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Efeitos do resveratrol sobre o metabolismo de carboidratos no fígado de ratos com artrite induzida por adjuvante

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas do Centro de Ciências Biológicas da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutor em Biologia das Interações Orgânicas.

Orientador: Prof. Dr. Jurandir Fernando Comar
Coorientadora: Profa. Dra. Lívia Bracht

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BIOGRAFIA

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APRESENTAÇÃO

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. A realização dos experimentos ocorreu no Laboratório de Metabolismo Hepático do Departamento de Bioquímica em parceria com o Laboratório de Inflamação do Departamento de Farmacologia e Terapêutica da Universidade Estadual de Maringá. A apresentação está na forma de dois artigos científicos originais, em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas.

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2) Simões, M.S.; Silva, F.M.S.; Ames-Sibin, A.P.; Sá-Nakanishi, A.B.; Bracht, L.; Bersani-Amado, C.A.; Bracht, A.; Comar, J.F. **Effects of resveratrol on inflammation and liver metabolism of healthy and arthritic rats.** SERÁ SUBMETIDO ao Journal of Traditional and Complementary Medicines (Cite score: 8.0).

RESUMO

INTRODUÇÃO E OBJETIVOS: O resveratrol é um polifenol que apresenta atividade imunomoduladora e anti-inflamatória e, por isso, tem sido investigado como um promissor agente antirreumático. De fato, o resveratrol reduz as manifestações da artrite reumatoide em modelos animais e pacientes com a doença. A artrite reumatoide é uma doença inflamatória crônica e sistêmica que afeta as articulações e outros órgãos, como o fígado. Alterações metabólicas também são proeminentes na doença, como a condição de perda muscular conhecida como caquexia reumatoide. As modificações metabólicas são também significativas no fígado de ratos com artrite induzida por adjuvante, os quais apresentam um alterado metabolismo de xenobióticos e reduzida gliconeogênese em decorrência de um estado mais oxidado e inflamado do órgão. O resveratrol é extensamente metabolizado no fígado à compostos biologicamente ativos. Nenhum estudo até o momento mostrou se o resveratrol modifica o metabolismo hepático e em que extensão ele é metabolizado no fígado de ratos artríticos. Assim, este estudo investigou os efeitos do resveratrol sobre o catabolismo do glicogênio e a gliconeogênese no fígado de ratos com artrite induzida por adjuvante, como um modelo para artrite reumatoide severa. As ações do resveratrol-3-O-glicuronídeo (R3G), o único metabólito do resveratrol que é significativamente produzido no fígado de roedores, foram avaliadas sobre as mesmas vias metabólicas no fígado. A biotransformação do resveratrol em R3G foi também avaliada no fígado saudável e artrítico. Por fim, avaliou-se os efeitos do resveratrol administrado por via oral sobre as manifestações artríticas e o metabolismo hepático de ratos artríticos.

MÉTODOS: A artrite foi induzida em ratos Holtzman com o adjuvante completo de Freund e animais saudáveis da mesma idade foram usados como controles. O fígado em perfusão isolada foi usado como ferramenta experimental. O catabolismo do glicogênio e a gliconeogênese a partir de lactato foram respectivamente avaliados no fígado de ratos alimentados e em jejum de acordo com dois protocolos: (1) resveratrol em concentrações na faixa de até 200 μM e R3G (200 μM) introduzidos no fígado perfundido de ratos controles e artríticos; e (2) perfusão de fígados isolados de ratos saudáveis e artríticos oralmente tratados com resveratrol em doses na faixa de até 500 mg/kg por 5 dias antes da indução da artrite e por adicionais 18 dias. Para a biotransformação do

resveratrol, os fígados de ratos alimentados e em jejum foram perfundidos conforme descrito acima e o resveratrol introduzido no órgão perfundido nas concentrações de 50, 100 e 200 μM. Amostras do fluido de perfusão efluente foram coletadas em intervalos regulares e analisadas para o seu conteúdo de glicose, lactato e piruvato. Resveratrol e R3G foram quantificados por HPLC. O consumo de oxigênio hepático foi monitorado por polarografia. Mitocôndrias hepáticas foram isoladas por centrifugação diferencial. A respiração foi medida polarograficamente, na presença de resveratrol, usando succinato e α-cetoglutarato (10 mM) como substratos. As velocidades de consumo de oxigênio foram medidas sob três condições: antes da adição de ADP (basal), após a adição de ADP (estado III) e após cessar o estímulo do ADP (estado IV). O controle respiratório e ADP/O foram calculados. As atividades da NADH-oxidase e succinato-oxidase foram medidas usando mitocôndrias rompidas. Edema da pata, escore artrítico e atividade da mieloperoxi-dase (MPO) hepática foram determinados para avaliar a inflamação.

RESULTADOS E DISCUSSÃO: A gliconeogênese hepática foi diminuída em ratos artríticos. O resveratrol inibiu a glicogenólise e glicólise quando infundido em concentrações acima de 25-50 μM e a gliconeogênese quando infundido mesmo na concentração de 10 μM diretamente em fígados perfundidos de ratos artríticos e saudáveis. Os fígados artríticos foram um pouco mais sensíveis ao resveratrol, principalmente em relação à gliconeogênese. A respiração mitocondiral estimulada por ADP (estado III), o controle respiratório (RC) e as atividades da NADH- e succinato-oxidase foram inibidas pelo resveratrol em concentrações acima de 100 μM e foram apenas em parte responsáveis pela inibição da gliconeogênese, já que a via foi fortemente inibida pelo resveratrol em concentrações tão baixas quanto 10 μM. O consumo hepático de oxigênio foi inibido pelo resveratrol em concentrações também acima de 100 μM e deve ser consequência da inibição da respiração mitocondrial em ratos saudáveis e artríticos. A inibição encontrada para o resveratrol no metabolismo hepático parece ser transitória e existe apenas quando o resveratrol está presente no órgão, uma vez que as alterações metabólicas tendem a retornar àquelas anteriores à introdução do composto. Por outro lado, esses efeitos não devem ser desprezíveis, pois concentrações significativas de resveratrol tem sido reladadas no plasma humano após sua administração oral. O resveratrol foi

significativamente metabolizado em R3G no fígado de ratos saudáveis e artríticos, no entanto, a formação de R3G foi menor em ratos artríticos quando introduzido em baixas concentrações e é provavelmente uma consequência da menor atividade das glucuronil transferases. Em altas concentrações, a formação de R3G foi consideravelmente inibida em fígados artríticos e saudáveis, mais para o artrítico, como consequência de uma menor disposição de glicose devido à inibição da gliconeogênese. O R3G também inibiu consideravelmente a gliconeogênese e o catabolismo do glicogênio no fígado de ratos artríticos e saudáveis. O consumo hepático de oxigênio, entretanto, não foi inibido pelo R3G, fenômeno esperado se for considerado que os glicuronídeos não devem permear a membrana mitocondrial interna. Por outro lado, o catabolismo do glicogênio foi inibido em maior extensão quando comparado ao resveratrol. Os efeitos do R3G também não devem ser desprezíveis porque esse metabólito também é encontrado em concentrações significativas no plasma de humanos, às vezes mais altas do que a molécula parental, como consequência da rápida glucuronidação após a ingestão oral de resveratrol. Para os animais tratados, o resveratrol diminuiu a atividade de MPO do fígado em doses acima de 100 mg/Kg, diminuiu o edema da pata contralateral em doses acima de 250 mg/Kg e retardou consideravelmente a evolução do escore artrítico e do edema da pata injetada em doses acima de 250 mg/Kg. O resveratrol não melhorou a reduzida gliconeogênese no fígado de ratos artríticos. No entanto, o resveratrol não modificou negativamente a gliconeogênese no fígado saudável e artrítico. O catabolismo do glicogênio foi apenas em parte modificado pelo resveratrol no fígado saudável e artrítico, mas esses efeitos foram não pronunciados e não dependentes da dose. Assim, não é provável que o resveratrol esteja afetando negativamente o metabolismo hepático de ratos tratados, ainda mais se for considerado que a gliconeogênese é extremamente sensível a alterações no ambiente e integridade celular e não foi significativamente prejudicada.

CONCLUSÃO: O resveratrol administrado por via oral diminui a inflamação articular e sistêmica de ratos artríticos e não modifica consideravelmente a gliconeogênese e o catabolismo do glicogênio no fígado artrítico e saudável. Por outro lado, o resveratrol e o R3G inibem o catabolismo do glicogênio quando introduzidos em altas concentrações (acima de 25 μM) e a gluconeogênese quando introduzidos em baixas concentrações (10 μM) em fígados perfundidos

de ratos saudáveis e artríticos. Esses efeitos do resveratrol parecem ser transitórios e existem apenas quando o resveratrol está presente no órgão, mas não devem ser desprezíveis, pois há relatos de concentrações similares encontradas no plasma após a ingestão oral de resveratrol por voluntários saudáveis. Esses resultados mostram que o resveratrol pode ser um adjuvante promissor nas atuais abordagens que visam o tratamento da artrite, contudo, é recomendado cautela tendo em vista seus efeitos transitórios no metabolismo hepático.

PALAVRAS-CHAVE: Artrite por adjuvante, artrite reumatoide, resveratrol, resveratrol glicuronídeo, metabolismo hepático, gliconeogênese.

ABSTRACT

BACKGROUND AND AIMS: Resveratrol is a dietary polyphenol that presents immune-modulatory and anti-inflammatory activity and, for this reason, it has been investigated as a promising antirheumatic agent. In fact, resveratrol lowers the rheumatoid arthritis manifestations in animal models and patients with the disease. Rheumatoid arthritis is a chronic and systemic inflammatory disease that affects the joints and many other organs. Metabolic alterations are also prominent in the disease, such as the muscle wasting condition known as rheumatoid cachexia. With regard to the liver, metabolic modifications are equally significant in rats with adjuvant-induced arthritis, which present altered metabolism of xenobiotics and reduced gluconeogenesis as a consequence of a more oxidized state and pronounced inflammation in the organ. No study until now has showed if resveratrol improves the hepatic metabolism of arthritic rats. In addition, resveratrol is extensively metabolized in the liver to metabolites which has been reported to be biologically active. Again, no study until now has showed if the hepatic metabolism of resveratrol is modified in the rheumatoid arthritis. Therefore, this study aimed to evaluate the effects of resveratrol on gluconeogenesis and glycogen catabolism in the liver of rats with adjuvant-induced arthritis, a model for severe rheumatoid arthritis. The actions of resveratrol-3-O-glucuronide (R3G), the only resveratrol metabolite significantly produced in the liver of rodents, on these same metabolic pathways were evaluated for comparative purposes. The biotransformation of resveratrol into R3G was further evaluated in the liver of arthritic and healthy rats. Finally, this study also evaluated the effects of the orally administered resveratrol on the arthritic manifestations and liver metabolism of arthritic rats.

METHODS: The induction of arthritis was performed in Holtzman rats with Freund's adjuvant and healthy animals with similar age were controls. The liver in isolated perfusion was used as experimental tool. The rate of gluconeogenesis from lactate and glycogen catabolism were evaluated respectively in the liver of fasted and fed rats according with two protocols: (1) resveratrol at concentrations in the range up to 200 μM and R3G (200 μM) directly introduced in the perfused liver of healthy and arthritic rats; and (2) free-drug perfused livers isolated of healthy and arthritic rats orally treated with resveratrol at doses in the range up to 500 mg/Kg for 5 days prior to the arthritis induction

and by additional 18 days after. For the biotransformation of resveratrol, the livers of fed and fasted rats were perfused as above described and resveratrol was directly introduced in the perfused organ at the concentrations of 50, 100 and 200 μM. Samples of the effluent perfusion fluid were collected at regular intervals and analyzed for their content of glucose, lactate and pyruvate by spectrophotometry, and resveratrol and R3G by HPLC. Hepatic oxygen uptake was monitored by polarography. Liver mitochondria were isolated by differential centrifugation. Respiration was measured by polarography, in the presence of resveratrol, using succinate and α-ketoglutarate (10 mM) as substrates. Oxygen uptake rates were measured under three conditions: before ADP addition (basal), after ADP addition (state III) and after cessation of the ADP stimulation (state IV). Respiratory control and ADP/O ratio were calculated. NADH oxidase and succinate oxidase activities were measured using disrupted mitochondria. Paw edema, arthritic score and liver myeloperoxidase (MPO) activity were measured to evaluate the arthritic inflammatory manifestations.

RESULTS AND DISCUSSION: The hepatic gluconeogenesis was decreased in arthritic rats. Resveratrol inhibited the glycogenolysis and glycolysis when directly infused at concentrations above 25-50 μM and gluconeogenesis when directly infused even at the concentration of 10 μM, in perfused livers of arthritic and healthy rats. Arthritic livers were a quite more sensitive to resveratrol, especially in relation to gluconeogenesis. The ADP-stimulated respiration (state III), respiratory control (RC) and NADH- and succinate-oxidase activities were inhibited by resveratrol at concentrations above 100 μM and they were only in part responsible for gluconeogenesis inhibition since the pathway was strongly inhibited by resveratrol at concentrations so low as 10 μM. The liver oxygen uptake was inhibited by resveratrol at concentrations also above 100 μM and it should be a consequence of the inhibition of mitochondrial respiration in both healthy and arthritic rats. However, the inhibition found for resveratrol on liver metabolism seem to be transitory and exist only when the resveratrol is present in the organ since the metabolic rates tend to return to those before the introduction of the compound. On the other hand, these effects must not be neglected because significant concentrations of resveratrol are reported to be found in the plasma of humans after its oral administration. Resveratrol was significantly metabolized to R3G in the liver of healthy and arthritic rats, however, the formation of R3G was lower in arthritic rats when introduced at

low concentrations and it is probably a consequence of the lower activity of glucuronyl transferases. At high concentrations, the formation of R3G was considerably inhibited in both arthritic and healthy livers, more for arthritic condition, as a consequence of a lower glucose disposal due to gluconeogenesis inhibition. R3G also inhibited considerably the gluconeogenesis and glycogen catabolism in the liver of arthritic and healthy rats. The oxygen uptake, however, was not inhibited by R3G, an expected situation considering that glucuronides should not permeate the inner mitochondrial membrane. On the other hand, the glycogen catabolism was inhibited in a higher extension when compared to that of resveratrol. The effects of R3G should not be also neglected because this metabolite is also reported to be found in significant concentrations in the plasma of humans, sometimes higher than the parental molecule, as a consequence of the rapid glucuronidation following oral ingestion of resveratrol. For treated animals, resveratrol decreased the liver MPO activity at doses above 100 mg/Kg, decreased the contralateral paw edema at doses above 250 mg/Kg, and considerably delayed the arthritic score and the injected paw edema at doses above 250 mg/Kg. Resveratrol did not improve the reduced gluconeogenesis in the liver of arthritic rats. However, resveratrol did not negatively modify the gluconeogenesis in the healthy and arthritic liver. Glycogen catabolism was only in part modified by resveratrol in the healthy and arthritic liver, but these effects were not pronounced and not dose-dependent. Thus, it is not probable that resveratrol is negatively affecting the liver metabolism, moreover if is taken in consideration that gluconeogenesis is extremely sensitive to alterations in the cellular behavior and integrity and it was not significantly impaired.

CONCLUSION: The orally administered resveratrol improves the articular and systemic inflammation in arthritic rats and does not modify considerably the gluconeogenesis and glycogen catabolism in the liver of arthritic and healthy rats. On the other hand, resveratrol and R3G inhibit the glycogen catabolism when directly introduced at high concentrations (above 25 μM) and gluconeogenesis when directly introduced even at low concentrations (10 μM) in perfused livers of healthy and arthritic rats. These effects of resveratrol seem to be transitory and exist only when the resveratrol is present in the organ, however, they should not be neglected because similar concentrations are reported to be achieved in the plasma after oral ingestion of resveratrol by healthy volunteers.

This results show that resveratrol may be a promising adjuvant to the most current approaches aiming at rheumatoid arthritis therapy, however, caution is recommended with respect to the transitory effects on the liver metabolism.

KEYWORDS: Adjuvant-induced arthritis, rheumatoid arthritis, resveratrol, Resveratrol glucuronide, liver metabolism, gluconeogenesis.

Resveratrol biotransformation and actions on the liver metabolism of healthy and arthritic rats

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Running head: Actions of resveratrol on the hepatic metabolism

ABSTRACT

This study investigated the effects of resveratrol on glycogen catabolism and gluconeogenesis in the perfused livers of healthy and arthritic rats. The actions of resveratrol-3-O-glucuronide (R3G) were also investigated for comparative purposes. The biotransformation of resveratrol into R3G was further evaluated in the livers of arthritic and healthy rats. Arthritis was induced with Freund's adjuvant. Resveratrol at concentrations in the range up to 200 μM and 200 μM R3G were introduced in perfused livers from fasted and fed rats. The liver output of glucose, lactate and pyruvate was measured in the outflowing perfusate by spectrophotometry and resveratrol and R3G quantified by HPLC. Oxygen uptake was monitored by polarography. Respiratory activity of isolated mitochondria was also investigated. Resveratrol inhibited the glycogenolysis and glycolysis when infused at concentrations above 25-50 μM and gluconeogenesis when infused even at the concentration of 10 μM in the liver of arthritic and healthy rats. Arthritic livers were a quite more sensitive to resveratrol, especially in relation to gluconeogenesis. The ADP-stimulated respiration and NADH- and succinate-oxidase activities were inhibited by resveratrol at concentrations above 100 μM and they were only partially responsible for gluconeogenesis inhibition. Resveratrol was significantly metabolized to R3G in the liver of healthy and arthritic rats, however, the formation of R3G was lower in arthritic livers and it must be a consequence of the lower glucuronyl transferases activities and the lower glucose disposal for glucuronidation. When compared to resveratrol, R3G inhibited the gluconeogenesis in a lower extension and glycogen catabolism in a higher extension. The effects of resveratrol and R3G tended to be transitory and existed only when the resveratrol is present in the organ, however, they should not be neglected because significant concentrations of both compounds are reported to be found in the plasma of humans after oral ingestion of resveratrol.

Keywords*: adjuvant-induced arthritis, rheumatoid arthritis, resveratrol, liver metabolism, resveratrol glucuronide, gluconeogenesis.*

INTRODUCTION

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) is a dietary polyphenol popularly known as the ingredient in red wine that prevents heart and other aging-associated diseases [Timmers et al., 2012]. In fact, studies with animal models and clinical trials have shown that resveratrol exhibits anticarcinogenic, antidiabetic, neuroprotective and cardioprotective actions [Ramirez-Garza et al., 2018; Zordoky et al., 2015]. Many of these therapeutic properties of resveratrol have been mainly associated with its antioxidant activity [Carrizzo et al., 2013] and therefore it has been added to nutritional supplements with the purpose of preventing aging-related diseases [Smoliga et al., 2011; Tomé-Carneiro et al., 2012; Bo et al., 2013].

Resveratrol has been reported also to exert beneficial effects by means of immunomodulatory and anti-inflammatory activity [Oliveira et al., 2017]. For this reason this polyphenol has been investigated as a promising antirheumatic agent [Nguyen et al., 2017]. In fact, resveratrol inhibits the synthesis of proinflammatory cytokines in synoviocytes isolated from arthritic rats and in human synoviocytes cell lines [Chen et al., 2013; Tian et al., 2013; Nakayama et al., 2012]. The dietary supplementation with resveratrol also lowers the rheumatoid arthritis manifestations in animal models and patients with the disease [Castro et al., 2020, Khojah et al., 2018; Rivieiro-Navieira et al., 2016; Xuzhu et al., 2012]. Resveratrol in association with fenofibrate improves the joint inflammation of rats with adjuvant-induced arthritis [Wahba et al., 2016]. This model of rheumatoid arthritis in rats is aggressive and share many features of advanced rheumatoid arthritis in humans [Stolina et al., 2009].

Rheumatoid arthritis is a chronic inflammatory disease that affects the joints and it is associated with progressive disability and premature mortality [Kitas & Gabriel, 2011]. Rheumatoid arthritis is systemic disease and affects other organs, such as brain, liver and lungs [Mcinnes & Schett, 2011]. Similarly, oxidative stress is increased systemically in animal models and patients with rheumatoid arthritis [Schubert et al., 2016; Bracht et al., 2016; Wendt et al., 2015; Stamp et al., 2012]. Metabolic alterations are also prominent in rheumatoid arthritis, particularly the muscle wasting condition known as rheumatoid cachexia [Roubenoff, 2009]. With respect to the liver, metabolic modifications are equally significant in rats with adjuvant-induced arthritis,

which present altered metabolism of xenobiotics, reduced gluconeogenesis and increased fat acids oxidation [Wendt et al., 2019; Castro-Ghizoni et al., 2017]. The metabolic modifications in the liver of arthritic rats have been shown to be a consequence of a more oxidized state associated with both accelerated body catabolism and pronounced oxidative stress in the organ [Wendt et al., 2019].

