## BASIC RESEARCH

# ATZ (3-amino-1,2,4-triazole) injected into the fourth cerebral ventricle influences the Bezold–Jarisch reflex in conscious rats

Vitor E. Valenti,[I] Luiz Carlos de Abreu,[II] Monica A. Sato,[II] Celso Ferreira[I]

[I]Universidade Federal de São Paulo (UNIFESP) - Departamento de Medicina, Disciplina de Cardiologia, São Paulo, São Paulo, Brazil. [II]Faculdade de Medicina do ABC - Departamento de Morfologia e Fisiologia, Santo André, São Paulo, Brazil.

**OBJECTIVES:** Many studies have investigated the importance of oxidative stress on the cardiovascular system. In this study we evaluated the effects of central catalase inhibition on cardiopulmonary reflex in conscious Wistar rats.

**METHODS:** Male Wistar rats were implanted with a stainless steel guide cannula in the fourth cerebral ventricle. The femoral artery and vein were cannulated for mean arterial pressure and heart rate measurement and for drug infusion, respectively. After basal mean arterial pressure and heart rate recordings, the cardiopulmonary reflex was tested with a dose of phenylbiguanide (PBG, 8 μg/kg, bolus). Cardiopulmonary reflex was evaluated before and μl15 minutes after 1.0 μL 3-amino-1,2,4-triazole (ATZ, 0.01g/100μL)0.01 g/100 μL) injection into the fourth cerebral ventricle. Vehicle treatment did not change cardiopulmonary reflex responses.

**RESULTS:** Central ATZ significantly increased hypotensive responses without influencing the bradycardic reflex.

**CONCLUSION:** ATZ injected into the fourth cerebral ventricle increases sympathetic inhibition but does not change the parasympathetic component of the cardiopulmonary reflex in conscious Wistar rats.

**KEYWORDS:** Reflex; Oxidative Stress; Catalase; Medulla Oblongata; Cardiovascular System.

## INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anions ($SO_2^-$) and hydrogen peroxide ($H_2O_2$), once thought only to be harmful byproducts of oxidative metabolism, are now recognized as critical second messengers in a wide range of physiological processes.[1–4] ROS are the result of incomplete reduction of oxygen to $O_2^-$, which is spontaneously or enzymatically dismutated to $H_2O_2$ by superoxide dismutase (SOD). $H_2O_2$ is transformed to $H_2O$ and $O_2$ under catalase activity.[5] 3-Amino-1,2,4-triazole (ATZ) is a heterocyclic organic compound that consists of a 1,2,4-triazole substituted with an amino group. ATZ is a competitive inhibitor of the product of the HIS3 gene, imidazoleglycerol-phosphate dehydratase. Imidazoleglycerol-phosphate dehydratase is an enzyme catalyzing the sixth step of histidine production. This drug also inhibits catalase activity, thus increasing endogenous $H_2O_2$.[5]

Previously, von Bezold and Hirt observed that an intravenous injection of veratrum alkaloids caused a decrease in blood pressure and heart rate, which was then named the Bezold–Jarisch reflex or cardiopulmonary reflex.[6] Chemical activation of the cardiopulmonary afferent endings with phenylbiguanide (PBG, 5-HT3 agonist) is a pharmacological approach that has been used by different laboratories to induce the cardiopulmonary reflex. This approach has been considered to be a valid index for the evaluation of the cardiopulmonary reflex.[7] The activity of the sympathetic and parasympathetic systems, which are both involved in the cardiopulmonary reflex, is under the control of a medullary circuitry comprising the nucleus of the solitary tract (NTS), rostral (RVLM) and caudal ventrolateral medulla (CVLM) and the nucleus ambiguous. Drug injection into the fourth cerebral ventricle (V4) may easily reach structures surrounding the ventricular system such as the area postrema, NTS, CVLM, RVLM and the dorsal motor nucleus of the vagus.[8]

Previous studies indicated that ROS are also related to impaired baroreflex.[9,10] It was found that increased cholesterol levels were associated with baroreflex gain (ΔHR/ΔMAP) dysfunction and an imbalance between oxidant and antioxidant forces.[10] However, to the best of our knowledge, no previous study has demonstrated the effects of ATZ, a

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catalase inhibitor drug, injected into central areas involved in cardiovascular reflex. Therefore, we investigated the effects of central ATZ on the cardiopulmonary reflex in conscious Wistar rats.

