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Dose and Time-Dependent Effects of Caffeine on Cardiovascular Changes Induced by Adenosine

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HIGHLIGHTS

- Adenosine induces a reduction in blood pressure, heart rate and renal blood flow.
- Caffeine (30 mg/kg) blocked all the cardiovascular changes induced by adenosine.
- The effect of caffeine lasts for 4 hours.

Abstract: Adenosine is an important regulator within the cardiovascular system and modulates various processes through four distinct G protein-coupled receptors (A_1, A_{2A}, A_{2B}, and A_3), causing hypotension and reduced renal blood flow, which contributes to ischemia tissue and organ dysfunction. Caffeine causes most of its biological effects by blocking adenosine receptors. Although caffeine is vastly used as a pharmacological tool in basic research, there is a lack of studies characterizing the caffeine doses necessary for blocking the cardiovascular effects of adenosine. Therefore, we evaluated the effect of different doses of caffeine (10, 30 and 100 mg/kg, s.c) on the cardiovascular changes induced by adenosine. In addition, the adenosine response was evaluated at different times (4 and 8 hours) after caffeine administration. The bolus injection of increasing doses of adenosine dose-dependently reduced blood pressure, renal blood flow and heart rate. The dose of 30 mg/kg was the most effective in blocking adenosine-induced cardiovascular events. However, the higher dose of caffeine (100 mg/kg) fails to block the adenosine effect on heart rate. The effect of caffeine lasts for 4 hours. Therefore, caffeine in the dose of 30 mg/kg can block the effects of adenosine for up to 4 hours. These data provide valuable information on the dose and frequency of caffeine administration for future studies in rats.

Keywords: Blood pressure; Renal blood flow; Heart rate.

INTRODUCTION

Adenosine is a ubiquitous endogenous nucleoside that acts on four evolutionarily rather well conserved G-protein coupled receptors denoted A_1, A_{2A}, A_{2B} and A_3 [1]. Caffeine is an antagonist of adenosine receptors and impacts a wide range of cardiovascular physiological processes. Caffeine is a purine alkaloid and binds with a very similar affinity to adenosine A_1 and A_{2A} receptors. At doses commonly consumed by humans, caffeine is able to impair the adenosine response in both receptors. On the other hand, the A_{2B} and A_3 receptors were initially shown to be little affected by caffeine [2].

The vascular effects of adenosine differ according to the receptor subtype activated, the type of vascular bed involved, and the species [3]. For example, A_{2A} adenosine receptors are highly expressed in the vasculature and cause vasorelaxation, and blood pressure reduction [4]. In contrast, in the renal vasculature, adenosine induces vasoconstriction of the afferent glomerular arteriole via A_1 receptors, reducing renal blood flow [5].

In non-clinical research, caffeine has been widely used as a pharmacological tool for the non-selective blockade of adenosine receptors. However, many results are conflicting and part of this is due to the different doses and frequency of administration of caffeine [2]. For example, caffeine produces biphasic effects on heart rate depending on the doses [6]. Moreover, caffeine has a short plasma half-life of approximately one hour [7], therefore, the plasma concentration of a single dose of caffeine, generally used in non-clinical studies, decreases rapidly and the effect may be time-dependent. Thus, the use of inappropriate doses and frequency of administration generates discrepant data in the literature.

In this study, we have investigated the effects of different doses of caffeine on cardiovascular responses to adenosine (blood pressure, heart rate and renal blood flow). Moreover, we evaluated the adenosine response at different times after caffeine administration. The determination of the correct dose of caffeine to block the effects of adenosine, as well as the duration of this blockade, is essential for the use of caffeine as a pharmacological tool.

MATERIAL AND METHODS

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 9770210519) and were in accordance with 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 [8].

Mean arterial pressure (MAP) measurement

The animals were anesthetized 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 the drug injections. The animals were allowed to breathe spontaneously via a tracheal cannula. 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 adenosine (1, 3, 10, 30, 100, and 300 nmol/kg, i.v.) was 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 adequacy of anesthesia was assessed by regular respiratory rate, heart rate monitoring, 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.

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 [9]. 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 phosphate-buffered 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 adenosine was also recorded.

