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PROSTANOIDS CONTRIBUTE TO ACETAMINOPHEN INDUCED VASCULAR DYSFUNCTION IN RATS

Hellen Karine Paes Porto [1], Mikaelle Costa Correia [1], Marcella Daruge Grando [2] and Matheus Lavorenti Rocha [*1]

Laboratory of Cardiovascular Pharmacology [1], Faculty of Pharmacy, Federal University of Goias, Goiânia/GO - Brazil.
Laboratory of Cardiovascular Pharmacology [2], Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences, FCFRP/USP, Ribeirão Preto, SP, Brazil.

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Correspondence to Author:
Matheus Lavorenti Rocha

UFG,
Laboratory of Cardiovascular Pharmacology, Faculty of Pharmacy, Federal University of Goias, Goiânia/GO - Brazil.

E-mail: rochaml@ufg.br.

ABSTRACT: Acetaminophen (APAP) is the most consumed potentially toxic drug in the world, and its cardiovascular injury has been emphasized. We evaluated the role of inflammatory mediators in the impaired vascular relaxation after APAP treatment. Rats were treated with APAP for 2 weeks (400 mg/Kg/day/p.o.) and after euthanasia, blood was collected for biochemical analysis (hepatic transaminases, lipid peroxidation and glutathione), and aortas were isolated for vascular reactivity, lipid peroxidation and biochemical analysis (glutathione, arachidonic acid, $TBXA_2$, PGD_2, 12-HETE and 15-HETE). Both blood and arteries presented increased levels of lipid peroxidation and decreased levels of glutathione. The vasodilation response to acetylcholine was impaired in the APAP group and restored after treatment with diclofenac (10 μM). In the arteries, levels of arachidonic acid were reduced while PGD_2 was increased ($TBXA_2$, 12- and 15-HETE remained significantly unchanged). Thus, oxidative stress and contractile prostanoids play a significant role in the impaired vascular relaxation caused by APAP treatment.

INTRODUCTION: Like a widespread drug throughout the world, acetaminophen (N-acetyl-p-aminophenol, paracetamol; APAP) also named paracetamol, has been considered effective and relatively safe, but due to its low price and easy availability, there has been a significant increase in poisonings due to misuse and overdose [1, 2]. Lately, studies have emphasized the toxic side effects and serious complications of APAP on the cardiovascular system [3-6].

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The precise mechanism of APAP-induced cardiovascular toxicity is still not well understood. Although the literature has shown a higher incidence of cardiovascular problems in APAP users, this relationship remains imprecise and controversial [3, 7, 8]. For example, the continued use of APAP can lead to impaired vasodilation and hypertension [3, 5, 6, 9]. On the other hand, studies have reported superior vasodilation and hypotension after APAP treatment [4, 10, 11].

The cardiovascular system is very susceptible to oxidative stress, leading to deficiency in vascular relaxation, increased levels of prostanoids, atherosclerosis and cardiovascular remodeling [12-14]. In this way, APAP treatment has been linked to vascular oxidative injuries and changes in vessel morphology [5, 6].

In addition to oxidative stress, the inflammatory process is a great determinant of endothelial dysfunction. Inflammation is a defense mechanism enabling the cell or tissue to respond to pathogenic challenges, metabolic changes and injuries induced by xenobiotics and drugs [13]. Although the cardiovascular effects of APAP are unclear, here we provide evidence that the inflammatory process plays an important role in the vascular alterations induced by this drug, which may be an appropriate parameter for future basic and clinical investigations.

MATERIALS AND METHODS:

Animals and Apap Treatment: Adult male Wistar rats (6 to 7 weeks old, 200–220g) provided by Central Bioterium of the Federal University of Goiás, were housed in a light- and temperature-controlled room (12h light/dark cycle; 22±2°C) in our laboratory with free access to rodent chow and filtered water. During acclimatization (one week), water intake was measured daily to know the water consumption and to evaluate the dose for APAP. Following one week, the rats treated with APAP (Anqiu Lu'an Farmacêutica Co Ltda, Shandong, China; purity ≥ 99.15% by TCL) received the drug dissolved in drinking water "*ad libitum*" for 14 days (400 mg/Kg/day) [5, 6]. The control group received only water.

