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Effect of adenosine receptor blockade on cardiovascular and renal changes induced by sepsis

Efeito do bloqueio de receptores de adenosina nas alterações cardiovasculares e renais induzidas pela sepse

Florianópolis
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Orientador: Prof. Dr. Daniel Fernandes.

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Este Trabalho de Conclusão de Curso foi julgado adequado para obtenção do título de Bacharel em Farmácia e aprovado em sua forma final pelo Curso de Graduação em Farmácia.

Florianópolis, 27 de junho de 2023.

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"A ciência nos ensina a duvidar. Ela nos ensina a questionar tudo, inclusive a nós mesmos."

(Yuval Noah Harari, 2020)

Este trabalho de conclusão de curso foi desenvolvido em formato de artigo, pois há interesse de publicação futura. Além disso, estudos complementares deverão ser feitos para compor os resultados, para que então seja submetido para a revista proposta.

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RESUMO

A sepse é caracterizada por uma perfusão microvascular inadequada dos tecidos, levando a disfunção de órgãos e morte. A adenosina é um importante regulador do sistema cardiovascular que por meio de quatro receptores acoplados à proteína G (A_1, A_{2A}, A_{2B} e A_3), causam hipotensão e redução do fluxo sanguíneo renal, contribuindo para disfunção tecidual e falência de órgãos. A cafeína é um antagonista não seletivo dos receptores de adenosina e pode prevenir o colapso cardiovascular na sepse. Nosso objetivo foi avaliar os efeitos da cafeína nos parâmetros cardiovasculares e na reatividade vascular em animais sépticos. Avaliamos a pressão arterial e fluxo sanguíneo renal induzida no modelo de CLP em animais que receberam doses de cafeína (30 mg/kg, s.c). A sepse resultou em hipotensão, hiporresponsividade aos vasoconstritores, redução do fluxo sanguíneo renal e aumento dos níveis de glicose e lactato no sangue. A cafeína manteve os níveis glicêmicos dentro dos limites normais mesmo após 24 horas de indução da sepse e aumentou a frequência cardíaca. Ela não alterou os níveis de ureia, creatinina, AST e ALT. Assim, as injeções repetidas de cafeína preservaram os níveis normais de glicose no sangue durante a sepse. No entanto, ela aumentou a frequência cardíaca e reduziu a sensibilidade aos vasoconstritores, o que pode comprometer alguns parâmetros cardíacos. Portanto, a cafeína não gera mudanças significativas durante a sepse.

Palavras-chaves: Infecção; Cafeína; Hipotensão; Reatividade vascular

ABSTRACT

Sepsis is characterized by inadequate microvascular tissue perfusion, leading to organ dysfunction and death. Adenosine is an important regulator of the cardiovascular system that, through four receptors coupled to the G protein (A1, A2A, A2B, and A3), cause hypotension and reduced renal blood flow, contributing to tissue dysfunction and organ failure. Caffeine is a non-selective adenosine receptor antagonist that may prevent sepsis's cardiovascular collapse. The effects of caffeine on cardiovascular parameters and vascular reactivity in septic animals were aimed to be evaluated. Blood pressure and renal blood flow induced in the CLP model were evaluated in animals that received doses of caffeine (30 mg/kg, s.c). Sepsis resulted in hypotension, hyporesponsiveness to vasoconstrictors, reduced renal blood flow, and increased blood glucose and lactate levels. Caffeine maintained blood glucose levels within normal limits even after 24 hours of sepsis induction and increased heart rate. It did not change the levels of urea, creatinine, AST, and ALT. Thus, repeated caffeine injections preserved normal blood glucose levels during sepsis. However, it increased heart rate and reduced sensitivity to vasoconstrictors, which can compromise some cardiac parameters. Therefore, caffeine does not generate significant changes during sepsis.

Keywords: Infection; Caffeine; Hypotension; Vascular reactivity.

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SUMÁRIO

1 INTRODUCTION

Sepsis is a condition that is associated with high mortality rates in ICU (Fleischmann-Struzek *et al.*, 2020). The estimated number of cases of sepsis worldwide is 19 to 48.9 million per year, leading to the death of 35 to 45% of patients (Rudd *et al.*, 2020). Despite this high negative impact in health care the pathophysiology of sepsis is not completely understood.

Over the last few decades, several important discoveries have demonstrated that adenosine plays an essential role as an extracellular signaling molecule (Zhang, Yu-Jing, Ma, 2022). Adenosine is an endogenous nucleoside formed mainly from the breakdown of ATP and ADP nucleotides. Interestingly, the extracellular concentration of ATP increases under conditions that are associated with sepsis, such as inflammation, ischemia, and hypoxia (Schmidt *et al.*, 1995). Extracellular adenosine exerts its actions through four distinct G protein-coupled receptors, A_1, A_{2A}, A_{2B}, and A_3. These receptors modulate multiple physiological functions, regulating the central, cardiovascular, peripheral, and immune nervous systems. However, extracellular adenosine binds with high affinity to A_1 and A_{2A} receptors (Borea *et al.*, 2018).

Interestingly, activation of the A2A receptor leads to vasodilation and a decrease in blood pressure. (Headrick *et al.*, 2011). The A_1 adenosine receptor, on the other hand, causes vasoconstriction in the renal vasculature and controls renal vascular tone (Vallon, Mühlbauer, Osswald, 2006). Therefore, adenosine may participate in the hemodynamic disturbances during sepsis.

Adenosine receptors are non-selectively antagonized by caffeine, and their blockade varies according to the receptor, with a higher affinity observed for A1 and A2A receptors. Since adenosine levels are upregulated in sepsis (Martin *et al.*, 2000), the simultaneous blockade of A_1 and A_2 receptors by caffeine could play an important role in the renal ischemia and hypotension induced by these receptors during sepsis, preventing organ dysfunction and increasing survival.

In this sense, several studies have evaluated the effect of caffeine in experimental sepsis models in animals (Bauza, Remick, 2015) and humans (Ramakers *et al.*, 2011). However, the results are contradictory and part of it is due to the different doses of caffeine used (ranging from 4 to 50 mg/kg). The dose of caffeine is usually based on the amount of caffeine present in popular beverages like coffee and tea. Although this approach may be suitable for studies that aim to evaluate the effects of chronic caffeine

exposure, it is not adequate for evaluating acute adenosine receptor blockade. Finally, these studies used a single dose (Bauza, Remick, 2015) or administration at 24-hour intervals (Verma *et al.*, 2009), but data in humans show that caffeine has short effects, with a half-life about 3 and 5 hours (Tavares, Sakata, 2012), and this may be even lower in animals. Therefore, the objective of this study was to evaluate the effect of caffeine on sepsis, using an appropriate dose and frequency of administration for the blockade of adenosine receptors (Albino *et al.*, 2023). Thus, we hypothesize that caffeine can modulate a response through adenosine receptors and promote a protective effect on cardiovascular changes.

2 MATERIAL AND METHODS

2.1 CHEMICAL COMPOUNDS

The following substances were used in this study: sodium heparin (Eurofarma, São Paulo, SP, Brazil), ketamine hydrochloride and xylazine (Syntec do Brasil Ltda, Cotia, SP, Brazil), caffeine, phenylephrine, and angiotensin II (Sigma Chemical Co., St. Louis, MO, USA), isoflurane (Instituto BioChimico, Penedo, RJ, Brazil), tramadol hydrochloride (Laboratório Teuto, Anápolis, GO, Brazil).

