

Increased sample asymmetry and memory of cardiac time-series following endotoxin administration in cirrhotic rats

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Abstract

Sepsis, and other causes of acute systemic inflammation, can reduce heart rate variability (HRV) and increase cardiac cycle regularity in mammals. Thus, HRV monitoring has been used for early detection of sepsis in adults and neonates. Liver cirrhosis is associated with reduced basal HRV and the development of tolerance to the cardiac chronotropic effects of bacterial endotoxin. This may pose limitations on the use of heart rate monitoring in early detection of sepsis in this patient population. In a study to develop a physiologic marker for the detection of sepsis in cirrhosis, we observed that endotoxin administration in adult cirrhotic rats leads to the development of transient heart rate decelerations, a phenomenon which has been reported in neonates with sepsis, and quantified using sample asymmetry analysis. In the present study, cirrhosis was induced by surgical ligation of the bile duct in rats. Cirrhotic rats were given intraperitoneal injections of either saline or endotoxin (1mg kg^{-1}). Changes in sample asymmetry and memory length of cardiac time-series were studied in conscious rats using implanted telemetric probes. Cirrhotic (but not control) rats exhibited increased sample asymmetry following endotoxin injection, which was consistent with the development of transient heart rate deceleration. Endotoxin administration in cirrhotic rats was associated with prolongation of memory length for observing decelerating perturbations in the cardiac rhythm. These findings may have application in the development of an HRV monitoring system for early detection of sepsis in cirrhosis.

Key words: Cirrhosis, Endotoxin, Heart rate variability, Memory length, Sample asymmetry, Sepsis

1. Introduction

Sepsis is the major cause of mortality in intensive care units, and early detection of sepsis in its initial stages is essential to reduce preventable deaths (Fairchild *et al* 2013). A variety of serum and physiological markers have been investigated for early detection of sepsis. Among the physiological markers, continuous heart rate variability (HRV) monitoring is shown to herald onset of sepsis in adults with neutropenia, as well as premature neonates (Griffin *et al* 2005, Ahmad *et al* 2009, Bravi *et al* 2012). Sepsis and other causes of systemic inflammation can reduce the instantaneous variability of the cardiac cycle and decrease its complexity (Garrard *et al* 1993, Lake *et al* 2002). Acute administration of a bacterial lipopolysaccharide (LPS) reduces HRV and increases the regularity of cardiac cycle in humans, rabbits, mice and rats (Godin *et al* 1996, Goldstein *et al* 1995, Fairchild *et al* 2009, Gholami *et al* 2012). Clinical and experimental evidence suggest that uncoupling of cardiovascular regulatory mechanisms and decreased controllability of cardiac pacemakers may mechanistically contribute to the loss of HRV in sepsis (Godin *et al* 1996, Hajiasgharzadeh *et al* 2011, Gholami *et al* 2012, Mazloom *et al* 2014).

Although a significant reduction in HRV is a consistent finding in patients and experimental models of acute inflammation, such changes are partially impaired when LPS is administered to subjects with chronic inflammation (Haddadian *et al* 2013, Meamar *et al* 2015). Underlying medical conditions, such as diabetes or liver cirrhosis, are associated with chronic inflammation and reduced basal HRV, probably due to autonomic neuropathy (González-Clemente *et al* 2007, Mani *et al* 2009). However, acute injection of LPS in either diabetic or cirrhotic rats has significantly less effect on conventional HRV indexes (*e.g.* standard deviation of R-R intervals and spectral indexes) than on control rats (Meamar *et al* 2015, Haddadian *et al* 2013). Although this phenomenon is interesting within the context of endotoxin tolerance, it suggests that HRV monitoring may have limitations in early diagnosis of sepsis in patients with specific underlying medical conditions, such as diabetes or cirrhosis. This is particularly important in patients with cirrhosis, as sepsis is the main cause of mortality in this patient population (Wong *et al* 2005). We have previously shown that HRV is decreased in patients, as well as rats, with cirrhosis (Mani *et al* 2006a, Mani *et al* 2009). Likewise, acute LPS injections could not markedly decrease conventional HRV from its basal level in cirrhotic rats (Haddadian *et al* 2013). Thus, the identification of a novel physiomerker may be useful for the application of heart rate monitoring in early detection of sepsis in cirrhosis.