## METHOD

### Animals

The experiments were performed in Wistar rats (n = 15, 300–400 g). Rats were housed individually in plastic cages under standard laboratory conditions. They were kept under a 12-h light/dark cycle (lights on at 06:30 h) and had free access to food and water. Housing conditions and experimental procedures were approved by our institution's Animal Ethics Committee. Efforts were made to minimize the number of animals used.

### Surgical Preparation

Five days before the experiment, the rats were anesthetized with ketamine (50 mg/kg i.p.) and xylazine (50 mg/kg i.m.).[11,12] After scalp anesthesia with 2% lidocaine, the skull was exposed and stainless steel guide cannulas (26 G) were implanted into the V4 1 mm above the injection site using a stereotaxic apparatus (David Kopf Instruments, USA). Stereotaxic coordinates for cannula implantation into the V4 were: AP, −12 mm from the bregma; L, 0 mm from the medial suture; V, –6.1 to 6.4 mm from the skull.[13] Cannulas were fixed to the skull with dental cement and one metal screw.

One day before the experiments, the rats were anesthetized with ketamine (50 mg/kg i.p.) and xylazine (50 mg/kg i.m.) and a catheter was inserted into the abdominal aorta through the femoral artery for blood pressure and heart rate recording. Another catheter was inserted into the femoral vein for drug administration. Catheters were made of 4-cm segments of PE-10 polyethylene (Clay Adams, USA) heat bound to a 13-cm segment of PE-50. The catheters were tunneled under the skin and exteriorized at the animal's dorsum.[11,12,14]

### Arterial pressure and heart rate recording

After surgery, the animals were kept in individual cages, which were used to transport them to the experimental room. Animals were allowed 60 minutes to adapt to the conditions of the experimental room, such as sound and illumination, before starting blood pressure and heart rate recording. The experimental room was acoustically isolated and had constant background noise produced by an air exhauster. At least another 20-minute period was allowed before beginning experiments. Pulsatile arterial pressure of freely moving animals was recorded using an HP-7754A preamplifier (Hewlett Packard, USA) and an acquisition board (MP100A, Biopac Systems Inc, USA) connected to a computer. Mean arterial pressure (MAP) and heart rate (HR) values were derived from the pulsatile arterial pressure (PAP) recordings and processed on-line.[15] No signs of pain or discomfort were observed.

### Cardiopulmonary reflex activation

Animals were connected to the acquisition system and allowed to recover from manipulation stress for at least 30 minutes. When MAP and HR were stable, 20–30 minutes baseline.were recorded for the HR and MAP baselines. The cardiopulmonary reflex was evaluated through i.v. bolus injection of phenylbiguanide (PBG, 8 µg/kg). Chemical activation of the cardiopulmonary afferent endings with PBG is a pharmacological approach that has been used by different laboratories to induce the cardiopulmonary reflex. This approach has been considered to be a valid index for the evaluation of the cardiopulmonary reflex.[7]

### Injections into the V4

Injections into the V4 were made with 10-µL Hamilton syringes connected by polyethylene tubing (PE-10) to an injector needle. The injector, when completely inserted, protruded 2 mm beyond the tip of the guide cannula. Injections into the V4 were 1.0 µL for about 5–10 s.[16]

### Experimental procedures

Central catalase activity was inhibited by injection of 3-amino-1,2,4-triazole (ATZ, 0.01 g/100 µL – catalase inhibitor) into the V4. To verify cardiopulmonary reflex after central catalase inhibition we tested cardiopulmonary reflex before (control) and 15 minutes after ATZ administration into the V4.

