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JAMIL SONI NETO

Ações do metil jasmonato sobre a inflamação e o estresse oxidativo sistêmicos em ratos com artrite induzida por adjuvante

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

Orientador: Prof. Dr. Jurandir Fernando Comar
Coorientadora: Profª. Drª. Anacharis Babeto de Sá Nakanishi

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BANCA EXAMINADORA

_____
Prof. Dr. Jurandir Fernando Comar
Universidade Estadual de Maringá

_____
Profª. Drª. Monique Cristine de Oliveira
Centro Universitário Ingá

_____
Profª. Drª. Maria Raquel Marçal Natali
Universidade Estadual de Maringá

_____
Profª. Drª. Cristiane Vizioli de Castro Ghizoni
Universidade Estadual de Maringá

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APRESENTAÇÃO

Este é um trabalho realizado no Laboratório de Metabolismo Hepático do Departamento de Bioquímica e no Laboratório de Inflamação do Departamento de Farmacologia e Terapêutica da Universidade Estadual de Maringá, apresentado na forma de um artigo científico original, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas.

Artigo original:

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Efeitos do metil-jasmonato sobre o estado oxidativo e inflamatório de ratos com artrite por adjuvante. Integrantes: Anacharis Babeto de Sá-Nakanishi (Doutora), Jurandir Fernando Comar (Doutor), Cristiane Vizioli de Castro Ghizoni (Doutora), Ciomar Aparecida Bersani-Amado (Doutora), Geferson de Almeida Gonçalves (Mestre), Jamil Soni Neto (Bacharel).

Biografia do autor:

Nascido em Apucarana/PR, onde se formou no ensino fundamental e parte do ensino médio. Foi para o Japão por intercâmbio e cursou os primeiros anos do ensino médio em uma escola em regime de internato. Informalmente, realizou um estágio de aproximadamente um mês no Laboratório de Nutrição, Universidade Kochi Joshi Daigaku hoje com o nome de Universidade de Kochi. Quando voltou para o Brasil, terminou o ensino médio em Curitiba/PR. Ingressou na Universidade Estadual de Londrina no curso de Ciências Biológicas nas modalidades de licenciatura e bacharelado, período de graduação no qual foi mantido um interesse em biologia molecular, genética e imunologia, sendo “estresse oxidativo” o primeiro tema que causou entusiasmo no seu primeiro estágio voluntário.

"When the war finally came to an end, I was at a loss as to what to do... . I took stock of my qualifications. A not-very-good degree, redeemed somewhat by my achievements at the Admiralty. A knowledge of certain restricted parts of magnetism and hydrodynamics, neither of them subjects for which I felt the least bit of enthusiasm. No published papers at all... . Only gradually did I realise that this lack of qualification could be an advantage. By the time most scientists have reached age thirty they are trapped by their own expertise. They have invested so much effort in one particular field that it is often extremely difficult, at that time in their careers, to make a radical change. I, on the other hand, knew nothing, except for a basic training in somewhat old-fashioned physics and mathematics and an ability to turn my hand to new things... . Since I essentially knew nothing, I had an almost completely free choice... ."

(FRANCIS CRICK, WHAT MAD PURSUIT)

LISTA DE ABREVIATURAS E SIGLAS

A/G ratio	Albumin/Globulin Ratio
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
COX2	Cyclooxygenase-2
DCFH	Reduced Dichlorofluorescein
DNPH	2,4-Dinitrophenylhydrazine
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
FRAP	Ferric Reducing Activity of Plasma
G6Pase	Glucose-6-Phosphatase
GK	Glucokinase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
IBU	Ibuprofen
IL-1	Interleukin 1
KC	Kupffer cell
LPS	Lipopolyssacharide
LYM	Lymphocyte
MeJA	Methyl Jasmonate
MPH	Macrophages
MPO	Myeloperoxidase
NEUTR	Neutrophil
NFκB	Nuclear factor Kappa B
EC50	Effective Dose of 50% Activity Inhibition
RC	Respiratory Control
OPT	o-Phthalaldehyde
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SYN	Synoviocyte
TBARS	Thiobarbituric Acid Reactive Substances
TNF-α	Tumoral Necrosis Factor Alpha

SUMÁRIO

RESUMO GERAL

INTRODUÇÃO

O metil jasmonato (MeJA) é uma ciclopentanona natural derivada de ácidos graxos que compartilha semelhanças estruturais com as prostaglandinas e, por isso, muitos estudos vêm sendo realizados no sentido de avaliar suas ações em células de mamíferos, principalmente as potenciais atividades anti-inflamatórias e anticancerígenas. O MeJA inibiu a proliferação de diversas linhagens celulares de câncer em murinos e humanos. As mitocôndrias parecem ser o alvo principal do MeJA, onde estimula a produção de espécies reativas de oxigênio (ROS), dissocia a hexocinase ligada a mitocôndria, estimula a depleção de ATP e causa morte celular. Em relação à ação anti-inflamatória, o MeJA foi capaz de inibir a produção mediada por NFκB de óxido nítrico, prostaglandina E, TNF-alfa, IL-1 e IL-6 em macrófagos de murinos ativados por lipopolissacarídeo (LPS) (RAW 264.7). Entretanto, estes efeitos foram demonstrados apenas em células isoladas e nenhum estudo até a presente data de acordo com nosso conhecimento, avaliou se MeJA é capaz de inibir *in vivo* a inflamação. O presente estudo, portanto, investigou a ação do MeJA sobre a inflamação e o estado oxidativo sistêmicos em ratos com artrite induzida por adjuvante, uma imunopatologia em ratos que compartilha muitas características da artrite reumatóide humana. Esses animais apresentam inflamação sistêmica que afetam outros órgãos além das articulações, como o fígado, que apresenta marcadas alterações metabólicas associadas a um pronunciado estresse oxidativo. O presente estudo também avaliou as ações do MeJA sobre produção de ROS e atividade respiratória em mitocôndrias isoladas de fígado. Por fim, foi investigado também o efeito do MeJA sobre a atividade da glicoquinase hepática, a enzima que catalisa o primeiro passo limitante da glicólise, uma via que é reportada estar mais acelerada no fígado artrítico.

MÉTODOS

A indução de artrite foi realizada em ratos Holtzman com adjuvante de Freund. Os animais foram distribuídos em sete grupos: controles (saudáveis), os quais receberam óleo de milho; controles tratados com MeJA 300 $mg \cdot Kg^{-1}$; ratos artríticos, os quais receberam óleo de milho; ratos artríticos tratados com MeJA nas doses de 75, 150 e 300 $mg \cdot Kg^{-1}$; e artríticos tratados com ibuprofeno 30 $mg \cdot Kg^{-1}$. O tratamento foi feito por via oral (gavagem) uma vez ao dia durante 5 dias antes da indução da artrite e 18 dias depois. O volume das patas foi medido por pletismografia e as lesões secundárias foram monitoradas entre os dias 10 e 18. Os leucócitos circulantes e os leucócitos recrutados na cavidade da articulação femorotibial foram

adicionalmente quantificados nos ratos artríticos. No dia 19, a cavidade peritoneal dos ratos anestesiados foi exposta, o sangue coletado da veia cava e o fígado removido para preparar o homogenato e isolar mitocôndrias. Os níveis de proteínas carboniladas, TBARS e ROS foram medidos no homogenato para avaliar o estresse oxidativo hepático. O conteúdo de glutationa oxidada (GSSG) e reduzida (GSH) e as atividades das enzimas catalase, SOD, mieloperoxidase (MPO) e glicoquinase foram medidas no sobrenadante do homogenato. A capacidade antioxidante do plasma (FRAP), grupos tiol, albumina, proteínas carboniladas e atividade das enzimas MPO, AST, ALT e fosfatase alcalina foram medidas no plasma. As mitocôndrias foram isoladas por centrifugação diferencial e utilizadas para investigar a produção de ROS e atividade respiratória. A respiração mitocondrial foi medida na presença (estado III) e ausência (basal e estado IV) de ADP e as atividades das enzimas NADH e succinato oxidases foram medidas em mitocôndrias rompidas. A produção de ROS e a atividade respiratória foram medidas em mitocôndrias hepáticas incubadas com MeJA na faixa de até 10 mM e também nas mitocôndrias isoladas de animais tratados com MeJA.

RESULTADOS

Os ratos artríticos desenvolveram uma intensa resposta inflamatória ao adjuvante nas patas injetada e contralateral. Em adição, estes animais apresentaram menor ganho de peso corporal e manifestações inflamatórias sistêmicas, conforme foi evidenciado pela maior atividade da MPO plasmática e hepática, pelos baixos níveis de albumina plasmática, surgimento e persistência de lesões secundárias à artrite e maior número de leucócitos no sangue e também recrutados para as cavidades articulares. O estresse oxidativo também foi pronunciado no plasma e no fígado de ratos artríticos, conforme evidenciado pelos maiores níveis de ROS, TBARS e proteínas carboniladas no fígado, diminuição da atividade da catalase e da razão GSH/GSSG no fígado, pelos maiores níveis de proteínas carboniladas no plasma e diminuição do FRAP e tióis no plasma. Os volumes das patas injetada e não injetada foram respectivamente 55% menores e não alterados nos ratos artríticos tratados com MeJA 300 $mg \cdot Kg^{-1}$, grupo no qual também houve uma diminuição do número de leucócitos no sangue e recrutados para as articulações. No entanto, o escore de lesões secundárias e os níveis de albumina plasmática em ratos artríticos não foram modificados pelo tratamento com MeJA. As atividades da MPO plasmática e hepática foram respectivamente 40 e 27% menores em ratos artríticos tratados com MeJA 300 $mg \cdot Kg^{-1}$. O tratamento de ratos controles e artríticos com MEJA não modificou as atividades da AST, ALT e fosfatase alcalina no plasma. O MeJA (150 e 300 $mg \cdot Kg^{-1}$) aboliu o aumento das proteínas carboniladas no plasma e no fígado, o teor de

ROS no fígado e diminuiu (apenas 300 mg·Kg^{-1}) a razão GSH/GSSG e atividade da catalase no fígado de ratos artríticos. Além disso, o MeJA aumentou os grupos tióis e FRAP plasmáticos. O MeJA adicionado exogenamente (1,25-10 μM) estimulou a produção de ROS e inibiu a respiração em mitocôndrias isoladas hepáticas de ratos controles e artríticos, mas apenas a produção de ROS mitocondrial foi estimulada nos ratos tratados com MeJA (300 mg·Kg^{-1}). As atividades da NADH e succinato oxidases foram inibidas em mitocôndrias isoladas hepáticas incubadas com MeJA e também em ratos artríticos e controles tratados com MeJA 300 mg·Kg^{-1}. A atividade da glicoquinase hepática foi 60% maior em ratos artríticos (comparada com controles) e o tratamento de ratos controles e artríticos (todas as doses) com MeJA inibiu a atividade da glicoquinase em aproximadamente 60%.Os resultados foram comparados com tratamento controle positivo de ibuprofeno 30 mg · Kg^{-1}.

DISCUSSÃO

O MeJA foi efetivo como anti-inflamatório, em particular na dose de 300 mg·Kg^{-1}, o qual foi capaz de diminuir o edema de pata, leucocitose e o recrutamento de leucócitos para as articulações, ações que foram semelhantes ao do ibuprofeno. O mecanismo de ação do MeJA parece estar associado à inibição da expressão de citocinas pró-inflamatórias induzidas por NFκB . O acentuado estresse oxidativo plasmático em ratos artríticos ocorre como consequência dos baixos níveis de albumina e tióis. Em relação ao fígado, o estresse oxidativo é acentuado como consequência de um impedido sistema antioxidante associado à aumentada produção de ROS, ambos induzidos por citocinas pró-inflamatórias de forma direta e também indireta ao estimular o metabolismo oxidativo hepático. Em adição, a glicólise acelerada no fígado artrítico aumenta o fluxo de equivalentes redutores do citosol para as mitocôndrias, tornando o citosol mais oxidado. O MeJA foi capaz de diminuir o estresse oxidativo no plasma e no fígado de ratos artríticos, um efeito que parece ser a consequência da supressão do sistema pró-oxidante induzido por citocinas e de um aumento da atividade antioxidante, em particular a razão GSH/GSSG. Embora o MeJA tenha estimulado a produção mitocondrial de ROS em ratos artríticos e controles, o estresse oxidativo não se modificou no fígado, um fenômeno que pode estar associado a uma diminuição do fluxo através da glicólise devido à inibição da glicoquinase. As doses efetivas de MeJA no presente estudo podem ser ainda relativamente altas, mas não tiveram efeitos tóxicos, o que faz do MeJA um ponto de partida importante para desenvolvimento de drogas anti-inflamatórias.

Palavras-chave::artrite reumatóide, produção mitocondrial de ROS, atividade da glicoquinase.

GENERAL ABSTRACT

BACKGROUND

Methyl jasmonate (MeJA) is a natural fatty acid-derived cyclopentanone which shares structural similarities with prostaglandins and therefore many studies have been carried out to evaluate their actions on mammal cells, specially the potential anti-inflammatory and anti-cancer activities. MeJA was able to inhibit the proliferation in various murine and human cancer cell lines and the mitochondria seem to be the main target of MeJA action, which stimulates the production of reactive oxygen species (ROS), bind to and detach the mitochondria-bound hexokinase, ATP depletion and finally cell death. Regarding the anti-inflammatory action MeJA was able to inhibit NFκB -mediated production of nitric oxide, prostaglandin E, TNF-α, IL-1 and IL-6 in LPS-activated murine macrophages (RAW264.7). However, these effects have been demonstrated only in isolated cells and no study has until now evaluated if MeJA is able to inhibit *in vivo* the inflammation. The present study therefore investigated the action of MeJA on the systemic inflammation and oxidative status in rats with adjuvant-induced arthritis, an experimental pathology in rats that shares many features with the human rheumatoid arthritis. Arthritic animals present systemic inflammation and in addition to articular sites other organs are affected, such as liver, which presents marked metabolic alterations associated to a pronounced oxidative stress. The present study has also evaluated the actions of MeJA on ROS production and respiratory activity of hepatic isolated mitochondria. Finally, this study also investigated the effect of MeJA on hepatic hexokinase (glucokinase) activity, enzyme that catalyzes the first rate-limiting step of glycolysis, which is reported to be accelerated in the arthritic liver.