A pioneering report by Griffin *et al* (2003) showed that reduced HRV is not the only feature in neonatal sepsis and transient heart rate deceleration is also a hallmark of sepsis in neonates. Thus, sepsis in neonates is associated with episodic heart rate decelerations prior to clinical illness and death, which has diagnostic and prognostic value (Fairchild *et al* 2013). In order to quantify transient heart rate decelerations, Kovatchev *et al* (2003) developed sample asymmetry as an analytic tool to detect the changes in the shape of the distribution of R-R intervals caused by transient decelerations and/or reduced accelerations of heart rate. Lear *et al* (2015) have recently applied sample asymmetry to show subclinical heart rate decelerations in preterm fetal sheep after acute on chronic LPS exposure. In a study to develop a physiomerker for the detection of sepsis in cirrhosis, we observed that LPS administration in adult cirrhotic rats is associated with transient heart rate decelerations, a phenomenon which is not observed in control rats challenged with LPS. This study reports our

investigations on episodic subclinical bradycardia and increased sample asymmetry in an experimental model of cirrhosis.

2. Methods

2.1. Induction of cirrhosis and telemetric recording of electrocardiogram (ECG) from conscious rats

Male Sprague–Dawley rats (body weight 230–250 g) were used in this study. All animal procedures were in accordance with recommendations established by the Animal Ethics Committee of Tarbiat Modares University, Home Office (UK) as well as the United States NIH guidelines (publication no. 85-23). Cirrhosis was induced by ligation of the bile duct as described before (Hajiasgharzadeh *et al* 2014). At the time of bile duct ligation, a dorsally mounted radio frequency transmitter and leads were implanted for ECG recording (Mazloom *et al* 2013). Four weeks after the operation, ECG was recorded using a telemetry system (Data Sciences International, St. Paul, Minnesota, USA) connected to a Powerlab data acquisition system (ADInstruments, Sydney, Australia) with a sampling frequency of 10 kHz. The animals received an intraperitoneal injection of saline or endotoxin (LPS; *Salmonella typhimurium* lipopolysaccharide, 1 mg kg⁻¹ dissolved in isotonic saline). All recordings were started at 8 a.m. and were continued for 5 h. Five to fourteen rats were used in each group.

2.2. Isolation of spontaneously beating heart (*ex vivo*)

Both autonomic nervous system and the intrinsic cardiac pacemaker dynamics contribute to HRV (Mazloom *et al* 2014). It is known that systemic inflammation can potentially affect both autonomic nervous system and cardiac pacemaker activity (Gholami *et al* 2012; Eftekhari *et al* 2013). Since telemetric studies cannot separate these effects from each other, isolated perfused hearts from control and cirrhotic rats were studied in order to investigate the effect of acute LPS injection on cardiac pacemaker dynamics. One hour after LPS or saline injection, a separate group of cirrhotic or control rats was anaesthetized with sodium thiopental (50 mg kg⁻¹, intraperitoneal) and the hearts were removed. The isolated hearts were cannulated for retrograde perfusion according to the Langendorff method with oxygenated physiological salt solution as described (Mazloom *et al* 2014). Two stainless-steel electrodes were placed to record ventricular electrical activities. To avoid artifacts evoked by dissection, a stabilization period of 30 min was allowed before evaluation of the spontaneous electrical activity. Seven to fourteen rats were used in each group.

