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Orexinergic Activity Modulates Altered Vital Signs and Pituitary Hormone Secretion in Experimental Sepsis

Clifford S. Deutschman, MS, MD, Nichelle R. Raj, MS, Erin O. McGuire, BS, Max B. Kelz, MD, PhD

Department of Anesthesiology and Critical Care and the Sepsis Research Program, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Abstract

Objectives: Sepsis is a common, lethal poorly understood disorder affecting nearly a million Americans annually. The syndrome is characterized by altered cardiodynamics, respiration, metabolism, pituitary function, arousal, and impaired interaction among organ systems. The immunologic and endocrine systems, which are in part responsible for organ–organ communication, have been studied extensively in sepsis. However, little is known about sepsis-induced changes in central nervous system activity.

Hypothesis: A defect in hypothalamic neurons secreting the neurotransmitter orexin modulates physiologic derangements in sepsis.

Design: Animal study.

Setting: University Research Laboratory.

Interventions: Male C57Bl6 mice were made septic using cecal ligation and puncture. Data were collected 24 or 48 hours later, blood was collected, animals were killed, and brain tissue was harvested, fixed, and sectioned. Hypothalamic sections were subjected to immunohistochemistry using antibodies to orexin and c-Fos, a marker of neuronal activity. In a separate cohort of mice, cannulas were placed in the right lateral cerebral ventricle. Cecal ligation and puncture was performed 1 week later. At 24 or 48 hours post–cecal ligation and puncture, vital signs were measured, and 1 μ L of saline with or without 3 nmol orexin-A was infused. Vital signs were repeated at 25 or 49 hours post–cecal ligation and puncture, blood was collected, animals were killed, and brains were removed, fixed, sectioned, and stained.

Measurements and Main Results: Orexinergic activity decreased six-fold following cecal ligation and puncture. This change was associated with decreases in arousal, temperature, and heart and respiratory rates. Levels of selected pituitary hormones increased 24 hours post–cecal ligation and puncture but were significantly lower than baseline at 48 hours. Injection of orexin-A increased vital signs to baseline levels. Hormone levels were unaffected at 25 hours but increased to supranormal levels at 49 hours.

Conclusions: Sepsis-induced changes in activity, vital signs, and pituitary hormones are modulated by the orexinergic system. This finding implicates central nervous system dysfunction

in the pathogenesis of sepsis, suggesting further study of neurological dysfunction to identify novel approaches to management.

Keywords

central nervous system; cecal ligation and puncture; hypothalamus; orexin-A; vital signs

Sepsis is the leading cause of death in critically ill patients. The disorder accounts for more than 200,000 deaths in the United States each year and millions of fatalities worldwide (1). Although mortality during acute hospitalization for sepsis has decreased, we have become increasingly aware of the long-term consequences of this disorder. About 75% of septic patients die within 5 years of their initial hospitalization (2, 3). Those who survive may suffer from life-altering respiratory, neurological, motor, and cognitive abnormalities (4–6). Further, the prevalence of sepsis is increasing; in 2008, nearly 1 million Medicare beneficiaries were acutely hospitalized for severe sepsis, up from 387,000 in 1996 (2, 3). The cost of caring for these patients is escalating; Medicare spending on hospitalization for severe sepsis in the same period increased from \$6.03 to \$15.75 billion (2, 3). Clearly, sepsis represents a public health problem of enormous scope and importance.

Some years ago, Godin and Buchman hypothesized that sepsis impaired normal, low-level, minute-to-minute biological variability (7). Loss of these small changes, which are responsible for the coordination and “fine-tuning” of cell-to-cell and organ-to-organ interactions, were hypothesized to reflect impairments in the endocrine, humoral, and neuronal pathways, which modulate organ/organ interaction. Such a defect might explain how septic shock or severe sepsis progress to the multiple organ dysfunction syndrome and chronic critical illness, syndromes in which sequential, persistent, and intractable abnormalities arise in most organ systems (7). Sepsis-induced changes in endocrine activity have been well described and include loss of minute-to-minute variability, absent diurnal rhythms, and an overall decrease in hormone production and secretion (8). Similarly, immunosuppression and impaired interaction between leukocytes and target cells or organs also characterize sepsis (9, 10). However, the effects of sepsis on the third biologic coupler, the central nervous system (CNS), are largely unexplored. The paucity of information on CNS modulation of sepsis/multiple organ dysfunction syndrome limits our understanding of septic pathobiology. This deficit represents a gap in our knowledge that must be rectified if we are to identify viable therapeutic approaches to this deadly disorder.