METHODS

The arthritis induction was performed in *Holtzman* rats with Freund's adjuvant. Animals were distributed into seven groups: controls (healthy), which received corn oil; controls treated with 300 mg·Kg^{-1} MeJA; arthritic rats, which received corn oil; arthritic rats treated with 75, 150 and 300 mg·Kg^{-1} MeJA; and arthritic rats treated with 30 mg·Kg^{-1} ibuprofen. The treatment was made orally (gavage) once a day for 5 days prior to arthritis induction and by additional 18 days after. Paw volume was measured by plethysmography and the score of secondary lesions was assessed from the 10th to 18th day. Circulating leukocytes and those recruited into the femorotibial joint cavities were further quantified in the arthritic rats. At day 19th, peritoneal cavity of anesthetized rats was exposed, the blood was collected from the cava vein and the liver was

removed to perform mitochondria isolation and homogenate preparation. Protein carbonyl groups, TBARS and ROS were measured in the homogenate to evaluate the liver oxidative stress. Oxidized (GSSG) and reduced (GSH) glutathione contents and activity of the enzymes catalase, SOD, myeloperoxidase (MPO) and glucokinase were measured in the homogenate supernatant. The ferric reducing ability of plasma (FRAP), thiol groups, albumin, protein carbonyl groups and the activities of MPO, AST, ALT and alkaline phosphatase were measured in the plasma. Mitochondria were isolated by differential centrifugation and used to measure real time ROS production and respiratory activity. Mitochondrial respiration was measured in the presence (state III) and absence (basal and state IV) of ADP and activities of the NADH and succinate oxidases were measured in disrupted mitochondria. ROS production and respiratory activity were measured in isolated hepatic mitochondria incubated with MeJA exogenously added in the range up to 10 mM and also in mitochondria isolated from animals treated with MeJA.

RESULTS

Arthritic rats developed an intense inflammatory response to adjuvant in both injected and contralateral paws. Animals furthermore presented very low body weight gain and systemic inflammatory manifestations, as evidenced by higher plasma and liver MPO activity, low levels of plasma albumin, severe secondary lesions to arthritis and higher number of total leukocytes in the blood and also recruited into the femorotibial joint cavities. Oxidative stress was also pronounced in the plasma and liver of arthritic rats, as evidenced by higher levels of ROS, TBARS and protein carbonyl groups, as well as decreased activity of catalase and GSH/GSSG ratio in the liver, whereas higher levels of protein carbonyl groups and decreased FRAP and thiols were verified in the plasma. Injected and non-injected paw volumes were respectively 55% lower and not altered in arthritic rats treated with 300 $mg \cdot Kg^{-1}$ MeJA, which also decreased the number of total leukocytes in the blood and recruited into the femorotibial joints. However, score of secondary lesions and plasma albumin levels in arthritic rats were not modified by MeJA treatment. MPO activities in the plasma and livers were respectively 40 and 27% lower in arthritic rats treated with 300 $mg \cdot Kg^{-1}$ MeJA. Treatment of control and arthritic rats with MEJA did not modify the AST, ALT and phosphatase alkaline activities in the plasma. MeJA (150 and 300 $mg \cdot Kg^{-1}$) abolished the increase of carbonylated proteins in the plasma and liver, and ROS content in the liver, and decreased (only 300 $mg \cdot Kg^{-1}$) the hepatic GSH/GSSG ratio and catalase activity in arthritic rats. In addition, MeJA increased FRAP and thiols groups in the plasma. MeJA exogenously added (1.25-10 mM) stimulated ROS production and inhibited

respiration in isolated hepatic mitochondria of control and arthritic rats, but only mitochondrial ROS production was stimulated when rats were treated with MeJA (300 mg·Kg^{-1}). The activities of NADH and succinate oxidases were inhibited in isolated hepatic mitochondria by MeJA exogenously added and also in arthritic and control rats treated with 300 mg·Kg^{-1} MeJA. Hepatic glucokinase activity was 60% higher in arthritic rats (compared to the controls) and treatment of control (only 300 mg·Kg^{-1}) and arthritic rats with MeJA (all doses) inhibited the glucokinase activity by approximately 60%.

DISCUSSION

MeJA was effective as an anti-inflammatory agent, particularly at the dose of 300 mg·Kg^{-1}, which was able to decrease the paw edema, leukocytosis and leukocytes recruitment into the femorotibial joints, actions that were similar to ibuprofen, a classical anti-inflammatory drug. The mechanism of MeJA action seems to be associated with inhibition of NFκB -induced expression and release of cytokines. Pronounced oxidative stress in the plasma of arthritic rats occurs as consequence of low levels of albumin and thiol groups. Only this last was improved by the remission of inflammation, but it was enough to decrease the plasma oxidative stress. Regarding the liver, oxidative stress is more pronounced as consequence of an impaired ROS scavenging system associated with increased production of ROS, which are induced by proinflammatory cytokines directly and indirectly by stimulating the liver oxidative metabolism. In fact, the accelerated glycolysis in the liver of arthritic rats lead to an increased flux of reducing equivalents from the cytosol to mitochondria, making the cytosol more oxidized. MeJA was able to diminish the oxidative stress in the liver of arthritic rats, an effect that seems to be the consequence of both a suppression of the cytokine-induced pro-oxidant system and an improvement of the antioxidant defense, particularly the GSH/GSSG ratio. Although MeJA has also stimulated production of ROS in the liver mitochondria of arthritic rats, oxidative stress was not modified in the organ, a phenomenon that could be associated to a diminished flux through the glycolysis due to glucokinase inhibition. The effective doses of MeJA in the present study may be still relatively high, but they had no toxic effects and make this compound a potentially important starting point for anti-inflammatory and anti-rheumatic drugs development.

***Key-words**:* rheumatoid arthritis, mitochondrial ROS production, glucokinase activity.

Anti-inflammatory action of methyl jasmonate is associated with oxidative modifications in the liver of arthritic rats

Jamil Soni Neto[1], Lucas S. Moreira[1], Geferson A. Gonçalves[1], Anacharis B. Sá-Nakanishi[1], Francielli M. S. Silva[2]; Lívia Bracht[1], Ciomar A. Bersani-Amado[2], Rosane M. Peralta[1]; Adelar Bracht[1], Jurandir F. Comar[1*]

[1] Department of Biochemistry, State University of Maringa, PR, Brazil

[2] Department of Pharmacology and Therapeutics, State University of Maringa, PR, Brazil

Address for correspondence:

*Jurandir Fernando Comar

Department of Biochemistry

University of Maringá

87020900 Maringá, Brazil

Email: jurandircomar@yahoo.com.br

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ABSTRACT

Methyl jasmonate (MeJA) is a fatty acid-derived cyclopentanone which shares structural similarities with prostaglandins and has been reported to have anti-inflammatory and anti-cancer activities. The present study investigated the action of MeJA on systemic inflammation and oxidative status in rats with adjuvant-induced arthritis, an experimental pathology in rats which shares many features of human rheumatoid arthritis. Glucokinase activity was assessed in the liver and production of reactive oxygen species (ROS) and respiratory activity were also performed in isolated hepatic mitochondria. Holtzman rats were distributed into seven groups: controls (healthy), which received corn oil; controls treated with 300 mg·Kg^{-1} MeJA; arthritic rats, which received corn oil; arthritic rats treated with 75, 150 and 300 mg·Kg^{-1} MeJA; and arthritic rats treated with 30 mg·Kg^{-1} ibuprofen. MeJA was orally administrated during 18 days after arthritis induction with Freund's adjuvant. Articular and systemic inflammation was greatly increased in arthritic rats and likewise oxidative stress in the plasma and liver. Hepatic glucokinase activity was 60% higher in arthritic rats. MeJA (300 mg·Kg^{-1}) decreased most inflammatory parameters, as paw edema, leukocytosis, leukocytes recruitment into the femorotibial joints and myeloperoxidase activity in the plasma and liver. MeJA (150 and 300 mg·Kg^{-1}) abolished the increase of carbonylated proteins in the plasma and liver, and ROS content in the liver, and decreased (only 300 mg·Kg^{-1}) the hepatic GSH/GSSG ratio and catalase activity in arthritic rats. MeJA exogenously added (1.25-10 mM) was able to stimulate ROS production and to inhibit respiration in isolated hepatic mitochondria of control and arthritic rats, however, only mitochondrial ROS production was stimulated when rats were treated with MeJA (300 mg·Kg^{-1}). This latter effect, however, was not able to modify the liver oxidative stress, a phenomenon that may be related to modifications of oxidative metabolism in the liver. Indeed, MeJA was able to inhibit the hepatic glucokinase activity, enzyme that catalyzes the first rate-limiting step of glycolysis, which is increased in the arthritic liver where it generates a more oxidizing cellular environment. Effective doses of MeJA had no toxic effects in the present work and make this compound a potential starting point for development of anti-inflammatory and anti-rheumatic drugs.

***Key-words**:* rheumatoid arthritis, mitochondrial ROS production, glucokinase activity.

INTRODUCTION

Jasmonates are fatty acid-derived cyclopentanones widely distributed in the plant kingdom, where they act as signaling molecules in responses to abiotic and biotic stresses and also in the regulation of plant growth and development [Wasternack, 2007]. Jasmonate family consists mainly of jasmonic acid, *cis*-jasmonate and methyl jasmonate (MeJA), which share structural similarities with prostaglandins, especially those that have known anti-inflammatory activities [Thoma et al., 2004]. For this reason many studies have been carried out to evaluate their actions on mammal cells. Jasmonates have been reported to have cytotoxic activity against cancer cells without affecting normal cells [Cesari et al., 2014; Raviv et al., 2013; Cohen & Flescher, 2009]. The anti-cancer activity of MeJA, however, showed to be superior to other jasmonates and therefore the pharmacological properties of MeJA and its synthesized derivatives have been lately under investigation as promising agents for cancer treatment [Cesari et al., 2014]. MeJA was able to induce apoptosis and to inhibit proliferation in various murine and human cancer cell lines, including those of breast, colon, prostate, neuroblastoma, lymphoma, and leukemia [Cesari et al., 2014; Tong et al., 2008]. In addition, MeJA was also able to increase the survival period of mice bearing EL-4 lymphoma and mice inoculated with multiple myeloma (MM.1S) cells [Klippel et al., 2012; Fingrut & Flescher, 2002].

Mitochondria of cancer cells seem to be the main target of MeJA action, where it stimulates the production of reactive oxygen species (ROS), bind to and detach the mitochondria-bound hexokinase, cytochrome *c* release, ATP depletion and, finally, cell death [Zhang et al., 2015; Raviv et al., 2013; Goldin et al., 2008]. On the other hand, the anti-cancer activity of MeJA is not restricted to its action on mitochondria but also seems to be related to anti-inflammatory actions. Association between cancer and chronic inflammation are widely accepted and both are associated with increased expression of proinflammatory enzymes (COX-2 and 5-lipoxygenase) and inflammation mediators, such as TNF-α, IL-1 and IL-6 [Reuter et al., 2012]. Previous studies showed that MeJA is able to inhibit the overexpressed proinflammatory enzyme 5-lipoxygenase (5-LOX) in cancer cell lines [Ezekwudo et al., 2007]. MeJA was also able to inhibit NFκB -mediated production of nitric oxide (NO), prostaglandin E, TNF-α, IL-1 and IL-6 in LPS-activated murine macrophages (RAW264.7) [Kim et al., 2016; Lee et al., 2011; Dang et al., 2008]. However, these anti-inflammatory properties of MeJA were demonstrated only *in vitro* using isolated cells and no study has evaluated until now if MeJA is able to inhibit inflammation *in vivo*.

Adjuvant-induced arthritis in rats is an experimental model of chronic and systemic inflammation which shares many features of human rheumatoid arthritis and has been widely used to evaluate anti-inflammatory and anti-rheumatic drugs [Hegen et al., 2008; Bendele et al., 1999; Szekanecz et al., 2000]. Rheumatoid arthritis affects 0.5-1.0% of the adult population worldwide and it is associated with an increased mortality rate [Uhlig *et al.*, 2014]. Both rheumatoid arthritis and adjuvant-induced arthritis affect primarily the joints, where occur an intense hyperplasia of synovial membrane and cartilage with participation of proinflammatory cytokines and overproduction of reactive species, which act as mediators of tissue injury [McInnes & Schett, 2011; Stolina et al., 2009]. Inflammatory manifestations are also significant systemically and affect other organs, such as brain, heart, liver and lungs [McInnes & Schett, 2011]. Metabolic alterations are also prominent in rheumatoid arthritis, as the muscle wasting condition known as rheumatoid cachexia, which affects approximately two-thirds of all patients [Roubenoff, 2009]. Metabolic modifications are equally significant in arthritic rats, which present in addition to cachexia substantial alterations in hepatic metabolism. Perfused livers from arthritic rats indeed presented higher rates of oxygen uptake as well as reduced gluconeogenesis from various substrates, increased glycolysis and reduced metabolism of xenobiotics [Roubenoff, 2009; Fedatto et al., 1999; Fedatto et al., 2000; Fedatto et al., 2002]. Similarly, oxidative stress is altered in the serum blood of patients [Lemarechal et al., 2006] and also in the liver, brain and heart of rats with arthritis by adjuvant [Schubert et al., 2016; Bracht et al., 2016; Wendt et al., 2015; Comar et al., 2013]. Particularly in the arthritic rat liver, where inflammation and metabolic alterations are prominent, oxidative stress is rather pronounced when compared to other organs and, in addition, it seems to be associated with the accelerated oxidative metabolism that occurs in the organ [Wendt et al., 2015; Comar et al., 2013].

Considering the above-mentioned actions of MeJA, specially the anti-inflammatory activity, it seems reasonable to hypothesize that it should be able to attenuate the articular and systemic inflammation that occurs in arthritis. As the generalized inflammatory process in arthritis is closely associated with oxidative stress, particularly in the liver, it is possible that MeJA is able to decrease inflammation and oxidative stress also systemically. Therefore, the present study was planned to investigate the action of MeJA, orally administrated, on systemic inflammation and oxidative status of rats with adjuvant-induced arthritis.

