

Pre-treatment and continuous administration of simvastatin during sepsis improve metabolic parameters and prevent CNS injuries in survivor rats

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Research Article

Keywords: experimental sepsis, metabolism, metabolic disorders, HMG-CoA inhibitors, cytokines, glia

Posted Date: December 28th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1202746/v1>

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Abstract

Sepsis causes overproduction of inflammatory cytokines, organ dysfunction and cognitive impairment in survivors. In addition to inflammation, metabolic changes occur according to the stage and severity of the disease. Understanding the role and place of metabolic disturbances in the pathophysiology of sepsis is essential to evaluate the framework of septic patients, predict the syndrome progress and define treatment strategies. We investigated the effect of simvastatin on the disease time course and on metabolic alterations, especially with respect to their possible consequences in the CNS of surviving rats. The animals of this study were weighed daily and followed for 10 days to determine the survival rate. In the first experiment, control or CLP-animals were randomized in 24 h, 48 h and 10 days after septic induction, for bacterial load determination and, quantification of cytokines. In the second experiment, control or CLP-animals were treated or not with simvastatin and randomized in the same three time points for cytokines quantification and assessment of their body metabolism and locomotor activity (at 48 h and 10 days), as well as the evaluation of cytoarchitecture and astrogliosis (at 10 days). The CLP-rats treated with simvastatin showed a reduction in plasma cytokines and improvement in metabolic parameters and locomotor activity, followed by minor alterations compatible with apoptosis and astrogliosis in the hippocampus and prefrontal cortex. These results suggest that the anti-inflammatory effect of simvastatin plays a crucial role in restoring energy production, maintaining a hypermetabolic state necessary for the recovery and survival of these CLP-rats.

Introduction

Sepsis has been the focus of intense investigation because of its clinical impact related to high mortality rates and associated morbidities that generate financial burden on the health care system [1]. Despite the intense and progressive effort in sepsis research, it is still considered a major health issue worldwide. Global data on sepsis incidence and mortality reported approximately 19.4 million cases and 5.3 million deaths annually [2]. With few exceptions, even developed countries present high mortality rates, leading to high costs (US\$24 billion annually, in the United States for example) [3, 4].

Due to the relevance of sepsis in clinical practice, the investigation of different aspects of sepsis pathophysiology through reliable experimental models is essential for the development new therapeutic approaches. Our research group has used the polymicrobial intra-abdominal infection model induced by cecal ligation and puncture (CLP) to investigate relevant aspects of sepsis, such as neuroendocrine and cognitive dysfunctions [5–7]. Using the CLP model, it is possible to modulate the severity of the ensuing infection by varying the amount and size of the needle used to puncture the cecum [8, 9]. In this study, we used a well-established model of sepsis survivor animals to monitor the evolution of peripheral infection and its impact or consequences on the CNS. Particularly, we were interested in the effects of simvastatin in this context.

In the CLP model, there is a progressive release of pro-inflammatory cytokines that will play an essential role in activating the neuroendocrine response, mediating metabolic changes, and altering

metabolism[10, 11]. In the course of sepsis, these metabolic changes occur according to the stage and severity of the disease, with a dysregulation in the metabolism of all macronutrients - carbohydrates, lipids, and proteins[10, 12]. In this study, we use indirect calorimetry to analyze whole-body energy metabolism by measuring energy expenditure (EE) and identifying the substrate consumed through calculating the respiratory quotient (RQ)[13]. Understanding the role and place of metabolic disturbances in the pathophysiology of sepsis is essential for better treatment management in patients and for discovering targets for potential new therapies[14].

Considering these inflammatory and metabolic parameters, we used a statin as a pharmacological tool due to its known pleiotropic effects [15]. Several studies have shown that HMG-CoA reductase inhibitors, such as simvastatin, have anti-inflammatory properties that may play a role in modulating the immune system and contribute to the treatment of even neuroinflammatory diseases [6, 16–21]. This pleiotropic effect occurs in part through molecular mechanisms related to a cellular signaling cascade triggered by the inhibition of isoprenoid compounds and G-protein prenylation [22]. Given the wide use of statins by the world population and its anti-inflammatory effect, here we investigate the simvastatin effect on the time course of CLP-induced sepsis, by analyzing metabolic changes and histopathological assessments in the CNS.

Material And Methods

Animals

Male Wistar (280 - 360 grams) provided by the Central Animal Facility of the University São Paulo, Ribeirão Preto Campus, were housed in collective cages under controlled temperature ($25 \pm 1^\circ\text{C}$) and photoperiodic (12:12 h night:day cycle) conditions with water and commercial diet *ad libitum* (Nuvilab CR-1 Autoclavable, NUVITAL Nutrients S/A). The animals were weighed every day for 10 days. All experiments were carried out according to the National Council of Animal Experiment Control (CONCEA) and with approval by the Institutional Animal Care and Use Committee at the School of Dentistry of Ribeirão Preto, University of São Paulo (protocol number: #2019.1.51.58.6). We used human endpoints in shock research [23] as criteria to euthanize cecal ligation and puncture (CLP) animals in high suffering, immediately before or soon after the studied time points [6].

Cecal ligation and puncture (CLP) surgery and drug administration

Polymicrobial sepsis was induced by CLP, as previously described [5, 18, 24]. This model has been widely used in our laboratory to investigate neuroimmunoendocrine alteration during sepsis. Briefly, animals were anesthetized with tribromoethanol (TBE) 2.5% (Sigma- Aldrich) in saline solution (250 mg/kg) and an abdominal incision of 2 cm was performed followed by exposure of the cecum and obstruction (partial ligation) of the ileocecal valve. The cecum was punctured once with a 14G needle and after

checking the extravasation of the fecal content, it was reintroduced into the peritoneal cavity and the incision was sutured. Sterile saline was applied subcutaneously as resuscitation fluid (20 ml/kg).

