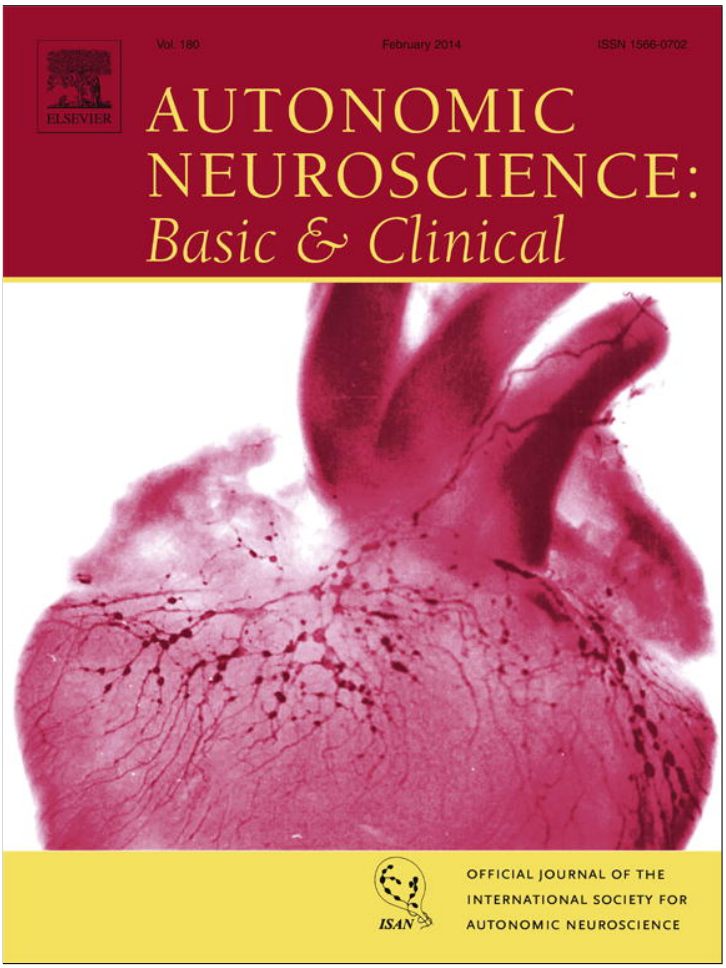

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Right atrium cholinergic deficit in septic rats

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ABSTRACT

Heart rate variability (HRV) is mainly determined by the influence of both branches of the Autonomic Nervous System over the sinus node. Low HRV has been associated with a worse prognosis in patients with sepsis. The objective of this study was to explain the reduction in HRV during experimental sepsis in adult rats. We recorded the heart's electrical activity by telemetry in conscious unrestrained male rats before and 1 day after the induction of peritonitis ($N = 39$) or sham peritonitis ($N = 15$). Then, we analyzed the chronotropic responsiveness of the isolated heart to the autonomic neurotransmitters and determined catecholamine concentrations in blood plasma and acetylcholine and choline concentrations in the right atrium. The surviving septic rats ($N = 33$) had increased heart rate (HR) and diminished HRV. Despite the higher HR in situ, the spontaneous basal HR in septic and sham isolated hearts was the same. The isolated septic hearts showed acetylcholine hypersensitivity ($\log(IC_{50},M) = -7.2 \pm 0.2$ vs. -6.0 ± 0.4, $P = 0.025$) and lower concentrations of choline in their right atriums (in nMol/mg protein: 0.6 ± 0.1 vs. 1.6 ± 0.6, $P = 0.013$). Norepinephrine concentration in blood plasma from septic rats was higher (in ng/ml: 29.2 ± 8.4 vs. 5.8 ± 4.1, $P = 0.019$). In conclusion, septic rats present a deregulation of the autonomic nervous system, not only sympathetic overexcitation but also parasympathetic dysfunction.

1. Introduction

Sepsis is a clinical syndrome defined by the presence of both infection and systemic inflammatory response (Levy et al., 2003). We have previously shown that in an otherwise similar group of septic patients, reduced heart rate variability (HRV) was associated with a worse prognosis, leading to multiple organ dysfunction syndrome and high mortality (Pontet et al., 2003).

HRV reflects the continuous oscillation of the RR intervals around its mean value, and it is mostly determined by the modulation of the sinus node activity by both branches of the autonomic nervous system. A lower HRV has been described in clinical and experimental sepsis (Pancoto et al., 2008). The reduction in HRV during sepsis has been ascribed to the uncoupling of the autonomic and cardiovascular systems (Godin et al., 1996). This could be due to refractoriness of the heart to the autonomic neurotransmitters (Hajiasgharzadeh et al., 2011; Haddadian et al., 2013) and/or to an altered activity of the autonomic nervous system (Werdan et al., 2009).

