UNIVERSIDADE ESTADUAL DE MARINGÁ
Programa de Pós-graduação em Ciências Biológicas
Área de concentração – Biologia Celular

Alessandra Salvador de Souza

As ações do extrato de *Arrabidaea chica* sobre o metabolismo hepático

Maringá
2007

Alessandra Salvador de Souza

As ações do extrato de *Arrabidaea chica* sobre o metabolismo hepático

Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Estadual de Maringá, área de concentração em Biologia Celular, para obtenção **do grau de Mestre**

Orientadora: Dra. Nair Seiko Yamamoto

Co-orientadora: Dra. Clairce Luzia Salgueiro Pagadigorria

Maringá
2007

Dados Internacionais de Catalogação-na-Publicação (CIP)
(Biblioteca Central - UEM, Maringá – PR., Brasil)

S729a

Souza, Alessandra Salvador de
As ações do extrato de *Arrabidaea chica* sobre o metabolismo hepático / Alessandra Salvador de Souza. -- Maringá : [s.n.], 2007.
38 f. : figs., tabs.

Orientador : Profª. Drª. Nair Seiko Yamamoto.
Co-orientadora : Profª. Drª. Clairce Luzia Salgueiro Pagadigorria.
Dissertação (mestrado) - Universidade Estadual de Maringá. Programa de Pós-graduação em Ciências Biológicas, 2007.

1. *Arrabidaea chica* - Metabolismo hepático. 2. Fígado - Alterações metabólicas. 3. Fígado em perfusão. 4. Mitocôndria. I. Universidade Estadual de Maringá. Programa de Pós-graduação em Ciências Biológicas. II. Título.

CDD 21.ed. 615

Este é um trabalho de equipe, realizado nos Laboratórios de Metabolismo Hepático e Oxidações Biológicas da Universidade Estadual de Maringá, baseado no artigo:

Alessandra Salvador de Souza, Claircе Luzia Salgueiro Pagadigorria, Emy Luiza Ishii-Iwamoto, Adelar Bracht, Diógenes Aparício Garcia Cortez, Gislaine Trassi, Nair Seiko Yamamoto. Effects of the *Arrabidaea chica* extract on energy metabolism in the rat liver. *Journal of Ethnopharmacology.*

Resumo

INTRODUÇÃO E OBJETIVOS — A bignoniaceae *Arrabidaea chica*, conhecida como carajuru, puca panga, chica ou pariri é uma trepadeira lenhosa distribuída na América tropical. Folhas desta planta são usadas como agentes antiinflamatório e adstringente, bem como remédios para cólica intestinal, diarréia sanguínea, leucorréia, anemia e leucemia na região amazônica. Um estudo fitoquímico das folhas da *A. chica* mostrou que a carajurina, uma 3-desoxiantocianidina, é responsável pela inibição do fator de transcrição NF-kB, fenômeno responsável pela atividade antiinflamatória. Antocianinas, flavonóides, taninos, fitosteróis e flavonas têm sido identificados no extrato preparado a partir das folhas da planta. Considerando-se que, além da atividade antioxidante, vários flavonóides, como a quercetina, podem exercer uma variedade de efeitos sobre o metabolismo hepático, a presença de flavonóides no extrato das folhas da *Arrabidaea chica* sugere que o extrato poderia também afetar o metabolismo energético celular. Esta possibilidade foi investigada no presente trabalho, no qual foram medidos os efeitos do extrato das folhas e do caule da *Arrabidaea chica* sobre o metabolismo energético celular em mitocôndrias isoladas e em fígados perfundidos de ratos.

MÉTODOS — Ratos machos Wistar, pesando 200 a 250 g, foram utilizados em todos os experimentos. O extrato hidroalcoólico de folhas e galhos da *Arrabidaea chica* foi filtrado, evaporado sob pressão reduzida e liofilizado para uso posterior. Mitocôndrias de fígado de rato foram isoladas por centrifugação diferencial para os testes polarográficos e enzimáticos. O consumo de oxigênio de mitocôndrias intactas foi medido polarograficamente adicionando-se os seguintes substratos ao meio de incubação: succinato, β-hidroxi-butirato e ADP. Mitocôndrias rompidas por congelamento foram usadas para os ensaios das atividades enzimáticas ligadas à membrana: NADH-oxidase, succinato-oxidase e oxidação de TMPD-ascorbato. Microssomos foram isolados por centrifugação diferencial para a determinação da atividade glicose-6-fosfatase e ATPase. Fígados isolados de ratos alimentados ou em jejum de 24 horas foram perfundidos no sistema não-recirculante. Glicose, lactato e piruvato liberados no perfusado foram medidos através de procedimentos enzimáticos.

RESULTADOS — As principais alterações promovidas pelo extrato de *Arrabidaea chica* (0,25-1,25 mg/ml) em **mitocôndrias isoladas** foram:

a) estimulação do consumo de oxigênio na ausência de ADP (respiração do estado IV) e na presença do substrato FAD-dependente (succinato) na concentração mais alta (1,25 mg/ml);

b) redução da respiração do estado III e decréscimo progressivo da razão de controle respiratório (RC) com substratos NAD^+-dependente (β-hidroxibutirato) e FAD-dependente (succinato);

c) decréscimo das atividades NADH-oxidase e succinato-oxidase;

d) ativação da atividade ATPásica.

Em **microssomos isolados** o extrato causou inibição da atividade da glicose 6-fosfatase. A concentração média que produziu 50% de inibição (ID_{50}) foi igual a 0,69±0,14 mg/ml.

Nos experimentos de **perfusão de fígado**, o extrato de *Arrabidaea chica* foi infundido nas concentrações de 0,25, 0,50 e 1,0 mg/ml. Nesta faixa de concentração o extrato causou:

a) estímulo do consumo de oxigênio;

b) inibição da gliconeogênese a partir de lactato e piruvato no estado de jejum;

c) redução da liberação de glicose a partir do glicogênio endógeno.

O conteúdo hepático de glicose 6-fosfato foi aumentado e o nível de ATP foi diminuído na presença do extrato (1,0 mg/ml).

CONCLUSÕES — Os dados obtidos permitem concluir que o extrato de *A. chica* afeta o metabolismo energético hepático. A natureza das alterações indica que o extrato contém substâncias que atuam como agentes desacopladores, assim como inibidores de atividades enzimáticas ligadas à cadeia respiratória e da atividade glicose 6-fosfatase. A ação desacoplante pode ser inferida dos seus efeitos sobre a respiração mitocondrial e sobre a atividade ATPásica. Mais especificamente da estimulação do estado IV da respiração, do decréscimo do controle respiratório e do aumento da hidrólise de ATP em mitocôndrias intactas. Os efeitos do extrato sobre a cadeia respiratória podem ser devidos à inibição das atividades da NADH-oxidase e da succinato-oxidase. O principal efeito verificado em fígados perfundidos foi a inibição da produção de glicose além da estimulação do consumo de oxigênio. Estes fenômenos são geralmente causados por agentes desacoplantes. A inibição da glicose 6-fosfatase é a provável causa do aumento da concentração celular de

glicose 6-fosfato, fenômeno que, por sua vez, pode exercer considerável influência sobre várias vias metabólicas relacionadas.

Abstract

INTRODUCTION AND AIMS — The bignoniaceous *Arrabidaea chica*, known as carajuru, puca panga, chica or pariri is a liana growing in tropical America. Leaves of this plant are used as anti-inflammatory and adstringent agents as well as remedies for intestinal colic, sanguine diarrhoea, leucorrhoea, anaemia and leukaemia in the Amazon region.