The volume of drinking solution was verified and changed daily to check that APAP was being intake regularly, according to protocols already established in our laboratory [5, 6]. This daily dose of APAP for rats was calculated following the formula [equivalent human dose (mg/Kg) = animal dose (mg/kg) x animal *K*m / human *K*m] postulated by Reagan-Shaw *et al.*[15], where the *K*m is the body weight (Kg) / surface area (m^2) [5, 6]. Following treatments, the rats were anesthetized and euthanized by cardiac punction and exsanguination. The blood samples were collected, centrifuged and the plasma and aorta samples were kept in a freezer (-80 ºC) for biochemical studies. All trials were accepted by the Animal Research Ethics Committee at the Federal University of Goiás, Goiânia, Brazil, in agreement with the internationally accepted principles for experimental animal use and care (Guide for the Care and Use of Laboratory Animals, under protocol number 116/20).

Biochemical Analysis: Serum contents of glutamic-oxalacetic transaminase (GOT), glutamate pyruvate transaminase (GPT) and glutathione (GSH, antioxidant enzyme aiding in redox homeostasis) were measured in plasma. Lipid peroxidation (TBARS reaction; malondialdehyde [MDA] level) and GSH were measured in arteries (n=6 for all groups) homogenized in lysis buffered solution by a kinetic colorimetric assay kit, according to manufacturer's specifications (Cayman Chemical, Ann Arbor, MI, US). Absorbances were measured in a semiautomatic spectrophotometer Multiskan Spectrum v1.2 (Thermo Fisher Scientific Oy, Vantaa, Finland).

Additionally, arachidonic acid (AA), tromboxane B_2 (TXB_2, stable metabolite of the tromboxane A_2), prostaglandin D_2 (PGD_2), 12- and 15-Hydroxyeicosatetraenoic acid (12-HETE and 15-HETE) were analyzed in arteries by reverse-phase HPLC coupled to electrospray ionization mass spectrometry (HPLC-MS/MS). The aorta were removed, cleaned and placed in tubes containing 0.5 mL of the same Krebs solution and conditions used for the vascular reactivity trials. The arteries were stimulated with Phe (0.1 μmol/L, 15 min) and the supernatant was used to quantify the levels of AA and TXB_2, PGD_2, 12-HETE and 15-HETE. An aliquot of each extracted sample (20 μL) was injected into an HPLC column (Accucore C18–50×3 mm, 2.6 μm; Thermo Scientific, Waltham, MA-USA) for HPLC analysis [16]. The HPLC system was directly interfaced with the electrospray source of a triple quadrupole mass spectrometer (API 4000; SCIEX, Framingham, MA), wherein MS analysis was carried out in the negative ion mode. Multiple reaction monitoring (MRM) of specific m/z transitions was used, and quantitation was accomplished by using standard isotope dilution. The results were calculated in pg/μL.

Vascular Reactivity: Thoracic aortic rings (± 3-4 mm) were prepared for isometric tension recordings in an isolated organ bath (basal tension = 1.5 g) containing modified Krebs solution (NaCl 130 mM, KCl 4.7 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 14.9 mM, glucose 5.5 mM, $CaCl_2$ 1.6 mM; pH 7.4, at 36 ± 1°C and aerated with carbogenic gas) and connected to a computerized system of data acquisition unit (DATAQ Instruments, Akron, OH, USA). Each rat

provided only one aortic ring for different trials. The aortic rings with functional endothelium (relaxation to acetylcholine ≥ 80%) were constricted with phenylephrine (0.1 µmol/L), and cumulative concentration-response curves for acetylcholine (ACh; 0.1 nmol/L to 10 µmol/L) were performed in the absence or presence (40 min) of the non-selective COX inhibitor, sodium diclofenac (10 µmol/L).

Statistical Analysis: The data are presented as the mean ± SEM. The distribution of data was analyzed by the Kolmogorov–Smirnov test. The statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software Corporation). Comparisons between groups were made using one-way ANOVA (post-test: Newman-Keuls) or the t-Student test, where p values <0.05 were considered significant.

RESULTS: Blood levels of GOT and GPT were determined as a degree of liver damage, which is the first sign after exposure to APAP (indirect APAP intake index). Both enzymes increased significantly ($p<0.001$) in the APAP group (269.7 ± 21.8%, n=6 and 308.2 ± 32.3%, n=6, respectively) compared to untreated controls (data not shown).