2.2 ANIMALS

Male Wistar rats (12-week-old, 358 ± 13 g) used in this study were housed in a temperature and light-controlled room (23 ± 2°C; 12-h light/dark cycle) and had free access to water and food (Biobase, Biotech line). Rats were kept in 45 x 34 x 16 cm plastic cages (5 rats per cage). All the experiments using rats were performed between 9:00 and 16:00 h. The procedures were previously approved by the University Institutional Ethics Committee (protocol number 301220221). They were following the Brazilian National Council of Animal Experimentation and the National Institutes of Health Animal Care Guidelines. In addition, animal studies are reported in compliance with the ARRIVE guidelines (Sert *et al.*, 2020).

2.3 CECAL LIGATION AND PUNCTURE (CLP)

CLP surgery was performed as previously described (Wichterman, Baue, Chaudry, 1980) with minor modifications. Five minutes before initiating the procedure, the rats were administered the opioid analgesic tramadol at a dose of 10 mg/kg (i.p). After this, the animals were anesthetized by inhalation of isoflurane in the mix of oxygen. For sedation, isoflurane 5% was used in a chamber of inhalation, then, isoflurane 3% was maintained in a mask inhalation. The cecum was exposed and partially occluded by a non-obstructing ligation right above the ileocecal valve. One transfixed puncture was made through the caecum with an 18-gauge needle and a small amount of cecal content was extravasated through the puncture. Finally, the cecum was replaced in the peritoneal cavity, and the muscles and skin of the

abdominal region of the animals were sutured. The sham-operated rats underwent a similar surgical procedure with cecal exposure but it was neither ligated nor punctured. All the animals received 50 ml/kg of saline (NaCl 0,9%) subcutaneously immediately after the procedure. Saline solution was administered for fluid resuscitation to reproduce clinical hemodynamic support and to induce a hyperdynamic phase circulatory state (Hubbard *et al.*, 2005)(Wichterman, Baue, Chaudry, 1980). Animals were placed in cages with a heating mat (37± 1 °C) and lighting for postoperative warming until they returned from anesthesia. Twelve hours after the surgical procedure the rats were again treated with tramadol at 5 mg/kg i.p. to maintain the analgesic effect.

2.4 MEASUREMENT OF MEAN ARTERIAL PRESSURE

Animals were submitted to anesthesia intramuscularly with ketamine and xylazine (90 and 10 mg/kg, respectively) and supplemented, when necessary, with ketamine during the complete experimental protocol. A heparinized PE-20 polyethylene catheter was inserted into the right jugular vein for drug injections. A tracheal cannula was used to allow animals to breathe. Finally, a heparinized polyethylene catheter PE-50 was inserted into the left carotid arteria and connected to a pressure transducer coupled to the PowerLab 8/30 (AD Instruments Pty Ltd., Castle Hill, Australia) running by an integration LabChart7® software for mean arterial pressure (in mmHg) and heart rate (in BPM) recording. After 15 minutes of stabilization, the basal values of mean arterial pressure and heart rate were recorded, and a dose-response curve to phenylephrine (1, 3, 10, and 30 nmol/kg, i.v.) and angiotensin II (1, 3, 10 and 30 pmol/kg, i.v.), were obtained. The doses were injected in a total volume of 250 µL (including washing of the catheter). The change in mean arterial pressure (in mmHg) was calculated and compared between the groups. The monitoring of anesthesia was assessed by regular respiratory rate, heart rate, and absence of withdrawal reflex upon hind toe pinching. The animals were kept on a heating mat with a controlled temperature (37± 1 °C) throughout the experiment.

2.5 MEASUREMENT OF RENAL BLOOD FLOW

Simultaneous to the mean arterial pressure measurement, renal blood flow (in perfusion units, PU) was determined in animals, as previously described (Kovalski *et al.*, 2017). Briefly, a transverse abdominal incision was performed in the anesthetized rats to assess the posterior left subhepatic space, allowing the visualization of the left kidney. Then, a laser probe (model VP3), connected to a laser Doppler blood flow monitor (moorVMSLDF2, Moor Instruments, England) was carefully placed directly on the left kidney. The laser Doppler monitor was also coupled to the PowerLab 8/30 and the renal blood flow (RBF; in perfusion unit, PU) was recorded in a computer by an integration LabChart7® software. The probe was kept in this position and the surgical incision was covered with gauze sponges soaked in sterile saline to protect the kidney from drying. An interval of 15 min was allowed before measuring the basal values of renal blood flow. In addition, the change in renal blood flow induced by phenylephrine and angiotensin II was also recorded.

2.6 MEASUREMENT OF BLOOD GLUCOSE AND LACTATE

Glycemia was measured at baseline, 6, 12, and 24 hours after sepsis induction from the blood tail of awake rats. A drop of blood was drawn from the tail vein of each rat to measure glucose levels using an automatic analyzer (Call on Plus II, Medlevensohn, São Paulo, Brazil). The blood lactate was measured only at 24 hours also from the tail blood The analysis was done using a kit measure of lactate Accutrend® plus (Roche, Mannheim, Germany).

2.7 MEASUREMENT OF UREA, CREATININE, ALT, AND AST LEVELS

Sample blood was collected from the carotid catheter used for blood pressure measurement in tubes containing 1µL of sodic heparin for each 1mL of blood. The material collected was centrifuged (1.500 x g; 10 min; 4 ºC) for obtained plasm. The plasma levels of urea, creatinine, aspartate aminotransferase (ALT), and alanine aminotransferase (AST) were measured using commercially available clinical assay kits (Labtest Diagnóstica S.A. Lagoa Santa, MG, Brazil).