Experimental protocol

The rats were prepared for mean arterial pressure and renal blood flow measurement as previously described. After 15 minutes of stabilization, basal values of mean arterial pressure, heart rate and renal blood flow were recorded, and dose-response curve to adenosine (1, 3, 10, 30, 100, and 300 nmol/kg, i.v.) was obtained. Ten minutes after the last adenosine dose, animals were randomly assigned to receive caffeine (10, 30 or 100 mg/kg, s.c). Sixty minutes after caffeine injection, the basal values of cardiovascular parameters were recorded and a second dose-response curve to adenosine was performed (Figure 1A).

In another set of experiments, the rats were randomized assigned to receive caffeine (30 mg/kg, s.c.) or vehicle (1 mL/kg, s.c.). The animals were prepared for mean arterial pressure and renal blood flow measurement one, four, or eight hours after treatment. After 15 minutes of stabilization, basal values of mean arterial pressure and renal blood flow were recorded, and a dose-response curve to adenosine (1, 3, 10, 30, 100, and 300 nmol/kg, i.v.) was obtained (Figure 1B). At the end of the experiments, the animals were killed by an anesthetic overdose.

The experiments were not done blindly and the experimenter was aware of the treatment of the animals. We have not adopted any criteria of inclusion or exclusion.

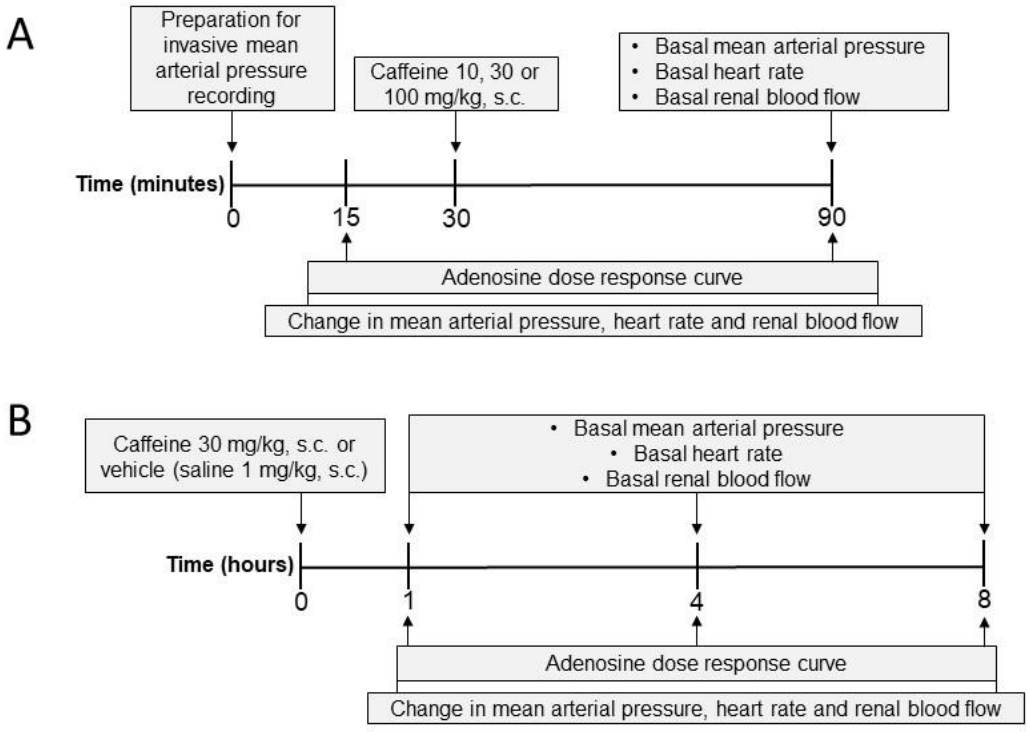

Figure 1. Timeline of the experimental protocol designed to study the effect of caffeine on adenosine cardiovascular effects. Following preparation for mean arterial pressure (MAP) recording, increasing bolus doses of adenosine were injected intravenously. (A) Rats received caffeine (10, 30, or 100 mg/kg, s.c.) or vehicle (saline, 1 mg/kg, s.c.), and one hour later the adenosine dose-response curve was repeated. (B). In a separate set of experiments**,** rats received caffeine (30 mg/kg, s.c.) or vehicle (saline 1 mg/kg, s.c.). One, four, or eight hours later, a dose-response curve of adenosine and cardiovascular analyses were performed.

