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CRISTIANE VIZIOLI DE CASTRO GHIZONI

EFEITOS DO ÓLEO DE COPAÍBA (*Copaifera reticulata*) SOBRE A INFLAMAÇÃO, METABOLISMO E ESTADO OXIDATIVO DE RATOS COM ARTRITE POR ADJUVANTE

Maringá
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Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de doutor.

Orientador: Dr. Jurandir Fernando Comar

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CRISTIANE VIZIOLI DE CASTRO GHIZONI

EFEITOS DO ÓLEO DE COPAÍBA (*COPAIFERA RETICULATA*) SOBRE A INFLAMAÇÃO, METABOLISMO E ESTADO OXIDATIVO DE RATOS COM ARTRITE POR ADJUVANTE

Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de doutor.

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BIOGRAFIA

Cristiane Vizioli de Castro Ghizoni nasceu em Birigui/SP em 12/05/1985. Possui graduação em Farmácia-Bioquímica (2009) e Mestrado em Biologia Celular e Molecular (2012) pela Universidade Estadual de Maringá. Durante a graduação foi contemplada com 3 bolsas de Iniciação Científica CNPQ, 2 na área de metabolismo hepático e bioenergética e 1 na farmácia e farmacologia. Foi monitora de Farmacologia. Atuou como docente (professor temporário) do Departamento de Bioquímica da Universidade Estadual de Maringá de 10/04/2014 a 19/03/2016. Ministrou a disciplina de Bioquímica básica para os cursos de Educação Física, Biomedicina, Biotecnologia, Zootecnia e Farmácia. Desenvolveu o trabalho de doutorado sob a orientação do professor Dr. Jurandir Fernando Comar, no Laboratório de Metabolismo Hepático, Departamento de Bioquímica.

Dedico este trabalho ao meu marido, minha mãe e minhas irmãs.

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"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê."

(Arthur Schopenhauer)

APRESENTAÇÃO

Este é um trabalho realizado no Laboratório de Metabolismo Hepático e Radioisótopos do Departamento de Bioquímica, Laboratório de Bioquímica de Microrganismos Laboratório de Bioquímica de Microrganismos, no Laboratório de Inflamação do Departamento de Farmacologia e Terapêutica e no Laboratório de Histologia Animal do Departamento de Ciências Morfológicas da Universidade Estadual de Maringá, apresentado na forma de dois artigos científicos originais, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas.

Artigo 1:

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Cristiane V. Castro-Ghizoni, Ana Paula A. Ames, Ciomar A. Bersani-Amado, Anacharis B. Nakanishi, Lívia Bracht, Maria R. M. Natali, Adelar Bracht, Jurandir F. Comar. **Actions of copaiba oil (*Copaifera reticulata*) on the liver metabolism of rats with adjuvant-induced arthritis.** Cell Biochemistry and Function, a ser submetido.

LIST OF ABBREVIATIONS AND ACRONYMS

ROS	Reactive oxygen species
GSH	Reduced glutathione
GSSG	Oxidized glutathione
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CEEA	Ethics Committee for Animal Experimentation
C	Controls rats
CCO 0.58	Controls rats treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$
CCO 1.15	Controls rats treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$
A	Arthritic rats
ACO 0.58	Arthritic rats treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$
ACO 1.15	Arthritic rats treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$
TAC	Total antioxidant capacity
Thiols	Protein sulfhidryl groups
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
OPT	o-phthalaldehyde
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
DTNB	5,5-dithiobis-2-nitrobenzoic acid
DNPH	2,4-dinitrophenylhydrazine
DCF	Oxidized dichlorofluorescein
MPO	Myeloperoxidase

RESUMO GERAL

INTRODUÇÃO: A artrite reumatoide é uma doença autoimune caracterizada por inflamação crônica e sistêmica que afeta as membranas sinoviais, cartilagens articulares e ossos. A cartilagem articular torna-se hiperplásica com a participação de células e citocinas pró-inflamatórias e acentuado aumento nos níveis de espécies reativas de oxigênio (ROS) e mediadores inflamatórios, que causam lesão tecidual. A artrite reumatoide é uma doença sistêmica e, além das articulações, outros órgãos são afetados, como cérebro e coração. A artrite induzida por adjuvante é uma imunopatologia experimental em ratos que compartilha muitas características da artrite reumatoide. Estes animais apresentam inflamação sistêmica, caquexia e, adicionalmente às articulações, outros órgãos são afetados, como o fígado, que apresenta alterações morfológicas e metabólicas associadas a um acentuado estresse oxidativo. O óleo de copaíba é um oleorresina extraído de árvores do gênero *Copaifera*, abundante na Amazônia brasileira e comercializada mundialmente para diversos fins terapêuticos, especialmente como anti-inflamatório. Nenhum estudo avaliou até o momento se o óleo de copaíba é capaz de inibir a inflamação crônica e sistêmica, como a que ocorre na artrite reumatoide. Desta forma, o presente estudo investigou a ação do óleo de copaíba (*C. reticulata* Ducke), contendo 37,6% de β-cariofileno, oralmente administrado sobre a inflamação sistêmica, o estado oxidativo sistêmico, a morfologia hepática e o metabolismo hepático de ratos com poliartrite induzida por adjuvante. Estudos anteriores também indicam que o tratamento de ratos com óleo de copaíba parece ser prejudicial ao fígado. Assim, alguns parâmetros plasmáticos foram determinados para avaliar a toxicidade do óleo no fígado. **MÉTODOS**: O óleo de copaíba foi fornecido pela EMBRAPA/PA e sua atividade antioxidante *in vitro* foi medida por ensaios de DPPH e ABTS. A poliartrite foi induzida em ratos Holtzman (180-200 g) com o adjuvante completo de Freund. Os ratos foram distribuídos em seis grupos: controles; controles tratados com óleo de copaíba nas doses de 0,58 e 1,15 $g \cdot Kg^{-1}$, artrite e artrite tratada com óleo de copaíba nas doses de 0,58 e 1,15 $g \cdot Kg^{-1}$. O óleo de copaíba foi administrado oralmente

(gavagem) uma vez ao dia durante 5 dias antes e 18 dias após a indução da artrite. O volume da pata foi monitorado por pletismografia. As lesões secundárias e o peso das glândulas adrenais, fígado e linfonodos foram também medidos. No 19° dia, a cavidade peritoneal dos ratos previamente anestesiados foi exposta, o sangue colhido da veia cava e o fígado removido e dividido em duas partes: uma usada para o processamento histológico e outra para a preparação do homogenato. Proteínas carboniladas e ROS foram medidos no homogenenato para avaliar o estresse oxidativo hepático. O conteúdo de glutationa oxidada (GSSG) e reduzida (GSH), assim como a atividade da catalase e superóxido dismutase (SOD) foram avaliados no sobrenadante do homogenenato. Também foram medidos a capacidade antioxidante total (TAC), tióis protéicos, proteínas carboniladas e atividade da mieloperoxidase (MPO) no plasma. Os níveis de bilirrubina e as atividades da AST, ALT e fosfatase alcalina foram medidos no plasma. A análise morfológica e morfométrica foi feita com imagens da região próxima à veia central do parênquima hepático. O protocolo de tratamento foi repetido mais três vezes para avaliar o metabolismo hepático. A glicogenólise, glicólise e gliconeogênese foram avalidos utilizando fígados em perfusão isolada. A atividade respiratória de mitocôndrias isoladas do fígado foi adicionalmente avaliada. **RESULTADOS**: ratos artríticos desenvolveram uma resposta inflamatória intensa ao adjuvante na pata injetada e na pata contralateral. Os animais também apresentaram baixo ganho de peso corporal e manifestações inflamatórias sistêmicas, como evidenciado pela maior atividade da MPO plasmática, baixos níveis de albumina plasmática, lesões secundárias à artrite na cauda e orelhas associadas ao aumento do peso das glândulas adrenais e linfonodos. Foram observadas alterações histológicas hepáticas, como dilatação sinusoidal, focos inflamatórios encapsulados e menor número de hepatócitos por área de fígado. Ratos artríticos apresentaram aumento do estresse oxidativo plasmático e no fígado, conforme evidenciado por maiores níveis de ROS e proteínas carboniladas no fígado, reduzida atividade da catalase e diminuídos níveis de GSH no fígado, aumento de proteínas carboniladas no plasma e diminuição da TAC e tióis no plasma. A glicogenólise e a glicólise de ratos com artrite não foram diferentes, mas a gliconeogênese foi 44% menor na artrite. Na presença de óleo de copaíba, o volume da pata foi 43 e

51% menor, respectivamente, para ratos artríticos tratados com doses de 0,58 e 1,15 $mg \cdot Kg^{-1}$ e, apenas na dose de 0,58 $mg \cdot Kg^{-1}$, foi capaz de diminuir o peso das adrenais e linfonodos entre 20-50%. O óleo de copaíba não modificou o estado oxidativo plasmático de ratos artríticos, mas diminuiu a atividade da MPO plasmática (-30%) na dose de 0,58 $mg \cdot Kg^{-1}$. O óleo de copaíba na dose de 1,15 $mg \cdot Kg^{-1}$ diminuiu os níveis de proteínas carboniladas e ROS no fígado para valores iguais aos controles. Ambas as doses aumentaram o conteúdo de GSH e a atividade da catalase no fígado. O óleo de copaiba 1,15 $g \cdot Kg^{-1}$ também diminuiu o ganho de peso corporal (-45%) e o número de hepatócitos (-20%), enquanto aumentou o peso do fígado (29%) e a área dos hepatócitos (13%). O óleo na dose de 1.15 $g \cdot Kg^{-1}$ diminuiu a glicólise (-65%), a glicogenólise (-58%) e gliconeogênese (-30%) no fígado de animais artríticos. No entanto, a gliconeogênese foi diminuída também no fígado de ratos controles tratados com ambas as doses do óleo de copaíba. A atividade respiratória das mitocôndrias isoladas praticamente não foi modificada. **CONCLUSÃO:** Os resultados do presente estudo revelaram que (I) a artrite induzida por adjuvante em ratos está associada a uma intensa resposta inflamatória sistêmica, particularmente modificações histológicas e metabólicas no fígado; (II) O óleo de copaíba apresentou efeitos anti-inflamatórios moderados sobre a indução da artrite e melhorou as manifestações sistêmicas na dose de 0,58 $g \cdot Kg^{-1}$; (III) o tratamento na dose de 1,15 $g \cdot Kg^{-1}$ foi efetivo em diminuir o estresse oxidativo hepático, uma ação que pode ser atribuída à estimulação do sistema antioxidante endógeno; (IV) o tratamento não foi eficaz em melhorar as alterações histológicas hepáticas de ratos artríticos; (V) o óleo também diminuiu a glicólise, a glicogenólise e gliconeogênese no fígado destes animais; mas (VI) diminuiu a gliconeogênese hepática também nos ratos controles; (VII) o óleo de copaíba não modificou a atividade respiratória nas mitocôndrias isoladas do fígado; e por fim (VIII), causou alterações histológicas no fígado de ratos controles, o que associado ao menor ganho de peso corporal e maior peso do fígado pode indicar uma possível ação nociva do óleo, como por exemplo uma colestase hepática. Assim, a utilização de óleo de copaíba no tratamento da artrite reumatoide deve ser considerada com cautela, pois pode ser também prejudicial, especialmente para o fígado, onde foram observadas grandes modificações.

GENERAL ABSTRACT

BACKGROUND: Rheumatoid arthritis is an autoimmune disease characterized by chronic and systemic inflammation that affects the synovial membranes, articular cartilages and bones. The articular cartilage becomes hyperplasic with participation of proinflammatory cells and cytokines, reactive oxygen species (ROS) and inflammatory mediators, which cause tissue injury. Rheumatoid arthritis is a systemic disease and in addition to the joints other organs are affected, such as brain and heart. Adjuvant-induced arthritis is an experimental immunopathology in rats that shares many features with rheumatoid arthritis in humans. These animals present systemic inflammation and additionally to articular sites other organs are affected, as the liver, which presents morphological and metabolic alterations associated to a pronounced oxidative stress. Copaiba oil is an oleoresin extracted from trees of the genus *Copaifera*, abundant in the Brazilian Amazon and worldly commercialized for several therapeutic purposes, specially anti-inflammatory. No study has until now evaluated if the copaiba oil is able to improve the chronic and systemic inflammation, such as that occurs in rheumatoid arthritis. The present study therefore was planned to investigate the action of copaiba oil (*C. reticulata* Ducke), containing 37.6% of *β*-caryophyllene, orally administrated on the systemic inflammation, oxidative status and liver histology and metabolism of rats with adjuvant-induced polyarthritis. Previous studies also indicate that the treatment of rats with copaiba oil seems to be harmful to the liver. Thus, plasma parameters were additionally accessed to evaluate potential toxicity of the oil on the liver, the first organ that receives the oil when ingested orally. **METHODS**: Copaiba oil was provided by EMBRAPA/PA and its antioxidant activity *in vitro* was measured by DPPH and ABTS assays. Induction of polyarthritis was performed in *Holtzman* rats (180-200 g) with Freund's adjuvant. The rats were distributed into six groups: controls; controls treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$, arthritis, and arthritis treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$. Copaiba oil was administrated orally once a day during 5 days prior and 18 days after arthritis induction. Paw volume was measured by

plethysmography. Secondary lesions and the weight of adrenals, liver and lymph nodes were additionally measured. At day 19th, peritoneal cavity of anesthetized rats was exposed, blood collected from the cava vein and the liver was removed and divided into two parts: one was used for histological processing and the other used for liver homogenate preparation. Protein carbonyl groups and ROS were measured in the homogenate to evaluate liver oxidative stress. Oxidized (GSSG) and reduced (GSH) glutathione content and catalase and SOD activity were evaluated in the homogenate supernatant as antioxidant parameters. Total antioxidant capacity (TAC), thiol groups, protein carbonyl groups and mieloperoxidase (MPO) activity were also measured in the plasma. Bilirubin levels and AST, ALT and alkaline phosphatase activities were measured in the plasma to evaluate the liver damage. Morphologic and morphometric analysis was done with images from the region near the central vein of liver parenchyma. The treatment protocol was repeated three times to evaluate the hepatic metabolism. Glycogenolysis, glycolysis and gluconeogenesis were measured in the isolated perfused livers. It was additionally investigated the respiratory activity of mitochondria isolated from livers. **RESULTS**: Arthritic rats developed an intense inflammatory response to adjuvant in the injected paw and also in the contralateral paw. Animals furthermore presented low body weight gain and systemic inflammatory manifestations, as evidenced by higher plasma myeloperoxidase activity, low levels of plasma albumin, severe secondary lesions to arthritis in the tail and ears associated with increased weight of adrenals and lymph nodes. It was observed histological alterations in the liver, as sinusoid dilatation, encapsulated inflammatory foci and lower number of hepatocyte per hepatic area. Arthritic rats presented an increased oxidative stress in the plasma and in the liver, as evidenced higher levels of ROS and protein carbonyl groups in the liver, decreased activity of catalase and GSH content in the liver, higher levels of protein carbonyl groups in the plasma and decreased TAC and thiols in the plasma. Glycogenolysis and glycolysis of arthritis rats were not different, but the gluconeogenesis was 44% lower in the arthritis. In the presence of copaiba oil, paw volume was 43 and 51% lower, respectively, for arthritic rats treated at a doses of 0.58 and 1.15 mg·Kg^{-1} and, only at a dose of 0.58 mg·Kg^{-1}, was able to decrease the weight of adrenals and lymph nodes

