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EFEITOS DA COMBINAÇÃO DE PARACETAMOL E CAFEÍNA SOBRE A BIOENERGÉTICA MITOCONDRIAL EM FÍGADO DE CAMUNDONGOS

Santa Maria, RS, Brasil

2017

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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Profa. Dra. Cristiane Lenz Dalla Corte

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“Tudo tem o seu tempo determinado, e há tempo para todo o propósito debaixo do céu. Há tempo de nascer, e tempo de morrer; tempo de plantar, e tempo de colher...”

(Eclesiastes 3:1,2)

“*Daria tudo o que sei pela metade do que ignoro*”

(René Descartes)

APRESENTAÇÃO

No item INTRODUÇÃO consta uma revisão sucinta da literatura sobre os temas trabalhados nesta dissertação.

A metodologia realizada e os resultados obtidos que fazem parte desta dissertação estão apresentados no item MANUSCRITO sob a forma de um manuscrito redigido em inglês conforme as normas do periódico ao qual foi submetido. No mesmo constam as seções: Introdução, Materiais e Métodos, Resultados, Discussão, Conflito de interesse, Apoio financeiro, Referências e Figuras e legendas.

Os itens CONCLUSÕES e PERSPECTIVAS, encontrados no final desta dissertação, apresentam conclusões gerais sobre os resultados do manuscrito presente neste trabalho e as perspectivas para futuros trabalhos.

As REFERÊNCIAS BIBLIOGRÁFICAS referem-se somente às citações que aparecem no item INTRODUÇÃO desta dissertação.

RESUMO

EFEITOS DA COMBINAÇÃO DE PARACETAMOL E CAFEÍNA SOBRE A BIOENERGÉTICA MITOCONDRIAL EM FÍGADO DE CAMUNDONGOS

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O paracetamol (acetaminofeno, ou APAP do inglês N-acetyl-p-aminofenol) é um fármaco, com propriedades analgésicas e antipiréticas, amplamente utilizado devido ao seu baixo custo, facilidade de aquisição e eficácia clínica. O consumo acidental ou intencional de altas doses de APAP pode causar insuficiência hepática. A cafeína (1,3,7 trimetilxantina) é um alcaloide do grupo das xantinas, utilizada como estimulante e encontrada em diversas bebidas e em preparações farmacológicas. Alguns medicamentos incluem a combinação de paracetamol com cafeína. O presente trabalho avaliou os efeitos decorrentes da combinação paracetamol e cafeína sobre parâmetros de bioenergética mitocondrial e estresse oxidativo em fígado de camundongos. Para a realização deste estudo foram utilizados camundongos swiss albino machos. Os animais receberam uma dose de 20 mg/kg de cafeína via i.p. e 30 minutos após receberam uma dose de 250 mg/kg de APAP via i.p. Os animais foram sacrificados 4 horas depois da administração do APAP. Foram avaliados parâmetros de funcionalidade mitocondrial e estresse oxidativo. O tratamento com APAP diminuiu pela metade o consumo de oxigênio celular além de prejudicar a atividade dos complexos mitocondriais, além disso o tratamento com APAP aumentou três vezes o inchaço em mitocôndrias isoladas de fígado de camundongos e também aumentou a peroxidação lipídica e a produção de espécies reativas em fígado de camundongos. Por outro lado cafeína associada ao APAP foi capaz de melhorar a função bioenergética mitochondrial, além de prevenir a peroxidação lipídica e diminuir produção de espécies reativas de oxigênio. Com os resultados desse trabalho foi possível relacionar os efeitos da combinação de cafeína e paracetamol, na bioenergética mitocondrial e em parâmetros de estresse oxidativo.

Palavras-chave: inchaço mitocondrial, estresse oxidativo, hepatotoxicidade, disfunção mitocondrial

ABSTRACT

EFFECTS OF ACETAMINOPHEN AND CAFFEINE COMBINATION ON MITOCHONDRIAL BIOENERGETICS IN MICE LIVER

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ADVISOR: Cristiane Lenz Dalla Corte

Paracetamol (acetaminophen, N-acetyl-p-aminofenol, APAP) is a drug with analgesic and antipiretic effects, widely used, presenting low cost, easier aquisition and clinical efficacy. Accidental or intentional use of high doses APAP may cause hepatic impairment. Caffeine (1,3,7-trimethylxanthine) is an alkaloid of xanthines group, used as stimulant and found in many beverages and pharmacological preparations. Some drugs include the combination of acetaminophen with caffeine. The present study evaluated the effects of paracetamol and caffeine combination on mitochondrial bioenergetics parameters and oxidative stress in mice liver. For this study it was used male swiss albine mice. The animals were treated with a caffeine dose of 20 mg/kg i.p and 30 minutes after were treated with APAP dose of 250 mg/kg APAP i.p. The animals were sacrificed 4 hours after APAP administration. We evaluated mitochondrial and stress oxidative parameters. APAP treatment halved cellular oxygen consumption, moreover impairment mitochondrial complex activities, furthermore, the treatment with APAP increased by three times the swelling in isolated mitochondria from mice liver. Treatment with APAP also increased lipid peroxidation and reactive species production, in livers of mice. On the other hand, caffeine associated with APAP was able to improve bioenergetics mitochondrial function, moreover prevent lipid peroxidation and decrease oxygen reactive species production in mice liver. The results of this work enable the link between effects of caffeine and APAP combination on mitochondrial bioenergetics and oxidative stress parameters.

Key-words: mitochondrial swelling, oxidative stress, hepatotoxicity, mitochondrial disfunction.

LISTA DE ILUSTRAÇÕES

Manuscrito

LISTA DE ABREVIATURAS

Introdução

APAP – Paracetamol

EROS – Espécies reativas de oxigênio

GSH- Glutationa reduzida

GSSG- Glutationa oxidada

IHA - Insuficiência hepática aguda

LPO – Lipoperoxidação lipídica

NAPQI - N-acetil-p-benzoquinona imina

OH- Radical Hidroxila

Manuscrito

ADP- Adenosine triphosphate

ALF- Acute liver failure

APAP- Acetaminophen

AST- Aspartate aminotransferase

ALT- Alanine aminotransferase

BHT- 3,5-ditert-4-butylhydroxytoluene

CI- Complex I

CII- Complex II

CYP450- Citochrome P-450

DCF- 2'7' dichlorodihydrofluorescein

GSH- Reduced glutathione

H_2DCF-DA- 2’7’ dichlorodihydrofluorescein diacetate

HRR- High resolution respirometry

NSAID- Non-steroidal anti-inflammatory drug

NAPQI- N- acetyl-p-benzoquinone imine

OH- Hydroxyl radical

OXPHOS- Oxidative phosphorylation

LPO- Lipid peroxidation

RCR- Ratio control respiratory

SUMÁRIO

1. INTRODUÇÃO

O paracetamol (acetaminofeno, N-acetil-p-aminofenol, APAP) é um fármaco utilizado como analgésico e antipirético não apresentando atividade anti-inflamatória clinicamente significativa (KHANDELWAL et al., 2011). A correta administração de doses terapêuticas de paracetamol é segura, todavia a overdose pode levar a falha hepática. A fácil aquisição e baixo custo do APAP aumentam os casos de intoxicação hepática aguda (IHA) devido ao consumo de altas doses desse medicamento, intencional ou acidentalmente.

Overdoses causadas por APAP correspondem à metade dos casos de intoxicação nos Estados Unidos e Grã-Bretanha, resultando em um alto custo no tratamento de pacientes intoxicados (LARSON; POLSON; FONTANA, 2005). Segundo dados do centro de informação toxicológica do Rio Grande do Sul (CIT-RS) entre os anos de 2005 e 2010 só no Rio Grande do Sul foram identificados 2572 casos de exposição tóxica ao APAP representando 63% dos casos de intoxicação por analgésicos.