### Histology

In order to confirm the placement of the injection needle, at the end of the experiments the animals were anesthetized with urethane (1.25 g/kg i.p.), and 200 nL of 1% Evan's blue dye was injected into the V4 as a marker of the injection site. The chest was surgically opened, the descending aorta occluded, the right atrium severed and the brain perfused with 10% formalin through the left ventricle. The brains were post fixed for 24 h at 4℃, and 40-µm sections were cut in a cryostat (model CM 1900, Leica, Germany). Brain sections were stained with 1% neutral red. The actual placement of the injection needles was verified in serial sections.[16]

### Statistical Analysis

The results are reported as means ± standard error of means. In order to compare variables (bradycardia and hypotension induced by PBG) before and 15 minutes after ATZ test.administration, we used the paired Student's t-test. Analyses of variance (ANOVA) for repeated measures followed by the Tukey post test were used for comparisons of MAP and HR before, 5, 15, 30, and 60 minutes after ATZ treatment. Differences were considered significant when the probability of a type I error was less than 5% ($p < 0.05$).

## RESULTS

### Histology

Fig. 1 is a photomicrograph showing the typical sites of the injections into the V4 in one rat representative of the rats used in our study. According to Paxinos and Watson,[13] these coronal sections are located approximately 13 mm caudal to bregma.

### Effects of ATZ on Basal MAP and HR

The vehicle-treated group did not present significant changes in relation to baseline MAP (Table 1). Conversely, we observed that injections of ATZ into the V4 produced a significant ($p < 0.05$) increase in basal MAP in conscious Wistar rats 60 minutes after injection (Table 1). The results presented in Table 1 are modest and the degree of the

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**Figure 1** - Histological image of a rat brain coronal slice showing the medulla and cerebellum (~13 mm caudal to bregma). V4: fourth cerebral ventricle.

alteration is not higher than the difference between vehicle and ATZ groups at 60 minutes: ATZ 106(113–119 mmHg or 5.0%) vs vehicle (101–106 mmHg or 5.1%) groups.

Basal HR was not significantly changed after vehicle injection into the V4 of Wistar rats (Table 1). On the other hand, central catalase inhibition significantly (p<0.05) increased basal HR in the ATZ-treated group during the first 15 minutes (Table 1).

## Effects of ATZ on Cardiopulmonary Reflex

The PBG dose of 8 µg/kg elicited a biphasic response with initial bradycardia and hypotension in both groups. In the ATZ-treated group the hypotension was significantly increased (p = 0.012) after catalase inhibition into the V4 (Fig. 2B). However, no significant alteration was observed regarding bradycardic reflex elicited by i.v. PBG (p = 0.2522). Bradycardic responses to i.v. PBG and the hypotension elicited by i.v. PBG were not significantly influenced by vehicle injection into the V4 (Fig. 2A).

## DISCUSSION

In this study we reported that injections of ATZ, a catalase inhibitor, into the V4 significantly increased basal MAP and HR. Moreover, it also increased hypotensive responses to cardiopulmonary reflex activation with i.v. PBG but not the bradycardic reflex. On the other hand, in rats treated with vehicle (saline 0.9%) we observed no significant changes regarding baseline MAP and HR and sympathetic and

parasympathetic components of the Bezold–Jarisch reflex (cardiopulmonary reflex). The lack of any change in the vehicles groups is consistent with this assumption. Owing to the anatomical extent of the V4, an effect on only one neuronal group is not an easy accomplishment; nevertheless, studiesprevious studies have suggested a preference for parasympathetic whichsystems modulateswhich modulate HR, such as the nucleus ambiguous and dorsal motor nucleus of the vagus, which receive glutamathergic projections from the nucleus tractus solitarius.[8]

We noted that baseline MAP and HR were enhanced after catalase inhibition into the V4. Cardoso et al.[16] showed that exogenous $H_2O_2$ caused pressor dose-dependent responses in conscious Wistar rats, and peripheral blockade of adrenoceptors$\alpha_1$-adrenoceptors with prazosin abolished those responses elicited by exogenous $H_2O_2$, suggesting that increased ROS in the brainstem stimulate sympathetic discharges causing vasoconstriction and an increase in arterial pressure. Considering that ATZ is a catalase inhibitor and consequently increases endogenous H2O2,17 we expected that ATZ injected into the V4 would cause the same responses observed in the research of Cardoso et al;[16] however, we did not report bradycardic responses. On the other hand, basal MAP was not similar between vehicle and ATZ-treated groups before treatment (101 mmHg vs 113 mmHg, respectively). Thus, we should be careful of interpreting this change observed in MAP after central ATZ treatment.