Many studies with animal models of hepatic insult and diseases have shown that resveratrol presents hepatoprotective action [Faghihzadeh et al., 2015]. Similarly, resveratrol has been reported to act on hepatic metabolism. In the livers of rodent models of steatosis, for example, resveratrol modifies the lipid metabolism and reduces the lipid accumulation [Aguirre et al., 2014]. However, no study until now has showed if resveratrol improves the hepatic metabolism of arthritic rats. Resveratrol is efficiently absorbed after oral administration in humans and rodents, however, it is extensively metabolized in the liver to resveratrol sulfate and glucuronide, a condition that limits its plasma bioavailability [Springer & Moco, 2019]. In addition, resveratrol metabolites are biologically active, although not as effective as the parent molecule [Xia et al., 2017]. Again, no study until now has showed if the hepatic metabolism of resveratrol itself is modified in the rheumatoid arthritis.

Considering all the above, this study aimed to evaluate the effects of resveratrol on gluconeogenesis and catabolism of glycogen in the liver of rats with adjuvant-induced arthritis, the first organ that receives the resveratrol after intestinal absorption. The effects of resveratrol-3-O-glucuronide, the main resveratrol metabolite in rats, on these same metabolic pathways were also evaluated for comparative purposes. The hepatic metabolism of resveratrol was further evaluated in arthritic rats.

The rat liver in isolated and non-recirculating perfusion was used as experimental tool. This is an excellent system for the study of hepatic metabolic pathways because it has the great advantage of preserving the organ microcirculation. This procedure also prevents the previous metabolism of resveratrol in the intestine or systemically because it is introduced into the portal vein of the perfused liver. Similarly, this procedure practically excludes the long-term effects of resveratrol, as for example, its actions on inflammation and gene expression. That is, in the present study, only the direct short-term actions of resveratrol were evaluated in the liver of arthritic rats, which in turn, should also allow extrapolations for patients with rheumatoid arthritis.

MATERIAL AND METHODS

Chemicals

Trans-resveratrol at 99% purity was purchased from Botica Ouro Preto (Maringá, PR, Brazil). Reservatrol-3-β-mono-D-glucoside at 95% purity, reservatrol-3-β-mono-D-glucoside at reference standard, enzymes and coenzymes were purchased from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were of analytical grade.

Animals and induction of arthritis

Male Holtzman rats weighting 200-220 g (50 days old) were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained under standard laboratory conditions at a temperature of 24 ± 3ºC under a regulated 12 h light/dark cycle. The animals were housed in conventional steel cages (3 rats/cage) and fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). Two hundred animals were used in six different experimental protocols followed throughout the study and, for each one, the animals were randomly distributed into control and arthritic groups. 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 [Bendele et al., 1999]. Animals were used for experiments at day 19th after adjuvant injection. Rats of similar age served as controls. The volume of both hind paws up to the tibiotarsal joint was measured daily by plethysmography. The 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 (CEUA) of the State University of Maringá (Protocol number CEUA 2495130916).

Characterization of the experimental model

The first measurements consisted in indicators of arthritis and liver damage in the rats after 21 days of adjuvant injection. The volume of paws was measured every 3 days by plethysmography. The body weight was checked every 3 days and the severity of secondary lesions was assessed daily from day

10 to 21 as previously described [Sá-Nakanishi et al., 2018]. The ALT and AST activities were assessed in the plasma to evaluate the degree of liver integrity. The myeloperoxidase (MPO) activity was measured in the plasma and liver to evaluate systemic inflammation.

Liver perfusion: gluconeogenesis and glycolysis

Hemoglobin-free non-recirculating liver perfusion was performed as previously described [Comar et al., 2003]. After cannulation of the portal and cava veins, the liver was removed and positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine serum albumin and saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 ºC. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France). Oxygen concentration in the venous perfusate was monitored by a teflon-shielded platinum electrode. Samples of the effluent perfusion fluid were collected at two minutes intervals and analyzed for their metabolite content. Substrates and resveratrol were added to the perfusion fluid according to the experimental protocols. Due to its low water solubility, resveratrol and resveratrol-3-O-glucuronide were added to the perfusion fluid as a dimethylsulfoxide solution to achieve the desired final concentration.

Glycolysis and glycogenolysis were measured in livers isolated from fed rats, which were perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. After stabilization of oxygen consumption, resveratrol was infused at concentrations in the range up to 200 µM during 20 minutes. Glucose, L-lactate and pyruvate were measured by standard enzymatic procedures [Bergmeyer, 1974]. At the steady-state, glycolysis = (lactate + pyruvate)/2 and glycogenolysis = glucose + [(lactate + pyruvate)/2].

Gluconeogenesis was measured in the perfused livers of 12 h fasted rats. These livers were initially perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. After stabilization of oxygen consumption, L-lactate (2 mM) was infused as a gluconeogenic substrate during 60 minutes. At 26 minutes of lactate infusion, resveratrol was infused together at concentrations in the range up to 200 µM for an additional period of 20 minutes. The final 14 minutes

of infusion occurred in the absence of resveratrol. Glucose and pyruvate were measured in the effluent perfusate.

Mitochondrial respiration

Hepatic mitochondria were isolated by differential centrifugation [Saling et al., 2011]. Mitochondrial oxygen consumption was measured by polarography using a teflon-shielded platinum electrode [Lima et al., 2006]. 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 EDTA and 10 mM Tris-HCl (pH 7.4). Succinate and α-ketoglutarate, both at a concentration of 10 mM, were used as substrates. Resveratrol was added at various concentrations in the range up to 500 µM. Rates of oxygen consumption were computed from the slopes of the recorder tracings and expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The respiration rates were measured under three conditions: (a) before the addition of ADP (substrate respiration or basal), (b) just after 0.125 mM ADP addition (state III respiration) and (c) after cessation of the ADP stimulation (state IV). The respiratory control (RC) was calculated as the state III/state IV ratio and the ADP/O ratio was determined according to Sá-Nakanishi et al., 2018.

The activities of NADH-oxidase and succinate-oxidase were measured by polarography using freeze-thawing disrupted mitochondria [Lima et al., 2006]. The incubation medium contained 20 mM Tris-HCl (pH 7,4) and, when appropriate, resveratrol was added at various concentrations in the range up to 500 µM. The reaction was started by the addition of substrates, 10 mM NADH and 10 mM succinate, for NADH-oxidase and succinate-oxidase, respectively. The couple TMPD-ascorbate was in addition used as electron donating substrate to cytochrome c/complex IV of the mitochondrial respiratory chain.

Hepatic biotransformation of resveratrol

The livers were perfused in the same way as above described for fed and fasted rats. These livers were initially perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. For fasted condition, after stabilization of oxygen consumption, L-lactate (2 mM) was infused during 60 minutes. At 26 minutes of lactate infusion, resveratrol was infused together at the

concentration of 200 μM for period of 20 minutes and, after ceasing the resveratrol infusion, only KH buffer + lactate was infused for an additional period of 10 minutes. For fed condition, after stabilization of oxygen consumption, resveratrol was infused at concentrations in the range up to 200 μM during 20 min and, after ceasing the resveratrol infusion, for an additional period of 10 minutes only with KH buffer. Samples of the effluent perfusion fluid were collected at 5 minutes intervals and analyzed for their content of resveratrol and resveratrol-3-O-glucuronide (R3G). The latter is the only resveratrol metabolite produced in significant quantities in the liver of rodents [Springer & Moco, 2019; Planas et al., 2012; Fig. S3]. The samples were then deproteinized by the addition of methanol (5:1), vortexed and centrifuged (6,000g/30 min). Supernatant was collected, filtered and frozen at -80 ºC. Resveratrol and R3G was quantified by HPLC. A reversed-phase C18 CLC-ODS column (5 μm, 250×4.6 mm i.d.; Shimadzu) protected with a CLC-ODS precolumn (5 μm, 4×3 mm i.d.; Phenomenex), was used with a mobile isocratic reversed-phase composed of water, methanol and acetic acid (50:45:5). The injected sample volume was 30 μL. The flow remained constant at 0.5 mL/min, temperature 30ºC, running time of 20 minutes and wavelength of 306 nm [Penalva et al., 2015]. The peaks were identified through the retention times collected by the injection of the resveratrol and R3G standards, being 12 and 7 minutes, respectively.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 8.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and the Tukeypost-hoc test was applied with the 5% level of significance ($p<0.05$). For comparison of two values the student's t test was applied with the 5% level ($p<0.05$).

RESULTS

Arthritis characterization

Adjuvant-induced arthritis has been extensively characterized in rats [Wendt et al., 2019; Castro-Ghizoni et al., 2017; Bracht et al., 2016; Hegen et al., 2008; Martín et al., 2008; Stolina et al., 2009]. For this reason, the results of arthritis characterization in the present study are shown as supplementary material (Fig. S1). Temporal evolution of body weight gain is shown in Fig. S1A. In practical terms, arthritic rats did not gain body weight during the experimental period compared to the healthy condition, thus, arthritis prevented a body mass gain of 40%. At day 21 the volume of the injected and contralateral paws had considerable increase in arthritic rats (Fig. S1B). Secondary lesions appeared at day 10 and reached the highest scores at day 15 (Fig. S1C). The activity of AST and ALT were accessed in the plasma as indicators for hepatic damage and the activity of MPO were accessed in the liver as indicator of liver inflammation. The activity of both AST and ALT in the plasma were not different for healthy and arthritic rats. Arthritis increased the MPO activity by 270% in the plasma and by 67% in the liver. The arthritic liver is approximately 30% heavier than healthy liver when expressed per g of body weight [Castro-Ghizoni et al., 2017].

Effects of resveratrol on glycogen catabolism and glycolysis

Livers from fed rats when perfused with substrate-free medium survive at the expense of glycogen degradation via glycolysis and oxidation of endogenous fatty acids [Comar et al., 2003]. Under these conditions the liver release glucose, lactate and pyruvate as a result of glycogen catabolism. Fig. 1A illustrates the responses of perfused livers from arthritic and healthy rats to resveratrol infusion at concentrations of 10 and 200 μM. It also illustrates a typical experimental protocol, which was used for all other resveratrol concentrations. After a pre-perfusion period of 10 minutes, resveratrol was infused during 20 minutes, followed by additional 10 minutes of resveratrol-free perfusion. Four parameters were measured: glucose release, lactate and pyruvate productions and oxygen consumption. As revealed by Fig. 1A all parameters were stable before the initiation of resveratrol infusion, but the

oxygen uptake, glucose release and pyruvate and lactate production were lower in the liver of arthritic rats. This is the consequence of a lower content of glycogen in the liver of arthritic rats [Fedatto et al., 2002]. Upon resveratrol infusion, oxygen uptake and glucose release remained unchanged at the concentration of 10 μM, but were inhibited at the concentration of 200 μM in both arthritic and healthy rats. There was practically no alteration of the lactate and pyruvate production after resveratrol infusion. After removing the resveratrol from the perfusion liquid, the parameters were different but not substantially modified.

Experiments like those illustrated in Fig. 1A were repeated with 25, 50 and 100 μM resveratrol in order to establish concentration dependences for the effects. For lactate and glucose the values represented in Fig. 1B and 1D are the rates before starting the resveratrol infusion (8-10 minutes perfusion time; zero resveratrol concentration) and the rates observed after stabilization of the changes induced by each concentration. For oxygen uptake the decreases produced by each concentration were represented, a procedure justified by the high basal rates (Fig. 1C). For healthy rats, resveratrol decreased equally by 45% the lactate production at the concentrations of 25, 50 and 100 μM, but no decrease was found at the concentration of 200 μM (Fig. 1B). For arthritic rats, resveratrol decreased the lactate production only at the concentration of 100 μM (-30%). The glucose overflow, which represents the excess of glycogenolysis not diverted into the glycolytic pathway, was decreased equally (-40%) by resveratrol at the concentrations of 25, 50, 100 and 200 μM for healthy rats, but was decreased only at the concentration of 200 μM for arthritic rats (-50%; Fig. 1D). Oxygen uptake was strongly inhibited by 100 μM resveratrol and suffered its greatest decrease at the concentration of 200 μM for both healthy and arthritic rats (Fig. 1C).

The rate of glycogenolysis and glycolysis are represented against the resveratrol concentration in Fig. 1E and F, respectively. In both healthy and arthritic livers, glycogenolysis and glycolysis were inhibited in a similar manner and resveratrol concentration that the glucose and lactate release, respectively. In Fig. 1G, the lactate to pyruvate ratio, an indicator for the cytosolic $NADH/NAD^+$ ratio [Scholz & Bücher, 1965], was plotted against the resveratrol concentration. In the absence of resveratrol, the $NADH/NAD^+$ ratio was 60% higher in the liver of arthritic rats. The latter has been already reported [Wendt

et al., 2019; Fedatto et al., 2002]. For healthy rats, the infusion of resveratrol did not modify the NADH/NAD^+ ratio, but in arthritic rats this ratio was decreased equally (-30%) by resveratrol at the concentrations of 50, 100 and 200 μM, which occurred due to increases of the pyruvate production in arthritic condition (results not shown).

Effects of resveratrol on gluconeogenesis

The effect of resveratrol on gluconeogenesis was investigated in perfused livers using lactate as precursor. The use of L-lactate as the gluconeogenic substrate presents some advantages [Castro-Ghizoni et al., 2017]: it is one of the main gluconeogenic substrates in humans and rodents; it is easily converted into pyruvate by the equilibrium cytosolic enzyme lactate dehydrogenase; and, consequently, allows to evaluate the complete gluconeogenic machinery going through all gluconeogenic steps from pyruvate up to glucose. Fig. 2A shows the time courses of the modifications caused by the infusion of 10 and 200 μM resveratrol in perfused livers from healthy and arthritic rats. It also illustrates a typical experimental protocol, which was used for all other resveratrol concentrations. Livers from 12 hours fasted rats were perfused in order to ensure low glycogen levels. Under such conditions the rate of glucose output reflects mainly the rate of gluconeogenesis [Comar et al., 2016]. Lactate infusion produced progressive increases in glucose and pyruvate productions and oxygen uptake. These increases tended to stabilize at 34 minutes perfusion time. The increment of glucose production was lower in the liver of arthritic rats, a phenomenon already reported by previous studies [Castro-Ghizoni et al., 2017; Ames-Sibin et al., 2018]. Upon resveratrol infusion, the lactate-induced stimulus in the oxygen uptake and glucose and pyruvate production were differently modified. At the concentration of 10 μM, resveratrol decreased the glucose production in the liver of arthritic and healthy rats and stimulated the pyruvate production only in the liver of arthritic rats. At the concentration of 200 μM, resveratrol strongly inhibited the glucose production and oxygen uptake in the liver of both arthritic and healthy rats, but inhibited the pyruvate production only in the liver of arthritic rats. After removing the resveratrol from the perfusion liquid, glucose production and oxygen uptake tended to return to their basal levels, but the pyruvate production was not considerably modified.

Experiments like those illustrated in Fig. 2A were repeated with 25, 50 and 100 μM resveratrol in order to establish concentration dependences for the effects. For pyruvate and glucose the values represented in Fig. 2B and 2D are the rate before starting the infusion of resveratrol (32-34 minutes perfusion time; zero resveratrol concentration) and the rates observed after stabilization of the changes induced by each concentration. For oxygen uptake the decreases produced by each concentration were represented (Fig. 2C). Resveratrol at low concentrations increased the stimulus of the pyruvate production by 40% in the liver of healthy rats (25 and 50 μM) and by 24% in the liver of arthritic rats (10 μM). At high concentrations resveratrol did not change the stimulus of pyruvate production and even inhibited it by 20% in the liver of arthritic rats (200 μM). On the other hand, the stimulus of the oxygen uptake and glucose production was strongly inhibited by resveratrol in a concentration dependent manner for both healthy and arthritic rats. The stimulus of oxygen uptake was inhibited by resveratrol from the dose of 50 μM and a half-maximal inhibition, as obtained by numerical interpolation, can be expected at a concentration of approximately 70 μM for both healthy and arthritic rats (Table 1). For glucose production the stimulus was inhibited by resveratrol already from the dose of 10 μM and a half-maximal inhibition can be expected at a concentration of 10 and 20 μM for healthy and arthritic rats, respectively (Table 1).

Respiration and membrane-bound enzymatic activities of isolated mitochondria

Considering that resveratrol inhibited the hepatic consumption of oxygen, experiments with isolated mitochondria were done with the purpose of facilitating the interpretation of the data obtained in perfused livers. Fig. S2 outlines the experimental approach used to evaluate the respiratory activity of phosphorylating liver mitochondria and the calculation procedures for obtaining the mitochondrial respiratory parameters. Fig. 3 shows the effects of resveratrol in the concentration range up to 500 μM on mitochondrial basal respiration, state III respiration and respiratory control (RC; state III/IV). No modification in basal respiration was found in hepatic mitochondria of arthritic and control rats, except by an increase of oxygen consumption at high concentrations (350-500 μM) when succinate was the respiratory substrate (Fig. 3A). State III respiration was diminished in a concentration dependent manner irrespective of

the substrate (succinate or α-ketoglutarate) and the conditions (healthy or arthritic; Fig. 3B). The half-maximal inhibition (IC_{50}) for state III respiration can be expected at high resveratrol concentrations and it was 34% lower for arthritic rats when α-ketoglutarate was the respiratory substrate and 32% higher when succinate was the substrate (compared to the controls; Table 1). The state IV respiration was practically not modified (results not shown) and, consequently, the respiratory control (RC) was diminished and even abolished with increasing resveratrol concentrations for both conditions and substrates (Fig. 3C). IC_{50} for RC can be expected also at high resveratrol concentrations and it was 42% lower for arthritic rats when α-ketoglutarate was the respiratory substrate and 15% higher when succinate was the substrate (compared to the controls; Table 1).

Considering that the mitochondrial respiratory activity, especially state III respiration, was inhibited when incubated with resveratrol, the NADH- and succinate-oxidase activities were further measured in disrupted mitochondria. The results are shown in Fig. 3D. NADH-oxidase activity was progressively diminished with increasing resveratrol concentrations in mitochondria from both control and arthritic rats, but succinate oxidase was inhibited only at a high concentration (350-500 μM). The IC_{50} values for NADH-oxidase was 32% lower for the arthritic condition.

Hepatic biotransformation of resveratrol

Resveratrol is extensively metabolized in the liver to resveratrol sulfate and glucuronide, which are biologically active [Springer & Moco, 2019; Xia et al., 2017]. Thus, the present study evaluated the biotransformation of resveratrol in perfused livers of healthy and arthritic rats. In humans resveratrol sulfates are important metabolites of resveratrol, however, in rats resveratrol-3-O-glucuronide (R3G) is the major conjugate and resveratrol sulfates had approximately 10 fold lower concentration [Rousova et al., 2016; Planas et al., 2012; Juan et al., 2010]. Fig. S3 shows the chromatograms of the outflowing perfusate from livers perfused with resveratrol and only resveratrol and R3G emerge in significant amount. For these reason only the biotransformation of resveratrol to R3G was evaluated in the present study. The levels of resveratrol and R3G were determined in the effluent perfusate according to the protocols illustrated in Fig. 1A (fed rats) and 2A (fasted rats). Fig. 4A shows the time

courses of the hepatic output of resveratrol and R3G caused by the infusion of 200 μM resveratrol in perfused livers from fed and fasted rats in both controls and arthritis. Fig. 4A illustrates only the perfusion time of Fig. 1A and 2A from that moment in which resveratrol is introduced in the liver, i.e., lactate infusion was omitted in the horizontal bars for fasted rats. Upon resveratrol infusion, the levels of itself and R3G progressively increased in the outflowing perfusate until 20 minutes perfusion time, when its introduction was interrupted. After this period, the hepatic output of resveratrol rapidly decrease while the R3G output continues to increase for arthritic and healthy livers of fed and fasted rats.

The rates of resveratrol and R3G releases in Fig 4A (0-30 minutes) can be best appreciated by evaluating the area under the curve (AUC), which correspond to the total amounts released during this period. Fig. 4B and C shows the amounts of resveratrol and R3G released in the outflowing perfusate of livers from fasted and fed rats, respectively. The hepatic output of resveratrol was higher and the formation of R3G was significantly lower in arthritic livers of both fed and fasted rats. In addition, the formation of R3G was higher in the livers of fed rats when compared with fasted rats. The latter probably occurs as a consequence of a higher glucose disposal from glycogen for glucuronidation of resveratrol in the fed state. For this reason, experiments like those illustrated in Fig. 4A were repeated with 50 and 100 μM resveratrol with fed rats in order to establish concentration dependences of its biotransformation. The rates of resveratrol and R3G releases (AUC: 0-30 minutes) are shown in Fig. 4D. The hepatic output of non-metabolized resveratrol was higher for arthritic rats in all concentrations. The formation of R3G was 44% lower in arthritic livers when 50 μM resveratrol was introduced, however, the R3G output was only 13% lower for arthritic livers when 100 μM resveratrol was introduced. For healthy livers the R3G output increased even more when resveratrol was infused at the concentration of 200 μM while in arthritic livers the formation of R3G was almost completely inhibited for this concentration. It is important to highlight that 200 μM resveratrol was the only concentration that inhibited the glucose release in the livers of fed arthritic rats (Fig. 1D).