Reagents

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 (Sigma Chemical Co., St. Louis, MO, USA) and adenosine (CAQ Ltda, Diadema, SP, Brazil).

Statistical analysis

The data were expressed as the mean ± standard error of the mean (SEM). The sample calculation was based on the magnitude of difference between the groups and the standard deviation obtained in the analysis of adenosine-induced reduction on mean arterial pressure (mmHg, primary outcome) from our previous pilot study (data not shown). Therefore, considering two experimental groups, α= 0.05 and a power of 80%, six animals in each group were required for statistical significance. The GPower 3.1.1 software was used to sample size calculation [10]. In order to account for potential technical loss, the n value was increased to nine. The final number (n) in each group is indicated in Figure legends. Normality and homogeneity of variance were verified through Shapiro-Wilk and Bartlett tests, respectively. The statistical significance was analyzed by two-way ANOVA, followed by Bonferroni's post hoc test.

RESULTS

Trace recording of a typical experiment showing adenosine response on cardiovascular parameters

Adenosine induces a dose-response reduction on mean arterial pressure (Figure 2A). Moreover, the adenosine caused a decrease in heart rate (Figure 2B) and renal blood flow (Figure 2C).

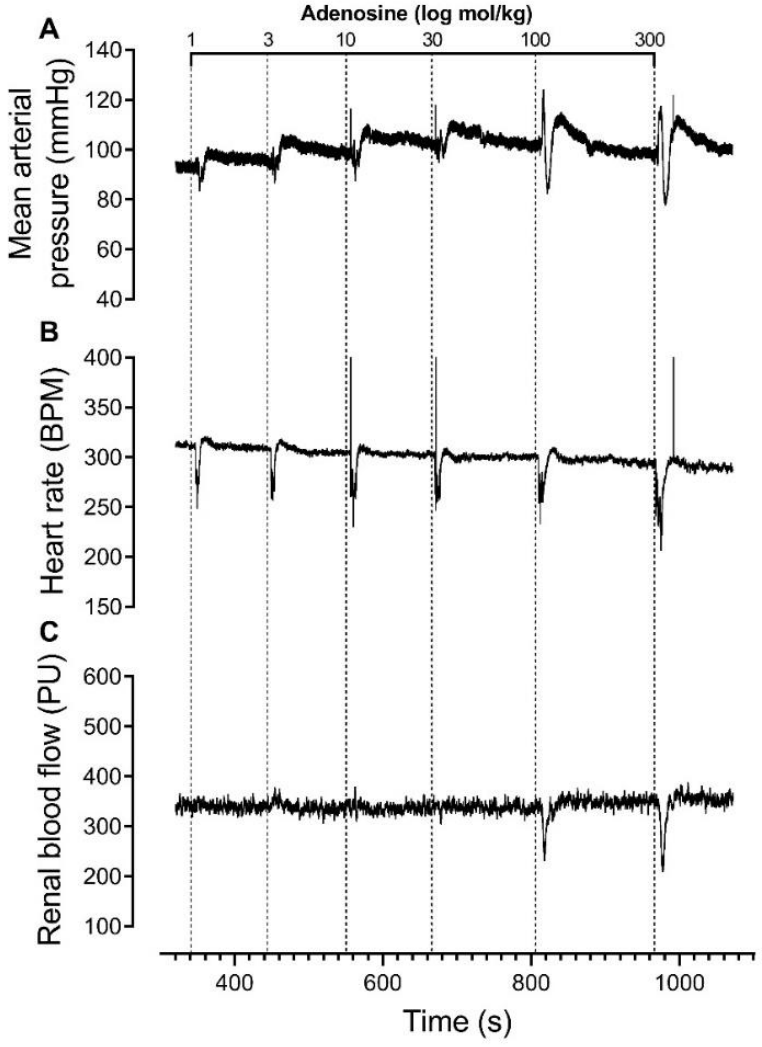

Figure 2. Trace recording of a typical experiment showing adenosine response on cardiovascular parameters. (A) Mean arterial pressure; (B) Heart rate; (C) Renal blood flow recording. Increasing intravenous bolus doses of adenosine (1, 3, 10, 30, 100, and 300 nmol/kg) were injected.