Simvastatin (trade name Zocor® - MSD, Merck Sharp & Dohme, UK) was dissolved in sterile saline (NaCl 0.9%) and administered by gavage at a dose of 20 mg/kg in according to our previous study [18]. The animals were treated with simvastatin or an equivalent volume of saline 4 days before and 24 h, 48 h, or 10 days after CLP surgery.

Experimental protocol

In the first experiment, control (naive = non-manipulated) or CLP-animals were randomized in three different time points (24 h, 48 h, and 10 days after CLP-surgery), to bacterial load determination and cytokines quantification. In the second experiment, CLP-animals were treated with simvastatin and randomized in the same three-time points to cytokines quantification, body metabolism assessment by indirect calorimetry and locomotor activity (48 h and 10 days) and, histological and immunohistochemical evaluation (10 days). In both experiments, all the animals were weighed every day and followed for 10 days to determine the survival rate.

Bacterial cultures

Brain, spleen and mesenteric lymph node (MLN) samples were cultured at the time points 24 h, 48 h, and 10 days after CLP-surgery [25]. Plates were incubated at 37°C in aerobic conditions for 48 h, and then the number of colony-forming units (CFU)/mg for each organ or tissue was counted.

Cytokines quantification

Plasma IL-1 β and IL-6 concentrations were determined using specific enzyme-linked immunosorbent assay (ELISA) kits for each cytokine (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and as described in previous work of this laboratory [5]. To interrupt the reaction, we added to each well 25 μ L of 2N sulfuric acid (H₂SO₄). The detection limits for IL-1 β and IL-6 specific ELISA kits were 5, 10, and 5 pg/mL, respectively. The absorbance was measured at 450 nm in a microplate reader (Synergy™ H1, BioTek® Instruments, Inc.).

Indirect calorimetry and locomotor activity

Analyses of the animals' body metabolism were performed at 48 h and 10 days after CLP-surgery by indirect calorimetry using the Oxylet Physiocage™ (PanLab, Barcelona, Spain) equipment. The experiment was carried in two metabolic boxes, with one animal per box, and recording for 14 h, of which the final 12 h were analyzed. The following metabolic variables were considered: the volume of carbon dioxide produced (VCO₂), the volume of oxygen consumed (VO₂) and energy expenditure (EE). The respiratory quotient (RQ) calculated by the relation [VCO₂/VO₂] was used for the estimation of macronutrient oxidation and locomotor activity (horizontal and vertical [rearing] movements).

Histological and immunohistochemical assays

The animals were perfused with 250 mL of PBS followed by 250 mL of fixative solution (4% paraformaldehyde in 0.1 mol/L phosphate buffer). Brains were post-fixed in paraformaldehyde (4%) for 2 days at 4 °C and then kept in alcohol at 70%. After stepwise dehydration and diaphanization, the brains were embedded in paraffin. Finally, the prefrontal cortex and hippocampus portions were coronally cut into 5 µm thick sections and mounted on gelatin-coated slides. Histological assessment was performed by hematoxylin and eosin staining in order to assess the general cytoarchitecture, structure distribution, and cellular density. Histological changes were examined by a pathologist who was blinded to the identification of the treatment groups.

Immunohistochemical assays were used to detect reactive astrocytes. Sections were incubated overnight at 4°C with an anti-GFAP (glial fibrillary acidic protein) polyclonal primary antibody generated in rabbits (Z0334 DAKO, Glostrup, Denmark) diluted 1:6,000 in bovine serum albumin (BSA). Subsequently, the sections were incubated with a biotinylated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology SC-2040) diluted 1:300 in BSA. Finally, the sections were incubated with HRP streptavidin reagent (dilution 1:400; Thermo Scientific JG 122591) and reacted with diaminobenzidine (DAB, Sigma D5905) for 1 min. Staining specificity was checked by the omission of the primary antibody in some sections, resulting in the complete elimination of the immunoreaction signal.

Images were captured using an AxioCam MRc system (Zeiss) coupled to a Zeiss KS300 microscope. The anatomical description of brain regions was done according to the rat brain atlas of Paxinos and Watson [26].

Statistical analysis

All results are expressed as mean±standard error of the mean (SEM). Cytokines, weight gain, and locomotor activity results were analyzed by One-way analysis of variance (ANOVA). Indirect calorimetry results were analyzed by Two-way ANOVA. Both ANOVAs were followed by a Tukey's *post-hoc* test for multiple comparisons. The survival rate was analyzed by the Log-rank (Mantel-Cox) test. All statistical analyses were performed using GraphPad©Prism software, version 9.0 (San Diego, CA, USA). Variables were compared and considered significant when $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

Results

Screening of sepsis severity through quantification of bacterial load and plasma cytokines during 10 days after CLP-surgery

The animals that underwent CLP-surgery showed typical signals of clinical sepsis, including lethargy, piloerection, and tachypnea. In this sepsis model, 50% of the animals died between 24 and 48 hours after CLP-surgery, with 30% at 48 hours. The mortality rate at the end of day 10 was 70% (Figure 1b).

At 24 and 48 hours, we observed statistically significant increase in the bacterial load present in the spleen (24 h: $P < 0.01$; 48 h: $P < 0.0001$) and mesenteric lymph node (MLN) (24 h: $P < 0.05$; 48 h: $P <$

0.0001) of septic animals compared to their controls. At 10 days after septic induction, no bacteria were found in any of the organs studied. No bacteria were found in the brain tissue at any of the time points studied (Figure 1c). We also found an increase in plasma cytokines at 24 h (IL-1 β : $P < 0.001$; IL-6: $P < 0.001$) and 48 h (IL-1 β : $P < 0.0001$; IL-6: $P < 0.0001$) in septic animals compared to control animals. At day 10 days after septic induction, there was a reduction of these plasma cytokines to levels similar to those of the controls (Figure 1d).

