

# Impairment of osmotic challenge-induced neurohypophyseal hormones secretion in sepsis survivor rats

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## Abstract

**Background/Purpose** Recent studies have reported that sepsis survivors show impaired central nervous system functions. The osmoregulation in this post-sepsis condition has not been well investigated. In the present study, we evaluated the secretion of neurohypophyseal hormones, arginine vasopressin (AVP) and oxytocin (OT), and water intake induced by osmotic challenge in survivor rats.

**Methods** Wistar rats were submitted to sepsis by cecal ligation and puncture (CLP). Five days after CLP surgery, the survivor and naive animals were stimulated with an osmotic challenge consisting of hypertonic saline administration. Thirty minutes later, blood and brain were collected for determination of osmolality, nitrite, interleukin (IL)-1 $\beta$ , IL-6, AVP and OT levels and *c-fos* expression analysis of hypothalamic supraoptic nuclei (SON), respectively. In another set of sepsis survivor animals, water intake was measured for 240 min after the osmotic stimulus.

**Results** High levels of nitrite and IL-1 $\beta$ , but not IL-6, were found in the plasma of sepsis survivors and this long-term systemic inflammation was not altered by the osmotic

challenge. Moreover, the AVP and OT secretion (but not the osmolality) and *c-fos* expression in SON were significantly attenuated in CLP survivor animals. Additionally, there was no alteration in the water intake response induced by osmotic challenge in the sepsis survivor group.

**Conclusion** The results suggest that the inflammatory components mediated a persistent impairment in the component of the osmoregulatory reflex affecting the secretion of neurohypophyseal hormones in sepsis survivor animals.

**Keywords** Cecal ligation and puncture · Water intake · c-Fos · Hypertonic saline · Cytokines

## Introduction

Sepsis is a systemic inflammatory syndrome characterized by exacerbated activation of the innate immune system followed by severe dysfunctions of vital organs [1]. Impairment of neurohypophyseal hormones secretion has been considered one of the main pathophysiological events in sepsis [2, 3]. These hormones are synthesized by magnocellular neurons in hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) and, in physiological conditions, are secreted in response to stimuli such as hyperosmolality, hypovolemia, stress and angiotensin II, playing a critical role in maintaining fluid and electrolytes homeostasis [4]. Recently, studies in sepsis models have shown alterations in the osmoregulatory neurons associated with arginine vasopressin (AVP) secretion and thirst [5]. Furthermore, during the early phase of sepsis, blood AVP and oxytocin (OT) levels are initially increased in response to hypotension/hypovolemia; but with the progression of the disease (late phase), these hormones are found in basal levels, despite the presence of a lasting hypotension [3, 6–8].

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It leads us to the hypothesis of a persistent impairment in central control of homeostasis.

Moreover, a particular attention has been given to patients who survived from sepsis, because they show pathophysiological dysfunctions in several organ systems. For example, long-term immunological alteration has been associated with an elevated susceptibility of acquiring secondary infections [9, 10]. In addition, besides the deleterious consequences in the periphery, dysfunctions in the central nervous system (CNS) are also observed in sepsis survivors, characterized by psychological disorders and long-term cognitive deficits [11].

Although evidence have also pointed to a persistent impairment in the osmoregulation in sepsis survivor patients after osmotic challenge [12, 13], this important secondary dysfunction has not received the merit attention and, therefore, has not been investigated. In this context, in the present study, we investigated the AVP and OT secretion and the water intake induced by an osmotic challenge in a sepsis survivor model.

## Materials and methods

### Animals

All experiments were performed using male Wistar rats with an average body weight of  $300 \pm 25$  g. The animals were obtained from the Central Animal Facility at the University of São Paulo, campus of Ribeirão Preto, and were housed in the same room under standard 12-h-light/12-h-dark cycle, room temperature ( $25 \pm 2^\circ\text{C}$ ) and *ad libitum* access to food (Nuvilab CR-1, NUVITAL) and water.