Similarly, an increase in MDA levels (the end product of lipid peroxidation) in the blood and arterial tissues were elevated by treatment with APAP (287.3 ± 19.5%, n=6 and 305.8 ± 27.3%, n=6, respectively), indicating an increase in oxidative stress **Fig. 1**. On the other hand, the GSH levels were reduced in both serum and artery tissues (48.8 ± 4.7%, n=6 and 53.6 ± 4.3% n=6, respectively) **Fig. 1.**

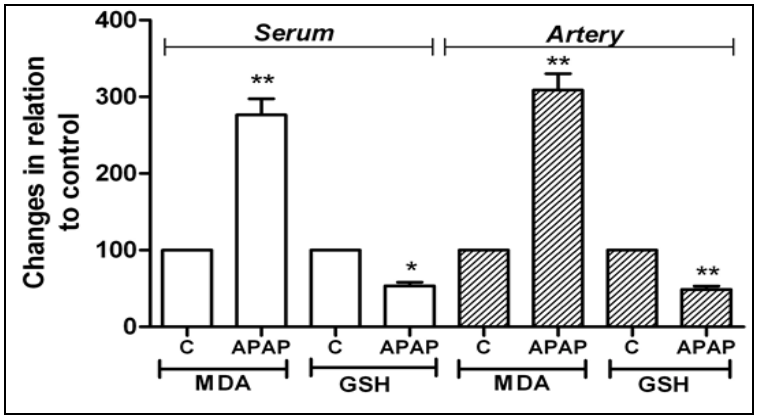

FIG. 1: EFFECT OF APAP TREATMENT (N=6 FOR EACH GROUP) ON SERUM AND VASCULAR (DASHED AREA) LEVELS OF MDA (TBARS REACTION, LIPID PEROXIDATION INDEX) AND GLUTATHIONE (GSH). DATA REPRESENT RELATIVE CHANGE (% IN RELATION TO THE CONTROL GROUP REPRESENTED BY LETTER C). SIGNIFICANT DIFFERENCE * P<0.05 AND **P<0.01 *VS*. CONTROL

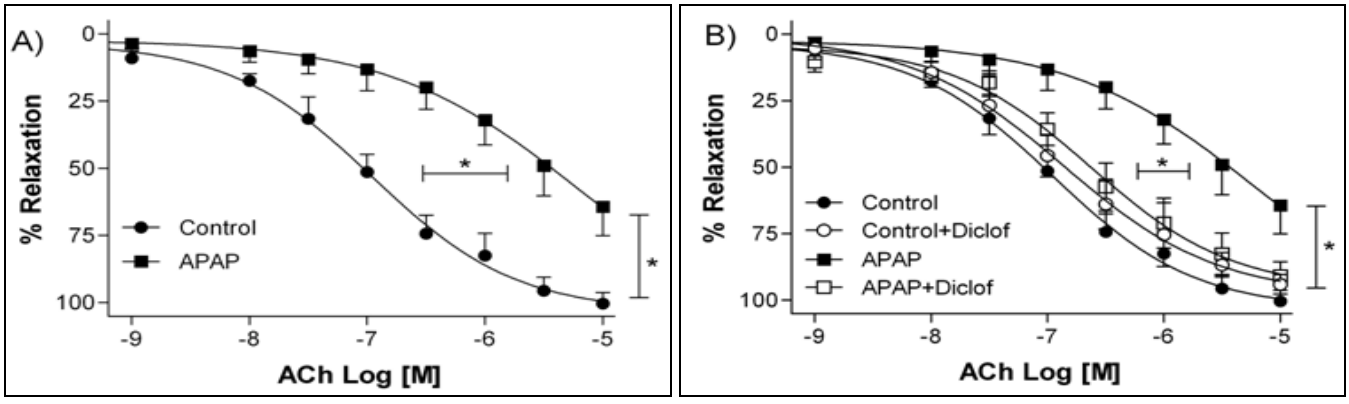

FIG. 2: ENDOTHELIUM-DEPENDENT VASCULAR RELAXATION IN RESPONSE TO ACH IN AORTA OF UNTREATED AND APAP-TREATED RATS BEFORE (A) AND AFTER (B) TREATMENT WITH SODIUM DICLOFENAC (10 MM, 40 MIN). DATA REPRESENTED AS MEAN ± SEM (N=6 FOR EACH GROUP) OF RELAXATION IN RELATION TO PHENYLEPHRINE-INDUCED PRECONTRACTION. *SIGNIFICANT DIFFERENCE IN PD_2 (HORIZONTAL) AND EMAX (VERTICAL) VS. CONTROL (P<0.001).