2.3. Data analysis

The signals were digitized at the sampling rate of 10 kHz and were displayed on a Powerlab system. The R peaks were detected and the R-R interval series was generated using an ad hoc computer program. The R-R interval series was visually inspected and 1750 artifact-free continuous R–R intervals were selected for HRV analysis. The standard deviation of the R-R intervals (SDNN) was calculated as a measure of total HRV. Unlike other measures of HRV, sample asymmetry and memory length allow separate quantification of the contribution of accelerations and decelerations (Kovatchev *et al* 2003, Shirazi *et al* 2013). In this context, memory was defined as a statistical feature that lasts for a period of time, and distinguishes the time-series from a random process. Thus, we measured sample asymmetry

and memory length in cardiac time-series as described by Kovatchev *et al* (2003) and Shirazi *et al* (2013) respectively.

2.4. Statistical analysis

Data were presented as the mean \pm standard error of the mean (SEM). Statistical comparison between the groups was performed using two-way ANOVA, followed by Sidak's multiple comparisons test. A P-value less than 0.05 was considered statistically significant.

3. Results

Bile duct ligated rats showed manifestation of cirrhosis, such as the development of splenomegaly and ascites, which was not observed in sham-operated control rats. Liver histology confirmed the development of extensive hepatic fibrosis and inflammation in cirrhotic rats. Four weeks after operation, control and cirrhotic rats were given a single dose LPS or saline injection and were then monitored for 24 hours. The animals used in this report were the same as those used and reported previously by us (Haddadian *et al* 2013). Therefore, the effect of LPS on conventional HRV indexes in cirrhotic rats can be found elsewhere (Haddadian *et al* 2013).

All cirrhotic and sham-operated rats survived when they were monitored for 24 hours after saline injection. All sham-operated control rats survived the LPS challenge, while all cirrhotic rats died within the first six hours of endotoxin injection ($P < 0.01$, Mantel–Cox test). All cirrhotic rats survived the initial two-hour lag following LPS injection, therefore heart rate dynamics were analyzed within two hours after endotoxin challenge. The mean heart rate of the control rats was 328 ± 5 beats/min, which changed to 396 ± 7 beats/min two hours following LPS injection ($P < 0.001$). The mean heart rate was 368 ± 6 beats/min in cirrhotic rats, which didn't change significantly after endotoxin injection (331 ± 17 beats/min, $P = 0.078$), a finding which corroborates previous reports on the development of tolerance to the cardiac chronotropic effects of endotoxin in rats (Jazaeri *et al* 2013, Haddadian *et al* 2013). All cirrhotic rats exhibited episodic heart rate deceleration following LPS injection (figure 1A), which was associated with a transient increase in sample asymmetry, as shown in figure 1B. Sample asymmetry of 1 (or close to 1) means that the time-series is symmetric around its median. Sample asymmetry greater than 1 is caused by reduced accelerations and/or transient decelerations of the heart rate.

Figure 2 shows heart rate dynamics in the experimental groups two hours after LPS. Control rats exhibited a statistically significant reduction in SDNN ($P < 0.05$), while cirrhotic rats had a low SDNN which did not further decrease following LPS injection (Figure 2A). Acute endotoxin challenge did not affect sample asymmetry in the control rats, while cirrhotic rats showed a significant elevation of sample asymmetry ($P = 0.0008$) even when the R-R time-series were selected from the segments without obvious episodic heart rate deceleration (figure 2B and * in figure 1B). Figure 1C indicates continuous monitoring of sample asymmetry in a representative healthy rat following saline injection. Sample asymmetry does not show a considerable fluctuation after injection of saline.

Mean memory length values for observing a decelerating rare event ($\text{jump} > \rho$) at different ρ levels (from σ to 3σ) are shown in figure 2C. There was no significant difference in memory length between control (saline) and control (LPS) groups. Cirrhotic (saline) rats showed similar average memory length when compared with control (saline) animals. The average memory length was higher in cirrhotic rats two hours following LPS administration, in comparison with the cirrhotic (saline) group for observing a

decelerating jump in the cardiac cycle which was greater than 2.5σ or 3σ (figure 2C). The results of memory length for observing an accelerating rare event ($\text{jump} \leq -\rho$) is shown in figure 2D. There was no statistically significant difference among the experimental groups.