Effect of caffeine on adenosine-induced cardiovascular changes

The lower dose of caffeine (10 mg/kg) reduced by 48% the adenosine-induced response on blood pressure (Figure 3A, *P*<0.05), while the highest doses (30 and 100 mg/kg) completely abrogated the effect of adenosine (Figures 3B-C, *P*<0.05). The dose of 10 and 30 mg/kg of caffeine reduced by 60% the adenosine effect on heart rate (Figures 3D-E, *P*<0.05). On the other hand, the highest dose of caffeine did not change the adenosine effect on this parameter (Figure 3F). All tested doses of caffeine reduced the adenosine effect on renal blood flow by around 55% (Figures 3G-I, *P*<0.05). Moreover, none of the caffeine doses changed basal mean arterial pressure (Figure 4A) or renal blood flow (Figure 4C) one hour after caffeine injection. However, the highest dose of caffeine increased the heart rate by 44 BPM (Figure 4B, *P*<0.05).

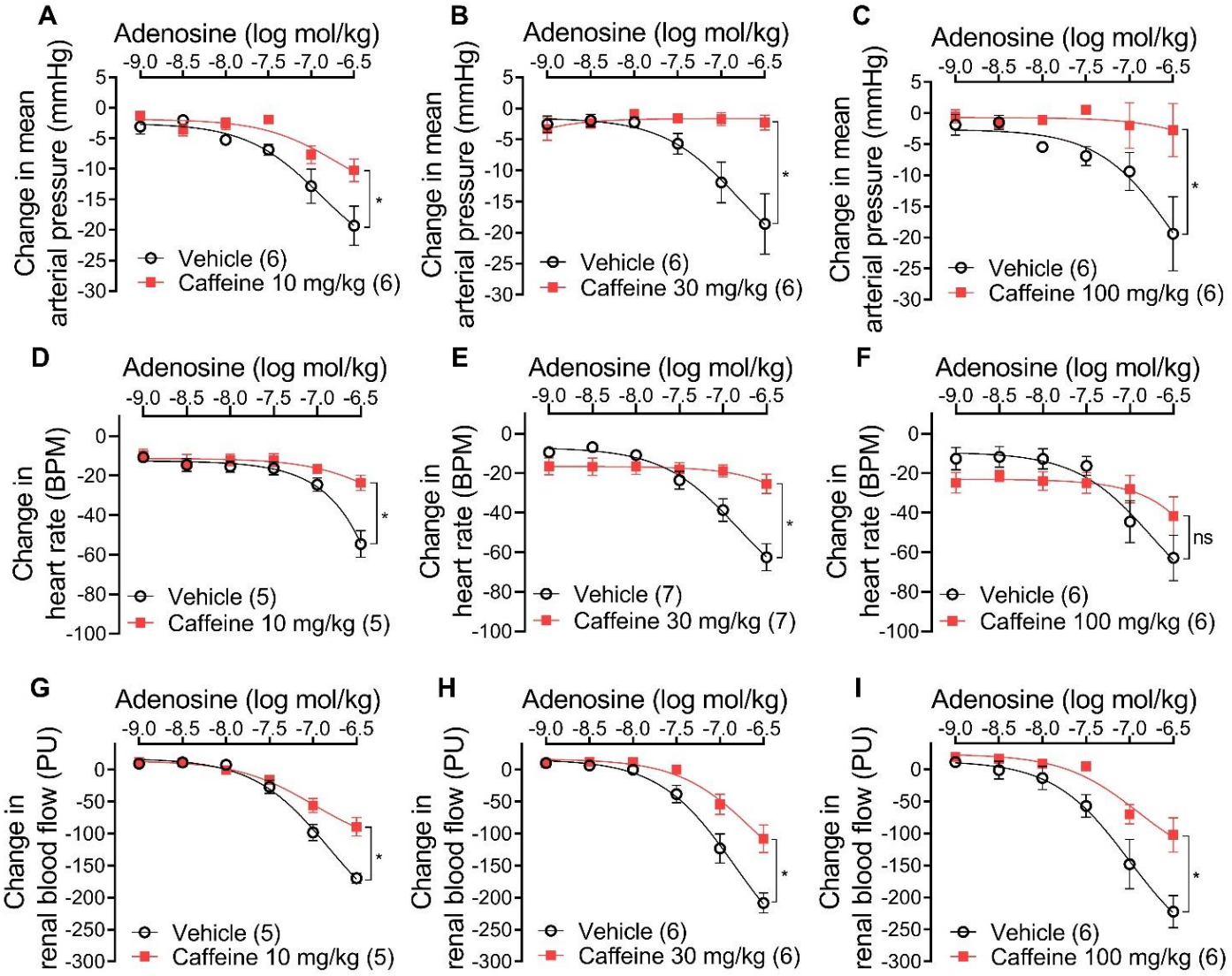

Figure 3. Effect of caffeine on adenosine-induced cardiovascular changes. The animals were anesthetized and prepared for the register of different parameters. (A-C) Mean arterial pressure; (D-F) Heart rate; (G-I) Renal blood flow. Then, increasing doses of adenosine were injected before and 90 min after a single dose of caffeine (10, 30, or 100 mg/kg, s.c.). The changes in the cardiovascular parameters were recorded. The results represent the mean ± SEM. The n of each group is indicated in the figures. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (*p<0.05, compared with the vehicle group).