### Sepsis model

Polymicrobial peritonitis was induced by the surgical procedure of cecal ligation and puncture (CLP). Briefly, the rats were anesthetized by an intraperitoneal (i.p.) injection of tribromoethanol (TBE) (250 mg/kg) and then a midline laparotomy was carried out under sterile conditions. The cecum was exposed, ligated below the ileocecal valve, and then gently punctured once with a sterile 14-gauge needle. Subsequently, the cecum was returned to the abdominal cavity and the incision was sutured in layers. These rats were given a subcutaneous injection of isotonic saline (0.9%; 5 mL/250 g) solution to compensate for fluid loss during surgery.

### Experimental design

Five days after CLP surgery, the survivor animals were submitted to an osmotic challenge consisting of hypertonic

saline administration (i.p.; 2 mol/L NaCl), in a volume corresponding to 1% of body weight. Isotonic saline was administrated in the control group (i.p.; 0.01 mol/L NaCl). After thirty minutes, the animals were anesthetized with TBE and blood was collected for plasma evaluation of osmolality, nitrite, IL-1 $\beta$ , IL-6 and, AVP and OT levels. Then, the same animals were perfused with 250 mL of PBS followed by 250 mL of fixative solution (4% paraformaldehyde in 0.1 mol/L phosphate buffer). The brains were removed, post-fixed in the fixative solution for 4 h, placed in PBS containing 30% sucrose and stored at  $4^\circ\text{C}$ . In another group, hypertonic saline was administrated in sepsis survivor animals and the water drinking was measured for 240 min.

### Plasma cytokines and nitrite quantification

The IL-1 $\beta$  and IL-6 plasma levels were determined using ELISA kits for each cytokine (R&D Systems, Minneapolis, Minn., USA) according to the manufacturer's instructions. The detection limits for IL-1 $\beta$  and IL-6 were 5 and 10 pg/mL, respectively. The determination of plasma nitrite was performed by the technique of chemiluminescence NO/ozone as described previously [14].

### Osmolality and plasma AVP and OT levels determination

AVP and OT were extracted from 500  $\mu\text{L}$  of plasma using acetone and petroleum ether. Dried sample extracts were then stored at  $-20^\circ\text{C}$  until quantification by ELISA (Enzo Life Sciences Inc. Farmingdale NY, USA). The AVP and OT ELISA sensitivities were 3.39 and 15.0 pg/mL, respectively. Plasma osmolality was determined by freezing point depression using an osmometer (Precision System, Inc., Natick, MA, USA).

### Immunohistochemistry

Brain coronal sections were cut at 40  $\mu\text{m}$  thickness in cryostat (Microm HM 505 E) and processed for immunocytochemistry. Free floating sections were washed three times in PBS (0.01 mol/L; pH 7.4) and submitted to antigen retrieval protocol: incubation in Tris/EDTA (10/1 mmol/L; pH 9.0) solution for 5 min and then heated for 30 min in water bath at  $70^\circ\text{C}$  in 10 mmol/L sodium citrate buffer (pH 6.0). After three new rinses, sections were incubated in PBS containing 1% hydrogen peroxidase for 10 min with PBS to block endogenous peroxidases. Nonspecific binding sites were then blocked for 60 min in PBS containing 5% normal goat serum (NGS) and 0.3% Triton X-100. Subsequently, sections were incubated for 24 h at room with: c-fos-antibody generated in rabbit (sc-52, Santa Cruz) diluted 1:100

in PBS containing 1% NGS, 1% bovine serum albumin and 0.3% Triton-X 100. After rising, the sections were incubated for 1.5 h at room temperature with biotinylated anti-rabbit IgG (1:200; Vector), washed again in PBS, placed for 30 min in avidin–biotin peroxidase complex (ABC, Vectastain). *C-fos* immunoreactivity was visualized by incubation with 0.02% 3,3'-diaminobenzidine tetrachloride and 0.2% hydrogen peroxide. The sections were mounted on gelatin-coated slides, dehydrated and cover slipped with Entellan (Merck). As a control for staining specificity, first antibody was omitted in some sections, resulting in the complete elimination for immunostaining. Since this antibody also recognizes Fos-related proteins, the staining we have obtained was described as Fos-like immunoreactivity.