Na exposição a doses controladas de APAP sua metabolização se dá no fígado via reações chamadas reações de fase II, ou também chamadas de reações de conjugação. Essas reações são consideradas rápidas e envolvem a conjugação de grupos substituintes endógenos que tornam, em geral, a molécula inicial mais polar e hidrofílica facilitando sua excreção. A molécula de APAP sofre reações de conjugação com grupos sulfato (sulfatação) e glicuronídeo (glicuronidação), formando metabólitos não tóxicos que são facilmente excretados pelo organismo. Porém, durante a overdose por APAP, as vias metabólicas do sulfato e glicuronídeo ficam saturadas e a metabolização do APAP é desviada para uma outra rota a qual envolve reações oxidativas de fase I, utilizando a via relacionada ao citocromo P-450, resultando na formação de um intermediário altamente reativo, o N-acetil-p-benzoquinona imina (NAPQI) (WALUBO et al., 2004).

A metabolização do NAPQI ocorre rapidamente em pH fisiológico envolvendo uma outra reação de fase II caracterizada pela conjugação com o grupo cisteína da glutationa reduzida (GSH), formando produtos não tóxicos (KOLING et al., 2007). A glutationa reduzida se conjuga com o APAP formando quantidades estequiométricas de seu produto oxidado GSSG (ALBANO et al., 1985).

Um excesso de NAPQI causa uma depleção dos níveis de GSH e um aumento dos níveis de GSSG no fígado. O não reestabelecimento de GSH faz com que o NAPQI se ligue covalentemente a proteínas celulares, modificando sua estrutura e função (MOYER et al., 2011), levando a uma perturbação da homeostase e consequente falha hepática.

Os efeitos decorrentes da metabolização de um excesso de paracetamol incluem a ligação de NAPQI a proteínas prejudicando seu correto dobramento e posterior funcionalidade o que ordena uma cascata de eventos que levam a formação de espécies reativas de oxigênio (EROs), as quais agravam a lesão hepática causando um aumento do estresse oxidativo e também lipoperoxidação (LPO) (JAESCHKE, 1990).

Uma importante via de formação ERO's no organismo de mamíferos inclui as mitocôndrias (FIGUEIRA et al., 2013). As mitocôndrias são as organelas responsáveis pela produção de moléculas de ATP as quais oferecem energia para as biossínteses. Essas organelas são formadas por uma membrana interna e a outra externa. Na membrana interna são encontrados diferentes complexos proteicos responsáveis pela oxidação de equivalentes redutores produzidos no ciclo de Krebs. Esses complexos proteicos compreendem: complexo I (NADH-desidrogenase), complexo II (succinato desidrogenase), complexo III (citocromo c-redutase), complexo IV (citocromo c-oxidase) e complexo V (ATP-sintase) (LAMPL et al., 2015). Os elétrons resultantes dessas oxidações são utilizados para formar um gradiente de prótons, que serão aproveitados como força motriz para a produção de ATP.

As mitocôndrias estão envolvidas no suporte bioenergético celular, e também desempenham papel central no metabolismo de fármacos como por exemplo o APAP (JAESCHKE; MCGILL; RAMACHANDRAN, 2012a; MCGILL et al., 2012). Alterações nas funções mitocondriais podem ser observadas no desenvolvimento e progressão de diversas doenças (LIAO; DONG; CHENG, 2017; MISHRA; KUMAR, 2014; REQUEJO-AGUILAR; BOLAÑOS, 2016). Já é bem descrito na literatura que doses excessivas de APAP podem causar modificações na morfologia das mitocôndrias e alterações nos complexos mitocondriais I e II, além de alterações no metabolismo do cálcio, interferindo na produção de ATP e comprometendo a funcionalidade mitocondrial (BURCHAM; HARMAN, 1991; HINSON; ROBERTS; JAMES, 2010; JAESCHKE; MCGILL; RAMACHANDRAN, 2012b).

Além disso, as interações entre proteínas celulares e NAPQI também resultam em uma série de eventos que desencadeiam o aumento do estresse oxidativo e levam à disfunção mitocondrial. Como consequência há um aumento da permeabilidade da membrana (CROMPTON, 1999). O aumento da permeabilidade da membrana mitocondrial ocasiona despolarização, desacoplamento do processo de fosforilação oxidativa, aumentando a entrada de íons e intermediários metabólicos resultando em inchaço mitocondrial (HINSON et al., 2004).

Os antioxidantes endógenos, assim como a utilização de antioxidantes exógenos podem proteger contra o dano celular induzido pelo estresse oxidativo mitocondrial (ZHAO; KALHORN; SLATTERY, 2002). Nesse sentido os antioxidantes exógenos, podem representar uma alternativa de proteção contra os danos derivados do estresse oxidativo. A cafeína surge como um potencial antioxidante exógeno (SHI; DALAL; JAIN, 1991). Essa substância é encontrada em muitas bebidas e alimentos comuns no cotidiano como, por exemplo, chá, chimarrão, café, chocolates, entre outros. O consumo de cafeína é comum na dieta da maioria das pessoas, o café, por exemplo, é uma bebida que possui ampla aceitação, sendo consumido diariamente.

A cafeína pertence ao grupo das metilxantinas e muitos estudos têm considerado o potencial antioxidante desta molécula (AOYAMA et al., 2011; ROSSOWSKA; NAKAMOTO, 1994; ZEIDÁN-CHULIÁ et al., 2013). O mecanismo antioxidante proposto envolve a capacidade *scavenger* da cafeína, particularmente associada ao sequestro de radicais hidroxila (OH-) (DEVASAGAYAM et al., 1996). Bioquimicamente a cafeína também se apresenta como antagonista dos receptores de adenosina do subtipo A2A, os quais estão ligados a respostas anti-inflamatórias (OHTA et al., 2007).

Além do consumo através de alimentos e bebidas a cafeína também é utilizada de forma combinada com diversos medicamentos, mais especificamente analgésicos, por sua capacidade de aumentar o efeito de analgesia desses fármacos (PALMER et al., 2010). Analgésicos como aspirina, diclofenaco e APAP são amplamente associadas à cafeína (LASKA et al., 1983) e constantemente comercializados em drogarias para o alívio de sintomas de dor corporal e febre (SAWYNOK; YAKSH, 1993). Estudos mostram que a associação de APAP e cafeína reduz em 40% a dose necessária para produzir o mesmo efeito analgésico, em comparação ao APAP sozinho (LASKA et al., 1983). A administração de APAP juntamente com a cafeína aumenta os níveis plasmáticos de APAP (ZHANG,

2001), sugerindo que essa combinação pode alterar a farmacocinética do medicamento em questão.

Nas formulações farmacêuticas é comum a associação de paracetamol e cafeína nos analgésicos disponíveis para venda em farmácias. Como exemplo podemos citar os fármacos com nome comercial de Tylenol ®, encapsulado em comprimidos contendo 750 mg de paracetamol e 65 mg de cafeína, e Cafelium® com comprimidos contendo 450 mg de paracetamol, 1g de mesilato de diidroergotamina, 75 mg de cafeína e 10 mg de cloridrato de metoclopramida. Tais medicamentos podem ser adquiridos sem receita médica.

Como previamente comentado já é conhecido o efeito antioxidante da cafeína. Todavia, existem poucos estudos mostrando possíveis consequências da cafeína na bioenergética mitocondrial. Além disso, o papel das mitocôndrias já é bastante descrito em protocolos de intoxicação por paracetamol, mostrando haver consequências do uso excessivo desse fármaco na bioenergética mitocondrial e estresse oxidativo. O uso rotineiro de analgésicos utilizando a combinação de APAP e cafeína traz a necessidade de se conhecer a interação desses componentes com foco na bioenergética mitocondrial, visto que, os estudos das consequências da co-administração de cafeína e APAP, são restritos à analgesia e ainda não foram descritas consequências relacionadas à mitocôndria e estresse oxidativo.