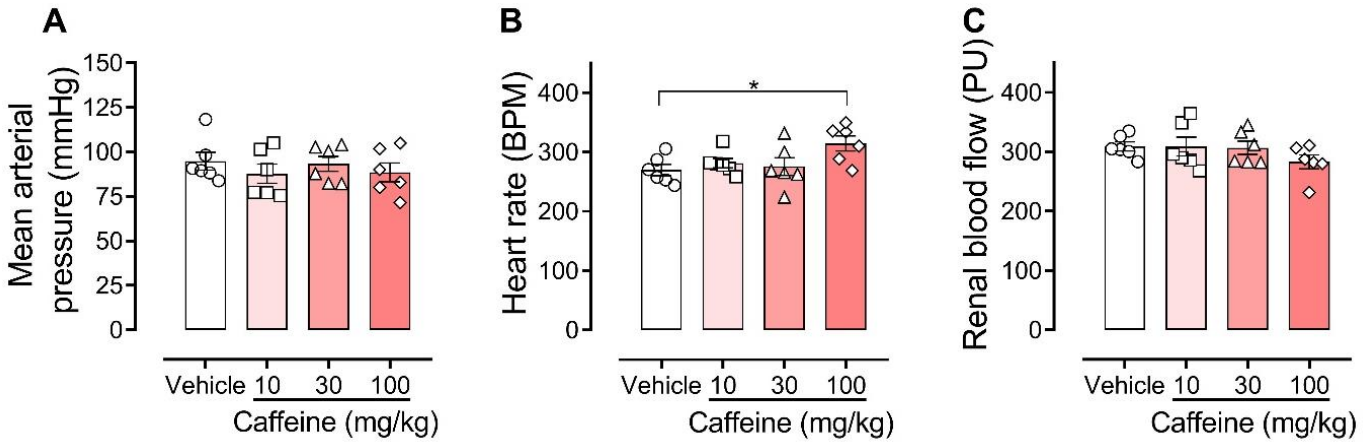

Figure 4. Cardiovascular and renal parameters induced by caffeine. One hour after the administration of the vehicle (saline 1 mL/kg, s.c.), or caffeine (10, 30, or 100 mg/kg, s.c.), the following parameters were recorded: (A) mean arterial pressure; (B) heart rate (C) renal blood flow. The results represent the mean ± SEM, n= 6 animals. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (*p<0.05, compared with the vehicle group).

Time course of caffeine effect on adenosine-induced cardiovascular changes

To evaluate the duration of the caffeine blockade, rats were injected with caffeine (30 mg/kg), and four and eight h after, they were anesthetized and instrumented for mean arterial pressure measurement. Four hours after caffeine administration, it was observed a reduction of 40% in the adenosine response in mean arterial pressure (Figure 5A, *P*=0.05), and 45% in renal blood flow (Figure 5C, *P*<0.05), but no changes in heart rate (Figure 5B, *P*=0.05). However, the effect of caffeine eight h later induced only a brief reduction of 17% in mean arterial pressure (Figure 5D *P*<0.05) but did not change the heart rate or renal blood flow (Figures 5E-F, respectively). Furthermore, the dose of 30 mg/kg did not change any of the basal cardiovascular parameters evaluated four or eight hours after caffeine treatment (Figures 6A-C, respectively).