A bioguided phytochemical study of *A. chica* leaves showed that carajurin, a 3-desoxyanthocyanidin is responsible for the transcription factor NF-kB inhibition involved in the anti-inflammatory activity of *A. chica* preparations. Anthocyanins, flavonoids, tannins and phytosterols have been identified in the extract prepared from leaves of the plant. Besides their antioxidant activities, several flavonoids like quercetin, can exert a variety of effects on liver metabolism. Its presence in *Arrabidaea chica* suggests, thus, that the extract could also be able to affect the hepatic metabolism. This possibility was investigated in the present work, in which the effects of leaves and stems extracts of *Arrabidaea chica* on the cellular energy metabolism were measured in isolated rat liver mitochondria and in the isolated perfused rat liver.

METHODS — Male Wistar rats weighing 200 to 250 g were used in all experiments. The hydroalcoholic extract of *Arrabidaea chica* leaves and stems was filtered, evaporated under reduced pressure, and lyophilized until further use. Rat liver mitochondria were isolated by differential centrifugation for polarographic and enzymatic assays. Oxygen consumption by intact mitochondria was measured polarographically adding the following substrates to the incubation medium: β-hydroxybutyrate, succinate and ADP. Freeze-thawing disrupted mitochondria were used for assaying membrane-bound enzymatic activities: NADH oxidase, succinate oxidase and TMPD-ascorbate oxidation. Microsomes were isolated by differential centrifugation for the determination of the glucose-6-phosphatase and the ATPase activities. Livers isolated from fed or 24-hours fasted rats were perfused in the non-recirculating mode. Glucose, lactate and pyruvate released into the perfusate were measured by standard enzymatic methods.

RESULTS — The main changes produced by the extract of *A. chica* (0.25-1.25 mg/ml) in isolated mitochondria incubations were:

a) stimulation of oxygen consumption in the absence of ADP (state IV respiration) and in the presence of a FAD-dependent substrate (succinate) at the highest concentration (1.25 mg/ml);

b) reduction of state III respiration and progressive decrease of the respiratory control ratios with both NAD^+-dependent (β-hydroxybutyrate) and FAD-dependent (succinate) substrates ;

c) decrease of both NADH oxidase and succinate oxidase activities;

d) activation of the ATPase activity.

In isolated microsomes the extract caused inhibition of the glucose 6-phosphatase activity. The mean concentration producing 50% inhibition (ID_{50}) was 0.69±0.14 mg/ml.

In the liver perfusion experiments, the *Arrabidaea chica* extract was infused at the concentrations of 0.25, 0.50 and 1.0 mg/ml. In this range of concentrations the extract caused:

a) stimulation of oxygen consumption;

b) inhibition of gluconeogenesis from lactate plus pyruvate in the fasted state;

c) reduction of glucose release from endogenous glycogen.

The hepatic content of glucose-6-phosphate was increased and the level of ATP was decreased in the presence of the extract (1.0 mg/ml).

CONCLUSIONS — The data allow to conclude that the *A. chica* extract affects the hepatic energy metabolism. The nature of the alterations indicates that the extract contains substances that act as uncouplers of oxidative phosphorylation, as inhibitors of enzymatic activities linked to the respiratory chain and of the glucose 6-phosphatase. The uncoupling action can be inferred from the extract effects on the mitochondrial respiration and on the ATPase activity. More specifically, from the stimulation of state IV respiration, the decrease of the respiratory control and from the increase in ATP hydrolysis in intact mitochondria. The effects of the extract on the respiratory chain were possibly caused by the inhibition of the NADH-oxidase and succinate-oxidase activities. The main effects found in the perfused liver were inhibition of glucose production and stimulation of oxygen consumption. These phenomena are generally caused by uncoupling agents. The inhibition of the glucose 6-phosphatase activity is the probable cause for the increased cellular concentrations of glucose-6-phosphate, a phenomenon which, in turn, could be exerting a considerable influence on several related metabolic pathways.

Effects of the *Arrabidaea chica* extract on energy metabolism in the rat liver

Alessandra Salvador de Souza, Clairce Luzia Salgueiro Pagadigorria, Emy Luiza Ishii-Iwamoto, Adelar Bracht, Diógenes Aparício Garcia Cortez, Gislaine Trassi and Nair Seiko Yamamoto

Laboratory of Liver Metabolism, University of Maringá, Brazil

Adressee for correspondence:

Nair Seiko Yamamoto
Email: nsyamamoto@uem.br
Fax: 55-44-32614896
Phone: 55-44-32614896
Laboratory of Liver Metabolism
Department of Biochemistry
University of Maringá
87020900 Maringá, Brazil

Abstract

The bignoniaceous *Arrabidaea chica,* popularly known as carajuru, puca panga, chica or pariri is very common in the Amazon region. Leaves of this plant are widely used as anti-inflammatory and adstringent agents as well as a remedies for intestinal colic, sanguine diarrhoea, leucorrhoea, anaemia and leukaemia. The action of the extract from leaves and stems of *Arrabidaea chica* on hepatic energy metabolism was investigated in the perfused rat liver and isolated mitochondria. In isolated mitochondria the *Arrabidaea chica* extract (0.25-1.25 mg/ml) decreased the respiratory coefficient with the substrates β-hydroxybutyrate and succinate. The activities of succinate-oxidase and NADH-oxidase were inhibited and the ATPase of intact mitochondria was stimulated. The extract also inhibited the glucose-6-phosphatase of isolated microsomes. The cellular content of glucose-6-phosphate was increased, while the level of ATP was reduced. In perfused livers the extract (0.25-1.0 mg/ml) caused stimulation of oxygen consumption, inhibition of gluconeogenesis from lactate and pyruvate and reduction of glucose release from endogenous glycogen. The results of this investigation suggest that the inhibitory effect of the extract on hepatic glucose production is mainly related to its actions on the mitochondrial energy metabolism.

Key words: *Arrabidaea chica*; Liver; Mitochondria; Metabolism; Bignoniaceae.

Introduction

The bignoniaceous *Arrabidaea chica* (Bonpl.) B. Verl. (syn. *Bignonia chica* (Bonpl.)) with the common names carajuru, puca panga, chica or pariri is a liana growing in tropical America (Barroso, 1986; Zorn et al., 2001). Although *A. chica* has a limited occurrence in the south of Brazil, Paraguai and northeast of Argentina, *A. chica* is very common in the Amazon region and leaves of this plant have been widely used as anti-inflammatory and adstringent agents as well as remedies for intestinal colic, sanguine diarrhoea, leucorrhoea, anaemia and leukaemia (Takemura et al., 1995).

In former times Indians of the Rio Meta and the Orinoco prepared a red pigment from the leaves for tattoos. It was found that the red colour was related to the 3-desoxyanthocyanidin named carajurin (6,7-dihydroxy-5,4'-dimethoxy-flavylium). A bioguided phytochemical study of *A. chica* leaves showed that carajurin is responsible for the transcription factor NF-kB inhibition by which the anti-inflammatory activity of *A. chica* preparations have been explained (Zorn et al., 2001). Additional studies of the leaves of *A. chica* revealed the presence of anthocyanins, flavonoids, tannins and phytosterols in addition to the 7,4'–dihidroxy-5-methoxyflavone (Takemura et al., 1995; Scogin, 1980).