2. OBJETIVOS

2.1 Objetivo Geral

Avaliar os efeitos da combinação de paracetamol e cafeína sobre a bioenergética mitocondrial e marcadores de estresse oxidativo em fígado de camundongos.

2.2 Objetivos específico

Avaliar possíveis alterações no funcionamento hepático em camundongos expostos à paracetamol e cafeína, assim como a atividade antioxidante da cafeína, através dos seguintes parâmetros bioquímicos:

- Transição de permeabilidade de membrana mitocondrial (inchaço mitocondrial).
- Respirometria de alta resolução, analisando a razão de controle respiratório (RCR), atividade dos complexos mitocondriais I e II quando acoplados a fosforilação oxidativa (OXPHOS).
- Estresse oxidativo avaliado pelos ensaios de peroxidação lipídica (TBARS) e oxidação de 2'7' diacetato de diclorofluoresceína (DCFH-DA).
- Níveis de glutationa reduzida (GSH).
- Atividade antioxidante total *in vitro* da cafeína e seus metabólitos pelo ensaio de atividade sequestradora de radicais avaliado pela redução do fosfomolibdênio.

3. MANUSCRITO

O manuscrito apresentado a seguir está submetido à revista Life Sciences, sob processo de revisão.

Caffeine and acetaminophen association: effects on mitochondrial bioenergetics

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3.1 Abstract:

Aims: Many studies have been demonstrating the role of mitochondrial function in acetaminophen (APAP) hepatotoxicity. Since APAP is commonly consumed in association with caffeine, this work aimed to evaluate the effect of APAP and caffeine combination on hepatic mitochondrial bioenergetics function in mice.

Main methods: Mice were treated with caffeine (20 mg/Kg, i.p.) or its vehicle and, after 30 minutes, APAP (250 mg/Kg, i.p.) or its vehicle. Four-hours later, livers were removed and the parameters associated to mitochondrial function and oxidative stress were evaluated. Hepatic mitochondrial oxygen consumption was evaluated by high resolution respirometry (HRR).

Key findings: APAP treatment decreased cellular oxygen consumption and mitochondrial complex activities in livers of mice. Besides, treatment with APAP increased swelling in isolated mitochondria from mice liver. On the other hand, caffeine associated with APAP was able to improve hepatic mitochondrial bioenergetics function. Treatment with APAP increased lipid peroxidation and reactive species production, and decreased the glutathione levels in livers of mice. Caffeine associated with APAP was able to prevent lipid peroxidation and reactive species production in mice livers, which may be associated with improvement of the mitochondrial function caused by caffeine treatment. **Significance:** we suggest that the antioxidant effects of caffeine and/ or its interactions with mitochondrial purinergic receptors, may be involved in its beneficial effects against APAP hepatotoxicity.

Keywords: Mitochondria, high resolution respirometry, oxidative stress, bioenergetics.

3.2 Introduction

Acetaminophen (paracetamol, N-acetyl-*p*-aminofenol, APAP) is a classical non-steroidal anti-inflammatory drug (NSAID), widely consumed as an analgesic and antipyretic medication. However, APAP overdose may cause hepatic injury leading to acute liver failure (ALF) [16]. Liver damage caused by high doses of APAP is associated with N-acetyl-*p*-benzoquinone imine (NAPQI) formation, a reactive metabolite which is formed by cytochrome P-450 (CYP450) enzyme family [31].

One important mechanism involved in APAP overdose is the disruption of hepatic mitochondrial metabolism [15,32]. Mitochondria has been demonstrated to be the main target of NAPQI [33] leading to functional alterations in calcium metabolism, complexes I and II inhibition and decreased ATP levels. The uncoupling oxidative phosphorylation process results in the entrance of ions and metabolic intermediates in mitochondria leading to mitochondrial swelling [19]. These events impair mitochondrial function and, ultimately, lead to cell death [16]. In this way, the mitochondrial permeability transition may be considered one of the mechanisms involved in APAP-induced impairment of hepatic mitochondria [32].

On the other side, endogenous antioxidants, such as glutathione, and exogenous antioxidant compounds can protect mitochondria against cellular oxidative damage as in APAP-induced stress process [34]. In this regard, caffeine, a methylxanthine alkaloid compound present in beverages (coffee, tea, energy drinks), food (chocolate, desserts) and medicines, has long been considered as an antioxidant molecule. Accumulating evidence has suggested a potential antioxidant role for caffeine [22–24]. Chemical studies have proposed ROS scavenging properties of caffeine, particularly on hydroxyl radical (OH·) [21,25,35,36]. However, the effects of caffeine on mitochondrial bioenergetics have been poorly investigated until now.

Caffeine is also frequently used as an analgesic drug adjuvant in several over-the-counter medications [30,37]. Drugs such as aspirin, diclofenac and APAP are largely associated

with caffeine [28] and constantly commercialized in drugstores for relief of pathological (or pathology-related) symptoms, such as body temperature dysregulation [38].

Studies about analgesic effects of drugs used for pain relief, like APAP, have shown that the association of APAP with caffeine reduce the drug dosage necessary to produce the same analgesic effect when compared to APAP alone [28,30,39]. However, few studies investigated the effects of caffeine associated with drugs such as APAP on mitochondrial liver function. Usually, studies about caffeine and APAP association focus on the analgesic effects [28,40,41]. In addition, the role of mitochondrial metabolism associated with APAP, mainly in poisoning, is already known but, the effect of caffeine and APAP association on mitochondria remains unclear [16,42]. Therefore, the aim of the present study is to investigate the effect of APAP and caffeine association on hepatic mitochondrial bioenergetics in mice.

3.3 Materials and methods

Animals

Forty male adult swiss albino mice (2 months old, 30-40 g) from our own breeding colony were used. The animals were kept on a separate animal room, on light/dark cycle of 12 h, at temperature of 22 ± 2 ºC, with free access to food and water. This study was approved by the Ethical and Animal Welfare Committee of Federal University of Santa Maria, Brazil, under the process number 3208150915/2015.

Experimental protocol

The animals were randomly divided into four groups, with three or four animals per group, depending on the analysis: (1) control saline, (2) caffeine, (3) APAP and (4) caffeine plus APAP. The doses of caffeine (20 mg/kg) and APAP (250 mg/kg) used in these procedures were described previously [26,43] and were administered by intraperitoneal injection (i.p.). The

APAP dose of 250 mg/kg was chosen because this dose produces mild hepatotoxicity and since the aim of this work was to evaluate the effect of APAP and caffeine combination on mitochondrial bioenergetics, a moderate mitochondrial damage is preferable in order to distinguish the caffeine effect from that APAP alone. To study the effects of APAP and caffeine association on mitochondrial bioenergetics the i.p. route of administration was chosen, since the compounds, both caffeine and APAP, would not suffer any alteration during its passage through the gastrointestinal tract. The animals were fasted for 16 hours [44] before starting the experimental protocol. Figure 1 describes the experimental protocol used in this study. The animals were pre-treated with caffeine or saline 30 minutes before APAP administration. Four hours after APAP administration, animals were killed and liver was immediately removed for biochemical analyses according to previous studies [45,46].