Whereas that MeJA is reported to stimulate production of reactive oxygen species (ROS) in mitochondria as a mechanism that induces apoptosis of cancer cells [Raviv et al., 2013], it seems contradictory to expect that it is able to decrease the tissue oxidative stress as a consequence of its anti-inflammatory activity. However, no study has evaluated until now if

MeJA affects the oxidative stress in chronic inflammation and if it can also stimulate production of reactive species in mitochondria isolated of non-cancer cells. Thus, the present study has also evaluated the actions of MeJA on ROS production in mitochondria isolated from livers of rats with adjuvant-induced arthritis. Finally, as MeJA was reported to interfere with the interaction of hexokinase with the external mitochondrial membrane it is possible that activity of this enzyme is also modified [Goldin et al., 2008]. Hexokinase is the enzyme that catalyzes the first rate-limiting step of glycolysis and both capacity of glucose phosphorylation and glycolytic flux are increased in the liver of arthritic rats [Fedatto et al., 2000]. Thus, this study also evaluated the effect of MeJA on hepatic hexokinase (glucokinase) activity.

The experimental model used in the current investigation is considered a severe arthritis model in rats which shows a widespread inflammatory response [Bracht et al., 2016]. 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 systemic effects of MeJA in rats with polyarthritis, which in turn, should also allow extrapolations for patients with rheumatoid arthritis, particularly for those that manifest more aggressive form of arthritis.

MATERIAL AND METHODS

Chemicals

Methyl jasmonate, o-dianisidine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), o-phthalaldehyde (OPT), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 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). All other chemicals were of analytical grade. Commercial kits for AST, ALT, alkaline phosphatase, creatinine, albumin and total proteins were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, MG, Brazil).

Animals and induction of arthritis

Male *Holtzman* rats weighting 170-180 g (50 days old) 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 24±3ºC under a regulated 12h light/dark cycle. Animals were housed in conventional steel cages (3 rats/cage) and were fed *ad libitum* with a standard laboratory diet (Nuvilab®, Colombo, Brazil). For arthritis induction, animals were injected subcutaneously in the left hind paw with 0.1 ml (500 µg) of Freund's adjuvant (heat inactivated *Mycobacterium tuberculosis*, derived from the human strain H37Rv), suspended in mineral oil [Bracht et al., 2016]. 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 number CEUA 6053280915).

Experimental design

Forty-nine rats were randomly distributed into seven groups: controls (C), to which corn oil was administered as pure vehicle; controls (C300) treated with MeJA at the dose of 300 $mg \cdot Kg^{-1}$; arthritic rats (A), to which corn oil was administered; arthritic rats (A75, A150 and A300) treated with MeJA respectively at the dose of 75, 150 and 300 $mg \cdot Kg^{-1}$; and arthritic rats (IBU) treated with ibuprofen at the dose of 30 $mg \cdot Kg^{-1}$. This procedure was repeated two times (98 animals in total) to evaluate all parameters of this study. Animals were treated once a day in the morning by oral administration (gavage) of MeJA, corn oil or ibuprofen for 5 days prior to the arthritis induction and by additional 18 days after. Daily doses of MeJA were established

considering the effective dose that caused no toxicity to rats as previously described [Umukoro et al., 2011].

Evaluation of inflammatory response

Evaluation of adjuvant-induced inflammatory response was carried out over a 18-day period. Following adjuvant inoculation, the volumes of both hind paws up to the tibiotarsal joint were measured by plethysmography. The results were expressed in terms of increased paw volume in relation to initial volume (volume at day 0). 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]. Blood was collected by means of tail incision to obtain total and differential count of circulating leukocytes. Total and differential count of leukocytes recruited into the femorotibial joint cavities was additionally performed in the arthritic rats at day 19^{th} as previously described [Estevão-Silva et al., 2016].

Blood collection and tissue preparation

Fasted (12 h) rats 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. The liver was subsequently 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 mitochondria isolation. Thereafter, the hind femorotibial joints were surgically exposed, articular cavities were washed with 40 µL of phosphate-buffered saline (PBS) solution containing 1mM EDTA and the exudates used for leukocytes count.

The blood was centrifuged at 3,000*g* for 10 min and the supernatant was separated as 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 supernatant separated as soluble fraction of the homogenate.

For mitochondria isolation, one part of the liver was 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. Tissue was minced and homogenized in Dounce homogenizer for lysing the cells. Mitochondria were then isolated by differential centrifugation [Saling et al., 2011].

Plasma analytical assays

Ferric reducing ability of plasma (FRAP), protein sulfhidryl groups (thiols), protein carbonyl groups and myeloperoxidase (MPO) activity were measured in the plasma to evaluate oxidative and inflammatory status. Albumin, total protein, creatinine, and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured in the plasma to evaluate liver and kidneys damage using commercial Kits (Gold Analisa®). Myeloperoxidase (MPO) activity was measured by the increase in absorbance due to oxidation of o-dianisidine in 460 nm [Bradley et al., 1982].

FRAP evaluates the ability of the plasma to convert Fe^{+2} to Fe^{+3}, i.e., the reducing capacity of the plasma and was measured by spectrophotometry (595 nm) using tripyridyltriazine (TPTZ) and ferric chloride ($FeCl_3$) [Benzie & Strain, 1996]. FRAP was calculated from a standard curve prepared with trolox and the results were expressed as nmol·(mL plasma)$^{-1}$.

Thiol contents were measured by spectrophotometry (412 nm) using DTNB (5,5'-dithiobis 2-nitrobenzoic acid) as previously described [Bracht et al., 2016]. Thiols contents were calculated using the molar extinction coefficient (ε) of 1.36×10^4 $M^{-1} \cdot cm^{-1}$ and the values expressed as nmol (mg protein)$^{-1}$. Protein carbonyl groups were measured by spectrophotometry using 2,4-dinitrophenylhydrazine [Sá-Nakanishi et al., 2014]. Levels of protein carbonyl groups were calculated using the molar extinction coefficient (ε) of 2.20×10^4 $M^{-1} \cdot cm^{-1}$ and the values expressed as nmol (mg protein)$^{-1}$.

Liver oxidative stress parameters

Protein carbonyl groups: levels of carbonylated proteins were measured in the supernatant of liver homogenate by spectrophotometry with DNPH, the same as described for the plasma [Sá-Nakanishi et al., 2014].

Lipid peroxidation: liver lipoperoxides were measured by means of the TBARS assay (thiobarbituric acid reactive substances) [Buege & Aust, 1978]. The amount of lipoperoxides was calculated from the standard curve prepared with 1,1',3,3'tetraethoxypropane and the values were expressed as nmol (mg protein)$^{-1}$.

Reactive oxygen species (ROS): levels of ROS were quantified in the supernatant of homogenate by spectrofluorimetry with the 2',7'-dichlorofluorescin diacetate (DCFH-DA) [Siqueira et al., 2005]. The assay quantifies the oxidation of DCFH-DA to the fluorescent 2',7'-dichlorofluorescin (DCF), in the presence of ROS. 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 protein)$^{-1}$. The rate of mitochondrial ROS production (real time ROS production) was estimated by measuring the linear fluorescence increase due to DCF formation [Biazon et al., 2016]. Acetate groups of DCFH-DA allow it to enter the organelle where these groups are removed by esterases producing the reduced DCFH within the mitochondria [Tarpey et al., 2004]. Briefly, intact mitochondria were suspended in 2 ml of a mixture containing 250 mM mannitol, 1.36 µM DCFA-DA, 10 mM Hepes buffer (pH 7.2), and 10 mM succinate as respiratory substrate. The reaction was initiated by addition of 0.4 µM horseradish peroxidase and the fluorescence recorded during 10 min under agitation. The results were expressed as nmol·min^{-1}·(mg protein)$^{-1}$ and, alternatively, as the effective concentration of MeJA that stimulates 50% (EC_{50}) of maximum ROS production. The EC_{50} was calculated by numerical interpolation using Stineman's interpolation formula [Stineman, 1980].

Glutathione assay: reduced (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)$^{-1}$.

Oxidative status enzymes assays: activities of catalase, superoxide dismutase (SOD) and myeloperoxidase (MPO) were assayed in the supernatant of 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]. Results 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)$^{-1}$. MPO activity was

measured as the same described for the plasma [Bradley et al., 1982]. Activity was calculated from the molar extinction coefficient (ε) of $11.3x10^3$ M^{-1} cm^{-1} and expressed as $\mu mol \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

Mitochondrial respiratory activity

Two protocols were used to evaluate mitochondrial respiratory activity: 1) mitochondria isolated from animals treated with MEJA and 2) mitochondria isolated from non-treated animals incubated with MeJA exogenously added. Oxygen consumption was measured in intact and additionally in freeze-thawing disrupted mitochondria (membrane-bound enzyme activities).

Mitochondrial oxygen uptake was measured polarographically using a Teflon-shielded platinum electrode [Saling et al., 2011]. Mitochondria were incubated in a closed oxygraph chamber in a medium (2.0 mL) containing 0.25M mannitol, 5 mM sodium diphosphate, 10 mM KCl, 0.2 mM EDTA, 10 mM Tris-HCl (pH 7.4) and two different substrates: succinate and α-ketoglutarate, both at a concentration of 10 mM. When appropriate, MeJA was added at various concentrations in the range up to 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 $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The respiration control ratio (RC), which is defined as the ability of activated respiration in state III to return to the less oxygen consuming state IV respiration and the phosphorylative activity ADP/O ratio that is the quotient of added ADP to the amount of oxygen uptake were calculated according to Chance & Willians [1955].

The activities of NADH oxidase and succinate oxidase were measured polarographically using freeze-thawing disrupted mitochondria [Saling et al., 2011]. Incubation medium was 20 mM Tris-HCl and, when appropriate, MeJA was added at various concentrations in the range up to 10 mM. The reaction was started by the addition of substrates, 1 mM NADH and 1 mM succinate, for NADH oxidase and succinate oxidase, respectively.

Hepatic glucose phosphorylation capacity (glucokinase activity)

The activity of glucokinase was measured in the liquid fraction obtained by additional centrifugation (1h at 105,000g) of the liver homogenate supernatant [Vilela et al., 2014]. The assay system was contained in a final volume of 1 ml: 100 mM Tris-HCl (pH 7.2), 20 mM glucose, 5 mM ATP, 10 mM $MgCl_2$, 1 mM NAD^+, 5 units glucose 6-phosphate dehydrogenase

from *Leuconostoc mesenteroides* and 20 µl of supernatant of homogenate. The increase in absorbance at 340 nm, resulting from NADH production, was measured during 3 min. Rates were evaluated from the slope of the recording traces and expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$.

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 ($p<0.05$). Where applicable the Student's t test was used for comparison between two data ($p<0.05$).

RESULTS

Effects of MeJA on the induction and development of adjuvant arthritis

Figure 1 shows the increase of the paw volumes (Panel A and B) and score of secondary lesions due to arthritis induction (Panel C). The initial volume of the hind paw before the adjuvant injection was 1.60 ± 0.10 ml. An inflammatory reaction in the injected paw was observed on the first day post-adjuvant injection equally in all groups (Panel A). These paw volumes remained relatively constant until the day 18 for arthritic rats treated with ibuprofen and 300 mg·Kg^{-1} MeJA, but they increased until the end of experimental period (at day 18) for arthritic rats treated with corn oil and 75 and 150 mg·Kg^{-1} MeJA. At day 18 the injected paw volume of arthritic rats treated with 300 mg·Kg^{-1} MeJA and ibuprofen was approximately 55% lower (compared to arthritic rats treated with corn oil). The volume of non-injected paw of rats treated with 300 mg·Kg^{-1} MeJA and ibuprofen were practically not modified (Panel B). However, for arthritic rats treated with corn oil and MeJA (75 and 150 mg·Kg^{-1}) the volumes increased progressively from day 10 until day 18, when was approximately 100% higher than those initial volumes. Secondary lesions appeared at day 10 and reached the highest scores at the 18th day after adjuvant injection (Panel C). Scores at day 18 were not different in all groups, although the treatment of arthritic rats with ibuprofen attenuated the secondary lesions between day 10 and 17.

Table I shows the number of leukocytes in the peripheral blood and those recruited into the femorotibial joint cavities. At day 18, the number of total blood leukocytes of non-treated arthritic rats was four times higher than the initial ones (day 0) and, additionally, polymorphonuclear leukocytes were found to be the predominant subpopulation. Treatment of animals with corn oil and MeJa (75 and 150 mg·Kg^{-1}) did not modify the number of total leukocytes in the blood, but treatment with 30 mg·Kg^{-1} ibuprofen and 300 mg·Kg^{-1} MeJA was able to decrease them by approximately 50%. Total leukocytes recruited into the articular cavity (joint of the injected paw) were approximately three times higher when compared to the contralateral joint. Treatment of animals with corn oil and 75 mg·Kg^{-1} MeJa did not modify the number of leukocytes recruited into the articular cavities, but treatment with ibuprofen, 150 mg·Kg^{-1} MeJA and 300 mg·Kg^{-1} MeJA was able to decrease leukocytes recruitment by 77%, 89% and 95%, respectively. Treatment with 30 mg·Kg^{-1} ibuprofen and 300 mg·Kg^{-1} MeJA were the only two able to decrease the number of leukocytes recruited into the femorotibial right joint.