between 20-50%. Copaiba oil did not modify the plasma oxidative status of arthritic rats, but it decreased the plasma myeloperoxidase activity (-30%) at a dose of 0.58 $mg \cdot Kg^{-1}$. Copaiba oil only at a dose of 1.15 $mg \cdot Kg^{-1}$ decreased the protein carbonyl groups and ROS in the liver to values close the control ones. Both doses increased the GSH content and catalase activity in the liver. Copaiba oil 1.15 $g \cdot Kg^{-1}$ also decreased the body weight gain (-45%) and number of hepatocytes (-20%) while increased the liver weight (29%) and the area of hepatocytes (13%) in arthritic and control rats. Copaiba oil 1.15 $g \cdot Kg^{-1}$ decreased the glycolysis (-65%), glycogenolysis (-58%) and gluconeogenesis (-30%) in the liver of arthritic animals. However, the gluconeogenesis was also diminished in the livers of treated control rats. The respiratory activity of isolated mitochondria was practically not modified by copaiba oil treatment. **CONCLUSION**: The results of the present study revealed that (I) adjuvant-induced arthritis in rats is associated to an intense inflammatory response with systemic manifestations, particularly histological and metabolic alterations in the liver; (II) copaiba oil presented a moderate anti-inflammatory effects on the arthritis induction and improved the systemic manifestation at a dose of 0.58 $g \cdot Kg^{-1}$; (III) treatment at a dose of 1.15 $g \cdot Kg^{-1}$ was effective to decrease the oxidative stress in the liver, one action that can be attributed to the stimulation in endogenous antioxidant system; (IV) treatment with copaiba oil 1.15 $g \cdot Kg^{-1}$ was not effective in improving the histological alterations in the liver of arthritic rats; (V) copaiba oil decreased the glycolysis, glycogenolysis and gluconeogenesis (-30%) in the liver of arthritic animals; however, (VI) it decreased the gluconeogenesis in the liver of control rats; (VII) copaiba oil did not modify substantially the respiratory activity in the isolated mitochondria; and finally (VIII), it caused histological alterations in the liver of control rats, which associated with the lower body weight gain, reduced gluconeogenesis in the liver and higher liver weight can indicate a possible harmful action of the oil, such as a liver cholestasis. Thus, the use of copaiba oil as an adjuvant in the treatment of rheumatoid arthritis in humans must be regarded with caution because it may additionally be harmful, specially for the liver, where great modifications were observed.

Anti-inflammatory and antioxidant actions of copaiba oil (*Copaifera reticulata*) are associated with histological modifications in the liver of arthritic rats

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ABSTRACT

Adjuvant-induced arthritis is an experimental immunopathology in rats that is often used as a model for studying autoimmune chronic inflammation. These animals present systemic inflammation and additionally to articular sites other organs are affected, as the liver, which presents morphological and metabolic alterations associated to a pronounced oxidative stress. The purpose of the present study was to investigate the action of copaiba oil (*C. reticulata* Ducke), containing 37.6% of *β*-caryophyllene, on the systemic inflammation, oxidative status and liver histology of rats with arthritis by adjuvant. The rats were distributed into six groups: controls; controls treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$, arthritis, and arthritis treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$. Copaiba oil was administrated orally (gavage) once a day during 5 days prior and 18 days after arthritis induction. Paw volume was 43 and 51% lower, respectively, for arthritic rats treated at a doses of 0.58 and 1.15 $mg \cdot Kg^{-1}$ and only 0.58 $mg \cdot Kg^{-1}$ was able to decrease the weight of adrenals and lymph nodes between 20-50%. Copaiba oil did not modify the plasma oxidative status of arthritic rats, but it decreased the plasma myeloperoxidase activity (-30%) at a dose of 0.58 $mg \cdot Kg^{-1}$. Copaiba oil only at a dose of 1.15 $mg \cdot Kg^{-1}$ decreased the protein carbonyl groups and reactive oxygen species in the liver to values close the control ones. Both doses increased the GSH content and catalase activity in the liver. Copaiba oil 1.15 $g \cdot Kg^{-1}$ also decreased the body weight gain (-45%) and number of hepatocytes (-20%) while increased the liver weight (29%) and the area of hepatocytes (13%) in the controls. The results reveal that the copaiba oil presented an systemic anti-inflammatory and liver antioxidant actions, however, the modifications in the liver histology of control rats in the presence of the oil seems to be associated with harmful effects.

Key words*: adjuvant-induced arthritis, copaiba oil, Copaifera reticulata, oxidative status, hepatic morphology.*

INTRODUCTION

Rheumatoid arthritis is an autoimmune and chronic inflammatory disease that primarily affects the small joints of the hands and feet. Rheumatoid arthritis occurs in 0.5-1.0% of the adult population worldwide and in addition to the osteoarticular manifestations it is associated with an increased mortality rate, mainly due to cardiovascular complications [Minichiello *et al.*, 2016; Uhlig *et al.*, 2014]. The pathophysiology of arthritis involves an intense hyperplasia of the synovial membrane and cartilage with participation of T and B lymphocytes, macrophages, fibroblasts and proinflammatory cytokines and overproduction of reactive species, such as superoxide anion ($O_2^{-}\cdot$), hydroxyl radical ($HO\cdot$), hydrogen peroxide (H_2O_2), and others, which act as mediators of tissue injury [Misko *et al.*, 2013; Kundu *et al.,* 2012]. Rheumatoid arthritis is a systemic disease and in addition to the joints other organs are affected, such as brain, heart, lungs and vascular tissue [Wartolowska *et al.*, 2012; Davis et al., 2011; Mcinnes & Schett, 2011; Voskuyl, 2006]. The oxidative status is likewise changed in the serum blood of patients with rheumatoid arthritis and also in the liver, brain, heart and vascular tissue of rats with adjuvant arthritis [Schubert *et al.*, 2016; Bracht *et al.*, 2015; Wendt *et al.*, 2015; Comar *et al.*, 2013; Stamp et al., 2012; Lemarechal *et al.*, 2006].

Metabolic alterations are also prominent in rheumatoid arthritis, as the muscle wasting condition known as rheumatoid cachexia, which occurs in approximately two-thirds of all patients and is mediated by proinflammatory cytokines [Roubenoff, 2009]. Metabolic changes are equally significant in the liver of rats with arthritis by adjuvant [Yassuda-Filho *et al.*, 2003; Fedatto *et al.*, 2002; Caparroz-Assef *et al.*, 1998; Toda *et al.*, 1994], where oxidative stress is also more pronounced when compared to other organs, such as brain and heart [Schubert *et al.*, 2016; Wendt *et al.*, 2015; Comar *et al.*, 2013]. The liver of arthritic rats presents higher levels of ROS, protein carbonyl groups and lipoperoxides in several subcellular fractions. These alterations are accompanied by lower activities of antioxidant enzymes and diminished levels of reduced glutathione [Comar *et al.*, 2013]. In addition, the alterations of both metabolism and

oxidative state of the liver seem to be associated with the metabolic alterations that occur in the body as consequence of the systemic inflammation.

Rheumatoid arthritis is non-curable and the treatment aims to induce remission of symptoms and to prevent relapse of the disease. The types of medications used depend on the severity of the symptoms but nonsteroidal anti-inflammatory drugs (NSAIDs), corticoids and disease-modifying antirheumatic drugs (DMARDs) are currently the main agents used for the treatment of rheumatoid arthritis [Uhlig *et al.*, 2014]. Herbal medicines have also been experienced as a natural alternative and complementary to conventional therapy. Green tea (*Camellia sinensis*), baswellic acid (*Boswellia serrata)* and ginger (*Zingiber officinale)* are some of the herbal medicines under investigation for treatment of arthritis and have shown promising efficacy [Gonçalves *et al.*, 2015; Al-Nahain *et al.,* 2014]

The copaiba oil is an oleoresin extracted from the trunk of *leguminous* trees of the genus *Copaifera*, which are native in the tropical regions of South America. The *Copaifera reticulata* Ducke is the most abundant species in the Brazilian Amazon and its oil is one important Amazonian herbal medicine worldly commercialized [Desmarchelier, 2010; Veiga Junior *et al.*, 2001]. The oil of diverse species of *Copaifera* has been used for several therapeutic purposes, some of them with established biological activity: anti-inflammatory, antioxidant, antitumoral, antibacterial, antipsoriasis, antinociceptive, neuroprotective and treatment of endometriosis [Borges et al., 2016; Destryana *et al.*, 2014; Leandro *et al.*, 2012; Gelmini *et al.*, 2013; Guimarães-Santos *et al.*, 2012; Gomes *et al.*, 2010].

The copaiba oil is a mixture of sesquiterpenes and diterpenes. The former accounts for approximately 80-90% of the oil composition and the β-caryophyllene is the main compound of the oil in most species of *Copaifera*, reaching up to 60% of the oil in some of them [Leandro *et al.*, 2012; Gomes *et al.*, 2007; Veiga Jr *et al.*, 2007]. The isolated β-caryophyllene shares many biological properties with copaiba oil, specially the well-documented anti-inflammatory activity [Leandro *el al.*, 2012; Desmarchelier, 2010]. The antioxidant properties of copaiba oil have also been investigated, specially for the species *C. langsdorffii* Desf, which showed antioxidant activity *in*

vitro (DPPH method) [Gelmini *et al.*, 2013] and also in rats with intestinal and skin oxidative stress induced by ischemia/reperfusion [Lima-Silva *et al.*, 2009; Paiva *et al.*, 2004], where the treatment of rats with the oil was able to improve several oxidative parameters, such as the activity of catalase and the levels of lipoperoxides and glutathione.

Considering the above-mentioned properties of the copaiba oil, it seems reasonable to hypothesize that the copaiba oil should be able to attenuate the articular and systemic inflammation that occur in the rheumatoid arthritis. Previous studies have already showed the anti-inflammatory effectiveness of copaiba oil *in vitro* and also *in vivo* by its ability to inhibit the acute inflammation in rats and mice [Destryana *et al.*, 2014; Kobayashi *et al.*, 2011; Veiga Jr *et al.*, 2007; Basile *et al.*, 1988]. However, no study has until now evaluated whether copaiba oil is able to inhibit the chronic and systemic inflammation, such as that occurs in rheumatoid arthritis [Ohrndorf & Backhaus, 2013]. The present study therefore was planned to investigate the action of copaiba oil, extracted from *C. reticulata* Ducke, orally administrated on the systemic inflammation, oxidative status and liver histology of rats with adjuvant-induced polyarthritis. The later is an experimental immunopathology in rats which shares many features of human rheumatoid arthritis and is often used as a model for evaluation of anti-inflammatory and anti-rheumatic drugs [Szekanecz *et al.*, 2000; Bendele *et al.*, 1999]. This model exhibit a strong and generalized inflammatory response and, therefore, allow to investigate not only the factors associated to pathogenesis at the joints, but also the systemic manifestations [Bendele *et al.*, 1999]. Previous studies also indicate that the treatment of rats with copaiba oil seems to be harmful to the liver [Botelho *et al.*, 2010; Brito *et al.*, 2000]. Thus, plasma parameters were additionally accessed in the present study to evaluate potential toxicity of the oil on the liver, the first organ that receives the oil when ingested orally. Considering that rheumatoid arthritis can range from a mild form to other more severe and disseminated form, the present study aims to provide data about the systemic effects of copaiba oil, including possible liver toxicity, in rats with polyarthritis, which in turn, should also allow extrapolations for the patients with rheumatoid arthritis, particularly for those that manifest the more aggressive form of arthritis.

MATERIAL AND METHODS

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH), o-phthalaldehyde (OPT), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 2,4-dinitrophenylhydrazine (DNPH), oxidized dichlorofluorescein (DCF), reduced glutathione (GSH), oxidized glutathione (GSSG), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial kits for AST, ALT, bilirubin, alkaline phosphatase, creatinine, albumin and total proteins were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, MG, Brazil). All other chemicals were of analytical grade.

Copaiba oil

Copaiba oil was provided by the Brazilian Enterprise for Agricultural Research (EMBRAPA), Center for Agroforestry Research of the Eastern Amazon, Brazil. The oleoresin was collected by means of artificial holes in the trunks of native adult trees of the species *Copaifera reticulata* Duke, which are localized at the km 67 of the National Forest of Tapajós (Belterra, PA, Brazil). A voucher specimen was deposited in the Herbarium IAN of Eastern Amazon EMBRAPA under the number 183939. The composition of the oleoresin was quantified by gas chromatography–mass spectrometry (GC/MS) and previously reported elsewhere [Ziech *et al.*, 2013] as follow: β-caryophyllene (37.6%), β-bisabolene (13.9%), *(E)*-α-bergamotene (9.3%), α-humulene + *(E)*-β-farnesene (5.3%), β-selinene (3.9%), β-elemene (3.3%), α-selinene (3.1%), α-bulnesene (2.1%), *(Z)*-α-bisabolene (1.8%), *(E)*-γ-bisabolene (1.3%), β-sesquiphellandrene (1.1%), another 14 different substances representing less than 1% each.

DPPH and ABTS radical scavenging activity assays

DPPH assay: the antioxidant activity was measured spectrophotometrically at 515 nm as the ability of copaiba oil in scavenging the DPPH radicals (1,1-diphenyl-2-picrylhydrazyl) [Carvajal *et al.*, 2012].

For DPPH assay the copaiba oil was previously dissolved in methanol. The antioxidant activity was calculated as the percentage of scavenging efficiency and the results expressed as the half-maximal effective concentration of the extract (EC_{50}).

ABTS assay: the capacity of copaiba oil in scavenging the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals was measured spectrophotometrically as previously described [Carvajal *et al.*, 2012]. The copaiba oil, dissolved in methanol, was allowed to react with 0.02 $mM \cdot L^{-1}$ ABTS solution during 1 h in the dark and after the absorbance was measured. The ABTS scavenging activity (%) was calculated as $100x(A_C - A_O)/A_C$, where A_C is the absorbance of control (water) and A_O is the absorbance of the oil. The results was expressed as the half-maximal effective concentration of the extract (EC_{50}).

Animals and induction of arthritis

Male *Holtzman* rats weighting 160-180 g were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained in standard laboratory conditions at a temperature of 22±3ºC under a regulated 12h light/dark cycle. The animals were housed in conventional steel cages (3 rats/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). For the induction of adjuvant arthritis, animals (50 days old) were injected subcutaneously in the left hind paw of 0.1 ml (500 µg) of Freund's adjuvant (heat inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil [Donaldson *et al.*, 1993]. Rats of similar ages served as controls. All procedures followed the guidelines of the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were previously approved by the Ethics Committee for Animal Experimentation of the State University of Maringá (Protocol 105/2014-CEEA).