At the critical time point of sepsis, both bacterial load (spleen: $P < 0.001$; MLN: $P < 0.01$) and cytokine levels (IL-1 β : $P < 0.001$; IL-6: $P < 0.05$) were higher at 48 h when compared to the 24 h time point after CLP-surgery (Figure 1c, d).

Analysis of survival curve and plasma levels of cytokines following simvastatin treatment

Considering 24 and 48 h as the period with the highest number of animal deaths, and the highest cytokines plasma levels, the reduction in these levels indicate a simvastatin effect. Simvastatin treatment given 4 days before and 10 days after CLP-surgery (Figure 2a) showed a survival rate that was 30% higher compared to the untreated septic animals. Only at the 48 h time point we observed deaths in the treatment group (Figure 2b). Simvastatin treatment reduced the plasma levels of IL-1 β ($P < 0.0001$) and IL-6 ($P < 0.001$) cytokines, but only at the 48 h time point after sepsis induction (Figure 2c). Additionally, the comparison of the simvastatin-treated groups for the two time points showed a decrease in the plasma levels of IL-1 β ($P < 0.001$) and IL-6 ($P < 0.05$) cytokines at 48 hours (Figure 2d)

Measurement of metabolic parameters and locomotor activity at 48 hours and 10 days after CLP-surgery on simvastatin-treated rats

Forty-eight hours after CLP-surgery, septic animals showed a reduced energy metabolism ($P < 0.0001$) and locomotor activity ($P < 0.01$). Simvastatin treatment restored all these metabolic parameters as VCO₂ ($P < 0.0001$), VO₂ ($P < 0.0001$), RQ ($P < 0.001$) and EE ($P < 0.0001$) compared to untreated septic rats (Figure 3b). Additionally, there was an increase in both horizontal movements ($P < 0.05$) and rearings ($P < 0.01$) of these animals (Figure 3c).

Ten days after CLP-surgery, sepsis survivor animals showed an increase in some metabolic parameters, such as VCO₂ ($P < 0.0001$) and RQ ($P < 0.0001$) (Figure 4c). However, there was a considerable reduction in body weight at the end of the experiment (Figure 4b). Simvastatin-treated animals remained with all metabolic parameters increased at the end of the experiment, but RQ values ($P < 0.01$) were lower when compared to untreated animals (Figure 4c). Simvastatin treatment restored body weight assessed at 10 days after sepsis induction ($P < 0.01$) (Figure 4b). Finally, there was only an increase in rearing movement ($P < 0.05$) of these treated animals (Figure 4d).

There was no difference in the plasma levels of IL-1 and IL-6 between the sepsis survivor groups studied (Figure 5b). In septic animals, the histopathological assessment in the prefrontal cortex and hippocampus (dentate gyrus and CA1 region) showed morphological characteristics suggestive of apoptosis, such as irregular cytoplasmic borders, in addition to nuclear fragmentation and condensation. These changes were less intense in septic animals treated with simvastatin (Figure 5c, d, e - upper squares). Reactive astrocytes stained with GFAP were more evident in the septic animals, showing hypertrophic processes. In contrast, simvastatin-treated rats showed scattered astrocytes, with thinner astrocytic processes (Figure 5c, d, e - lower squares).

Discussion

Despite recent medical advances, the incidence of sepsis has increased, especially after data collection in regions with a lower Socio-demographic Index (SDI) [27]. The lack of knowledge about the evolution of the disease and the difficulty in providing an early diagnosis contribute to increase this trend and the mortality of patients [28, 29]. Current therapeutic strategies for treating sepsis depend on antibiotics, fluid replacement, and symptomatic therapy [30]. Although there is no specific treatment for sepsis, several experimental studies have proposed potential new drugs or adjuvant therapies. In the present report, we show that the use of simvastatin 4 days before and 10 days after CLP-surgery improved metabolic parameters and prevented CNS changes in sepsis survivor animals.

Here, we show that at 24 and 48 hours after septic induction there is a progressive increase in bacterial load in the spleen and mesenteric lymph node of septic animals, coinciding with the time points of high mortality. Furthermore, we observed that bacterial dissemination increases in parallel with the increase of pro-inflammatory cytokines in the plasma of these animals. Recent studies have shown similar results by correlating severity and mortality scores with bacterial dissemination and cytokine levels in different organs in a pneumonia-induced sepsis model [31]. Although we did not adopt a score to assess sepsis severity, our animals showed typical signs, such as lethargy, piloerection, and tachypnea, which are parameters considered reliable to attest the severity of the disease [32, 33]. Forty-eight hours after sepsis induction, the animals had a higher bacterial load in organs studied, higher plasma levels of proinflammatory cytokines, and a higher mortality rate. Other studies using the CLP model showed similar results, demonstrating that this appears to be the critical time point of sepsis in Wistar rats [24, 34].

The severity of sepsis has been linked to plasma cytokine concentrations for a long time [35]. Considering 24 and 48 hours as the period with the highest number of animal deaths, the action of simvastatin was analyzed by quantifying the plasma levels of cytokines. With the simvastatin administration, survival was 100% at 24 hours and 60% at 48 hours after sepsis induction, remaining unchanged up to 10 days. It is likely that this maintenance of survival rate is related to the considerable

decrease in IL-1b and IL-6 levels observed at this time point in animals treated with simvastatin. One of the biggest challenges in controlling the progression of sepsis is to modulate the 'cytokine storm' in the early stages of the disease [36, 37]. Cytokines are often responsible for triggering the inflammatory cascade and production of mediators such as nitric oxide, chemokines, and free radicals, leading to organ dysfunction and death [38, 39]. Previous work has shown that this anti-inflammatory effect of simvastatin, by decreasing the overproduction of pro-inflammatory cytokines, was able to limit the release of other mediators, such as nitric oxide and reactive oxygen species, and to increase the survival of septic animals [18, 40]. This pleiotropic effect of statins is extremely relevant, considering that several clinical and preclinical studies have used plasma levels of cytokines as surrogates for diagnosis and prognosis of organ dysfunction and mortality [37, 41–44].