Biochemical parameters in the plasma

The activities of AST, ALT and alkaline phosphatase (ALP) were measured in the plasma to evaluate if treatment with MeJA was able to cause liver damage and the plasma creatinine was measured in order to evaluate renal damage. Albumin, globulins levels and MPO activity in the plasma were used as systemic inflammation parameters. The results are shown in Table II. Regarding the liver damage, arthritis induction increased the plasma activity of AST (+100%) and ALP (+130%). Treatment of arthritic rats with MeJA, but not with ibuprofen, was able to decrease AST and ALP activities to values close to the control ones. Plasma levels of creatinine were not different for control and arthritic rats treated or not treated with MeJA, however, they were 40% higher in the arthritic animals treated with ibuprofen. Arthritis induction increased the plasma MPO activity (+400%) and globulins (+30%) while it decreased the plasma levels of albumin (-44%) and A/G ratio (-60%). Treatment of arthritic rats with MeJA was not able to increase plasma albumin, globulin and A/G ratio, however, MPO activity was approximately 40% lower when arthritic rats were treated with 300 $g \cdot Kg^{-1}$ MeJA or ibuprofen. No plasma biochemical parameter was modified in controls treated with MeJA (compared to controls treated with corn oil).

Oxidative status in the plasma

Levels of protein carbonyl groups, thiol groups, and the ferric reducing ability of plasma (FRAP) are shown in Figure 2. Levels of protein carbonyl groups, a pro-oxidant parameter, were 25% higher in the plasma of non-treated arthritic rats (compared to the controls; Figure 2A). Treatment of arthritic rats with 150 and 300 $mg \cdot Kg^{-1}$ MeJA were able to decrease protein carbonyl contents to levels close to the control ones. Plasma thiol groups, an antioxidant parameter, were only 40% of those in the controls (Figure 2B). Treatment of arthritic animals with 75 and 150 $mg \cdot Kg^{-1}$ MeJA was not able to increase thiol groups, but treatment with the dose of 300 $mg \cdot Kg^{-1}$ increased them in the plasma (+60%). FRAP, an antioxidant parameter, was 33% lower in the plasma of non-treated arthritic rats (compared to the controls; Figure 2C). Treatment of arthritic animals with 75 and 150 $mg \cdot Kg^{-1}$ MeJA was not able to increase FRAP, but treatment with the dose of 300 $mg \cdot Kg^{-1}$ increased the plasma reducing power (+44%). Protein carbonyl groups, thiol groups and FRAP were not modified in the plasma of arthritic rats treated with ibuprofen and in controls treated with MeJA. These parameters were not different in controls treated with corn oil.

Liver oxidative stress

Levels of protein carbonyl groups in the liver homogenate were 80% higher in arthritic rats (compared to the controls; Figure 2D). Levels of carbonyl groups were 20, 26 and 43% lower in the liver of arthritic rats treated with 75, 150 and 300 mg·Kg^{-1} MeJA, respectively, and 20% lower when treated with ibuprofen. Levels of TBARS were 36% higher in the liver of arthritic rats (compared to the controls; Figure 2E). Treatment of arthritic animals with 75 and 150 mg·Kg^{-1} MeJA was not able to decrease these levels, but treatment with the dose of 300 mg·Kg^{-1} decreased TBARS to levels close to the control values. Tissue oxidative injury is normally caused by oxygen reactive species (ROS) and its content in the liver were 155% higher in arthritic rats (compared to the controls; Figure 2F). Treatment of arthritic rats with MeJA at the doses of 150 and 300 mg·Kg^{-1} was able to decrease ROS content in the liver by approximately 30%. ROS content was 25% lower in arthritic rats treated with ibuprofen. Hepatic oxidative status was not affected by corn oil (compared to saline; results not shown).

Mitochondrial ROS generation in real time

The rate of ROS production (real time ROS production) was measured in fresh mitochondria isolated from livers and the results are shown in Figure 3. Figure 3A shows the concentration dependence of MeJA exogenously added on mitochondrial ROS generation. In the absence of MeJA the ROS production was 23% higher in arthritic rats ($p=0.014$; compare to the controls, Student's t test) and MeJA stimulated it in both conditions. The effective concentration of MeJA that stimulates 50% (EC_{50}) of maximum ROS generation is shown in Table 4 and it was 82% higher in the arthritic condition. This shows that ROS production in control mitochondria were more sensible to MeJA stimulation. Production of ROS was also measured in isolated hepatic mitochondria of rats treated with MeJA (Figure 3B). Mitochondrial ROS generation was 58% and 28% higher, respectively, in control and arthritic rats treated with 300 mg·Kg^{-1} MeJA (in relation to corn oil treatment). However, there was no difference between control and arthritic rats treated with corn oil.

Antioxidant and inflammatory status of the liver

Levels of glutathione and activities of catalase and superoxide dismutase (SOD) in the liver are shown in Table III. Levels of GSH in the liver of arthritic rats were 38% lower than those of the controls. Treatment of arthritic rats with 75 and 150 mg·Kg^{-1} MeJA was not able to increase the hepatic GSH levels, but treatment with the dose of 300 mg·Kg^{-1} increased it to

levels close to the control ones. GSSG levels were similar for all groups. GSH/GSSG ratio was 45% lower in the arthritis condition when compared to the controls and treatment with 300 $mg \cdot Kg^{-1}$ MeJA was effective in re-establishing the control levels. Catalase activity in the liver of arthritic rats was only 20% of that in control rats. Treatment of arthritic rats with MeJA increased hepatic catalase activity only at the dose of 300 $mg \cdot Kg^{-1}$. MPO activity was 38% higher in the liver of arthritic rats (compared to the controls). Treatment of arthritic rats with MeJA at the doses of 150 and 300 $mg \cdot Kg^{-1}$ increased hepatic MPO activity by 18 and 27%, respectively.

Respiratory activity in isolated liver mitochondria

Considering that ROS production and respiratory activity are associated phenomena in mitochondria, it seems reasonable to verify if MeJA affects respiratory activity of isolated liver mitochondria. Figure 4C outlines the experimental approach used to evaluate the respiratory activity of phosphorylating liver mitochondria. Succinate and α-ketoglutarate were respectively used as electron donor substrates for complex I and II of the mitochondrial electron transport chain and ADP was added in appropriate times. Respiration rates were measured under three conditions: (a) before the addition of ADP (substrate respiration or basal), (b) just after ADP addition (state III respiration) and (c) after cessation of ADP stimulation (state IV). Respiratory control (RC) was calculated as state III/state IV ratio. Respiratory activity of isolated mitochondria was evaluated initially with MeJA exogenously added in the range up to 10 mM. Basal respiration in the absence of MeJA was 50% (with succinate) and 60% (with α-ketoglutarate) lower in mitochondria of arthritic rats ($p<0.05$; compared to the controls). In the presence of MeJA basal respiration was diminished only in the arthritic condition with the substrate α-ketoglutarate (Figure 4A). On the other hand, state III respiration was diminished with increasing MeJA concentration with the two substrates for both control and arthritic rats (Figure 4B). The effective concentration of MeJA that inhibits 50% (EC_{50}) of state III respiration was not different for control and arthritic rats (Table IV). State IV respiration was less sensible to inhibition by MeJA, since it was inhibited in lesser degree when compared to state III (Figure 5A). Mitochondrial respiratory control (RC), on the other hand, was diminished and even abolished with increasing MeJA concentrations for both conditions and substrates (Figure 5B).

Respiratory activity was also measured in hepatic isolated mitochondria of rats treated with MeJA. The results are shown in Figure 6. Basal respiration in non-treated animals (corn oil) was 37% (with succinate) and 58% (with α-ketoglutarate) lower in mitochondria of arthritic

rats (compared to the controls). Treatment with 300 mg·Kg^{-1} MeJA was able to modify the basal respiration, but in different ways for control and arthritic animals (Figure 6A). For controls, basal respiration was diminished (-30%) using succinate solely as substrate and, for arthritic rats, basal respiration was stimulated (+110%) when α-ketoglutarate was the substrate. State III respiration was not modified in mitochondria from control rats treated with MeJA, but it was stimulated (+90%) in mitochondria from arthritic rats when α-ketoglutarate was the substrate (Figure 6B). State IV respiration was practically not modified by treatment, except by a discrete inhibition (with succinate) in arthritic rats treated with 75 mg·Kg^{-1} MeJA (Figure 6D). Respiratory control (RC) was not modified by treatment with MeJA (Figure 6C). The increment in RC of control rats observed in Figure 6C should be considered an artifact because maximum RC is already observed in control mitochondria. The ADP/O ratio was not modified by MeJA treatment (not shown).

NADH and succinate oxidases activities in hepatic isolated mitochondria

Considering that mitochondrial respiratory activity, especially state III respiration, was inhibited when incubated with MeJA, NADH and succinate oxidases activities were further measured in disrupted mitochondria. Figure 7A shows the effects of MeJA exogenously added in the range up to 10 mM. Activities of both NADH oxidase and succinate oxidases were diminished with increasing MeJA concentration in both control and arthritic rats. EC_{50} for succinate oxidase was similar in control and arthritic rats, but EC_{50} of NADH oxidase in controls was only one third of that found in arthritic rats (Table IV). However, it is important to highlight that NADH oxidase activity in the absence of MeJA was already 30% lower in arthritic rats ($p=0.0036$; compared to the controls). Panel C in Figure 7 shows the effects of MeJA on oxygen consumption of disrupted mitochondria using the couple TMPD-ascorbate as substrate, which donate electrons directly to cytochrome c/complex IV of the mitochondrial respiratory chain. In this condition, oxygen consumption was not modified by MeJA in neither control nor arthritic rats.

Activities of NADH and succinate oxidases were also measured in disrupted mitochondria of rats treated with MeJA. The results are shown in Figure 7B. Succinate oxidase activity was not different between non-treated control and arthritic rats, but NADH oxidase activity was 30% lower in arthritic rats (compared to the controls). Treatment of control rats with 300 mg·Kg^{-1} MeJA was able to inhibit succinate oxidase and NADH oxidase by approximately

50%. Activity of both enzymes was also inhibited by MeJA in arthritic rats, which achieved the same levels of those in treated control rats.

Glucokinase activity

Figure 8 shows the effects of MeJA on the liver hexokinase (glucokinase) activity in controls and arthritic rats. Figure 8A shows the effects of MeJA exogenously added in the range up to 10 mM. Glucokinase activity in the absence of MeJA was 33% higher in the liver of arthritic rats ($p=0.0225$; compared to the controls). MeJA was able to diminish glucokinase activity in both control and arthritic rats, but only at the concentration of 10 mM. Figure 8B shows glucokinase activity in rats treated with MeJA. Glucokinase activity was 60% higher in non-treated arthritic rats (compared to the controls). Glucokinase activity was 60% lower in control rats treated with 300 $mg \cdot Kg^{-1}$ MeJA and approximately 70% lower in arthritic rats treated with all doses of MeJA.

DISCUSSION

The anti-inflammatory action

The experimental model of chronic inflammation used in the present study was induced by a high dose of Freund's adjuvant (500 ug; see Methods) and is considered a severe arthritis model in rats, which shares features of advanced human rheumatoid arthritis [Bracht et al., 2016; Hegen et al., 2008]. In this model, animals develop an intense inflammatory response to adjuvant in the contralateral paw (polyarthritis) and furthermore present signs of cachexia and generalized inflammatory manifestations [Bracht et al., 2016; Comar et al., 2013]. Systemic inflammation was observed in arthritic animals used in this work, as evidenced by increased plasma and hepatic MPO activity, decreased levels of plasma albumin, leukocytosis and severe secondary lesions to arthritis.

MeJA was effective as an anti-inflammatory agent, particularly at the dose of 300 $mg \cdot Kg^{-1}$. This can be concluded from the lesser contralateral paw edema and lower number of total leukocytes in the peripheral blood and those recruited into the femorotibial joints and also for the lower MPO activity in the plasma and liver. These actions of MeJA were similar to ibuprofen, a classical anti-inflammatory drug used to treat rheumatoid arthritis. On the other hand, MeJA was not able to diminish secondary lesions decurrent from arthritis nor able to increase the plasma albumin levels. However, it is important to highlight that this model of arthritis is severe and even the ibuprofen was not effective to diminish the score of secondary lesions. The anti-inflammatory activity of MeJA was already showed previously, but only *in vitro* using isolated cells, specifically using LPS-activated murine macrophages (RAW264.7) [Kim et al., 2016; Lee et al., 2011; Dang et al., 2008]. Thus, the results of the current study show that MeJA is an effective anti-inflammatory agent also *in vivo*. Regarding the anti-inflammatory mechanism it has not yet been completely clarified, but it has been demonstrated that MeJA is able to inhibit the NFκB pathway and by extension to inhibit the expression and release of proinflammatory cytokines [Kim et al., 2016; Lee et al., 2011].

The doses of MeJA that were effective as anti-inflammatory (150-300 $mg \cdot Kg^{-1}$) may be considered relatively elevated for clinical studies, however, they are similar of those previously used to evaluated the antitumoral properties of MeJA, which were in the range between 0.5-10.0 mM for *in vitro* studies and 50-1000 $mg \cdot Kg^{-1}$ for *in vivo* studies [Cesari et al., 2014]. On the other hand, it has been reported notable absence of MeJA toxicity in these doses, including an intravenous dose similar to the highest dose orally administrated in the present study [Cesari

et al., 2014; Umukoro & Olugbemide, 2011; Fingrut & Flescher, 2002]. In addition, the plasma markers of hepatic and renal damage were not modified by treatment, on the contrary, MeJA at the doses of 150 and 300 $mg \cdot Kg^{-1}$ improved the slightly increased activity of plasma phosphatase alkaline (ALP) observed in arthritic rats.

The antioxidant action

Oxidative stress is significantly altered systemically in rats with Freund's adjuvant-induced arthritis and it was also observed in the present study for plasma and liver, which will be separately discussed as follows. In the plasma, higher levels of carbonyl groups indicate that oxidative stress is occurring in a place where antioxidant enzymes and glutathione contribute poorly and the antioxidant activity depends mainly on thiol groups (sulfhydryl groups) of albumin [Bracht et al., 2016]. Levels of albumin, thiols and antioxidant capacity (FRAP) in the plasma were decreased by arthritis in this work and also previously [Bracht et al., 2016]. Treatment of arthritic rats with MeJA was able to decrease the plasma levels of carbonyl protein groups, but it was not able to increase albumin content. On the other hand, thiol groups and antioxidant capacity were both increased in the plasma. The anti-inflammatory action of MeJA was therefore not enough to increase albumin levels in the plasma, but it was able to increase thiol groups of the albumin and, consequently, the plasma antioxidant capacity. It is important to highlight that systemic inflammation was only partially improved by MeJA treatment.