As mentioned before, preparations of the crude plant are used for treating several diseases, but biochemical and physiological studies have not yet been carried out. However, the presence of flavonoids in the extract from leaves of *Arrabidaea chica* strongly suggest that the extract may act on liver cell energy metabolism, because several flavonoids, like quercetin, can exert a variety of effects on liver metabolism (Gasparin et al., 2003[a,b]). The present work was thus undertaken to investigate

possible actions of the extract on glycogen and glucose metabolism in perfused rat livers and on the respiratory activity and several membrane-bound enzymatic activities in isolated mitochondria and microsomes.

Materials and methods

Plant material

Leaves and stems of *Arrabidaea chica* were collected in April 2004, near Colorado do Oeste (Rondônia, Brazil) and identified by Dr O. A. Guimarães of the Botany Department at the Federal University of Paraná-UFPR, Brazil. A voucher specimen is deposited at the Botanic Department Herbarium of the State University of Maringá-UEM, Brazil, under registration number SP 11428.

Extract preparation

Leaves and stems of *Arrabidaea chica* (220.4 g) were air dried at room temperature, powdered and extracted by maceration with an ethanol-water mixture (8:2) according to the method described by Prista et al. (1975). The hydroalcoholic extract was filtered, evaporated under reduced pressure and lyophilized yielding 83.35 g of crude extract (37.81%). The lyophilized extract of the leaves and stems of *Arrabidaea chica* was stored at −20 °C until further use.

Isolation of mitochondria

Rat liver mitochondria were isolated by differential centrifugation in a mannitol-sucrose medium, according to Voss et al. (1961). Freeze-thawing disrupted mitochondria were used for the assay of membrane-bound enzymes. Intact mitochondria were frozen in liquid nitrogen and thawed rapidly at 37°C. This procedure was repeated three times. The

resulting disrupted mitochondria were maintained at 0-4°C for use. Protein contents were measured using the method of Lowry et al. (1951). The standard was bovine serum albumin.

Determination of oxygen consumption, ADP/O ratio and respiratory control ratio (RC)

Oxygen consumption by intact mitochondria was measured polarographically using an incubation medium containing 5 mM potassium phosphate, 10 mM TRIS-HCl (pH 7.4), 0.2 mM EGTA, 10 mM potassium chloride, 250 mM mannitol and 50 mg% fatty acid-free bovine serum albumin. The following substances were added to the incubation medium when required: succinate (10 mM), β-hydroxy-butyrate (10 mM) and ADP (125 μM). The ADP/O ratio was calculated according to Chance and Williams (1955) and it represents the amount of ADP added to the incubation system divided by the amount of oxygen consumed during the active phase of respiration (state III respiration). The respiratory control ratio (RC) is given by the rate of active respiration which follows ADP addition divided by the rate of respiration after ADP exhaustion (state IV respiration) (Chance and Williams, 1955). In this and in other experiments with isolated mitochondria, the extracts were added to the incubation medium in the concentration range between 0.25 to 1.25 mg/ml. After preincubation with mitochon-dria for a period of two minutes, the reactions were initiated by the addition of specific substrates.

ATPase activity

The ATPase activity was assayed by measuring phosphate release (Pullman et al., 1960; Caparroz-Assef et al., 2001). For intact mito-

chondria the reaction medium contained: 200 mM sucrose, 10 mM TRIS-HCl (pH 7.4), 50 mM KCl and, when required, 100 μM 2,4-dinitrophenol. When freeze-thawing disrupted mitochondria were used as enzyme source, the medium contained: 20 mM TRIS-HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP and stopped, after 20 min of incubation at 37°C, by the addition of ice-cold 5% trichloroacetic acid. Phosphate was measured as described by Fiske and Subbarow (1925).

Mitochondrial membrane-bound enzymatic activities

Freeze-thawing disrupted mitochondria were used as enzyme source for assaying membrane-bound enzymatic activities. NADH-oxidase and succinate-oxidase activities were assayed polarographically using a 20 mM TRIS-HCl (pH 7.4) medium (Singer, 1974; Caparroz-Assef et al., 2001). A polarographic assay was also run with TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine) plus ascorbate as substrates. The reactions were started by the addition of 10 mM NADH, 10 mM succinate or 0.2 mM TMPD + 5 mM ascorbate.

Isolation of microsomes

Microsomes were isolated by differential centrifugation according to Mihara and Sato (1972). The pellet of the last 105000g centrifugation, containing the microsomal fraction, was suspended in cold medium (4 °C) containing 150 mM KCl and 10 mM Tris (pH 7.4). The volume was adjusted to achieve a protein concentration of 30 mg ml^{-1}. Protein content was measured using the method of Lowry et al. (1951). The standard was bovine serum albumin.

The glucose 6-phosphatase activity was measured at 37 °C in a medium containing 100 mM KCl, 20 mM Tris-HCl (pH 6.5), 10 mM

glucose 6-phosphate and 0.2-0.3 mg microsomal protein. The medium was supplemented with 5 mM $MgCl_2$ and 5 mM ATP to measure the ATPase activity. Phosphate release was measured according to the method of Fiske and Subbarow (1925).

Liver perfusion

Male Wistar rats (weighing 200-250 g) were used in all experiments. All experiments were done in accordance with the world-wide accepted ethical guidelines for animal experimentation. For the surgical procedure, the animals were anesthetized by i.p. injection of sodium pentobarbital (50 mg/kg). Haemoglobin-free, non-recirculating perfusion was undertaken as described elsewhere (Kelmer-Bracht et al., 1984; Bracht et al., 2003). The perfusion fluid was Krebs/Henseleit-bicarbonate buffer, pH 7.4 (Krebs and Henseleit, 1932), saturated with an oxygen/carbon dioxide mixture (95/5%). The fluid was pumped through a temperature-regulated (37°C) membrane oxygenator prior to entering the liver via a cannula inserted in the portal vein. The perfusion flow was constant in each individual experiment and it was adjusted between 30 and 35 ml min^{-1}, depending on the liver weight. Samples of the effluent perfusion fluid were collected at 2-minutes intervals and analyzed for their metabolite content. When glycogen catabolism was measured, the livers of fed rats were used in the experiments. Livers from 24-hours fasted rats were used for the measurement of gluconeogenesis. The *A. chica* extract was dissolved into the perfusion fluid.

Analytical

Lactate and pyruvate were measured by standard enzymatic methods, using lactate dehydrogenase (Gutmann and Wahlefeld, 1974; Czok and Lamprecht, 1974). Glucose was measured by an enzymatic-colorimetric method using glucose-oxidase (Bergmeyer and Bernt, 1974). The oxygen concentration in the venous perfusate was monitored continuously employing a teflon-shielded platinum electrode. Metabolic rates were calculated from the input-output differences and the total flow rates and were referred to the wet weight of the liver.

The hepatic contents of ATP and glucose 6-phosphate were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralized with K_2CO_3 and assayed by means of standard enzymatic procedures (Lamprecht and Trautschold, 1974; Lang and Michal, 1974).

Treatment of data

The statistical significance of the differences between parameters was evaluated by means of Student's *t* test or Student-Newman-Keuls test. The latter was applied after submitting the data to variance analysis. The results are mentioned in the text as the *p* values; $p < 0.05$ was adopted as a criterion of significance.