### Quantification of Fos-like immunoreactivity (FLI)

The brain nuclei exhibiting FLI were identified and delimited according to the rat brain atlas of Paxinos and Watson [15]. The number of FLI nuclear profiles in the sections was counted at one level of the SON (−1.4 mm from the bregma). Nuclei with FLI were quantified using a system that includes a Zeiss microscope equipped with a digital camera. Images were digitalized and analyzed using Image J (version 1.49v—National Institute of Health, USA) software.

### Metabolic cages

In order to accurately assess the water intake, in the third day after CLP surgery, naive or sepsis survivor animals were housed in metabolic cages for adaptation, with food and water *ad libitum*. Two days later, they were submitted to an injection of hypertonic saline (i.p.), and water intake was assessed (30, 60, 120, 180 and 240 min).

### Statistical analysis

All results are expressed as mean ± SEM. For statistical analysis, we used two-way ANOVA followed by post hoc Tukey test. P values of <0.05 were considered statistically significant in all cases. The software used was GraphPad Prism version 5.0 (GraphPad Software, Inc. 2007).

## Result

### Neurohypophyseal hormones secretion is attenuated in sepsis survivor rats after osmotic challenge

We assessed the plasma neurohypophyseal hormones, cytokines and nitrite levels and osmolality after 30 min of administration of hypertonic saline in sepsis survivor rats

(survival rate ~70%—Fig. 1a). We detected a persistent increased plasma level of IL-1 $\beta$  and nitrite, but not IL-6, five days post-CLP surgery (Fig. 1b, c, d, respectively). The hypertonic saline injection did not alter the plasma IL-1 $\beta$  and nitrite levels in naive or septic animals, when compared with the respective isotonic saline-treated control groups. In addition, as expected, the osmotic challenge increased the plasma osmolality (Fig. 2a) and AVP and OT secretion, in naive and sepsis survivor rats (Fig. 2b, c). However, both hormones (but not the osmolality) were significantly attenuated in survivor group as compared with the control (Fig. 2b, c). Additionally, we evaluated whether the AVP and OT secretion and thirst are similarly affected, since under normal conditions, both can be stimulated via osmoreceptors during systemic hypertonicity. Here, we observed that the osmotic challenge increased water intake in both naive and survivor rats, but without differences between the groups (Fig. 2d).