Regarding the liver, oxidative stress was reported to be more pronounced when compared to other tissues of arthritic rats and additionally it seems to be associated with the prominent metabolic modifications that occur in the organ [Wendt et al., 2015; Comar et al., 2013]. Liver of arthritic rats presents higher levels of ROS, lipoperoxides and protein carbonyl groups in various subcellular fractions (mitochondria, peroxisomes and cytosol), which are accompanied by marked deficiency of catalase activity and very low GSH/GSSG ratios [Comar et al., 2013]. This altered oxidative stress in the arthritic liver was attributed not only to an impaired ROS scavenging system but also to an increased production of ROS, which are both direct and indirectly mediated by pro-inflammatory cytokines. Indeed, TNF-α and IL-1 were reported directly to stimulate mitochondrial ROS production and to diminish catalase activity in the liver [Kastl et al., 2014; Yasmineh et al., 1991]. Pro-inflammatory cytokines, including those released from rheumatic joints, indirectly also stimulate oxidative metabolism in the liver of arthritic rats, a phenomenon which generates a more oxidizing environment and a more intense production of ROS [Comar et al., 2013; Fedatto et al., 2000; Fedatto et al., 1999]. The scheme

in Figure 9 (black arrows) depicts events in the liver of arthritic rats and illustrates the relation between metabolic modifications and oxidative stress. Livers from arthritic rats present higher activity of glucokinase and higher rates of glycolysis, which lead to an increased flux of reducing equivalents from the cytosol to mitochondria, making cytosol more oxidized [Castro-Ghizoni et al., 2017; Comar et al., 2013]. The very low GSH/GSSG ratios reflect the more oxidized state of the arthritic liver. The fact that no increased oxygen consumption was found in isolated hepatic mitochondria from arthritic rats does not disprove that the phenomenon may occur in the liver, because incubations of isolated organelles do not reproduce exactly the conditions observed *in vivo* or even in the intact organ. In fact, perfused livers of arthritic animals present higher rates of oxygen uptake under several conditions [Comar et al., 2013; Fedatto et al., 2002].

MeJA was able to decrease oxidative stress in the liver of arthritic rats, as evidenced from the lower hepatic levels of ROS content, protein carbonyl groups and lipoperoxides. Figure 9 (red arrows) illustrates as MeJA would modify the oxidative stress in the liver of arthritic rats. There are two possible mechanisms: (1) decreasing the inflammatory process; or (2) stimulating the endogenous antioxidant system. With respect to mechanism (1) it is a general notion that inflammatory cells are responsible for production of an excess of reactive species and other inflammatory mediators, which may cause oxidative and inflammatory injuries. In addition, cytokines released from inflammatory cells also stimulate directly ROS production in other cellular sites, such as mitochondria. Thus, if inflammation is diminished, oxidative stress is also decreased. In the present study, the inflammatory process was decreased by MeJA (300 $mg \cdot Kg^{-1}$) in both plasma and liver of arthritic rats and lowered MPO activity in these tissues reflects the lower number of inflammatory cells. With respect to mechanism (2), the antioxidant system was also improved by MeJA treatment, as verified by increases in catalase activity and GSH/GSSG ratio, which can be at least in part attributed to inhibition of proinflammatory cytokines release.

Mitochondrial ROS production and respiration

MeJA stimulated ROS production in isolated hepatic mitochondria of control and arthritic rats and it also affected mitochondrial respiratory activity. Both events seem to be apparently associated and will be discussed in the following paragraphs. MeJA is reported to stimulate production of ROS in mitochondria as a mechanism that induces apoptosis of cancer cells, which are more sensitive than normal cells to higher ROS content [Cesari et al., 2013]. In addition, MeJA was reported to interact more selectively with mitochondria of cancer cells where

it affects respiratory activity and provoke ATP depletion as a mechanism that also induces apoptosis [Raviv et al., 2013]. In the present study, mitochondrial ROS production was equally stimulated by MeJA (exogenously added) in control and arthritic rats in a range of concentration that was similar to those previously used for cancer cell lines [Figure 3A; Milrot et al., 2012]. This latter observation does not allow to absolutely affirm that MeJA interacts more selectively with mitochondria of cancer cells, at least to stimulate ROS production, because it occurred similarly in mitochondria of normal cells. When compared to arthritis, isolated hepatic mitochondria of control rats were more sensible to MeJA action, as evidenced by its lower EC_{50} to stimulate ROS production. Thus, ROS-mediated cytotoxicity is probably related with the ability of cells to scavenge ROS whose production is similarly stimulated by MeJA in different cells.

Mitochondrial respiration, particularly in the presence of ADP, was inhibited by MeJA (exogenously added) proportionally to increase of ROS production in both control and arthritic rats, and in the same range of MeJA concentration. In other words, ATP depletion in mitochondria occurred simultaneously to ROS production stimulus and these are possibly associated events. In this context, it is already firmly established that inhibition of mitochondrial electron transport increases mitochondrial membrane potential ($\Delta\psi_m$) and stimulates ROS production [Cadenas & Boveris, 2011]. The present results not only corroborate this mechanism but they additionally show that MeJA inhibits mitochondrial electron transport in one or more points situated between the complex I and III of the respiratory chain. This can be concluded from the strong inhibition of mitochondrial NADH oxidase (complex I) and succinate oxidase (complex II) activities without inhibiting the oxygen consumption of disrupted mitochondria using TMPD-ascorbate as electron donor to cytochrome c (complex IV).

The production of ROS was stimulated in mitochondria incubated with MeJA exogenously added and also in mitochondria isolated from animals treated with MeJA (300 $mg \cdot Kg^{-1}$), but the respiratory activity was significantly inhibited only in mitochondria incubated with MeJA. On the contrary, respiratory activity was discreetly stimulated in mitochondria isolated from arthritic animals treated with MeJA when α-ketoglutarate was the substrate (Figure 6A and B). In addition, mitochondrial ATP synthesis (ADP/O) was not modified in control and arthritic rats treated with MeJA, a condition that is not apparently compatible with ATP depletion, at least not at the expense of oxidative phosphorylation in isolated mitochondria. These results seem also to be contradictory, but it is important to mention that respiration of mitochondria with exogenous MeJa sets up an acute exposition in which the drug is readily available to the organelle. On the other hand, isolated mitochondria from treated animals are no longer in contact with the MeJA because it even if residual was probably lost during the organelle

isolation. Thus, it is possible that the altered mitochondrial respiratory activity may be not permanent but transient modifications that occurred only when in contact with MeJA. If this is true then it is important to consider that the transient action of MeJA could be extrapolated for studies using isolated cells and additional endeavor should be made to confirm them *in vivo*. On the other hand, these findings allow concluding that the increase of mitochondrial ROS generation may be at least partially dissociated from the inhibition of mitochondrial respiration because it occurred equally in treated animals. In other words, mitochondrial ROS generation would be caused by a mechanism other than this. In this sense, it was reported that when attached to external mitochondrial membrane hexokinase decreases mitochondrial membrane potential ($\Delta\psi_m$) and suppresses ROS generation, while MeJA was reported to bind hexokinase and detach it from mitochondria, a condition that should stimulate ROS production [Mailloux et al., 2011; Goldin et al., 2008]. Even so, inhibition of respiratory chain should not be underestimated and it is possible that is even minimally occurring *in vivo* and contributing to stimulate mitochondrial ROS production.

Not all results of this work can be fully explained, as in the case of succinate oxidase and NADH oxidase inhibition in mitochondria of control and arthritic rats treated with 300 $mg \cdot Kg^{-1}$ MeJA, a situation in that mitochondrial respiration was not affected. A plausible explanation would be that inhibition of these mitochondrial enzymes was not limiting to modify the mitochondrial respiratory activity. The reason is that the activity of these enzymes is measured in disrupted mitochondria without having the limits imposed by the coupling of intact mitochondria, as proton gradient, for example, are absent. Again, these results suggest that *in vivo* these enzymes may be inhibited by MeJA and additionally indicate that the mitochondrial respiratory chain may be even minimally inhibited and so contributing to stimulate mitochondrial ROS production.

Glucokinase activity and antioxidant mechanism

MeJA diminished oxidative stress in the liver of arthritic rats, but at the same time it stimulated ROS production in isolated hepatic mitochondria of arthritic and control rats. These results seem apparently contradictory, but careful analysis of our results suggests that they may in fact be compatible. MeJA was able to diminish inflammation and consequently it also diminished the production of ROS by inflammatory cells and also that production stimulated by pro-inflammatory cytokines. At the same time MeJA stimulated mitochondrial ROS production but not enough to cause oxidative stress in the liver because the antioxidant system was improved

to such extent that it was able to scavenge excess of ROS. In fact, catalase activity was improved by some extent in addition to complete restoration of the GSH/GSSG ratio. The first effect just mentioned may be partially explained by decreased inhibitory action of TNF-α and IL-1, but restoration of the GSH/GSSG ratio may have been favored by a possible modification in the hepatic metabolism as a consequence of glucokinase inhibition (see Figure 8A). The reason is that inhibition of glucokinase leads to lower levels of glucose 6-phosphate and to lower rates of glycolysis, a condition that could be diminishing the flux of reducing equivalents from cytosol to mitochondria. This phenomenon should generate a more reducing environment, which is compatible with the normalization of the GSH/GSSG ratio and low rates of ROS production in the liver of arthritic rats. The results of the present work show that in addition to detachment of hexokinase (glucokinase) from mitochondrial membrane MeJA was also able to inhibit its activity.

CONCLUDING REMARKS

In conclusion it can be said that MeJA was able to diminish the articular and systemic inflammation in rats with severe adjuvant-induced arthritis. MeJA was similarly able to diminish the pronounced oxidative stress in the plasma and liver of arthritic rats, an effect that seems to be the consequence of both a suppression of the cytokine-induced pro-oxidant system and an improvement of the antioxidant defense, particularly the GSH/GSSG ratio. Although MeJA has also stimulated ROS production in the liver mitochondria of arthritic and control rats, oxidative stress was not modified in the organ, a phenomenon that could be associated to a diminished flux through glycolysis due to glucokinase inhibition. The effective doses of MeJA in the present work may be still relatively high, however, they had no toxic effects and make this compound a potentially important starting point for anti-inflammatory and anti-rheumatic drugs development.

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COMPETING INTERESTS

The authors declare that no competing interest exists and that they all approved the final manuscript.

AUTHORS' CONTRIBUTIONS

JFC conceived and designed the experiments. CABA and JSN induced the arthritis and treated the animals. JSN and FMSS performed the experiments of inflammation. ABSN, LSM and GAG performed the experiments of oxidative stress and mitochondrial respiratory activity. LB measured the glucokinase activity. RMP provided the techniques for measurement of the plasma antioxidant activity. JFC wrote the paper. AB reviewed the paper. All authors read and approved the final version of the paper.

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TABLES AND FIGURES

TABLE I. ***Number of leukocytes in the peripheral blood and femorotibial joint cavities.*** The number of leukocytes in the peripheral blood was measured before adjuvant induction (initial, at day 0) in the arthritic group (A) and at day 18 in all groups. The number of articular leukocytes was measured in the femorotibial hind joints at day 18. A, non-treated arthritic rats; A corn oil, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 $mg \cdot Kg^{-1}$; IBU, arthritic rats treated with ibuprofen at the dose of 30 $mg \cdot Kg^{-1}$.

Parameters	**Initial**	**A**	**A corn oil**	**A75**	**A150**	**A300**	**IBU**
	(at day 0)	blood leukocytes (at day 18)					
Total leukocytes ($x10^3$) $(mm^3)^{-1}$	13.1 ± 0.5^a	66.7 ± 12.1^b	47.2 ± 1.3^b	52.8 ± 6.4^b	52.8 ± 6.4^b	32.6 ± 2.4^c	34.6 ± 2.2^c
PMN cells (%)	15 ± 2^a	70 ± 3^b	64 ± 4^b	66 ± 3^b	66 ± 3^b	48 ± 6^c	62 ± 5^b
		Articular leukocytes (hind left joint)					
Total leukocytes ($x10^4$) $(mm^3)^{-1}$	---	16.8 ± 3.2^a	13.7 ± 1.7^a	16.1 ± 1.8^a	2.0 ± 0.7^b	0.9 ± 0.3^c	3.9 ± 1.1^b
PMN cells (%)	---	53 ± 3^a	69 ± 6^a	62 ± 2^a	58 ± 2^b	61 ± 3^b	65 ± 3^b
		Articular leukocytes (hind right joint)					
Total leukocytes ($x10^4$) $(mm^3)^{-1}$	---	6.9 ± 0.7^a	6.1 ± 1.5^a	6.5 ± 0.7^a	7.0 ± 1.5^a	2.3 ± 0.2^b	1.1 ± 0.4^b
PMN cells (%)	---	53 ± 1^a	61 ± 8^a	69 ± 2^a	60 ± 1^a	74 ± 3^a	69 ± 4^a

Data are the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line are different.

TABLE II. ***Plasma parameters of inflammation and liver/renal damage.*** C, controls treated with corn oil; C300, control treated with MeJA at the dose of 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}; IBU, arthritic rats treated with ibuprofen at the dose of 30 mg·Kg^{-1}.