Mitochondria isolation from livers of mice

Mice liver mitochondria were isolated at 4ºC by differential centrifugation [47]. After removal, livers were immersed and homogenized in ice-cold at 1:10 (weight/volume) isolation buffer I containing 320 mM sucrose, 1 mM EDTA, 1mM EGTA and 10 mM TRIZMA-base, pH 7.4. The resulting suspension was centrifuged for 10 minutes at 2500 x g in a Hitachi CR 21E centrifuge. Supernatant was centrifuged at 10,000 x g for 10 minutes. The pellet was re-suspended in isolation buffer I with bovine serum albumin (BSA) containing 320 mM sucrose, 1 mM EDTA, 1mM EGTA, 0.1% free fat acid BSA and 10 mM TRIZMA-base, pH 7.4. The resulting supernatant was decanted, and the final pellet was gently washed and re-suspended in isolation buffer II containing 250 mM sucrose, and 10 mM TRIZMA base, pH 7.4. The pellet was washed three times with isolation buffer II, finally, the pellet was re-suspend in 200 µL, also in isolation buffer II to get intact mitochondria. Isolated mitochondria was used in the mitochondrial swelling assay and mitochondrial GSH content assay.

Reactive Oxygen Species (ROS) production assay

ROS generation was determined spectrofluorimetrically in liver homogenate, using 2'7'dichlorodihydrofluorescein diacetate (H_2DCF-DA) (1 mM). The oxidation of H_2DCF to 2'7'dichlorofluorescein (DCF) is used as an index of the peroxide production by cellular components [48]. Briefly, liver homogenate were added to the standard medium and the fluorescence was determined at 488 nm for excitation and 525 nm for emission, with slit widths of 3 nm.

Measurement of lipid peroxidation (LPO)

Lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) [49]. An aliquot (200 µL) of liver homogenate was mixed with 500 µL thiobarbituric acid (0.6%), 200 µL sodium dodecyl sulphate (SDS) 8.1%, and 500 µL acetic acid (pH 3.4) and incubated at 90 ºC for 1 h. TBARS levels were measured at 532 nm using a standard curve of malondialdehyde (MDA) and the results were reported as nmol MDA/mg protein. LPO and RS production were evaluated in tissue homogenate to obtain an overview of the cellular oxidative stress associated with mitochondrial impairment [35].

High Resolution Respirometry (HRR) assay

For the respirometry determination, mice liver was weighed and homogenized in 2 mL of cold buffer containing 5 mM Tris-HCl, 250 mM sucrose and 2 mM EGTA (pH = 7.4) and transferred into the Oxygraph-2 k (O2k, OROBOROS INSTRUMENTS, Innsbruck) 2 ml-chambers. Oxygen polarography was performed at 37 °C and the oxygen flux was recorded at real-time using DatLab software. In the present protocol, the liver homogenate (0.1 mg/mL) was added to the chamber containing the respiration medium-MIR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60

mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 0.1 mg/ml free fat acid BSA). After signal stabilization, the experimental protocol of high-resolution respirometry was performed by sequential addition of 10 mM glutamate, 2 mM malate, 2.5 mM ADP, 10 mM succinate, 0.5 µM rotenone and 2.5 µM antimycin A[50–52].We used tissue homogenates for analysis of mitochondrial respiration, instead of isolated mitochondria, because the mitochondrial isolation process may remove more than 60% of mitochondrial population compared to tissue homogenate, impairing the mitochondria structure and functionality, besides disrupting mitochondrial network [53].

Mitochondrial swelling assay

Measurement of mitochondrial swelling was performed in RF- 5301 Shimadzu spectrofluorometer at 600 nm and slit 1.5 nm for excitation and emission. Mitochondria (0.1 mg protein) were incubated in buffer II containing 250 mM sucrose, and 10 mM TRIZMA base at pH 7.4 in presence of 100 µM Ca^{2+}[54]. This mitochondrial swelling assay requires the use of isolated mitochondria in order to obtain more reliable results [55].

Fluorimetric assay of mitochondrial reduced (GSH) glutathione

GSH levels were determined also in isolated mitochondria by fluorescence detection, using a method previously described [56]. Isolated liver mitochondria (0.5 mg protein) re-suspended in 1.5 mL phosphate-EDTA buffer and 500 µl H_3PO_4 (4.5%) were rapidly centrifuged at 100,000 x g (Hitachi, TL-100 ultracentrifuge) for 30 min. For GSH determination, 100 µL of supernatant was added to 1.8 mL phosphate buffer and 100 µL OPT. After mixing and incubating at room temperature for 15 min, the solution was transferred to a quartz cuvette and the fluorescence was measured at 420 nm and 350 nm of emission and excitation wavelengths, respectively. GSH levels were determined from comparisons with a linear GSH standard curve.

Total antioxidant capacity assay

The total antioxidant potential of caffeine and its metabolites, theophylline and theobromine, were evaluated by the phosphomolybdenium method [57]. Caffeine, theophylline, theobromine and 3,5-Di-tert-4-butylhydroxytoluene (BHT, used as positive control) were tested at concentrations of 1, 10, 100 and 1000 µM. The vials were capped and incubated at 95 ºC for 90 min. After cooling the mixture to room temperature, the absorbance was measured at 695 nm using Spectra Max Plate Reader® M2 (Molecular Devices), Sunnyvale, California, USA.

Protein determination

Protein was determined by Bradford's test [58] using serum albumin as standard.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Newman–Keul's test for post-hoc comparison. Data are expressed as means ± S.E.M. Values of $p<0.05$ were considered significant.

3.4 Results

Caffeine improve hepatic mitochondrial bioenergetics in APAP treated mice liver

The effect of caffeine, APAP and its combination on hepatic mitochondrial bioenergetics was investigated by HRR. LEAK state (Fig 3) was assessed in the absence of ADP leading to consequent OXPHOS inactivation. Coupled respiration was measured using different mitochondrial respiratory chain substrates and saturated concentration of ADP (Fig 3), enabling oxidative phosphorylation (OXPHOS). This protocol allowed to demonstrate the mitochondrial bioenergetics functionality, by evaluating the oxygen consumption with different mitochondrial complex substrates in coupled respiratory chain and the oxidative phosphorylation process [59].

Our protocol for respiration consisted of titration with different substrates and inhibitors that allowed specific responses on coupled mitochondrial respiratory chain. Figure 2 depicts the HRR protocol used with representative results of each group, highlighting oxygen consumption curve in response to addition of different substrates.

Results presented in Figure 3 demonstrated the effect of caffeine and/ or APAP treatments in the LEAK state which is the mitochondrial function in the absence of ADP and, consequently, without oxidative phosphorylation. Treatments with caffeine, APAP and caffeine plus APAP did not alter this parameter when compare to the control group. When we observed OXPHOS state, caffeine treatment did not alter the OXPHOS with saturated concentrations of CI and CII substrates and ADP. Treatment with APAP caused a significant decrease in respiration CI-linked, CII-linked and CI&CII-linked. Treatment with caffeine plus APAP recovered respiration CII-linked and CI&CII-linked, but not the CI-linked, when compared to control group.

The ratio control respiratory (RCR) values (Table 1) compares the mitochondrial function, in the presence of different substrates and saturated ADP concentrations (OXPHOS) (Fig 3), with the mitochondria state in which there is no oxidative phosphorylation (LEAK) (OXPHOS/LEAK) (Fig 3). Treatment with APAP did not significantly alter RCR values when compared with the control group. However, treatment with caffeine plus APAP caused a significant increase in RCR values when compared to the control and APAP groups in CI&CII-RCR. Since the RCR is an indicative of mitochondrial functionality, the increase in RCR values in caffeine plus APAP groups is associated with an increase in hepatic mitochondrial respiration.

Caffeine decreased mitochondrial swelling in APAP treated mice liver

Hepatic mitochondrial swelling (Fig. 4) was not altered by treatment with caffeine alone when compared to the control group. However, the APAP group presented a marked increase in mitochondrial swelling when compared to the control group. In liver of mice treated with caffeine plus APAP, mitochondrial swelling levels were undistinguishable from values of the control group.