Experimental design

Forty-two rats were randomly distributed into six groups (n=7 per group): controls (C), to non-treated; treated controls (CCO 0.58), which were treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$; treated controls (CCO 1.15), treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$; arthritic

rats (A), to non-treated; treated arthritic rats (ACO 0.58), which were treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$; and treated arthritic rats (CCO 1.15), which were treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$. Controls and arthritic treatment were also done with corn oil, horewer the results were very similar to those not treated, thus we obtained to leave untreated animals at work.

Rats were treated once a day in the morning by oral administration (gavage) of the copaiba oil solution for 5 days prior to the induction of arthritis and by additional 18 days after. The daily doses of copaiba oil were established considering the anti-inflammatory effective dose that caused no toxicity as previously described [Sachetti *et al.*, 2009].

Evaluation of inflammatory response

The weight of animals and the evaluation of the adjuvant-induced inflammatory response were carried out over a 18-day period. Following adjuvant inoculation, the volume of both hind paws up to the tibiotarsal joint was measured by plethysmography, as previously described [Bracht *et al.*, 2012]. The results were expressed in terms of increased paw volume in relation to the initial volume (volume at day 0). The appearance and severity of secondary lesions were also assessed from 10^{th} day to 18^{th} day as the following score graded from 0 to 5: (+1) appearance of nodules in the tail; (+1 or +2) appearance of nodules in one or both ears; and (+1 or +2) appearance of swelling in one or both forelimbs [Bracht *et al.*, 2012]. It was also weighed the adrenal glands, lymphatic, popliteal and inguinal nodules, which were removed immediately after the sacrifice of animals.

Blood collection and tissue preparation

Rats fasted for 12 h were deeply anesthetized with sodium thiopental ($100\ mg \cdot kg^{-1}$) plus lidocaine ($10\ mg \cdot Kg^{-1}$) and the peritoneal cavity was surgically exposed. Blood was then collected from the cava vein and deposited into tubes with 100 IU mL^{-1} of sodium heparin. Subsequently the liver was removed and divided into two parts: one was immediately freeze-clamped and stored in liquid nitrogen for oxidative status assessment and the other was used for histological processing.

The blood was centrifuged at 3,000*g* for 10 min and the supernatant was separated as the plasma fraction. For preparing the liver homogenate, the freeze-clamped portion of tissue was homogenized in a Van Potter-Elvehjem homogenizer with 10 volumes of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and an aliquot was separated for use as total homogenate. The remaining homogenate was centrifuged at 11,000*g* during 15 min and the supernatant separated as the soluble fraction of the homogenate.

For histological processing, the liver samples were fixed in 10% Bouin solution, dehydrated in graded ethanol, cleared in xylol and embedded in paraffin blocks. Semi-serial 6 µm thick cross-sections of liver were prepared with a rotary microtome (Leica RM2245), mounted on slide and stained with hematoxylin-eosin to determine morphology.

Histological analysis

The morphologic and morphometric analysis was done with images from the region near the central vein of the liver parenchyma. The images were captured from an optical microscope (Olympus BX41®, Japan) with a QColor3® camera (Olympus American INC, Canada), coupled to a software Q-Capture® [Almeida *et al.*, 2013]. The hepatocytes number and area was performed using the program Image-Pro Plus® 4.5 (Media Cibernetics). For number of hepatocytes, 50 images per animal were counted in an area of 329,972.45 μm^2 per image, totalling 250 images per group. For hepatocytes area were measured 200 hepatocytes per animal, totalling 1000 hepatocytes per group (μm^2).

Plasma analytical assays

The total antioxidant capacity (TAC), protein sulfhidryl groups (thiols), protein carbonyl groups and myeloperoxidase (MPO) activity were measured in the plasma. Albumin, bilirubin, creatinine, and aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were measured in the plasma to evaluate the liver and kidneys damage using commercial Kits (Gold Analisa®).

The TAC of plasma was measured by spectrophotometry using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)or ABTS [Erel, 2004]. The

total antioxidant capacity was calculated from the standard curve prepared with trolox (6-hydroxy-2,5,7,8-tetramethylchloraman-2-carboxylic acid), a water-soluble analog of vitamin E, and the results were expressed as nmol (mL plasma)$^{-1}$.

The thiol content was measured by spectrophotometry (412 nm) using DTNB (5,5'-dithiobis 2-nitrobenzoic acid) as previously described [Faure & Lafond, 1995]. The thiols contents were calculated using the molar extinction coefficient (ε) of 1.36×10^4 $M^{-1} \cdot cm^{-1}$ and the values were expressed as nmol (mg protein)$^{-1}$. The protein carbonyl groups content was measured by spectrophotometry using 2,4-dinitrophenylhydrazine [Levine *et al.*, 1990]. The protein carbonyl groups contents were calculated using the molar extinction coefficient (ε) of 2.20×10^4 $M^{-1} \cdot cm^{-1}$ and the values were expressed as nmol (mg protein)$^{-1}$.

Myeloperoxidase (MPO) activity was measured in the plasma by the increase in absorbance due to oxidation of *o*-dianisidine in 460 nm were expressed as μmol·min^{-1}·(mg protein)$^{-1}$ [Bradley *et al.*, 1982]. The activity was calculated from the molar extinction coefficient (ε) of 11.3×10^3 $M^{-1} \cdot cm^{-1}$.

Liver oxidative stress parameters

Protein carbonyl groups: the levels of carbonylated proteins were measured in the supernatant of liver homogenate by spectrophotometry with DNPH same as described for the plasma [Levine *et al.*, 1990].

Reactive oxygen species (ROS): the levels of ROS were quantified by spectrofluorimetry with the 2',7'-dichlorofluorescin diacetate (DCFH-DA) [Siqueira *et al.*, 2005]. The assay quantify the oxidation of DCFH-DA to the fluorescent 2',7'-dichlorofluorescin (DCF), in the presence of sterases and and oxygen reactive species (ROS). The formation of DCF was measured after stopping the reaction with ice using a spectrofluorimeter RF-5301 (Shimadzu) with the excitation and emission wavelengths at 504 and 529 nm, respectively. A standard curve with oxidized dichlorofluorescein (DCF) was used to express the results as nmol·(mg of protein)$^{-1}$.

Glutathione assay: reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured spectrofluorimetrically (excitation at 350 nm and emission at 420 nm) by means of the *o*-phthalaldehyde (OPT)

assay as previously described [Hissin & Hilf, 1976]. The fluorescence was estimated as GSH. For the GSSG assay, the sample was previously incubated with 10 mM N-ethylmaleimide and subsequently with a mixture containing 1 M NaOH and 0.4 µM OPT to detect the fluorescence. Standard curves were prepared with GSH or GSSG and the contents were expressed as nmol·(mg protein)$^{-1}$.

Antioxidant enzymes assays: activity of catalase and superoxide dismutase (SOD) were assayed in the supernatant of the liver homogenate. Catalase activity was estimated by measuring the change in absorbance at 240 nm using H_2O_2 as substrate and expressed as mmol·min^{-1}·(mg protein)$^{-1}$ [Bergmeyer, 1974]. The result were calculated using the molar extinction coefficient (ε) of 9.6 *x* 10^{-3} M^{-1}·cm^{-1}. SOD activity was estimated by its capacity to inhibit the pyrogallol autoxidation in alkaline medium at 420 nm [Marklund & Marklund, 1974]. One SOD unit was considered the quantity of enzyme that was able to promote 50% inhibition and the results were expressed as units·(mg protein)$^{-1}$. Total protein was assayed as described by Lowry *et al.* [1951].

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of GraphPad Prism Software (version 5.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and a Newman-Keuls post-hoc test was applied with the 5% level ($p<0.05$). For hepatocyte area were done Kruskal-wallis post test.

RESULTS

Antioxidant activity in vitro

The EC_{50} of the copaiba oil measured by DPPH and ABTS assays were, respectively, 19.3 ± 2.5 and 9.9 ± 0.6 $mg \cdot ml^{-1}$, which are consistent with a low antioxidant activity *in vitro*.

Effects of copaiba oil on the development of adjuvant arthritis

Figure 1 shows the increase of volume (edema) in the injected paw (Panel A) and non-injected paw (Panel B). The initial volume of the hind paw before the injection was 1.51 ± 0.08 ml. An inflammatory reaction in the injected paw was observed on the first day post-adjuvant injection for non-treated arthritic rats (+106%) and arthritic rats treated with the oil at dose of 0.58 $g \cdot Kg^{-1}$ (+110%) and 1.15 $mg \cdot Kg^{-1}$ (+93%). These paw volumes remained relatively constant until the seven day and after increased progressively until the end of the experimental period (18 days in Figure 1A). At 18 day, the paw volume of non-treated arthritic rats was 274% higher than those at day one. The increase of paw volume of arthritic rats treated with the oil at a dose 1.15 $g \cdot Kg^{-1}$ at day 18 was 29% lower than the non-treated arthritic rats, but it was not different for arthritic rats treated with the oil at a dose of 0.58 $g \cdot Kg^{-1}$.

The volume of non-injected paw of treated and non-treated rats did not modified significantly until the day 10 (Figure 1, Panel B), when started to increase progressively until day 18 for non-treated arthritic rats (+126% compared to those at day one). At 18 day, the increase of non-injected paw volumes were 43 and 51% lower, respectively, for arthritic rats treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$ (compared with non-treated arthritis).

The secondary lesions appeared at day 10 and reached the highest scores at day 18 after adjuvant injection, when reached the score of 5 for arthritic non-treated animals (Figure 1C). The scores at the end of experimental period were not different for treated arthritic rats. However, the treatment of arthritic animals with both doses of copaiba oil was able to attenuate the secondary lesions between the day 10 and 15 (Figure 1C).

The body weight of the rats was monitored before starting the treatment and at the day 18 after adjuvant injection. The mean of the initial weight of animals was 170 ± 3.0 g and the values of final weight is shown in Table 1. The weight of non-treated controls was 67% higher at the day 18. The body weight gain was similar for controls treated with the oil at a dose of 0.58 $g \cdot Kg^{-1}$, but the weight gain was lower (+40%) for controls treated at a dose of 1.15 $g \cdot Kg^{-1}$. Non-treated and treated arthritic rats practically did not gain body weight during this period. The liver weight of control and arthritic rats treated with both doses of copaiba oil were approximately 30% higher than the non-treated rats (Table 1). The weight of adrenals and popliteus and inguinal lymph nodes of arthritic rats were greatly increased when compared to the controls (Table 1). The treatment of control rats with the copaiba oil did not modify the weight of these organs, but the treatment of arthritic rats with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$ was able to decrease the weight of them between 20-50%, except the weight of the inguinal lymph node left. However, the treatment of arthritic rats with the oil at a dose of 1.15 $g \cdot Kg^{-1}$ did not modify the weight of these organs.

Plasma oxidative status

The plasma oxidative status was evaluated by measuring protein carbonyl groups, total antioxidant capacity (TAC), protein thiol groups and myeloperoxidase activity. This last is also a pro-inflammatory parameter. The results are shown in Figure 2. The levels of protein carbonyl groups and myeloperoxidase activity, two pro-oxidant parameters, were 40 and 63% higher in the plasma of non-treated arthritic rats when compared to the controls. The treatment of arthritic rats with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$ was able to decrease the myeloperoxidase activity (-30%; Figure 2A), but did not modified the levels of carbonyl protein groups (Figure 2B). The total antioxidant capacity and thiols groups, two antioxidant parameters, in the plasma of arthritic rats were 39 and 58% lower than the controls and the treatment with copaiba oil was not able to increase these parameters (Figure 2C and D).

Liver oxidative stress

Oxidative injury of the liver was evaluated by measuring the levels of protein carbonyl groups in the homogenate, which were 25% higher in the rats with adjuvant arthritis (compared to the controls; Figure 3A). The treatment of arthritic animals with copaiba oil 0.58 $g \cdot Kg^{-1}$ was not able to decrease the levels of protein carbonyl groups, but the treatment with the oil at a dose of 1.15 $g \cdot Kg^{-1}$ was able to decrease the protein carbonyl groups to values close the control ones. Treatment of control rats did not modify the levels of protein carbonyl groups in the liver.

Tissue oxidative injury is normally caused by increases in the levels of oxygen reactive species (ROS), whose liver contents were 84% higher in the rats with adjuvant arthritis (compared to the controls; Figure 3B). The treatment of arthritic animals with copaiba oil 0.58 $g \cdot Kg^{-1}$ was not able to decrease the levels of ROS in the liver, but the treatment with the oil at a dose of 1.15 $g \cdot Kg^{-1}$ was able to decrease them to values close to the control ones.

Liver antioxidant status

The antioxidant status of the liver was evaluated by means of glutathione levels and activity of the enzymes catalase and superoxide dismutase (SOD). The results are shown in Table 2 and 3. The levels of GSH in the liver of arthritic rats were only one third of those in the controls (Table 2). The treatment of arthritic rats with copaiba oil at both doses was able to increase the GSH levels to values higher than the controls. The GSH levels in the liver of control rats were 58% higher at a dose of 1.15 $g \cdot Kg^{-1}$. The GSSG levels in the liver were similar for all groups. The GSH/GSSG ratio was 73% lower in the arthritis when compared to the controls, and the treatment was effective in re-established the control levels. The catalase activity in the liver of arthritic rats was only 20% of those in the control rats (Table 3). The treatment with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$ was able to increase the catalase activity in the arthritic rats at 68 and 136%, respectively.

Plasma biochemical parameters

The plasma AST, ALT and alkaline phosphatase (ALP) activities and the levels of plasma albumin of control and arthritic rats were measured to

evaluate if the treatment with copaiba oil is able to cause liver damage. Plasma creatinine levels was assessed as parameter of renal damage. The results are shown in Table 4. The arthritis induction only diminished the albumin levels (-32%). The treatment with copaiba oil 0.58 $g \cdot Kg^{-1}$ increased only discretely the plasma activity of ALT in arthritic and control rats and the alkaline phosphatase in the control rats. The creatinine levels was also increased in the control rats treated with copaiba oil at both doses. The alteration on the plasma creatinine was discrete and only in the control treated with copaiba oil. Therefore, it is not enough to affirm renal damage.

Liver histology

The lower body weight gain, the increased liver weight and the discrete alterations of plasma enzymes activity in the treated control rats may indicate a possible toxic effect of the copaiba oil, especially on the liver. Therefore, morphological and morphometric analysis was performed in the liver of control and arthritic rats non-treated and treated with copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$. Figure 4 depicts the morphology of liver sections stained with hematoxilin-eosin. Non-treated control rats showed a normal histological structure as noted by typical central vein and portal space, and hepatocytes arranged in slightly cords with central nucleus and preserved cytoplasm. Non-treated arthritic rats showed some alterations, as sinusoid dilatation, distortion of the normal architecture of hepatocytes and encapsulated inflammatory foci (yellow arrow in Figure 4). Controls and arthritic rats treated with copaiba oil showed histological alterations as sinusoid dilatation, distortion of the normal architecture of hepatocytes, loss of arrangement in cords and greater presence of Kupffer cells. In addition, treated arthritic rats showed inflammatory, however, lower than the non-treated arthritic rats (Figure 4).