Recent studies on arousal have identified a discrete system of hypothalamic neurons that secrete the neurotransmitter orexin, also known as *hypocretin* (11–13). This system receives input from multiple cephalic and diencephalic centers and projects to the entire neuroaxis (14). The brain stem and diencephalic nuclei modulated by the orexinergic system regulate not only arousal but also basic physiologic functions (15) and pituitary hormone secretion (16). Thus, the orexinergic system uniquely connects and modulates many basic metabolic and physiologic variables that become dysfunctional in sepsis. Therefore, in this study, we tested the hypothesis that orexinergic dysfunction contributes to some of the physiologic and endocrine abnormalities characteristic of sepsis.

METHODS

Animals

Studies were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and were conducted in accordance with National Institutes of Health guidelines. All experiments were performed on C57Bl6 mice obtained from Charles River. Mice were housed in a climate-controlled room, subjected to standard 12:12 light:dark cycles, and were acclimated for 1 week prior to their initial procedure. Acclimatization included preparing mice to wear a collar that recorded heart and respiratory rates (STARR clip -Starr Life Corps, Oakmont, PA). Halothane anesthesia was used for all procedures because this agent does not inhibit the orexinergic system (17). Delta-phase heating pads (Braintree Scientific, Braintree, MA) were placed under the animal during surgery or under the cage for 12 hours later. Animals were given free access to food and water after intracerebroventricular (ICV) catheter placement, but food was restricted after cecal ligation and puncture (CLP) or sham operation (SO).

Placement of Catheters in the Lateral Cerebral Ventricle

One week prior to intraventricular injection experiments, mice were anesthetized and placed in a stereotactic frame. A 20-gauge stainless guide cannula (Plastics One, Roanoke, VA) was positioned stereotactically above the right lateral cerebral ventricle at the following coordinates: 1.25 mm right of the midline, 0.4 mm posterior to the bregma (18). The underlying skin was prepped and incised, a hole was drilled, the ventricle was accessed, the guide was placed and secured with dental cement (Parkell, Inc, Edgewood, NY), and the incision was closed. Postoperative analgesia was provided with bupivacaine 0.25% (0.25 mg/kg). Animals were resuscitated with 1 cc of saline injected subcutaneously and allowed to awaken. At least 1 week was allowed to elapse before further experimentation.

Induction of Sepsis Using CLP

Severe sepsis was induced in C57Bl6 mice via CLP under halothane anesthesia as previously described (19, 20). Control mice were subjected to SO. At the end of the procedure, the animal was placed on its belly, an area of the lower back was lightly scrubbed with alcohol, and an electronic temperature probe (Bio Medic Data Systems, Inc, Seaford, DE) was injected into the muscle. Bupivacaine 0.25% (0.25 mg/kg ~ 0.05 mL) was injected around the incisions for perioperative analgesia, and animals were fluid resuscitated with 80 mL/kg sterile saline subcutaneously.

Orexin-A or Saline Injection

Animals were assigned to groups for examination at either 24 or 48 hours after CLP or SO. At the designated time of intervention, heart rate (HR), respiratory rate (RR), and temperature (Temp) were determined. The ICV catheters were then perfused at 0.5 μ L/min with 1 μ L of saline with or without 3 nmol orexin-A, an amount previously shown to reverse narcolepsy in orexin-deficient mice (21). One hour later, measurements were repeated, and animals were killed as described below.