Caffeine decreased oxidative stress markers in APAP treated mice liver

Treatment with caffeine alone did not alter lipid peroxidation levels (Fig. 5A) in liver tissue. On the other hand, treatment with APAP caused a significant increase in hepatic lipid peroxidation levels when compared to the control group. In livers of mice treated with caffeine plus APAP, lipid peroxidation levels were similar to those of the control group.

Reactive species production (DCFH oxidation, Fig. 5B) in liver of mice treated with caffeine did not differ from the control group. Treatment with APAP significantly increased the reactive species production in mice liver, and treatment with caffeine plus APAP did not present differences in RS production when compared to the control group.

Figure 6 shows the GSH content in isolated mitochondria from liver of mice treated with caffeine and/ or APAP. The group treated with caffeine did not present differences in mitochondrial GSH content when compared to the control group. However, APAP alone and caffeine plus APAP groups presented a significant decrease in mitochondrial GSH levels when compared to the control group.

Caffeine and its metabolites presented *in vitro* antioxidant activity

The scavenger ability of caffeine, theobromine and theophylline was evaluated in comparison with a known scavenger of reactive species, using the total antioxidant activity assay [57]. The results (Table 2) demonstrated that caffeine and its metabolites, theobromine and theophylline,

presented antioxidant activity when compared to a positive control. These results suggest that both caffeine and its metabolites present scavenger activity independent of the concentration tested, since 1, 10, 100 and 1000 µM produce similar scavenger activity when compared to positive control.

3.5 Discussion

The present study aimed to investigate the effect of caffeine and APAP association in the hepatic mitochondrial bioenergetics functionality. Mitochondrial dysfunction is an extensively studied mechanism in APAP-induced hepatotoxicity [11]. The effects of APAP overdose include: inhibition of mitochondrial complexes, especially complex I and II; decrease of ATP levels; and mitochondrial swelling [16]. Other studies demonstrated the role of NAPQI binding with cellular structures in APAP toxicity, since it can form mitochondrial adducts leading to mitochondrial dysfunction [17].

The APAP dose (250 mg/Kg) used in the present study was not capable of causing evident hepatotoxicity, which was observed by the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (data not show). However, this dose clearly influenced mitochondrial function, interfering with the respiratory chain complexes, respiratory control ratio and, cellular oxidative stress markers. Therefore, this dose was useful for the purpose of this work that was to assess the influence of caffeine and APAP association on mitochondrial bioenergetics parameters.

Our analysis of mitochondrial complexes interdependent pathways was realized based on OXPHOS (Fig 3). Our results are in agreement with previous studies, which demonstrate the inhibitory effect of APAP on mitochondrial complex I and complex II from rat hepatocytes [15]. It is interesting to note that caffeine seems to induce a compensatory response mediated by mitochondrial complex II, increasing mitochondrial respiration to the same levels of the control group. Therefore, the decrease of complex I by APAP was counterbalanced by a significant

increase in respiration related to the complex II that maintained the phosphorylation at the same levels of the control group. The same effect was also observed in relation to complex III. RCR results (Table 1) emphasize the modulatory effect of caffeine to improve hepatic mitochondrial function [51,60,61], demonstrated by an increase in oxygen consumption in livers of mice treated with caffeine plus APAP.

In general, this result indicates an improvement in the hepatic mitochondrial respiratory function by caffeine, preventing the mitochondrial oxidative phosphorylation inhibited by APAP. To the best of our knowledge, this is the first time that the effect of caffeine in combination with APAP on hepatic mitochondrial respiration is investigated. Caffeine health benefits has been attributed to its antioxidant and ergogenic properties [22–24], although, there are few studies relating caffeine and mitochondrial bioenergetics[13,62].

Considering the common association of caffeine with other drugs [28], it is relevant to investigate the effects of this combination on mitochondrial [13] parameters. Caffeine is a known antagonist of purinergic receptors[63,64], which justify the use caffeine as support analgesic. On the other hand, caffeine also seems to interfere in mitochondrial metabolism [13]. Other studies demonstrate that caffeine can improve mitochondrial function[62], increasing metabolic capacity and mitochondrial biogenesis [61]. Caffeine effects on mitochondrial bioenergetics could occur through antagonism of specific purinergic receptors, such as P2Y [65], which are found in mitochondrial membrane and is involved with mitochondrial calcium uptake. The antagonism of P2Y receptors cause decrease of mitochondrial calcium uptake leading to metabolic effects [66]. Calcium uptake regulates the stimulation of respiratory chain enzymes, the increase in ATP production and the opening of permeability transition pore, increasing mitochondrial swelling and leading to apoptosis [67]. According to our results, is possible to verify a protective effect of caffeine on APAP-induced mitochondrial swelling, suggesting a possible antagonistic effect of caffeine on P2Y pathway, decreasing calcium

uptake, preventing the transition pore opening and stimulating the respiratory chain as observed by HRR evaluation.

In addition, mitochondrial swelling may be related with the oxidative stress induced by APAP. The overproduction of reactive species impairs mitochondrial respiration resulting in mitochondrial permeability transition and increased calcium uptake by mitochondria [16]. In this way, caffeine´s protective effect on APAP-induced oxidative stress and mitochondrial damage could also be associated with its antioxidant effects.

The increase of reactive species production leading to cellular oxidative stress is known [68] to interfere in mitochondrial function, decreasing ATP generation and in extreme cases leading to cell death [69]. In order to observe if the protective effect of caffeine could be associated with its antioxidant properties, we decided to evaluate oxidative stress markers in livers of mice treated with caffeine and/or APAP. Indeed, APAP treatment enhanced reactive species production (DCFH oxidation, Fig. 5B) and increased TBARS levels (Fig. 5A) in liver of mice. Association with caffeine protected from APAP-induced reactive species production and increase TBARS levels in mice livers. This result corroborate the antioxidant effect of caffeine and its metabolites [25,35], which was previously demonstrated by other studies, and has been attributed to the scavenger ability of these molecules. The scavenger ability of caffeine and its metabolites was confirmed by the *in vitro* total antioxidant activity assay (Table 2), which demonstrated that both caffeine and its metabolites present antioxidant activity when compared to a positive control.

Mitochondrial antioxidant defenses are also very important to the mitigation of oxidative damage. In this way, GSH is an important cellular antioxidant defense that can preserve mitochondrial functionality in APAP–induced hepatotoxicity by reducing the toxic metabolite NAPQI [6]. We analyzed the effect of caffeine and APAP association on mitochondrial GSH levels (Fig. 6). GSH depletion is a classic consequence of APAP-

hepatotoxicity, since GSH is responsible for the neutralization of NAPQI [17]. Our results demonstrate that APAP treatment decreased hepatic mitochondrial GSH levels, and the association of caffeine with APAP did not prevent mitochondrial GSH depletion, indicating that caffeine did not interfere in APAP metabolism and NAPQI levels. These data allowed to conclude that the action of caffeine in reduce APAP-induced oxidative stress in liver is not linked with a decrease of NAPQI metabolite levels, or increase of endogenous antioxidant mitochondrial defenses, such as GSH. Instead, we suggest that the antioxidant effect of caffeine observed here is probably related with the cellular scavenge of reactive species [25].

Overall, results presented here demonstrate that APAP treatment interferes in hepatic mitochondrial functionality by decreasing mitochondrial respiration and impairing mitochondrial complexes activity, mainly of the complex I. These events can provoke additional increase of reactive species production in liver. Caffeine, on its turn, prevented hepatic oxygen consumption decrease induced by APAP treatment, by the improvement of the mitochondrial respiration in the complex II, being able to maintain complex III activity. Interestingly, caffeine also prevented mitochondrial swelling caused by APAP in liver of mice. Caffeine promoted the decrease of hepatic reactive species production and lipid peroxidation induced by APAP, which may be associated with its well-known antioxidant properties. Furthermore, caffeine treatment increased the RCR ratio of mitochondria from liver of mice indicating an improvement of the mitochondrial respiration. This effect of caffeine needs to be investigated in detail in further studies.