Partial uncoupling of isolated atria from endotoxemic rats to cholinergic stimulation has been described recently using non-lethal doses of lipopolysaccharide (LPS) (Gholami et al., 2012).

The enhanced sympathetic activity in sepsis has been time correlated with tachycardia in LPS treated rats (Vayssettes-Courchay et al., 2005). In accordance with this, a huge increase in plasma norepinephrine levels at 24 h after cecal ligation and puncture (CLP) in rats has been reported (Berger et al., 2011; Kovarik et al., 1987).

The activation of vagal afferent and efferent signaling has been related to the inflammatory reflex that attenuates the host response to pathogens that might itself become deleterious if left uncontrolled (Tracey, 2007). Lack of this activation or even depression of the cholinergic activity (e.g., by desensitization (Fairchild et al., 2011)) might contribute to decreased HRV and worse prognosis in severe sepsis.

Our main objective was to explain the reduction in HRV during sepsis. We first measured HRV in conscious rats, before and 1 day after the induction of polymicrobial sepsis by peritonitis. Then, we analyzed the chronotropic responsiveness of their isolated hearts to autonomic neurotransmitters or indirectly assessed their autonomic nervous system efferent activity by determining the catecholamine concentrations in their plasma and the acetylcholine and choline concentrations in their right atria.

2. Materials and methods

2.1. Animals

This study was approved by the Comité de Ética en el Uso de Animales (CEUA, Uruguay), protocol number # A5373-01. Animal care

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and handling were in accordance with the National Institutes of Health Guidelines.

Fifty-four adult Wistar derived albino male rats (13.5 ± 0.3 weeks old and weighing 312 ± 4 g) were provided by the *vivarium* located in the same building where the laboratory is situated. Each week, two rats were brought to the lab and housed in individual cages at controlled room temperature and natural photoperiod conditions. The two rats were randomly assigned to the sepsis ($N = 39$) or sham protocols ($N = 15$). Rodent chow and water were available ad libitum during the entire experimental period (4 days).

2.2. In vivo study: heart rate and HRV evaluation in conscious unrestrained rats

We recorded the heart electrical activity (1-lead ECG) of all the rats included in this study ($N = 54$). A dorsally mounted radiofrequency transmitter (CA-F40, Data Sciences International DSI, St. Paul, MN) with two wire leads was implanted subcutaneously in the rats under anesthesia with isoflurane (2.5% at 0.5 lpm O_2). The electrodes were sutured to the chest muscles (lead II-like configuration) and the skin was closed with metal clips. The rats were allowed to recover for 48 h.

The ECG of the conscious, freely moving rats was recorded for 60 min in the morning (8–11 am) before and 1 day after the induction of sepsis.

We used a 1208 FS A/D converter card (Measurement Computing, Norton, MA) and homemade software. The sampling frequency was 1000 Hz. Automatic detection of R waves and measurement of RR intervals were performed offline with Spike2 software (version 6.07, Cambridge Electronic Design, Cambridge, UK). The detection was visually inspected and corrected if necessary. Another software was used for the filtering of the RR time series (Machado et al., 2000) and for the calculation of the mean heart rate (HR) and statistical HRV indices. The absence of outliers after appropriate filtering was confirmed by graphical representation of all RR intervals considered for HRV analysis. Each RR interval was plotted as a function of time (tachogram) or as a function of the preceding RR (Poincaré plot). We calculated SDNN (the standard deviation of all normal RR intervals) and RMSSD (square root of the mean squared successive differences of RR intervals) (Aubert et al., 1999). SDNN is an index of global variability, while RMSSD evaluates short-term variability on a beat-to-beat basis (parasympathetic influences (Hill and Siebenbrock, 2009)). As SDNN is affected by HR, the coefficient of variation (CV) was also calculated as SDNN (ms) / RR interval (ms) × 100.

Although frequency-domain analysis is generally used for autonomic nervous system evaluation, we did not use it for the following reasons: the procedure for the estimation of these indices is not standardized for rodents, non-stationary time-series yield non-reliable results and besides, it has been argued that this method does not provide additional information beyond that obtained by statistical indices in rodent sepsis (Fairchild et al., 2011; Stauss, 2003). Furthermore, Stauss has reported that these indices do not always reflect autonomic nervous system activity, and that simple statistics of HRV reliably predict the prognosis of various diseases (Stauss, 2003).

2.3. Sepsis induction

Intra-abdominal sepsis was induced by cecal ligation and fecal inoculum (CLF, $N = 39$). Under anesthesia with isoflurane, an abdominal incision was made and the cecum was exposed. Double ligation of the cecum was performed to provide a source of necrotic tissue that is often found in clinical sepsis (Hubbard et al., 2005). The bowel was returned without perforation. Instead, fecal slurry (300 mg of autologous feces in 5 ml of sterile saline solution) was spilled in the peritoneum (300 mg/kg) (Chopra and Sharma, 2007; Chopra et al., 2011). The abdominal cavity was closed in two layers.