Results

Effect of the extract of *Arrabidaea chica* on oxygen consumption in isolated mitochondria

The effect of the extract of *Arrabidaea chica* on oxygen consumption by intact mitochondria was measured using NAD^+-dependent (β-hydroxybutyrate) and FAD-dependent (succinate) substrates in the presence of exogenously added ADP (state III respiration) or after ADP exhaustion (state IV respiration). The extract of the *Arrabidaea chica* was added to the incubation medium for final concentrations between 0.25 and 1.25 mg/ml as shown in Figure 1. The mitochondrial respiration driven by succinate in the presence of ADP (state III respiration) was decreased by the extract. Statistically the decreases were significant only at the concentrations of 1.0 and 1.25 mg/ml of the extract (Figure 1B). With β-hydroxybutyrate as substrate (Figure 1A) the state III respiration was not significantly affected up to the concentration of 1.25 mg/ml of the extract. At this highest concentration of the extract the state III respiration was decreased by 57% ($p < 0.01$). State IV respiration tended to increase with increasing extract concentrations, but statistical significance was lacking in the range up to 1.25 mg/ml. The respiration of mitochondria uncoupled with 2,4-dinitrophenol, however, was gradually inhibited by increasing concentrations of the extract with both, β-hydroxybutyrate and succinate as oxidative substrates as illustrated in the Figure 2.

Table 1 shows the effects of the *Arrabidaea chica* extract on the ADP/O ratios and the respiratory control ratios (RC). The respiratory control (RC) was progressively decreased by increasing concentrations of the extract with both substrates, β-hydroxybutyrate and succinate. With succinate as the substrate no respiratory control at all was found at

the concentration of 1.25 mg/ml. At this concentration, evidently, no ADP/O ratio could be evaluated, but at lower extract concentrations the ADP/O ratio was not significantly decreased.

Effects of the *Arrabidaea chica* extract on the activities of membrane-bound enzymes

The effects of several concentrations of the *Arrabidaea chica* extract on NADH-oxidase activity, succinate-oxidase activity, and TMPD-ascorbate oxidation were measured in disrupted mitochondria and the mean values are summarized in Figure 3. The extract promoted inhibition of both NADH-oxidase and succinate-oxidase activities in a dose-dependent manner. The mean concentration producing 50% inhibition (ID_{50}) of succinate-oxidase activity was 0.79±0.13 mg/ml. The maximal inhibition of the NADH-oxidase activity did not exceed 40%. TMPD-ascorbate oxidation was not significantly affected by the extract.

The effects of the *Arrabidaea chica* extract on the ATPase activity were measured in intact mitochondria either in the absence (coupled mitochondria) or in the presence of 2,4-dinitrophenol (uncoupled mitochondria), as shown in Figure 4. The ATPase activity of coupled mitochondria was increased in a dose-dependent manner; maximal stimulation of 525% (p=0.001) was achieved at an extract concentration of 1.0 mg/ml. When uncoupled mitochondria were used as the enzyme source, the ATPase activity was not significantly affected by the extract.

Effects of the *Arrabidaea chica* extract on the microsomal ATPase and glucose 6-phosphatase activities

The substantial stimulation of the mitochondrial ATPase activity raised the question about the possible effects of the extract on the

microsomal ATPase and glucose 6-phosphatase activities. The results illustrated in the Figure 5 reveals that the microsomal ATPase activity was not affected. Glucose-6-phosphatase activity, however, was inhibited in a dose-dependent manner by the extract. The mean concentration producing 50% inhibition (ID_{50}) of glucose-6-phosphatase activity was 0.69 ± 0.14 mg/ml.

Effects of the *Arrabidaea chica* extract on hepatic ATP and glucose 6-phosphate levels

Since the glucose 6-phosphatase activity was inhibited by the *Arrabidaea chica* extract, alterations in the glucose 6-phosphate levels can also be expected (Kelmer-Bracht et al., 2003). In the present work the levels of glucose 6-phosphate were measured before and after 30 minutes of 1.0 mg/ml extract infusion into perfused livers isolated from fed rats as shown in Table 2. The glucose 6-phosphate levels were increased by 65%. The levels of ATP which were measured under the same conditions, on the other hand, were reduced by 24%.

Effects of the *Arrabidaea chica* extract on glycogen catabolism and oxygen consumption in livers from fed rats

The actions of the *Arrabidaea chica* extract on oxygen consumption, glycogenolysis, glycolysis and gluconeogenesis from lactate and pyruvate were investigated in the perfused rat liver. Several experiments were undertaken in which the *Arrabidaea chica extract* was infused in the range between 0.25 and 1.0 mg/ml during 20 minutes. Figure 6A shows the results of representative experiments performed with livers from fed rats. These livers were perfused with substrate-free perfusion fluid, in an open system. Under these conditions, the livers release glucose, lactate and pyruvate as a result of glycogen catabolism

(Scholz and Bücher, 1965). As shown in Figure 6A, the infusion of 0.5 mg/ml of *Arrabidaea chica extract* at 10 min of perfusion produced decreases in glucose release. At the end of the infusion, glucose release was reduced by 22% (p = 0.003) when compared with the rates measured before the infusion of the extract. The extract also increased oxygen consumption (18%, p=0.002) and lactate production (18%, p=0.009). When the infusion of the *Arrabidaea chica* was interrupted at 30 min, the metabolic fluxes tended to return slowly to the rates before infusion (basal rates). Experiments like those represented in Figure 6A were repeated with several extract concentrations and the mean values of glucose and lactate release and oxygen uptake at the end of the infusion period are summarized in Figure 6B. The maximal stimulation of oxygen uptake, as well as the maximal inhibition of glucose release were obtained with the concentration of 0.50 mg/ml.

Effects of the *Arrabidaea chica* extract on oxygen consumption and gluconeogenesis in the livers from fasted rats

Glucose production from lactate and pyruvate was evaluated according to the protocol illustrated by Figure 7A in perfused livers from 24-h fasted rats. As expected, during the time period before lactate (2.0 mM) plus pyruvate (0.2 mM) infusion, glucose release was minimal because both the glycogen stores and the levels of endogenous gluconeogenic substrates are very low in livers from 24-hours fasted rats (Williamson and Brosnan, 1974). The addition of the exogenously supplied gluconeogenic substrates immediately increased glucose production and oxygen consumption which stabilized after 20 min of infusion. The introduction of 1.0 mg/ml of *Arrabidaea chica* extract increased further the oxygen consumption (15%, p=0.009). Glucose release, on the other hand, was diminished in the presence of the

extract. At the end of the infusion, glucose release was reduced by 52% (p = 0.014) when compared with the rates measured before the infusion of the extract. These effects were reversible, that is, when the infusion of the *Arrabidaea chica* extract was interrupted at 50 min, the metabolic fluxes tended to return slowly to the rates before extract infusion.

Figure 7B allows an evaluation of the changes caused by several concentrations of the *Arrabidaea chica* extract at the end of the infusion period on oxygen uptake and gluconeogenesis. The *Arrabidaea chica* extract was infused in the range of 0.25 to 1.0 mg/ml concentrations. Oxygen uptake was increased in a dose-dependent manner by the extract. Inhibition of gluconeogenesis was already evident at the concentration of 0.25 mg/ml, but increased considerably when the concentration of the extract was raised to 1.0 mg/ml.

Discussion

The results of the present work reveal that the extract of *Arrabidaea chica* is able to influence liver metabolism. The data obtained with isolated mitochondria showed that the extract inhibits the respiration coupled to ADP phosphorylation, reduces the respiratory control ratios and increases ATP hydrolysis of intact coupled mitochondria. These effects are characteristic of uncouplers such as 2,4-dinitrophenol (Hopfer et al., 1968; Hanstein, 1976) and non-steroidal anti-inflammatory drugs like piroxicam (Salgueiro-Pagadigorria et al., 1996) and diclofenac (Petrescu et al., 1997). It is thus probable that the extract also acts as an uncoupler of oxidative phosphorylation. This conclusion is corroborated by the absence of stimulation of the mitochondrial ATPase activity of 2,4-dinitrophenol uncoupled mitochondria.