The morphometric analysis of the histological sections was additionally performed and the values are shown in Table 5. The number and area of hepatocytes were, respectively 12% lower and 9% higher in the non-treated arthritic rats when compared to the controls. The number of hepatocytes was 20 and 13% lower, respectively, in the treated control and arthritic rats (compared to the non-treated rats). The hepatocytes area was 12 and 17% higher, respectively, in the treated control and arthritic rats.

DISCUSSION

The results of the present investigation will be discussed considering four main points: first, the features of the experimental model of arthritis by complete Freund's adjuvant, specifically in relation to systemic inflammation, systemic oxidative stress and morphology; second, the effects of the treatment with copaiba oil on the inflammation of arthritic rats; third, the effects of treatment with copaiba oil on the oxidative state of plasma and liver of arthritic rats; and finally, the effects of the treatment with copaiba oil on the morphology of liver from control and arthritic rats. The potential hepatotoxicity of the copaiba oil will be briefly discussed in this last topic. These four points will be discussed separately in the following paragraphs.

The arthritis by complete Freund's adjuvant in rats shares many features of human rheumatoid arthritis, as sinovial hyperplasia, systemic inflammation and cachexia [Stolina *et al.*, 2009; Szekanecz *et al.*, 2000]. The experimental model used in the present study is induced by a high dose of adjuvant (500 ug; see Methods) and is considered a severe arthritis model in rats, which shows a widespread inflammatory response [Bracht *et al.*, 2015; Comar *et al.*, 2013; Bracht *et al.*, 2012]. Our results showed that arthritic rats developed an intense inflammatory response to adjuvant in the injected paw and also in the contralateral paw (polyarthritis). The animals furthermore presented signs of cachexia and systemic inflammatory manifestations, as evidenced by higher plasma myeloperoxidase activity, low levels of plasma albumin, severe secondary lesions to arthritis in the tail and ears associated with increased weight of adrenals and lymph nodes. In addition, the histological alterations observed in the liver corroborate previous findings [Ritter *et al.*, 2013]. Our results also showed that arthritic rats presented an increased oxidative stress in the plasma and in the liver, which were already previously described [Bracht *et al.*, 2015; Comar *et al.*, 2013].

The results of the present work also show that arthritic rats presented an increased oxidative stress in the plasma and in the liver, which were already described by previous studies [Bracht *et al.*, 2015; Comar *et al.*,

2013]. The oxidative stress is increased in several organs of arthritic rats, but it is quite pronounced in the liver, which presents higher levels of ROS and protein carbonyl groups associated with decreased activity of antioxidant enzymes, specially catalase, and diminished levels of reduced glutathione [Comar *et al.*, 2013]. The oxidative state has already been described as strongly changed in the plasma of rats with adjuvant-induced polyarthritis, which presented increased levels of protein carbonyl groups associated to decreased total antioxidant capacity and protein thiol groups [Bracht *et al.*, 2015].

Concerning the anti-inflammatory effects of copaiba oil on the arthritis induction and its systemic manifestations, the treatment was only partially effective since it was able to decrease the contralateral paw edema, but it was not able to diminish the secondary lesions to arthritis in the tail and ears. In addition, at a dose of 0.58 $g \cdot Kg^{-1}$ was able to decrease considerably the weight of adrenals and lymph nodes, and the plasma myeloperoxidase activity. It is important considerate that the adjuvant arthritis used in the present study is severe with a generalized inflammatory response. Therefore, the only moderate effect of copaiba oil on arthritis observed cannot be underestimated and even reinforce the anti-inflammatory actions previously demonstrated: improvement of carrageenan-induced paw edema in rats [Basile *et al.*, 1988], inhibition of the lipopolysaccharide (LPS)-induced nitric oxide production in macrophages [Destryana *et al.*, 2014; Veiga Jr *et al.*, 2007], inhibition of the zymozan-induced pleurisy in mice and rats [Kobayashi *et al.*, 2011; Veiga Jr *et al.*, 2007], inhibition of the LPS-stimulated proinflammatory cytokines (IL-1β, IL-6 and TNF) release by HTP1 human monocytes in culture [Gelmini *et al.*, 2013], and inhibition of the myeloperoxidase activity in ischemia-reperfusion skin flaps in rats [Lima-Silva *et al.*, 2009].

The copaiba oil component that was responsible by the anti-inflammatory effect has not been actually confirmed but it is very probable to be the *β*-caryophyllene since it presents a well-documented anti-inflammatory activity [Calleja *et al.*, 2013; Leandro *et al.*, 2012; Legault & Pichette, 2007] and it accounts for exactly 37.6% of the oil composition that was used in the present study [Ziech *et al.*, 2013]. Similarly, the anti-inflammatory mechanism of copaiba oil has not also been actually

demonstrated, however, it has been attributed to the inhibition of NF-κB translocation to the nucleus and, consequently, inhibition of expression and releasing of proinflammatory cytokines [Gelmini *et al.*, 2013].

The copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$ was effective in decreasing the weight of adrenal glands and lymph nodes, but it was not effective at a dose of 1.15 $g \cdot Kg^{-1}$. This result seems apparently contradictory, however, the treatment of control rats with copaiba oil 1.15 $g \cdot Kg^{-1}$ also decreased the body weight gain and the number of hepatocytes while increased the liver weight and the area of hepatocytes. Control rats treated with copaiba oil 1.15 $g \cdot Kg^{-1}$ additionally showed histological alterations in the liver, such as sinusoid dilatation, distortion of the normal architecture of hepatocytes and loss of arrangement in cords. These modifications in the control rats seem indicate a harmful action of the oil at a dose of 1.15 $g \cdot Kg^{-1}$. These findings deserve an additional attention and some comments about them will be made further with the comments about the liver metabolism alterations.

Regarding the action of copaiba oil on oxidative stress, the treatment was effective in decreasing the oxidative stress in the liver only at a dose of 1.15 $g \cdot Kg^{-1}$. There are three possible mechanisms to the copaiba oil improve the oxidative stress in the liver: (1) decrease the inflammatory process; (2) direct antioxidant activity of the copaiba oil constituents; or (3) its capacity of stimulating the endogenous antioxidant system. In relation to the mechanism (1) it is a general notion that inflammatory cells are responsible for the production of an excess of reactive species and other inflammatory mediators, which may cause oxidative and inflammatory injuries. Thus, if the inflammation is diminished, consequently, the oxidative injuries also is decreased. However, the copaiba oil at the dose of 0.58 $g \cdot Kg^{-1}$ was no able to decrease the oxidative stress in the liver of arthritic rats, but it was more effective as anti-inflammatory than the dose of 1.15 $g \cdot Kg^{-1}$. Therefore, it is improbable that the liver oxidative stress is been decreased by anti-inflammatory action. This suggests that copaiba oil can be acting via the other two mechanisms: direct antioxidant activity via free radical scavenging (2) and/or stimulating the endogenous antioxidant system (3). Both mechanisms act by neutralizing the reactive species released by the inflammatory cells which avoid the oxidative injury in the liver.

The isolated β-caryophyllene showed an effective direct antioxidant action via free radical scavenging against hydroxyl radical, superoxide anions and lipid peroxides [Calleja *et al.*, 2013]. As copaiba oil used in the present study contains a higher amount of β-caryophyllene, it would be expected an effective free radical scavenging activity for the copaiba oil. However, its antioxidant activity *in vitro* was low when compared with the isolated β-caryophyllene [Calleja *et al.*, 2013] or with other compounds which present a well documented free radical scavenging activity [Biazon *et al.*, 2016]. On the other hand, the antioxidant system, specifically the liver GSH content and catalase activity, were increased by copaiba oil treatment in both doses. So, it is possible that copaiba oil is stimulating the endogenous antioxidant system in the liver. This hypotheses is still reinforced by ineffectiveness of copaiba oil in decreasing the oxidative stress in the plasma, where the enzymes and glutathione contribute poorly and the antioxidant activity depends mainly of albumin thiol groups, which is diminished in the arthritis as consequence of severe chronic inflammation [Bracht *et al.*, 2015].

The histological alterations in the liver of control rats treated with copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$ can be associated with the others alterations observed in this study, such as the lower body weight gain and the higher weight of adrenals and lymph nodes, which reinforce the hypothesis that copaiba oil was harmful for the rats. On the other hand, the plasma markers of hepatic damage were not changed or only slightly increased by treatment with the oil, specifically the alkaline phosphatase activity, and were not sufficiently high so as to state conclusively that liver damage occurred, however, they are by no means negligible, especially when associated with liver histological alterations. Moderate increases in the plasma alkaline phosphatase were also previously observed, in which health rats were treated with copaiba oil and it was associated with a possible impairment of bile secretion, such as an initial cholestasis or hepatic vascular congestion [Brito *et al.*, 2000]. In addition, previous work demonstrated that a single copaiba oil dose of 5.5 $g \cdot Kg^{-1}$ was sufficient to increase 30 times the plasma activity of alkaline phosphatase, however, without altering the values of plasma AST and ALT activities, an effect that was attributed to cholestasis development [Botelho *et al.*, 2010]. It is

import emphasize that the doses used in the present work exceed approximately 10 times the recommended for humans [Brito *et al.*, 2000], however, the safe use of copaiba oil at doses lower than 2.0 $g \cdot Kg^{-1}$ as demonstrated in previous work [Sachetti *et al.*, 2009; Basile *et al.*, 1988] should be viewed with caution and more investigations is needed in order to stipulate a real safe dose for the copaiba oil.

In summary, it can be said that the results of the present study revealed that (I) adjuvant-induced arthritis in rats is associated with an intense inflammatory response with systemic manifestations; (II) Copaiba oil presented a moderate anti-inflammatory effects on the arthritis induction and improve the systemic manifestation at a dose of 0.58 $g \cdot Kg^{-1}$ (III) the treatment at a dose of 1.15 $g \cdot Kg^{-1}$ was effective to decrease the oxidative stress in the liver, one action that can be attributed to the stimulation in endogenous antioxidant system; (IV) treatment with copaiba oil 1.15 $g \cdot Kg^{-1}$ was not effective in improving the histological alterations in the liver of arthritic rats; and finally (V), it caused histological alterations in the liver of control rats, which associated with the lower body weight gain and higher liver weight can indicate a possible harmful action of the oil. Thus, the use of copaiba oil as an adjuvant in the treatment of rheumatoid arthritis in humans must be regarded with caution because it may additionally be harmful, specially for the liver, where great modifications were observed.

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Table 1. Body and organs weight of control and arthritic rats treated with copaiba oil. The mean of the initial weight of animals was 170 ± 3.0 g. C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}.

Parameter	**Groups**					
	C	**CCO 0.58**	**CCO 1.15**	**A**	**ACO 0.58**	**ACO 1.15**
Body weight (g)	284.7 ± 7.6^{a}	282.0 ± 7.9^{a}	238.3 ± 5.8^{b}	186.1 ± 5.8^{c}	198.5 ± 5.7^{c}	204.8 ± 3.5^{c}
Liver (g)	7.7 ± 0.4^{a}	10.9 ± 0.4^{b}	9.9 ± 0.7^{b}	6.8 ± 0.3^{a}	9.8 ± 0.3^{b}	9.5 ± 0.5^{b}
	weight (mg) of fresh organ per 100 g animal body weight					
Popliteus lymph nodes right	5.2 ± 0.8^{a}	4.5 ± 0.2^{a}	5.1± 0.6^{a}	47.8 ± 4.9^{b}	30.6 ± 4.5^{c}	42.3 ± 8.6^{b,c}
Popliteus lymph nodes left	5.4 ± 0.6^{a}	4.2 ± 0.3^{a}	4.9 ± 0.4^{a}	93.5 ± 15.0^{b}	42.7 ± 7.8^{a}	80.9 ± 3.5^{b}
Inguinal lymph nodes right	8.6 ± 0.1^{a}	6.6 ± 0.8^{a}	6.5 ± 0.2^{a}	19.7 ± 2.2^{b}	12.8 ± 2.3^{a}	15.3 ± 2.2^{a,b}
Inguinal lymph nodes left	8.9 ± 0.6^{a}	6.6 ± 0.4^{a}	7.0 ± 0.5^{a}	83.1 ± 6.7^{b}	99.3 ± 15.5^{b}	109.2 ± 8.9^{b}
Adrenal gland right	8.9 ± 0.3^{a}	8.5 ± 0.5^{a}	12.2 ± 0.4^{b}	19.9 ± 0.7^{c}	16.8 ± 0.6^{d}	18.1 ± 0.3^{d}
Adrenal gland left	9.0 ± 0.3^{a}	8.6 ± 0.5^{a}	12.3 ± 0.2^{a}	22.6 ± 2.1^{b}	16.9 ± 0.7^{c}	18.0 ± 0.5^{c}

The data are the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line are different ($p < 0.05$).

Table 2. Effects of copaiba oil on the levels of reduced (GSH) and oxidized (GSSG) glutathione in the liver of control and arthritic rats. C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}.

Parameter	**Groups**					
	C	**CCO 0.58**	**CCO 1.15**	**A**	**ACO 0.58**	**ACO 1.15**
GSH (nmol·mg protein^{-1})	11.0 ± 0.7^{a}	9.7 ± 0.5^{a}	17.4 ± 2.8^{b}	3.7 ± 0.3^{c}	11.7 ± 1.3^{a}	19.1 ± 2.4^{b}
GSSG (nmol·mg protein^{-1})	1.4 ± 0.2^{a}	0.9 ± 0.1^{a}	1.8 ± 0.3^{a,b}	1.8 ± 0.3^{a,b}	1.4 ± 0.2^{a,b}	2.5 ± 0.3^{b}
GSH + 2GSSG (nmol GSH units·mg protein^{-1})	13.7 ± 2.2^{a,b}	11.2 ± 0.5^{a,b}	19.8 ± 3.3^{a}	7.5 ± 2.0^{b}	15.7 ± 1.9^{a,b}	20.0 ± 2.8^{a}
GSH/GSSG ratio	9.0 ± 1.3^{a,b}	11.6 ± 1.1^{a}	10.3 ± 2.4^{a}	2.4 ± 1.2^{b}	7.9 ± 1.2^{a}	7.2 ± 1.4^{a}

The data are the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line are different ($p < 0.05$).

Table 3. Effects of copaiba oil on the activity of catalase and superoxide dismutase (SOD) in the liver of control and arthritic rats. C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}.

Parameter	**Groups**					
	C	**CCO 0.58**	**CCO 1.15**	**A**	**ACO 0.58**	**ACO 1.15**
Catalase (mmol·min^{-1}·mg protein^{-1})	1.13 ± 0.05^{a}	0.97 ± 0.03^{b}	1.09 ± 0.08^{a,b}	0.22 ± 0.02^{c}	0.37±0.02^{d}	0.52 ± 0.03^{e}
SOD (U·mg protein^{-1})	2.9 ± 0.2^{a}	2.3 ± 0.1^{a}	3.0 ± 0.3^{a}	2.5 ± 0.1^{a}	2.3 ± 0.1^{a}	2.9 ± 0.2^{a}

The data are the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line are different ($p < 0.05$).

Table 4. Effects of copaiba oil on the plasma markers of liver and renal damage. C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}.