Sham animals ($N = 15$) were submitted to laparotomy and the cecum was manipulated, but not tied. An equivalent volume of normal saline was spilled in the peritoneum instead.

At the end of the surgery, all rats received a subcutaneous injection of normal saline (Hubbard et al., 2005) to reach a dose of volume replacement therapy of 10 ml/kg.

2.4. In vitro study: chronotropic responsiveness of isolated hearts to Autonomic Nervous System neurotransmitters

2.4.1. Chronotropic responsiveness of isolated hearts to norepinephrine

We evaluated the effect of sepsis on the chronotropic responsiveness of isolated hearts to norepinephrine stimulation 1 day after the induction of sepsis and sham sepsis ($N = 10$ and 5 respectively) once the in vivo ECG was recorded.

Under anesthesia with isoflurane, the thorax was opened and the heart was exposed. The heart was excised and mounted in a Langendorff setup for perfusion through the aorta (constant flow of 9 ml/min) with Tyrode solution (composition in mmol/l: NaCl, 140; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 2; NaH_2PO_4, 0.33; glucose, 10; and HEPES, 10; pH adjusted to 7.4 with NaOH at 37 °C). The solution was gassed with 100% O_2.

Two wire electrodes were attached to the right atrium and pulmonary cone, and the spontaneous electrical activity of the heart was recorded using a Tecnomed electrical amplifier, LabMaster A/D converter, and Axotape software (the sampling frequency was 500 Hz). Recordings were analyzed offline with Spike2 software. Automatic detection of the first wave of the electrical recording was performed to consider the sinus rhythm.

Dose–response results were obtained by sequential perfusion with five norepinephrine solutions at increasing concentrations for 5 min each (10^{-8}–10^{-4} M). Adrenergic responsiveness was examined in the presence of 10^{-6} M atropine (15 min before the first dose and during the doses). The cholinergic antagonist was added to prevent effects of possible local neurotransmitter release (Hicks et al., 1997), and it had no effect on basal pacemaker rate. The maximum HR reached with each dose of norepinephrine was considered for analysis. Basal HR (spontaneous beating rate) was considered as the average HR for the last 5 min of the 25-min stabilization period (before changing to the antagonist). Control HR for dose–response analysis was calculated as the average HR for the 5-min period prior to perfusion with the lowest dose of neurotransmitter (after 10 min with atropine).

2.4.2. Chronotropic responsiveness of isolated hearts to acetylcholine

We evaluated the effect of sepsis on the chronotropic responsiveness of isolated hearts to acetylcholine stimulation 1 day after the induction of sepsis and sham sepsis ($N = 10$ and 5 respectively) once the in vivo ECG was recorded.

The protocol was the same as described above for norepinephrine, but the dose–response results were obtained by sequential perfusion with three increasing doses of acetylcholine (10^{-8}–10^{-6} M) for 5 min each. Cholinergic responsiveness was examined in the presence of an adrenergic antagonist (10^{-6} M nadolol). The minimum HR achieved with each dose was considered for analysis.

2.5. Quantification of autonomic nervous system neurotransmitters

Fifteen rats were included in this protocol, 1 day after the induction of sepsis and sham sepsis ($N = 10$ and 5, respectively) once the in vivo ECG was recorded.

The rats were anesthetized with isoflurane and their hearts were exposed. Blood samples for epinephrine and norepinephrine quantification were collected from the right ventricles. The rats were euthanized when the right atria were isolated from the hearts for

quantification of acetylcholine and choline (acetylcholine precursor and metabolite (Tsai, 2000)).

The atria were immediately frozen in liquid nitrogen and were stored at −80 °C. Frozen tissue samples were sent to the Center for Molecular Neuroscience Neurochemistry Core Lab at Vanderbilt University (Nashville, TN, USA) for determination of acetylcholine and choline concentrations by high-performance liquid chromatography (HPLC) with detection using a post-column enzyme reactor (Damsma et al., 1985). Acetylcholine and choline values were normalized to the protein concentrations in samples. For details on the method, see the supplementary data.

Blood was centrifuged at 2400 rpm for 10 min to separate the plasma. The plasma was stored at −80 °C until catecholamines were determined by HPLC (Foti et al., 1987) at the Departamento de Neuroquímica and Plataforma HPLC, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay.