In addition to the uncoupling action the extract is also able to affect electron flow as indicated by the inhibition of NADH and succinate oxidation in disrupted mitochondria. The exact site of this action cannot be inferred from the data. The extract could be acting at any point on the electron transport chain between complex I and cytochrome c. The existence of more than one site of action is equally possible. Probably the inhibitory effect of the extract on the electron transport chain prevented stimulatory actions on state IV respiration which usually occur in the presence of mitochondrial uncouplers (Hopfer et al., 1968; Hanstein, 1976).

The data of rat liver perfusion provided evidence that the extract is also active on intact cells. It should be remarked that the intact liver differs from the system containing isolated mitochondria. While in an incubation system with isolated mitochondria the extract interacts

directly with these organelles, in the case of the intact liver the access of any compound to the mitochondria is influenced by several factors including plasma membrane transport, biotransformation, binding to intracellular components, etc.

The addition of the extract to the perfused livers caused activation of oxygen consumption either in livers from fed or fasted rats. Oxygen consumption stimulation could be the consequence of the uncoupling of oxidative phosphorylation which reduces the net rates of ATP production. Moreover, the lower rates of glucose production observed in the presence of the extract in livers from fasted rats could be, partly at least, due to depressed gluconeogenesis, since this pathway is strictly dependent on mitochondrial ATP production. On the other hand, an uncoupling action should equally produce stimulation of glycogenolysis and glycolysis (Kemmelmeier and Bracht, 1989; Nascimento et al., 1992; Constantin et al., 1995; Constantin et al., 1995; Salgueiro-Pagadigorria et al., 1996). Lactate production was indeed slightly increased, suggesting some degree of glycolysis stimulation. However, glucose release from endogenous glycogen was not stimulated, actually it was decreased, whereas the opposite normally occurs when uncouplers are supplied to the liver cells (Kemmelmeier and Bracht, 1989; Nascimento et al., 1992; Constantin et al., 1995; Salgueiro-Pagadigorria et al., 1996). This observation suggests that the extract of *Arrabidaea chica* exerts other effects in addition to mitochondrial uncoupling. One such effect was actually detected in the present work, namely the glucose 6-phosphatase inhibition. Inhibition of this enzyme alone, however, should not in principle be responsible for the decreases in glucose release as indicated by experiments performed with livers from arthritic rats and other inhibitors of glucose 6-phosphatase. In arthritic rats, glucose production from glycerol is normal (Fedatto-Junior et al., 1999) in spite of a 60% reduced activity of glucose 6-

phosphatase in these rats (Kelmer-Bracht et al., 2003). Moreover, inhibition of the enzyme by isosteviol does not avoid a severalfold stimulation of glucose release derived from glycogenolysis stimulation, which is also caused by the former compound (Ishii and Bracht, 1987). This occurs because inhibition of glucose 6-phosphatase shifts the steady-state glucose 6-phosphate concentration to higher levels, restoring the flux through the enzyme to levels reflecting the steady-state rates of glucose 6-phosphate production (Ishii and Bracht, 1987). An increase in the cellular glucose 6-phosphate concentration was indeed observed in the present work with the *Arrabidaea chica* extract. The same occurs in livers from arthritic rats (Kelmer-Bracht et al., 2003) and in perfused livers when isosteviol is infused (Ishii and Bracht, 1987). It could be, however, that glycogenolysis stimulation by the *Arrabidaea chica* extract was not as intense as necessary for supplying the glycolytic route with the amounts of glucose 6-phosphate required to maintain the energy status of the hepatocytes. In this case, competition between glucose 6-phosphatase and glycolysis would not allow the glucose 6-phosphate concentration to raise sufficiently so as to overcome inhibition. This interpretation needs, thus, the combination of three events: (1) increased glycolysis as a compensatory phenomenon for the diminished efficiency of mitochondrial energy transduction, (2) inhibition of glucose 6-phosphatase and (3) some mechanism that does not allow glycogenolysis to be increased at the necessary levels. Events (1) and (2) were corroborated experimentally in the present work, but event (3) must be regarded here as a working hypothesis still to be confirmed by future experiments. Future experiments must also provide an answer regarding the nature of the active substances implicated in the metabolic effects of the *Arrabidaea chica extract*. Only the testing of pure substances will allow definitive conclusions about the precise molecular mechanisms of action of *Arrabidaea chica* preparations.

Acknowledgements

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and from the Programa Nacional de Núcleos de Excelência (PRONEX).

References

Barroso, G. M., 1986. Sistemática de Angiospermas do Brasil, Vol. 3, Viçosa, Brasil, pp.155-165.

Bergmeyer, H.U., Bernt, E., 1974. Determination of D-glucose with glucose oxidase and peroxidase. In: Bergmeyer H. U. (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, pp. 1205-1215.

Bracht, A., Ishii-Iwamoto, E. L., Kelmer-Bracht, A. M., 2003. O estudo do metabolismo no fígado em perfusão. In: Bracht, A., Ishii-Iwamoto, E. L. (Eds), Métodos de Laboratório em Bioquímica. Editora Manole Ltda., São Paulo, pp. 275-288.

Caparroz-Assef, S. M., Salgueiro-Pagadigorria, C. L., Amado, C. A., Bracht, A., Kelmer-Bracht, A. M., Ishii-Iwamoto, E. L., 2001. The uncoupling effect of the non-steroidal antiinflammatory drug nimesulide in liver mitochondria from adjuvant-induced arthritic rats. Cell Biochemistry and Function 19, 117-124.

Chance, B., Williams, G.R., 1955. A simple and rapid assay of oxidative phosphorylation. Nature 175, 1120-1121.

Constantin, J., Ishii-Iwamoto, E.L., Suzuki-Kemmelmeier, F., Yamamoto, N.S., Bracht, A., 1995. Bivascular liver perfusion in the anterograde and retrograde modes: zonation of the response to inhibitors of oxidative phosphorylation. Cell Biochemistry and Function 13, 201–209.

Czok, R., Lamprecht, W., 1974. Pyruvate, phosphoenolpyruvate and D-glycerate 2-phosphate. In: Bergmeyer H. U. (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, pp. 1446-1451.

Fedatto-Junior, Z., Ishii-Iwamoto, E.L., Bersani-Amado, C., Vicentini, G.E., D'Urso-Panerari, A., Bracht, A., Kelmer-Bracht, A.M., 1999. Gluconeogenesis in the liver of arthritic rats. Cell Biochemistry and Function 17, 271-278.

Fiske, C.H., Subbarow, Y., 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Gasparin, F. R. S., Salgueiro-Pagadigorria, C. L., Ishii-Iwamoto, E. L., Bracht, A. and Constantin, J., 2003a. Action of quercetin on glycogen catabolism in the rat liver. Xenobiotica, 33 (6), 587-602.

Gasparin, F. R. S., Salgueiro-Pagadigorria, C. L., Ishii-Iwamoto, E. L., Bracht, A. and Constantin, J., 2003b. Actions of quercetin on gluconeogenesis and glycolysis in rat liver. Xenobiotica, 33 (9), 903-911.