3.6 Conclusions

In conclusion, the results presented here suggest that association of caffeine with APAP does not increase the hepatoxicity induced by APAP but, present a beneficial effect on mitochondrial bioenergetics improving the hepatic mitochondrial respiration possibly through caffeine´s antioxidant properties and/ or via its interactions with mitochondrial purinergic receptors.

3.7 Conflict of interest

The authors have declared no conflicts of interest

3.8 Funding

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3.10 Figures and captions

TABLE 1. Effect of administration of caffeine, APAP and caffeine plus APAP on RCR ratio.

	CI	**CII**	**CI + CII**
Control	5,85 ±1,70	14,63 ± 6,57	13,55 ± 6,28
Caf.20	7,59 ±3,81	18,67 ± 4,65	13,13 ± 6,70
APAP	6,53 ± 2,17	10,09 ± 1,40	6,04 ± 3,20
Caf.20 + APAP	12,48 ± 2,37	23,41 ± 9,24	37,02 ± 12,34$^{*\#}$

Ratio control respiratory - RCR (OXPHOS/CI-LEAK) using different substrates. Respiratory complexes are indicated by CI, CII or CI + CII. Values are the mean ± SEM of five different experiments n=5.

* Indicates $p<0.05$ as compared to control.

# Indicates $p<0.05$ as compared to APAP.

TABLE 2. Total antioxidant capacity (%) of different concentrations of caffeine, theophylline and theobromine measured by the phosphomolybdenum method.

[ ] µM	1	10	100	1000
Caffeine	31,03 ± 11,35	24,23 ± 5,92	23,93 ± 2,51	53,70 ± 16,73
Theophylline	19,71 ± 8,73	20,86 ± 1,82	16,98 ± 4,38	23,26 ± 3,48
Theobromine	24,61 ± 7,43	22,87 ± 1,72	21,55 ± 4,35	30,51 ± 4,41

Percentage was calculated using BHT 1000 µM as 100 %. The results are presented as mean ± S.E.M of three different experimental assays.

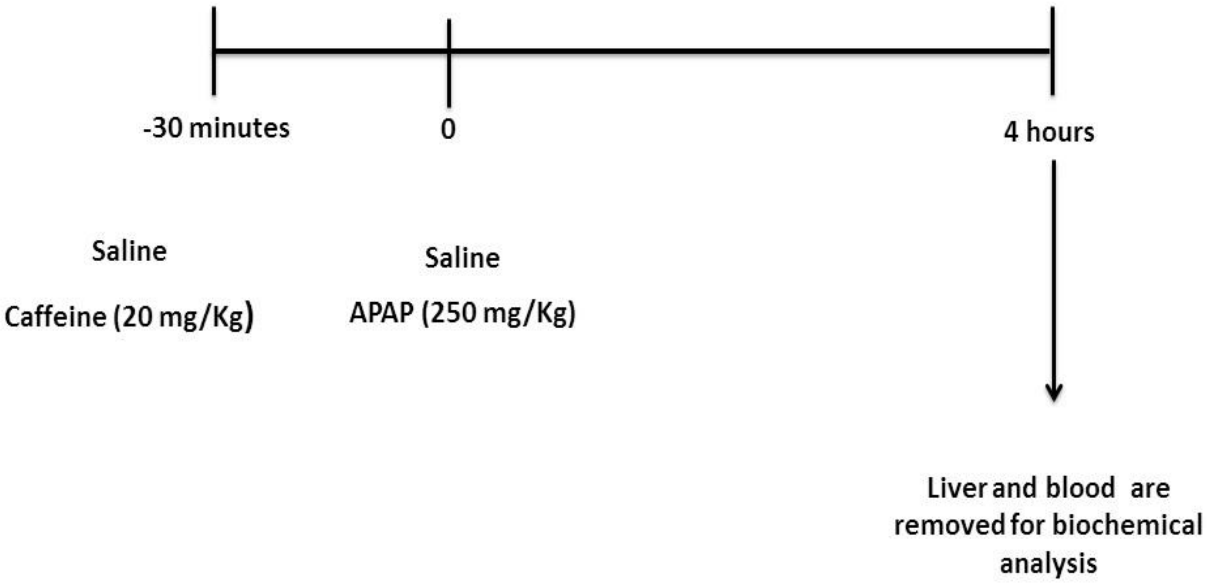

FIGURE 1. Description of experimental protocol used in this study.

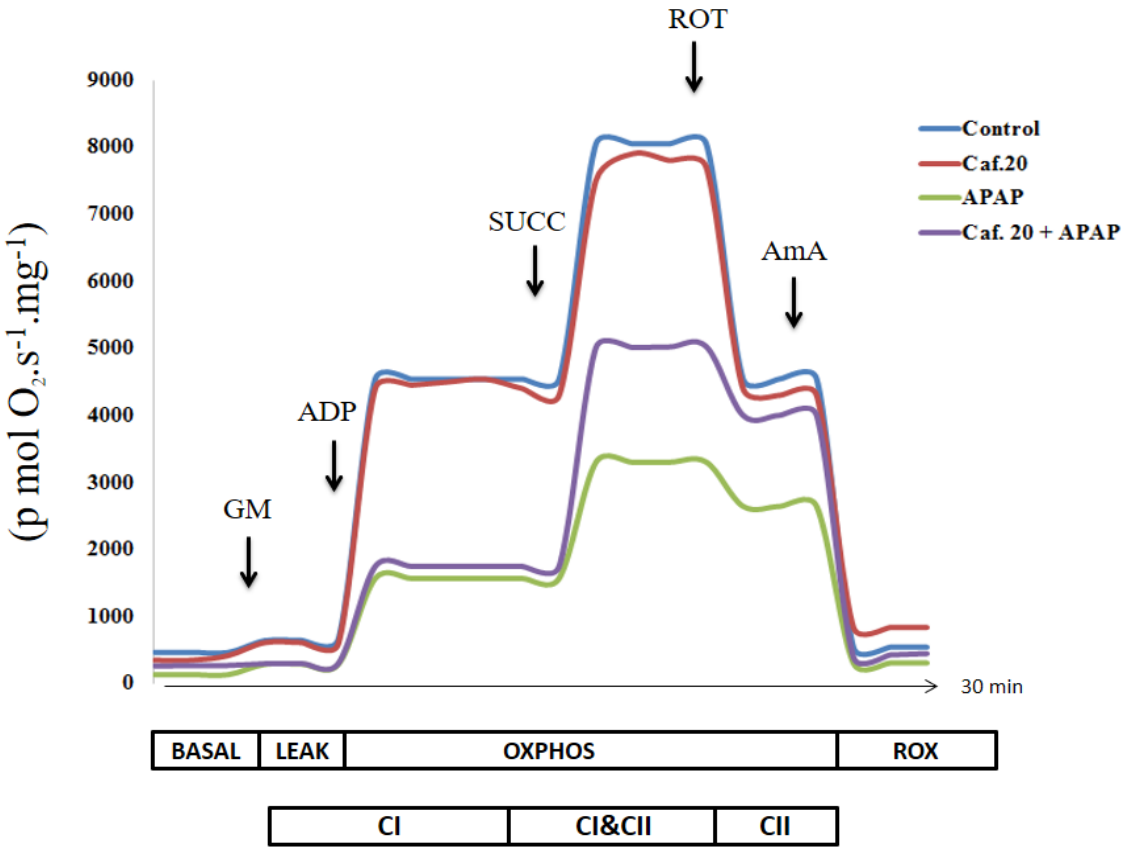

FIGURE 2**. Representative figure of the real-time, high-resolution respirometry experiments for each treatment group.** Basal respirometry (BASAL), indicates the state without oxidative phosphorylation. LEAK is the state with addition of glutamate and malate (GM). Oxidative phosphorylation (OXPHOS) starts by addition of ADP, the increase in respiration observed is related to complex I (CI) activity, then it was added succinate and, the increase in oxygen consumption observed is related to complex I and II (CI&CII) activities. Rotenona (ROT), a complex I inhibitor, was added to observe the respiration activity only by complex II (CII). Lastly, it was added antimycin A (AmA), an inhibitor of cytochrome c reductase, to obtain the residual oxygen consumption (ROX).