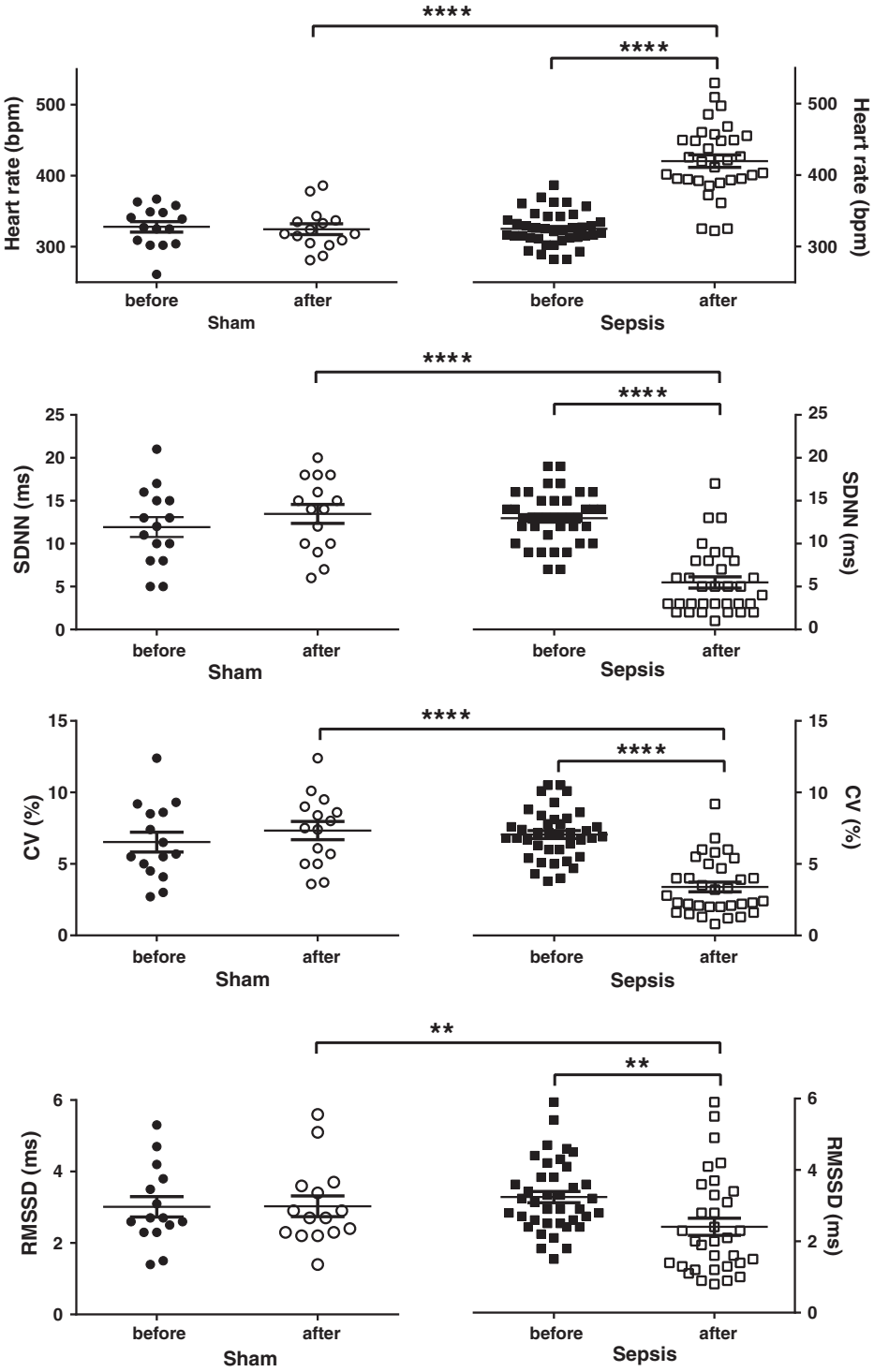

Fig. 1. In vivo results for sham (circles, $N = 15$) and septic (squares, $N = 39$) rats. The mean HR for the 60-min ECG and the HRV indices (SDNN, CV, and RMSSD) for each rat are presented before (filled symbols) and after (empty symbols) the induction of sepsis (or sham sepsis). In the septic group, the number of rats after the induction of sepsis is 33 due to the mortality in this group (15%). The horizontal bars represent the mean value ± SEM for each group. $P < 0.0001$ (****) or < 0.01 (**) for comparisons within each group (Wilcoxon matched paired signed rank test) and between groups (Mann–Whitney test).