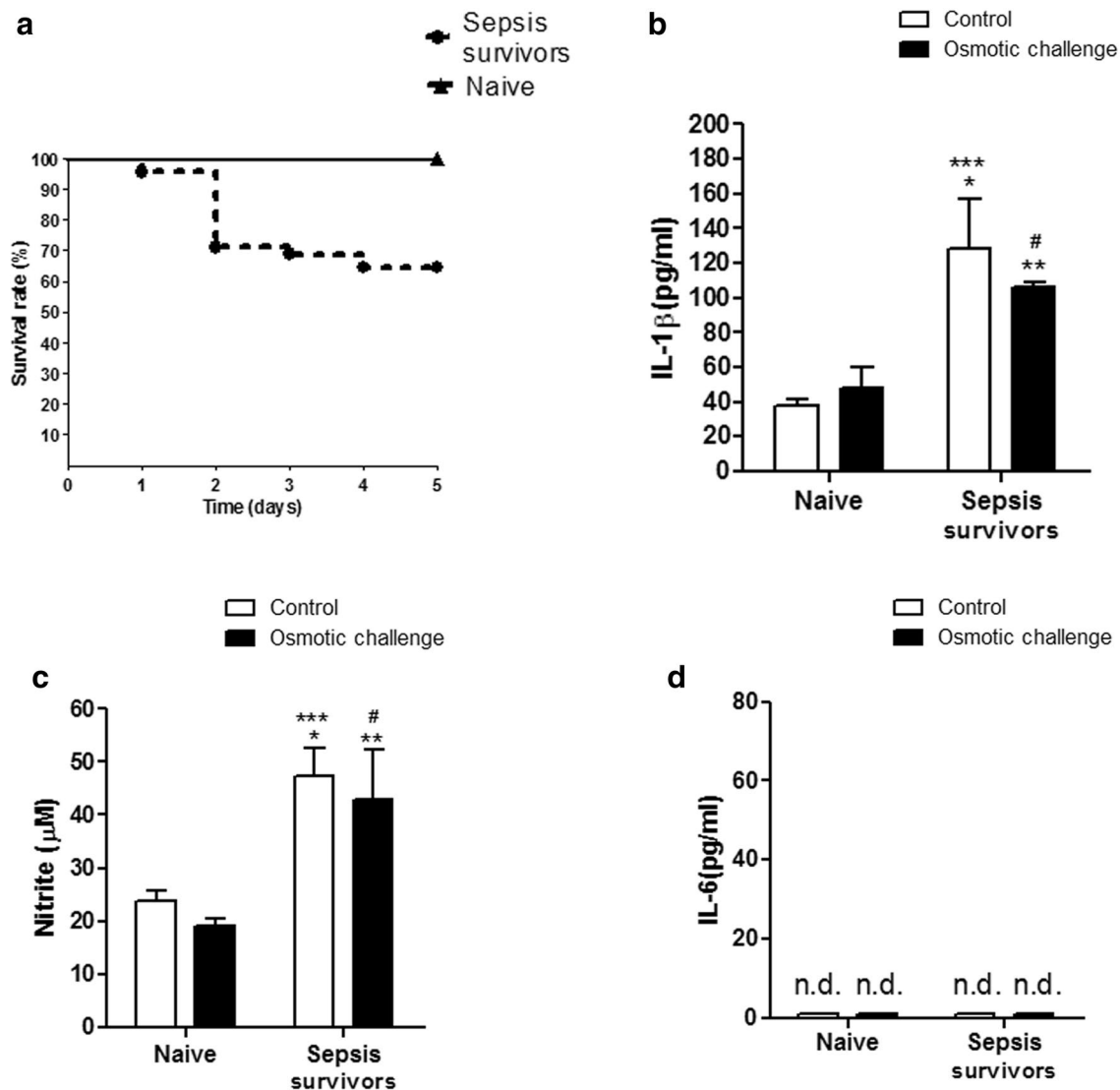
### Neuronal activation is attenuated in sepsis survivor rats after osmotic challenge

Following, we assessed a possible hypothalamic dysfunction in sepsis survivor through *c-fos* expression analysis, an immediate-early gene used as a marker of activated vasopressinergic and oxytocinergic neurons by osmotic stimulation [16, 17]. As expected, we observed that in naive rats, saline hypertonic injection increased significantly the number of Fos-labeled neurons in SON as compared with isotonic saline administration (Fig. 3a, b, e). However, in the sepsis survivors, the osmotic challenge did not enhance the *c-fos* expression in SON showing a degree of activation similar to the saline-treated group (Fig. 3a, d, e).

## Discussion

In the current study, we showed a long-term neuroendocrine dysfunction in an animal model of sepsis characterized by an impairment of AVP and OT secretion following an acute osmotic stimulus associated with abnormal activation of hypothalamic neurons.