Data Collection and Killing

One group of animals (T0 controls) was killed prior to any intervention to obtain blood samples and normal brain, lung, and liver tissue. In a second group, Temp, HR, and RR were determined prior to intervention, and animals were subjected to either CLP or SO. At either 24 or 48 hours after CLP/SO, Temp, HR, and RR were remeasured, animals were deeply anesthetized with halothane, the laparotomy incision was re-opened, and blood was withdrawn from the inferior vena cava for determination of hormone levels. Once the heart had stopped, the scalp was incised, the skull opened, and the brain removed and fixed as previously described (22). Lungs and liver were also harvested for histologic examination. In a third group, ICV catheters were placed, animals were allowed to recover for at least 1 week and were then assigned to receive either saline or orexin and were designated for killing at either 25 or 49 hours. Temp, HR, and RR were measured, and animals were subjected to CLP. Temp, HR, and RR were again determined at either 24 or 48 hours and either saline or orexin was injected. Vital signs were repeated 1 hour later (either 25 or 49 hours after CLP), mice were deeply anesthetized with halothane, the laparotomy incision was re-opened, and blood for hormone assays was withdrawn until the animal died. Brain, heart, and lung tissues were harvested.

Brain Fixation and Staining

The brain was fixed, and 10 μ m sections were generated. As in prior studies, this provided six sections containing orexinergic nuclei from each mouse (17, 22). These sections were treated with a primary antibodies to orexin (mouse anti-orexin, 1:8000 dilution, MAB763, R&D Systems, Minneapolis, MN) and c-Fos (rabbit anti-c-Fos, 1:1000 dilution, PC05, Calbiochem; EMD Biosciences, La Jolla, CA). Detection involved a goat antimouse IgG conjugated to Alexa 488 (A11029 Invitrogen, Carlsbad, CA) for orexin and a biotinylated goat antirabbit IgG (Chemicon AP132BMI, Millipore, Temecula, CA) and streptavidin/HRP/Texas Red (NEL721001EA, Perkin Elmer, Waltham, MA) for c-Fos.

Statistical Analysis

Significance was determined using two-way analysis of variance (time and SO/CLP or time and saline/orexin) with Sidak's correction for multiple comparisons. Because multiple blood draws are not feasible in septic mice, hormone data prior to injection of saline/orexin were made in mice without ventricular catheters. These data were compared with values obtained from the second cohort after saline/orexin injection. Statistics were reviewed by E. Andrew Ochroch, MD, our departmental expert.

RESULTS

Our experiments used a well-validated, well-accepted model of severe sepsis, fluid-resuscitated CLP in C57BL6 mice (23). To limit stimuli that affect orexinergic activity, procedures were performed under halothane anesthesia (17), interventions and data collection occurred at night when activity is maximal (24), and access to food was restricted (25). As in previous studies, mortality following CLP was significant. In the initial experiments involving untreated mice, 2/6 (33%) mice designated for killing at 24 hours died while 10/14 (72%) mice designated for killing at 48 hours succumbed. In the studies

involving orexin or saline injection into the cerebrospinal fluid (CSF), 3/7 (43%) mice designated for killing at 24 hours died, and 9/13 (77%) mice designated for killing at 48 hours did not survive. Thus, overall survival at 24 hours was 62% (8/13) and at 48 hours was 30% (8/27). No animals died following ICV injection. Pathological evaluation of lung and liver tissue was consistent with previous reports (26, 27)

We first compared orexinergic activity in brain tissue harvested 24 or 48 hours after CLP with tissue from unoperated (T0) and SO controls. Hypothalamic sections were co-immunostained with antibodies to orexin and to c-Fos, a marker of neuronal activation (22). Sections (Fig. 1, A and B) from control mice contained 21 ± 4 orexin-containing cells, and 13 ± 4 of these expressed c-Fos. Although CLP (Fig. 1C) did not alter the number of orexin-positive cells at either time point, the number of c-Fos-positive neurons was substantially reduced at both. This decrease was significantly different from T0 and SO (Table 1) both 24 and 48 hours after CLP. In addition, the number of c-Fos-positive neurons observed 48 hours after CLP was significantly less than the number at 24 hours after CLP. This reduction in orexin activity was associated with statistically significant decreases in temperature (Temp), HR, and respiratory rate (RR) both 24 and 48 hours after CLP (Fig. 2). As previously described, CLP induced lethargy and a loss of response to loud noises or tactile stimulation (26).