Gutmann, I., Wahlefeld, A.W., 1974. L(+) Lactate determination with lactate dehydrogenase and NAD^+. In: Bergmeyer H. U. (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, pp. 1464-1466.

Hanstein, W., 1976. Uncoupling of oxidative phosphorylation. Biochim. Biophys. Acta 456, 129-148.

Hopfer, U., Lehninger, A. L., Thompson, T. E., 1968. Protonic conductance across phospholipid bilayer membranes induced by uncouplers of oxidative phosphorylation. Proc. Natl. Acad. Sci. (USA) 59, 484-490.

Ishii, E.L., Bracht, A., 1987. Glucose release by the liver under conditions of reduced activity of glucose 6-phosphatase. Brazilian Journal of Medical and Bological Research 20, 837-843.

Kelmer-Bracht, A. M., Ishii, E. L., Andrade, P. V. M., Bracht, A.,1984. Construction of a liver perfusion apparatus for studies on metabolic regulation and mechanisms of drug action. Arquivos de Biologia e Tecnologia 27, 419-438.

Kelmer-Bracht, A.M., Barbosa-Santos, C.P., Ishii-Iwamoto, E.L., Broetto-Biazon, A.C., Bracht, A., 2003. Kinetic properties of the glucose 6-phosphatase of the liver from arthritic rats. Biochimica et Biophysica Acta 1638, 50-56.

Kemmelmeier, F.S., Bracht, A., 1989. Effects of the non-steroidal antiinflammatory mefenamic acid on energy metabolism in the perfused rat liver. Biochemical Pharmacology 38, 823–830.

Krebs, H. A., Henseleit, H., 1932. Untersuchungen tiber die Harnstoffbildung im Tier-körper. Z. Physiol. Chem. 210, 33.

Lamprecht, W. and Trautschold, I., 1974. Adenosine-5'-triphosphate. Determination with hexokinase and glucose-6-phosphate dehydrogenase. In H. U. Bergmeyer (ed). Methods of Enzymatic Analysis (New York: Academic Press), 2101-2110.

Lang, G. and Michal, G.1974. D-glucose-6-phosphate and D-fructose-6-phosphate. In H. U. Bergmeyer (ed). *Methods of Enzymatic Analysis* (New York: Academic Press), pp. 1238-1242.

Lowry, O.H., Rosebrough, N.J., Farr, A.C., Randal, R.L.J., 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Mihara, K., Sato, R., 1972. Partial purification of NADH-cytochrome b5 redutase from rabbit liver microsomes with detergents and its properties. Journal of Biochemistry 71, 725-735.

Nascimento, E.A., Kelmer-Bracht, A.M., Bracht, A., Ishii-Iwamoto, E.L., 1992. Activation of hepatic glycogenolysis by non-steroidal antiinflammatories is independent of Ca^{2+}. Pharmacology Communications 3, 129–138.

Petrescu, I.; Tarba, C., 1997. Uncoupling effects of diclofenac and aspirin in the perfused liver and isolated hepatic mitochondria of rat. Biochem. Biophys. Acta 1318: 385-404.

Prista, L. N.; Alves, A. C.; Morgado, R., 1975. Técnica Farmacêutica e Farmácia Galénica. v1. 2ª ed. Editora Fundação Calouste Gulbenkan, Lisboa, Portugal. 1211p.

Pullman, M.E., Penefski, H.S., Datta, A., Racker, E., 1960. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphate. Journal of Biological Chemistry 235, 3322–3329.

Salgueiro-Pagadigorria, C. L., Kelmer-Bracht, A. M., Bracht, A. and Ishii-Iwamoto, E. L., 1996. Effects of the nonsteroidal anti-inflammatory drug piroxicam on rat liver mitochondria. Comp. Biochem. Physiol. 113C, 85-91.

Scholz, R.Bücher, T., 1965. Hemoglobin-free perfusion of rat liver. In: Chance, B., Estabrook, R. W., Willianson, J. R. (Eds), Control of Energy Metabolism. Academic Press, New York, pp. 393-414.

Scogin, R., 1980. Anthocyanins of the bignoniacea. Biochemical Systematics and Ecology 8, 273-276.

Singer, T.P., 1974. Determination of activity of succinate, NADH, choline and glycerophosphate dehydrogenase. Methods of Biochemical Analysis 22, 125–175.

Takemura, O.S., Iinuma, M., Tosa, H., Miguel, O. G., Moreira, E. and Nozawa, Y., 1995. A flavone from leaves of *Arrabidaea chica f. cuprea.* Phytochemistry 38, 1299-1300.

Voss, D. O., Campelo, A. P., Bacila, M. 1961. The respiratory chain and the oxidative phosphorylation of rat brain mitochondria. Biochem. Biophys. Res. Commun. 4, 48-51.

Williamson, D. H., Brosnan J. T. 1974. Concentration of metabolites in animal tissues. In: Bergmeyer H. U. (Ed.), Methods of Enzymatic Analysis, Academic Press, New York, 2266-2302.

Zorn, B., Garcia-Piñeres A. J., Castro V., Murillo, R., Mora G. and Merfort I.,2001. 3-Desoxyantocyanidins from *Arrabidaea chica.* Phytochemistry 56 (8), 831-835.

Table 1

The action of the *Arrabidaea chica* extract on mitochondrial respiration driven by β-hydroxybutyrate and succinate in the presence and absence of exogenously added ADP. Mitochondria (0.25-2.5 mg/ml) were incubated in reaction medium as described in Materials and methods. Oxygen consumption was recorded polarographically. ADP (125 μmols) was added at appropriate times. The respiratory control coefficients (RC) and ADP/O ratios were calculated according to Chance and Williams (1955). $^{+}$ $p < 0.05$; ** $p < 0.01$; * $p < 0.001$, ANOVA with Newman-Keuls test.

	β-Hydroxybutyrate (n=6)		Succinate (n=6)	
Extract (mg/ml)	ADP/O	RC	ADP/O	RC
0.0	3.00 ± 0.01	6.26 ± 0.73	1.82 ± 0.07	5.67 ± 0.28
0.25	2.89 ± 0.30	5.07 ± 0.51	1.69 ± 0.08	4.28 ± 0.24**
0.50	2.74 ± 0.22	4.62 ± 0.23^{+}	1.72 ± 0.07	3.65 ± 0.24*
0.75	2.57 ± 0.25	3.97 ± 0.37**	1.83 ± 0.06	3.34 ± 0.16*
1.00	2.75 ± 0.17	3.07 ± 0.21*	1.85 ± 0.07	2.53 ± 0.14*
1.25	-	1.26 ± 0.17*	-	≈1.00*

Table 2

Adenine nucleotide and glucose-6-phosphate contents of livers from fed rats in the presence and absence of the *A. chica* extract. Isolated perfused livers from fed rats were freeze-clamped and extracted as described in Materials and methods. Assays were undertaken by standard enzymatic techniques. *p* refers to the Student's *t*-test. Error parameters are standard errors of the mean. * $p < 0.001$; ** $p < 0.01$; $^{+}$ $p < 0.05$.