Moreover, we observed persistent increased plasma levels of IL-1 $\beta$  and nitrite, but not IL-6, in these septic survivors animals. Here, we used the systemic cytokine levels to define the animals as sepsis survivors. For example, high IL-6 levels have a positive association with elevated sepsis mortality and, therefore, its presence has been considered a good predictor of death [18, 19]. However, in our study, IL-6 was not detected in the plasma of surviving animals in contrast with the persistent high IL-1 $\beta$  levels. The same pattern of blood cytokines in sepsis survivor rats was already reported



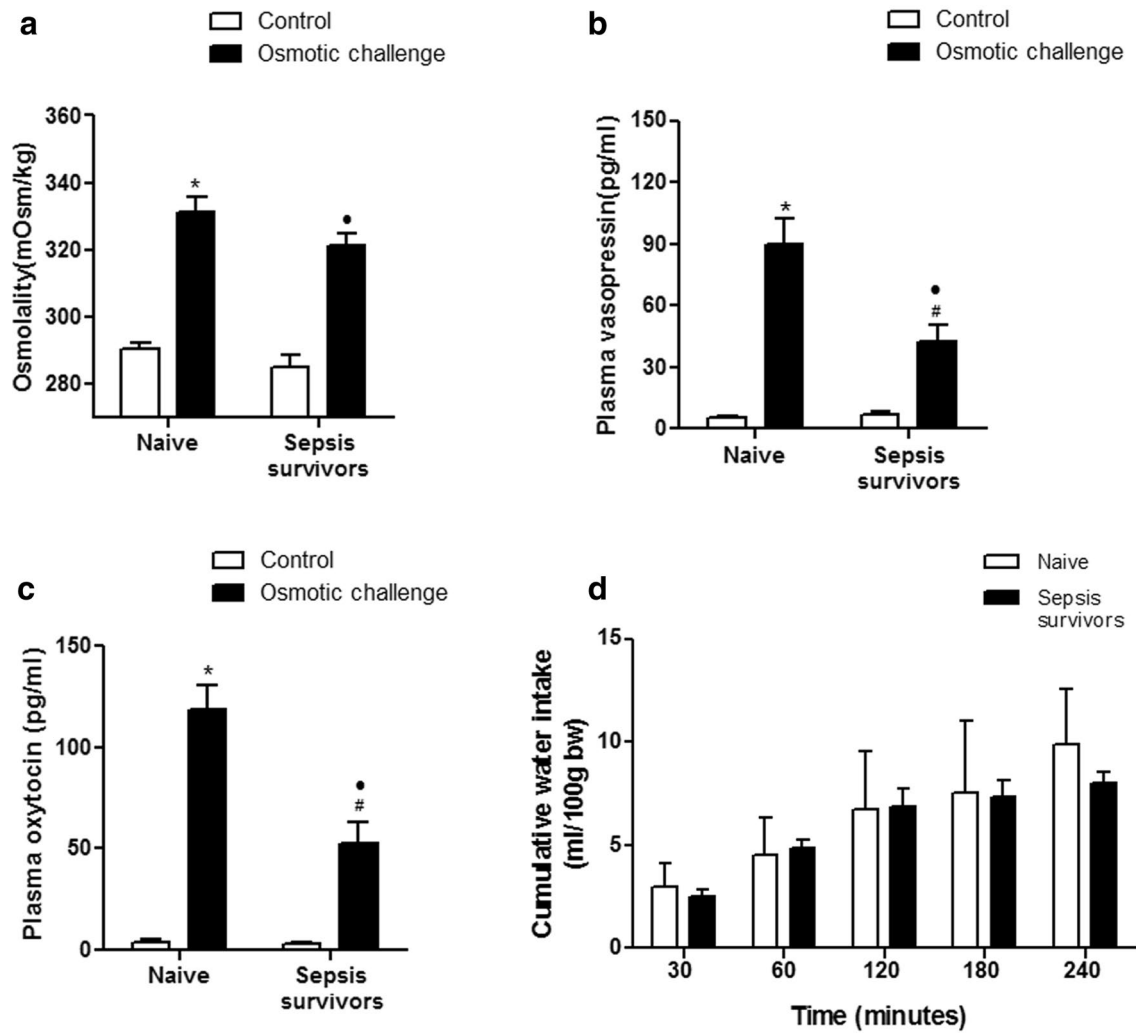
**Fig. 1** Persistent inflammatory mediators in sepsis survivors. **a** Survival curve from naive rats or submitted to CLP surgery ( $n=14\text{--}20$  animals/group). After 5 days, the survivor animals were challenged with isotonic (control) or hypertonic saline administration. **b** Plasma IL-1 $\beta$ , **c** Nitrite, **d** IL-6. The data represent the means  $\pm$  SEM of 5–8