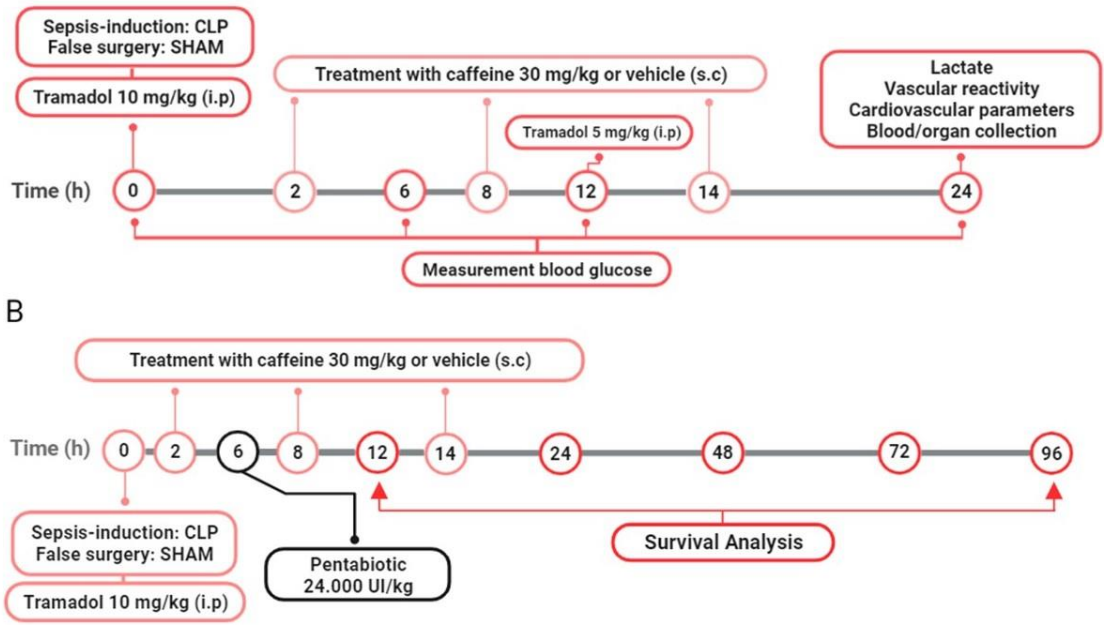
2.8 EXPERIMENTAL PROTOCOL

Rats were distributed into twice groups and were subjected to either a sham or a CLP procedure. The CLP group was randomized to receive caffeine or vehicle. The caffeine (30 mg/kg, s.c) or vehicle (saline 1 mL/kg, s.c) were administered two, eight, and fourteen hours after the procedure. The sham group was treated only with vehicle (saline 1 mL/kg, s.c) at the same time as the CLP group. Twenty-four hours after the procedure the animals were prepared for the measurement of mean arterial pressure and renal blood flow as previously described. At the end of the experiment, sample blood was collected to measure the levels of urea, creatinine, AST, and ALT (Fig. 1A). Posteriorly the animals were killed by anesthetic overdose. The experiments were not done blindly and the experimenter was aware of the treatment of the animals. It was not adopted criteria for the inclusion or exclusion of the animals.

In another set experiment, it was assessed the effect of caffeine on the sepsis survival rate. Rats were randomly distributed into twice groups and were subjected to either a sham or a CLP procedure. The CLP group was randomized to receive caffeine or vehicle. The caffeine (30 mg/kg, s.c) or vehicle (saline 1 mL/kg, s.c) were administered two, eight, and fourteen hours after the procedure. The sham group was treated only with vehicle (saline 1 mL/kg, s.c) at the same time as the CLP group. The CLP also received a single dose of a broad-spectrum antibiotic (24.000 UI/kg; Veterinary Pentabiotic, Zoetis) six hours after the procedure. The sham group was treated only with vehicle (saline 1 mL/kg, s.c). The survival of the animals was observed until 96 hours (Fig. 1B).

The dose of caffeine and the frequency of administration were based on pharmacokinetic, and toxicologic tests (Arnaud, 2011), and in previous studies (Albino *et al.*, 2023).

Figure 1: Experimental protocol designed to study the effect of caffeine on sepsis.



Note: (A) Two, eight, and fourteen hours after the CLP or sham procedure rats were assigned to receive either caffeine (30 mg/kg, s.c.) or vehicle (saline, 1 ml/kg, s.c.). Twenty-four hours after the CLP or sham procedure the analyses were performed. Five minutes before the procedure the animals received tramadol (10 mg/kg) for analgesia and, 12 hours after the procedure the animals received a reinforcement of 5mg/kg to maintain the analgesia. (B) For survival analysis, the animals were submitted to sepsis using the ligation and puncture model of the cecum CLP or Sham. Six hours after the procedure, the animals received Pentabiotic in a dose of (24.000 UI/kg, i.m). The animals were monitored for 96 hours, and survival was monitored every 12 hours.

2.9 STATISTICAL ANALYSIS

The sample calculation was based on the standard deviation (SD) and the magnitude of difference between the groups obtained in the analysis of blood pressure (in mmHg) from our previous studies (Kovalski et al., 2017). Thus, considering 3 experimental groups, α= 0.05, a power of 80%, and an effect size of 0.52 (f), 12 animals in each group are required for statistical significance. This sample size maintains the power of at least 80% also for the other cardiovascular parameters. To account for the mortality rate of 25 % of the CLP model in 24 h or a potential technical loss, 15 animals were included in the CLP groups. The final number (n) in each group is indicated in Figure legends. The GPower 3.1.1 software was used to sample size calculation (Faul F, Erdfelder E, Lang AG, 2007). For the survival analysis, a sample size of 30 animals

per group was calculated based on the expected rate of mortality reduction of 35%, with a power of 80% and α =0.05. The Primer of Statistics version 7 software was used to sample size calculation of survival experiments.

The data were expressed as the mean ± standard error of the mean (SEM). The statistical significance was analyzed by two-way ANOVA, followed by Bonferroni's post hoc test. Normality and homogeneity of variance were verified through Shapiro-Wilk and Bartlett tests, respectively. Agonist concentration-response curves were fitted using nonlinear regression.

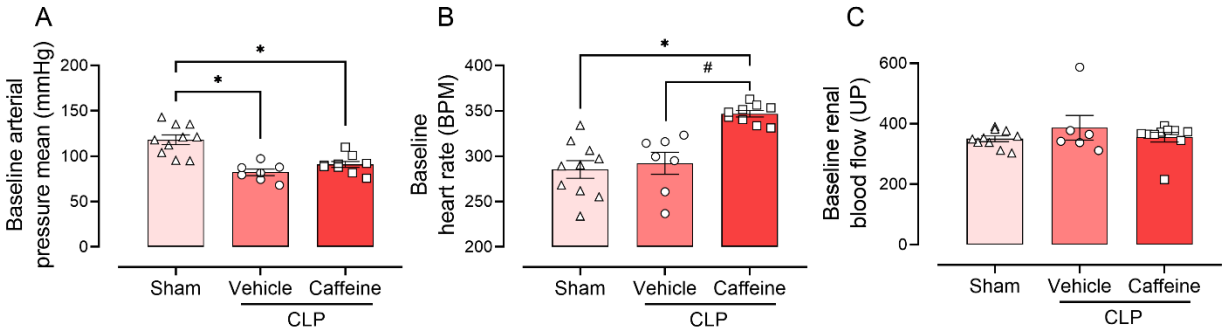
3 RESULTS

3.1 EFFECT OF CAFFEINE ON CLP-INDUCED CARDIOVASCULAR CHANGES.

Twenty-four hours after sepsis induction, the animals presented decreased mean arterial pressure by 36 mmHg (Fig. 2A, $p<0.05$). Caffeine treatment failed to restore sepsis-induced hypotension. The heart rate increased by 60 BPM in the animals treated with caffeine (Figure 2B, $p<0.05$). On the other hand, there was not a significant change in renal blood flow (Figure 2C). The CLP vehicle group did not present a hyporeactive response to phenylephrine (Fig. 3A-B). However, the CLP caffeine group presented a decreased response to phenylephrine compared to the CLP vehicle and Sham (Fig. 3A-B, $p<0.05$). However, the CLP animals presented an impairment in angiotensin II response, but not changed the time response (Fig. 3C and D, $p<0.05$). Although the CLP procedure reduced the angiotensin II induced increase in blood pressure, it did not change the time response (Fig. 3D, $p<0,05$).