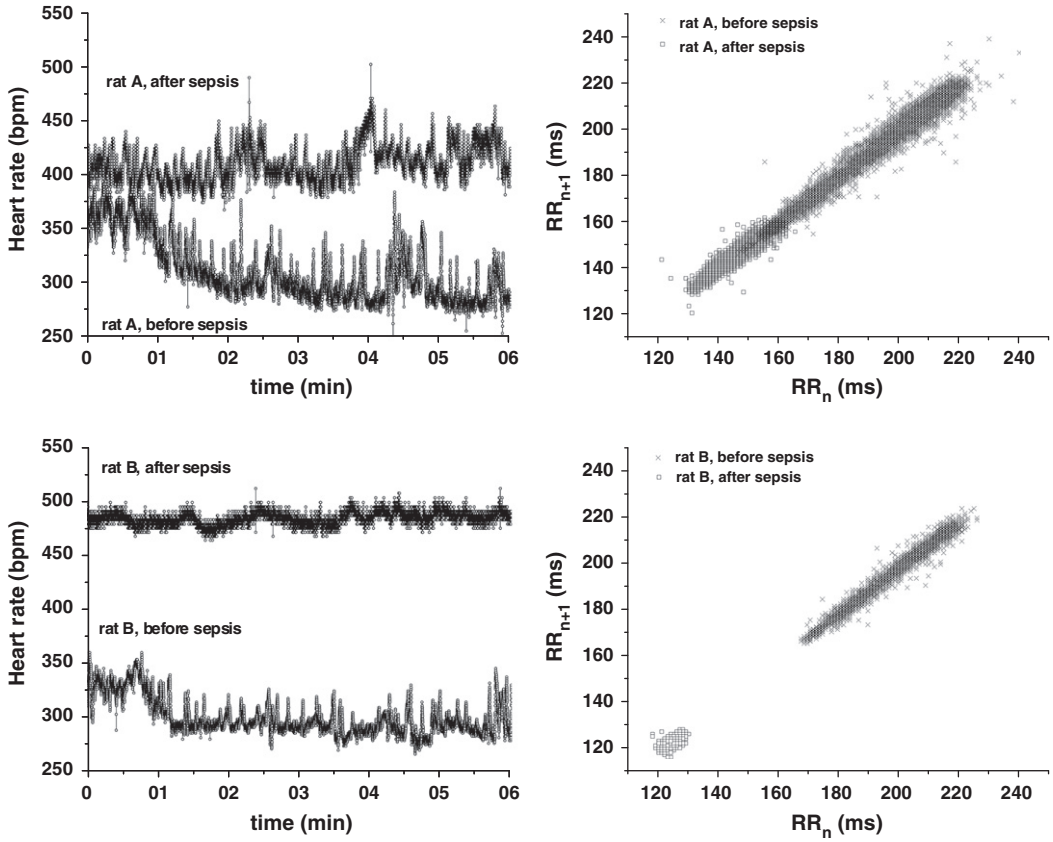

Fig. 2. Tachograms and Poincaré plots for two septic rats before and after sepsis induction. Tachograms (graphs on the left) represent instantaneous heart rate as a function of time. Poincaré plots (right graphs) also show all the data recorded in 1-hour ECG but in this case each RR interval (RR_{n+1}) is plotted as a function of the preceding one (RR_n). Both the rats exhibited increased HR and reduced HRV (SDNN, CV, and RMSSD). The mean HR for the 60-min ECG and HRV values (before vs. after sepsis induction) are as follows: Rat A (top panel), heart rate (bpm) 310 vs. 410, SDNN (ms) 17 vs. 6, CV (%) 9 vs. 4, RMSSD (ms) 3.0 vs. 1.6; Rat B (bottom panel), heart rate (bpm) 302 vs. 485, SDNN (ms) 11 vs. 2, CV (%) 5 vs. 1, RMSSD (ms) 1.5 vs. 1.3.

The laboratories that performed the HPLC measures were blinded to any information about this study.

2.6. Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The analysis was performed using GraphPad Prism (version 6.00, GraphPad Software, La Jolla, CA).

Nonparametric tests were used for all comparisons: Wilcoxon test was employed for paired comparisons (before vs. after induction of sepsis and sham sepsis) and the Mann–Whitney test was used for comparisons between groups. Two-tailed P values < 0.05 were considered statistically significant.

An individual log IC_{50} or EC_{50} (log concentration of neurotransmitter that produced half-maximal response) was obtained by nonlinear regression analysis of the normalized dose–response results for each heart. Then, log IC_{50} or EC_{50} was compared between the two groups. Besides, the average response for each dose was compared between groups and used to obtain a representative dose–response curve to be shown in the figures.

3. Results

Mortality rates were evaluated 1 day after the procedure to induce sepsis and sham sepsis at the time of the ECG recording. The mortality rate was 0% for the sham rats and 15% for the septic rats (6 out of 39 septic rats died). Necropsies were similar in all septic rats: inflated abdomens, peritoneal cavity with cloudy fluid, gangrenous cecum, and sticky abdominal organs. No abnormalities were found in the sham rats.

The sham rats did not exhibit changes in their HR or HRV indices after the sham induction of sepsis (see left graphs in Fig. 1). However, the septic rats had increased HR and diminished HRV. Fig. 1 shows that these changes were statistically significant when compared to the values of the same rats before the induction of sepsis or with those of the sham rats (after the induction of sham sepsis). The range of HR and HRV values became wider in the septic rats after the induction of sepsis. Fig. 2 shows two examples of the tachograms and Poincaré plots before and after sepsis for two rats from the septic group.