The results of cardiac cycle variability analysis in isolated perfused hearts are presented in table 1. The mean heart rate in the control group was 311 ± 11 beats/min, which did not change following either development of cirrhosis and/or LPS administration. SDNN of the isolated hearts (*ex vivo*) in the control group was 3.54 ± 0.33 msec. There were no significant differences in SDNN among the experimental groups. The SDNN of the isolated hearts of the control group was lower than the SDNN of the control group recorded from conscious healthy rats (5.96 ± 0.63 msec, $P < 0.05$), which is probably due to the effect of denervation of the heart and lack of autonomic control of cardiac pacemaker (Mani *et al* 2006b).

4. Discussion

Sepsis is a major cause of death in patients with cirrhosis (Wong *et al* 2005). Increased mortality of cirrhotic rats in response to endotoxin has been reported previously (Harry *et al* 1999, Haddadian *et al* 2013) and goes along with clinical reports, which shows that mortality in cirrhotic patients with sepsis is markedly higher compared to that of septic patients without cirrhosis (O'Brien *et al* 2012). Thus, development of a physiomarker for early diagnosis of sepsis in patients with cirrhosis may have potential clinical application in prevention of high mortality by starting early treatment in this patient population.

To the best of our knowledge, increased sample asymmetry and transient heart rate decelerations have only been reported in neonates with sepsis (Kovatchev *et al* 2003; Flower *et al* 2010) and have not been reported within the context of sepsis and cirrhosis. This finding may be important since other conventional HRV indexes (such as SDNN) do not show a marked reduction in cirrhotic rats after administration of LPS. Despite this report, the value of sample asymmetry in early detection of sepsis in patients with cirrhosis is unknown and requires investigation in the future. Unlike most other measures of HRV, sample asymmetry allows separate quantification of the contribution of accelerations and decelerations (Kovatchev *et al* 2003). Likewise, inverse statistical analysis can also provide information on accelerations and decelerations separately (Ebadi *et al* 2011, Shirazi *et al* 2013). The concept of memory length in physiological time-series was introduced by Shirazi *et al* (2013) and measures the time period, during which rare events within a physiological time-series do not appear randomly. This method is based on the calculation of the distribution of the waiting time to observe a jump in a time-series. A jump from a given point in a time-series means finding the point that becomes ρ units slower (or faster) than the R-R interval of the given point. The level of 'rarity' of such events depends on the definition of ρ . For example, if σ is the standard deviation of a normalized R-R time-series, the jump can be defined to be greater than σ , 2σ or 3σ for decelerating rare events. Alternatively, jumps can be defined to be less than $-\sigma$, -2σ or -3σ for accelerating rare events (Ebadi *et al* 2011, Shirazi *et al* 2013). This method compares the distribution of these waiting times for the main time-series and its shuffled version. Previous analysis has shown that the difference between the original and shuffled distributions occurs only within small regions, and afterwards the two distributions cross and there is no statistically

significant difference between the distributions after the cross. This interval can be interpreted as the 'memory length' of the time-series (Shirazi *et al* 2013).

We showed that the memory length was higher in cirrhotic rats two hours following LPS administration, in comparison with the cirrhotic (saline) group for observing a jump in the cardiac cycle which was greater than 2.5σ or 3σ (figure 2C). This observation may lead us to assume that the effect of environmental or physiological decelerating perturbations on the heart rate last longer in cirrhotic rats than healthy rats. The prolongation of memory is indirectly linked to a reduced controllability in a complex system; therefore our data may provide evidence for a reduced controllability in cardiac rhythm following LPS injection in cirrhotic rats. The memory length for observing an accelerating rare event (jump $\leq -\rho$) was also calculated. In this case, there was no statistically significant difference among the experimental groups. Therefore, that there is an asymmetric behavior in prolongation of memory with respect to decelerating or accelerating rare events in endotoxemic cirrhotic rats. In other words, memory is prolonged only after decelerating perturbation and not after accelerating events in endotoxemic rats with cirrhosis. This may explain why sample asymmetry is increased in this experimental model, although the physiological mechanism of this phenomenon needs clarification.