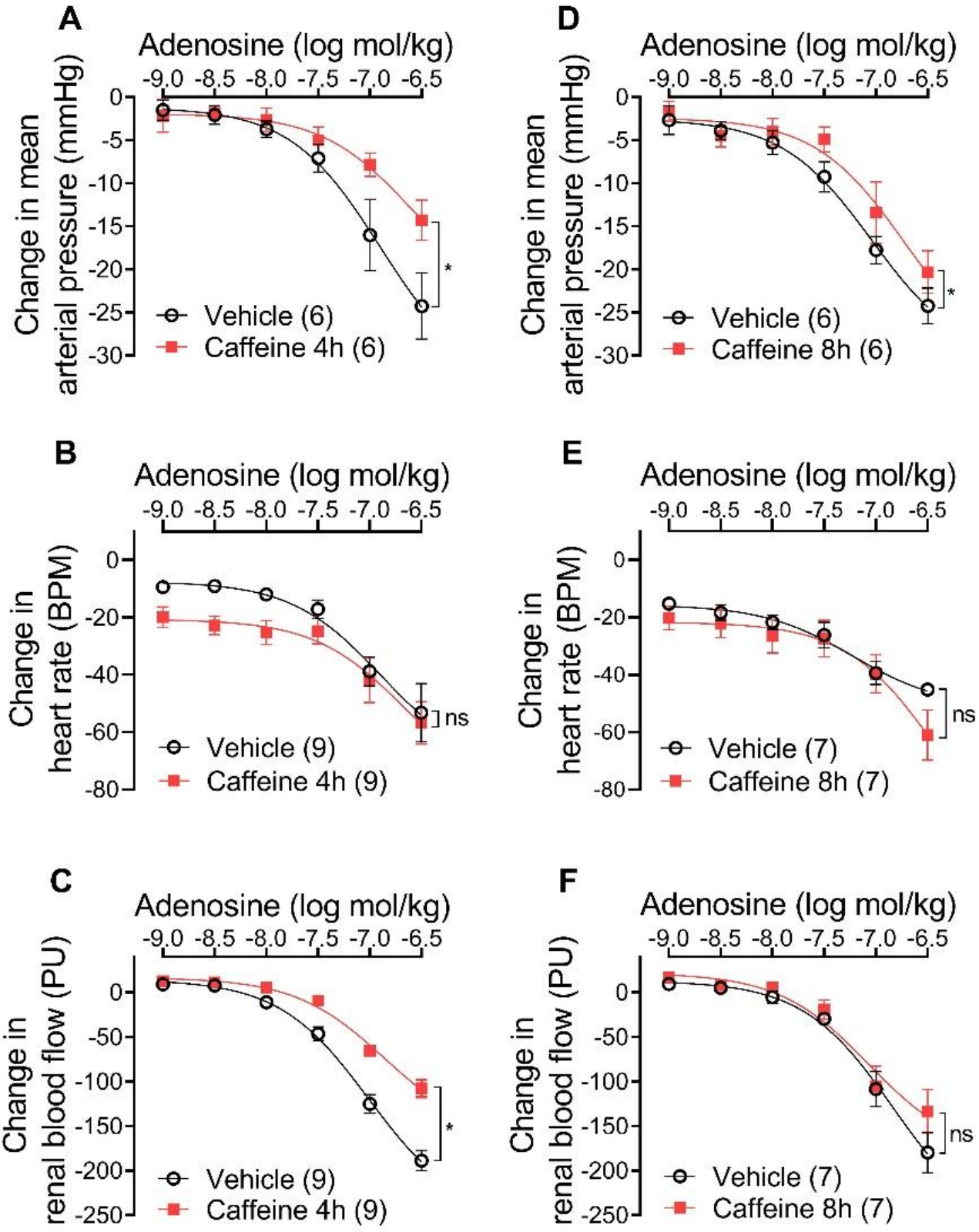

Figure 5. Time course of caffeine effects on adenosine-induced cardiovascular changes. Four or eight hours after caffeine (30 mg/kg, s.c.) or vehicle (saline1 mL/kg, s.c.) treatment, animals were anesthetized and prepared for: (A;D) Mean arterial pressure; (B;E) Heart rate; (C;F) Renal blood flow measurement. Increasing doses of adenosine were injected (i.v.), and changes in cardiovascular were recorded. The results represent the mean ± standard error of the mean. The n of each group is indicated in the figures. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. (*$p<0.05$, compared with the vehicle group).