Extract (mg/ml)	ATP μmol x (g liver)$^{-1}$	Glucose 6-phosphate μmol x (g liver)$^{-1}$
0	2.01 ± 0.05	0.20 ± 0.02
1.0	1.53 ± 0.06*	0.33 ± 0.02^{+}

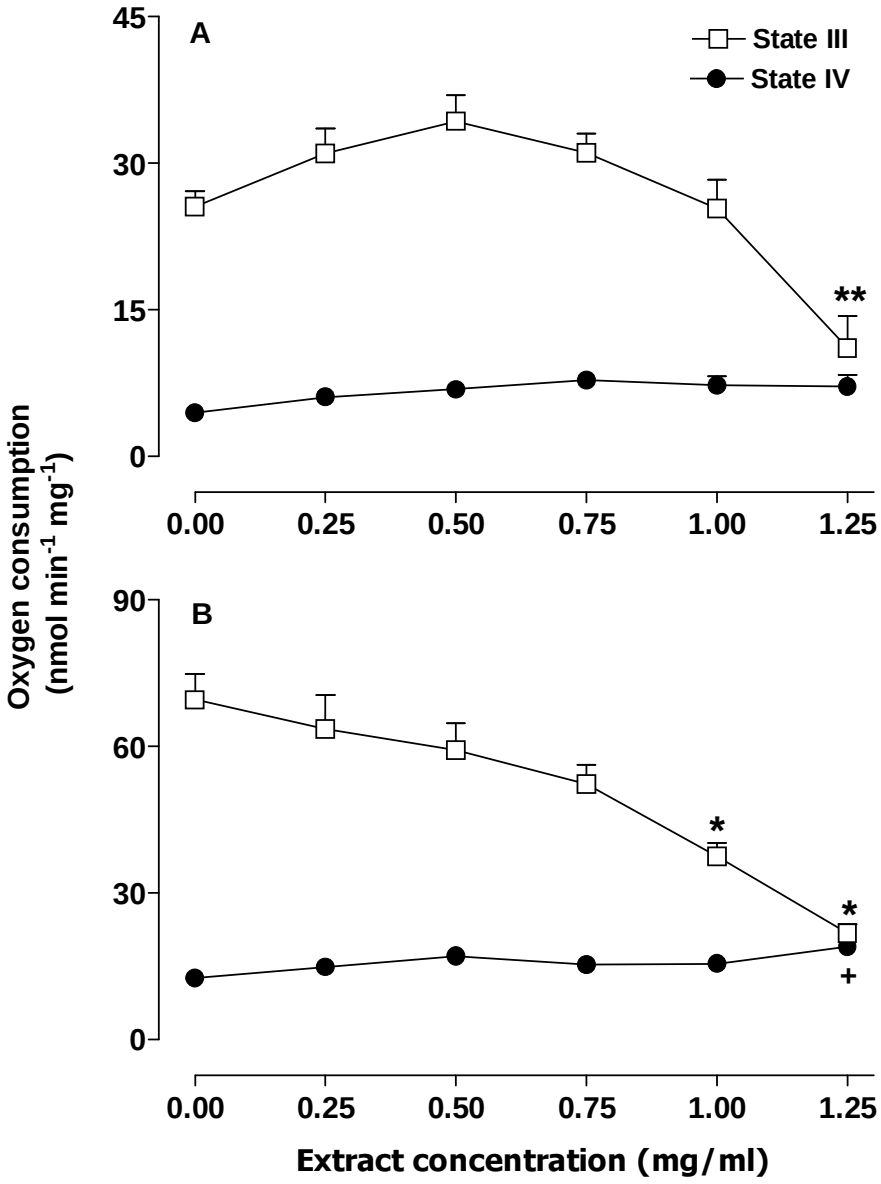

Fig. 1. Effects of the extract of *Arrabidaea chica* on the respiratory activity of isolated rat liver mitochondria. Mitochondria (0.5-1.0 mg/ml) were added to the reaction medium in the closed vessel of the oxygraph. The reaction was initiated by the addition of β-hydroxybutyrate (A) or succinate (B) and the oxygen consumption was followed polarographically for 5 min. After this time 0.125 mmol of ADP were added. Rates of oxygen consumption were computed from the slopes of the polarographic records. Each data point is the mean ± SEM of 6 independent experiments. $^{+}$ $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, ANOVA with Newman-Keuls test.

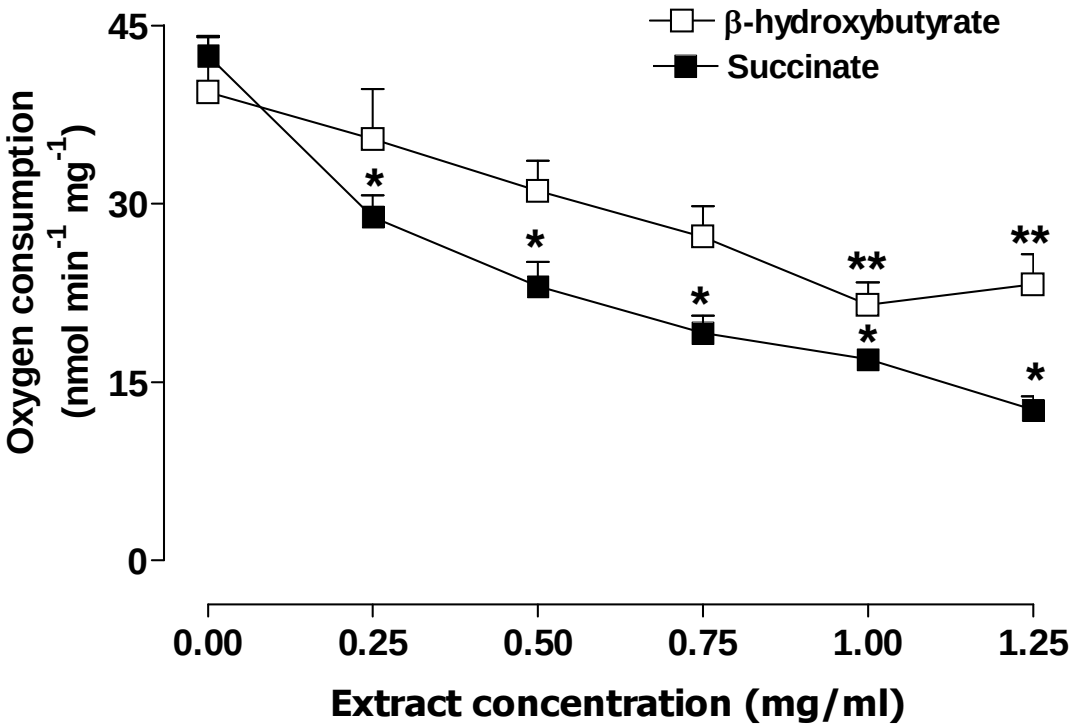

Fig. 2. Effects of the extract of *Arrabidaea chica* on the respiratory activity of isolated rat liver mitochondria in the presence of dinitrophenol. Mitochondria (0.5-1.0 mg/ml) were added to the reaction medium in the closed vessel of the oxygraph. The reaction was initiated by the addition of β-hydroxybutyrate or succinate and the oxygen consumption was followed polarographically for 5 min. Rates of oxygen consumption were computed from the slopes of the polarographic records. Each data point is the mean ± SEM of 6-9 independent experiments. ** $p < 0.01$, * $p < 0.001$, ANOVA with Newman-Keuls test.