As shown in Fig. 4A, the hepatic output of R3G continues to increase after the cessation of resveratrol introduction and the amount of R3G + resveratrol in the outflowing perfusate (AUC: 0-30 minutes) shown in Fig. 4B, C and D corresponds to only a part of the introduced resveratrol. The average amount

of resveratrol inflow in the liver (AUC: 0-20 minutes) and the percentage of R3G and resveratrol + R3G output are shown in Table 2. Considering that the formation of other resveratrol metabolites in significant levels is improbable, the liver retained resveratrol that has not been processed yet, except in that situation in which the liver glucose is deficient. The latter is the case of arthritic rats when 200 μM resveratrol is introduced in the fed and fasted livers and healthy rats when 200 μM resveratrol is introduced in the fasted livers.

Effects of resveratrol-3-O-glucuronide on glycogen catabolism

Considering that R3G was significantly formed from the biotransformation of resveratrol in the liver, experiments like those illustrated in Fig. 1A were done with this compound at the concentration of 200 μM to evaluate its effects on glycogen catabolism. The same procedure of Fig. 1B-G was adopted to represent the results in Fig. 5: for glucose and lactate the values represented in Fig. 5A and B are the rates before starting the infusion of R3G (8-10 minutes perfusion time; zero concentration) and the rates observed after stabilization of the changes induced by its infusion (28-30 minutes perfusion time; 200 μM concentration); For oxygen uptake the decreases produced by 200 μM R3G were represented. In order to compare the actions of R3G with those found for its parental molecule, the results of 200 μM resveratrol in Fig. 1 were again plotted alongside those found for R3G in Fig. 5. The liver output of glucose was considerably more inhibited by R3G in healthy and arthritic rats when compared with resveratrol (Fig. 5A). The lactate production was also significantly inhibited by R3G in both conditions while it was not modified by resveratrol infusion at the concentration of 200 μM (Fig. 5B). Oxygen uptake was not inhibited by 200 μM R3G while it was strongly inhibited by resveratrol at the concentration of 200 μM for both healthy and arthritic rats (Fig. 5C). Glycogenolysis was considerably more inhibited by R3G in healthy and arthritic rats when compared with resveratrol (Fig. 5D). In both healthy and arthritic livers, glycolysis was modified in a similar manner that the lactate release, i.e., not inhibited for 200 μM resveratrol and strongly inhibited by R3G (Fig. 5E). In the absence of resveratrol, the $NADH/NAD^+$ ratio was 60% higher in the liver of arthritic rats and only resveratrol decreased this value to that found in control rats (Fig. 5F).

Effects of resveratrol-3-O-glucuronide on gluconeogenesis

Experiments like those illustrated in Fig. 2A were done with R3G at the concentration of 200 μM to evaluate its effects on gluconeogenesis. The same procedure of Fig. 2B-D was adopted to represent the results in Fig. 5G, H and I: for pyruvate and glucose productions the values are the rate before starting the infusion of R3G (32-34 minutes perfusion time in Fig. 2A; zero R3G concentration) and the rates observed after stabilization of the changes induced by 200 μM R3G. For oxygen uptake the decreases produced by R3G were represented. Similarly, the results of 200 μM resveratrol in Fig. 2 were again plotted alongside those found for R3G in Fig. 5 for comparison purpose. In both control and arthritic livers, the lactate-induced stimulus of glucose production was inhibited by R3G, but in lesser extension than that inhibition caused by 200 μM resveratrol (Fig. 5G). R3G increased the stimulus of the pyruvate production while 200 μM resveratrol inhibited it (Fig. 5H). The same effect of 200 μM R3G on pyruvate production was found for resveratrol at lower doses (Fig. 2C). In contrast to 200 μM resveratrol, R3G did not modify the increment of the oxygen uptake in the liver of both healthy and arthritic rats (Fig. 5I).

DISCUSSION

The results shows that resveratrol inhibits the glycogen catabolism when introduced at high concentrations and gluconeogenesis when introduced even at low concentrations (10 μM) in the liver of healthy and arthritic rats. The arthritic livers seem to be more sensitive to resveratrol, particularly in relation to gluconeogenesis, and the reason can be the more undermined situation of the arthritic organ. In fact, glycogen content and gluconeogenesis are reduced in the liver of arthritic rats and have been shown to be a consequence of a more pronounced oxidative stress and inflammation in the organ [Wendt et al., 2019; Castro-Ghizoni et al., 2017; Bracht et al., 2016; Stolina et al, 2011; Fedatto et al., 2000]. An antioxidant action of resveratrol, however, is unexpected because the direct ROS scavenging action of this compound is rather poor and its effects against oxidative stress *in vivo* have been related to actions as a gene regulator [Li et al., 2012; Xia et al., 2014; Xia et al., 2017]. In addition, resveratrol has shown pro-oxidant and cytotoxic effects *in vitro* at high concentrations and even levels so low as 10-20 μM exerts pro-oxidant effects against endothelial and hepatic stellate cells [Shaito et al., 2020; Martins et al., 2014]. The inhibition of mitochondrial state III respiration and membrane-bound enzymes activity in the present study was found only at resveratrol concentrations above 100 μM and can be related to mitochondriotoxic effects. The latter was previously found for endothelial cell mitochondria [Posadino et al., 2015]. Moreover, 50 μM resveratrol inhibits the complex I from mice brain mitochondria by competitively occupying the nucleotide pocket on this complex [Gueguen et al., 2015] and, also at the concentration of 50 μM, it inhibits state III respiration of mitochondria from rat brain, but not from rat liver [Moreira et al., 2013]. However, 25 μM resveratrol inhibits the NADH oxidase activity from both liver and brain disrupted mitochondria [Moreira et al., 2013], showing that the liver organelle is less susceptible to resveratrol and its respiratory activity should be significantly inhibited only at higher concentrations, as those used in this study, which inhibited also the mitochondrial succinate oxidase activity.

The liver oxygen uptake was inhibited by resveratrol at concentrations above 100 μM and it should be a consequence of the inhibition of mitochondrial respiration in both healthy and arthritic rats. This should be certainly contributing to reduce the gluconeogenesis from lactate. In fact, mitochondrial

respiration was inhibited when ADP was added (state III respiration), i.e., exactly when the mitochondrial oxidative phosphorylation is necessary to support the energy requirement for the costly gluconeogenesis from lactate. However, gluconeogenesis was inhibited by resveratrol at concentrations so low as 10 μM, which did not inhibit the liver oxygen consumption. Thus, resveratrol must be inhibiting the gluconeogenesis by another additional mechanism that still require further investigation, such as a direct inhibition of gluconeogenic enzymes activity. It is also at low concentrations that resveratrol increases the liver output of pyruvate in fasted healthy and arthritic rats, showing that resveratrol does not inhibit the transformation of lactate into pyruvate. On the contrary, the liver output of pyruvate increases probably as result of its non-use in the gluconeogenesis, which does not occur at high resveratrol concentrations, when the mitochondrial respiratory activity and the liver oxygen uptake is also inhibited and the cellular NADH/NAD^+ increases. This phenomenon shifts the near-equilibrium of the lactate dehydrogenase reaction from pyruvate toward lactate [Sies, 1982].

The inhibition found for resveratrol on liver gluconeogenesis, however, seem to be transitory and exist only when the resveratrol is present in the organ since the metabolic rates tend to return to those before the introduction of the compound (see Fig. 2A). On the other hand, these effects must not be neglected because significant concentrations of resveratrol are found in the plasma of humans after its oral administration[Xia et al., 2017; Cai et al., 2015; Rousova et al., 2016; Sergides et al., 2015]. Although reports about the levels of resveratrol in the plasma present many inconsistencies, it has been already reported a maximum peak plasma concentration (C_{max}) of 137 μM after oral ingestion of a single 1 g resveratrol dose by healthy volunteers, and circulating resveratrol at concentration of 14 μM was still detectable as late as 24 h after the compound administration [Xia et al., 2017; Cai et al., 2015]. In addition resveratrol can accumulate in specific compartments such as liver and kidney at a concentration higher than in plasma [Vitrac et al., 2003].

In relation to the hepatic biotransformation of resveratrol, the formation of R3G was lower in arthritic rats and two factors can be contributing to that: (1) lower activity of xenobiotics metabolizing enzymes in the arthritic liver and (2) lower glucose disposal in the arthritic livers in both fasted and fed states. In fact, metabolization of xenobiotics is reported to be relatively impaired in the

liver of arthritic rats as a consequence of a reduced activity of cytochrome P450 enzymes [Sanada et al., 2011; Morton & Chatfield, 1970; Ling & Jamali, 2005]. In addition, the activity of the enzyme UDP-glucuronyl-transferase is decreased in the liver microsomes of rats with adjuvant-induced arthritis [Kimura et al., 2017]. Considering that resveratrol is mainly metabolized by phase II reactions and no phase I metabolites has been found at significant concentrations [Springer & Moco, 2019], the lower activity of glucuronyl transferases seems to be limiting the hepatic transformation of resveratrol when it is introduced in the liver at concentrations of 50 and 100 μM. However, the glucose disposal should be limiting the glucuronidation at the concentration of 200 μM. This because the difference in the formation of R3G is relatively lower between healthy and arthritic livers for 100 μM resveratrol and it is strongly decreased in fasted rats for both conditions, more for arthritis, when the glucose production from lactate was considerably reduced, also more for arthritis.

Resveratrol was significantly converted into R3G in the liver of both arthritic and healthy rats, which has been reported to be biologically active, although not as effective as the parent molecule [Xia et al., 2017]. In fact, R3G strongly inhibited the gluconeogenesis when infused in the liver, but in a lower extension than that of resveratrol. The oxygen uptake, however, was not inhibited by R3G, an expected situation considering that glucuronides should not permeate the inner mitochondrial membrane. In this situation, the additional reduction of gluconeogenesis caused by resveratrol (compared with R3G) should be a consequence of the mitochondrial activity impairment. In addition, if R3G and resveratrol inhibit gluconeogenesis from lactate by similar mechanisms, then, this should be in some point outside the mitochondrial matrix, i.e., some point above the enzyme PEPCK. The latter requires further investigation, however, the lower effect of R3G on glucose production from lactate can also be associated to a lower hepatic uptake of R3G or at least a lower liver retention of the compound, a phenomenon that can be indirectly inferred from the spectrophotometric absorption behavior of resveratrol and R3G in the liver outflowing perfusate (see Fig. S4). When compared to resveratrol, the hepatic output of R3G increased more rapidly after its introduction into the liver and also decreased more rapidly when its introduction is stopped. However, the real inhibition of gluconeogenesis by R3G can be even higher because the infusion of R3G into the perfused liver does not reproduce

the real condition in which a great part of R3G is formed already into the cells. Thus, the previous comparison of the effectivity between R3G and resveratrol should be viewed with caution, at least for the negative effects on glucose metabolism. This is particularly true for the glycogen catabolism in the liver of healthy and arthritic fed rats, in which R3G inhibited more strongly the liver output of glucose, lactate and the rate of glycogenolysis and glycolysis. Once more the oxygen uptake was not inhibited by R3G and shows that the inhibition of glycogen catabolism is not associated with mitochondrial impairment. It is important to highlight that R3G is also reported to be found in significant concentrations in the plasma of humans as a consequence of the rapid glucuronidation and, following a single oral dose of resveratrol, it often presents a C_{max} greater than the parental molecule [Rousova et al., 2016; Sergides et al., 2015].

CONCLUSION

The results show that resveratrol inhibits the glycogen catabolism when introduced at high concentrations and gluconeogenesis when introduced even at low concentrations (10 μM) in the liver of healthy and arthritic rats. The arthritic livers seem to be a quite more sensitive to resveratrol, particularly in relation to gluconeogenesis, and the reason can be the more undermined situation of the arthritic organ. These effects of resveratrol seem to be transitory and exist only when the resveratrol is present in the organ, however, they should not be neglected because similar concentrations are achieved in the plasma after oral ingestion of resveratrol by healthy volunteers. Resveratrol was significantly metabolized to resveratrol-3-O-glucuronide (R3G) in the liver of healthy and arthritic rats, however, the formation of R3G was lower in arthritic rats when introduced at low concentrations and it is probably a consequence of the lower activity of glucuronyl transferases. At high concentrations, the formation of R3G was considerably inhibited in both arthritic and healthy livers, more for arthritic condition, as a consequence of a lower glucose disposal due to gluconeogenesis inhibition. R3G also inhibits considerably the gluconeogenesis and glycogen catabolism in the liver of arthritic and healthy rats. The oxygen uptake was not modified by R3G and the glycogen catabolism was inhibited in a higher extension when compared to that of resveratrol. The effects of R3G should not be also neglected because this metabolite is also found in significant concentrations in the plasma of humans, sometimes higher than the parental molecule, as a consequence of the rapid glucuronidation following oral ingestion of resveratrol.

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Competing interests

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

Authors' contributions

JFC conceived and designed the experiments. CABA and MSS induced arthritis and treated the animals. MSS, ABSN and LB performed the liver perfusion experiments. APAS performed HPLC analysis. JFC and RMP wrote the paper. AB reviewed the paper. All authors have read and approved the final version of the paper.

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TABLE 1. ***Half-maximal inhibitory concentration (IC50) of resveratrol on gluconeogenesis, state III mitochondrial respiration, NADH oxidase activity and succinate oxidase activity in livers of control and arthritic rats.*** Half-maximal inhibitory concentration (IC50) was obtained by numerical interpolation from data of Figures 2 and 3. Resv 0 μM is the basal rate of metabolites in the absence of resveratrol expressed as $\mu mol \cdot min^{-1} \cdot g^{-1}$ for perfused livers and $nmol \cdot min^{-1} \cdot mg^{-1}$ for mitochondrial state III respiration and membrane-bound enzymes activity. Respiratory control (RC) = state III/state IV and does not contain units.

Parameter	Control		Arthritis	
	Resv. 0 μM	IC_{50} (μM)	Resv. 0 μM	IC_{50} (μM)
	Gluconeogenesis			
Gluconeogenesis	0.826 ± 0.022	10 μM	0.436 ± 0.030*	20 μM
Δ Oxygen uptake	0.606 ± 0.048	69 μM [a]	0.515 ± 0.029[a]	68 μM [a]
	State III respitation			
Oxygen uptake (α-ketoglutarate)	31.70 ± 2.02	424 μM	23.51 ± 1.03	279 μM
Oxygen uptake (Succinate)	78.19 ± 1.50	168 μM	90.230 ± 5,66	221 μM
	Respiratory control (RC)			
α-Ketoglutarate	2.77 ± 0.12	304 μM [b]	1.88 ± 0.11	175 μM [b]
Succinate	4.46 ± 0.40	87 μM [b]	3.76 ± 0.13	100 μM [b]
	Mitochondrial membrane-bound enzymes activity			
NADH oxidase	47.42 ± 4.02	244 μM	50.97 ± 5.56	165 μM
Succinate oxidase	58.81 ± 4.17	--- [c]	42.98 ± 9.72	--- [c]

The data are the mean ± standard error of the mean of 5 animals.

[a] *The value of* Δoxygen uptake *for 200 μM resveratrol was used as maximal inhibition.*

[b] *RC = 1 is the maximal inhibition.*

[c] *Succinate oxidase was not inhibited in a concentration-dependent manner.*

TABLE 2. ***Biotransformation of resveratrol into resveratrol-3-O-glucuronide (R3G) in perfused livers of heathy and arthritic rats.*** Livers from fed and 12 h fasted rats were perfused with resveratrol as illustrated in Fig 4A. Resveratrol inflow are expressed as AUC (0-20 minutes; $\mu mol \cdot g^{-1}$) and corresponded to the average of total amount introduced in the liver. R3G output is expressed as AUC (0-30 minutes) and corresponded to the average percentage of the total amount of resveratrol introduced in the liver. Resveratrol + R3G output are expressed as AUC (0-30 minutes) and corresponded to the average percentage of the total amount of resveratrol introduced in the liver. Data of resveratrol inflow are the mean ± SEM obtained from 4 animals for each condition.

Parameter	**Resveratrol inflow (AUC: 0 → 20 min)**	**R3G output (AUC: 0 → 30 min)**	**Resveratrol + R3G output (AUC: 0 → 30 min)**
Units	$\mu mol \cdot g^{-1}$	% of resveratrol inflowed	% of resveratrol inflowed
200 μM Resveratrol (Fast controls)	14.00 ± 0.38	10	77
200 μM Resveratrol (Fast arthritis)	15.90 ± 0.77	2	72
200 μM Resveratrol (Fed controls)	12.00 ± 0.16	23	57
200 μM Resveratrol (Fed arthritis)	11.37 ± 0.51	3	50
100 μM Resveratrol (Fed controls)	7.98 ± 0.08	20	46
100 μM Resveratrol (Fed arthritis)	7.73 ± 0.16	17	54
50 μM Resveratrol (Fed controls)	3.72 ± 0.18	53	61
50 μM Resveratrol (Fed arthritis)	3.41 ± 0.11	32	72

FIGURE CAPTIONS

Fig. 1. *The concentration-dependent effects of resveratrol on glycogen catabolism in the perfused livers of healthy and arthritic rats.* **Panel A:** time courses of glucose, lactate and pyruvate production and oxygen consumption. Livers from fed rats were perfused with resveratrol (RESV) at the concentration range up to 200 μM as indicated by the horizontal bar in Panel A. The outflowing perfusate was sampled every 2 minutes and analysed for their contents of metabolites. Oxygen uptake was monitored by polarography. Data are the mean ± SEM obtained from 5 animals for each condition. The values in **Panels B**, **D, E, F** and **G** are the rates before starting the resveratrol infusion (8-10 minutes perfusion time; zero resveratrol concentration) and the rates observed after stabilization of the changes induced by each concentration. For oxygen uptake **(Panel C)** the decreases produced by each concentration were represented. The values in **Panels E**, **F** and **G** were calculated from the steady-state rates of glucose, lactate and pyruvate production. Glycogenolysis = glucose + 1/2(lactate + pyruvate) and glycolysis = 1/2(lactate + pyruvate). Each datum represents the mean of 5 liver perfusion experiments. *$p<0.05$: different from 0.0 μM resveratrol in controls; **$p<0.05$: different from 100 μM resveratrol in controls; $^{\#}p<0.05$: different from 0.0 μM resveratrol in arthritis; $^{\#\#}p<0.05$: different from 100 μM resveratrol in arthritis (ANOVA ONE-WAY and the Tukey post-hoc); $^{a}p<0.05$: for difference between 0.0 μM resveratrol in controls and 0.0 μM resveratrol in arthritis (Student's t test).

Fig. 2. *The concentration-dependent effects of resveratrol on gluconeogenesis from lactate in the perfused livers of healthy and arthritic rats.* **Panel A:** time courses of glucose and pyruvate production and oxygen consumption due to lactate infusion. Livers from 12 h fasted rats were perfused with 2 mM L-lactate and resveratrol as indicated by the horizontal bar in Panel A. The outflowing perfusate was sampled every 2 minutes and analysed for their contents of glucose and pyruvate. Oxygen uptake was monitored by polarography. The values in **Panels B** and **D** are the rate before starting the infusion of resveratrol (32-34 minutes perfusion time in Fig. 2A; zero resveratrol concentration) and the rates observed after stabilization of the changes induced by resveratrol (54-56 min perfusion time in Panel A). For oxygen uptake **(Panel C)** the decreases

produced resveratrol were represented. Data are the mean ± SEM obtained from 5 animals for each condition. *p, **p and ***p<0.05: different respectively from 0.0, 100 and 200 μM resveratrol, in controls; $^{\#}$p, $^{\#\#}$p, and $^{\#\#\#}$p<0.05: different respectively from 0.0, 100 and 200 μM resveratrol in arthritis (ANOVA ONE-WAY and the Tukey post-hoc); ^{a}p<0.05: for difference between 0.0 μM resveratrol in controls and 0.0 μM resveratrol in arthritis (Student's t test).

Fig. 3. *The concentration-dependent effects of exogenously added resveratrol on the respiratory activity of isolated hepatic mitochondria.* Isolated or disrupted mitochondria (1.0 mg·mL^{-1}) were incubated at 37ºC in a closed oxygraph chamber containing 2 mL of the reaction medium and resveratrol at the concentration range up to 500 μM. The experimental protocol and calculation procedures are shown in Fig. S2. Mitochondrial respiration driven by α-ketoglutarate or succinate in the absence **(A; basal)** and presence **(B; state III)** of 0.125 mM ADP was followed polarographically. **(C),** respiratory control (RC). **(D; disrupted mitochondria),** concentration dependence of inhibition of the mitochondrial membrane-bound NADH oxidase and succinate oxidase activities. Data represent the mean ± SEM of 6 animals. *p<0.05: different from 0.0 μM RESV in controls; $^{\#}$p<0.05: different from 0.0 μM RESV in arthritis (ANOVA ONE-WAY and the Tukey post-hoc); **, ***, $^{\#\#}$, $^{\#\#\#}$p<0.05: different from previous values in the same group.

Fig. 4. *Biotransformation of resveratrol into resveratrol-3-O-glucuronide (R3G) in perfused livers of heathy and arthritic rats.* **Panel A:** time courses of resveratrol and R3G output in perfused livers of fed and fasted rats. Livers from fed and 12 h fasted rats were perfused with 200 μM resveratrol exactly as illustrated in Fig 1A and 2A, respectively. The outflowing perfusate was sampled every 5 minutes and analyzed for their contents of resveratrol and R3G by HPLC. **Panel A** illustrates only the perfusion time of Fig. 1A and 2A from that moment in which resveratrol is introduced in the liver. Lactate infusion was omitted in horizontal bars for fasted rats. The values in **Panels B**, **C** and **D** are the area under the curve (AUC: 0-30 minutes) and correspond to the total amounts of resveratrol and R3G released during this period in Panel A. Data are the mean ± SEM obtained from 5 animals for each condition. *p<0.05: for difference between control and arthritis (Student's t test).