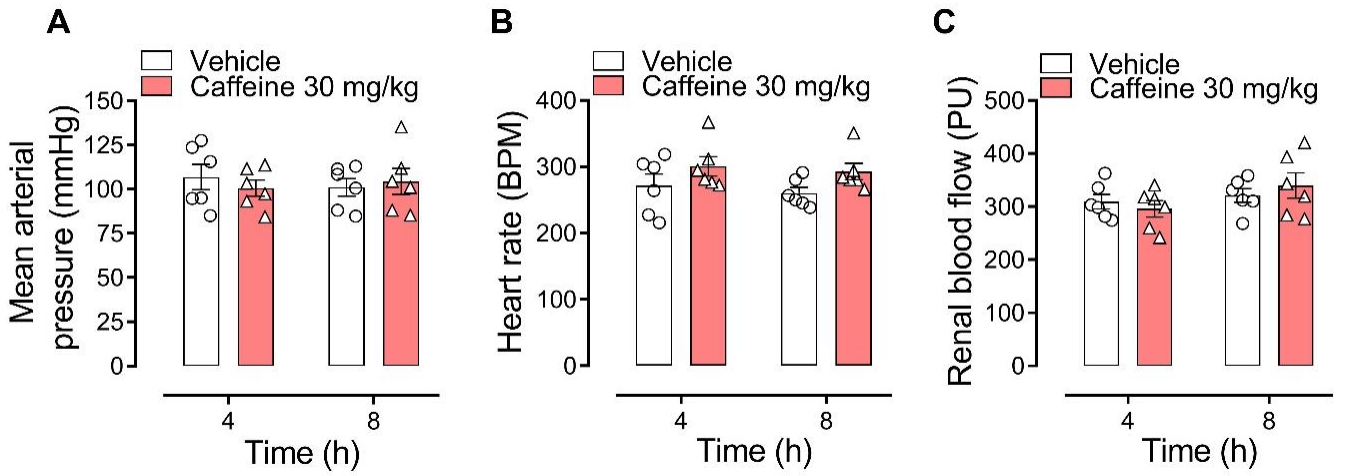

Figure 6. Cardiovascular and renal parameters induced by caffeine. Four or eight hours after the administration of vehicle (saline 1 mL/kg, s.c.), or caffeine (30 mg/kg, s.c.), the animals were anesthetized and prepared to register: (A) mean arterial pressure; (B) heart rate; (C) renal blood flow. The results represent the mean ± standard error of mean, n= 6 animals. Statistical analyses were performed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (*p<0.05, compared to the vehicle group).

DISCUSSION

In the present study, we have shown that intravenous injection of adenosine dose dependently decreases blood pressure, renal blood flow and heart rate. All these adenosine induced-cardiovascular changes were blocked by caffeine.

Adenosine has a biphasic effect on blood pressure with an initial transient increase in blood pressure followed by a reduction. This effect is more evident at doses of 100 and 300 nmol/kg (Figure 2) and agrees with previous studies showing that adenosine induces initial transient vasoconstriction mediated by A_1 receptors followed by vasodilation mediated by A_2 receptors [11].

Despite the reduction on blood pressure which could increase heart rate by reflex control, adenosine induces a reduction in heart rate which is probably mediated by the stimulation of A_1 receptors on the sinus and atrioventricular (AV) node, slowing impulse conduction. The effect of adenosine on nodal tissues is dose-dependent and of very short duration. This rapid effect of adenosine in the heart is the basis for the clinical administration of intravenous adenosine by bolus injection as an antiarrhythmic agent [12].