Parameter	Groups					
	C	**CCO 0.58**	**CCO 1.15**	**A**	**ACO 0.58**	**ACO 1.15**
AST (U·L^{-1})	80.6 ± 1.4^{a}	58.2 ± 2.5^{a}	72.9 ± 3.0^{a}	90.8 ± 7.2^{a}	94.7 ± 16.2^{a}	84.6 ± 5.9^{a}
ALT (U·L^{-1})	39.9 ± 1.5^{a,c}	60.8 ± 3.9^{b}	54.1 ± 8.9^{a,b}	30.7 ± 5.0^{c}	69.0 ± 8.9^{b}	39.9 ± 4.3^{a,c}
Alkaline phosphatase (U·L^{-1})	65.6 ± 8.0^{a}	192.1 ± 6.3^{b}	84.2 ± 9.2^{a,c}	98.4 ± 14.0^{a,c}	135.3 ± 9.6^{c}	120.8 ± 23.0^{c}
Plasma protein (mg·dL^{-1})	5.5 ± 0.2^{a}	5.7 ± 0.1^{a,b}	6.1 ± 0.2^{b}	6.3 ± 0.2^{b}	6.4 ± 0.2^{b}	6.1 ± 0.2^{b}
Plasma albumin (mg·dL^{-1})	2.2 ± 0.1^{a}	2.6 ± 0.1^{b}	2.5 ± 0.1^{a,b}	1.5 ± 0.1^{c}	2.4 ± 0.1^{a,b}	1.7 ± 0.1^{c}
Creatinine (mg·dL^{-1})	0.11 ± 0.01^{a,d}	0.36 ± 0.04^{b}	0.22 ± 0.02^{c,e}	0.05 ± 0.03^{a}	0.31 ± 0.05^{b}	0.17 ± 0.02^{d,e}

The data represent the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line differ statistically ($p < 0.05$).

Table 5. Morphometric analysis: number and area of hepatocytes. C, non-treated controls; CCO 1.15, controls treated with copaiba oil at a dose of 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}. Hematoxylin-eosin staining (x200).

Parameter	Groups			
	C	**CCO 1.15**	**A**	**ACO 1.15**
Number of hepatocyte	1090.5 ± 5.3^{a}	875.6 ± 2.9^{b}	962.9 ± 5.5^{c}	837.4 ± 4.4^{d}
Hepatocyte Area (μm^2)	159.3 ± 0.9^{a}	177.9 ± 0.9^{b}	174.1 ± 0.9^{c}	202.9 ± 1.2^{d}

Number of hepatocyte in useful area of 329972.45 μm^2. The data represent the mean ± standard error of the mean of 5 animals. The number of hepatocytes has 250 image per group and the area has 1000 hepatocytes per group. Values with different superscript letters in the same line are different ($p < 0.05$).

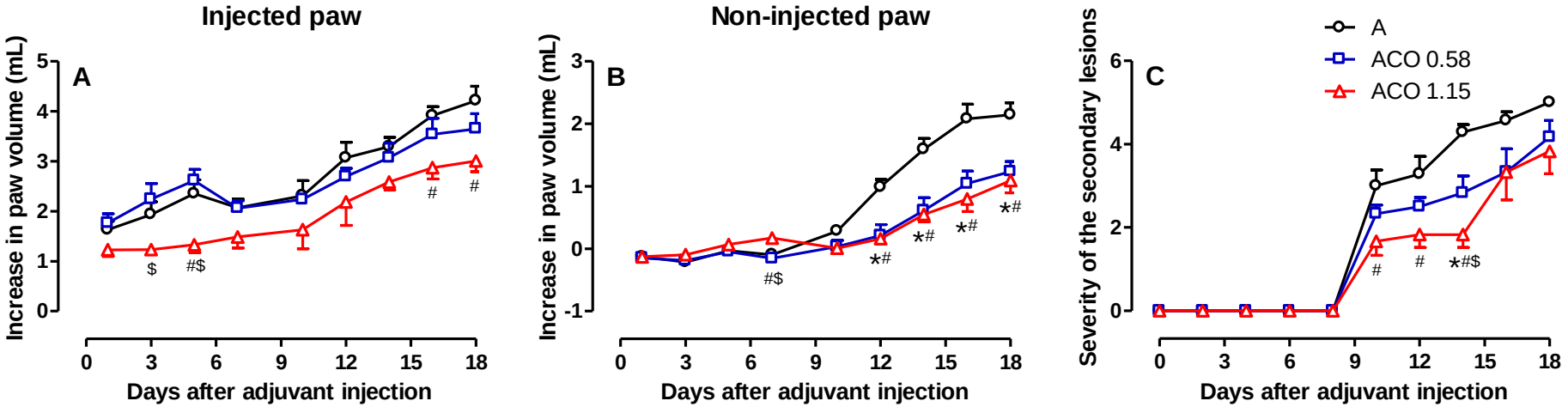

Figure 1. Effects of copaiba oil treatment on the development of the inflammatory response to Freund's adjuvant in arthritic rats. (A) edema of injected paw; **(B)** edema of non-injected paw; **(C)** score of secondary lesions. The volume of the paws was monitored by plethysmography and the values are expressed as the increase in the paw volume. The score of severity of the secondary lesions was monitored as described in Methods. A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at dose of 1.15 g·Kg^{-1}. Each point represents the mean ± standard error of the mean of 4-7 animals. *p<0.05 for difference between A and ACO 0.58; $^{\#}$p<0.05 for difference between A and ACO 1.15; and $^{\$}$p<0.05 for difference between ACO 0.58 and ACO 1.15.

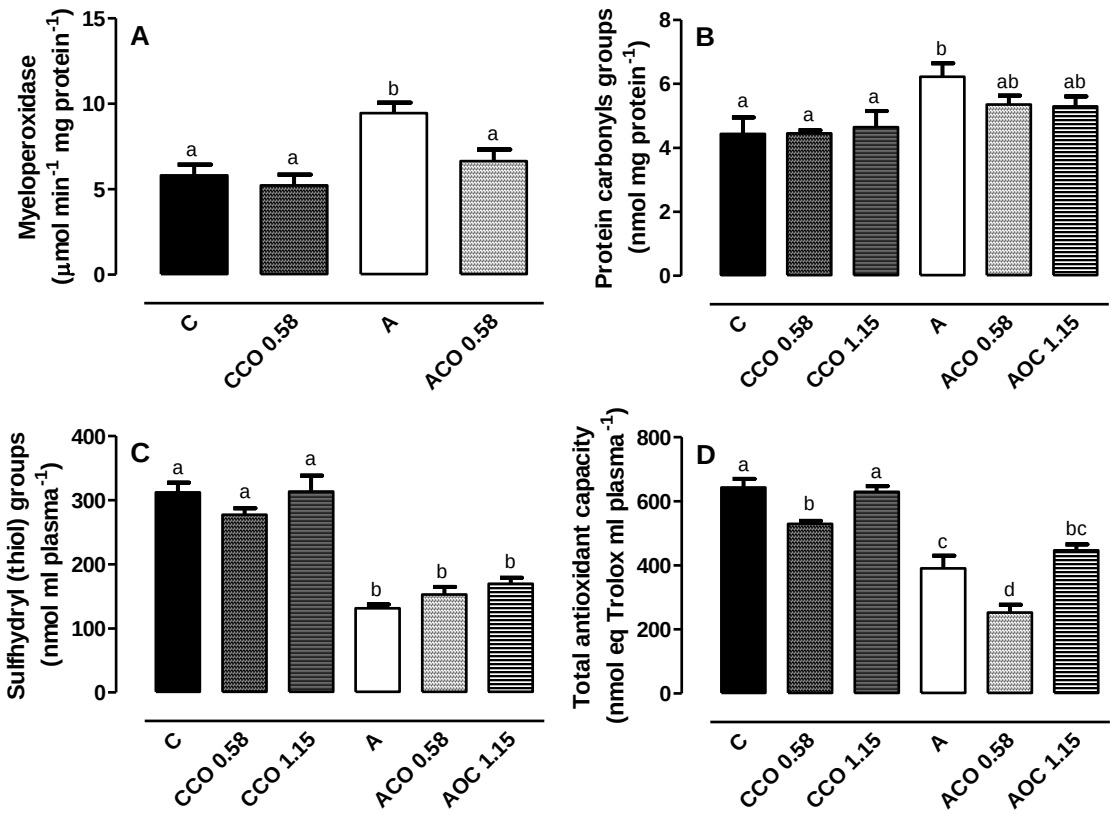

Figure 2. Effects of copaiba oil in the plasma oxidative stress of control and arthritic rats. (A) myeloperoxidase activity; **(B)** levels of protein carbonyl groups; **(C)** protein sulfhydryl (thiol) groups; and **(D)** total antioxidant capacity (TAC). The letters under each column represent: C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; and ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}. The data represent the mean ± standard error of the mean of 3-7 animals. Values with different superscript letters are different ($p < 0.05$).

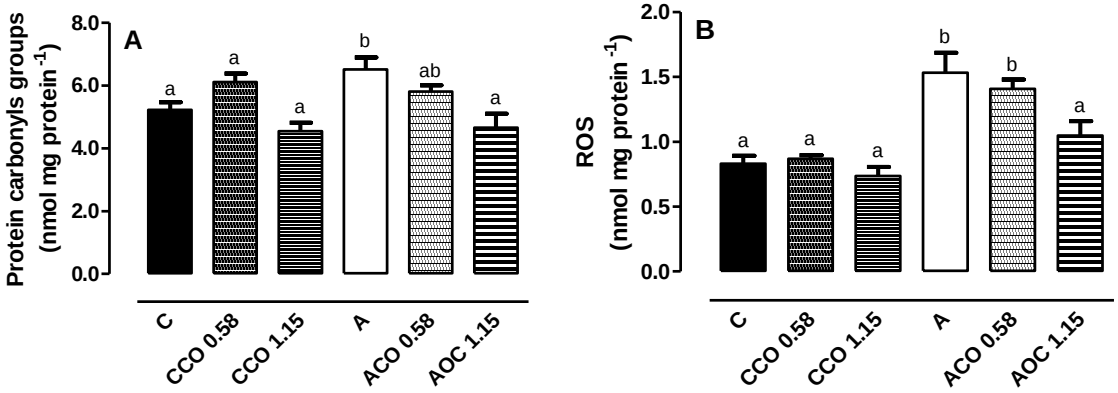

Figure 3. Effects of copaiba oil on the liver oxidative stress. (A) protein carbonyl groups; **(B)** oxygen reactive species (ROS). The letters under each column represent: C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$; CCO 1.15, controls treated with copaiba oil 1.15 $g \cdot Kg^{-1}$; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$; and ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$. The data represent the mean ± standard error of the mean of 4-9 animals. Values with different superscript letters are different ($p < 0.05$).

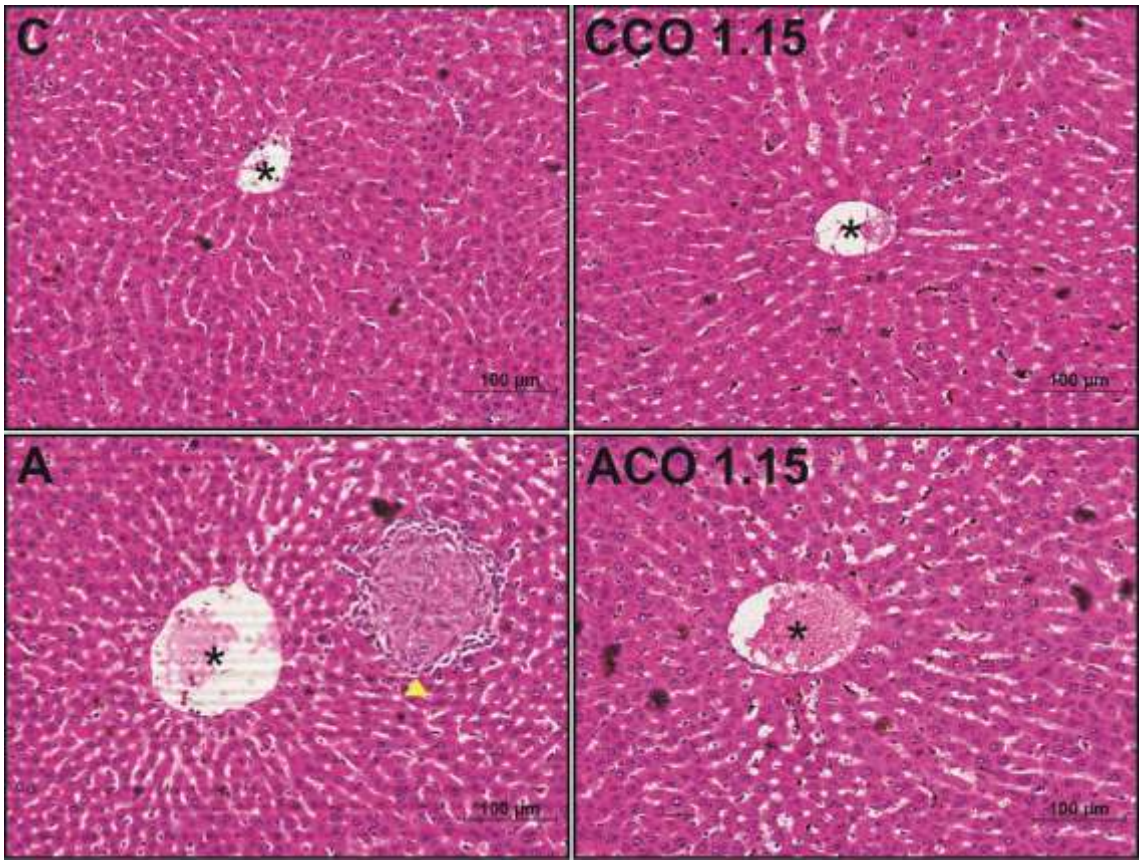

Figure 4. Photomicrographs of liver sections of control and arthritics rats non-treated and treated with copaiba oil. C, non-treated controls; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; and ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}. Hematoxylin-eosin staining (x200). Scale = 100 µm. *Indicates vein center lobular. The arrow indicates inflammatory focus.

Actions of copaiba oil (*Copaifera reticulata*) on the liver metabolism of rats with adjuvant-induced arthritis

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ABSTRACT

Adjuvant-induced arthritis is an experimental immunopathology in rats that is often used as a model for studying autoimmune chronic inflammation and inflammatory cachexia. In these animals the metabolism is altered in the liver. Therefore, the purpose of the present study was investigate the actions of copaiba oil (*Copaifera reticulata*) on the glycogenolysis, glycolysis and gluconeogenesis in the livers of arthritic rats where morphological alterations has been reported to occurs in the presence of this oil. It was additionally investigated the respiratory activity of mitochondria isolated from livers of the rats. Holtzman rats were distributed into six groups: controls; controls treated with copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$, arthritics, and arthritics treated with copaiba oil 0.58 and 1.15 $g \cdot Kg^{-1}$. The oil was administrated orally once a day during 5 days before and 18 days after arthritis induction. Glycogenolysis and glycolysis of non-treated control and arthritis rats were not different, but the gluconeogenesis was 44% lower in the arthritic rats. Copaiba oil only at a dose of 1.15 $g \cdot Kg^{-1}$ decreased the glycolysis (-65%), glycogenolysis (-58%) and gluconeogenesis (-30%) in the liver of arthritic animals. However, the gluconeogenesis was diminished in the livers of control rats treated with both doses of copaiba oil. The respiratory activity of isolated mitochondria was practically not modified by copaiba oil treatment. The results showed that hepatic metabolism in the liver of arthritic rats was modified by copaiba oil, however, the lower gluconeogenesis observed in the liver of treated control rats seems to be associated with harmful effects.