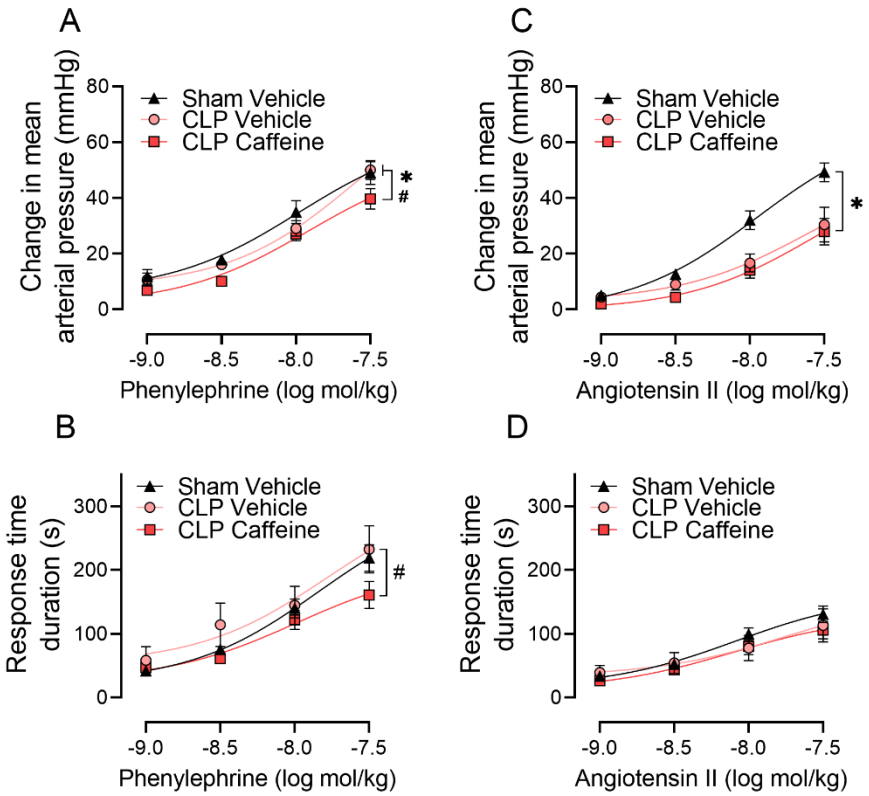
The vasopressors (phenylephrine and angiotensin II) induced a dose-response reduction in renal blood flow (Fig.4). The CLP group animals present impairment in phenylephrine and angiotensin II response when compared to the Sham group (Fig. 4A and B, $p<0.05$), respectively. The treatment with caffeine did not exercise an effect on the renal flow.

Figure 2: Effect of CLP and caffeine on basal values of blood pressure, renal blood flow, and heart rate.



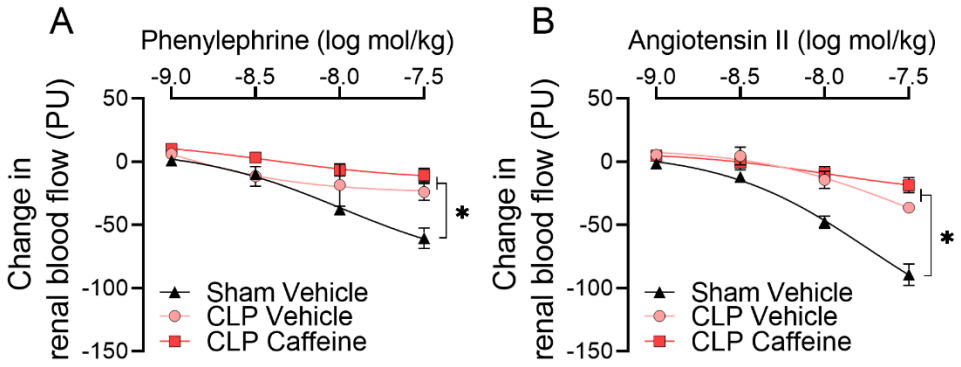
Note: Two, eight, and fourteen hours after the CLP or sham procedure the rats were assigned to receive vehicle (saline 1 mL/kg, s.c.), or caffeine (30 mg/kg, s.c.). Twenty-four after surgery the following parameters were recorded: (A) mean arterial pressure; (B) heart rate, and (C) renal blood flow. Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey posthoc test. The results represent the mean ± SEM, n= 6-10 animals. *$p<0.05$, CLP compared with the Sham group. $^{\#}p<0.05$ CLP treated compared group CLP vehicle.

Figure 3: Effect of caffeine on the blood pressure and hyporesponsiveness of animals submitted to CLP surgery.



Note: The animals were submitted to sepsis using the model of ligation and puncture of the cecum CLP or Sham. Twenty-four hours after sepsis induction, the animals were anesthetized and prepared for in vivo vascular reactivity assessment with non-cumulative increasing doses of phenylephrine (A-B) and angiotensin II (C-D). The results represent the mean ± SEM, n= 7-10 animals. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. *p<0.05, CLP compared with the Sham group. #p<0.05 CLP treated compared group CLP vehicle.

Figure 4: Effect of CLP and caffeine on renal blood flow.



Note: The animals were submitted to sepsis using the model of ligation and puncture of the cecum CLP or Sham. Twenty-four hours after sepsis induction, the animals were anesthetized and prepared for in vivo vascular reactivity assessment with non-cumulative increasing doses of phenylephrine (A) and angiotensin II (B). The results represent the mean ± SEM, n= 7-10 animals. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. *p<0.05, CLP compared with the Sham group. $^{\#}$p<0.05 CLP treated compared group CLP vehicle.

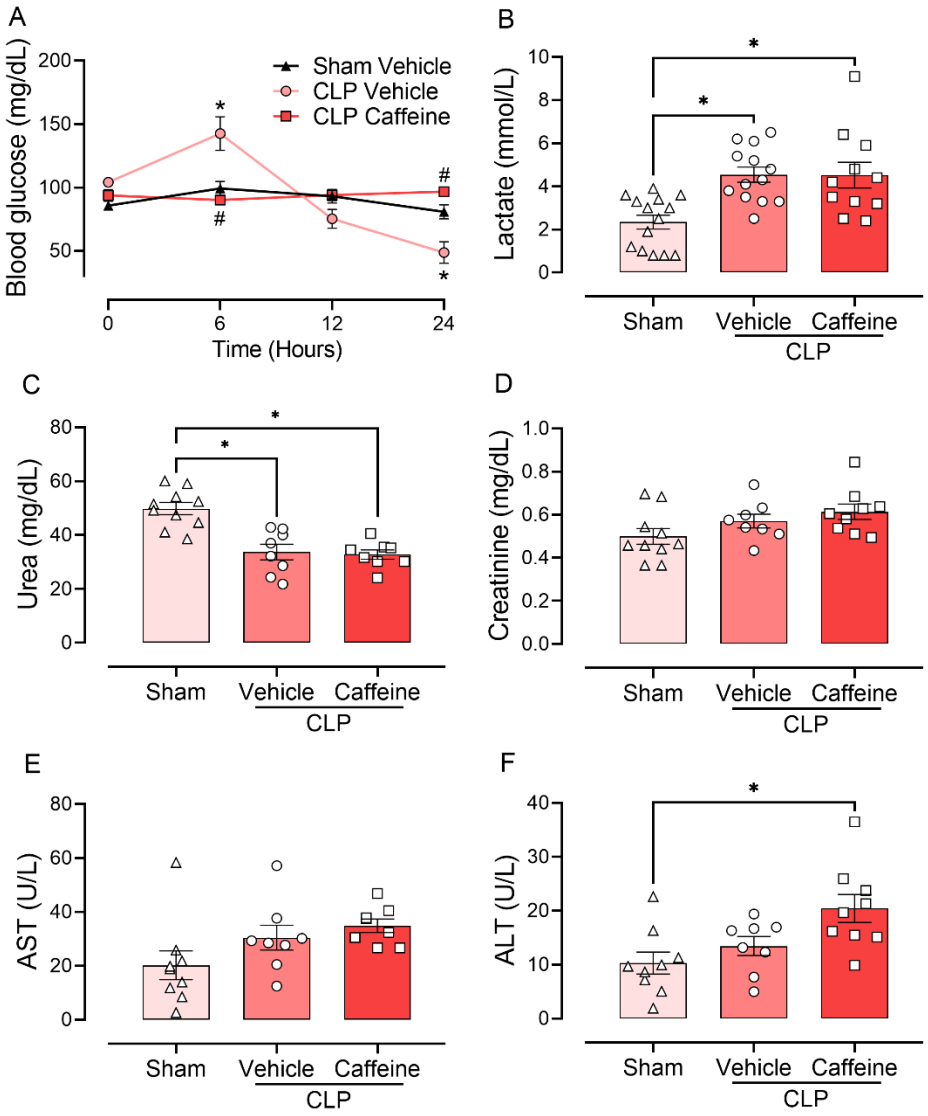
3.2 SEPSIS INDUCES VARIATION IN BLOOD GLUCOSE LEVELS.