The endothelium-dependent relaxation induced by ACh was significantly reduced ($p<0.001$) in APAP group by an E_{max}: 66.8 ± 6.1%, n=6, vs. control group's E_{max}: 102.7 ± 5.2%, n=6. Furthermore, the potency of ACh in inducing relaxation was greater in the arteries from control rats (pD_2: 7.18 ± 0.41) than in APAP treated rats (pD_2: 6.01 ± 0.30; $p<0.001$) **Fig. 2A**. Incubation of rat aorta with diclofenac significantly ($p<0.001$) improved the maximum vascular relaxation and potency of ACh in arteries from APAP-treated rats **Fig. 2B.**

The AA concentration was significant reduced and its subproduct PGD_2 was increased, respectively, after APAP treatment **Fig. 3.** Although not significant, it was observed a tendency to increase TXB_2 production in APAP-treated group.

The lipoxygenase-derived metabolites 12-HETE and 15-HETE were unchanged after APAP treatment (96.15 ± 6.8 %, n=5 and 104.8 ± 7.3 %, n=5, in relation to the control group, respectively).

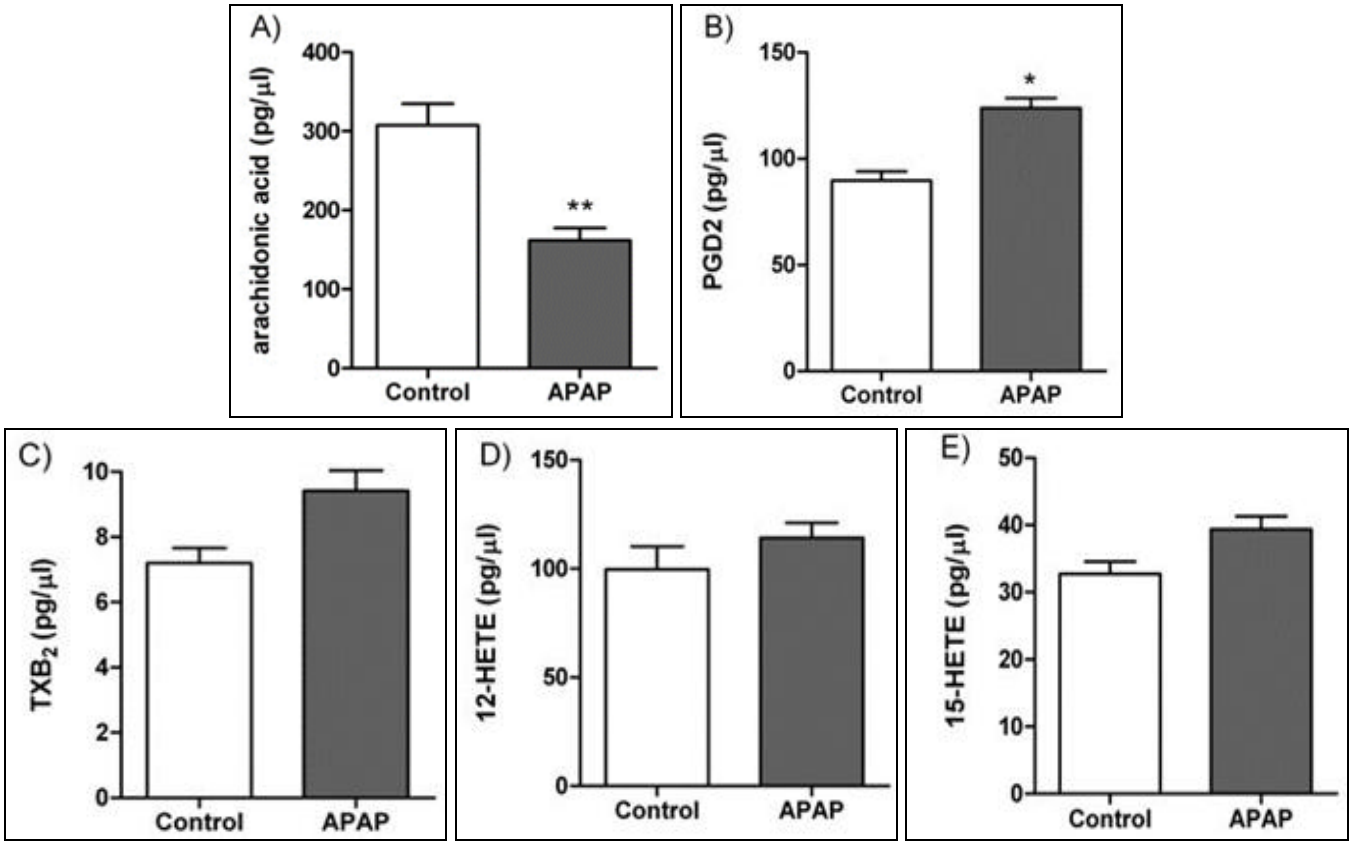

FIG. 3: EFFECT OF APAP TREATMENT (N=6 FOR EACH GROUP) ON VASCULAR LEVELS OF ARACHIDONIC ACID (AA), PROSTAGLANDIN D_2 (PGD_2), TROMBOXANE B_2 (TBX_2); 12- AND 15-HYDROXYEICOSATETRAENOIC ACID (12- AND 15-HETE). SIGNIFICANT DIFFERENCE * P<0.05 AND **P<0.01 *VS*. CONTROL

DISCUSSION: The cardiovascular toxicity induced by APAP is currently standing out in the literature. As it is one of the most consumed over-the-counter medications in the world and can lead to serious side effects, it is essential to understand its consequences on the cardiovascular system.

While the specialized literature has shown that APAP users are more likely to develop cardiovascular complications, interventional and observational studies verifying the effect of APAP on blood pressure have shown conflicting results [3, 7]. To date, most but not all observational studies indicate that medium- to long-term APAP use increases the risk of developing hypertension [9, 17]. This apparent increase in arterial pressure could have severe population-based consequences.

Over-production of reactive oxygen species (ROS) in the cardiovascular system triggers deficiency in vasodilation induced by nitric oxide (NO), since ROS reacts NO neutralizing its effects [5, 18]. Our results show that APAP treatment increases lipid peroxidation in the vascular system, leading to

impaired endothelium-dependent vasodilation by overproducing prostanoids (mostly PGD_2) as observed with other drugs and conditions that induce increased oxidative stress [14, 16, 18, 19].