animals per group. \* $P<0.01$  (c) and 0.001 (b) versus Naive-control; \*\* $P<0.05$  (c) and 0.01 (b) versus Naive-control; \*\*\* $P<0.01$  (b) and 0.001 (c) versus Naive-osmotic challenge; # $P<0.05$  (b, c) versus Naive-osmotic challenge

in another study using the same sepsis model suggesting that, in our conditions, the levels of cytokines are reproducible but not an isolated data [20]. Although high IL-1 $\beta$  levels are not associated with high mortality of septic animals, this cytokine could contribute to the dysfunction of neurohypophysial hormones secretion as we already observed in septic rats [14, 21]. IL-1 $\beta$  can reach the CNS through the circumventricular organs, structures lacking a blood–brain barrier, modifying the function of magnocellular neurons present in the SON [2]. Our group already reported an increase of IL-1 $\beta$  and NO production in hypothalamus associated with the impairment of AVP secretion one day after sepsis induction

and that IL-1 $\beta$  can also induce central iNOS expression amplifying mechanisms that results in the activation of apoptotic pathways [7, 14]. In this study, we observed increased nitrite plasma levels in all survivor animals groups, without difference between groups. Nitrite is a metabolite, that indirectly indicates the formation of NO [22]. Peripherally, high levels of NO results in decreased blood pressure and a deficient response to vasoconstrictors, contributing to the cardiovascular failure during septic shock [7, 23].

We observed that this impaired hormonal secretion was accompanied by an attenuated neuronal activation in SON. It may reflect an overall decrease in the activation



**Fig. 2** Neurohypophyseal hormones secretion is attenuated in sepsis survivor rats after osmotic challenge. **a** Osmolality, **b** AVP and **c** OT levels were evaluated 30 min after hypertonic saline administration. **d** Cumulative water intake was measured during 240 min after osmotic

challenge in sepsis survivor animals. Naive rats were used as the control group. The data represent the means  $\pm$  SEM of 4–8 animals per group. \* $P < 0.001$  (**a**, **b**, **c**) versus naive-control; # $P < 0.001$  (**b**, **c**) versus naive-osmotic challenge; • $P < 0.01$  versus CLP-control

of both vasopressinergic and oxytocinergic magnocellular neurons harbored in SON even in the presence of osmotic stimulation.