The CLP vehicle animals displayed hyperglycemia in six hours and hypoglycemia in twenty-four hours (Fig. 5A, p<0.05). The CLP caffeine animals maintained normal glycemia levels until twenty-four hours following sepsis-induced. Twenty-four hours after CLP surgery the levels of plasma lactate increased compared to sham animals (Fig. 5B, p<0.05). Treatment with caffeine did not affect lactate levels.

3.3 EFFECT OF CAFFEINE ON CLP-INDUCED ORGAN DYSFUNCTION AND SYSTEMIC INFLAMMATION PARAMETERS.

The CLP animals exhibited a decrease in levels of plasma urea compared with sham animals, but the treatment with caffeine was not able to affect urea levels (Fig. 5C, p<0.05). CLP procedure did not change levels of plasma creatinine (Fig. 5D). The CLP animals presented increased levels of plasma AST, but not statistically significant (Fig. 5E). On the other hand, was possible to observe an increase in plasmatic levels of the ALT in animals treated with caffeine (Fig. 5F, p<0.05).

Figure 5: Effect of CLP and caffeine in blood glucose, lactate, and plasmatic markers of organ dysfunction and inflammation.

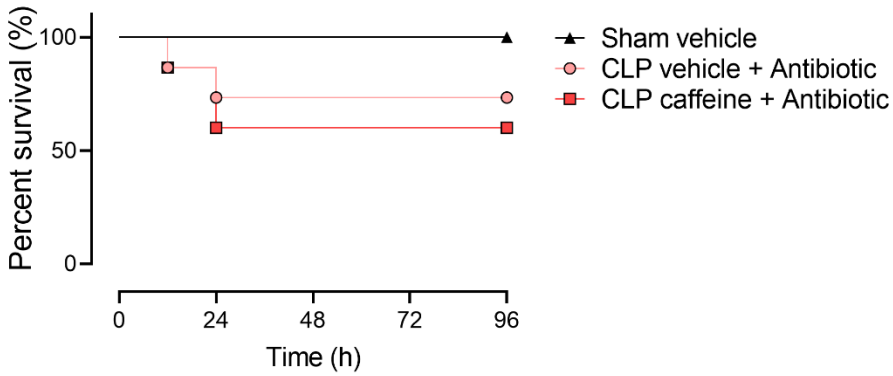


Note: (A) The glycemic levels were evaluated in times 0, 6, 12, and 24 hours. The animals have submitted a cut at the caudal end to obtain a drop of blood. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by the Tukey posthoc test. The results represent the mean ± SEM, n=5-10. (B) The lactate levels were measured only at the 24 hours' time. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by a Tukey posthoc test. The results represent the mean ± SEM, n=11-14. Two, eight, and fourteen hours after the CLP or sham procedure the rats were assigned to receive vehicle (saline 1 mL/kg, s.c.), or caffeine (30 mg/kg, s.c.). Twenty-four h after surgery, blood was obtained to assess urea (C), creatinine (D), ALT (E), and AST (F). Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by the Tukey posthoc test. The results represent the mean ± SEM, n= 8-9 animals. *$p<0.05$, CLP compared with the Sham group. $^{\#}p<0.05$ CLP treated compared group CLP vehicle.

3.4 EFFECT OF CAFFEINE ON SURVIVAL RATE.

Survival of the CLP rats declined in the first 24 h and remained constant up to 96h (Fig. 6). Caffeine did not change the mortality when compared with the CLP control group (Fig. 6).

Figure 6: Effect of caffeine on sepsis-induced mortality.



Note: The CLP animals were randomly assigned to receive caffeine (30 mg/kg, s.c) or vehicle (1 mL/kg, s.c) at two, eight, and fourteen hours after to procedure. The animals were submitted to sepsis using the model of ligation and puncture of the cecum CLP or Sham. The mortality rate was monitored and recorded for a period of four days. The antibiotic was administered six hours after to procedure (24.000 UI/kg, i.m). CLP n=30 animals and Sham n=7 animals. Data were analyzed using the Log-rank test (Mantel-Cox).

4 DISCUSSION

In our current study, we conducted experiments to investigate the effects of non-selective inhibition of adenosine receptors by caffeine on septic animals. Our findings revealed that caffeine administration successfully maintained normal blood glucose levels and led to an increase in heart rate. However, we did not observe significant changes in hemodynamic parameters or survival rates in septic animals following caffeine treatment. These results suggest that while caffeine may help preserve glycemic levels during sepsis, it may have adverse effects by increasing heart rate. Consequently, in our experimental conditions, we have concluded that caffeine, as a non-selective antagonist, does not provide substantial improvement in the treatment of sepsis.

Previous studies have already evaluated the effects of caffeine on experimental sepsis. For example, using the CLP model Verma and colleagues evaluate the effect of caffeine on cardiac function during sepsis. Interestingly, caffeine increased left ventricular pressure (Bedet *et al.*, 2020). However, they used a low dose of caffeine (7.5 mg/kg, the equivalent of 1-1.5 cups of coffee) administrate late on sepsis (24 and 48h after the CLP procedure). However, according to our previous data, this low dose of caffeine and frequency of administration are not suitable for blocking adenosine receptors efficiently (Albino *et al.*, 2023). Furthermore, the delayed assessment of sepsis parameters makes the comparison with our data difficult. In another study conducted by Bauza and Remick, mice were administered a single dose of 20 mg/kg (s.c) immediately after the cecal ligation and puncture (CLP) procedure. Alternatively, an osmotic pump was subcutaneously placed the day before CLP, providing a continuous infusion of caffeine at a rate of 10 mg/kg/h for a total duration of 24 hours. Notably, this continuous caffeine infusion appears to effectively block caffeine receptors, ensuring adequate receptor blockade in this experimental context (Bauza, Remick, 2015). However, apart from heart rate, the study lacks a comprehensive evaluation of cardiovascular parameters.