Metabolic responses to sepsis are often difficult to measure, as they depend on several factors, including the body nutritional status and hormonal aspects [45, 46]. Furthermore, the stage of disease during which the patient or animal is studied must be considered and may explain the variability of results reported in the literature [47, 48]. In this work, we used indirect calorimetry to assess the energy metabolism in sepsis survivor rats treated with simvastatin. Our septic animals evaluated 48 hours after CLP-surgery had considerably lower calorimetric parameters (RQ = 0.90) compared to treated animals (RQ = 1.02). This discrepancy in the RQ values indicates that the animals of this experimental groups consume different substrates as an energy source, since higher RQ values suggest a predominant use of carbohydrates as an energy substrate [49]. Moreover, our results showed an increase in the measurement of EE and locomotor activity of septic animals treated with simvastatin compared to those not treated, suggesting a restoration of energy production. Taken together, these results lead us to infer that the simvastatin administration may have contributed to the production of a hypermetabolic state, necessary for the recovery of these animals. Several authors agree that the absence or premature terminus of a hypermetabolic state is associated with severe prognosis [48, 50, 51]. Higher mortality was observed in septic animals that remained in a hypometabolic state and were unable to transition from lipid utilization to carbohydrates [50]. Similarly, preterminal patients have hypofunctional changes in metabolism, such as decreased oxygen consumption, triglyceride clearance, and hepatic mitochondrial activity [47, 52].

The inability to sustain a hypermetabolic state and the incapacity to transit to carbohydrate utilization is associated with a dysregulation of the energy metabolism caused by mitochondrial dysfunction [53]. Thus, it is possible to infer that the improvement in metabolic parameters and the restoration of energy production is related to the antioxidant action of statins [54, 55]. Previous studies have shown that treatment with statins reduced glial activation and oxidative stress at 48 hours after sepsis induction [17, 18]. It is likely that this effect is related to the inhibition of the synthesis of isoprenoid compounds and prenylation of small G proteins. The occurrence of these events limits the production of inflammatory mediators and reactive oxygen species by negatively modulating the binding of these G proteins to the plasma membrane to trigger the cell signaling cascade [22, 56].

Here, we observed that sepsis survivor animals evaluated 10 days after CLP-surgery showed an increase in RQ and EE values, suggesting success in the transition from lipid substrate to carbohydrates and

reaching the hypermetabolic state. However, these animals were unable to restore body weight over the 10 days of evaluation. In experimental sepsis, the reduction in food and water consumption, even temporarily for 2 – 3 days post-CLP, is sufficient to maintain the animals body weight below baseline values for 7 days [15]. In our study, it is likely that the severity of sepsis has produced a decrease in the search for food with consequent fasting and triggering of the mechanisms that promote lipolysis [57, 58]. Although we have noticed an improvement in the horizontal movements of the untreated survivors, the number of vertical movements remained lower compared to the treated survivors. This can be explained by the attempt to minimize energy expenditure in face of foraging activities necessary for survival, inasmuch as vertical movements are costly [59, 60]. The restoration of energy production at early time points provided simvastatin-treated rats with greater locomotor activity at both time points evaluated, in particular, an increase in vertical movements, observed even after 10 days of septic induction.

Patients that develop severe sepsis and septic shock have organ dysfunction and high levels of plasma cytokines, which can lead to death. Even with early intervention, patients who survive this condition are likely to develop neurocognitive impairments [35]. Cognitive deficits found in sepsis survivor animals are due to neurodegenerative processes associated with sustained neuroinflammation [6]. In this study, we performed morphological assessments in the hippocampus and prefrontal cortex, which are brain regions responsible for memory and cognition. We observed that animals surviving sepsis showed morphological characteristics suggestive of astrocytic activation and apoptosis. In sepsis, alterations in blood-brain barrier (BBB) permeability allow peripheral inflammatory mediators to reach the CNS [61, 62]. These inflammatory mediators play a key role in sustained glial activation, which, in turn, contributes to the perpetuation of a neuroinflammatory environment with consequent cell death and cognitive impairment [6]. Our sepsis survivor animals did not show alterations in the plasma levels of IL-1 and IL-6, demonstrating that, although peripheral inflammation has been resolved, glial activation persists up to 10 days after sepsis. Other authors have reported long-term brain dysfunction in surviving animals using the CLP model [15, 34, 63].

Modulating sustained glial activation appears to be critical for containing persistent neuroinflammation in sepsis survivors [64–67]. Here, animals treated with simvastatin showed less noticeable morphological alterations in both investigated brain regions. Previous studies from our laboratory observed a decrease in Iba-1 expression accompanied by a reduction in cytokines and cleaved caspase-3, and an increase in Bcl-2 in the brain of the simvastatin-treated animals. The likely mechanism proposed for this anti-inflammatory effect of simvastatin is its role in the NF- κ B (nuclear factor-kappaB)/SIRT1 (silent information regulator 1) signaling pathway by inhibiting the M1 microglia phenotype [68–72]. Since this microglial activation induces the neurotoxic phenotype of astrocytes through the release of large amounts of cytokines, suppressing this activation may explain the decrease in astrogliosis observed in treated animals [73, 74].