The reason for transient heart rate deceleration in neonatal sepsis is not understood. Such decelerations may be linked to episodic discharges of the vagus nerve or altered electrical function within the cardiac conducting system (Eftekhari *et al* 2013). We looked at R-R variability analysis of isolated perfused hearts and measured SDNN, sample asymmetry and memory length two hours after injection of LPS or saline. The results showed that there was no significant difference in these variability indexes between the experimental groups. Although these results do not rule out the possibility of an intrinsic cardiac dysfunction in the development of transient heart rate deceleration in endotoxemic cirrhotic rats, they do show that increased sample asymmetry and memory cannot be explained simply by impaired pacemaker dynamics in cirrhosis. Cirrhosis is associated with disordered central cardiovascular regulation and impaired Fos staining at the nucleus of the solitary tract and ventrolateral medulla in the brain stem (Song *et al* 2001). Future studies on the contribution of central cardiovascular regulation may pave the way to elucidate the mechanism of transient heart rate decelerations in neonatal sepsis, as well as endotoxemia in rats with cirrhosis.

We report that endotoxin administration in adult cirrhotic rats is associated with transient heart rate decelerations, increased sample asymmetry and prolongation of memory in cardiac time-series. These finding may have application in the development of monitoring systems for early detection of sepsis in cirrhosis.

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Figure 1. A. A representative sample recorded from ECG of a cirrhotic rat after endotoxin (LPS) administration showing a transient heart rate deceleration. **B.** Continuous monitoring of sample asymmetry in a representative cirrhotic rat following bacterial lipopolysaccharide (LPS) injection. * indicates the segment where the time-series was used for calculation of sample asymmetry in figure 2B. **C.** Continuous monitoring of sample asymmetry in a representative control rat following saline injection.