The cecal ligation and puncture (CLP) model is widely recognized as the most commonly used model for studying sepsis, as it accurately reproduces the course and characteristics of human sepsis, including the hemodynamic and metabolic phases (Buras, Holzmann, Sitkovsky, 2005). In our study, we utilized the CLP model, and the animals did not exhibit organic dysfunction. However, we were able to observe the progression and development of sepsis through the evaluation of cardiovascular

parameters such as hypotension and increased lactate levels (Fernandes et al., 2009), as well as increased heart rate (Bedet et al., 2020).

In sepsis, cardiovascular dysfunction is characterized by hypotension that often necessitates the use of vasopressor therapy. In the CLP model, animals exhibit significant hypotension due to various cardiovascular abnormalities (Zaky et al., 2014). However, in our study, caffeine treatment did not successfully prevent hypotension. Interestingly, previous studies have demonstrated that the use of non-selective receptor antagonists can improve cardiac function (Tofovic et al., 2001).

It is well-known that patients with sepsis often experience persistent tachycardia, which is associated with an increased risk of mortality (Hasegawa et al., 2021). In our study, animals treated with caffeine exhibited an increase in heart rate. This effect of caffeine on heart rate can be attributed to its ability to stimulate adrenal chromaffin cells, leading to increased secretion of catecholamines by raising intracellular calcium levels in these cells Furthermore, several studies have documented elevated levels of catecholamines in both septic patients and animal models (Hahn et al., 1995). Therefore, caffeine may indirectly contribute to the positive inotropic and chronotropic effects through the activation of β-adrenergic receptors by increasing the release of catecholamines.

Adenosine is involved in the reduction of heart rate through activation of the A_1 receptors on the sinus and atrioventricular node, slowing impulse conduction (Funakoshi *et al.*, 2007). Several studies have demonstrated an association with increased heart rate in late-onset neonatal sepsis (Fairchild, O'Shea, 2010). On the other hand, caffeine can inhibit non-selectively the adenosine receptors, including the A1 receptor, thereby diminishing the protective effect triggered by its activation. Thus, the utilization of selective antagonist or non-selective adenosine receptor A_1 for control of heart rate can be deleterious effects.

In clinical, the vasopressors are utilized to keep the organ perfusion pressure, to maintain a MAP ≥ 65 mmHg (Singer *et al.*, 2016). In addition to the baseline changes, was observed in septic animals an impairment in phenylephrine and angiotensin II response which is a hallmark of septic shock. Added to this, the animals treated with caffeine showed had response prejudiced against the vasoconstrictors. This manifestation clinical can be explained through the inhibition of the phosphodiesterase enzyme in smooth musculature, that when inhibited triggered arteriolar vasodilatation (Ribeiro, Sebastio, 2010). Thus, the vasodilatory effect of

caffeine on smooth muscle can interfere with the normal response to vasoconstrictors, such as phenylephrine and angiotensin II, resulting in a reduced blood pressure response. This highlights the potential negative impact of caffeine on maintaining adequate perfusion pressure in septic animals.

The reduction of renal blood flow induced by sepsis contributes to impaired perfusion in the kidneys, ultimately leading to acute kidney injury. This process is a consequence of sepsis and its detrimental effects on renal function. The pathophysiology of acute kidney damage involves tissue hypoperfusion, which leads to ischemia and acute tubular necrosis (Schrier, Wang, 2004). In our study, we did not observe an improvement in renal blood flow in the animals treated with caffeine. However, it is important to note that other studies have shown that despite an increase in glomerular filtration rate following the ingestion of 250 mg of caffeine, the overall plasma renal flow remained preserved (Passmore, Kondowe, Johnston, 1987). These findings suggest that caffeine may have variable effects on renal blood flow in different experimental models or dosages. Further investigation is needed to elucidate the precise impact of caffeine on renal blood flow and its implications for septic renal dysfunction.

Sepsis can induce a metabolic derangement leading to hyper and /or hypoglycemia. (Neyens, Gaskill, Chalela, 2018). Interestingly, hyperglycemia during sepsis is associated with a major risk of mortality, mainly in patients with early-stage sepsis (Dungan, Braithwaite, Preiser, 2009). The CLP caffeine group maintained normal glycemic levels for up to twenty-four hours. This finding is consistent with previous studies that have demonstrated the ability of caffeine ingestion to decrease glycemia levels. This effect may be attributed to increased expression of GLUT4 in skeletal muscle and adipocytes, resulting from elevated intracellular calcium levels and enhanced expression of the enzyme MAPK. (Park *et al.*, 2009). In addition, the hypoglycemic state observed in the late phase of sepsis is also associated with a higher mortality rate (Wang *et al.*, 2021). On the other hand, caffeine administration had a protective effect on hypoglycemia observed in septic animals. This effect may be due to stimulation in the release of counter-regulatory hormones (adrenaline and cortisol) to hypoglycemia by caffeine administration (Debrah *et al.*, 1996). Hence, caffeine shows promise in its potential to regulate blood glucose levels over an extended period. However, further studies are required to fully explore this effect

Lactate is a widely used clinical parameter that serves as a marker of tissue perfusion and plays a crucial role as a predictor of mortality in patients with sepsis (Liu et al., 2019). In line with expectations, the CLP group in our study exhibited increased lactate levels. However, caffeine treatment did not have an impact on lactate levels. The inability of caffeine to influence lactate levels implies that it does not directly affect tissue perfusion or metabolic processes associated with lactate production and clearance. While caffeine may have other effects, such as modulating heart rate or blood glucose levels, its influence on lactate levels appears to be limited.

It is interesting to note that no significant difference in plasma urea levels was observed in the CLP group in our study. However, previous research has shown that elevated urea levels are associated with increased severity of neonatal sepsis(Li et al., 2021). Furthermore, we measured plasma AST and ALT levels, which are transaminase enzymes involved in hepatic dysfunction caused by poor liver perfusion in sepsis (Li et al., 2018). In our study, the CLP animals treated with caffeine exhibited an increase in plasma AST levels. It is important to consider that this increase could be attributed to the potential hepatotoxic effect of caffeine (Sato *et al.*, 1985). On the other hand, other studies have reported improvements in AST and ALT levels following treatment with caffeine, although the underlying molecular mechanisms by which caffeine exerts beneficial effects on the liver are not well-defined (Arauz et al., 2014). These contrasting findings suggest that the effects of caffeine on hepatic function may vary depending on the specific experimental conditions and dosage used.

In our study, it was determined that repeated administration of caffeine does not have an impact on survival rates during the acute phase of sepsis. These findings are consistent with previous research that utilized the cecal ligation and puncture (CLP) model and demonstrated that both a single dose of caffeine and continuous infusion did not affect survival rates (Bauza, Remick, 2015). Therefore, while caffeine may enhance glucose blood levels, it does not alter the response to vasoconstrictors, markers of organ dysfunction, blood pressure, or survival rates.

It is important to emphasize that our study has certain limitations. Caffeine acts as a non-selective antagonist and affects various molecular targets, which complicates the understanding of its mechanism of action. Therefore, the use of selective antagonists of adenosine receptors could be considered as a future option to further investigate its effects. Additionally, we did not evaluate the inflammatory profile of the animals in our study. Furthermore, the survival analysis was conducted with a small

number of replicates, which may limit the generalizability of the results. Therefore, further experiments are necessary to assess other important parameters related to sepsis.