In sepsis, although organ system dysfunction is more easily evaluated through routine laboratory tests, there are still no well-defined and practical biological markers in the CNS that can be targeted for therapeutic interventions. Therefore, studies that investigate the course of disease evolution and its

relationship with the neuroinflammatory response are needed to clarify the brain dysfunction observed in sepsis survivors. Here we demonstrate that simvastatin administered 4 days before and 10 days after CLP-surgery improved metabolic parameters by sustaining a hypermetabolic state necessary for animal survival, reduced levels of pro-inflammatory cytokines, and prevented damage to areas responsible for memory and cognition of survivor rats. We believe that the pre-treatment was crucial, because in a pilot study we did not obtain satisfactory results when administering this drug only after septic induction. Other authors reported similar results using statins before and after sepsis [40]. As statins are widely used by the world population, further studies are needed to consider not interrupting this drug during the treatment of sepsis and to assess its likely potential as adjuvant neuroprotective therapy.

Declarations

Acknowledgements

The authors thank Nadir Fernandes for the technical support and Dr Klaus Hartfelder for his assistance with English language.

Authors' contributions

CHRC and MJAR conceived and designed research, with input from AOS, NNSJ and LHAC. CHRC, AOS, NNSJ, LHAC and JRS performed the experiments, analyzed data and drafting parts of the paper. CHRC, LCA and MJAR wrote the final manuscript and revised statistical analyses. All authors read and approved the final version of the manuscript.

Funding

The study was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP-grant 2017/12462-0) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Availability of data and materials

Data available on request from the authors.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

The study procedures were performed according to the National Council of Animal Experiment Control (CONCEA) and with approval by the Institutional Animal Care and Use Committee at the School of Dentistry of Ribeirão Preto, University of São Paulo (protocol number: #2019.1.51.58.6).

Consent to participate

All authors meet the qualifications for authorship and had an opportunity to read and comment the manuscript.

Consent for publication

All authors support publication of the manuscript in Molecular and Cellular Biochemistry.

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Figures

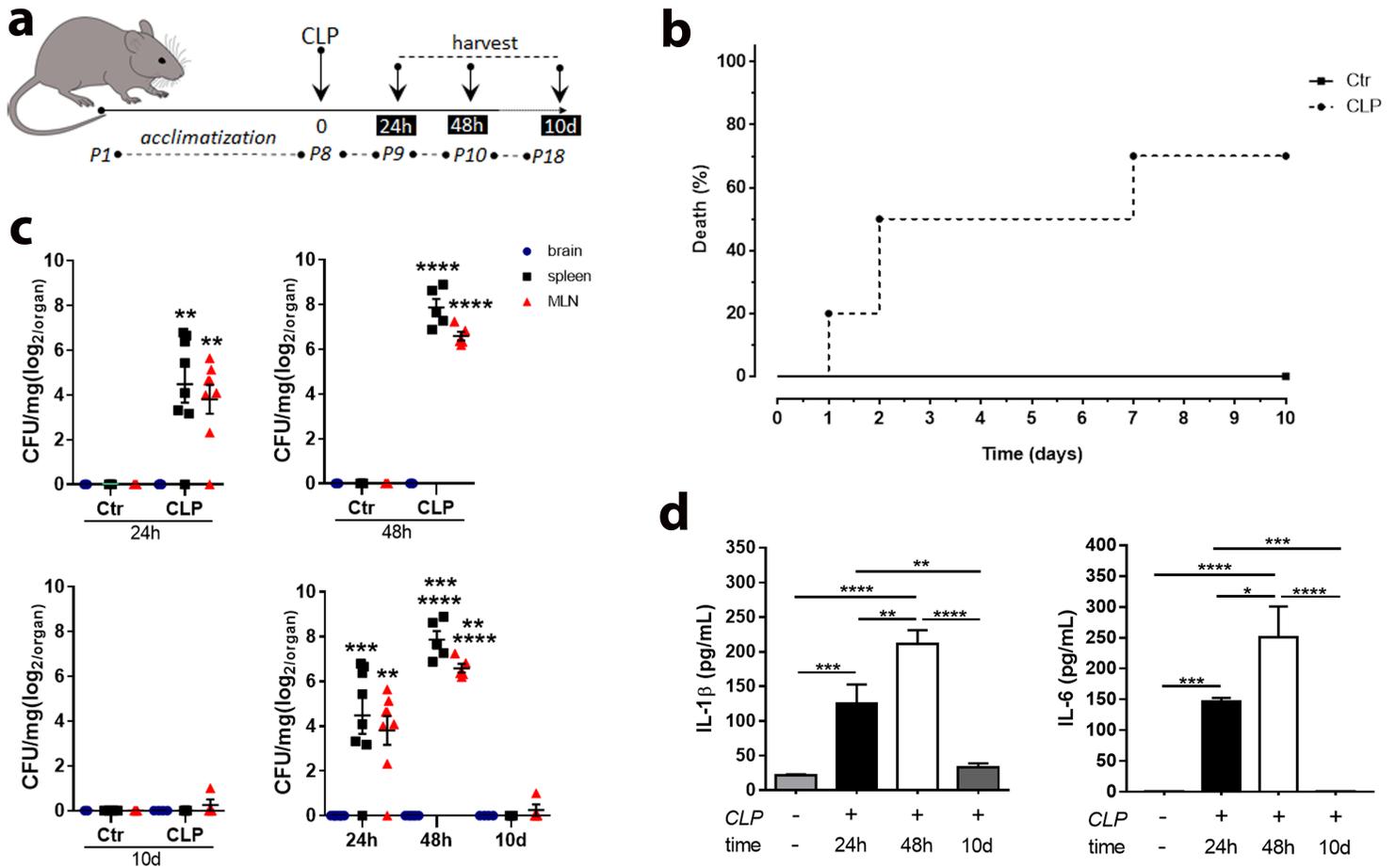


Figure 1

Analysis of the evolution of sepsis induced by cecal ligation and puncture (CLP) during 10 days after surgery. Experimental protocol detailing the time points studied as 24 h, 48 h and 10 days (**a**). Septic animals presented mortality of 70% over a span of 10 days and the crucial death time point occurred at 48 hours (30%) (**b**). At this same time point, there was a significant increase in bacterial load (**c**) and IL-1 β and IL-6 cytokines (**d**). Ten days after CLP surgery, there was sepsis recovery, as demonstrated by the absence of bacteria (**c**) and normalization of plasma levels of cytokines (**d**). Bars indicate mean \pm SEM ($n = 5-8$ animals per group). One-way ANOVA with Tukey's Multiple Comparison Test correction.