That changes in orexinergic activity can alter Temp, HR, RR, and arousal in normal animals and humans is well established (15). Our initial experiments demonstrated that Temp, HR, and RR were not altered at 24 or 48 hours after SO (data not shown). However, all three variables were significantly altered 24 and 48 hours after CLP. To further address causality, we rescued sepsis-induced decrements in orexinergic activity in a cohort of septic mice. To accomplish this, at 24 or 48 hours after CLP, Temp, HR, and RR were measured, behavior was assessed, and a previously implanted right lateral cerebral ventricular catheter was injected with either saline or 3 nmol of orexin-A. One hour after injection, vital signs were remeasured, and the animals were killed. Injection of either saline or orexin-A did not change the number of cells that showed orexin immunofluorescence. Saline administration did not alter Temp, HR, or RR (Fig. 2). However, injection of orexin-A significantly altered each variable, attenuating CLP-induced changes at both 24 and 48 hours (Fig. 2). Indeed, orexin injection 48 hours after CLP restored Temp, HR, and RR to levels indistinguishable from baseline. Behaviorally, orexin-injected mice became agitated and hyper-responsive to noise and tactile stimulation. These data suggest that CLP-induced changes in three basic physiologic variables and in behavior are mediated by altered activity in the orexinergic nervous system.

Sepsis also is associated with changes in endocrine activity (8). The orexinergic system is known to effect pituitary and hypothalamic activity and thus could modulate these changes (16). Therefore, we measured the serum concentrations of the pituitary hormones adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), growth hormone (GH), prolactin (PRO), and follicle stimulating hormone (FSH) 24 and 48 hours after SO and CLP. ACTH, TSH, GH, PRO, and FSH levels increased 24 hours after both SO (data not shown) and, as depicted in Figure 3 (light gray bars), CLP. Forty-eight hours after SO, hormone levels had returned to baseline (data not shown), but in animals subjected to CLP

(Fig. 3, light gray bars), levels of all five hormones were significantly lower than both baseline and SO measurements. To solidify the cause-and-effect relationship between loss of orexinergic activity and altered endocrine responses, we examined changes in hormone levels 1 hour after injection of either orexin-A or saline into the CSF. Saline injection (Fig. 3, dark gray bars) did not alter the level of any hormone at either time point. With injection 24 hours after CLP and blood sampling one hour later, orexin significantly increased ACTH levels relative to T0 above the increase induced by CLP alone. This increase resulted in ACTH levels significantly higher than those observed following injection with saline. In contrast, when we injected orexin 48 hours after CLP and again sampled blood 1 hour later, levels of all five hormones were significantly higher than values at T0 and in untreated CLP mice or saline-injected CLP mice (Fig. 3). These data demonstrate that injection of orexin reversed some aspects of CLP-induced changes in pituitary function, particularly at later time points. Overall, our findings implicate the loss of orexinergic activity in the development of late sepsis-induced endocrine abnormalities.

DISCUSSION

The data presented here document that murine sepsis induced by CLP decreases activity in the orexinergic nervous system. The loss of activity is progressive and more pronounced at 48 hours than at 24 hours after CLP. Altered orexinergic activity was associated with decreases in Temp, HR, and RR (15). More interestingly, serum levels of ACTH, TSH, PRO, GH, and FSH were increased 24 hours after CLP but dropped precipitously by 48 hours. Injection of orexin-A into the CSF of septic animals attenuated or reversed the changes in Temp, HR, and RR at both the 24- and 48-hour time points and restored to baseline the reduced pituitary hormone levels observed at 48 hours.

Surprisingly few investigations have examined the effects of sepsis on the CNS. This deficit identifies an important gap in our understanding of septic pathophysiology. Although encephalopathy, alterations in the blood-brain barrier, and minor functional/electrical changes have long been recognized as components of sepsis (28, 29), only studies by Tracey and colleagues have examined sepsis-induced changes in specific CNS pathways. This group has described a vagally mediated nicotinic anti-inflammatory pathway (30) and implicated HMGB-1-induced change in hippocampal structural abnormalities (31). There remains, however, a pressing need to explore interactions between sepsis and other CNS pathways.

A role for the orexinergic system in the regulation of temperature, cardiorespiratory function, and hypothalamic/pituitary activity is well established (32–34). The link between these changes and sepsis, however, is less clear. Administration of bacterial lipopolysaccharide (LPS) suppresses orexinergic activity, likely by an indirect, cytokine-mediated mechanism (35–38). These alterations have also been linked to behavioral changes collectively referred to as *sickness behavior* that include anorexia, lethargy, and reduced movement (35, 37–40). At one time used to model sepsis, LPS administration is now felt to reflect a toxic injury that lacks many of the characteristic features of the clinical disorder. Indeed, the changes provoked by LPS, including sickness behavior, more closely resemble a low-grade bacterial or viral infection. That said, alterations in orexinergic activity in both sepsis and endotoxemia suggest the presence of some common pathogenic mechanisms.