Fig. 5. *The effects of resveratrol-3-O-glucuronide (R3G) on glycogen catabolism and gluconeogenesis in the perfused livers of healthy and arthritic rats.* Livers from fed and fasted rats were perfused with 200 μM R3G in the same manner as resveratrol in Fig. 1A and 2A. The outflowing perfusate was sampled every 2 minutes and analyzed for their contents of metabolites. Oxygen uptake was monitored by polarography. For fed rats, the values in **Panels A**, **B, D, E** and **F** are the rates before starting the R3G infusion (8-10 minutes perfusion time in Fig. 1A; zero R3G concentration) and the rates observed after stabilization of the changes induced by R3G. For oxygen uptake **(Panel C)** the decreases produced by each concentration were represented. For fasted rats, the values in **Panels G** and **H** are the rate before starting the infusion of R3G (32-34 minutes perfusion time in Fig. 2A; zero R3G concentration) and the rates observed after stabilization of the changes induced by 200 μM R3G. For oxygen uptake **(Panel I)** the decreases produced R3G were represented. For comparison purpose the results of 200 μM resveratrol in Fig. 1 and 2 were again plotted alongside those found for R3G. Each datum represents the mean of 5 liver perfusion experiments. Values with superscript bars are statistically different ($p<0.05$: Student's t test) and the numbers above the bars correspond to the percentage of decline or increment in relation to 0.0 μM resveratrol or R3G.

Fig. S1. *Characterization of the experimental model.* **A:** Body weight gain (g); **B:** Increases in paw volumes (mL); **C:** score of secondary lesions due arthritis; **D:** Activity of aminotransferases in the plasma and myeloperoxidase (MPO) in the plasma and liver. The mean initial weight of the animals was 200 ± 5 g. The volume of both hind paws up to the tibiotarsal joint was measured by plethysmography. The mean initial paw volume was 1.6 ± 0.1 mL. The appearance and severity of secondary lesions were also assessed from the 9th to the 21th day according to the following score graded from 0 to 5: (+1) nodules in the tail; (+1 or +2) nodules in one or both ears; and (+1 or +2) swelling in one or both forelimbs. The activity of MPO is expressed per mg of tissue or plasma protein and the activity of AST and ALT is expressed as units per liter. The values represent the mean ± standard error of the mean of 5 animals. For comparison of two values the student's t test was applied with the

5% level ($p<0.05$). $^{*}p<0.05$ for difference from controls, $^{\#}p<0.05$ for difference from controls

Fig. S2. Oxygraph (A) and experimental approach used to determine the respiratory activity of isolated mitochondria (B).

Fig. S3. *Chromatographic profile of resveratrol and resveratrol-3-O-glucuronide (R3G) in the outflowing perfusate from livers.* **A**: perfusion fluid contaminated with 50 µM resveratrol; **B**: outflowing perfusate from the livers of fed arthritic rats after introduction of 100 µM resveratrol (30 minutes perfusion time in Fig. 4A). **C**: perfusion fluid (Krebs/Henseleit bicarbonate buffer) contaminated with 50 125 µM resveratrol-3-O-glucuronide (R3G). Peak 1: R3G; Peak 2: resveratrol.

Fig. S4. *Spectrophotometric profile (time courses) of resveratrol and resveratrol-3-O-glucuronide (R3G) output in livers perfused with resveratrol or R3G.* Healthy and arthritic livers from fasted **(A)** and fed **(B)** rats were perfused with 200 µM resveratrol or R3G as indicated by horizontal bars. The outflowing perfusate was sampled in regular intervals and the absorbance measured at 340 nm, which is the wavelength of maximum absorption of resveratrol and R3G. Data are the mean ± SEM of 3-5 experiments for each condition.

Fig. 1

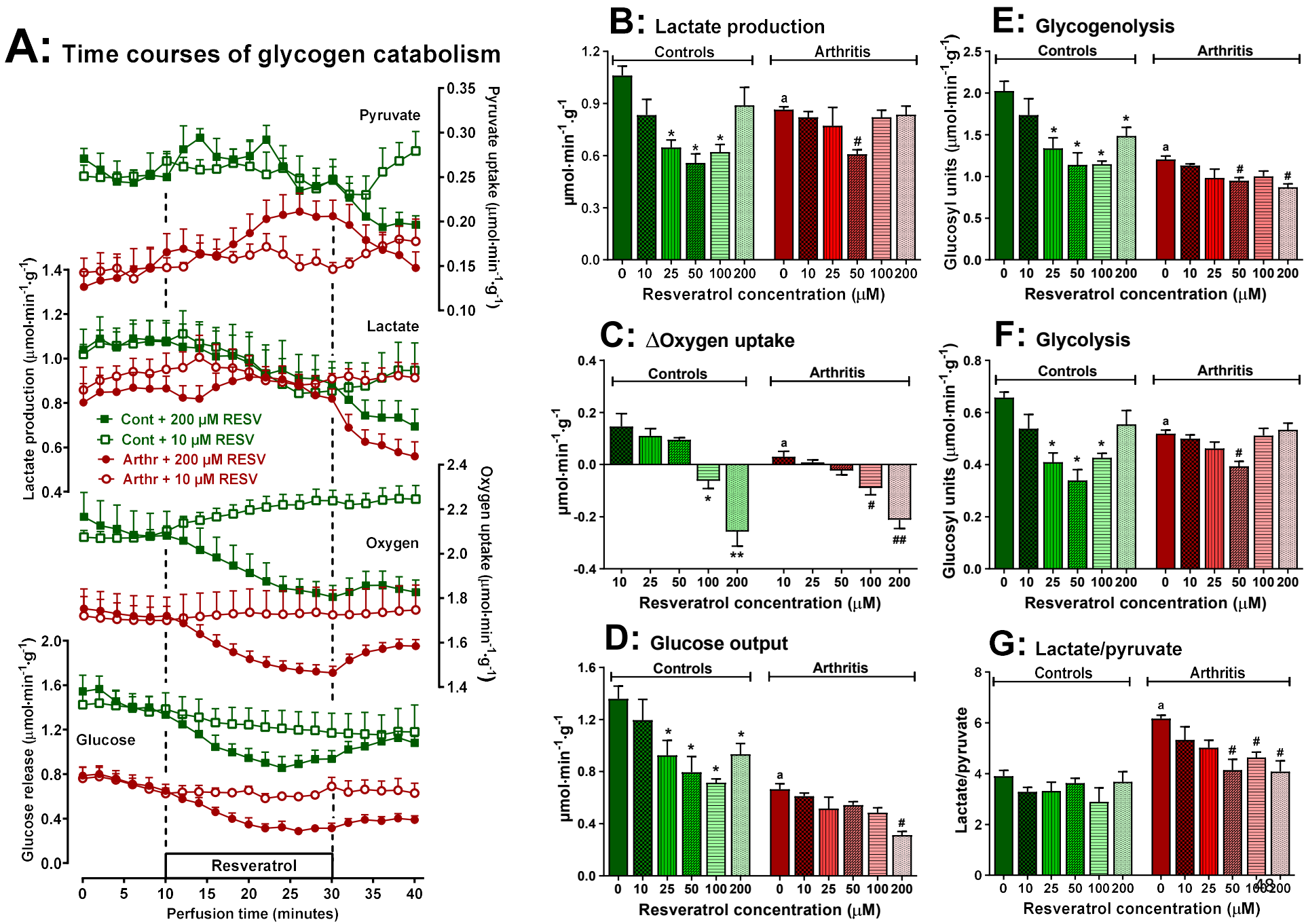

A: Time courses of glycogen catabolism
Pyruvate
Lactate
Oxygen
Glucose
Pyruvate uptake (µmol·min⁻¹·g⁻¹)
Lactate production (µmol·min⁻¹·g⁻¹)
Oxygen uptake (µmol·min⁻¹·g⁻¹)
Glucose release (µmol·min⁻¹·g⁻¹)
Cont + 200 µM RESV
Cont + 10 µM RESV
Arthr + 200 µM RESV
Arthr + 10 µM RESV
Resveratrol
Perfusion time (minutes)
B: Lactate production
C: ΔOxygen uptake
D: Glucose output
E: Glycogenolysis
F: Glycolysis
G: Lactate/pyruvate
Controls
Arthritis
Resveratrol concentration (µM)
Glucosyl units (µmol·min⁻¹·g⁻¹)
Lactate/pyruvate

Fig. 2

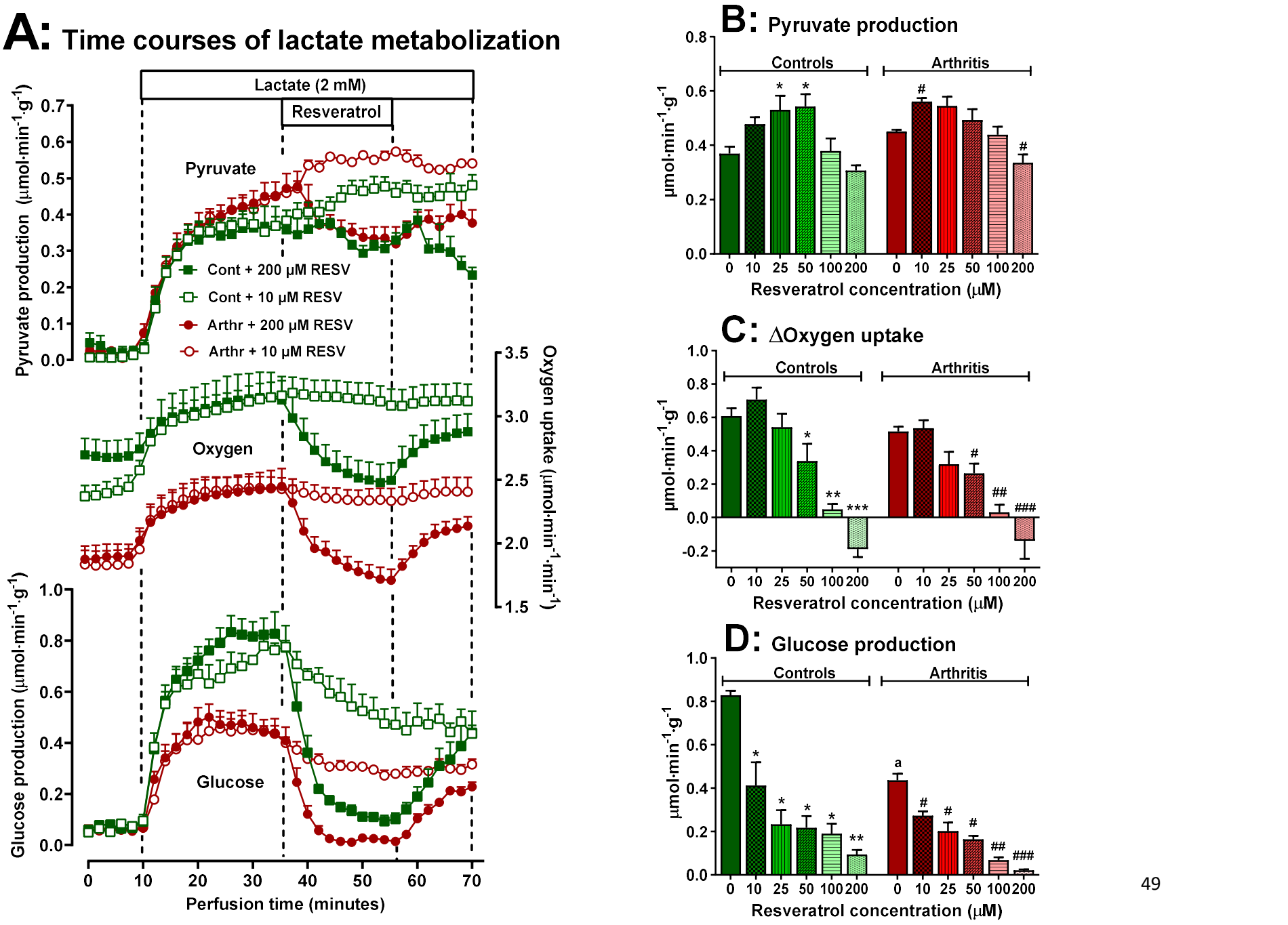

A: Time courses of lactate metabolization
Lactate (2 mM)
Resveratrol
Pyruvate
Oxygen
Glucose
Cont + 200 µM RESV
Cont + 10 µM RESV
Arthr + 200 µM RESV
Arthr + 10 µM RESV
Pyruvate production (µmol·min-1·g-1)
Oxygen uptake (µmol·min-1·min-1)
Glucose production (µmol·min-1·g-1)
Perfusion time (minutes)
B: Pyruvate production
Controls
Arthritis
µmol·min-1·g-1
Resveratrol concentration (µM)
C: ΔOxygen uptake
Controls
Arthritis
µmol·min-1·g-1
Resveratrol concentration (µM)
D: Glucose production
Controls
Arthritis
µmol·min-1·g-1
Resveratrol concentration (µM)

Fig. 3

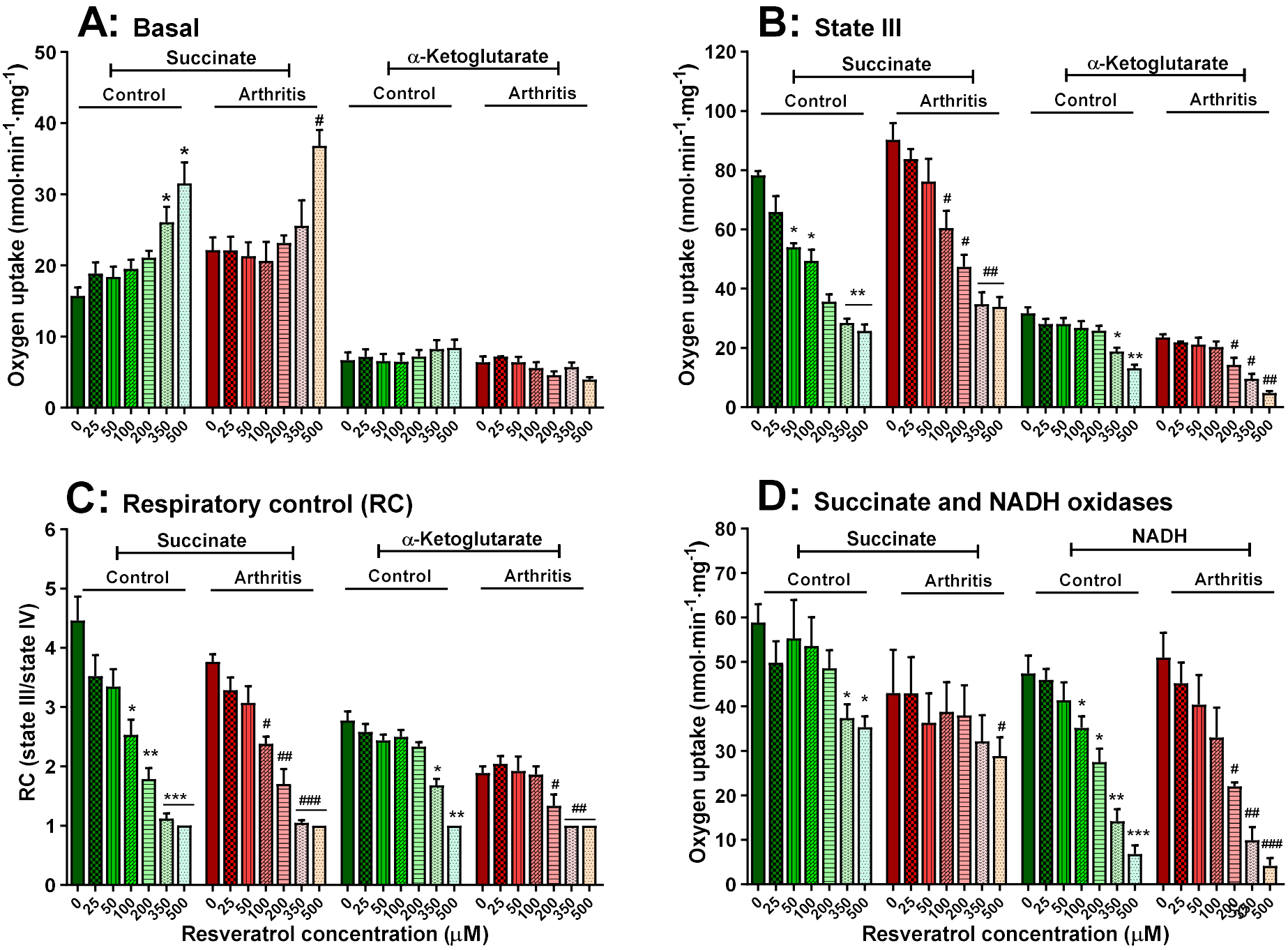

A: Basal
Succinate
α-Ketoglutarate
Control
Arthritis
Control
Arthritis
Oxygen uptake (nmol·min-1·mg-1)
0 10 20 30 40 50
0 25 50 100 200 350 500
B: State III
Succinate
α-Ketoglutarate
Control
Arthritis
Control
Arthritis
Oxygen uptake (nmol·min-1·mg-1)
0 20 40 60 80 100 120
0 25 50 100 200 350 500
C: Respiratory control (RC)
Succinate
α-Ketoglutarate
Control
Arthritis
Control
Arthritis
RC (state III/state IV)
0 1 2 3 4 5 6
0 25 50 100 200 350 500
Resveratrol concentration (μM)
D: Succinate and NADH oxidases
Succinate
NADH
Control
Arthritis
Control
Arthritis
Oxygen uptake (nmol·min-1·mg-1)
0 10 20 30 40 50 60 70 80
0 25 50 100 200 350 500
Resveratrol concentration (μM)