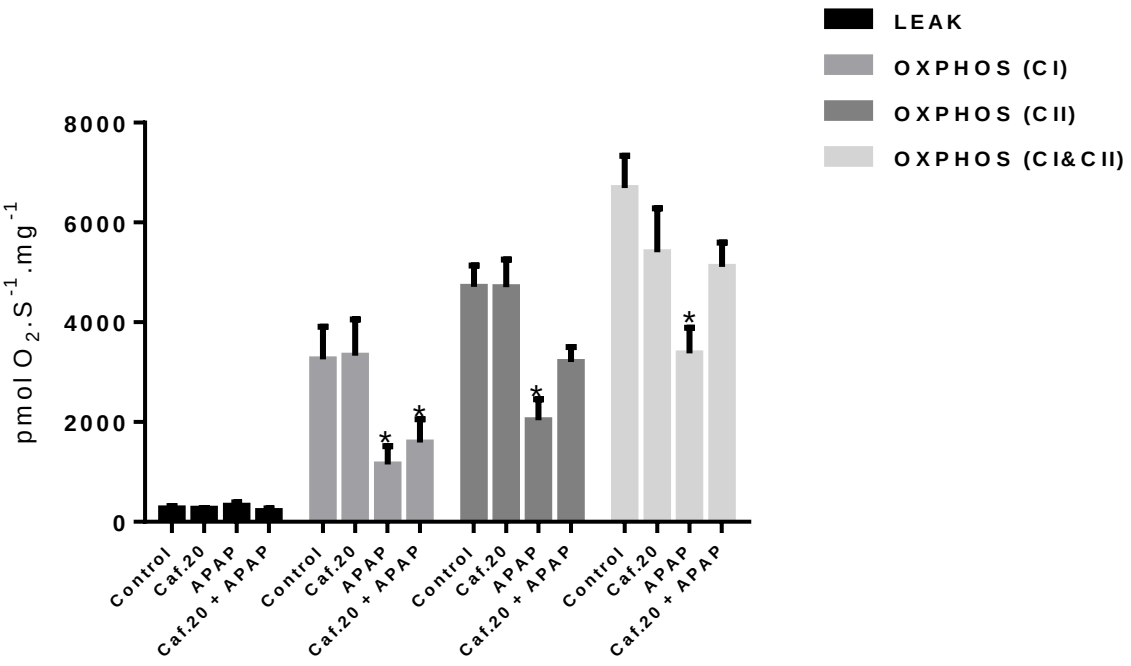

FIGURE 3. **Effect of administration of caffeine, APAP and caffeine plus APAP on HRR in liver of mice.** The LEAK stage was demonstrated in the inactive state of phosphorylation system (without ADP), but in presence of CI substrates (glutamate and malate). OXPHOS is the oxygen consumption in oxidative phosphorylation with saturated concentrations of substrates and ADP. OXPHOS was measured by sequential addition of glutamate, malate, ADP (CI-linked), succinate (CI&CII-linked) and rotenone (CII-linked). Data are reported as mean±S.E.M., n=5. Significance was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls's test for post hoc comparison. *Indicates $p<0.05$ as compared to the control group.

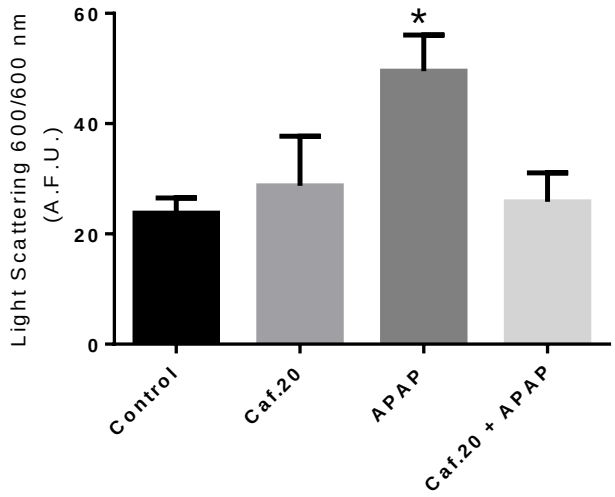

FIGURE 4. **Effect of caffeine, APAP or caffeine plus APAP on the mitochondrial swelling from liver mitochondria of mice.** Mitochondrial swelling. Mitochondria (0.1 mg/mL) were incubated in the reaction medium and energized by 5 mM glutamate and 5 mM succinate. The light scattering was monitored for five minutes. Data are expressed as means±S.E.M., n=4. Significance was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by *$p<0.05$ when compared with control group.

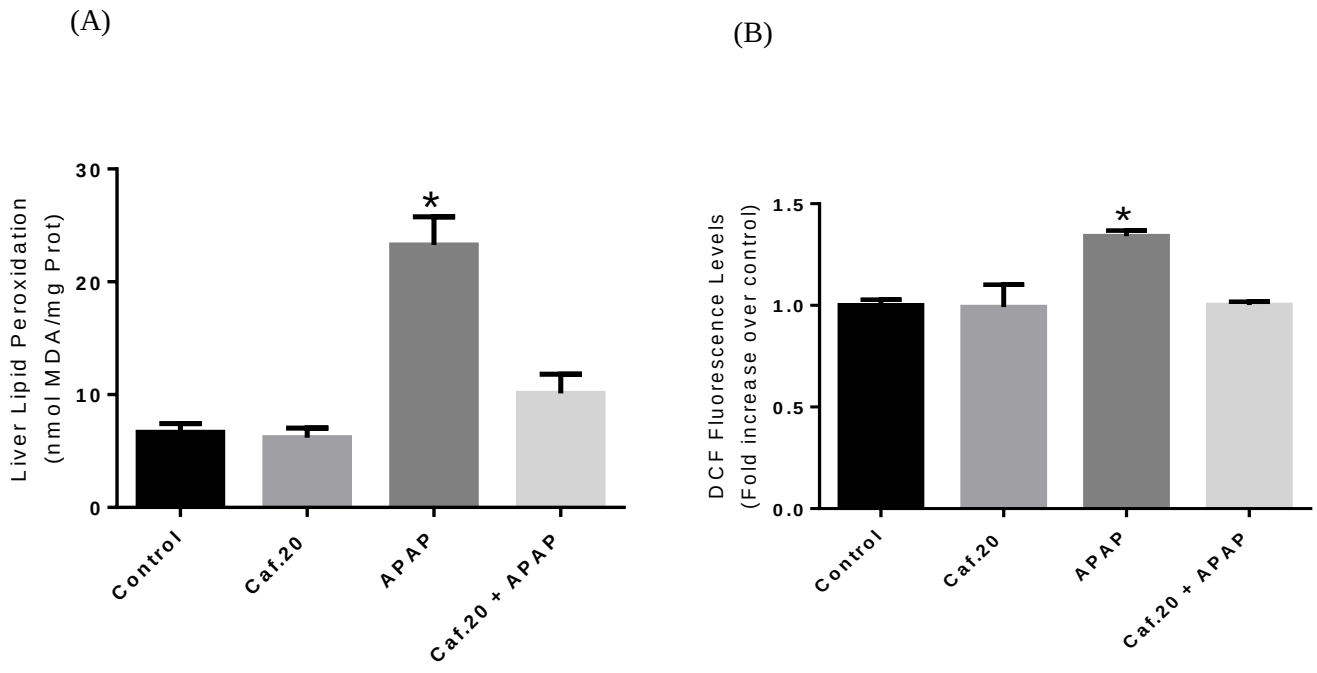

FIGURE 5. **Effect of caffeine, APAP or caffeine plus APAP on oxidative damage markers in liver of mice.** (A) Lipid peroxidation (TBARS). (B) Reactive species production (DCFH oxidation). Data are expressed as means±S.E.M., n=4. Significance was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by *$p<0.05$ when compared with control group.