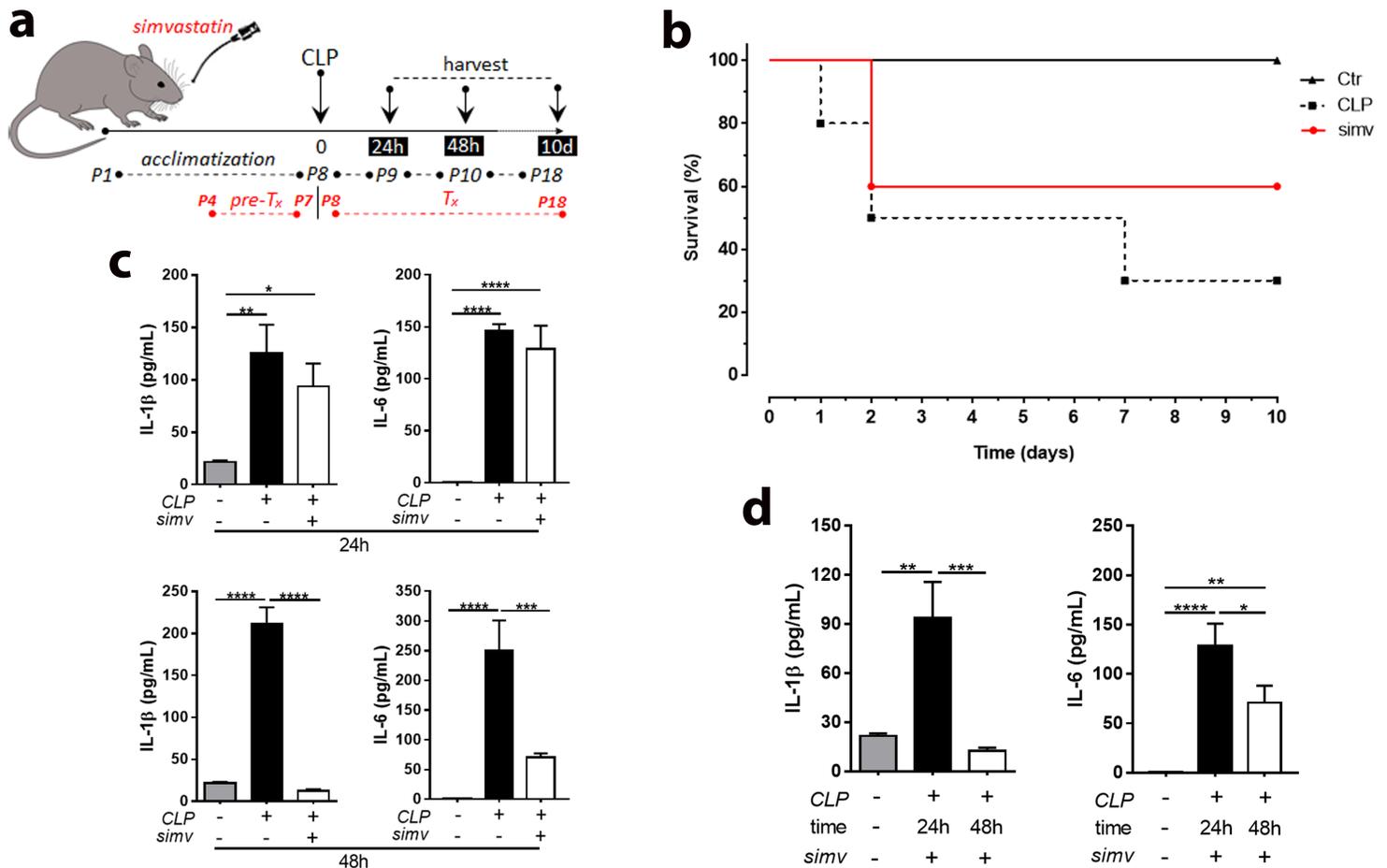


Figure 2

Effect of treatment with simvastatin (20 mg/kg, p.o.) 4 days before and 24 h, 48 h and 10 days after CLP-surgery on proinflammatory plasma cytokine levels and survival rate. Experimental protocol detailing the time points studied (**a**). Simvastatin-treated rats showed survival of 60% after 10 days CLP-surgery (**b**). Sepsis caused an increase in IL-1 β and IL-6 levels in the plasma. Simvastatin treatment prevented the increase of these cytokines only at 48 h (**c**). At this same time point, there was a significant reduction in these proinflammatory cytokines when compared with 24 h time point (**d**). Bars indicate mean \pm SEM (n = 5-8 animals per group). One-way ANOVA with Tukey's Multiple Comparison Test correction.