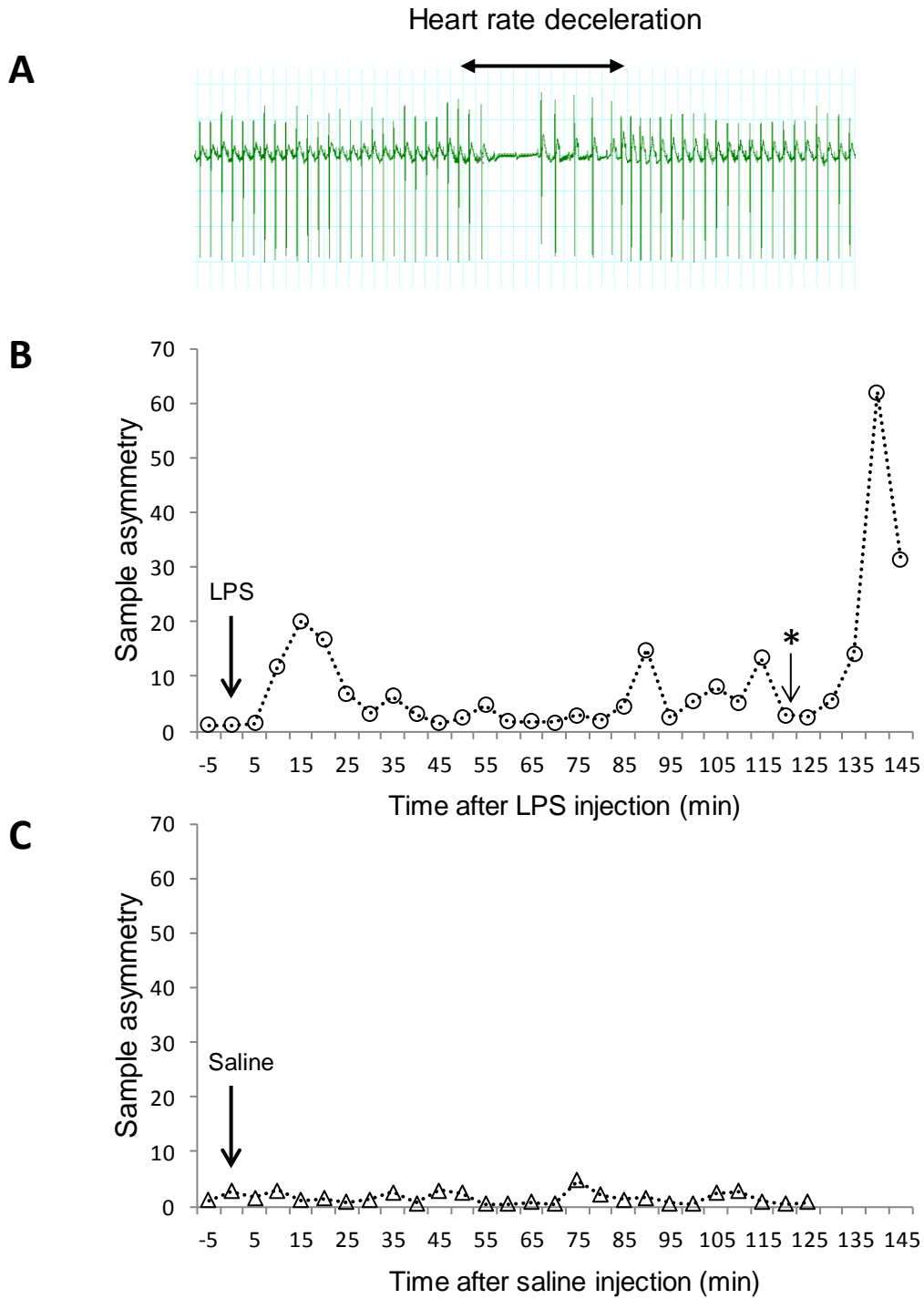


Figure 2. HRV indexes in control and cirrhotic rats two hours after endotoxin (LPS) or saline challenge. **A.** The effect of LPS on SDNN in control or cirrhotic rats two hours after endotoxin challenge. **B.** Comparison of sample asymmetry among the experimental groups. **C.** Comparison of memory length for observing decelerating events (ΔB) with varying thresholds (from σ to 3σ) among control and cirrhotic rats given saline or LPS. **D.** Comparison of memory length for observing accelerating events (ΔB) with varying thresholds (from σ to 3σ) among the experimental groups. Data are shown as mean \pm SEM. * $P < 0.05$, + $P < 0.01$ in comparison with the cirrhotic (saline) group, # $P < 0.05$ in comparison with the control (saline) group.