Adenosine plays a critical role in the regulation of renal hemodynamics. The A_1 receptors are widely expressed in the kidney and regulate vascular tone [13]. Here we have demonstrated that a bolus injection of adenosine reduces renal blood flow. Activation of renal A_1 receptors reduces renal blood flow and some studies have implicated this effect in reducing renal function due to ischemia and sepsis [14].

Caffeine, the most widely consumed psychoactive drug globally, is a non-selective adenosine receptor antagonist [15]. Therefore, in addition to being popular, caffeine is an important pharmacological tool for studying adenosine receptors. But surprisingly, there is a lack of studies evaluating the effect of caffeine on adenosine-induced cardiovascular changes. Most studies use an empirical dose, without prior evaluation. In addition, they generally use once-daily dosing, despite the well-established short half-life of caffeine [16]. Here we evaluated the effect of different doses of caffeine on adenosine-induced cardiovascular changes. Moreover, we evaluated the duration of the caffeine effect.

Caffeine was able to block all adenosine-induced cardiovascular changes. Although the caffeine dose of 10 mg/kg is widely used in the literature in rodent studies, the dose of 30 mg/kg was more effective in blocking the cardiovascular effects of adenosine. The dose of 100 mg/kg of caffeine failed to block the adenosine-induced reduction in heart rate, and this can be explained by the increases in basal heart rate induced by the highest dose of caffeine itself. This effect, which is not due to adenosine antagonism, counteracts the response caused by adenosine. At high doses caffeine can inhibit phosphodiesterases and increases cAMP, which may account for caffeine cardiostimulatory actions.

Furthermore, the direct effects of caffeine can be observed on endothelial and vascular smooth muscle cells, mainly through modulation of calcium signaling in the cells, which is associated with the control of blood pressure, vascular tone, and peripheral blood flow. These effects depend on the inhibition of voltage-dependent calcium channels in the cellular plasmatic membrane and the increase of calcium release by the endoplasmic reticulum. Caffeine raises calcium release by the endoplasmic reticulum by activating ryanodine

receptors [17], generating a slight transitory contraction in the vessels [18]. On the other hand, caffeine exerts indirect effects on the calcium release by the endoplasmic reticulum in endothelial cells, forming the calcium-calmodulin complex, which activates the endothelial nitric oxide synthase enzyme (NOS3) and triggers the synthesis of nitric oxide (NO), generating vasodilation response [19]. Although these effects of caffeine are useful as a pharmacological tool, high concentration of caffeine are required (mM scale), which is hardly obtained in *in vivo* experiments [20]. In addition, part of the caffeine effect on vessels is related to the release of catecholamines. It has also been demonstrated that caffeine increases intracellular calcium in the adrenal chromaffin cells inducing the secretion of catecholamines [21–23]. The catecholamines, through activation of the β-adrenergic receptor, can induce heart effects related to inotropic and chronotropic positive responses [24]. However, these effects of high doses of caffeine *in vivo* need further exploration, which is beyond the scope of this study.

Nevertheless, as the objective of the work is to characterize the best dose of caffeine for blocking the adenosine receptor, the dose of 30 mg/kg of caffeine was the dose chosen for treatment. The blockade of caffeine in the adenosine-induced reduction in blood pressure and renal blood flow lasts about 4 hours, which agrees with caffeine half-life [16]. Some studies have shown that in humans, the peak plasma concentration of caffeine occurs between 1 to 2 hours after administration, and the half-life is between 2.5 and 5 hours [25]. Therefore, for a proper blockade of the cardiovascular effects of adenosine, caffeine administration should be done every 4-6 hours. Thus, our data are opposed to studies that use single or daily doses of caffeine as a treatment, as these protocols may have no therapeutic effects as caffeine is short-lived.

Under normal physiological conditions, extracellular adenosine levels are low (20- 300 nM), rising to high micromolar levels (30 μM) in ischemic conditions [1]. Therefore, the high levels of extracellular adenosine could play an important role in the cardiovascular changes induced by inflammatory and ischemic conditions such as sepsis [26,27]. Thus, it is essential to characterize the cardiovascular effects of adenosine as well as its blockade by antagonists. Therefore, the present study took a step forward on the understanding of the role of adenosine in cardiovascular alterations.

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Conflicts of Interest: The authors declare no conflict of interest.

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