Parameter	**Groups**						
	C	**C300**	**A**	**A75**	**A150**	**A300**	**IBU**
AST (U·L^{-1})	37.6 ± 1.6^{a}	36.6 ± 3.9^{a}	77.4 ± 2.8^{b}	51.7 ± 1.6^{a,c}	49.3 ± 5.5^{a,c}	52.4 ± 1.6^{a,c}	60.6 ± 3.6^{c}
ALT (U·L^{-1})	18.9 ± 0.8	18.6 ± 0.6	16.5 ± 1.1	19.2 ± 1.2	20.0 ± 1.1	19.0 ± 0.6	26.1 ± 0.6
ALP (U·L^{-1})	60.7 ± 7.7^{a}	59.0 ± 5.4^{a}	139.5 ± 11.6^{b}	114.4 ± 11.3^{b}	70.2 ± 6.2^{a}	81.1 ± 8.1^{a}	151.1 ± 5.9^{b}
Total protein (g·dL^{-1})	5.92 ± 0.33	5.82 ± 0.26	5.92 ± 0.08	6.06 ± 0.13	6.35 ± 0.14	5.58 ± 0.13	5.81 ± 0.20
Albumin (g·dL^{-1})	2.35 ± 0.21^{a}	2.30 ± 0.02^{a}	1.33 ± 0.03^{b}	1.17 ± 0.07^{b}	1.58 ± 0.12^{b}	1.60 ± 0.09^{b}	2.11 ± 0.06^{a}
Globulin (g·dL^{-1})	3.56 ± 0.53^{a}	3.40 ± 0.10^{a}	4.59 ± 0.10^{b}	4.88 ± 0.18^{b}	4.77 ± 0.14^{b}	3.97 ± 0.16^{a}	3.69 ± 0.16^{a}
Albumin/globulin (A/G ratio)	0.70 ± 0.14^{a}	0.68 ± 0.02^{a}	0.29 ± 0.01^{b}	0.24 ± 0.02^{b}	0.33 ± 0.04^{b}	0.41 ± 0.04^{b}	0.58 ± 0.02^{a}
Creatinine (mg·dL^{-1})	0.37 ± 0.08^{a}	0.38 ± 0.1^{a}	0.55 ± 0.01^{a}	0.58 ± 0.02^{a}	0.50 ± 0.02^{a}	0.55 ± 0.02^{a}	0.77 ± 0.09^{b}
MPO activity (nmol·min^{-1}·mg^{-1})	2.30 ± 0.35^{a}	3.11 ± 0.24^{a}	14.74 ± 1.41^{b}	13.90 ± 1.89^{b}	14.93 ± 1.61^{b}	8.44 ± 1.10^{c}	8.32 ± 0.87^{c}

Data are the mean ± standard error of the mean of 4-7 animals. Values with different superscript letters in the same line are different.

TABLE III. ***Effects of MeJA on antioxidant and inflammation parameters in the livers of control and arthritic rats.*** C, controls treated with corn oil; C300, control treated with MeJA at the dose of 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, 150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}; AIBU, arthritic rats treated with ibuprofen at the dose of 30 mg·Kg^{-1}. All parameters are referred per mg of protein.

Parameter	**Groups**						
	C	**C300**	**A**	**A75**	**A150**	**A300**	**IBU**
GSH (nmol·mg^{-1})	11.42 ± 0.61^{a}	12.03 ± 1.54^{a}	7.17 ± 0.32^{b}	8.63 ± 0.89^{b}	7.81 ± 0.78^{b}	12.28 ± 0.85^{a}	8.70 ± 0.68^{b}
GSSG (nmol·mg^{-1})	1.47 ± 0.18^{a}	1.30 ± 0.15^{a}	1.69 ± 0.17^{a}	1.42 ± 0.233^{a}	1.65 ± 0.33^{a}	1.40 ± 0.29^{a}	1.66 ± 0.24^{a}
GSH + 2GSSG (nmol GSH units·mg^{-1})	14.36 ± 0.83^{a}	14.63 ± 1.49^{a}	10.55 ± 0.34^{b}	11.49 ± 1.27^{b}	11.10 ± 0.84^{b}	14.13 ± 0.50^{a}	12.02 ± 0.39^{a,b}
GSH/GSSG ratio	8.75 ± 0.20^{a}	10.80 ± 1.20^{a}	4.84 ± 0.44^{b}	6.33 ± 0.60^{a,b}	6.17 ± 0.58^{a,b}	8.25 ± 1.11^{a}	6.56 ± 1.02^{a,b}
Catalase activity (mmol·min^{-1}·mg^{-1})	1.12 ± 0.09^{a}	1.01 ± 0.02^{a}	0.24 ± 0.02^{b}	0.21± 0.02^{b}	0.25± 0.01^{b}	0.41 ± 0.04^{c}	0.26 ± 0.03^{b}
SOD activity (U·mg^{-1})	1.88 ± 0.08^{a}	2.16 ± 0.17^{a}	1.76 ± 0.20^{a}	1.93 ± 0.18^{a}	2.35 ± 0.10^{a}	2.26 ± 0.22^{a}	2.41 ± 0.27^{a}
MPO activity (nmol·min^{-1}·mg^{-1})	16.70 ± 0.08^{a}	16.01 ± 0.06^{a}	23.12 ± 0.82^{b}	20.88 ± 1.27^{b}	18.92 ± 1.61^{a}	16.90 ± 0.08^{a}	18.43 ± 1.62^{a}

Data are the mean ± standard error of the mean of 3-7 animals. Values with different superscript letters in the same line are different.

TABLE IV. **Effective concentration of MeJA that causes 50% (EC_{50}) of maximum stimulus or inhibition on liver parameters of arthritic and control rats.** The EC_{50} of MeJA exogenously added was calculated for the stimulation in mitochondrial ROS generation (Figure 3A), inhibition of mitochondrial state III respiration and respiratory control (Figures 4B and 5B), inhibition of mitochondrial NADH oxidase and succinate oxidase activities (Figure 7A) and inhibition of glucokinase activity (Figure 8A). The EC_{50} was calculated by numerical interpolation using Stineman's interpolation formula.

Parameter	MeJA EC_{50} (mM)		
	Control	Arthritis	p
Mitochondrial ROS generation	0.60 ± 0.12	1.09 ± 0.12	0.0188*
State III (succinate)	2.21 ± 0.31	2.58 ± 0.38	0.4791
State III (α-ketoglutarate)	1.06 ± 0.06	0.83 ± 0.06	0.0351
RC (succinate)	1.53 ± 0.17	3.03 ± 0.08	0.0002*
RC (α-ketoglutarate)	0.69 ± 0.06	0.70 ± 0.09	0.9263
NADH oxidase	0.43 ± 0.04	1.34 ± 0.12*	<0.0001*
Succinate oxidase	1.15 ± 0.20	0.93 ± 0.14	0.4427
Glucokinase	2.20 ± 0.60	6.60 ± 0.80*	0.0108*

Data are the mean ± standard error of the mean of 4-5 animals. Values with superscript asterisk (*) are statistically different.

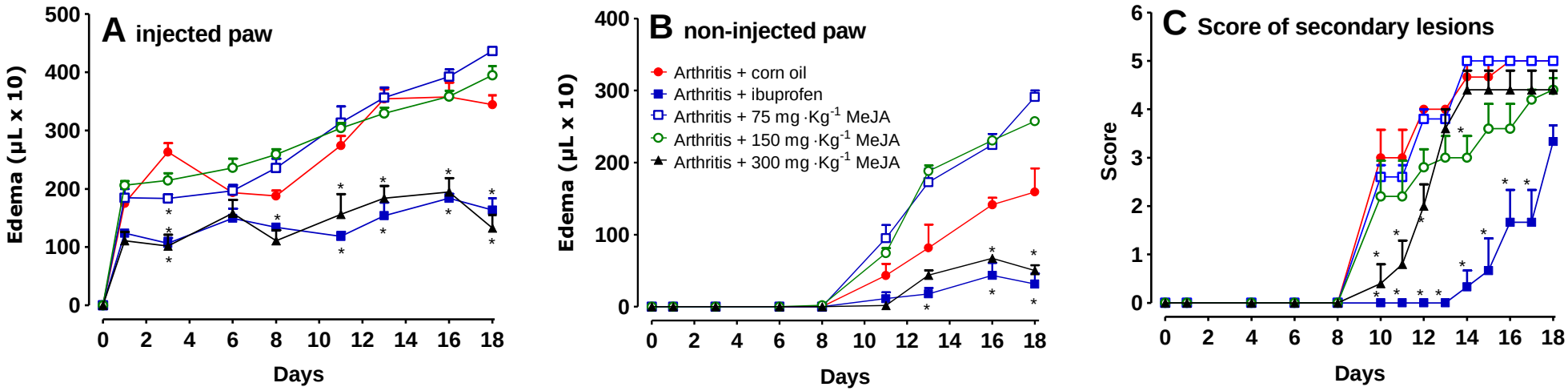

Figure 1. **Effects of methyl jasmonate on the inflammatory response to Freund's adjuvant in Holtzman rats. (A)** edema in the injected paw; **(B)** edema in the posterior non-injected paw; and **(C)** score of secondary lesions. The rats were treated with corn oil, MeJA or ibuprofen as described in Methods. The volume of paws was monitored by plethysmography and the values are expressed as the increase in paw volume. Score of secondary lesions (arthritic score) are defined in Methods. Each point represents the mean ± standard error of the mean of 5-6 animals. Asterisks indicate statistical significance ($p<0.05$) relative to arthritic rats treated with corn oil.

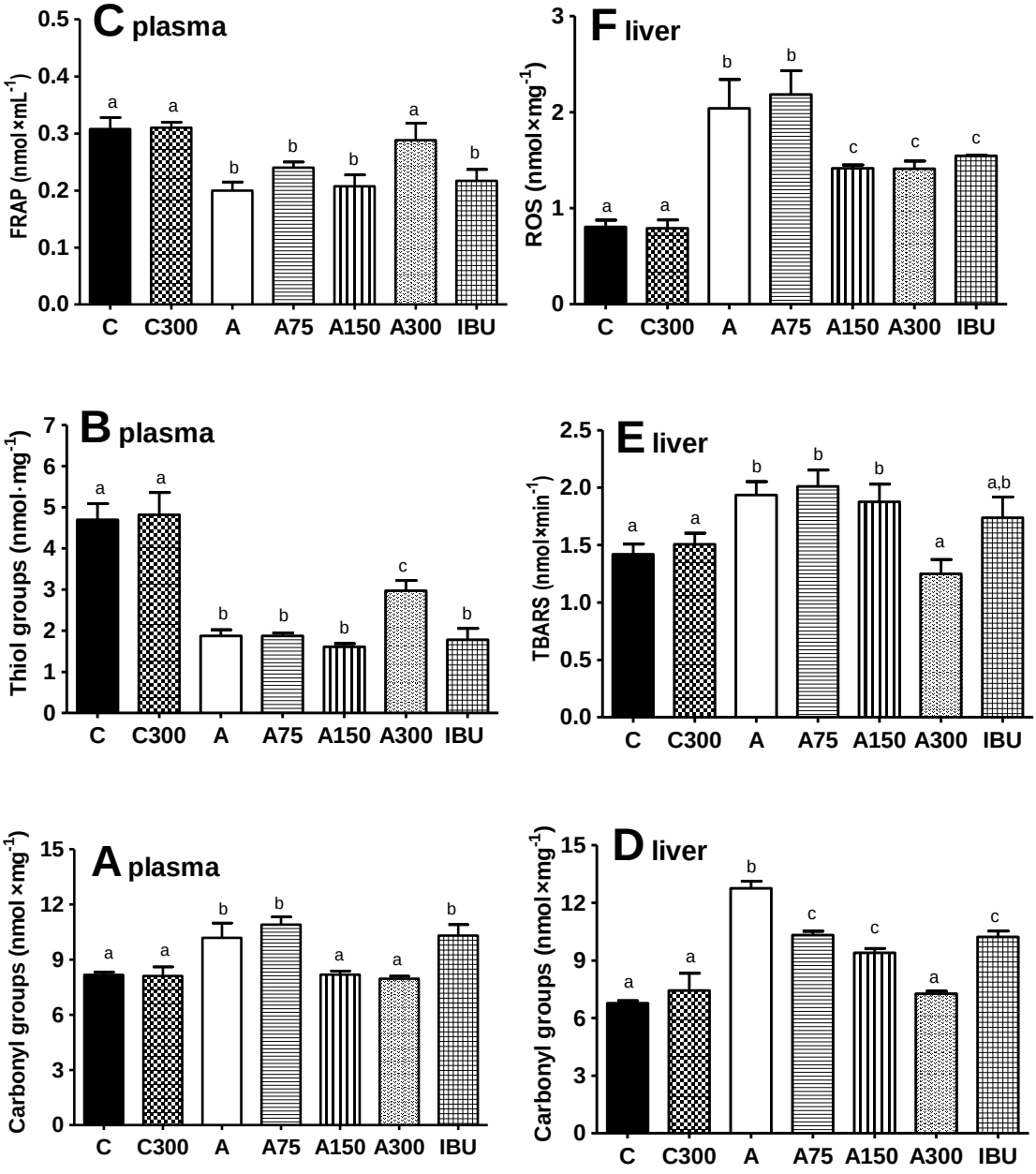

Figure 2. ***Effects of MeJA on oxidative state in the plasma and liver of arthritic rats.*** **(A)** Plasma protein carbonyl groups; **(B)** Plasma thiol groups; **(C)** Ferric reducing ability of plasma (FRAP); **(D)** Hepatic protein carbonyl groups; **(E)** Hepatic TBARS levels; **(F)** Hepatic oxygen reactive species (ROS). C, controls treated with corn oil; C300, control treated with MeJA at the dose of 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}; IBU, arthritic rats treated with ibuprofen (30 mg·Kg^{-1}). Data represent the mean ± SEM of 3-7 animals. Values with different superscript letters are statistically different ($p < 0.05$).

