympathetic overactivity and inflammation, LLTS may offer a therapeutic advantage in the treatment of POTS. The rabbit model provides a convenient means for induction of autoantibodies and has proven useful for examination of cardiac arrhythmias associated with autoantibodies. However, the hemodynamic parameters including HRV are different between the rabbit and human.[38] Therefore, species differences should be taken into consideration when the efficacy of LLTS in this animal model and human POTS is evaluated and compared. Further studies of sufficient size and duration with appropriate sham controls are needed to confirm these promising findings. Assessment of BP variability, which can provide further insight into cardiovascular autonomic modulation, will also be of interest.

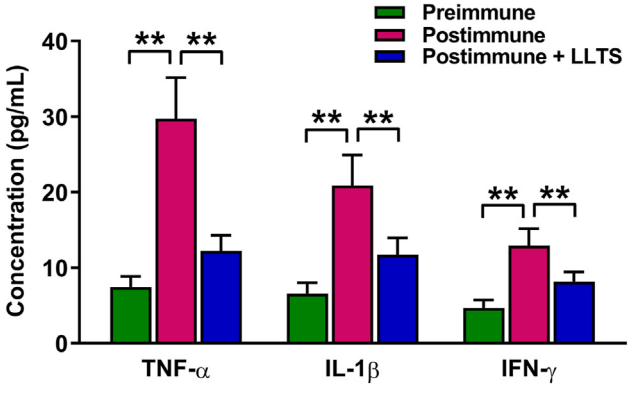

Figure 6 Serum levels of inflammatory cytokines in the rabbit measured by enzyme-linked immunosorbent assay. Serum concentrations of the inflammatory cytokines tumor necrosis factor α (TNF-α), interleukin (IL)-1β, and interferon γ (IFN-γ) were significantly increased after immunization. Low-level tragus stimulation (LLTS) largely suppressed these elevated cytokines. $**P < .01$. n = 6.

Conclusion

Neuromodulation with LLTS improved cardiac autonomic function in a recently developed rabbit model of antiadrenergic autoimmune POTS by normalizing sympathovagal balance and decreasing inflammatory cytokines. These findings provide the basis for further studies exploring the effectiveness of noninvasive neuromodulation in the treatment of POTS.

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Authorship: All authors attest they meet the current ICMJE criteria for authorship.

Ethics Statement: This study protocol was approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center, and conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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