Fig. 4

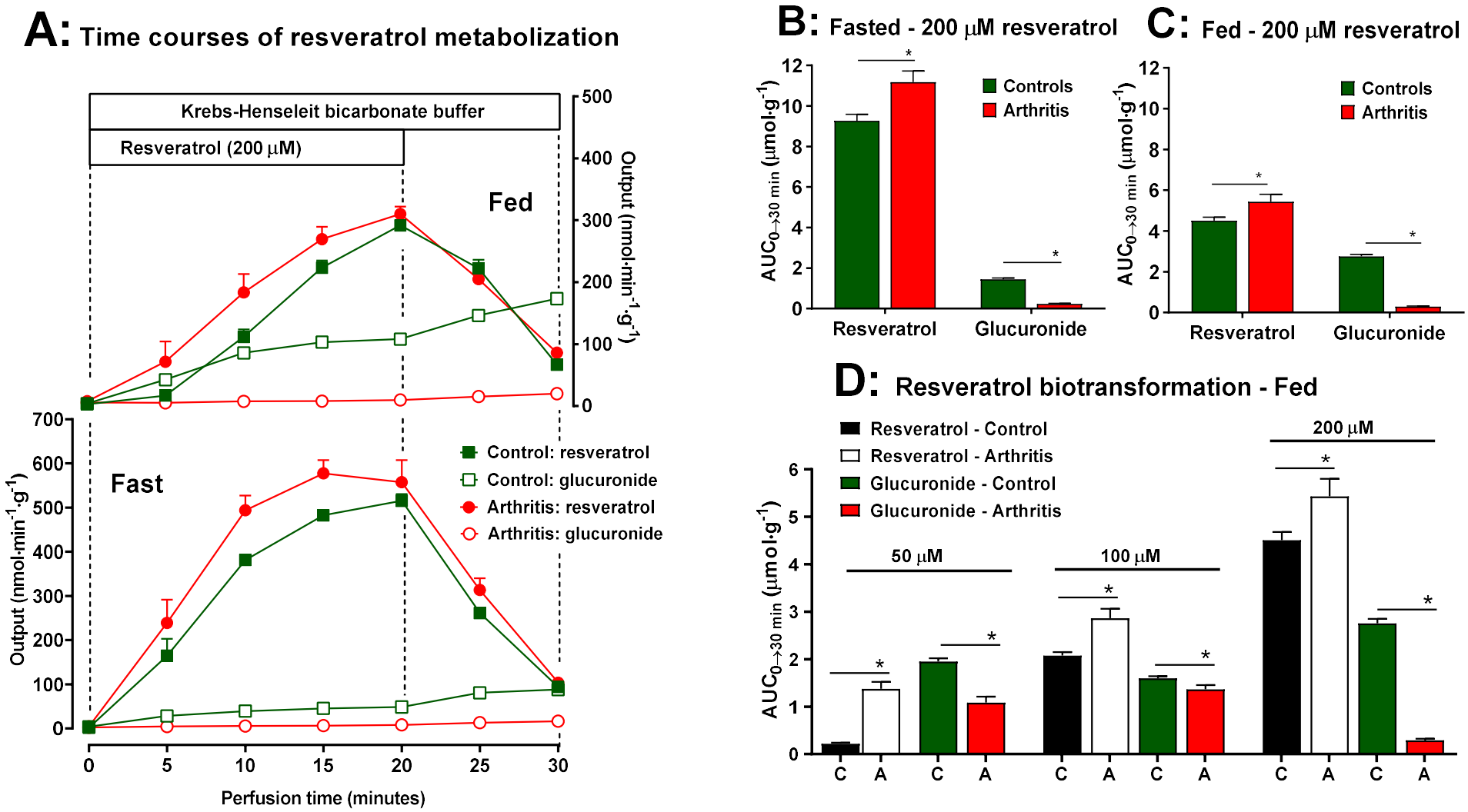
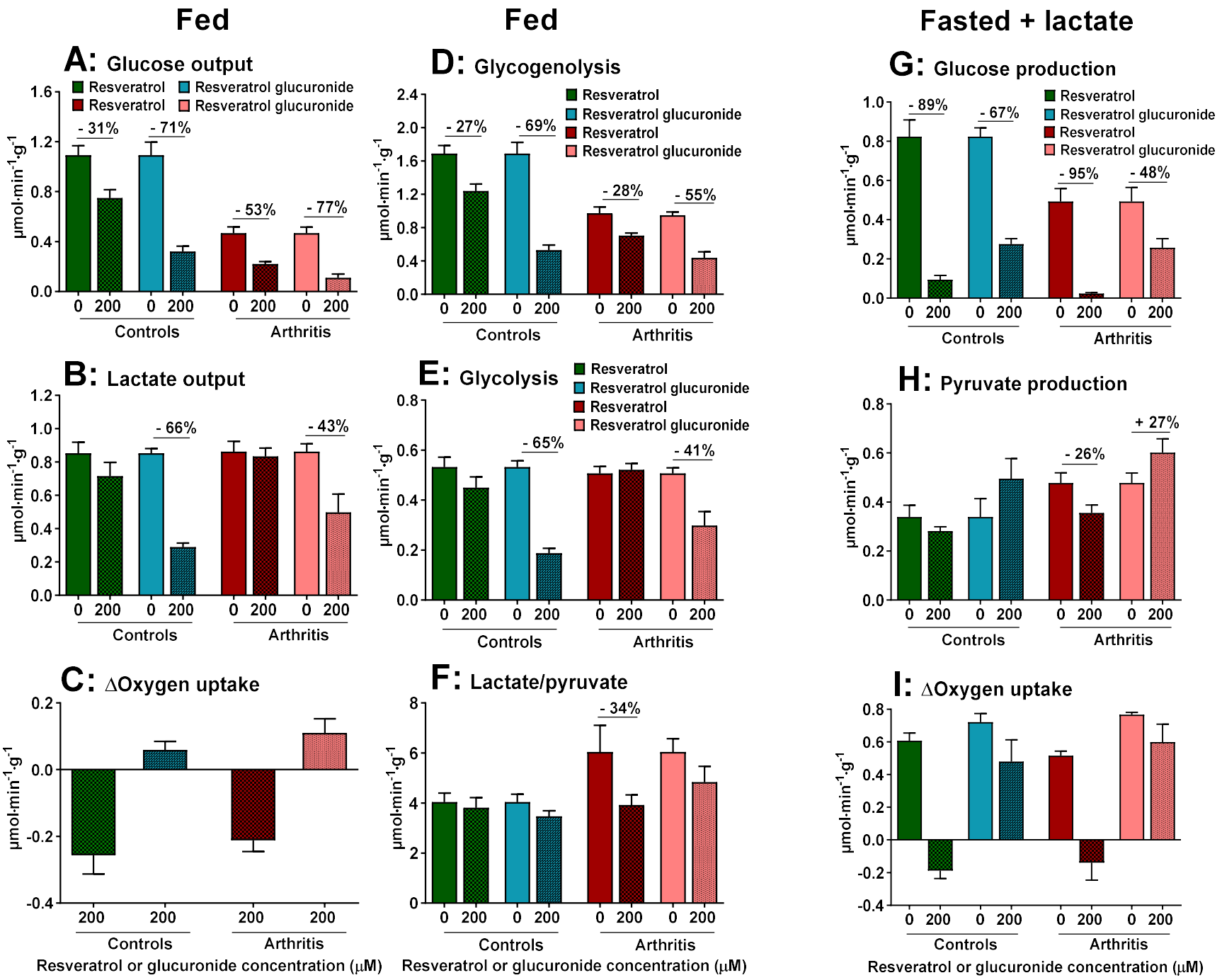

A: Time courses of resveratrol metabolization
Krebs-Henseleit bicarbonate buffer
Resveratrol (200 μM)
Fed
Fast
Control: resveratrol
Control: glucuronide
Arthritis: resveratrol
Arthritis: glucuronide
Output (nmol·min-1·g-1)
Perfusion time (minutes)
B: Fasted - 200 μM resveratrol
C: Fed - 200 μM resveratrol
Controls
Arthritis
AUC0→30 min (μmol·g-1)
Resveratrol
Glucuronide
D: Resveratrol biotransformation - Fed
Resveratrol - Control
Resveratrol - Arthritis
Glucuronide - Control
Glucuronide - Arthritis
50 μM
100 μM
200 μM
C
A

Fig. 5


Fed
Fed
Fasted + lactate
A: Glucose output
D: Glycogenolysis
G: Glucose production
B: Lactate output
E: Glycolysis
H: Pyruvate production
C: ΔOxygen uptake
F: Lactate/pyruvate
I: ΔOxygen uptake
Resveratrol
Resveratrol glucuronide
Controls
Arthritis
µmol·min-1·g-1
Resveratrol or glucuronide concentration (µM)

SUPPLEMENTARY MATERIAL

Fig. S1.

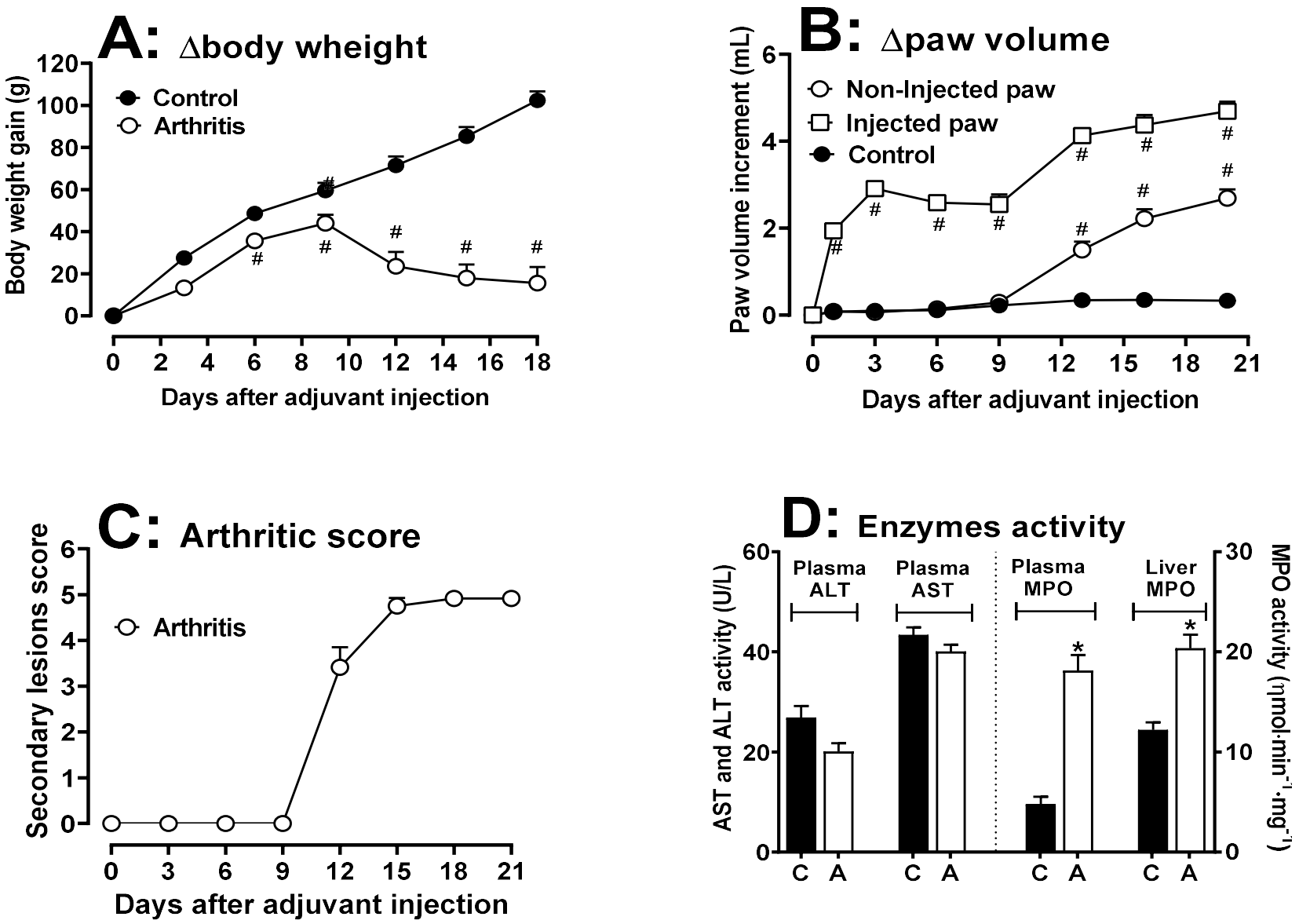


Fig. S2

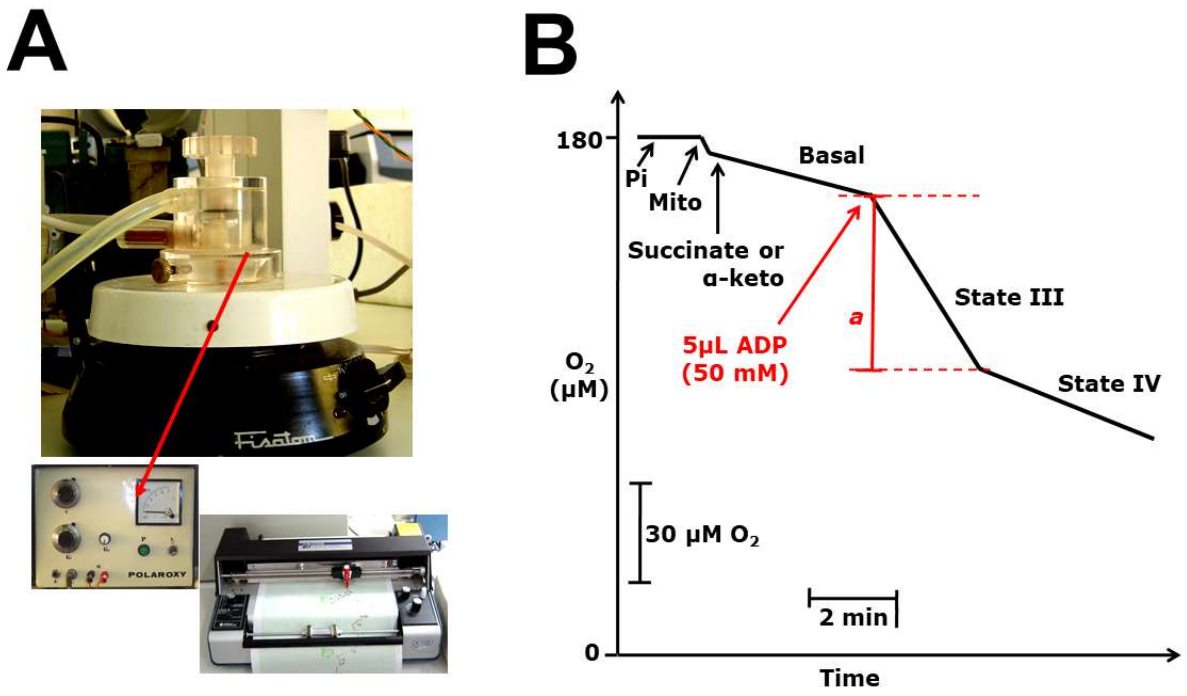


Fig. S2. Oxygraph (A) and experimental approach used to determine the respiratory activity of isolated mitochondria (B).

Fig. S3

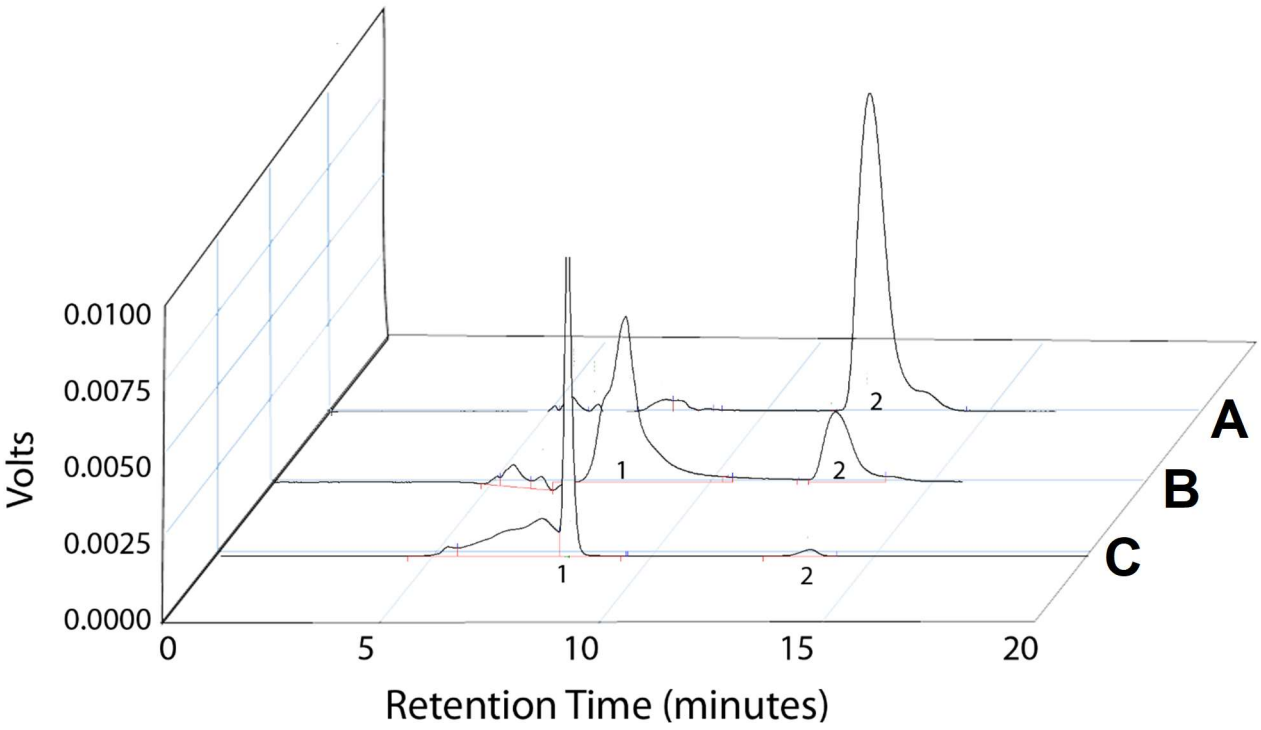


Fig. S3. *Chromatographic profile of resveratrol and resveratrol-3-O-glucuronide (R3G) in the outflowing perfusate from livers.* **A**: perfusion fluid contaminated with 50 µM resveratrol; **B**: outflowing perfusate from the livers of fed arthritic rats after introduction of 100 µM resveratrol (30 minutes perfusion time in Fig. 4A). **C**: perfusion fluid (Krebs/Henseleit bicarbonate buffer) spiked with 125 µM resveratrol-3-O-glucuronide (R3G). Peak 1: R3G; Peak 2: resveratrol.

Fig. S4

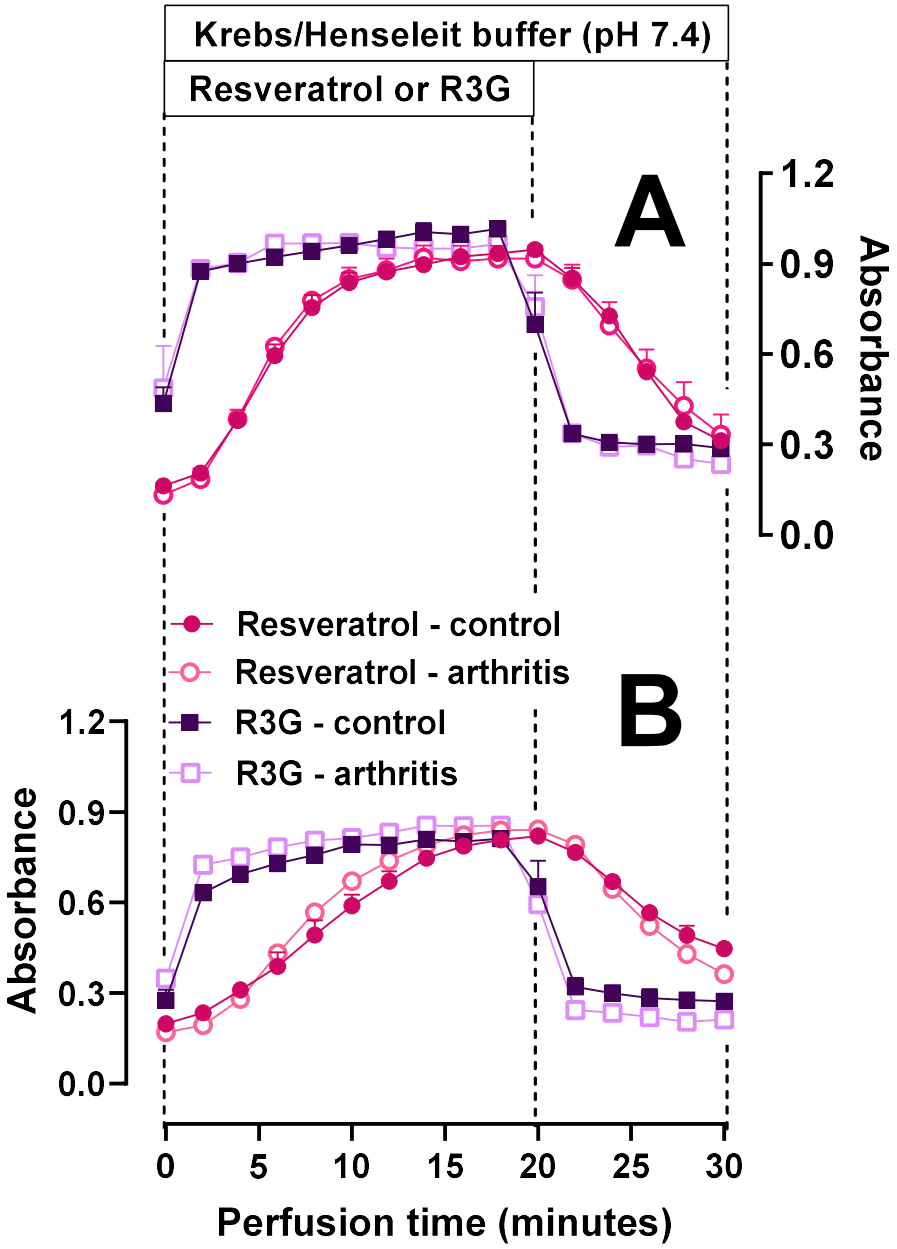


Fig. S4. *Spectrophotometric profile (time courses) of resveratrol and resveratrol-3-O-glucuronide (R3G) output in livers perfused with resveratrol or R3G.* Healthy and arthritic livers from fasted **(A)** and fed **(B)** rats were perfused with 200 μM resveratrol or R3G as indicated by horizontal bars. The outflowing perfusate was sampled in regular intervals and the absorbance measured at 340 nm, which is the wavelength of maximum absorption of resveratrol and R3G. Data are the mean ± SEM of 3-5 experiments for each condition.

Effects of resveratrol on inflammation and liver metabolism of healthy and arthritic rats

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ABSTRACT

Background and Aim: Resveratrol has been investigated as a promising antirheumatic agent; however, this polyphenol inhibits the gluconeogenesis and glycogen catabolism when infused in perfused livers of arthritic and healthy rats. Therefore, this study investigated the effects of orally administered resveratrol on the arthritic manifestations and liver metabolism of rats with Freund's adjuvant-induced polyarthritis, a model for severe rheumatoid arthritis.

Experimental procedure: Holtzman healthy and arthritic rats were orally treated with resveratrol at doses in the range up to 500 mg/Kg for 5 days prior to the arthritis induction and by additional 20 days after. Paw edema, arthritic score and liver myeloperoxidase (MPO) activity were measured to evaluate the inflammation. Glycogen catabolism and gluconeogenesis from lactate were respectively evaluated in perfused livers from fed and fasted rats.

Results: resveratrol decreased the liver MPO activity at doses above 100 mg/Kg, decreased the contralateral paw edema at doses above 250 mg/Kg, and considerably delayed the arthritic score and the injected paw edema at doses above 250 mg/Kg. The hepatic gluconeogenesis was decreased in arthritic rats and resveratrol did not improve it. However, resveratrol did not negatively modify the gluconeogenesis in the healthy and arthritic liver. Glycogen catabolism was only in part modified by resveratrol in the healthy and arthritic liver, but these effects were not pronounced and not dose-dependent. Thus, it is not probable that resveratrol is negatively affecting the liver metabolism, moreover if is taken in consideration that gluconeogenesis is extremely sensitive to alterations in the cellular behavior and integrity.