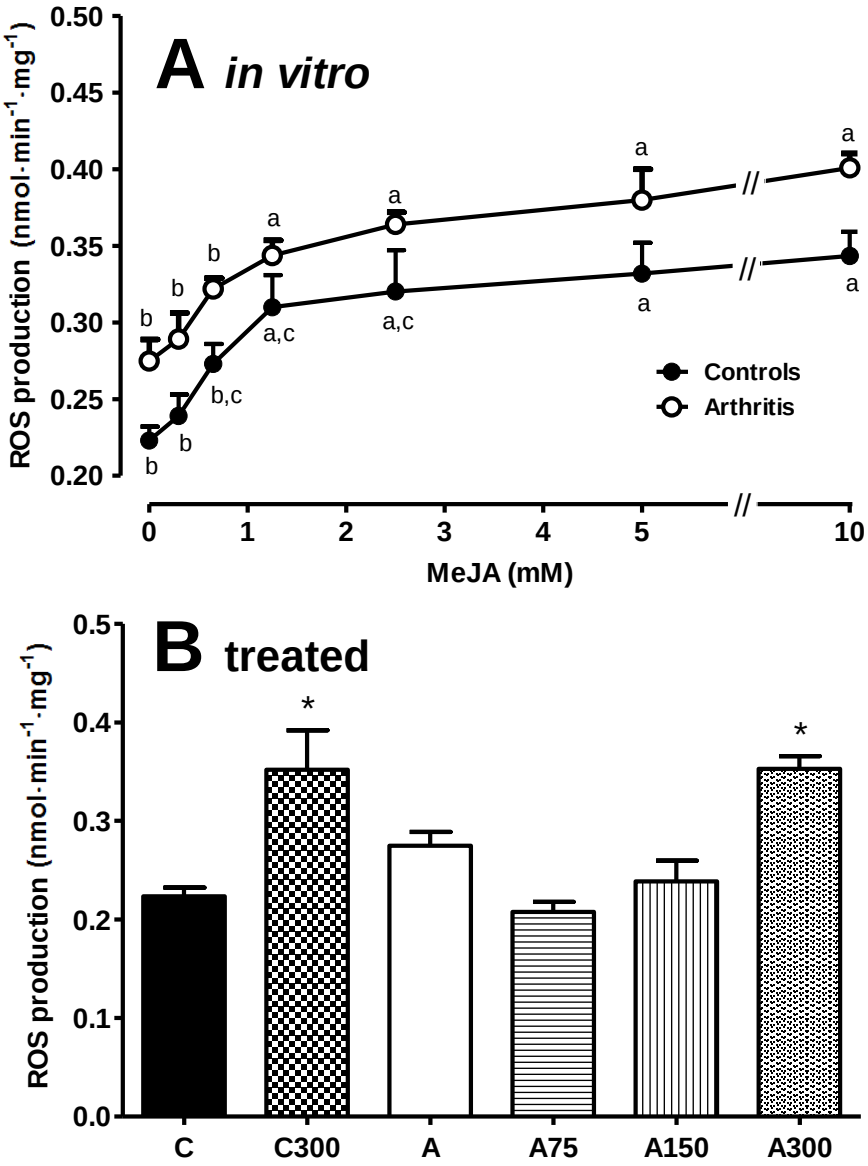

Figure 3. ***Effects of MeJA on ROS generation in mitochondria isolated from liver of control and arthritic rats.*** **(A)** Concentration dependence on the stimulation of mitochondrial ROS generation by MeJA exogenously added; **(B)** Mitochondrial ROS generation in rats treated with MeJA. ROS generation was measured as described in the experimental section. C, controls treated with corn oil; C300, control treated with MeJA at the dose of 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}. Data represent the mean ± SEM of 4-7 animals. Values with different superscript letters in the same condition are statistically different ($p < 0.05$) in Panel A. *$p<0.05$: different from the others in Panel B.

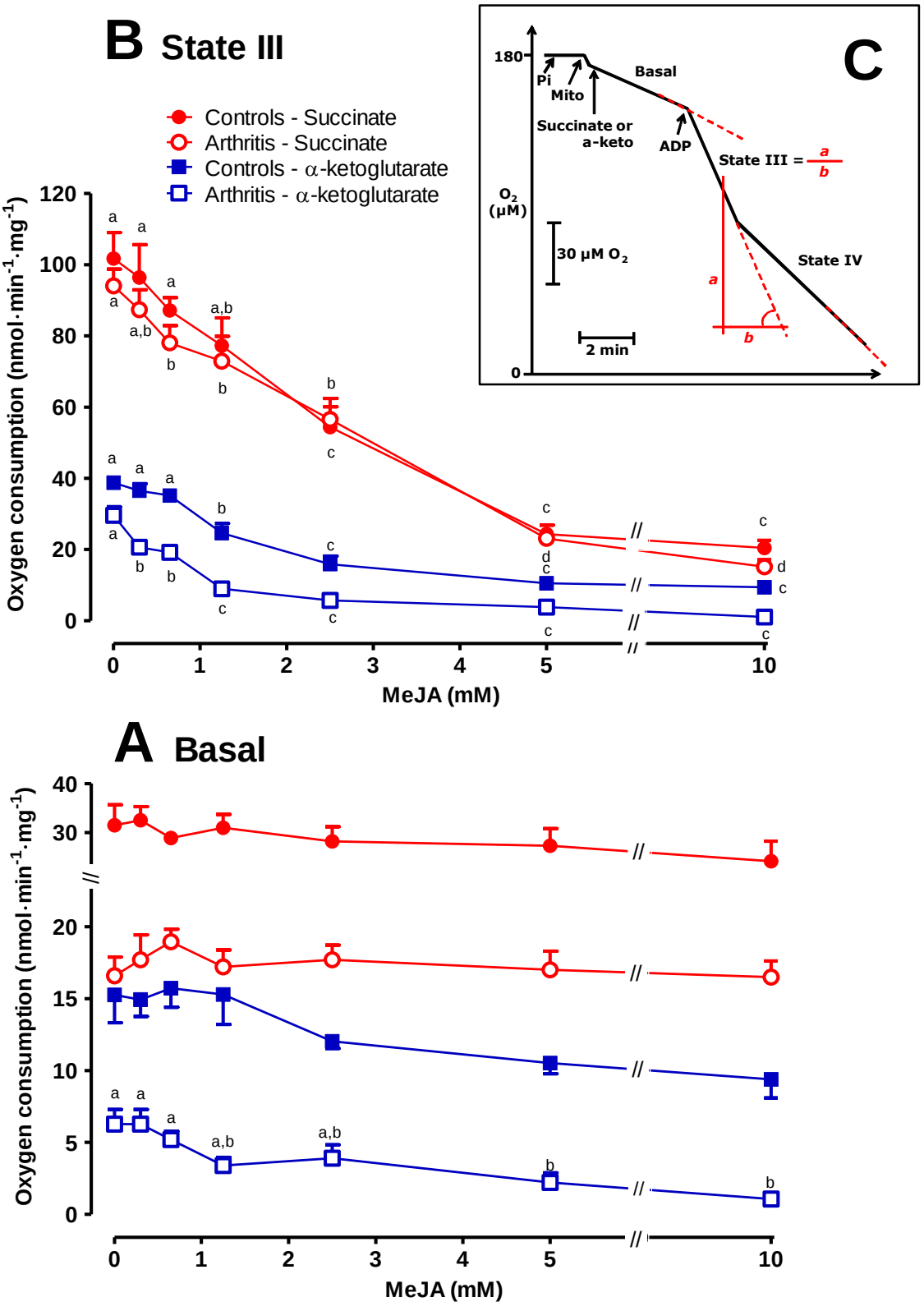

Figure 4. ***Effects of MeJA on the respiratory activity in mitochondria isolated from liver.*** Concentration dependence of the inhibition of basal respiration **(A)** and state III respiration **(B)** by MeJA exogenously added. **Panel C**: Experimental protocol and oxygen consumption calculation. Intact mitochondria were incubated in a closed oxygraph chamber as described in methods. Succinate (10 mM) and α-ketoglutarate (10 mM) were used as substrate. Data represent the mean ± SEM of 4-5 animals. Values with different superscript letters in the same condition are different.

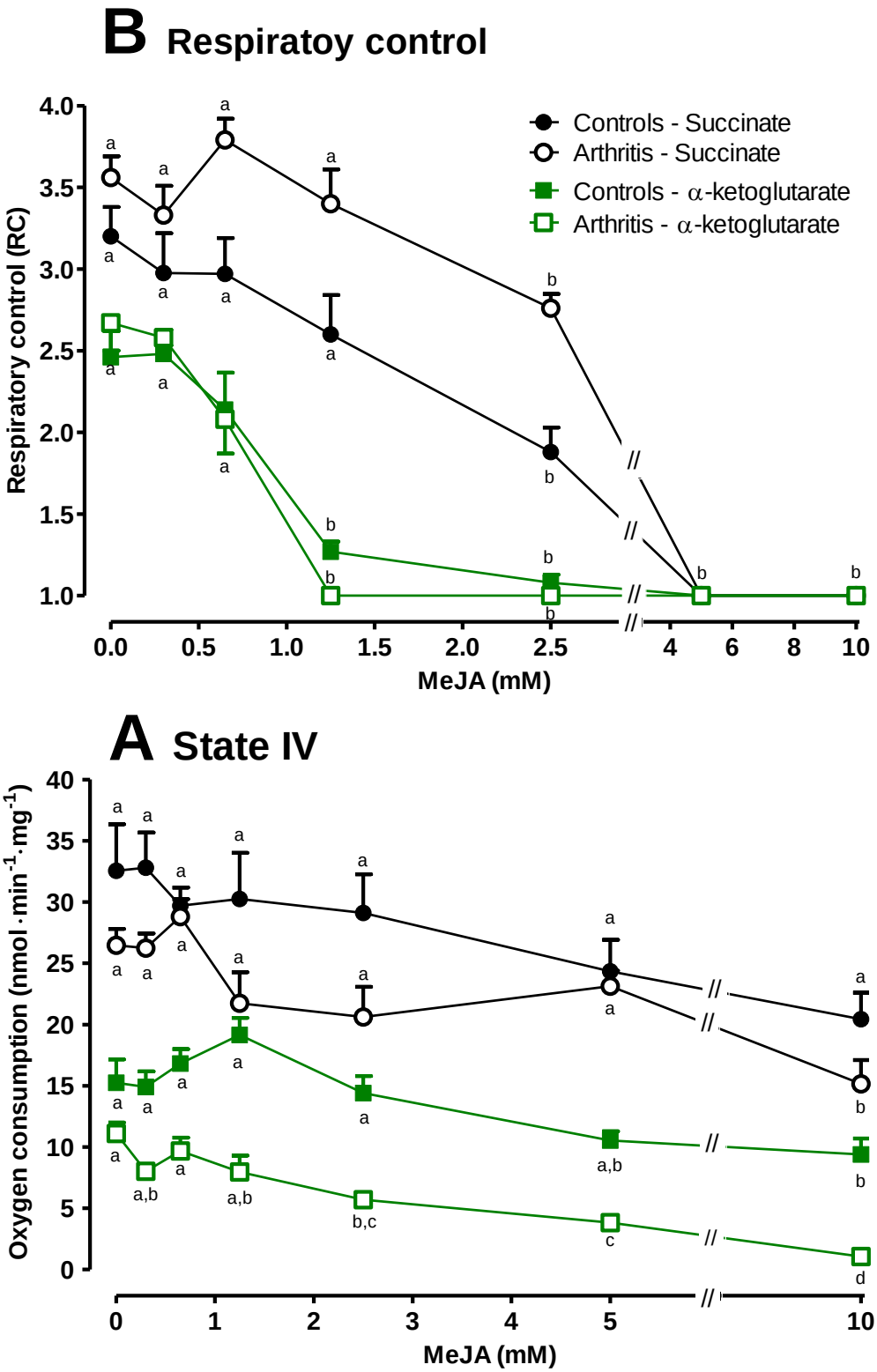

Figure 5. ***Effects of MeJA on the respiratory activity in mitochondria isolated from liver.*** Concentration dependence of the inhibition of state IV respiration **(A)** and respiratory control **(B)** by MeJA exogenously added. Intact mitochondria were incubated in a closed oxygraph chamber as described in methods. Succinate (10 mM) and α-ketoglutarate (10 mM) were used as substrate. Respiratory control (RC) was calculated as state III/state IV ratio. Data represent the mean ± SEM of 4-5 animals. Values with different superscript letters in the same condition are different.

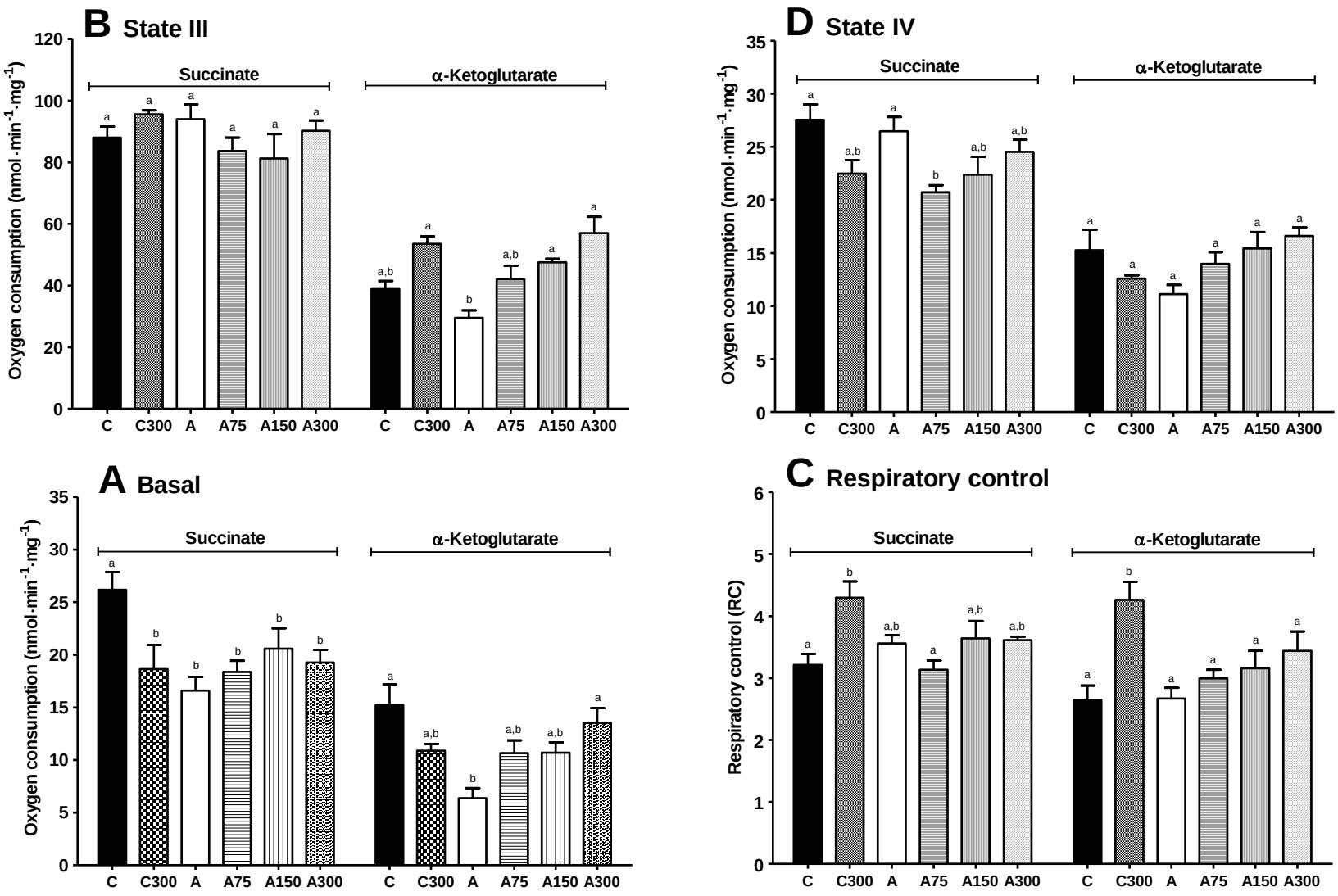

Figure 6. ***Effects of MeJA treatment on respiratory activity of intact isolated hepatic mitochondria.*** C, controls treated with corn oil; C300, control treated with 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}. Data represent the mean ± SEM of 4-5 animals. Values with different superscript letters are statistically different ($p < 0.05$).