Based on our findings, increased basal HR during the first 15 minutes after ATZ injection into the V4 indicates sympathetic overactivity. Furthermore, antioxidant treatment[18] in RVLM blunted the elevated $O_2^-$ and $H_2O_2$ in RVLM, leading to reduction in sympathetic vasomotor outflow and vasodepression in hypertensive animals. Thus, our findings are in agreement with the literature.

The hypotensive response to i.v. PBG represents sympathetic inhibition while bradycardic reflex response to the Bezold–Jarisch reflex indicates parasympathetic activation.[19] Many previous studies have already shown the effects of oxidative stress on the cardiovascular reflex. Zanzinger and Czachurski[20] demonstrated that SOD injected into the RVLM decreased sympathetic nerve activity in swine. Several groups have now shown that ROS stimulate central sympathetic outflow.[21,22] Campese et al[22] provided evidence that the lack of low-density lipoprotein (LDL) receptor-enhanced cholesterol blood levels increased oxidative stress and impaired baroreflex sensitivity. Monahan et al[23] supported the hypothesis that oxidative stress contributes to age-associated decreases in cardiovagal baroreflex sensitivity in healthy men. On the

**Table 1** - Baseline level of mean arterial pressure (MAP) and heart rate (HR) in Wistar rats treated with vehicle (vehicle group: N = 7) and Wistar rats treated with ATZ (ATZ group: N = 8).

| Vehicle group | Variable | Control | 5 minutes | 15 minutes | 30 minutes | 60 minutes |
|---|---|---|---|---|---|---|
| | MAP (mmHg) | 101 + 3 | 101.4 + 2 | 100 + 1 | 103 + 5 | 106.2 + 4 |
| | HR (bpm) | 354 + 6 | 343.7 + 5 | 354.7 + 4.9 | 365.9 + 5 | 370 + 2 |
| **ATZ group** | **Variable** | **Control** | **5 minutes** | **15 minutes** | **30 minutes** | **60 minutes** |
| | MAP (mmHg) | 113.1 + 1.6 | 123.2 + 1.1* | 121.9 + 1.07* | 124.2 + 2.8* | 118.75 + 0.2* |
| | HR (bpm) | 354.4 + 9 | 433.75 + 12.6* | 437.25 + 14.7* | 386.4 + 17.8 | 347.74 + 11.5 |

Means + SEM.
*p<0.05: different from control. Means + SEM.

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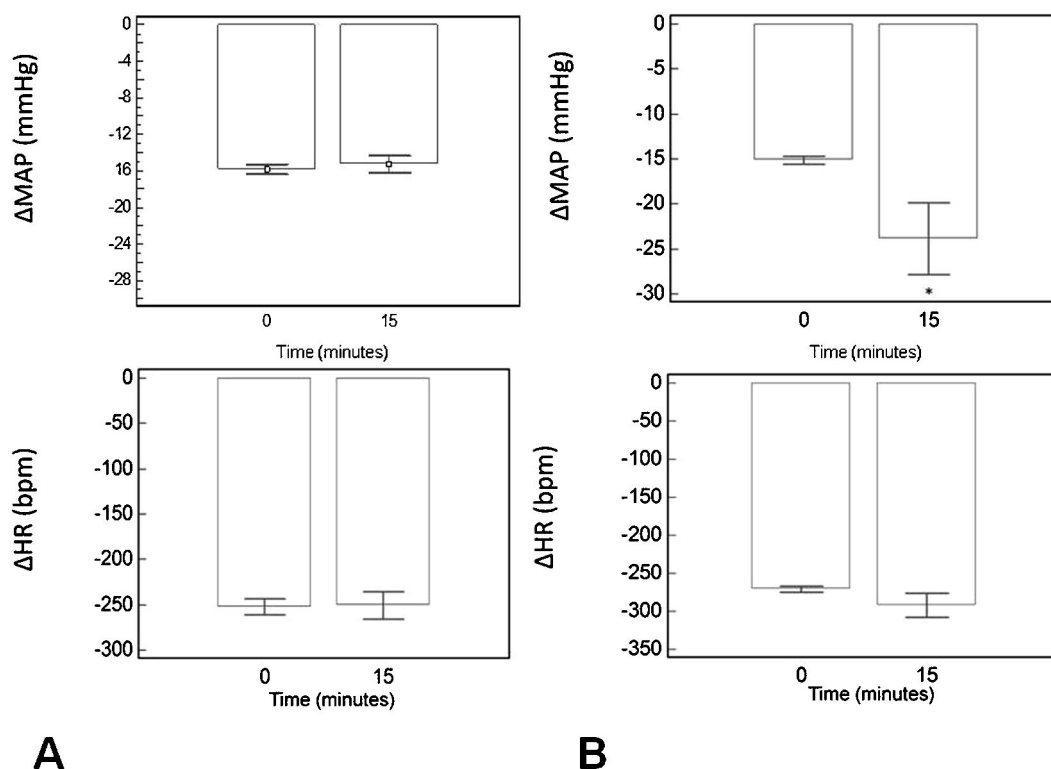Valenti VE et al.