Conclusion: the orally administered resveratrol improves the articular and systemic inflammation in arthritic rats and does not modify considerably the liver metabolism of arthritic and healthy rats. This results show that resveratrol may be a promising adjuvant to the most current approaches aiming at rheumatoid arthritis therapy, however, caution is recommended with respect to the previously reported transitory effects on the liver metabolism.

Keywords*: adjuvant-induced arthritis, resveratrol, liver metabolism, inflammation, hepatic gluconeogenesis, rheumatoid arthritis.*

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 progressive disability and premature mortality an increased mortality rate, mainly due to cardiovascular complications [Uhlig et al., 2014; Kitas & Gabriel, 2011]. 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 [Misko et al., 2013]. Rheumatoid arthritis is a systemic disease and in addition to the joints other organs are affected, such as brain, heart, lungs and vascular tissue [Castro et al., 2020; Mcinnes & Schett, 2011; Schubert et al., 2016; Bracht et al., 2016; Wendt et al., 2015; Comar et al., 2013].

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 rats with arthritis induced by adjuvant, which present in addition to cachexia substantial alterations in the liver metabolism, such as a higher rates of oxygen uptake, reduced gluconeogenesis, increased glycolysis, increased fatty acid metabolism and reduced metabolism of xenobiotics [Wendt et al., 2019; Ames-Sibin et al., 2018; Sá-Nakanishi et al., 2018; Castro-Ghizoni et al., 2017; Kimura et al., 2017; Fedatto et al., 2002; Morton & Chatfield, 1970]. The metabolic modifications in the liver of arthritic rats have been shown to be a consequence of a more oxidized state associated with accelerated body catabolism, pronounced oxidative stress and systemic inflammation [Wendt et al., 2019; Comar et al., 2013].

Resveratrol (3,5,4’-trihydroxy-*trans*-stilbene) is a dietary polyphenol popularly known as the ingredient in red wine that prevents heart and other aging-associated diseases [Timmers et al., 2012]. This polyphenol has been reported to present anticarcinogenic, neuroprotective, cardioprotective and antidiabetic activities [Ramirez-Garza et al., 2018; Zordoky et al., 2015]. Many beneficial effects of resveratrol have been also attributed to its immuno-

modulatory and anti-inflammatory activity [Oliveira et al., 2017]. For this reason, resveratrol has been investigated as a promising antirheumatic agent [Nguyen et al., 2017]. In fact, resveratrol inhibits the synthesis of proinflammatory cytokines in synoviocytes isolated from arthritic rats and in human synoviocytes cell lines [Chen et al., 2013; Tian et al., 2013; Nakayama et al., 2012]. The dietary supplementation with resveratrol also lowers the rheumatoid arthritis manifestations in animal models and patients with the disease [Khojah et al., 2018; Rivieiro-Navieira et al., 2016; Xuzhu et al., 2012]. Resveratrol in association with fenofibrate improves the joint inflammation of rats with adjuvant-induced arthritis [Wahba et al., 2016].

Many studies with animal models of hepatic insult and diseases have shown that resveratrol presents hepatoprotective action [Faghihzadeh et al., 2015]. Similarly, resveratrol has been reported to act on hepatic metabolism. In the livers of rodent models of steatosis, for example, resveratrol modifies the lipid metabolism and reduces the lipid accumulation [Aguirre et al., 2014]. However, no study until now has showed if resveratrol improves the hepatic metabolism of arthritic rats. On the contrary, resveratrol has been shown to inhibit the glycogen catabolism when introduced at concentrations above 25 μM and gluconeogenesis when introduced even at a low concentration of 10 μM in perfused livers of healthy and arthritic rats [to be published]. Moreover, when compared to healthy livers, the arthritic liver seems to be a quite more sensitive to resveratrol and the reason can be the more undermined situation of the arthritic organ [to be published]. On the other hand, these effects found for resveratrol seem to be transitory and exist only when the resveratrol is present in the organ, i.e., when it is directly infused in the perfused liver. However, these actions should not be neglected because similar concentrations are achieved in the plasma after oral ingestion of resveratrol by healthy volunteers [Xia et al., 2017; Cai et al., 2015; Rousova et al., 2016].

Considering all the above, the present study investigated the effects of orally administered resveratrol on gluconeogenesis and catabolism of glycogen in the liver of rats with adjuvant-induced arthritis. These effects were compared with those of healthy rats orally treated with resveratrol. The effects of resveratrol on inflammation were additionally assessed in arthritic rats. The results of the present study should allow extrapolations to patients with severe rheumatoid arthritis.

MATERIAL AND METHODS

Chemicals

Trans-resveratrol at 99% purity was purchased from Botica Ouro Preto (Maringá, PR, Brazil). Enzymes and coenzymes were purchased from Sigma Chemical Co (St. Louis, MO, USA). Commercial kits for AST and ALT were purchased from Gold Analisa Diagnóstica Ltda (Belo Horizonte, MG, Brazil). All other chemicals were of analytical grade.

Animals and induction of arthritis

Male Holtzman rats weighting 200-220 g (50 days old) were obtained from the Center of Animal Breeding of the State University of Maringá (UEM) and maintained under standard laboratory conditions at a temperature of 24 ± 3ºC under a regulated 12 h light/dark cycle. The animals were housed in conventional steel cages (3 rats/cage) and fed ad libitum with a standard laboratory diet (Nuvilab®, Colombo, Brazil). Two hundred animals were used in six different experimental protocols followed throughout the study and, for each one, the animals were randomly distributed into control and arthritic groups. 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 [Bendele et al., 1999]. Animals were used for experiments at day 21th after adjuvant injection. Rats of similar age served as controls. The volume of both hind paws up to the tibiotarsal joint was measured every three days by plethysmography. The 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 (CEUA) of the State University of Maringá (Protocol number CEUA 2495130916).

Experimental design

Animals were randomly distributed into controls (C) and arthritis (A). Both groups orally received resveratrol at the dose of 0, 25, 50, 100, 250 and 500 mg/kg. Animals were treated once a day in the morning by oral administration (gavage) of resveratrol vehiculated in carboxymethylcellulose

(CMC) for 5 days prior to the arthritis induction and by additional 18 days after. The procedures were repeated three times to evaluate all parameters of this study. Considering that rheumatoid arthritis is intermittent and adjuvant-induced arthritis is a model of severe arthritis, a simultaneous preventive and curative treatment protocol has been used. Daily doses of resveratrol were established considering the doses previously used in mice and humans volunteers [Rousova et al., 2016; Xuzhu et al., 2016; Khojah et al., 2018] after converting into rat doses as given by the body surface area normalization method [Reagan-Shaw et al., 2007].

Evaluation of the inflammatory response

Animals were weighed and evaluated for their adjuvant-induced inflammatory response over 18-days period. Following adjuvant inoculation, the volume of both hind paws up to the tibiotarsal joint was measured by plethysmography. 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 the 10^{th} to the 18^{th} day according to 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 [Sá-Nakanishi et al., 2018]. The ALT and AST activities were assessed in the plasma to evaluate the liver integrity. The myeloperoxidase (MPO) activity was measured in the supernatant of liver homogenate to evaluate systemic inflammation [Bradley et al., 1982].

Blood collection and tissue preparation

Rats were anesthetized with an overdose of sodium thiopental (100 $mg \cdot kg^{-1}$) plus lidocaine (10 $mg \cdot Kg^{-1}$) and the peritoneal cavity was surgically exposed. Blood was collected from cava vein. Next, the liver was removed and divided into two parts: one was stored in liquid nitrogen for MPO assessment and the other was used for mitochondria isolation. Blood was centrifuged (3,000*g*/15 min) to separate the plasma fraction. The liver homogenate was prepared by the homogenization of a freeze-clamped tissue in a type Potter homogenizer with 10 volumes 0.1 M potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged (11,000*g*/15 min) for obtaining the soluble

fraction of the homogenate. Fresh liver was used for mitochondria isolation by differential centrifugation as described elsewhere [Lima et al., 2006].

Liver perfusion: gluconeogenesis and glycolysis

Hemoglobin-free non-recirculating liver perfusion was performed as previously described [Comar et al., 2003]. After cannulation of the portal and cava veins, the liver was removed and positioned in a plexiglass chamber. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine serum albumin and saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37 ºC. The flow was maintained constant by a peristaltic pump (Minipuls 3, Gilson, France). Oxygen concentration in the venous perfusate was monitored by a teflon-shielded platinum electrode. Samples of the effluent perfusion fluid were collected at two minutes intervals and analyzed for their metabolite content.

Glycolysis and glycogenolysis were measured in livers isolated from fed rats, which were perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. After stabilization of oxygen consumption, samples of the effluent perfusion fluid were collected during 20 minutes. Glucose, L-lactate and pyruvate were measured by standard enzymatic procedures [Bergmeyer, 1974]. At the steady-state, glycolysis = (lactate + pyruvate)/2 and glycogenolysis = glucose + [(lactate + pyruvate)/2].

Gluconeogenesis was measured in the perfused livers of 12 h fasted rats. These livers were initially perfused with Krebs/Henseleit buffer in the absence of exogenous substrates. After stabilization of oxygen consumption, L-lactate (2 mM) was infused as a gluconeogenic substrate during 30 minutes. Glucose and pyruvate were measured in the effluent perfusate.

Mitochondrial respiration

Mitochondrial oxygen consumption was measured by polarography using a teflon-shielded platinum electrode [Saling et al., 2011]. 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 EDTA and 10 mM Tris-HCl (pH 7.4). Succinate and α-ketoglutarate, both at a concentration

of 10 mM, were used as substrates. Rates of oxygen consumption were computed from the slopes of the recorder tracings and expressed as $nmol \cdot min^{-1} \cdot (mg\ protein)^{-1}$. The respiration rates were measured under three conditions: (a) before the addition of ADP (substrate respiration or basal), (b) just after 0.125 mM ADP addition (state III respiration) and (c) after cessation of the ADP stimulation (state IV). The respiratory control (RC) was calculated as the state III/state IV ratio and the ADP/O ratio was determined according to Sá-Nakanishi et al., 2018.

Statistical analysis

The parameters presented in graphs and tables are means ± standard errors of the means. Statistical analysis was done by means of the GraphPad Prism Software (version 8.0). The statistical significance of the data was analyzed by means of ANOVA ONE-WAY and the Tukey post-hoc test was applied with the 5% level of significance ($p<0.05$). For comparison of two values the student t test was applied with the 5% level ($p<0.05$).

RESULTS

Effects of resveratrol on inflammation

Fig. 1A shows the time-courses of the volume increment (edema) in the injected paws. Inflammatory reactions in the injected paws were observed at the first day and they were equal in all arthritic groups until the day 9, when progressively increased until the day 20. From the 13th day the paw volume of the 500 mg/Kg resveratrol-treated rats tended to be smaller, but it was not significant at the day 20. For comparison purposes, Fig. 1B shows the values of the injected paw edema at day 13 and 20 of all groups. The increment of paw volume in arthritic rats were 30% lower at day 13 when treated with 500 mg/Kg resveratrol and 14% lower at day 20 when treated with 250 mg/Kg resveratrol.

The time-course of the volume increment and the values of volume increment at day 13 and 20 of the non-injected hind paws (contralateral paws) are shown, respectively, in Fig. 1C and D. The volume of the contralateral paw of all groups was not modified until day 10, when the paw volume of arthritic rats progressively increased until day 20. The treatment of the arthritic animals with both 250 and 500 mg/Kg resveratrol caused smaller increments (by 27% at day 20) in the contralateral paw volumes (Fig. 1D).

The secondary lesions appeared at day 10 and reached the highest scores at day 20 after adjuvant injection, when reached the score of 5 for arthritic non-treated animals (Fig. 2A). The scores at the end of experimental period were not different for treated arthritic rats. However, the treatment of arthritic animals with 250 and 500 mg/Kg resveratrol was able to attenuate the secondary lesions between the day 10 and 18 (Fig. 2B).

The body weight before starting treatment was 200 ± 5 g and control rats progressively gained weight (Fig. 2C). Following the adjuvant injection, the body weight of all arthritic groups practically did not change until day 18, when they were around 28% lower than the control animals (Fig. 2D).

The activity of AST and ALT were accessed in the plasma as indicators for hepatic damage and the activity of MPO were accessed in the liver as indicator of liver inflammation. Arthritis increased the MPO activity by 66% in the liver and the treatment with 100, 250 and 500 mg/Kg completely abolished this increment (Fig. 3A). The activity of both AST and ALT in the plasma were not increased for treated and non-treated arthritic rats (Fig. 3B).

Effects of resveratrol on glycogen catabolism and glycolysis

Livers from fed rats when perfused with substrate-free medium survive at the expense of glycogen degradation via glycolysis and oxidation of endogenous fatty acids [Comar et al., 2003]. Under these conditions the liver release glucose, lactate and pyruvate as a result of glycogen catabolism. Fig. 4A illustrates the time-courses of metabolic modifications of perfused livers from arthritic and healthy rats non-treated and treated with 25 mg/Kg resveratrol. It also illustrates a typical experimental protocol, which was used for all other resveratrol doses. Four parameters were measured: glucose release, lactate and pyruvate productions and oxygen consumption. As revealed by Fig. 4A all parameters were stable and achieved the steady-state at the 30 minutes perfusion time, when no difference was observed between non-treated control and arthritic rats, except by a discrete reduction in the glucose output in arthritic livers. The latter is the consequence of a lower content of glycogen in the liver of arthritic rats [Fedatto et al., 2002]. The liver output of glucose, pyruvate and lactate, however, were lower at 30 minutes perfusion time in the liver of control and arthritic rats treated with 25 mg/Kg resveratrol. The oxygen uptake was lower only in the liver of arthritic rats treated with 25 mg/Kg resveratrol.

Experiments like those illustrated in Fig. 4A were repeated with rats treated with 25, 50, 100, 250 and 500 mg/Kg resveratrol in order to establish dose dependences for the effects. Fig 5A, B and C shows respectively the rates of oxygen uptake, glucose output and lactate output in perfused livers at 30 minutes perfusion time in Fig 4A. The treatment did not modify the oxygen uptake of control rats, but it was decreased by 14% in arthritic rats treated with 25 and 50 mg/Kg resveratrol (compared to the non-treated arthritic rats; Fig. 5A). For healthy rats, 25 and 500 mg/Kg resveratrol decreased equally by 30% the lactate production and 100 mg/Kg resveratrol decreased it by 56% (compared to the non-treated control rats; Fig. 5C). The treatment did not modify the lactate production in the liver of arthritic rats. The glucose overflow, which represents the excess of glycogenolysis not diverted into the glycolytic pathway, was decreased equally by approximately 40% by resveratrol at the dose of 25 mg/Kg in control livers and at the dose of 25 and 50 mg/Kg in arthritic livers. On the other hand, the glucose output was 30% higher in the liver of control rats, but not arthritic rats treated with 500 mg/Kg resveratrol (compared

to the non-treated controls).

The rate of glycogenolysis and glycolysis are represented against the resveratrol concentration in Fig. 5D and E, respectively. Glycogenolysis was approximately 40% lower in the liver of control rats treated with 25 mg/Kg resveratrol and arthritic rats treated with 25 and 50 mg/Kg resveratrol (Fig. 5D). Glycolysis was 40% lower in the liver of control rats treated with 25 mg/Kg resveratrol (Fig. 5E). The treatment did not modify the glycolysis of arthritic rats. In Fig. 5F, the lactate to pyruvate ratio, an indicator for the cytosolic NADH/NAD^+ ratio [Scholz & Bücher, 1965], was not modified by the treatment of arthritic and control rats, despite reducing the lactate production in the liver of control rats. This occurred because the pyruvate production was also decreased (results not shown).

Effects of resveratrol on liver gluconeogenesis from lactate

The effect of resveratrol on gluconeogenesis was investigated in perfused livers using lactate as precursor. Fig. 4B shows the time courses of pyruvate and glucose production and oxygen consumption in perfused livers of control and arthritic rats non-treated and treated with 25 mg/Kg resveratrol. It also illustrates a typical experimental protocol, which was used for all other resveratrol doses. Livers from 12 hours fasted rats were perfused in order to ensure low glycogen levels. Under such conditions the rate of glucose output reflects mainly the rate of gluconeogenesis [Comar et al., 2016]. Lactate infusion produced progressive increases in glucose and pyruvate productions and oxygen uptake. These increases tended to stabilize at 30-35 minutes perfusion time. The increment of glucose production and oxygen uptake was lower and the pyruvate production was higher in the liver of arthritic rats, a phenomenon already reported by previous studies [Castro-Ghizoni et al., 2017; Ames-Sibin et al., 2018]. The treatment with 25 mg/Kg resveratrol decreased the oxygen consumption and glucose production only of control rats.

Experiments like those illustrated in Fig. 4B were repeated with 25, 50, 100, 250 and 500 mg/Kg resveratrol in order to establish concentration dependences for the effects. Fig 6A, B and C shows respectively the rates of oxygen uptake, glucose output and pyruvate output in perfused livers at 36 minutes perfusion time in Fig 4B. The oxygen uptake was approximately 15%

lower in the liver of control rats treated with all doses of resveratrol, except 100 mg/Kg (compared to the nontreated controls). The treatment did not modify the oxygen uptake in the livers of arthritic rats (Fig. 6A). The stimulus of the glucose production was lower in the liver of rats treated with resveratrol, but only in the controls treated with the dose of 50 mg/Kg (-24%; Fig. 6B). The treatment did not modify the stimulus of the pyruvate production in the liver of both arthritic and control rats (Fig. 6C).

Effects of resveratrol on the respiratory activity of hepatic isolated mitochondria

Fig. S1 outlines the experimental approach used to evaluate the respiratory activity of phosphorylating liver mitochondria and the calculation procedures for obtaining the mitochondrial respiratory parameters. Fig. 7 shows the dose dependency of the effects of resveratrol on mitochondrial basal respiration, state III respiration, respiratory control (RC; state III/IV) and ADP/O ratio. All parameters were not modified in the liver of arthritic and control rats, except by an increase of the RC of control rats treated with 100 mg/Kg resveratrol (only when succinate was the respiratory substrate) and a decrease of RC of arthritic rats treated with the same dose, but only when α-ketoglutarate was the substrate (Fig. 7C).

DISCUSSION

The experimental model of chronic inflammation used in the present study is considered severe in rats and it shares features of advanced rheumatoid arthritis [Stolina et al., 2009]. In this model, animals develop an intense inflammatory response to the adjuvant in the paws (polyarthritis) and present cachexia and generalized inflammatory manifestations [Stolina et al., 2009]. In this study, these signals were evidenced by the increased hepatic MPO activity, paw edema and severe secondary lesions to arthritis. The animals did not gain body weight during the experimental period of 21 days and, in addition, the hepatic gluconeogenesis was reduced in the arthritic liver, which corroborate previous findings [Ames-Sibin et al., 2018; Castro-Ghizoni et al., 2017; Bracht et al., 2016; Comar et al., 2013].

With respect to the effect of resveratrol on arthritis, the compound was only partially effective to decrease the articular and systemic inflammation. This because resveratrol decreased the MPO activity in the liver and the contralateral paw edema, but it was not able to diminish the secondary lesions decurrent from arthritis in the tail, ears and forelimbs. The latter, however, was considerably delayed by resveratrol at the dose of 250 and 500 mg/Kg (Fig. 2A and B) and even the edema of injected paw was considerably lower at the day 20 for 250 mg/Kg resveratrol (Fig. 1A and B). One point in the present study that drew attention is that, even without to considerably improve arthritic score at the day 20, arthritic rats treated with resveratrol at the doses of 250 and 500 mg/kg exhibited a better physical condition at the day 13, particularly by improving joint movement and body mobility (see supplementary video S2). It is important to highlight that adjuvant-induced arthritis in rats is associated with pain, joint stiffness and limitation of movements. Therefore, resveratrol could be in addition promoting analgesia and pain relief in the arthritic rats, a find that requires future investigation.

The anti-inflammatory activity of resveratrol was already previously shown *in vitro* by inhibiting the synthesis of proinflammatory cytokines in synoviocytes isolated from arthritic rats and in human synoviocytes [Chen et al., 2013; Tian et al., 2013; Nakayama et al., 2012]. In addition, the dietary supplementation with resveratrol also lowers the arthritis manifestations in rodents with adjuvant-induced mild arthritis and in patients with rheumatoid arthritis [Khojah

et al., 2018; Rivieiro-Navieira et al., 2016; Xuzhu et al., 2012]. Previous study has been already shown that resveratrol at the dose of 10 mg/Kg improves the serum biomarkers of arthritis and knee joints inflammation of rats with adjuvant-induced arthritis [Wahba et al., 2016]. Our results reveal that resveratrol additionally decreases the MPO activity in the liver at doses above 100 mg/Kg and improves the pronounced paw edema of arthritic rats, but at doses above 250 mg/kg. However, the doses of 250 and 500 mg/Kg would correspond to a human dose of 40-80 mg/kg, respectively, as given by the body surface area normalization method, a value within the recommended limit of resveratrol for adults [Patel et al., 2011].