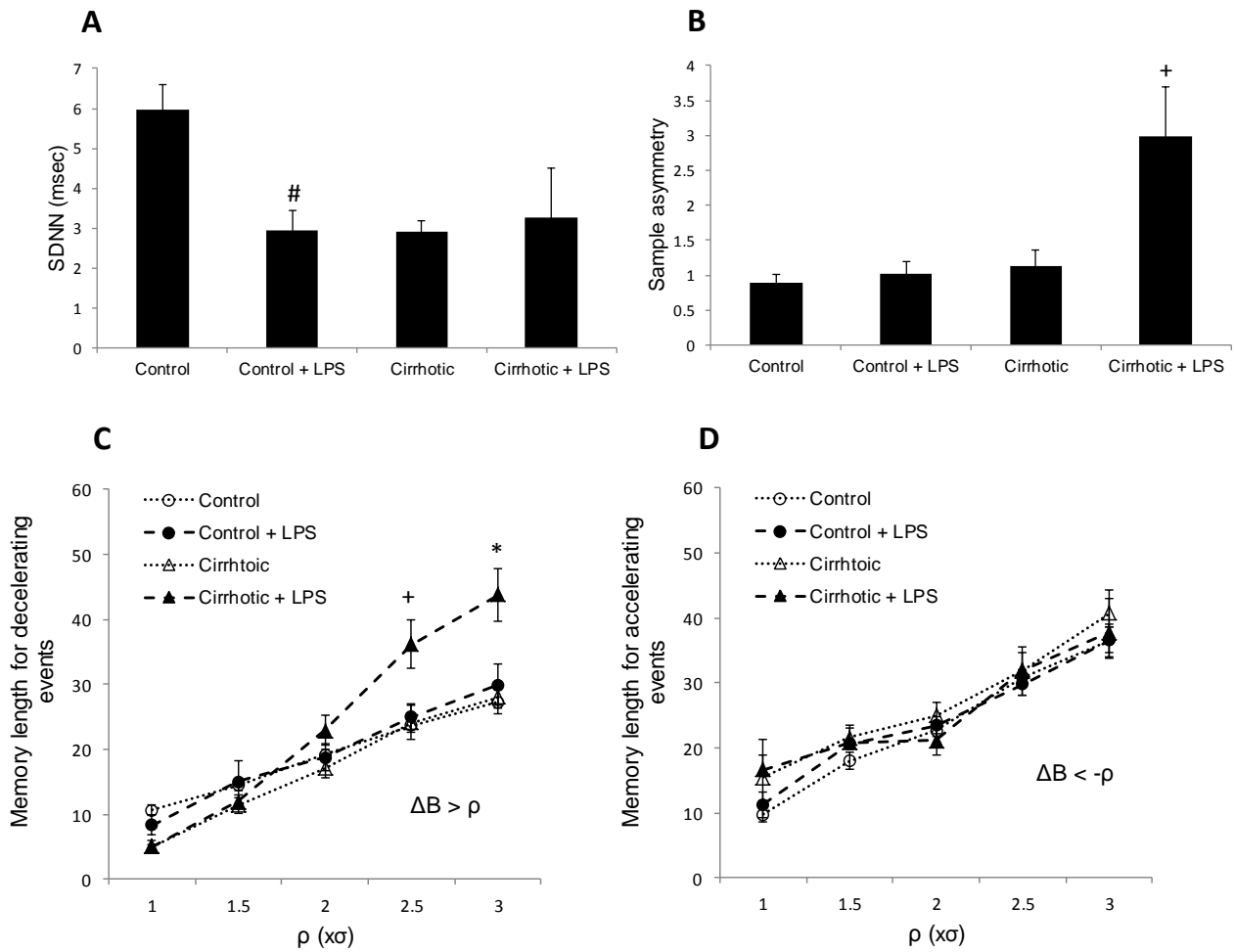


Table 1. Beating rate, SDNN, sample asymmetry and memory length in isolated perfused hearts in control or cirrhotic rats given saline or endotoxin (LPS). Data are shown as mean \pm SEM.

Cardiac rhythm index (in vitro)	Control + Saline	Control + LPS	Cirrhotic + Saline	Cirrhotic + LPS
Heart rate (beat/min)	311 \pm 8	313 \pm 8	291 \pm 19	304 \pm 16
SDNN (msec)	3.54 \pm 0.33	4.17 \pm 0.52	3.93 \pm 0.47	4.49 \pm 0.90
Sample asymmetry	2.95 \pm 0.75	2.41 \pm 0.83	1.62 \pm 0.78	1.63 \pm 0.14
Memory length (for decelerating events, $\rho > 3\sigma$)	26.32 \pm 2.51	27.70 \pm 3.68	26.83 \pm 2.52	27.04 \pm 1.25
Memory length (for accelerating events, $\rho < -3\sigma$)	21.93 \pm 2.52	24.78 \pm 2.64	24.06 \pm 2.57	21.67 \pm 1.81