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**Figure 2** - Mean arterial pressure (MAP, mmHg) and heart rate (HR, bpm) responses elicited by activation of the cardiopulmonary reflex by i.v. PBG injections in the vehicle-treated (A, N = 9) and groupATZ-treated groups (B, N = 8) before (0 minutes) and 15 minutes after ATZ administration into the V4. *p<0.05: different from control (0 minutes).

other hand, Wright et al[24] indicated that in male smokers, circulating antioxidants had no effect on baroceptor reflex function, and minor effects on the cardiovascular system were seen following acute fat and vitamin ingestion. To the best of our knowledge, no previous has study investigated the effects of ATZ injected into the V4 on cardiopulmonary reflex. Our data support the hypothesis that endogenous $H_2O_2$ in the regions surrounding the V4 is acutely associated with influencing the sympathetic inhibition component of the cardiopulmonary reflex in conscious rats.

As a main result, our data showed that catalase inhibition into the V4 increased hypotension responses to cardiopulmonary reflex activation without change bradycardic reflex. A recent study[9] indicated that reduced oxidative stress due to exercise training is associated with attenuated cardiac sympathetic modulation and increased cardiopulmonary reflex in spontaneously hypertensive rats. Taken together, it is suggested that central ROS increases the hypotensive response. We believe that increased $H_2O_2$ caused by ATZ treatment into the V4 influences cardiopulmonary reflex due to a possible effect on the RVLM, since it controls the sympathetic discharges activating pre-ganglionic sympathetic neurons in the spinal cord.[8] We suggest that ATZ increases endogenous $H_2O_2$ and decreases synaptic release of glutamate in RVLM neurons[25] and as a consequence it increases baseline HR and the sympathetic inhibition component of the cardiopulmonary reflex, as observed in our study.

The dose of ATZ injected into the V4 used in our work was able to influence sympathetic inhibition during cardiopulmonary reflex activation. Previous investigations have used ATZ as a catalase inhibitor.[26] Moreover, it was demonstrated that catalase activity can be significantly inhibited by the irreversible antagonist ATZ.[26] Therefore, based on previous studies, we consider this drug as an effective catalase inhibitor.

We tested the cardiopulmonary reflex in conscious rats, since cardiorespiratory reflex activity is attenuated under anesthesia[27] reducing the range of HR, which results in an analysis of a restricted portion of the cardiovascular response. Hence, we believe that our research provides reliable information regarding the effects of the catalase inhibitor ATZ, which increases endogenous $H_2O_2$, injected into the V4, on the sympathetic inhibition component of the Bezold–Jarisch reflex in Wistar rats. These data present relevant information; currently the cardiovascular reflex is largely studied in different models and strains of rats with the aim of preventing hypertension development in humans, because impaired cardiovascular reflex function is indicative of cardiovascular disease.[28-]disease.[28–31]

One point from our investigation that should be addressed: we did not measure the concentration of $H_2O_2$ or other ROS inside the V4 before and after the injection of ATZ. It would significantly strengthen the impact of our results to show that $H_2O_2$ or some other ROS is actually altered in the V4 with this duration and level of treatment. Unfortunately, we did not measure endogenous $H_2O_2$ because of the lack of such equipment in our laboratory.

In conclusion, ATZ administration into the V4 increases the sympathetic inhibition response to i.v. PBG (hypotension) without significantly changing the parasympathetic component of the Bezol-Jarisch reflex (bradycardic response). We confirm the significance of ATZ at this particular dose on cardiopulmonary reflex.

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