The anti-inflammatory mechanism of resveratrol has not yet been completely clarified, but it is known that it suppresses the factor nuclear *kappa* B *(*NF-kB)-mediated expression of proinflammatory enzymes and cytokines [Yang et al., 2017; Wahba et al., 2016; Chen et al., 2014]. At this respect, resveratrol has been reported to inhibit the cyclooxygenase 2 (COX-2) transcription by up-regulating sirtuin 1 (SIRT-1) that in turn suppresses the acetylation of the transcription factors AP-1 (activator protein 1) and NF-κB in human rheumatoid arthritis synovial fibroblasts [Yang et al., 2017]. In fact, resveratrol inhibits the production of prostaglandin E, matrix metalloprotinease-3 (MMP-3), tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1) and the expression of COX-2 in synovial tissues of arthritic rats [Rivieiro-Navieira et al., 2016; Wahba et al., 2016; Chen et al., 2014]. In addition, resveratrol inhibits the production of TNF-α and nitric oxide (NO) and the expression of COX-2 and inducible nitric oxide synthase (iNOS) in rotenone-exposed primary microglia of rats [Chang et al., 2013]. The results of the present study shows additionally that resveratrol inhibits the activity of MPO in the liver, which is associated with reduction of the neutrophils infiltration in the liver of arthritic rats, i.e., is associated with reduction of the liver inflammation.

Resveratrol did not increase the body weight of rats with adjuvant-induced arthritis, which shows that the chronic inflammation inherent to this model of arthritis was not improved completely. However, the adjuvant-induced arthritis is severe and even ibuprofen, indomethacin and dexamethasone did not improve the body weight [Bendele et al., 1999]. On the other hand, no increases of the plasma AST and ALT activity was found in rats treated with resveratrol, which shows that no liver damage was associated with the compound.

With respect to the liver metabolism, resveratrol was not effective to improve the gluconeogenesis in the liver of arthritic rats. This phenomenon occurs even when the organ inflammation is decreased, as revealed by the reduction of the MPO activity. On the other hand, the compound did not negatively modify the gluconeogenesis in the liver of control and arthritic rats, except by a discrete reduction in control rats treated with the dose of 50 mg/Kg. In contrast, resveratrol considerably inhibits the gluconeogenesis when directly introduced even at the low concentration of 10 μM in the perfused liver of healthy and arthritic rats, an action that seems to be transitory and exist only when the resveratrol is present in the organ [to be published]. Thus, the results of the present study show that the inhibition of liver gluconeogenesis by resveratrol is really transitory and that the administration of the compound in rats did not modify the hepatic gluconeogenesis. However, the transitory effects previously found must not be neglected because significant concentrations of resveratrol are found in the plasma of humans after its oral administration [Xia et al., 2017; Cai et al., 2015; Rousova et al., 2016; Sergides et al., 2015].

In contrast to gluconeogenesis, the glycogen catabolism was at least in part modified by resveratrol in the liver of control and arthritic rats. However, the effects were not pronounced and were not notedly dose-dependent. For this reason, it is not probable that these effects are negatively affecting the liver metabolism. The same can be said about the stimulus of the hepatic oxygen uptake of control rats, which was also discretely inhibited by resveratrol at several doses, an effect that was not found for hepatic isolated mitochondria in the present study. In this regard, gluconeogenesis is extremely sensitive to alterations in the cellular integrity because the pathway occurs in different intracellular compartments and requires energy from an aerobic system associated to membranes [Castro-Ghizoni et al., 2017; Ames-Sibin et al., 2018]. Thus, the possible negative actions of resveratrol on hepatic metabolism should be certainly involving the modification of gluconeogenesis.

CONCLUSION

The results show that the orally administered resveratrol partially improves the articular and systemic inflammation in rats with adjuvant-induced arthritis, which corroborates previous findings. In addition, this compound does not modify considerably the liver metabolism of both arthritic and healthy rats, showing that the strong inhibition of resveratrol on the hepatic gluconeogenesis and mitochondrial respiration previously found is transitory and exist only when the resveratrol is present in the organ. Overall, the results show that resveratrol may be a promising adjuvant to the most current approaches aiming at rheumatoid arthritis therapy, however, caution is recommended with respect to the transitory effects on the liver metabolism.

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Competing interests

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

Authors' contributions

JFC and AB conceived and designed the experiments. CABA and MSS induced arthritis and treated the animals. MSS and LB performed the liver perfusion experiments. FMSS and MSS performed the inflammation experiments. JFC wrote the paper. AB reviewed the paper. All authors have read and approved the final version of the paper.

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FIGURE CAPTIONS

Fig. 1. ***Effects of resveratrol on evolution of paw volume of arthritic rats.*** Treatment of the animals was initiated 5 days before arthritis induction and maintained for 18 days after. The volume of both hind paws up to the tibiotarsal joint was measured by plethysmography. The mean initial paw volume was 1.6 ± 0.1 mL. Δ volume of paws (paw edema) = volume at day x - initial volume (day zero). Controls received vehicle and arthritis received resveratrol at the dose of 0, 100, 250 and 500 mg/Kg. The values are the mean ± standard error of the mean of 5-6 animals. The statistical significance of the data was analysed by means of ANOVA ONE-WAY, for comparison of two values the student's t test was applied with the 5% level of significance ($p<0.05$). PANEL A and C: *$p<0.05$: for difference from controls; $^{\#}p<0.05$: for difference from arthritis. PANEL B and D: Values with different superscript letters in the same group are different.

Fig. 2. ***Effects of resveratrol on body weight and arthritis score of arthritic rats.*** Treatment of the animals was initiated 5 days before arthritis induction and maintained for 18 days after. Controls received vehicle and arthritis received resveratrol at the dose of 0, 100, 250 and 500 mg/Kg. The mean initial weight of the animals was 200 ± 5 g. The appearance and severity of secondary lesions were also assessed from the 9th to the 21th day according to the following score graded from 0 to 5: (+1) nodules in the tail; (+1 or +2) nodules in one or both ears; and (+1 or +2) swelling in one or both forelimbs. The values are the mean ± standard error of the mean of 5-6 animals. PANEL A and B: *$p<0.05$: for difference from controls; $^{\#}p<0.05$: for difference from arthritis. PANEL C and D: Values with different superscript letters in the same group are different.

Fig. 3. ***Effects of resveratrol on hepatic myeloperoxidase (MPO) activity and plasma AST and ALT.*** The activity of MPO is expressed per mg of protein and the activity of AST and ALT is expressed as units per liter. Treatment of the animals was initiated 5 days before arthritis induction and maintained for 18

days after. Controls received vehicle and arthritis received resveratrol at the dose of 0, 25, 100, 250 and 500 mg/Kg. The values represent the mean ± standard error of the mean of 5 animals. The statistical significance of the data was analysed by means of ANOVA ONE-WAY and the Newman-Keuls post-hoc test was applied with the 5% level of significance. Values with different superscript letters in the same group are different ($p<0.05$).

Fig. 4. Time courses of glycogen catabolism and gluconeogenesis in the perfused livers of control and arthritic rats treated with vehicle. (A) Glycogen catabolism: livers from fed rats were perfused with Krebs/Henseleit bicarbonate-buffer as shown in the horizontal bar. **(B)** Gluconeogenesis: livers from fasted rats were perfused with Krebs/Henseleit-buffer and L-lactate as shown in the horizontal bar. The effluent perfusate was sampled in 2-min intervals and analysed for glucose, lactate, and pyruvate. Oxygen uptake was monitored by polarography. Data points represent the means ± standard errors of 5-6 livers.

Fig. 5. ***The concentration-dependent effects of resveratrol on glycogen catabolism in the perfused livers of healthy and arthritic rats.*** Livers from treated fed rats were perfused as indicated by the horizontal bar in Fig. 4A. The outflowing perfusate was sampled in regular intervals and analysed for their contents of metabolites. Oxygen uptake was monitored by polarography. The values in **Panels A, B** and **C** are the rates observed after stabilization of the changes induced by each treatment (28-30 minutes perfusion time). The values in **Panels E**, **F** and **G** were calculated from the steady-state rates of glucose, lactate and pyruvate production. Glycogenolysis = glucose + 1/2(lactate + pyruvate) and glycolysis = 1/2(lactate + pyruvate). Each datum represents the mean of 5 liver perfusion experiments. *$p<0.05$: different from 0 mg/Kg resveratrol in controls; $^{\#}p<0.05$: different from 0 mg/Kg resveratrol in arthritis; (ANOVA ONE-WAY and the Newman-Keuls post-hoc); $^{a}p<0.05$: for difference between 0 mg/Kg resveratrol in controls and arthritis (Student's t test).

Fig. 6. ***The concentration-dependent effects of resveratrol on gluconeo-genesis from lactate in perfused livers of healthy and arthritic rats.*** Livers from 12 h fasted rats were perfused with 2 mM L-lactate as indicated by

the horizontal bar in Fig. 4B. The outflowing perfusate was sampled in regular intervals and analysed for their contents of glucose and pyruvate. Oxygen uptake was monitored by polarography. The values in **Panels A, B** and **C** are the rate observed after stabilization of the changes induced by lactate (36-38 minutes perfusion time in Fig. 4B). Data are the mean ± SEM obtained from 5 animals for each condition. *$p<0.05$: different from 0 mg/Kg resveratrol in controls; $^{\#}p<0.05$: different from 0 mg/Kg resveratrol in arthritis; (ANOVA ONE-WAY and the Newman-Keuls post-hoc); $^{a}p<0.05$: for difference between 0 mg/Kg resveratrol in controls and arthritis (Student's t test).

Fig. 7. ***The concentration-dependent effects of resveratrol on the respiratory activity of isolated hepatic mitochondria.*** Isolated mitochondria (1.0 $mg \cdot mL^{-1}$) were incubated at 37ºC in a closed oxygraph chamber containing 2 mL of the reaction. The experimental protocol and calculation procedures are shown in Fig. S1. Mitochondrial respiration driven by α-ketoglutarate or succinate in the absence **(A; basal)** and presence **(B; state III)** of 0.125 mM ADP was followed polarographically. **(C),** respiratory control (RC); **(D),** ADP/O ratio. Data represent the mean ± SEM of 5 animals. *$p<0.05$: different from 0 mg/Kg resveratrol in controls; $^{\#}p<0.05$: different from 0 mg/Kg resveratrol in arthritis; (ANOVA ONE-WAY and the Newman-Keuls post-hoc).

Fig. S1. Oxygraph (A) and experimental approach used to determine the respiratory activity of isolated mitochondria (B).

Fig. 1

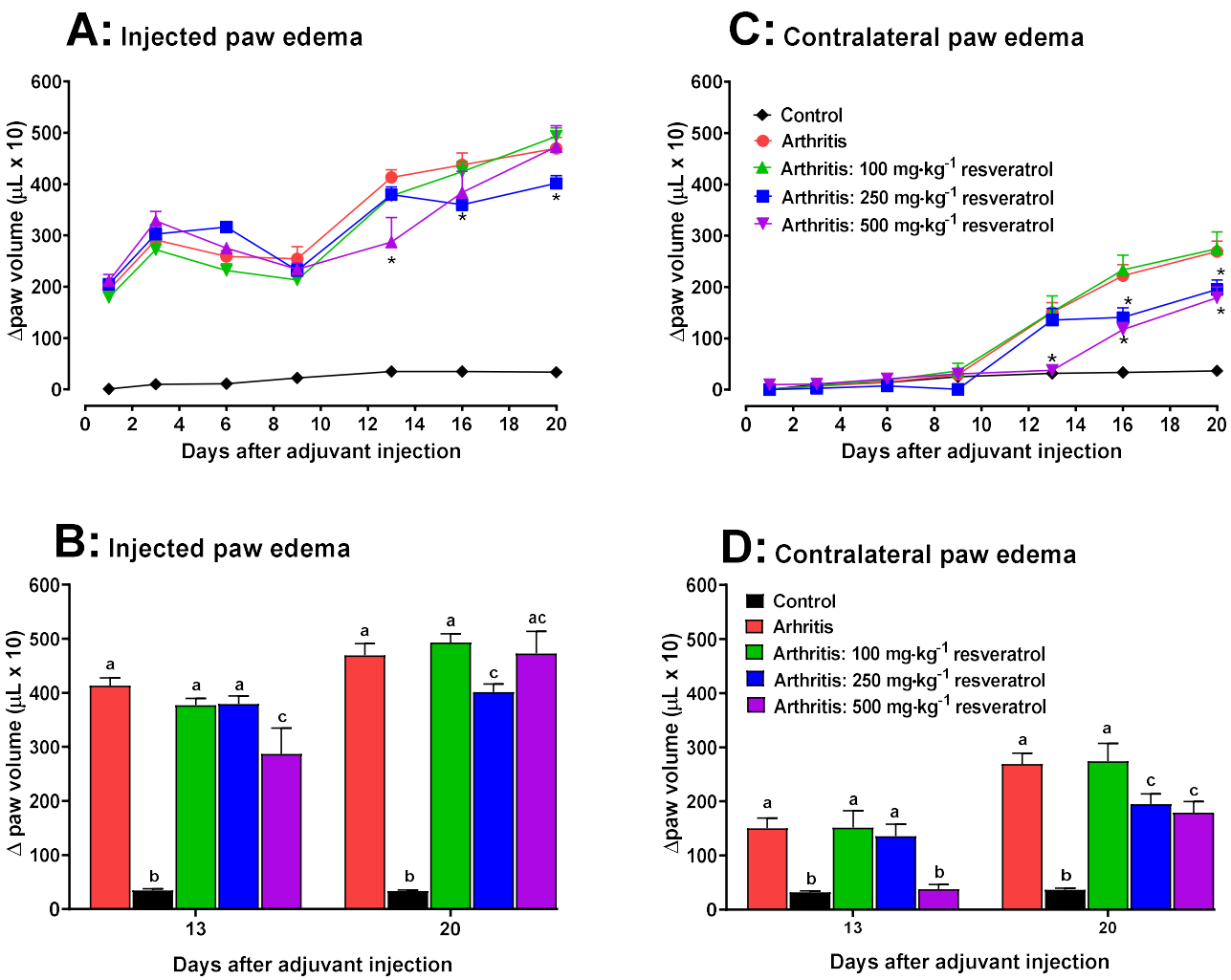

A: Injected paw edema
Δpaw volume (µL x 10)
Days after adjuvant injection
C: Contralateral paw edema
Control
Arthritis
Arthritis: 100 mg·kg-1 resveratrol
Arthritis: 250 mg·kg-1 resveratrol
Arthritis: 500 mg·kg-1 resveratrol
B: Injected paw edema
Δ paw volume (µL x 10)
D: Contralateral paw edema
Arhritis

Fig. 2

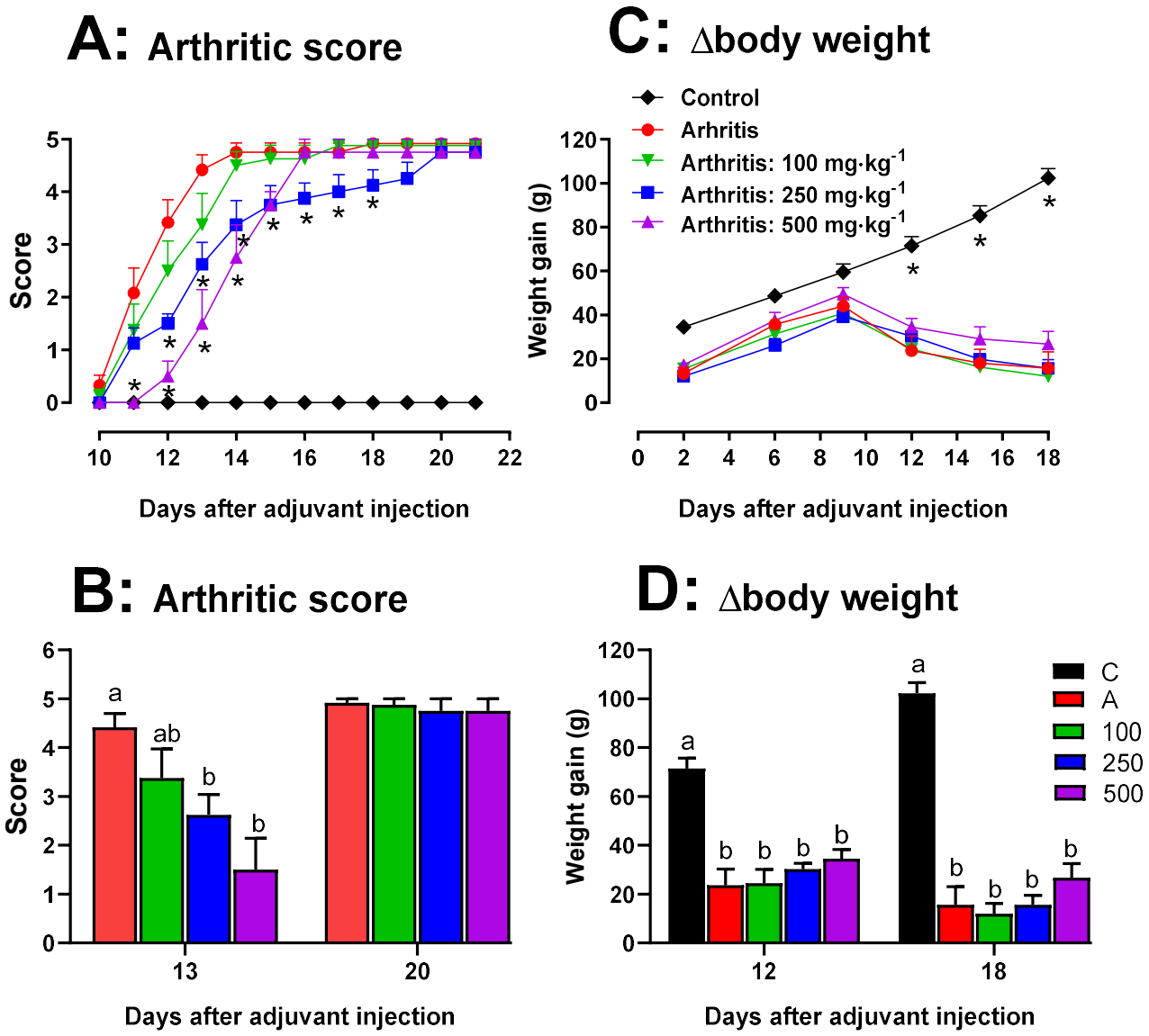

A: Arthritic score
Score
Days after adjuvant injection
C: Δbody weight
Control
Arhritis
Arthritis: 100 mg·kg-1
Arthritis: 250 mg·kg-1
Arthritis: 500 mg·kg-1
Weight gain (g)
Days after adjuvant injection
B: Arthritic score
Score
Days after adjuvant injection
D: Δbody weight
Weight gain (g)
C
A
100
250
500
Days after adjuvant injection

Fig. 3.

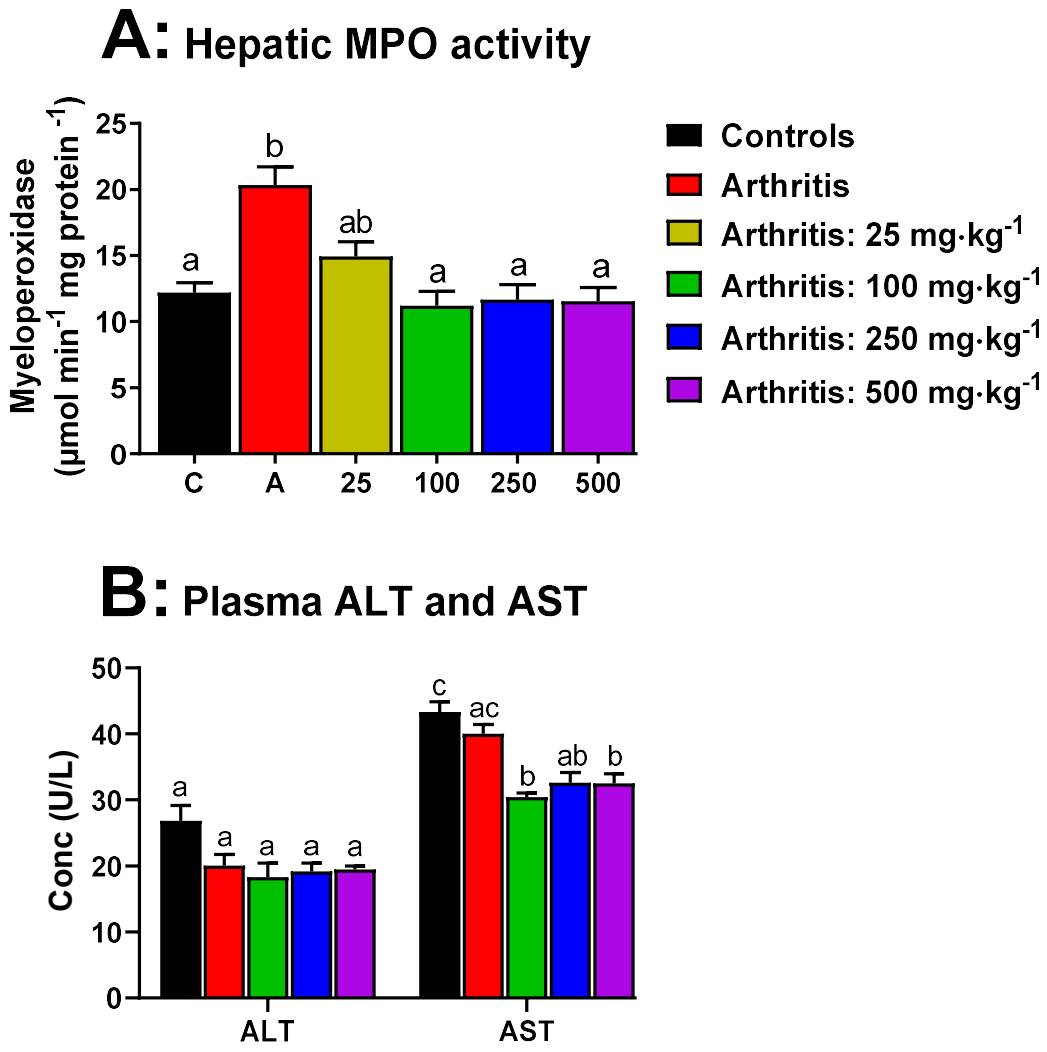

A: Hepatic MPO activity
Myeloperoxidase (µmol min-1 mg protein-1)
25
20
15
10
5
0
a
b
ab
a
a
a
C
A
25
100
250
500
Controls
Arthritis
Arthritis: 25 mg·kg-1
Arthritis: 100 mg·kg-1
Arthritis: 250 mg·kg-1
Arthritis: 500 mg·kg-1
B: Plasma ALT and AST
Conc (U/L)
50
40
30
20
10
0
a
a
a
a
a
c
ac
b
ab
b
ALT
AST

Fig. 4.