Despite the higher HR in septic rats, their isolated hearts presented similar spontaneous beating rates than those from sham rats (Fig. 3).

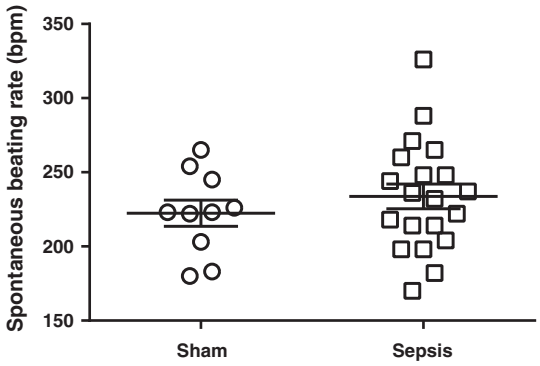

Fig. 3. In vitro results: the spontaneous beating rate of hearts isolated from sham (circles) and septic (squares) rats ($N = 10$ and 20 respectively). The horizontal bars show the mean value ± SEM for each group. No significant P value was obtained.

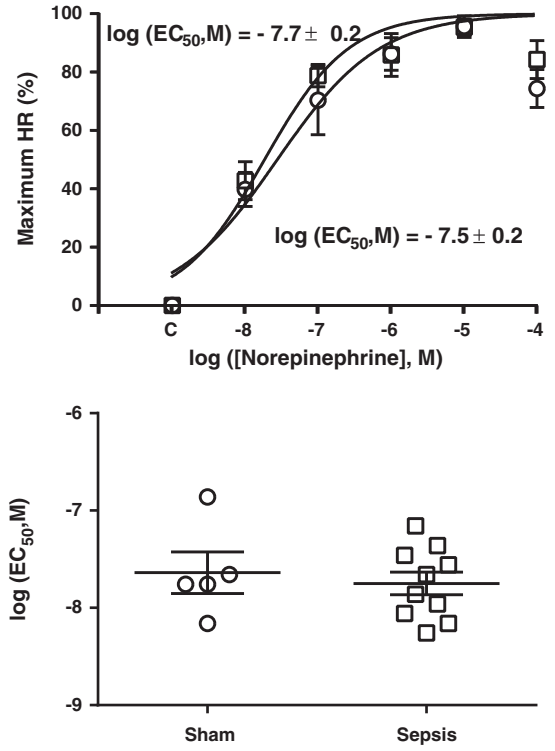

Fig. 4. In vitro results: chronotropic responsiveness of the isolated hearts to norepinephrine. Dose–response curves for sham (circles) and septic (squares) groups ($N = 5$ and 10, respectively) obtained by non-linear fit of the average response (top graph). The log EC_{50} from these curves is similar to the one obtained by averaging the individual log EC_{50} for each heart. Bottom graph: Norepinephrine log EC_{50} for each sham (circles) and septic (squares) heart. No significant P values were obtained.

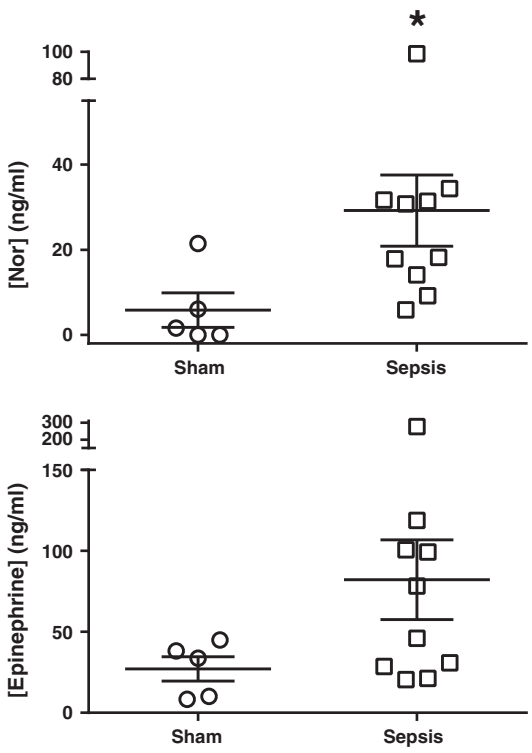

Fig. 6. Quantification of catecholamines in blood plasma. Norepinephrine (Nor, top graph) and epinephrine (bottom graph) concentrations in blood plasma for each sham (circles) and septic (squares) rat. The horizontal bars show the mean value ± SEM for each group. *: P value = 0.019 (Mann–Whitney test).