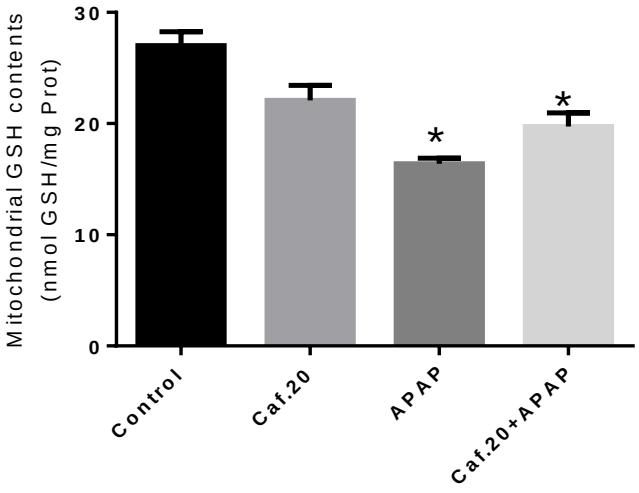

FIGURE 6. **Effect of caffeine, APAP or caffeine plus APAP on reduced glutathione (GSH) levels in liver mitochondria of mice.** Data are expressed as means±S.E.M., n=4. Significance was assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls's test for post hoc comparison. Significant differences are indicated by * $p<0.05$ when compared with control group.

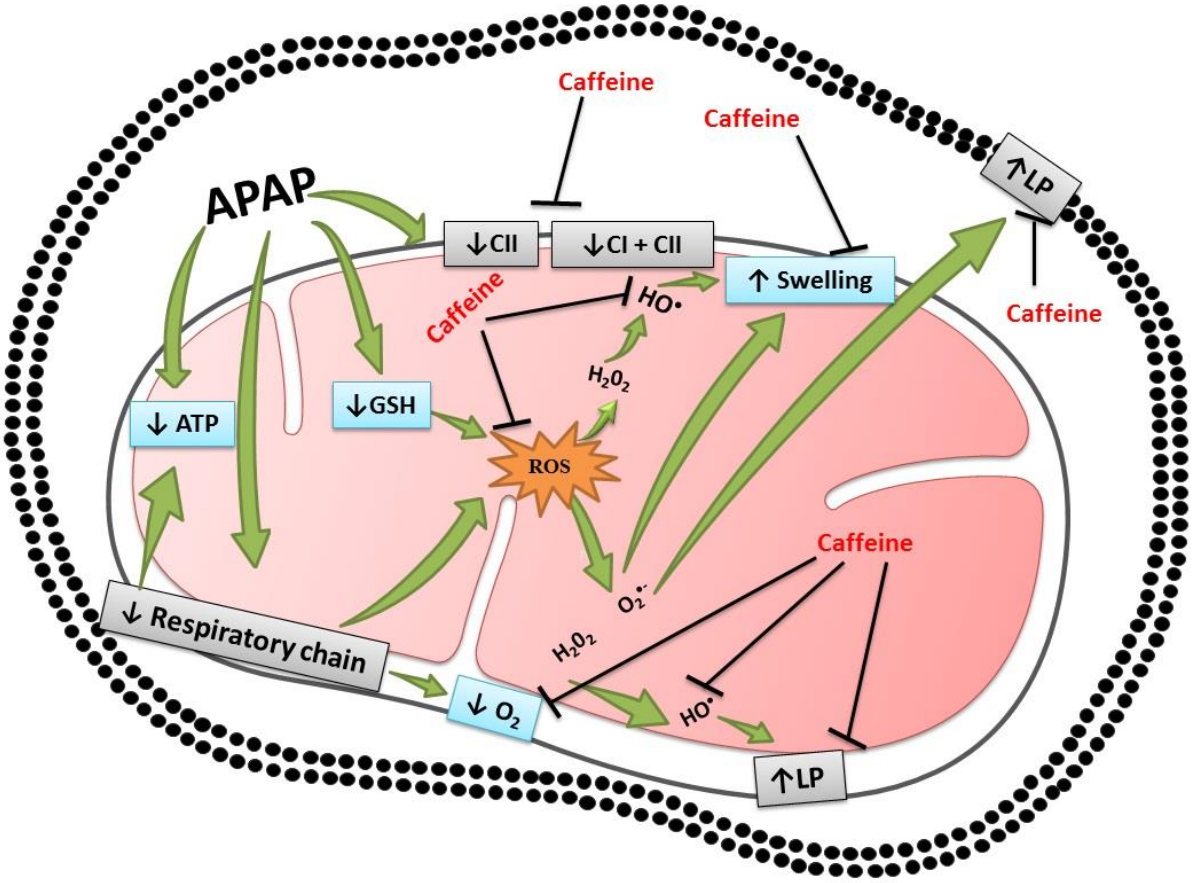

FIGURE 7. **Graphical abstract.** The figure describe the graphical abstract of this work, demonstrating pathways and possible mechanisms involved in caffeine response when associated to acetaminophen.

4. CONCLUSÕES

Os resultados apresentados nesse trabalho, ressaltam aspectos importantes com foco na bioenergética mitocondrial e no estresse oxidativo relacionado a agentes farmacológicos contendo a combinação de paracetamol e cafeína. Tal combinação é encontrada em diversos fármacos utilizados como analgésicos e disponíveis para livre aquisição no mercado farmacêutico.

Em geral, os estudos com foco em fármacos contendo a combinação de paracetamol e cafeína relacionam-se a analgesia e a possíveis alterações na absorção desses fármacos. As conclusões dos resultados obtidos nesse trabalho, trazem um foco novo de avaliação.

É possível perceber que a combinação de paracetamol e cafeína foi capaz de interferir em parâmetros mitocondriais. Quando comparamos APAP sozinho com a associação de cafeína e APAP, percebemos que a combinação dessas duas substâncias promoveu um aumento da atividade do complexo II, aumentou a razão de controle respiratório (RCR), diminuiu o inchaço mitocondrial induzido por APAP, e apresentou também resultados positivos relacionados aos parâmetros de estresse oxidativo, como a diminuição da peroxidação lipídica e diminuição dos níveis de diclorofluoresceína oxidada induzidos por APAP.

Em suma, destacamos os resultados encontrados nesse trabalho como inéditos visto que, a interferência da combinação de cafeína e paracetamol na bioenergética mitocondrial, assim como, em parâmetros relacionados a disfunção mitocondrial ainda não havia sido descrita na literatura científica. Associamos esses efeitos à diminuição do estresse oxidativo pela cafeína, ou, a uma possível interação da cafeína com receptores de adenosina encontrados na membrana mitocondrial com efeitos modulatórios sobre a bioenergética mitocondrial.

5. PERSPECTIVAS

A partir dos resultados obtidos, mais estudos são necessários para identificar precisamente o mecanismo de ação pelo qual a combinação de cafeína e paracetamol interfere na bioenergética mitocondrial. Citamos como exemplos experimentos

relacionados aos receptores de adenosina, em especial, os receptores A2A que são possivelmente antagonizados pela cafeína e, de acordo com estudos já citados anteriormente, são encontrados nas membranas mitocondriais. Dessa forma, poderia se compreender melhor o papel da cafeína combinada ao APAP com foco nas mitocôndrias.

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