The experiments detailed here are hampered by certain technical limitations. Our investigation involved only animals surviving until the designated time point for killing, adding a bias. We administered only a single dose of orexin into a lateral cerebral ventricle and observed changes 1 hour later. This approach precluded us from examining the effects of sustained restoration of orexinergic tone. We, thus, could not examine the contribution-restored orexinergic activity made to sepsis-induced changes in organ function or histology, or on mortality. The use of a continuous orexin infusion is prohibitively expensive. Further, orexin delivery into the CSF does not truly correct the pathologic defect as the activity of the orexinergic fibers themselves is unaffected. At this time, there are no agents available that directly stimulate orexinergic neurons. We are exploring transgenic approaches that may allow us to directly stimulate orexinergic neurons for use in upcoming studies.

Vanhorebeek and Van den Berghe have documented endocrine alterations in the critically ill that change over time. In “early” critical illness, target-tissue responses to hormones are reduced. To compensate for this “peripheral-resistance”, secretion of hormones is markedly increased (8). With progression of critical illness to a chronic state, Vanhorebeek and Van den Berghe observed “central suppression,” where secretion of pituitary hormones is dramatically reduced (8). Our data reveal increases in each of the pituitary hormones studied at 24 hours after CLP, a finding consistent with peripheral hormonal resistance, and significantly reduced hormone levels at the 48-hour time point, as one would in the central endocrine suppression characteristic of a chronic, later phase of critical illness (16). These finding may, therefore, extend the clinical relevance of the CLP model.

Future experiments will allow us to explore interventions that may have clinical implications. For example, we will need to evaluate how sepsis effects the interactions between the orexinergic neurons and the cholinergic neurons implicated in Tracey’s studies on the “inflammatory reflex.” Orexin stimulates cholinergic fibers terminating in the dorsal raphe and laterodorsal tegmentum, areas that likely affect vagal tone (41) and most tellingly basal forebrain (39, 40) fibers that project to the hippocampus and modulate memory and learning (42). This pathway may modulate sepsis-induced cognitive dysfunction (31). Further, orexinergic neurons are themselves stimulated by acetylcholine-secreting neurons arising throughout the brain stem, including the nuclei that supply the vagus nerve (43). Thus, sepsis-induced changes in the two systems could amplify each other; loss of orexinergic tone would decrease cholinergic activity, an alteration that would further decrease orexinergic output. The reciprocal cholinergic-orexinergic innervation could link neuronal changes to inflammation, arousal, and regulation of temperature, hemodynamics, and endocrine activity.

In addition to cholinergic inputs, the orexinergic system is modulated by several important neuronal systems that involve many well-described neurotransmitters and thus may be amenable to pharmacologic manipulation. This possibility suggests some intriguing therapeutic options. Several agents that are used clinically are known to modulate orexinergic activity and might be used to manage sepsis. For example, recent studies in our laboratory indicate that caffeine, which modulates orexinergic activity, is beneficial in CLP (20).

CONCLUSIONS

In summary, the findings reported here demonstrate a specific, sepsis-induced change in the activity of the CNS pathway modulated by the neurotransmitter orexin. These data suggest that further investigation of the role of the CNS in septic pathophysiology is warranted.

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Figure 1.

Effects of cecal ligation and puncture–sepsis on orexinergic activity. Representative sections stained for orexin and c-Fos. Tissue harvested from four surviving mice 48 hr after the induction of sepsis via cecal ligation and two 23-gauge punctures or from four unoperated or four sham-operated controls. Orexin-containing neurons (*fluorescent green*), c-Fos containing neurons (*fluorescent red*). *Green arrows* indicate orexin-positive, and c-Fos-negative neurons. *Yellow arrow* depicts orexin-negative, and c-Fos-positive neurons. *White arrows* indicate neurons positive for both c-Fos and orexin. **A**, Section from unoperated control. **B**, Section of tissue harvested 48 hr after sham operation. **C**, Section of tissue harvested 48 hr after 2CLP. CLP = cecal ligation and puncture.