Key words*: adjuvant-induced arthritis, copaiba oil, Copaifera reticulata, oxidative status, liver metabolism, gluconeogenesis*

INTRODUCTION

Rheumatoid arthritis is an autoimmune disease characterized by chronic and systemic inflammation that affects the synovial membranes, articular cartilages and bones. Rheumatoid arthritis occurs in 0.5-1.0% of the adult population worldwide and it is associated with an increased mortality rate [Minichiello *et al.*, 2016; Uhlig *et al.*, 2014]. The pathophysiology of arthritis involves an intense hyperplasia of the synovial membrane and cartilage with participation of T and B lymphocytes, macrophages, fibroblasts, proinflammatory cytokines and overproduction of reactive species which act as mediators of tissue injury [Misko *et al.*, 2013; Kundu *et al.,* 2012]. Rheumatoid arthritis is a systemic disease and in addition to the joints other organs are affected, such as brain, heart, lungs and vascular tissue [Wartolowska *et al.*, 2012; Mcinnes & Schett, 2011; Davis et al., 2011; Voskuyl, 2006].

Metabolic alterations are equally prominent, as for example, the muscle wasting condition known as rheumatoid cachexia, which is mediated by TNF–α and IL–1β and occurs in approximately two-thirds of all patients with rheumatoid arthritis [Roubenoff, 2009]. With respect to the liver, several alterations caused by arthritis have been reported occur. Perfused livers from rats with adjuvant-induced arthritis present reduced gluconeogenesis from various substrates, increased glycolysis, modifications in the urea cycle, modifications on the calcium homeostasis and reduced metabolism of xenobiotics [Utsunomiya *et al.*, 2013; Yassuda-Filho *et al.*, 2003; Fedatto *et al.*, 2002; Fedatto *et al.*, 2000; Toda *et al.*, 1994]. In addition, the alterations of the liver metabolism is associated with pronounced oxidative stress and seem to be the consequence of systemic inflammation [Comar *et al.*, 2013].

Herbal medicines have also been largely used as complementary therapy with the aims to relieve the symptoms of rheumatoid arthritis. Among them, green tea (*Camellia sinensis*), baswellic acid (*Boswellia serrata)* and ginger (*Zingiber officinale)* have been reported to be useful in the management of rheumatoid arthritis [Gonçalves *et al.*, 2015; Al-Nahain *et al.,* 2014]. Copaiba oil is a natural compound that have a well-

documented anti-inflammatory activity and have been reported to be effective in improving some symptoms of arthritis by adjuvant in rats [Castro-Ghizoni *et al.*, 2017]. The copaiba oil is an oleoresin extracted from trees of the genus *Copaifera*, abundant in the Brazilian Amazon and worldly commercialized for several therapeutic purposes, some of them with established biological activity: anti-inflammatory, antioxidant, antitumoral, antibacterial, antipsoriasis, antinociceptive, neuroprotective and treatment of endometriosis [Borges *et al.*, 2016; Destryana *et al.*, 2014; Gelmini *et al.*, 2013; Guimarães-Santos *et al.*, 2012; Leandro *et al.*, 2012; Gomes *et al.*, 2010; Desmarchelier, 2010].

Previous study showed that copaiba oil administrated orally was only partially effective in improving the inflammatory response in rats with adjuvant-induced arthritis, an experimental model that shares many features of human rheumatoid arthritis, particularly those forms that are more severe and aggressive [Castro-Ghizoni *et al.*, 2017; Szekanecz *et al.*, 2000]. Copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$ was able to decrease the paw edema in both injected and contralateral paw, to decrease the induced-swelling of adrenals and lymph nodes, and to decrease the plasma myeloperoxidase activity. In addition, copaiba oil treatment was able to decrease the oxidative stress in the liver, an effect that was attributed to capacity of stimulating the endogenous antioxidant system, as evidenced by increments in the GSH content and catalase activity. On the other hand, copaiba oil was not able to diminish the secondary lesions due to arthritis induction in the tail and ears and, additionally, controls and arthritic rats treated with copaiba oil showed histological alterations in the liver, as sinusoid dilatation, distortion of the normal architecture of hepatocytes, loss of arrangement in cords and lower number of hepatocytes per liver area. These effects in the control rats were attributed to a possible harmful action of the oil [Castro-Ghizoni *et al.*, 2017].

Considering that control and arthritic rats treated with copaiba oil showed histological alterations in the liver [Castro-Ghizoni *et al.*, 2017], it seems reasonable to hypothesize that the hepatic metabolism should be even more altered in the liver of arthritic rats and additionally in the controls in the presence of copaiba oil. The present study was therefore planned to investigate the action of copaiba oil, extracted from *C. reticulata*

Ducke, orally administrated on the glycogenolysis, glycolysis and gluconogenesis in the livers of controls and arthritic rats. It was additionally investigated the respiratory activity of mitochondria isolated from livers of arthritic rats treated with copaiba oil. In addition to histological alterations, previous studies also indicate that the treatment of rats with copaiba oil was able to increase the plasma levels of bilirubin and activity of alkaline phosphatase without modifications in the activity of plasma ALT and AST, a phenomenon that was associated with a possible impairment of bile secretion, such as an initial cholestasis [Botelho *et al.*, 2010; Brito *et al.*, 2000]. Thus, the present study will allow to evaluate if these hepatic alterations are associated with modifications in the liver function.

MATERIAL AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Enzymes and coenzymes used in the enzymatic assays were purchased from Sigma-Aldrich Co (St. Louis, USA). All other chemicals were from the best available grade (98-99.8% purity).

Copaiba oil

Copaiba oil was provided by the Brazilian Enterprise for Agricultural Research (EMBRAPA), Center for Agroforestry Research of the Eastern Amazon, Brazil. The oleoresin was collected by means of artificial holes in the trunks of native adult trees of the species *Copaifera reticulata* Duke, which are localized at the km 67 of the National Forest of Tapajós (Belterra, PA, Brazil). A voucher specimen was deposited in the Herbarium IAN of Eastern Amazon EMBRAPA under the number 183939. The composition of the oleoresin was quantified by GC/MS and previously reported elsewhere [Ziech *et al.*, 2013] as follow: β-caryophyllene (37.6%), β-bisabolene (13.9%), *(E)*-α-bergamotene (9.3%), α-humulene + *(E)*-β-farnesene (5.3%), β-selinene (3.9%), β-elemene (3.3%), α-selinene (3.1%), α-bulnesene (2.1%), *(Z)*-α-bisabolene (1.8%), *(E)*-γ-bisabolene (1.3%), β-sesquiphellandrene (1.1%), another 14 different substances representing less than 1% each.

Animals and induction of arthritis

Male *Holtzman* rats weighting 160-180 g were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained in standard laboratory conditions at a temperature of 22±3ºC under a regulated 12h light/dark cycle. The animals were housed in conventional steel cages (3 rats/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). For the induction of adjuvant arthritis, animals (50 days old) were injected subcutaneously in the left hind paw of 0.1 ml (500 µg) of Freund's adjuvant (heat inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv),

suspended in mineral oil [Donaldson *et al.*, 1993]. Rats of similar ages served as controls. All procedures followed the guidelines of the Brazilian Council for the Control of Animal Experimentation (CONCEA) and were previously approved by the Ethics Committee for Animal Experimentation of the State University of Maringá (Protocol 105/2014-CEEA).

Experimental design

Forty-two rats were randomly distributed into six groups (n=7 per group): controls (C), to non-treated; treated controls (CCO 0.58), which were treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$; treated controls (CCO 1.15), treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$; arthritic rats (A), to non-treated; treated arthritic rats (ACO 0.58), which were treated with copaiba oil at dose of 0.58 $g \cdot Kg^{-1} \cdot day^{-1}$; and treated arthritic rats (CCO 1.15), which were treated with copaiba oil at dose of 1.15 $g \cdot Kg^{-1} \cdot day^{-1}$. This procedure was repeated three times (126 animals in total) to evaluate all parameters of this study. Rats were treated once a day in the morning by oral administration (gavage) of the copaiba oil solution for 5 days prior to the induction of arthritis and by additional 18 days after. The daily doses of copaiba oil were established considering the anti-inflammatory effective dose that caused no toxicity as previously described [Castro-Ghizoni *et al.*, 2017; Sachetti *et al.*, 2009].

Liver perfusion

The glycolysis, glycogenolysis and gluconeogenesis were evaluated in the perfused rat livers [Bracht *et al.*, 2003]. The experiments were performed in the 19^{th} days after the arthritis induction. For the surgical procedure, animals were anaesthetized with intraperitoneal injection of a mixture containing sodium thiopental (100 $mg \cdot kg^{-1}$) and lidocaine (10 $mg \cdot Kg^{-1}$). Briefly, after the cannulation of the portal and cava veins, the liver was removed and positioned in a plexiglass chamber. The perfusion fluid was a Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with a simultaneous temperature adjustment to 37ºC. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France) and was adjusted to between 30 and 33 $mL \cdot min^{-1}$, depending on the rat

weight. Samples of the effluent perfusion fluid were collected at 2 minute intervals and analyzed for their metabolite content. At the end of the perfusion, the liver was removed and weighed to allow precise metabolic calculations.

Analytical

In the effluent perfusion fluid the following compounds were assayed by means of standard enzymatic procedures: glucose, lactate and pyruvate, [Bergmeyer, 1974]. The oxygen concentration in the outflowing perfusate was monitorated continuously, employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber at the exit of the perfusate [Scholz & Bücher, 1965]. Metabolic rates were calculated from input-output differences and the total flow rates and were referred to the wet weight of the liver.

The glycolysis and glycogenolysis were evaluated in the liver of fed rats, which were perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. After stabilization in oxygen consumption, perfusate effluent samples were collected during 30 minutes. In the fed state, liver glycogen is broken down to glucose (glycogenolysis) that is in part released in the perfusate effluent and part is metabolized by the glycolysis forming lactate and pyruvate, which may also be released into the effluent perfusate. At the stead state, the glycolysis = (lactate + pyruvate)/2 and glycogenolysis = glucose + [(lactate + pyruvate)/2].

Gluconeogenesis was evaluated in the perfused liver of 12 h fasted rats, which were initially perfused with Krebs/Henseleit buffer in the absence of exogenous substrate and, after stabilization in the oxygen consumption, L-lactate (2 mM) was added to the perfusion fluid as substrate. Glucose and pyruvate were measured in the effluent perfusate for 30 minutes.

Mitochondria isolation and measurement of respiratory activity

Treated and non-treated rats were decapitated and their livers removed immediately and placed in ice-cold buffer containing 200 mM mannitol, 75 mM sucrose, 0.2 mM ethylene glycol tetraacetic acid (EGTA), 2 mM tris(hydroxymethyl) aminomethane (Tris-HCl), pH 7.4 and 50 mg%

bovine serum albumin. The tissue was minced, washed with the buffer and homogenized in the same medium by means of a Dounce homogenizer for lysing the cells. After the homogenization, the mitochondria were isolated by differential centrifugation [Bracht *et al.*, 2003] and suspended in the same medium, which was kept at 0-4°C.

Oxygen uptake by isolated mitochondria was measured polarographically using a Teflon-shielded platinum electrode [Bracht *et al*., 2003]. Mitochondria were incubated in the closed oxygraph chamber in a medium (2.0 mL) containing 0.25 M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 25mg% fatty acid-free bovine serum albumin, 10 mM Tris-HCl (pH 7.4) and three different substrates: succinate, alpha-ketoglutarate and L-glutamate + L-malate, all of them at a concentration of 10 mM. ADP, for a final concentration of 0.125 mM, was added in appropriate times. Rates of oxygen uptake were computed from the slopes of the recorder tracings and expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$ protein. The respiration control ratio (RC) and the ADP/O ratio were calculated according to Bracht *et al.* [2003].

Protein content of the mitochondrial suspension were measured by means of the method described by Lowry *et al.* [1951], using the Folin-phenol reagent and bovine serum albumin as standard.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of GraphPad Prism Software (version 5.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and Duncan's post test was applied with the 5% level ($p<0.05$).

RESULTS

Liver perfusion: glycolysis and glycogenolysis

The first experiments were planned in order to evaluate the effects of copaiba oil treatment on glycogen catabolism and glycolysis. Liver from fed rats when perfused with substrate-free medium survive at the expense of glycogen degradation via glycolysis and oxidation of endogenous fatty acids [Scholz & Bücher, 1965]. Under these conditions the livers release glucose, lactate and pyruvate as a result of glycogen catabolism. Figure 1 illustrate the time courses of the glycogen catabolism in the perfused livers from control and arthritic rats treated with copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$ and it represents also a typical experimental protocol, which was used for the rats treated with a dose of 0.58 $g \cdot Kg^{-1}$. After stabilization of oxygen consumption, the livers were perfused during 30 minutes with Krebs/Henseleit-bicarbonate buffer. Four parameters were measured: glucose release, lactate and pyruvate production and oxygen consumption. As noted in Figure 1, all parameters changed only minimally after 10 minutes of perfusion time.

In order to compare the groups, the rate of each parameter were taken in the steady state (20 minutes of perfusion time in Figure 1) and the values are shown in Table 1. The glucose production and the oxygen consumption were 35 and 24% lower, respectively, in the arthritic rats when compared to the controls. The pyruvate and lactate production were not different for control and arthritic rats. The treatment of control rats with copaiba oil did not modify any parameter. The treatment of arthritic rats with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$ did also not modify any parameter, but the treatment at a dose of 1.15 $g \cdot Kg^{-1}$ was able to cause a marked decrease in the glucose release (-48%), pyruvate production (-53%) and lactate production (-67%).

The values of Table 1 can be explored better if the glucose release and pyruvate + lactate production are used to calculate the glycolysis (pyruvate + lactate) and glycogenolysis [glucose + (lactate + pyruvate)/2], which were represented against the copaiba oil dose (Figure 2). As noted, the treatment of control rats did not modify both glycolysis and

glycogenolysis, but the treatment of arthritic rats with copaiba oil at a dose of 1.15 g·Kg^{-1} decreased both glycolysis (-65%) and glycogenolysis (-58%).