The main finding of this study is that the impairment of vascular relaxation caused by APAP treatment is totally reversed by COX inhibition. These results link impaired vascular relaxation to increased prostanoids production following APAP exposure as a result of redox balance dysfunction.

The increased production of contractile prostanoids has been found in the arteries from rats exposed to high oxidative stress conditions [16, 18, 19]. The production of prostanoids initiates with the recruitment of AA from the membrane by phospholipase A_2. Both COX isoforms can metabolize AA generating different prostanoids, including the PGD_2 [19, 20]. Our analysis have shown increased production of PGD_2 and reduced AA levels in arteries from rats exposed to APAP.

Lipoxygenases and their products contribute to the maintenance of vascular tone and other pathological processes such as atherosclerosis and vascular inflammation [14, 21]. In our experiments, the major vascular lipoxygenase-derived metabolites (12-HETE and 15-HETE) remained unchanged after APAP treatment, reinforcing that the reduction in AA levels could be related to increased production of contractile prostanoids in the treated group.

Accumulating evidences have shown that PGD_2 plays important roles in regulating cardiovascular function, in addition to being a potent contractile agent in arteries [19, 22-24]. Moreover, increased expression of PGD synthase and PGD_2 overproduction have been reported in conditions of high oxidative stress [24, 25]. The increase in PGD_2 synthesis may be one of those responsible for the impaired vascular relaxation after APAP exposure.

CONCLUSION: These observations suggest that treatment with APAP impairs endothelium-dependent vasorelaxation, which can be totally reversed by COX inhibition. Vascular lipid peroxidation is increased after APAP treatment, which also generates an overproduction of PGD_2 accompanied by a reduction in AA. Since APAP treatment may be associated with an increased risk of cardiovascular problems, further research to explore the clinical importance of these findings is clearly important.

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Declarations Sections

Authors' Contributions: HKPP, MCC, MDG Planned, conducted the experiments, analyzed the results, and wrote the manuscript. MLR analyzed the results and wrote the manuscript. The manuscript was read and approved by all authors.

Ethical Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (protocol number 116/2021).

CONFLICT OF INTEREST: The authors declare no conflict of interest, financial or otherwise.

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