Figure 2.

Changes in physiologic variables in awake mice. *White bars* = T₀ control; *cross-hatched bars* = value prior to saline injection into lateral ventricle; *light gray bars* = value prior to orexin injection; *dark gray bars* = value 1 hr after saline injection; *black bars* = value 1 hr after orexin injection; *n* = 4 for data depicted on each bar. Data are mean ± sd. X-axis on all graphs: time following sham operation/2CLP. Statistical significance was determined using two-way (time, SO/CLP) analysis of variance. Multiple comparisons were assessed using Sidak test. **A**, Temperature (Temp). Y-axis: Temp in degrees Celcius. Analysis of variance (ANOVA): time, *p* < 0.0001; treatment (saline/orexin), *p* = 0.0087; interaction, *p* < 0.0001. **B**, Heart rate (HR). Y-axis: beats/min. ANOVA: time, *p* < 0.0001; treatment (saline/orexin), *p* < 0.0027; interaction, *p* < 0.0001. **C**, Respiratory rate (RR). Y-axis: breaths/min. ANOVA: time, *p* < 0.0001; treatment (saline/orexin), *p* = 0.0037; interaction, *p* = 0.0002. * = Significantly different than value at T₀. † = Significantly different than value prior to injection. ^ = Significantly different than value at same time point following saline injection. SO = sham operation, CLP = cecal ligation and puncture.

Figure 3.

Changes in pituitary hormones in awake mice. *White bars* = T0 control, *gray bars* = value 1 hr after injection of saline, *black bars* = value 1 hr after injection of orexin. Data are mean $\pm$ sd; n = at least four for data depicted on each bar. X-axis on all graphs: time following sham operation/cecal ligation and puncture. **A**, Adrenocorticotrophic hormone (ACTH). Y-axis: pg/mL. Analysis of variance (ANOVA): time, $p < 0.0001$; treatment (saline/ orexin), $p < 0.0001$; interaction, $p < 0.0001$. **B**, Thyroid stimulating hormone (TSH). Y-axis: ng/mL. ANOVA: time, $p = 0.034$; treatment (saline/orexin), $p < 0.0001$; interaction, $p < 0.0001$. **C**, Growth hormone (GH). Y-axis: ng/mL. ANOVA: time, $p = 0.0073$; treatment (saline/orexin), $p < 0.0001$; interaction, $p < 0.0001$. **D**, Prolactin (Pro). Y-axis: ng/mL. ANOVA: time, $p = 0.0019$; treatment (saline/orexin), $p < 0.0001$; interaction, $p < 0.0001$. **E** Follicle stimulating hormone (FSH). Y-axis: ng/mL. ANOVA: time, $p < 0.0001$; treatment (saline/ orexin), $p < 0.0001$; interaction, $p < 0.0001$. ANOVA: time, $p = 0.011$; treatment (saline/ orexin), $p < 0.0001$; interaction, $p < 0.0001$. * = Significantly different than value at T0. † = Significantly different than uninjected value. ^ = Significantly different than value at same

time point following saline injection. § = Significantly different than value following injection of saline at 24 hr.

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Table 1.

Percentage of Orexin-Containing Neurons at Baseline and Following Sham Operation and Cecal Ligation and Puncture

T0	SO24	SO48	CLP24	CLP48
61 ± 11	58 ± 3	58 ± 11	27 ± 4 ^{*^}	8 ± 1 ^{*†^}

SO = sham operation, CLP = cecal ligation and puncture.

In each animal, all slides containing orexinergic neurons were evaluated in a blinded manner by three individuals, including two of the authors (N.R.R., E.O.M.). The total number of cells fluorescing both red and green was divided by the sum of all cells containing both red and green fluorescence and cells containing green fluorescence only. Data are mean ± sd. Statistical significance determined using two-way (time, SO/CLP) analysis of variance. Time: $p = 0.0088$, SO/CLP: $p = 0.0054$, interaction: $p = 0.0061$. Multiple comparisons assessed using Sidak multiple-comparison test.

* = Significantly different than value at T0.

† = Significantly different than value for same intervention at T24.

^ = Significantly different value at same time point following saline injection.