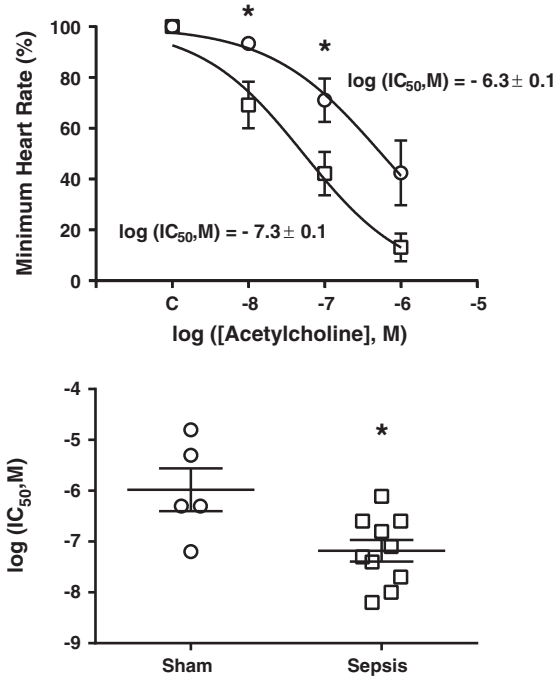

Fig. 5. In vitro results: chronotropic responsiveness of the isolated hearts to acetylcholine. Dose–response curves for sham (circles) and septic (squares) groups ($N = 5$ and 10, respectively) obtained by non-linear fit of the average response (top graph). The log IC_{50} from these curves is similar to the one obtained by averaging the individual log IC_{50} for each sham (circles) and septic (squares) heart (see bottom graph in this figure and text). Bottom graph: acetylcholine log IC_{50} for each sham (circles) and septic (squares) heart. The horizontal bars show the mean value ± SEM for each group. *: $P < 0.05$ for comparisons between groups (Mann–Whitney test).

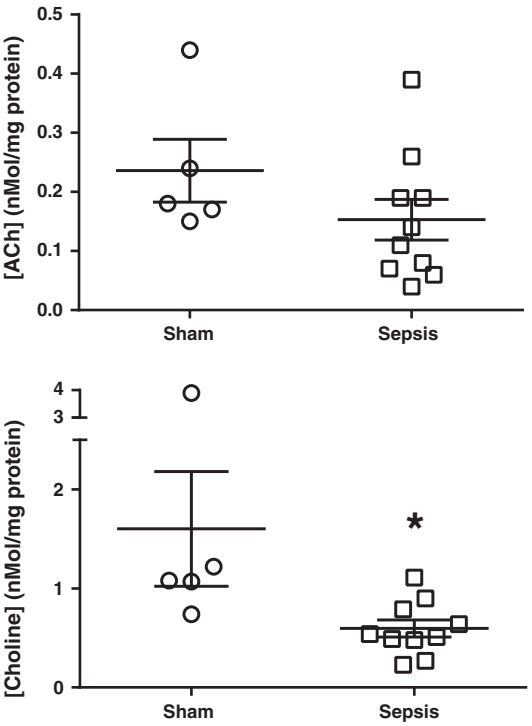

Fig. 7. Quantification of acetylcholine and choline in the right atria. Acetylcholine concentrations (ACh, top graph) and choline concentrations (bottom graph) are shown for the sham (circles) and septic (squares). The horizontal bars show the mean value ± SEM for each group *: P value = 0.013 (Mann–Whitney test).

The norepinephrine log EC_{50} did not show statistically significant differences between the hearts from the septic and sham rats (Fig. 4). Moreover, the maximum HR achieved was similar in both groups (358 ± 19 vs. 370 ± 18 bpm in the sham and septic groups, respectively).

The acetylcholine log IC_{50} was significantly lower in the hearts from the septic rats (Fig. 5). The difference between the mean log (IC_{50},M) in the septic and sham rats was 1.2 (−7.2 and −6.0, respectively). Transformation of this difference to its antilog turns it into a potency ratio of $10^{1.2}$, which is equal to 16, that is, acetylcholine was 16 times more potent in the septic than in the sham rats (Motulsky, 2010).

Epinephrine concentrations in blood plasma did not show statistically significant differences between the septic and sham rats, but norepinephrine concentrations were statistically significantly higher in the plasma from septic rats than those in the plasma from sham rats (Fig. 6).

Choline concentration in the right atriums of septic rats was statistically significantly lower than that in the right atriums of sham rats (Fig. 7).

4. Discussion

Our main finding is that septic rats have an altered autonomic function, not only with higher norepinephrine levels in the blood plasma but also with lower choline concentrations in the right atriums.

Despite the higher HR in situ, the results obtained in hearts isolated from septic rats showed that intrinsic HR was not altered. The spontaneous beating rates of isolated sham and septic hearts were similar, and lower than the HR in situ in both the groups before the induction of sepsis, indicating a possible predominance of sympathetic tone in basal conditions. The lack of tachycardia in the isolated septic heart shows that this effect of sepsis on HR requires the conditions present in the septic organism, for example, reflex mechanisms elicited by diminished preload, increased catecholamine levels and/or other inflammatory mediators (Takayama et al., 2005).