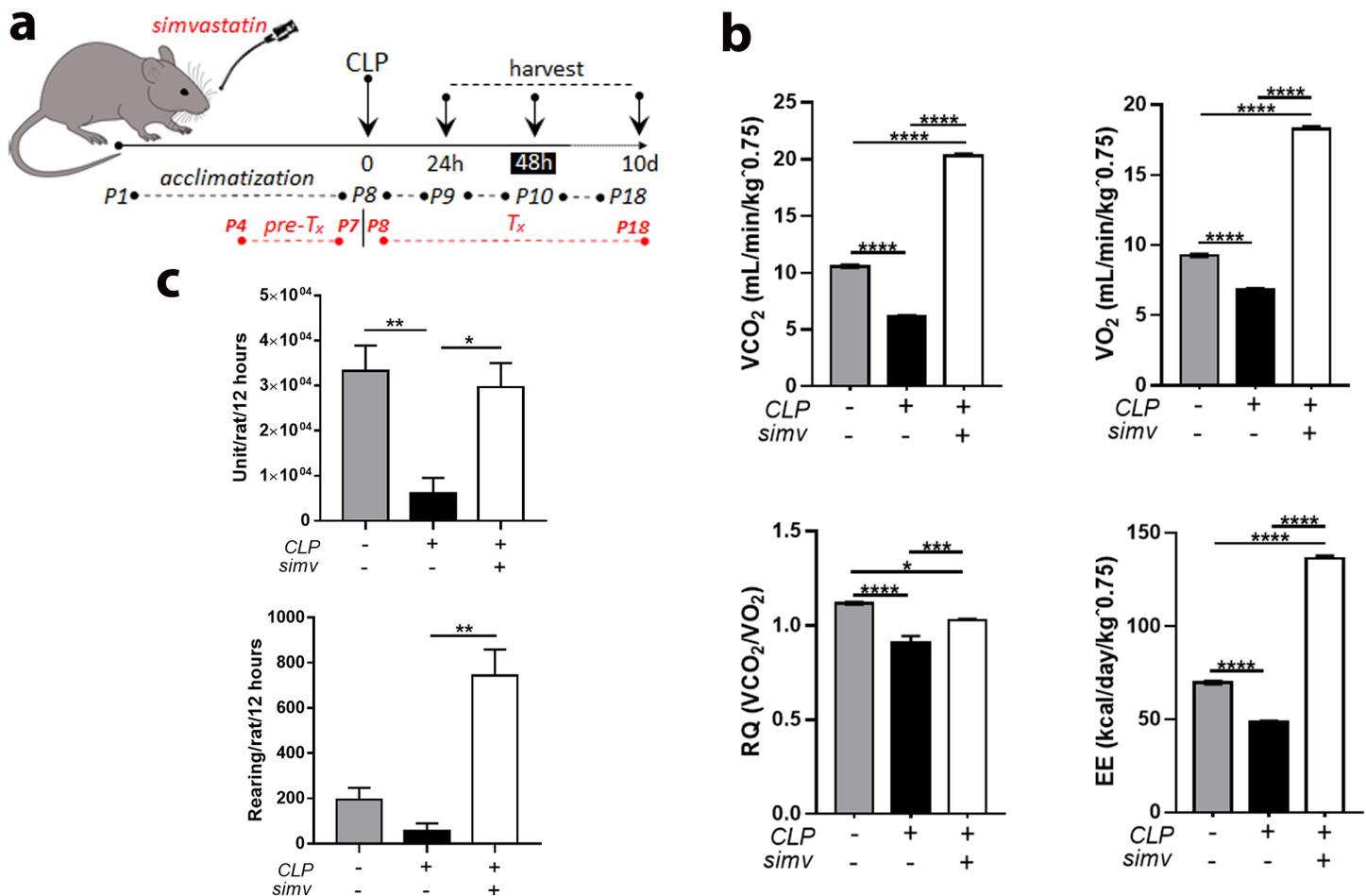


Figure 3

Effect of treatment with simvastatin (20 mg/kg, p.o.) 4 days before and 48 h after CLP-surgery on body metabolism and locomotor activity. Experimental protocol **(a)**. Sepsis caused a decrease in all the metabolic parameters analyzed. Simvastatin administration caused an increase of body metabolism in the treated rats by elevation these parameters **(b)**. Septic rats showed a decrease in locomotor activity while simvastatin-treated animals had an increase in both horizontal and vertical movements **(c)**. Bars indicate mean \pm SEM ($n = 5$ animals per group). One-way ANOVA with Tukey's Multiple Comparison Test correction.