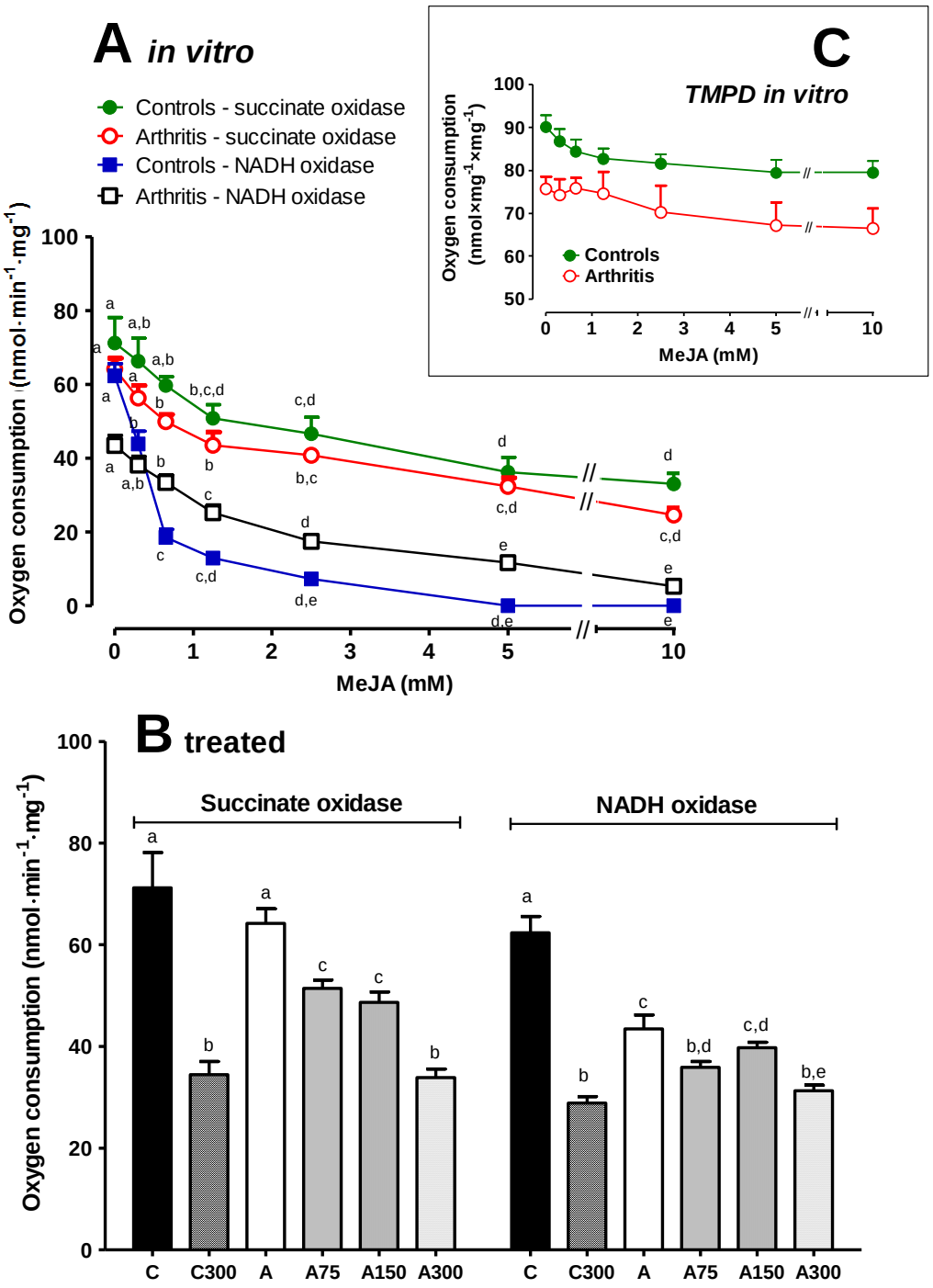

Figure 7. ***Effects of MeJA on mitochondrial membrane-bound enzyme activities.*** Freeze-thawing disrupted mitochondria were incubated in a closed oxygraph chamber as described in methods. **(A)** Concentration dependence of the inhibition of succinate oxidase and NADH oxidase activity by MeJA exogenously added; **(B)** Enzyme activities in mitochondria isolated from livers of rats treated with MeJA; **(C)** Oxygen consumption in the presence of TMPD-ascorbate. C, controls; C300, control treated with 300 mg·Kg^{-1} MeJA; A, arthritic rats; A75, 150 and 300, arthritic rats treated with 75, 150 and 300 mg·Kg^{-1} MeJA. Data represent the mean ± SEM of 4-7 animals. Panel A: values with different superscript letters are different in the same condition ($p<0.05$) Panel B: different superscript letters are different.

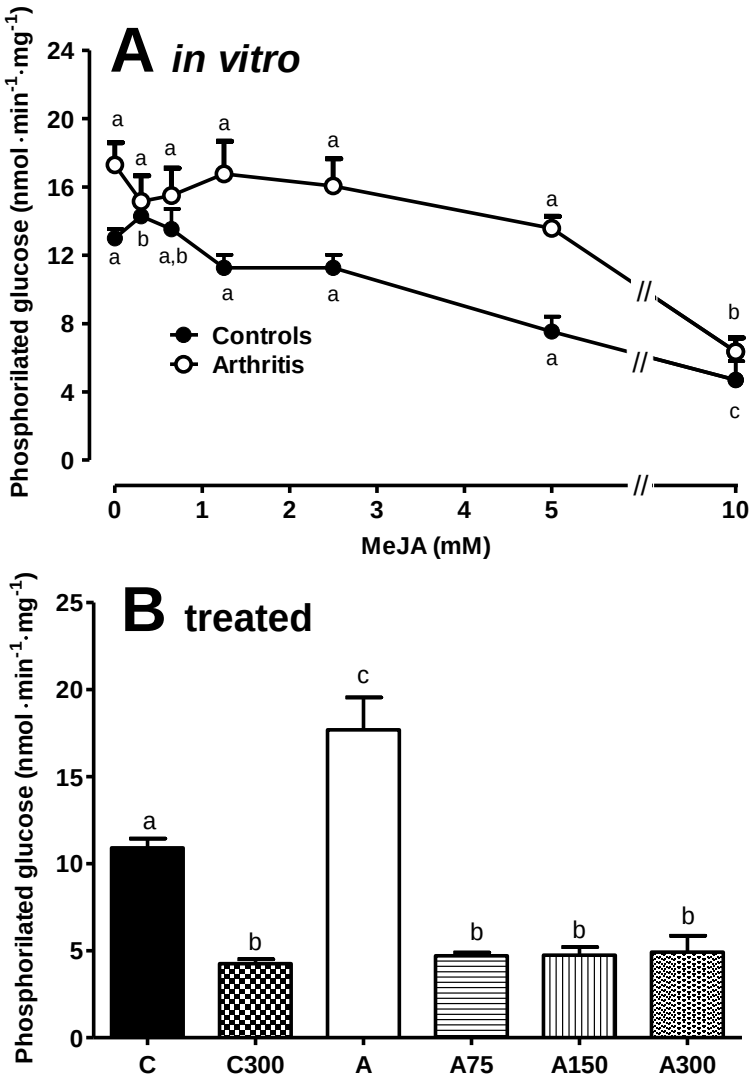

Figure 8. ***Effects of MeJA on glucokinase activity in the liver of control and arthritic rats.*** **(A)** Concentration dependence of the inhibition of glucokinase activity by MeJA exogenously added; **(B)** Glucokinase activity in the livers from rats treated with MeJA. Glucokinase activity was measured in the supernatant of liver homogenate as described in methods. C, controls treated with corn oil; C300, control treated with MeJA at the dose of 300 mg·Kg^{-1}; A, arthritic rats treated with corn oil; A75, A150 and A300, arthritic rats treated with MeJA at the doses of 75, 150 and 300 mg·Kg^{-1}. Data represent the mean ± SEM of 4-5 animals. Panel A: values with different superscript letters in the same condition are statistically different ($p < 0.05$). Panel B: values with different superscript letters are statistically different ($p<0.05$).

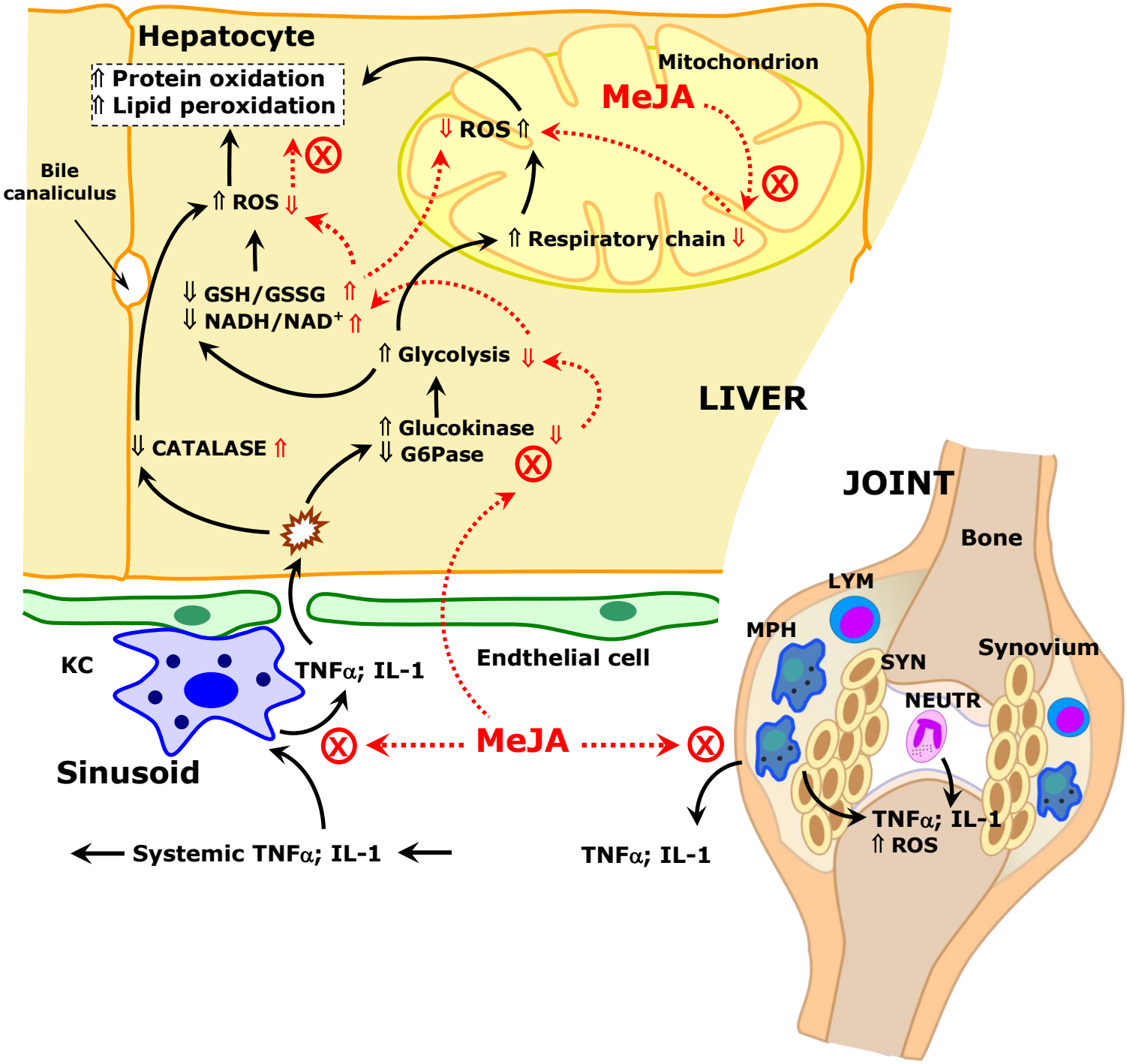

Figure 9. ***Schematic representation of events leading to increased oxidative stress in the liver and joints of rats with adjuvant arthritis and the actions of MeJA.*** The scheme is discussed in the text and is based on the results of the current work and on previously published data. The symbol ⇑ means up-regulation and ⇓ down-regulation. Black arrows indicate the events in the absence of MeJA and red arrows indicate the effects of MeJA. Abbreviations: TNF-α, tumor necrosis factor alpha; IL-1,

interleukin 1; GSH, reduced glutathione; GSSG, oxidized glutathione; MPH, macrophages; KC, Kupffer cell; LYM, lymphocytes; NEUTR, neutrophils; SYN, synoviocytes; ROS, reactive oxygen species; GK, glucokinase; G6Pase, glucose 6-phosphatase.