5 CONCLUSION

In summary, our study demonstrated that repeated injections of caffeine, a non-selective antagonist of adenosine receptors, effectively regulate glycaemic levels during sepsis. However, no change was observed after caffeine administration on vascular response or renal blood flow induced by vasoconstrictor. Thus, while caffeine administration does not elicit significant changes during sepsis, it does impact cardiovascular function to some extent, highlighting the need for further investigation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERÊNCIAS

Albino LB, Sordi R, Oliveira JG, Fernandes D. Dose and time-dependent effects of caffeine on cardiovascular changes induced by adenosine. Brazilian Arch Biol Technol. 2023;66(1).

Arauz J, Zarco N, Segovia J, Shibayama M, Tsutsumi V, Muriel P. Caffeine prevents experimental liver fibrosis by blocking the expression of TGF-β. Eur J Gastroenterol Hepatol. 2014;26(2):164–73.

Arnaud MJ. Pharmacokinetics and metabolism of natural methylxanthines in animal and man. Vol. 200, Handbook of Experimental Pharmacology. 2011.

Bauza G, Remick D. Caffeine improves heart rate without improving sepsis survival. Shock. 2015;44(2):143–8.

Bedet A, Voiriot G, Ternacle J, Marcos E, Adnot S, Derumeaux G, et al. Heart rate control during experimental sepsis in mice comparison of ivabradine and β-blockers. Anesthesiology. 2020;132(2):321–9.

Borea PA, Gessi S, Merighi S, Vincenzi F, Varani K. Pharmacology of adenosine receptors: The state of the art. Physiol Rev. 2018;98(3):1591–625.

Debrah K, Sherwin RS, Murphy J, Kerr D. Effect of caffeine on recognition of and physiological responses to hypoglycemia in insulin-dependent diabetes. Lancet. 1996;347(8993):19–24.

Dungan KM, Braithwaite SS, Preiser JC. Stress hyperglycemia. Lancet. 2009;373(9677):1798–807.

Fairchild KD, O'Shea TM. Heart Rate Characteristics: Physiomarkers for Detection of Late-Onset Neonatal Sepsis. Clin Perinatol. 2010;37(3):581–98.

Faul F, Erdfelder E, Lang AG BA. G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods. 2007;2(39):175–91.

Fernandes D, Sordi R, Pacheco LK, Nardi GM, Heckert BT, Villela CG, et al. Late, but not early, inhibition of soluble guanylate cyclase decreases mortality in a rat sepsis model. J Pharmacol Exp Ther. 2009;328(3):991–9.

Fleischmann-Struzek C, Mellhammar L, Rose N, Cassini A, Rudd KE, Schlattmann P, et al. Incidence and mortality of hospital- and ICU-treated sepsis: results from an updated and expanded systematic review and meta-analysis. Intensive Care Med. 2020;46(8):1552–62.

Funakoshi H, Zacharia LC, Tang Z, Zhang J, Lee LL, Good JC, et al. A1 adenosine receptor upregulation accompanies decreasing myocardial adenosine levels in mice with left ventricular dysfunction. Circulation. 2007;115(17):2307–15.

Hahn PY, Wang P, Tait SM, Ba ZF, Reich SS, Chaudry IH. Sustained elevation in circulating catecholamine levels during polymicrobial sepsis. Shock. 1995;4(4):269–73.

Hasegawa D, Sato R, Prasitlumkum N, Nishida K, Takahashi K, Yatabe T, et al. Effect of Ultrashort-Acting β-Blockers on Mortality in Patients With Sepsis With Persistent Tachycardia Despite Initial Resuscitation: A Systematic Review and Meta-analysis of Randomized Controlled Trials. Chest. 2021;159(6):2289–300.

Headrick JP, Peart JN, Reichelt ME, Haseler LJ. Adenosine and its receptors in the heart: Regulation, retaliation, and adaptation. Biochim Biophys Acta - Biomembr. 2011;1808(5):1413–28.

Hubbard WJ, Choudhry M, Schwacha MG, Kerby JD, Rue LW, Bland KI, et al. Cecal ligation and puncture. Shock. 2005;24(SUPPL. 1):52–7.

Kovalski V, Prestes AP, Oliveira JG, Alves GF, Colarites DF, Mattos J EL, et al. Protective role of cGMP in early sepsis. Eur J Pharmacol. 2017;807(2016):174–81.

Li JL, Li G, Jing XZ, Li YF, Ye QY, Jia HH, et al. Assessment of clinical sepsis-associated biomarkers in a septic mouse model. J Int Med Res. 2018;46(6):2410–22.

Li X, Li T, Wang J, Dong G, Zhang M, Xu Z, et al. Higher blood urea nitrogen level is independently linked with the presence and severity of neonatal sepsis. Ann Med. 2021;53(1):2192–8.

Liu Z, Meng Z, Li Y, Zhao J, Wu S, Gou S, et al. Prognostic accuracy of the serum

lactate level, the SOFA score, and the qSOFA score for mortality among adults with Sepsis. Scand J Trauma Resusc Emerg Med. 2019;27(1):1–10.

Martin C, Leone M, Viviand X, Ayem ML, Guieu R. High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock. Crit Care Med. 2000;28(9):3198–202.

Neyens RR, Gaskill GE, Chalela JA. Critical Care Management of Anti-N-Methyl-. 2018;(4):1–8.

Park S, Scheffler TL, Gunawan AM, Shi H, Zeng C, Hannon KM, et al. Chronically elevated calcium blocks AMPK-induced GLUT-4 expression in skeletal muscle. Am J Physiol - Cell Physiol. 2009;296(1).

Passmore AP, Kondowe GB, Johnston GD. Renal and cardiovascular effects of caffeine: A dose-response study. Clin Sci. 1987;72(6):749–56.

Ramakers BP, Riksen NP, van den Broek P, Franke B, Peters WHM, van der Hoeven JG, et al. Circulating adenosine increases during human experimental endotoxemia but blockade of its receptor does not influence the immune response and subsequent organ injury. Crit Care. 2011;15(1):1–10.

Ribeiro JA, Sebastio AM. Caffeine and adenosine. J Alzheimer's Dis. 2010;20(SUPPL.1).

Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. Lancet. 2020;395(10219):200–11.

Sato C, Izumi N, Nouchi T, Hasumura Y, Takeuchi J. Increased hepatotoxicity of acetaminophen by concomitant administration of caffeine in the rat. Toxicology. 1985;63(34):95–101.

Schmidt H, Siems WG, Grime T, Ludwig E, Singen H, Julius H, et al. It. 1995;23:167–74.

Schrier RW, Wang W. Mechanisms of disease: Acute renal failure and sepsis. N Engl J Med. 2004;351(2):159–69.

Sert NP, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. The arrive guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 2020;18(7):1–12.

Singer M, Deutschman CS, Seymour C, Shankar-Hari M, Annane D, Bauer M, et al. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA - J Am Med Assoc. 2016;315(8):801–10.

Tavares C, Sakata RK. Cafeína para o tratamento de dor. Rev Bras Anestesiol. 2012;62(3):394–401.