Liver perfusion: gluconeogenesis

The effects of treatment with copaiba oil on the gluconeogenesis was also evaluated in the perfused livers of fasted rats. Figure 3 shows the time courses of glucose production, pyruvate production and oxygen consumption of control and arthritic rats non-treated and treated at a dose of 1.15 g·Kg^{-1}. The Figure 3 also illustrate a typical experimental protocol, which was also used for the rats treated with a dose of 0.58 g·Kg^{-1}. As noted, after stabilization of oxygen consumption, the livers were additionally perfused with Krebs/Henseleit-bicarbonate buffer in the absence of exogenous substrate during 10 minutes (basal period) followed by 30 minutes in the presence of 2 mM L-lactate (gluconeogenic substrate). The basal rates of glucose and pyruvate production were minimal and similar for all groups, however, the basal oxygen consumption was 22% lower in the arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}. After the introduction of L-lactate all parameters increased, however, differently for the groups. The glucose production initially was similar for non-treated arthritic and control rats, however, at 30 minutes of lactate infusion the glucose production was lower for arthritic rats when compared to the controls (Figure 3). The glucose production for control and arthritic rats treated with copaiba oil at dose of 1.15 g·Kg^{-1} were lower than the non-treated rats initially and were equal to non-treated arthritics at the end of perfusion time (30 minutes from L-lactate infusion). The oxygen consumption after the lactate infusion was similar for non-treated control and arthritic rats but it was lower for both treated control and arthritic rats.

The Table 2 shows the increment in each parameter due to 2 mM L-lactate infusion and the values were calculated as [final values at the end of the infusion period with lactate] - [basal rates before infusion of lactate] in the Figure 3. The increment of glucose production was 44% lower in the non-treated arthritic rats when compared to non-treated controls. The increment in the glucose production was 35% and 58% lower in the liver of control rats treated with copaiba oil at a dose 0.58 and 1.15 g·Kg^{-1}, respectively. The increment in the glucose production was not different for

non-treated arthritic rats and arthritic rats treated with the oil at both doses (compared to the already decreased increment of non-treated arthritis). No difference was observed in the increment of pyruvate production in the non-treated arthritic rats when compared to controls. The increment in the pyruvate production was approximately 30% lower in the control rats treated with both doses of copaiba oil. The treatment of arthritic rats with copaiba oil did not modified the increment in the pyruvate production (compared to non-treated arthritics). No difference was observed in the increment of oxygen consumption of non-treated arthritic rats when compared to controls. The oxygen consumption was approximately 50% lower in livers of control rats treated with both doses of copaiba oil (compared to non-treated controls). The increments of oxygen consumption decreased at 31 to 59% in the arthritic rats treated with the oil at both doses.

Respiratory activity of isolated mitochondria

Histological and metabolic alterations, specifically the reduced gluconeogenesis, in the livers of controls and arthritic rats treated with copaiba oil may be associated with mitochondrial dysfunctions. Therefore, experiments with isolated mitochondria of livers from rats treated with copaiba oil 1.15 $g \cdot Kg^{-1}$ were carried to evaluate the respiratory activity and ADP consumption. Succinate, alpha-ketoglutarate and L-glutamate + L-malate were used as substrates in presence or absence of ADP. Table 3 shows the rates of oxygen consumption in absence (basal or substrate respiration) and in presence of ADP (state III respiration or coupled respiration) for all substrates. The oxygen uptake also was evaluated after the consumption of the ADP added (state IV respiration or recovering of basal respiration).

All parameters of mitochondrial respiration were not substantially changed by arthritis induction and by treatment of the animals. As noted, the basal respiration and the ADP/O ratio was not different for all groups (Table 3). The state III respiration was also not different, except by a slightly increase in the oxygen consumption in the arthritic condition (compared to controls) when L-malate + L-glutamate was used as substrate. The state IV respiration was higher in the arthritic condition

(compared to controls) only when alpha-ketoglutarate and L-glutamate + L-malate were substrates and the RC when L-glutamate + L-malate were substrates.

DISCUSSION

The arthritis by complete Freund's adjuvant in rats shares many features of human rheumatoid arthritis, as sinovial hyperplasia, systemic inflammation and cachexia [Stolina *et al.*, 2009; Szekanecz *et al.*, 2000]. The experimental model used in the present study is induced by a high dose of adjuvant (500 ug; see Methods) and is considered a severe arthritis model in rats, which shows a widespread inflammatory response [Bracht *et al.*, 2015; Comar *et al.*, 2013; Bracht *et al.*, 2012]. In this model, animals develop an intense inflammatory response to adjuvant in the contralateral paw (polyarthritis) and furthermore present signs of cachexia and systemic inflammatory manifestations, as evidenced by increased plasma myeloperoxidase activity, decreased levels of plasma albumin, severe secondary lesions to arthritis in the tail and ears associated with increased weight of adrenals and lymph nodes [Castro-Ghizoni *et al.*, 2017; Bracht *et al.*, 2015; Comar *et al.*, 2013]. The oxidative stress is increased in several organs of arthritic rats, but it is quite pronounced in the liver [Comar *et al.*, 2013], where metabolic changes were also already previously showed [Fedatto *et al.*, 2002; Fedatto *et al.*, 1999]. In addition, these animals present histological alterations in the liver, as sinusoid dilatation, encapsulated inflammatory foci and lower number of hepatocyte per hepatic area [Castro-Ghizoni *et al.*, 2017]. Metabolic changes in the liver of arthritic rats observed in the present study, specifically the lower gluconeogenesis from L-lactate, had already been previously demonstrated [Fedatto *et al.*, 2002; Fedatto *et al.*, 1999].

Concerning the liver metabolism, only gluconeogenesis was decreased in the liver of arthritic animals (compared to controls) in the present study. The glucose release in the perfused livers of fed rats was lower, but the glycogenolysis, calculated as glucose + [(lactate + pyruvate)/2], was not different for arthritic and control rats (Figure 2). The treatment with copaiba oil 1.15 $g \cdot Kg^{-1}$ decreased the glucose release, lactate and pyruvate production in the perfused livers from fed arthritic rats, but it did not modify these parameters in controls or even in arthritic rats treated with the dose of 0.58 $g \cdot Kg^{-1}$. However, the glucose release is

already diminished in non-treated arthritis and this drop of glucose release only in the arthritic condition can be associated to a possible harmful effect of copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$. The treatment can be causing an impairment of the liver function or at least an impairment of the liver glycogenolytic machinery in the arthritic animals, which would make the liver more vulnerable to copaiba oil harmful actions. Liver glycogen content (lower in the arthritic condition; Fedato *et al.*, 2000) is the main difference between control and arthritic rat livers in the fed state. Thus, it is possible suppose that the higher glycogen content may be protecting the liver of control rats against possible harmful effects of copaiba oil. It has been showed that high concentration of liver glycogen protects the liver against toxic agents-induced injury, such as that induced by carbon tetrachloride [Krahenbuhl *et al.*, 1991]. However, this can not be inferred from the present data and more investigation is needed in order to clarify this point.

The lower gluconeogenesis from L-lactate in the perfused livers of fasted arthritic rats was already observed from a variety of other precursors and it was attributed mainly to lower activity of gluconeogenic key enzymes, such as phosphoenolpyruvate carboxykinase and/or pyruvate carboxylase, associated to low availability of reducing equivalents in the cytosol [Fedatto *et al.*, 1999]. The last point reflects a lower $NAD(P)H/NAD(P)^+$ ratio and GSH/GSSG ratio in the arthritic condition, which are also responsible by the higher oxidative stress in the liver of arthritic rats [Comar *et al.*, 2013]. Treatment of arthritic rats with copaiba oil at both doses practically did not modify the liver gluconeogenesis, however, it was already decreased in the non-treated arthritic rats. On the other hand, the gluconeogenesis and oxygen consumption were greatly reduced in control rats treated with both doses of copaiba oil. These results seem to be consequence of harmful effects of copaiba oil, including at the dose of 0.58 $g \cdot Kg^{-1}$. It is important to highlight that the gluconeogenic pathway is extremely sensible to cellular integrity because the pathway occurs in different intracellular compartments and require energy from an aerobic system associated to membranes [Soares *et al.*, 2013]. Therefore, the histological alterations previously observed in the livers of treated control rats may be associated with cell structure alterations caused by the copaiba oil and may apparently explain the lower gluconeogenesis. However, there

was no difference between the respiratory activity and ADP/O ratio of mitochondria isolated from the livers of treated and non-treated controls, which indicate that the mitochondrial integrity and functionality was not affected by copaiba oil. However, the respiratory activity was evaluated in isolated mitochondria and it do not predict the actual mitochondrial function into the intact liver, which can be affected by other mechanisms even external to the organ. The possible impairment of the mitochondrial function caused by the copaiba oil on the intact liver can be evidenced by the lower oxygen consumption in the perfused liver, which diminished proportionally to the gluconeogenesis. Another point that can explain, at least in part, the lower gluconeogenesis and oxygen consumption of treated control rats is the lower number of hepatocytes per area of liver tissue in animals treated with copaiba oil [Castro-Ghizoni *et al.*, 2017].

The altered liver metabolism in control rats treated with copaiba oil corroborates not only the histological alterations previously observed in the liver, but also the decrease of body weight gain and increases in the weight of adrenals and lymph nodes, which reinforce the hypothesis that copaiba oil was harmful for the rats [Castro-Ghizoni *et al.*, 2017]. Plasma markers of hepatocytes damage were not changed or only slightly increased by treatment with the oil, specifically the alkaline phosphatase activity, and were not sufficiently high so as to state conclusively that hepatocytes damage occurred, however, they are by no means negligible, especially when associated with histological and metabolic alterations in the liver [Castro-Ghizoni *et al.*, 2017]. The mitochondrial integrity in the presence of the oil observed in our study partially explain other results, which showed that histological alterations in the liver were associated to normal plasma AST and ALT activity [Castro-Ghizoni *et al.*, 2017; Barbosa *et al.*, 2017; Botelho *et al.*, 2010]. Disruption of mitochondrial membranes in the liver are associated to higher activity of these enzymes in the plasma and are also associated to hepatocytes damage. Increases in the plasma alkaline phosphatase without altering the values of plasma AST and ALT activities have been reported to occur in rats treated with copaiba oil, which were attributed to cholestasis development or hepatic vascular congestion [Barbosa *et al.*, 2017; Botelho *et al.*, 2010]. Thus, it is possible that the decreased gluconeogenesis in the treated rats is associated with a hepatic

cholestasis process. Previous study has already showed that the gluconeogenic pathway is decreased in the cholestatic livers [Roselino *et al.*, 1992].

It is import emphasize that the doses used in the present work exceed approximately 10 times the recommended for humans [Brito *et al.*, 2000], however, the safe use of copaiba oil at doses lower than 2.0 $g \cdot Kg^{-1}$ as demonstrated in previous work [Basile *et al.*, 1988; Sachetti *et al.*, 2009] should be viewed with caution and more investigations is needed in order to stipulate a real safe dose for the copaiba oil.

In summary, it can be said that the results of the present study revealed that (I) Adjuvant-induced arthritis in rats is associated with an alterations in the liver metabolism, specifically the reduced gluconeogenesis from L-lactate; (II) Copaiba oil only at a dose of 1.15 $g \cdot Kg^{-1}$ decreased the glycolysis, glycogenolysis and gluconeogenesis in the liver of arthritic animals; (III) Copaiba oil at a dose of 0.58 and 1.15 $g \cdot Kg^{-1}$ decreased the gluconeogenesis in the liver of control rats (IV) Copaiba oil was not modified substantially the respiratory activity in the isolated mitochondria from livers of control and arthritic rats; (V) it is possible conclude that the metabolic changes in the liver metabolism of control and arthritic rats can be indicate a possible harmful action of the oil at a doses of 0.58 and 1.15 $g \cdot Kg^{-1}$. Thus, the use of copaiba oil as an adjuvant in the treatment of rheumatoid arthritis in humans must be regarded with caution because it may additionally be harmful, specially for the liver, where great modifications were observed.

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Table 1. Effects of copaiba oil treatment on the glycogen catabolism and oxygen consumption in the perfused liver of control and arthritic rats. The liver of control and arthritic rats in the fed state were perfused with Krebs/Henseleit bicarbonate as described in the Methods and the values are the rate in the steady-state of each parameter in the Figure 1 (20 minutes of perfusion time). C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$; CCO 1.15, controls treated with copaiba oil 1.15 $g \cdot Kg^{-1}$; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 $g \cdot Kg^{-1}$; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 $g \cdot Kg^{-1}$.

Groups	Parameter			
	Glucose release	Pyruvate production	Lactate production	Oxygen uptake
	$\mu mol \cdot min^{-1} \cdot g^{-1}$			
C	0.95 ± 0.06^{a}	0.19 ± 0.01^{a}	1.34 ± 0.32^{a}	1.77 ± 0.07^{a}
CCO 0.58	1.00 ± 0.15^{a}	0.20 ± 0.02^{a}	1.86 ± 0.24^{a}	$1.58 \pm 0.02^{a,b}$
CCO 1.15	$0.80 \pm 0.11^{a,b}$	0.17 ± 0.02^{a}	1.42 ± 0.12^{a}	1.71 ± 0.09^{a}
A	0.62 ± 0.05^{b}	0.17 ± 0.01^{a}	1.46 ± 0.17^{a}	1.35 ± 0.07^{b}
ACO 0.58	$0.63 \pm 0.04^{b,c}$	0.16 ± 0.01^{a}	1.51 ± 0.15^{a}	$1.55 \pm 0.12^{a,b}$
ACO 1.15	0.32 ± 0.07^{c}	0.08 ± 0.03^{b}	0.48 ± 0.06^{b}	1.66 ± 0.16^{a}

The data represent the mean ± standard error of the mean of 4-5 animals. Values with different superscript letters in the same column differ statistically ($p < 0.05$).

Table 2. Effects of copaiba oil treatment on the increments of glucose and pyruvate production and oxygen consumption in the perfused rat liver of fasted control and arthritic rats. The liver of fasted control and arthritic rats were perfused as the experimental protocol in the Figure 2 and the values were calculated as [final values at the end of the infusion period with lactate] - [basal rates before infusion of lactate]. C, non-treated controls; CCO 0.58, controls treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, non-treated arthritic rats; ACO 0.58, arthritic rats treated with copaiba oil at a dose of 0.58 g·Kg^{-1}; ACO 1.15, arthritic rats treated with copaiba oil at a dose of 1.15 g·Kg^{-1}.

Groups	Parameter		
	Glucose production	Pyruvate production	Oxygen uptake
		μmol·min^{-1}·g^{-1}	
C	0.71 ± 0.04^{a}	0.37 ± 0.05^{a}	0.54 ± 0.06^{a}
CCO 0.58	0.46 ± 0.05^{b}	0.25 ± 0.04^{b}	0.26 ± 0.06^{b}
CCO 1.15	0.30 ± 0.03^{c}	0.25 ± 0.01^{b}	0.20 ± 0.05^{b}
A	0.40 ± 0.07^{b,c}	0.44 ± 0.04^{a}	0.51 ± 0.04^{a}
ACO 0.58	0.38 ± 0.04^{b,c}	0.39 ± 0.03^{a}	0.35 ± 0.06^{b}
ACO 1.15	0.28 ± 0.05^{c}	0.32 ± 0.02^{a,b}	0.21 ± 0.05^{b}

The data represent the mean ± standard error of the mean of 4-5 animals. Values with different superscript letters in the same column differ statistically ($p < 0.05$).