In our study, we assumed that a reliable determination of both acetylcholine and choline concentrations in tissues is valuable for the analysis of cholinergic neurotransmission (Tsai, 2000). However, this approach has some limitations. For example, parallel changes in synthesis and release of acetylcholine might fail to alter its concentration in tissues (Blusztajn and Wurtman, 1983). We found that the choline concentration in septic atria was statistically significantly lower than in sham atria. This difference suggests an altered cholinergic function. Changes in choline availability have been shown to affect tissue concentrations of choline and acetylcholine synthesis and release (Crivello et al., 2010).

Our finding of acetylcholine hypersensitivity of isolated septic hearts seems paradoxical with the results obtained in vivo (higher HR and lower HRV). The enhanced response to acetylcholine in isolated hearts might be the compensatory consequence of impaired parasympathetic function in septic rats.

The enhanced response to acetylcholine found in isolated hearts from septic rats might be caused by the up-regulation of muscarinic receptors. Dong et al. (2000) described an overexpression of muscarinic M2 receptors in the cardiac ventricles of septic rats during the hyperdynamic phase of sepsis, which is consistent with our results (although they described the overexpression 9 h after CLP, followed by underexpression).

Gholami et al. (2012) recently explained the reduction in HRV in septic rats by hyporesponsiveness of the isolated atria to cholinergic stimulation after 5 h of LPS injection. This discrepancy with our results might be explained by the numerous differences between both the studies, such as the method of inducing sepsis. Our results were obtained in an experimental model that mimics gut-derived sepsis in humans (Deitch, 2005), combining tissue injury with polymicrobial infection.

The impaired parasympathetic activity in septic rats might be determined by desensitization of the activation of the "inflammatory" reflex (Fairchild et al., 2011), or the severe disease itself, might have produced retraction of parasympathetic nerve fibers. It has also been described that the co-transmitter neuropeptide Y released during prolonged cardiac sympathetic nerve stimulation reduced the release of acetylcholine, acting via presynaptic receptors (Herring et al., 2008). Since septic rats stop eating after the surgery to induce sepsis, it is possible to speculate that the choline reduction in the right atrium is a consequence of fasting. It has been reported that short-term nutritional changes in rats affect choline and acetylcholine metabolism in the peripheral nervous system (Crivello et al., 2010).

The analysis of catecholamine levels in blood plasma from septic rats showed statistically significant higher concentrations of norepinephrine, but not epinephrine compared to that in sham rats. This agrees with the results described by Kovarik et al. (1987) because the elevation in plasma epinephrine was found after 5 h of CLP in rats, but not after 20, 24, or 41 h. However, Hahn et al. (1995) described sustained elevation in circulating catecholamine levels after 20 h of CLP.

We did not find altered adrenergic responsiveness in isolated septic hearts compared to that of sham rats. Other authors have found contradictory changes in relation to sepsis (Bernardin et al., 2003; Tang et al., 1998). As mentioned above, the different models to induce sepsis must be considered when comparing these data. For example, it has been published recently that the hyporesponsiveness to adrenergic stimulation described in isolated atria from rats after 5 h of LPS injection was lost if LPS was injected into cirrhotic rats (Jazaeri et al., 2013).

Many therapeutic trials are in accordance with this autonomic phenotype of sympathetic hyperactivation and vagal reduced activity. For example, Setoguchi et al. (2012) recently reported that the injection of an acetylcholinesterase inhibitor after CLP in rats prevented HRV reduction and reduced the increase in cytokines and catecholamine serum levels. However, the survival rate did not improve. Kessler et al. (2012) found that subdiaphragmal vagotomy increased mortality in another model of polymicrobial sepsis in mice, although treatment with nicotine did not improve survival. However, the beneficial effects of nicotine treatment were found in LPS-injected rats (Kojima et al., 2011). In addition, beta-1 adrenoceptor blockade was evaluated as a possible therapeutic maneuver (Ackland et al., 2010).

4.1. Limitations of the study

Since most sepsis models result in substantial fluid deficits and we did not record hemodynamic data, it cannot be determined whether we are actually exploring hypovolemia rather than sepsis per se. Further analysis is needed to differentiate the contribution of hypovolemia to the autonomic changes in sepsis.

We used a lethal model to induce severe sepsis but only surviving septic rats were analyzed.

5. Conclusion

The paradox of hypersensitivity to acetylcholine in hearts isolated from septic rats, argues in favor of impaired vagal activity, instead of refractory septic hearts to acetylcholine to explain the reduction in HRV during sepsis. Impaired vagal function is further supported by lower concentrations of choline in the septic right atria compared to that in the sham atria. In conclusion, in experimental sepsis in rats, there is an altered activity of both branches of the autonomic nervous system, that is, sympathetic overexcitation (increased norepinephrine plasma concentration) and parasympathetic dysfunction (decreased choline concentration in the right atria).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.autneu.2013.10.002.

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