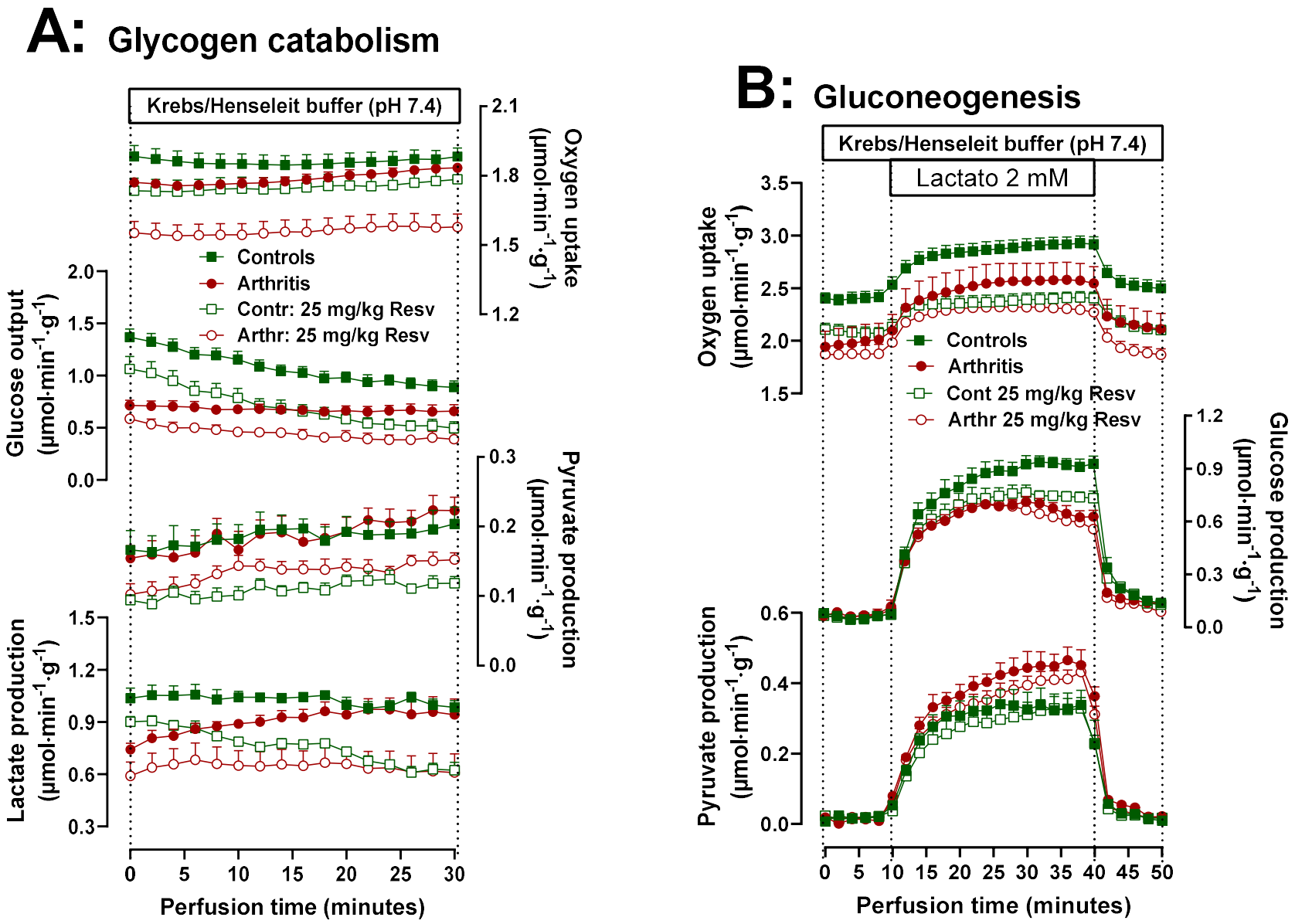

A: Glycogen catabolism
Krebs/Henseleit buffer (pH 7.4)
Controls
Arthritis
Contr: 25 mg/kg Resv
Arthr: 25 mg/kg Resv
Glucose output (µmol·min-1·g-1)
Oxygen uptake (µmol·min-1·g-1)
Pyruvate production (µmol·min-1·g-1)
Lactate production (µmol·min-1·g-1)
Perfusion time (minutes)
B: Gluconeogenesis
Krebs/Henseleit buffer (pH 7.4)
Lactato 2 mM
Cont 25 mg/kg Resv
Arthr 25 mg/kg Resv
Oxygen uptake (µmol·min-1·g-1)
Glucose production (µmol·min-1·g-1)
Pyruvate production (µmol·min-1·g-1)
Perfusion time (minutes)

Fig. 5

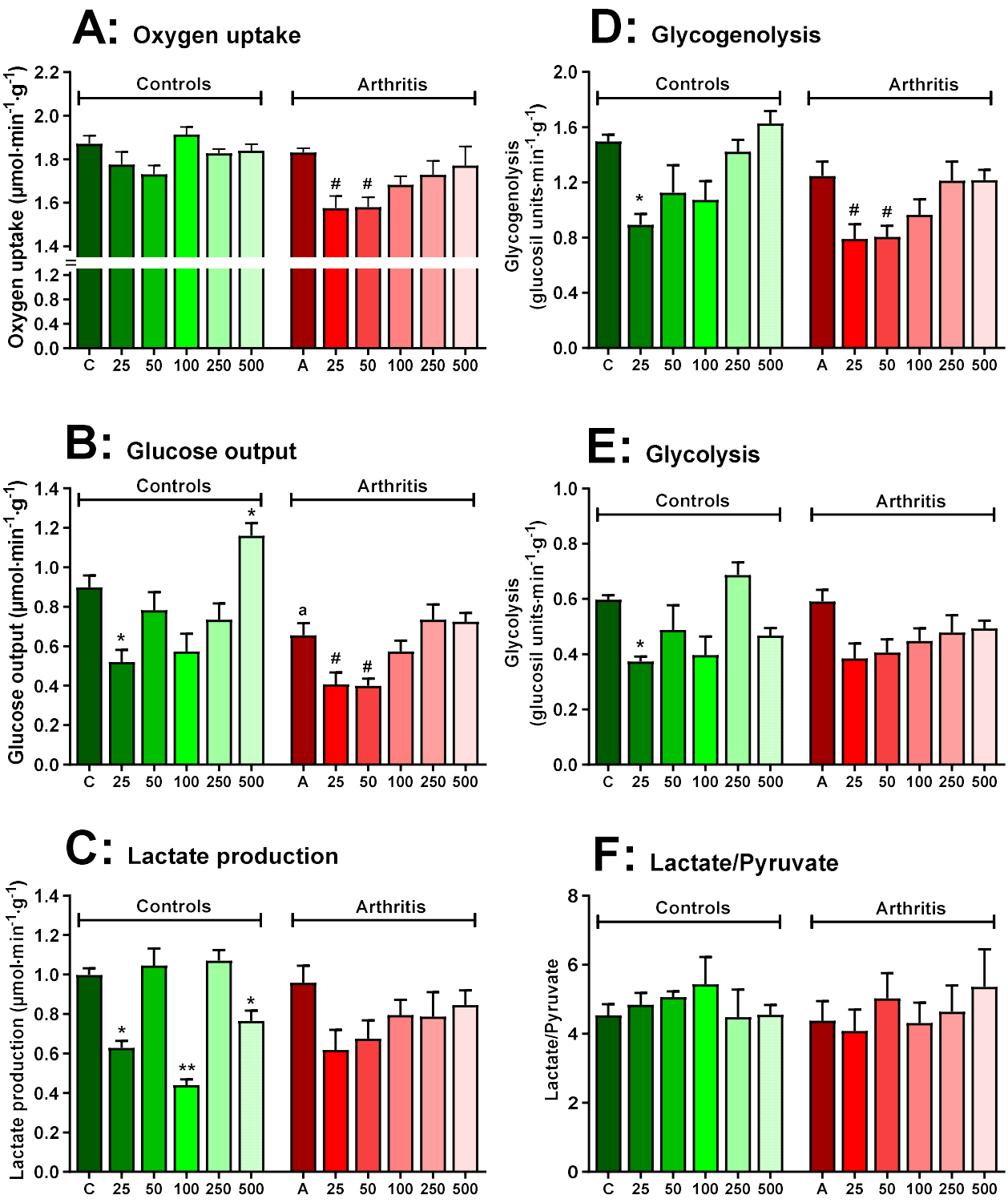

A: Oxygen uptake
Oxygen uptake (µmol·min-1·g-1)
Controls
Arthritis
C 25 50 100 250 500 A 25 50 100 250 500
D: Glycogenolysis
Glycogenolysis (glucosil units·min-1·g-1)
B: Glucose output
Glucose output (µmol·min-1·g-1)
E: Glycolysis
Glycolysis (glucosil units·min-1·g-1)
C: Lactate production
Lactate production (µmol·min-1·g-1)
F: Lactate/Pyruvate
Lactate/Pyruvate

Fig. 6

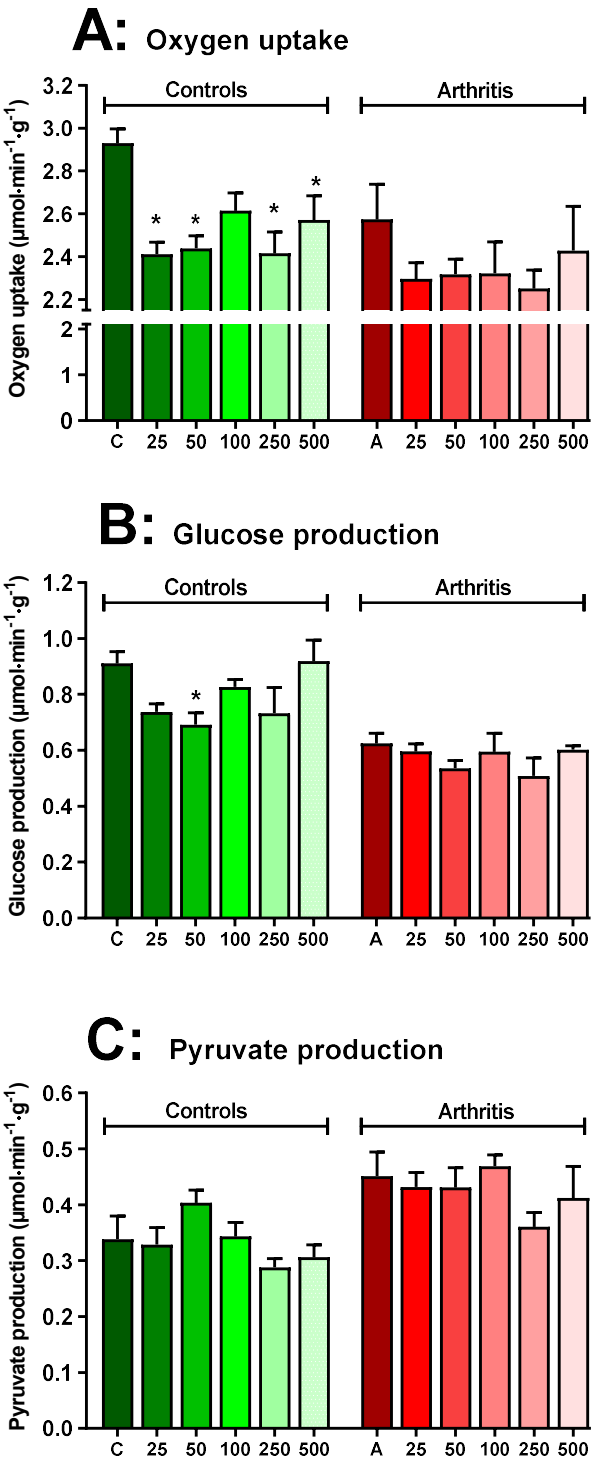
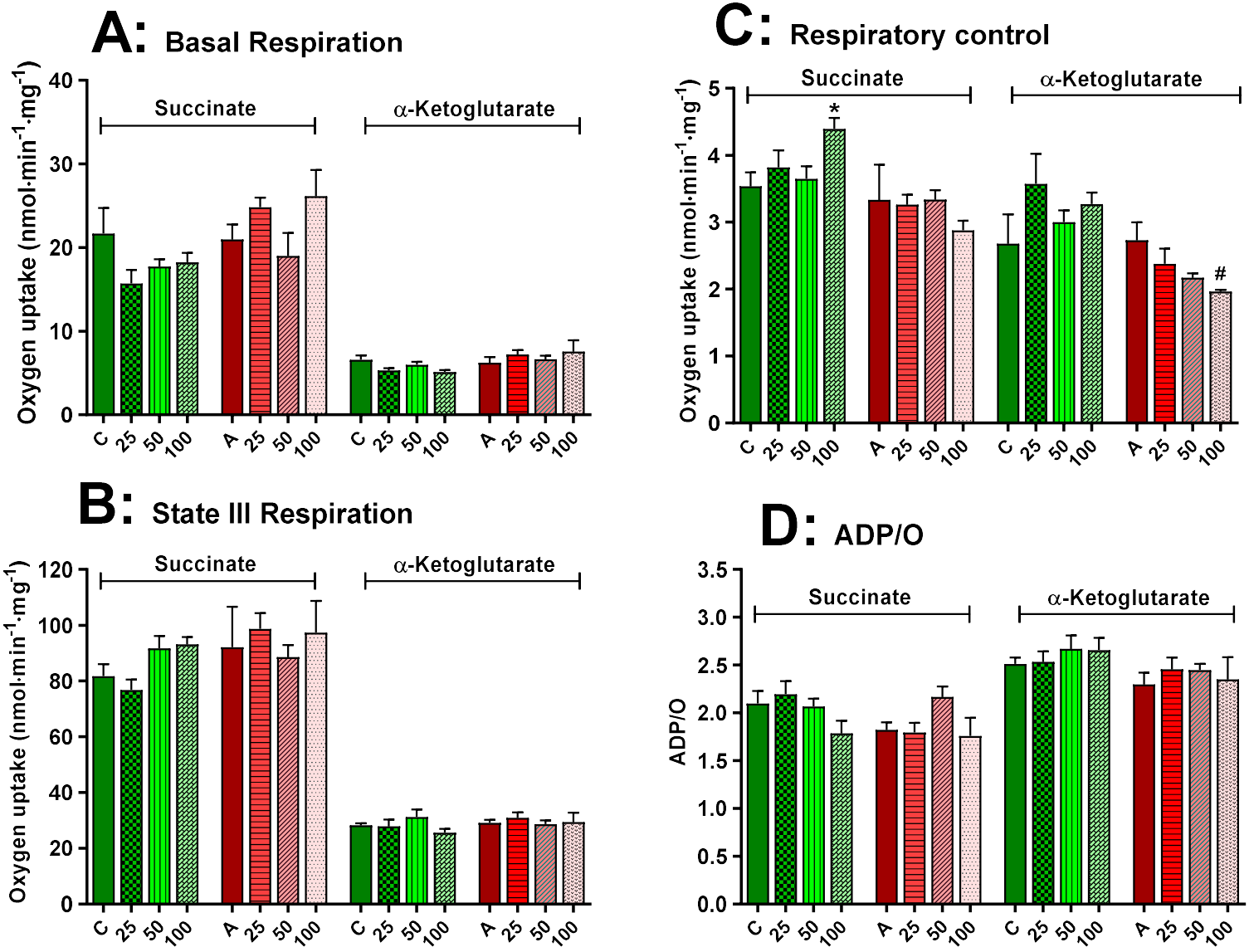

A: Oxygen uptake
Controls
Arthritis
Oxygen uptake (µmol·min-1·g-1)
3.2
3.0
2.8
2.6
2.4
2.2
2
1
0
C 25 50 100 250 500 A 25 50 100 250 500
B: Glucose production
Glucose production (µmol·min-1·g-1)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
C: Pyruvate production
Pyruvate production (µmol·min-1·g-1)
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Fig. 7


A: Basal Respiration
Oxygen uptake (nmol·min-1·mg-1)
Succinate
α-Ketoglutarate
C 25 50 100 A 25 50 100 C 25 50 100 A 25 50 100
B: State III Respiration
Oxygen uptake (nmol·min-1·mg-1)
Succinate
α-Ketoglutarate
C 25 50 100 A 25 50 100 C 25 50 100 A 25 50 100
C: Respiratory control
Oxygen uptake (nmol·min-1·mg-1)
Succinate
α-Ketoglutarate
*
#
C 25 50 100 A 25 50 100 C 25 50 100 A 25 50 100
D: ADP/O
ADP/O
Succinate
α-Ketoglutarate
C 25 50 100 A 25 50 100 C 25 50 100 A 25 50 100

SUPPLEMENTARY MATERIAL

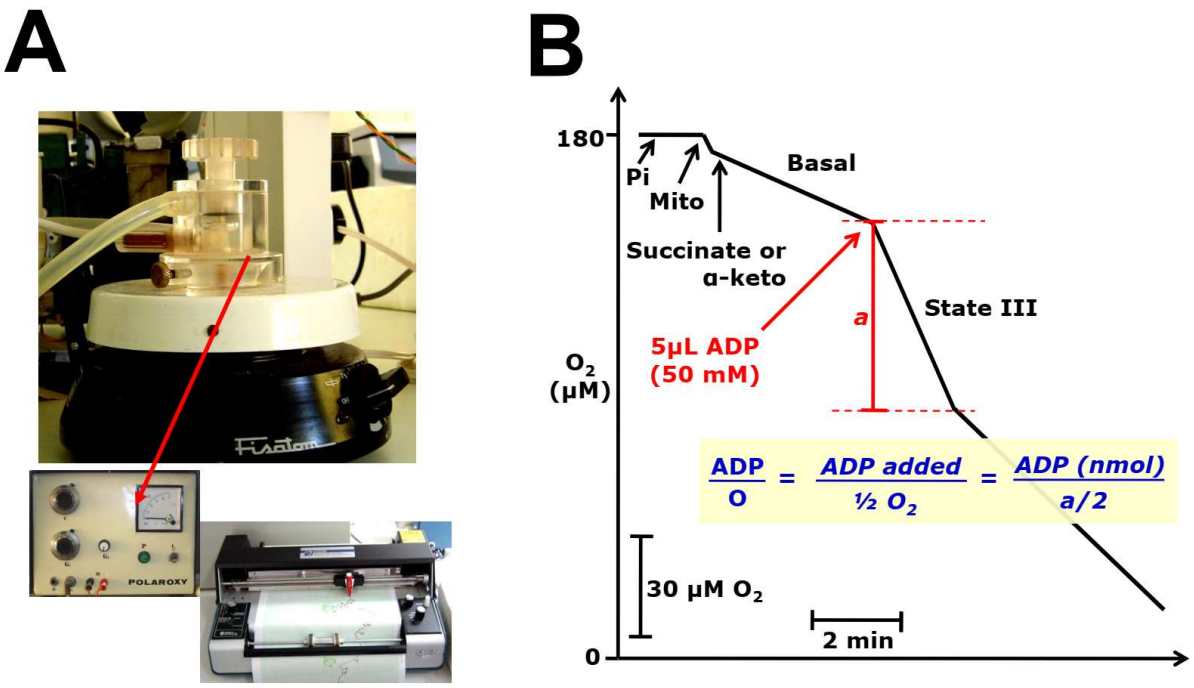


Fig. S1. Oxygraph (A) and experimental approach used to determine the respiratory activity of isolated mitochondria (B).