Table 3: Effects of copaiba oil treatment on the respiratory activity of mitochondria isolated from control and arthritic rats. The concentrations of succinate, α-ketoglutarate and malate + glutamate were 10mM. The concentration ADP were 125 μM. Basal, state III, state IV as expressed as nmol·min^{-1}·(protein mg)$^{-1}$. ADP/O ratio and respiratory control (RC) were calculated as described in Methods. C, controls; CCO 1.15, controls treated with copaiba oil 1.15 g·Kg^{-1}; A, arthritic rats; ACO 1.15, arthritic rats treated with copaiba oil g·Kg^{-1}.

Parameter	Groups			
	C	**CCO 1.15**	**A**	**ACO 1.15**
		succinate		
Basal	18.9 ± 1.1^{a}	19.1 ± 1.1^{a}	23.2 ± 1.2^{a}	23.6 ± 3.1^{a}
State III	95.7 ± 2.6^{a}	102.5 ± 4.2^{a}	118.8 ± 6.3^{a}	120.9 ± 14.5^{a}
State IV	24.9 ± 1.3^{a,b}	22.4 ± 1.8^{a}	31.6 ± 2.4^{b}	27.2 ± 2.9^{a,b}
ADP/O ratio	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	2.1 ± 0.2^{a}	2.1 ± 0.1^{a}
RC	3.9 ± 0.2^{a}	4.7 ± 0.3^{a}	3.8 ± 0.3^{a}	4.5 ± 0.2^{a}
		α-ketoglutarate		
Basal	6.2 ± 0.7^{a}	6.3 ± 0.7^{a}	5.9 ± 0.7^{a}	8.1 ± 0.7^{a}
State III	31.5 ± 1.9^{a}	37.2 ± 4.1^{a}	36.1 ± 3.9^{a}	37.1 ± 3.3^{a}
State IV	11.4 ± 0.7^{a,b}	9.2 ± 0.8^{a}	13.7 ± 1.2^{b}	11.8 ± 0.6^{a,b}
ADP/O ratio	3.0 ± 0.2^{a}	3.0 ± 0.1^{a}	2.8 ± 0.2^{a}	2.9 ± 0.1^{a}
RC	2.8 ± 0.2^{a}	4.1 ± 0.5^{b}	2.7 ± 0.3^{a}	3.1 ± 0.2^{a}
		Glutamate + malate		
Basal	6.1 ± 0.5^{a}	6.4 ± 0.8^{a}	8.7 ± 0.6^{a,b}	9.4 ± 1.0^{b}
State III	57.5 ± 0.8^{a}	63.1 ± 5.2^{a,b}	74.7 ± 3.6^{b}	70.3 ± 6.0^{a,b}
State IV	10.6 ± 0.5^{a,c}	9.6 ± 0.4^{a,c}	18.4 ± 2.0^{b}	13.5 ± 1.7^{c}
ADP/O ratio	3.2 ± 0.2^{a}	3.3 ± 0.2^{a}	3.0 ± 0.2^{a}	3.3 ± 0.3^{a}
RC	5.5 ± 0.2^{a,b}	6.6 ± 0.5^{a}	4.3 ± 0.4^{b}	5.4 ± 0.5^{a,b}

The data represent the mean ± standard error of the mean of 5-6 animals. Values with different superscript letters in the same line are different ($p < 0.05$).

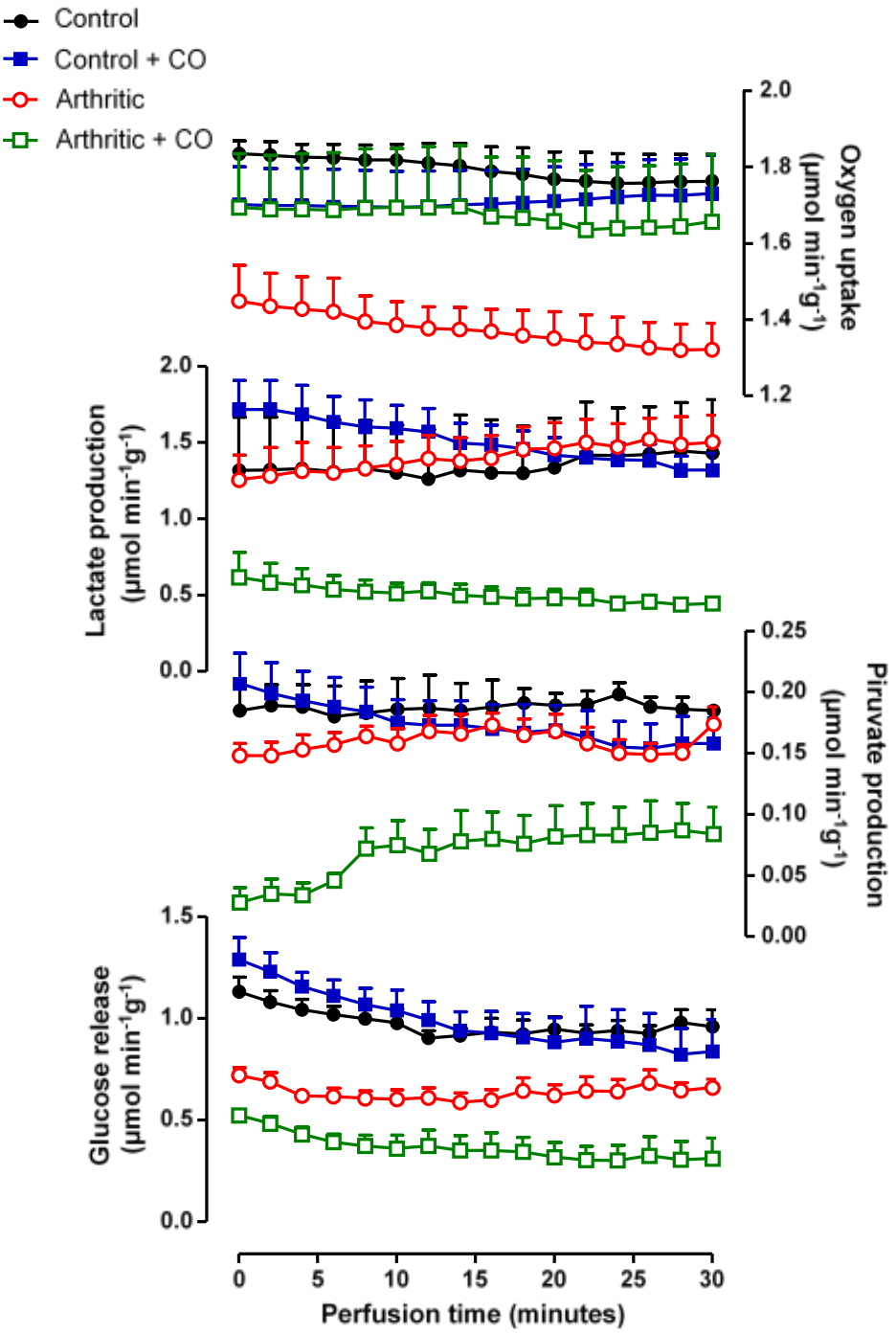

Figure 1. Time courses of the glycogen catabolism in the perfused livers from control and arthritic rats treated with copaiba oil. Control and arthritic rats were treated with copaiba oil (CO) at a dose of 1.15 g·Kg^{-1} as described in the Methods. Livers from fed rats were perfused with Krebs/Henseleit bicarbonate buffer. The effluent perfusate was sampled in 2-min intervals during 30 min and analyzed for glucose release, lactate and pyruvate production. Each data point represents the means of 4-5 liver perfusion experiments. Bars are standard errors of the mean.

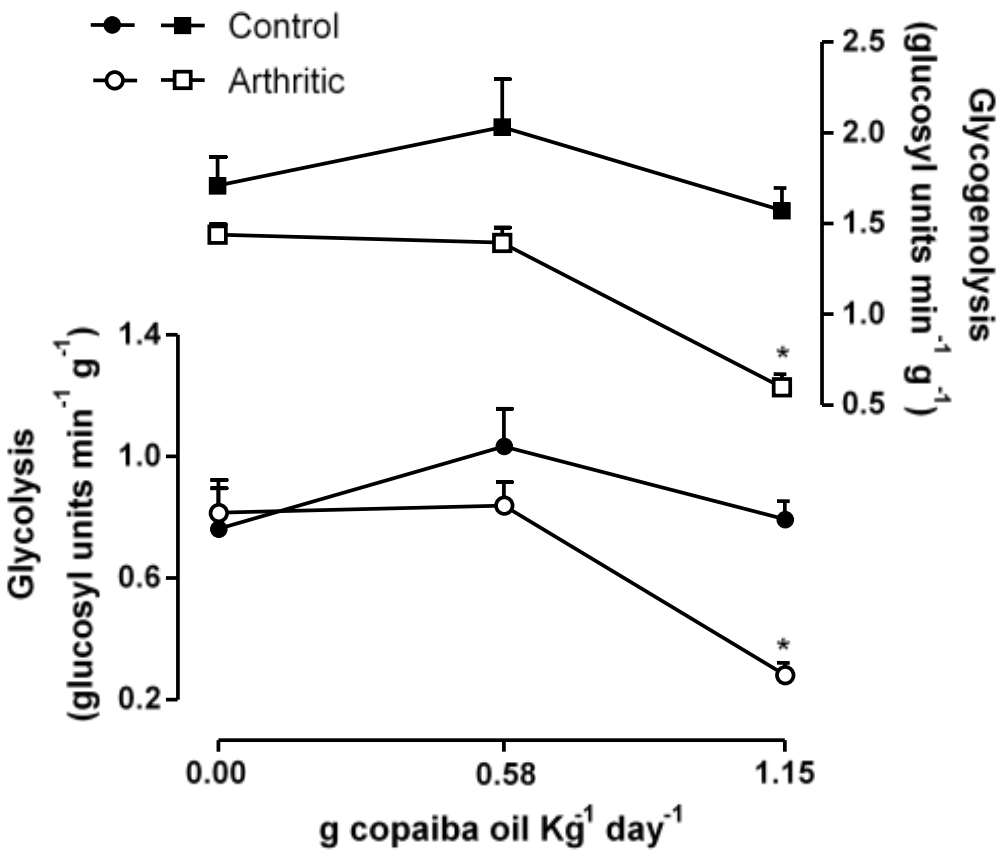

Figure 2. Dose dependences of the effects of copaiba oil treatment on the glycogen catabolism and glycolysis in the perfused livers of control and arthritic rats. Livers from fed rats were perfused with Krebs/Henseleit-bicarbonate buffer as the experimental protocols illustrated in Figure 1. Rates of glycogenolysis and glycolysis were calculated from glucose, lactate and pyruvate production. Glycogenolysis and glycolysis are expressed as glucosyl units and were calculated as: glucose+1/2 (lactate+pyruvate) and lactate+pyruvate, respectively. Each datum point represents the means of 4-5 liver perfusion experiments. Bars are standard errors of the mean. *Indicate statistical difference in relation to other groups. $p<0.05$.

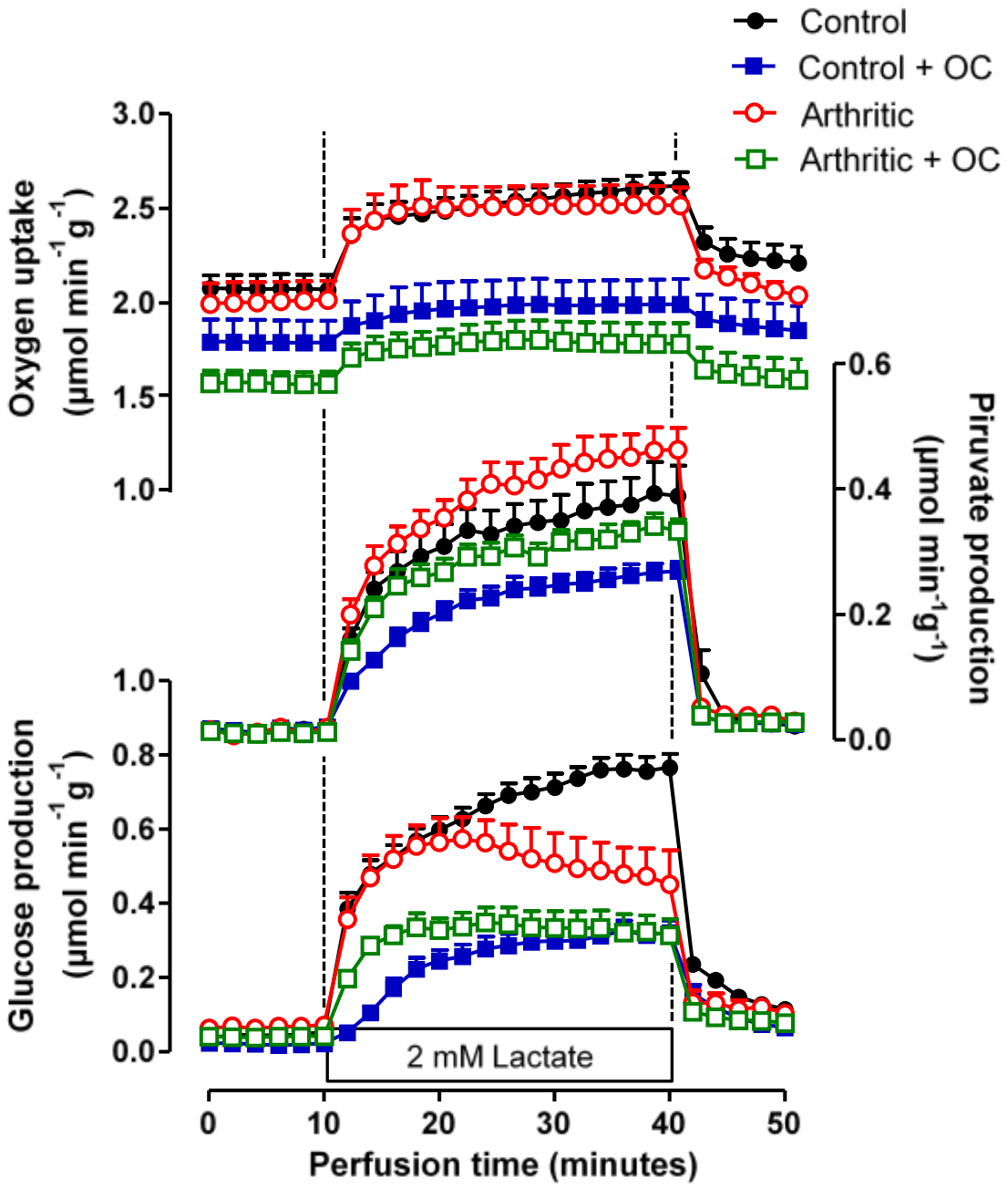

Figure 3. Effects of the copaiba oil treatment on the liver gluconeogenesis of control and arthritic rats. Control and arthritic rats were treated with copaiba oil (CO) at a dose of 1.15 g·Kg^{-1} as described in the Methods. Livers from 12 h fasted rats were perfused with Krebs/Henseleit bicarbonate buffer and 2 mM L-lactate as indicate by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analyzed for glucose and pyruvate. Oxygen consumption was monitored by polaroghraphy. Data are the mean values obtained from 4-5 animals. Vertical bars represent the standard errors of the means.