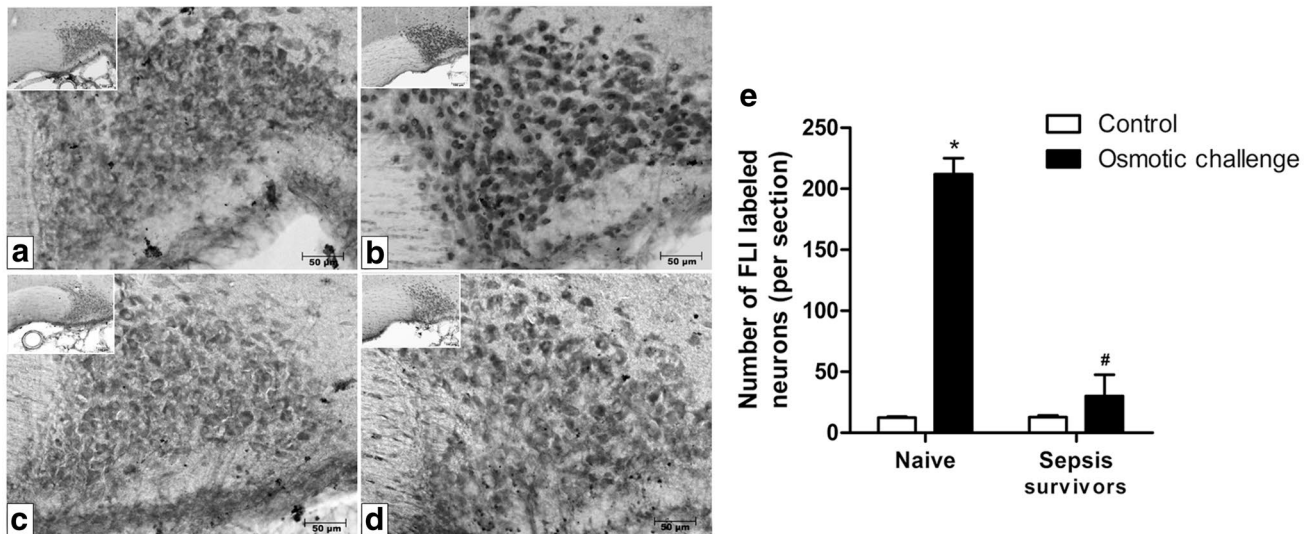
In the present study it was not evidenced an alteration in thirst in sepsis survivor rats, suggesting that only the endocrine component of osmoregulatory reflex is impaired. Differential disruptions in the components of osmoregulatory response have been evidenced during sepsis in an experimental model [5]. Therefore, the components of the osmoregulatory reflex, that include also organum vasculosum laminae terminalis and median preoptic nucleus, could have different recovery times. The neuronal dysfunction has been observed in human and experimental septic shock, where the AVP post-transcriptional synthesis is decreased mainly in the SON [24–26]. Recently we demonstrated in this nucleus the presence

of cellular death markers specifically in vasopressinergic neurons [27].

Post-sepsis clinical evidences pointed to the impairment of AVP secretion following an acute osmotic challenge [12, 13]. Herein, we provide the first experimental data demonstrating a persistent impairment of neurohypophyseal hormones in CLP survivor animals mimicking secondary neuroendocrine dysfunctions observed in patients. Alterations in this neuroendocrine function may decrease the life quality of survivor patients and, therefore, this long-term model could be useful to comprehend pathological events involved in this hypothalamic hormones dysfunction.

In conclusion, our results showed that persistent inflammatory mediators may contribute for the alterations in hypothalamic neuronal activation, providing a hypothetical mechanistic explanation for the impairment in the





**Fig. 3** Neuronal activation is attenuated in sepsis survivor rats after osmotic challenge. Photomicrographs illustrating nuclear labelling of FLI neurons in the SON. The number of FLI labelled neurons were performed 30 min after the **a, b** naive and **c, d** sepsis survivor groups

received, **a, c** isotonic saline or **b, d** osmotic challenge. The data represent the means  $\pm$  SEM of 5–8 animals per group. \*\*\* $P < 0.001$  (e) versus naive-control; # $P < 0.001$  (e) versus naive-osmotic challenge. Objective magnification:  $\times 40$  (oil immersion). Scale bar = 50  $\mu$ m

endocrine component of the osmoregulatory reflex in sepsis survivors. Further studies are necessary to determine the cause of this disturbance in the hypothalamic function. It is possible that neurodegeneration or synaptic dysfunction may be involved in long-term brain events after sepsis and that neurohypophyseal hormones secretion deficiency is just part of vast brain function impairment.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures were done following the Ethics Committee of Animal Experimentation at the University of São Paulo (CEUA)-Campus Ribeirão Preto.

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