Tofovic SP, Zacharia L, Carcillo JA, Jackson EK. Inhibition of adenosine deaminase attenuates endotoxin-induced release of cytokines in vivo in rats. Shock. 2001;16(3):192–202.

Vallon V, Mühlbauer B, Osswald H. Adenosine and kidney function. Physiol Rev. 2006;86(3):901–40.

Verma R, Huang Z, Deutschman CS, Levy RJ. Caffeine restores myocardial cytochrome oxidase activity and improves cardiac function during sepsis. Crit Care Med. 2009;37(4):1397–402.

Wang J, Zhu CK, Yu JQ, Tan R, Yang PL. Hypoglycemia and mortality in sepsis patients: A systematic review and meta-analysis. Heart Lung. 2021;50(6):933–40.

Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock—A review of laboratory models and a proposal. J Surg Res. 1980;29(2):189–201.

Zaky A, Deem S, Bendjelid K, Treggiari MM. Characterization of cardiac dysfunction in sepsis: An ongoing challenge. Shock. 2014;41(1):12–24.

Zhang T, Yu-Jing L, Ma T. The immunomodulatory function of adenosine in sepsis. Front Immunol. 2022;13(July):1–11.

ANEXO I

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Most articles published in BJPS will be organized into the following sections:
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Discussion should interpret the results and assess their significance in relation to existing knowledge. Speculation not warranted by actual data should be avoided. The Discussion should spell out the major conclusions and interpretations of the work including some explanation of the significance of these conclusions.

ACKNOWLEDGMENTS

When appropriate, briefly acknowledge technical assistance, advice, and contributions from colleagues. People who contributed to the work but do not fit the criteria for authors should be listed in the Acknowledgments section, along with their contributions. Donations of animals, cells, or reagents should also be acknowledged. You must also ensure that anyone named in the Acknowledgments agrees to being so named. Financial support for the research and fellowships should be acknowledged in this section (agency and grant number).

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Examples:

(Zhang, 2017)

(Ima, Souza, 2015)

(Fujisawa, Atsumi, Kadoma, 1989)(Aviral *et al*., 2009)

(Liu *et al.,* 2011a)(Liu *et al.,* 2011b)

References

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Abe T, Fukushima N, Brune K, Boehm C, Sato N, Matsubayashi H, et al. Genome-Wide allelotypes of familial pancreatic adenocarcinomas and familial and sporadic intraductal papillary mucinous neoplasms. Clin Cancer Res. 2007;13(20):6019-25.

Ali A, Iqbal F, Taj A, Iqbal Z, Amin MJ, Iqbal QZ. Prevalence of microvascular complications in newly diagnosed patients with Type 2 diabetes. Pak J Med Sci. 2013,29(4): 899-902.

Calvo A, Gimenez MJ. Ex Vivo Serum Activity (Killing Rates) After Gemifloxacin 320 mg Versus Trovafloxacin 200 mg Single Doses Against Ciprofloxacin-Susceptible and -Resistant Streptococcus pneumonia. Int J Antimicr Ag. 2007;20:144-6.

Article accepted for publication but not yet published: First 6 authors followed by et al. Title. Journal (abbreviation in normal font), Year of expected publication (in press) at the end of the citation.

Janiszewski M, Lopes LR, Carmo AO, Pedro MA, Brandes RP, Santos CXC, et al. Regulation of NAD(P)H oxidase by associated protein disulfide isomerase in vascular smooth muscle cells. J Biol Chem. 2005 (in press).

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Brasil. Ministério da Saúde, Secretaria de Vigilância em Saúde. Leishmaniose visceral grave: normas e condutas [Internet]. Brasília (DF): Ministério da Saúde, 2006. [citado 2008 Jan 7]. 60 p. (Série A. Normas e Manuais Técnicos). Disponível em: http://dtr2001.saude.gov.br/editora/produtos/livros/ pdf/06_0072_M.pdf

CAPES Statistics. [cited 2006 Mar 16]. Available from: http:// www.capes.gov.br/capes/portal.

Developmental toxicology. [cited 2015 Apr 10]. Available from: http://www.devtox.org/nomenclature/organ.php.

Whole Book: Authors, Book title, Edition, City, Publisher, Year.

Hewitt W. Microbiological assay for pharmaceutical analysis: a rational approach. Boca Raton: CRC Press; 2003.

Jenkins PF. Making sense of the chest x-ray: a hands-on guide. New York: Oxford University Press; 2005. 194 p.

Laws:

Agência Nacional de Vigilância Sanitária (Brasil). Resolução nº. 259, de 20 de setembro de 2002. Regulamento Técnico para Rotulagem de Alimentos Embalados. Diário Oficial da União 23 set 2002; Seção 1.

Milech A, et al., Oliveira JEP, Vencio S, organizadores. Diretrizes da Sociedade Brasileira de Diabetes. São Paulo: A.C. Farmacêutica;2016.

Book Chapter: Authors, Chapter Title, Editors, Book title, Edition, City, Publisher, Year, Pages of citation.

Conference or Symposium Proceedings: Cite papers only from published proceedings.

Hejzlar RM, Diogo PA. The use of water quality modelling for optimizing operation of a drinking water reservoir. In: Proceedings of the International Conference Fluid Mechanics and Hydrology. 1999 Jun 23-26; Prague. Prague: Institute of Hydrodynamics AS CR; 1999. p 475-482.

Report

Rojko JL, Hardy WD Jr. Feline leukemia virus and other retroviruses. In: Sherding RG, editor. The cat: diseases and clinical management. New York: Churchill Livingstone; 1989. p. 229-332.

World Health Organization. WHO. Department of Mental Health and Substance Abuse. Mental health atlas 2005. Geneva: World Health Organization; 2005. 409 p.

Proceedings of the 10th annual meeting of the Canadian Society for Pharmaceutical Sciences. *J Pharm Pharm Sci*. 2007 Dec 3;10(4):1s-186s.

Audiovisual Material

Physician's Desk Reference (PDR). Release 2003.1AX. [CD- ROM]. Montvale: Thomson PDR; 2003.

Computer Program

Dean AG, Dean JA, Coulombier D, Brendel KA, Smith DC, Burton AH, et al. *Epi info, version 6.04: a word processing database and statistics program for public health on IBM- compatible microcomputers*. [Computer program]. Atlanta: Centers of Disease Control and Prevention;1998. World Health Organization. WHO. Working to overcome the global impact of neglected tropical diseases, First WHO report on neglected tropical diseases. Geneva, Switzerland: WHO Press; 2010.

Patent

Larsen CE, Trip R, Johnson CR. Methods for procedures related to the electrophysiology of the heart. Patent No.5.529.067. Novoste Corporation; 1995.

Thesis and Dissertations

Joselevitch C. Visão no ultravioleta em Carassius auratus (Ostariophysi, Cypriformes, Cyprinidae):

estudo eletrofisiológico do sistema cone - células horizontais. [Master's dissertation]. São Paulo: Instituto de Psicologia, USP; 1999.

Marcolongo R. Dissolução de medicamentos: fundamentos, aplicações, aspectos regulatórios e perspectivas na área farmacêutica. [dissertação]. São Paulo: Universidade de São Paulo, Faculdade de Ciências Farmacêuticas; 2003.

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