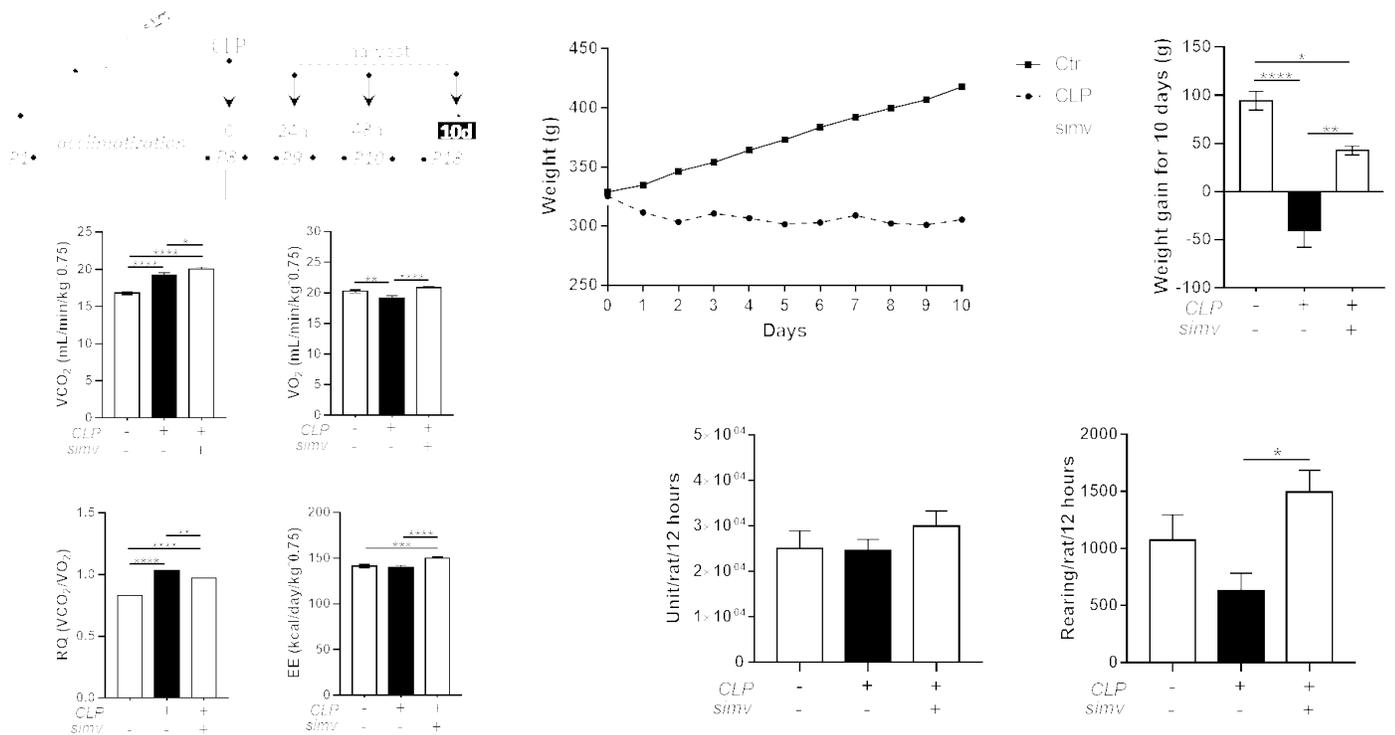


Figure 4

Effect of treatment with simvastatin (20 mg/kg, p.o.) 4 days before and 10 days after CLP-surgery on body metabolism, locomotor activity and weight. Experimental protocol **(a)**. There was a considerable reduction in body weight of the sepsis survivor animals at the end of the experiment and simvastatin recovered the weight of the treated rats **(b)**. Simvastatin-treated animals remained with all metabolic parameters increased at the end of the experiment except for the RQ values that were lower compared to untreated animals **(c)**. There was only an increase in vertical movements (rearing) of these treated animals **(d)**. Bars indicate mean \pm SEM (n = 5 animals per group). One-way ANOVA with Tukey's Multiple Comparison Test correction.

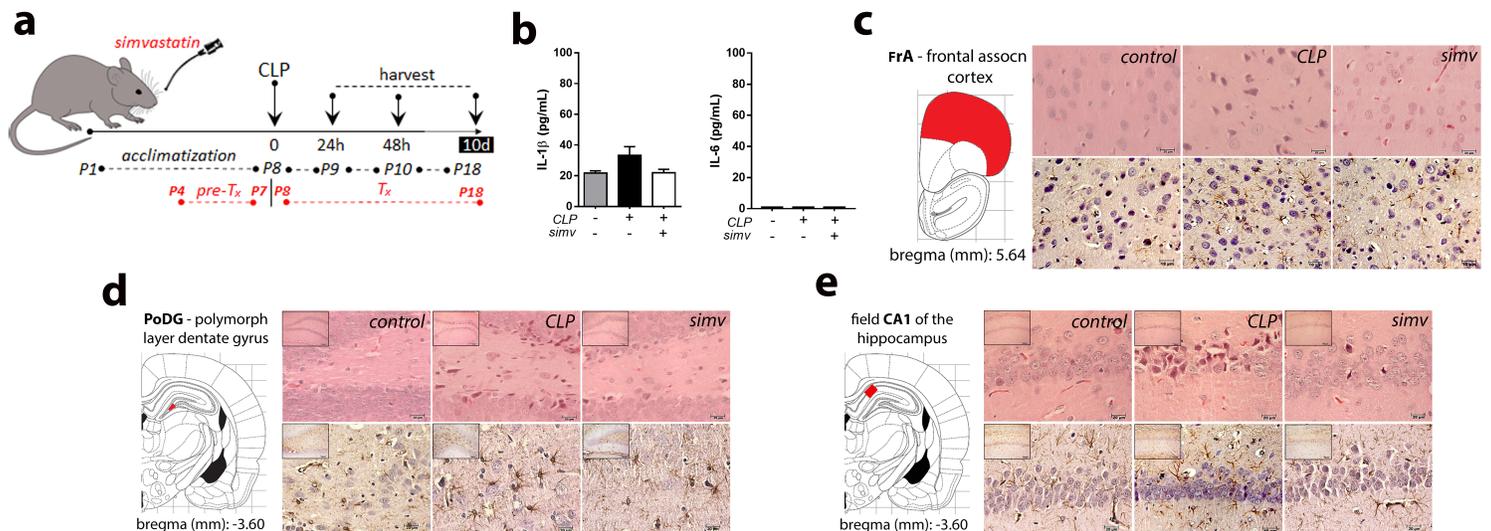


Figure 5

Effect of treatment with simvastatin (20 mg/kg, p.o.) 4 days before and 10 days after CLP-surgery on proinflammatory plasma cytokine levels and histopathological alterations. Experimental protocol **(a)**. There was no difference in levels of plasma of IL-1 β and IL-6 cytokines in any of the groups **(b)**. Photomicrographs of different regions of rat brains stained for hematoxylin-eosin (HE) (upper squares) and immunostained for GFAP (lower squares): prefrontal cortex **(c)**, dentate gyrus **(d)** and CA1 region **(e)** of the hippocampus. In septic animals, HE-stained brain regions showed cellular morphology with characteristics suggestive of apoptosis and astrocytes GFAP-immunostained with hypertrophic processes. In contrast, simvastatin-treated rats showed discrete morphological alterations and scattered astrocytes with thin astrocyte processes. Objective magnification: 40 \times (oil immersion). Scale bar, 20 μ m.