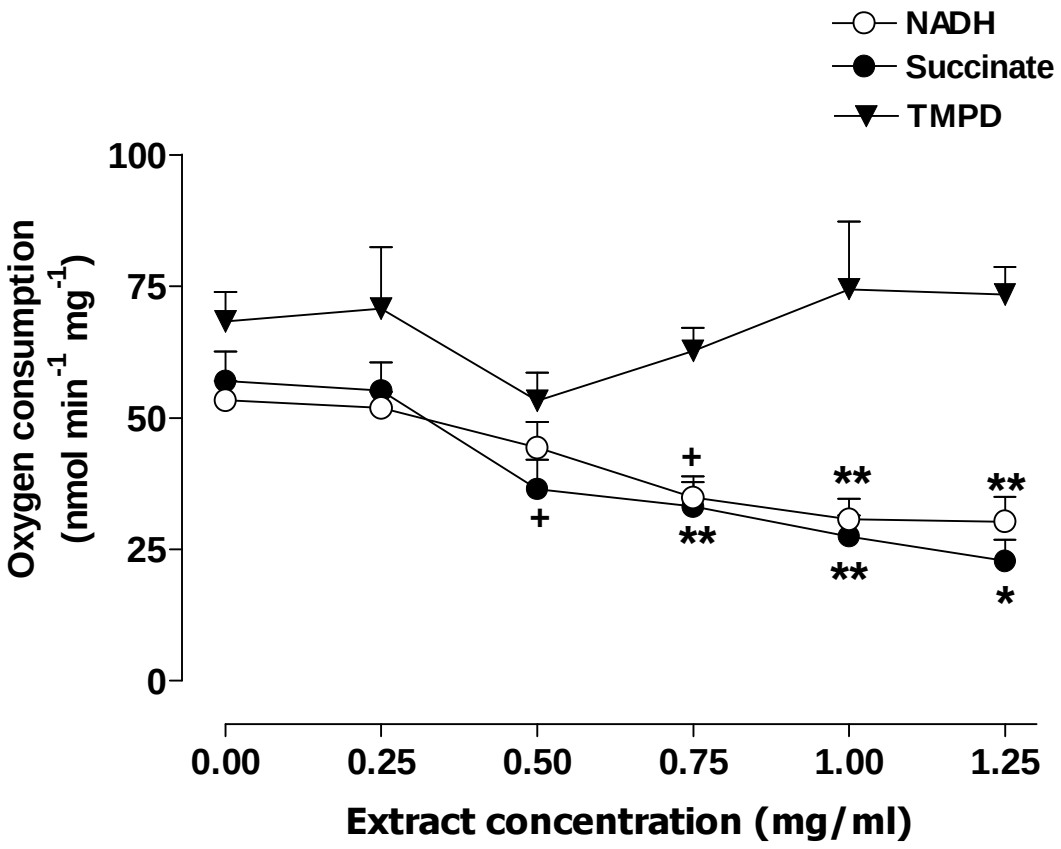

Fig. 3. Effects of the extract of *Arrabidaea chica* on several membrane-bound enzymatic activities in rat liver mitochondria. NADH-oxidase, succinate-oxidase activities and TMPD-ascorbate oxidation were measured with freeze-thawing disrupted mitochondria, incubated at 37º C in reaction medium as described in Materials and methods. Each data point is the mean ± SEM of 5 independent experiments. $^{+}$ $p < 0.05$, ** $p < 0.01$, * $p < 0.001$, ANOVA with Newman-Keuls test.

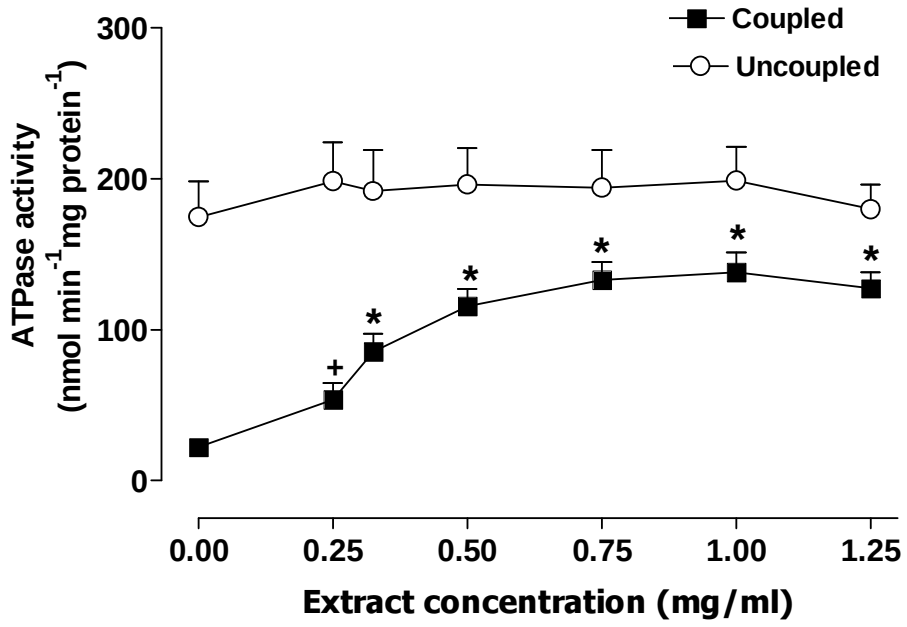

Fig. 4. Effects of the extract of *Arrabidaea chica* on the ATPase activity of coupled and uncoupled mitochondria. The mitochondria were incubated at 37° C in reaction medium as described in Materials and methods. Each assay point represents the mean of 5-8 independent experiments and the bars are SEM. $^{+}$ $p < 0.05$, * $p < 0.001$, ANOVA with Newman-Keuls test.

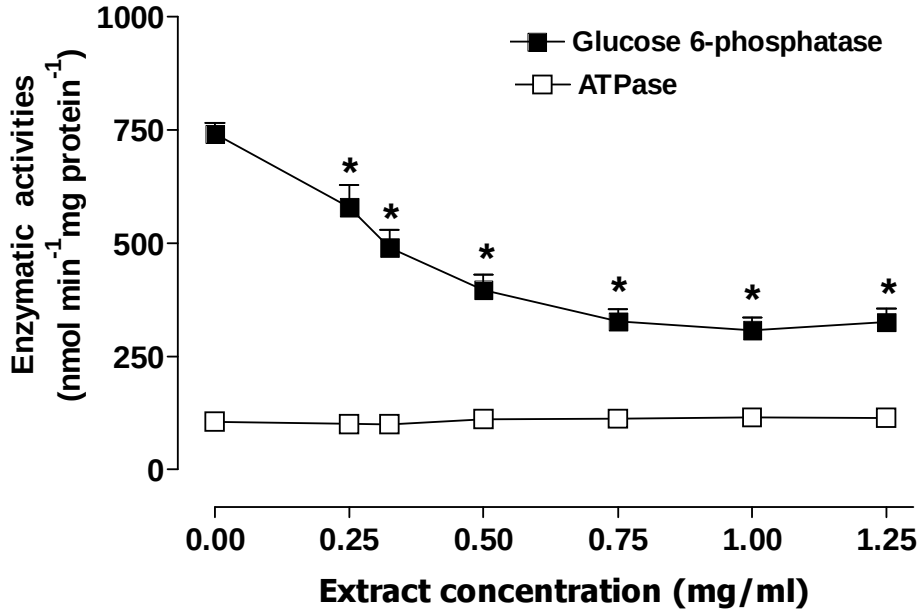

Fig. 5. Actions of the *Arrabidaea chica* extract on ATPase and glucose 6-phosphatase activities. Livers from fasted rats were homogenized and subjected to differential centrifugation as described in Materials and methods. The microsomal fraction was used for glucose 6-phosphatase and ATPase assays. Initial rates were measured at various extract concentrations. The data points are the means of five determinations. Bars are standard errors of the mean. * $p < 0.001$, ANOVA with Newman-Keuls test.

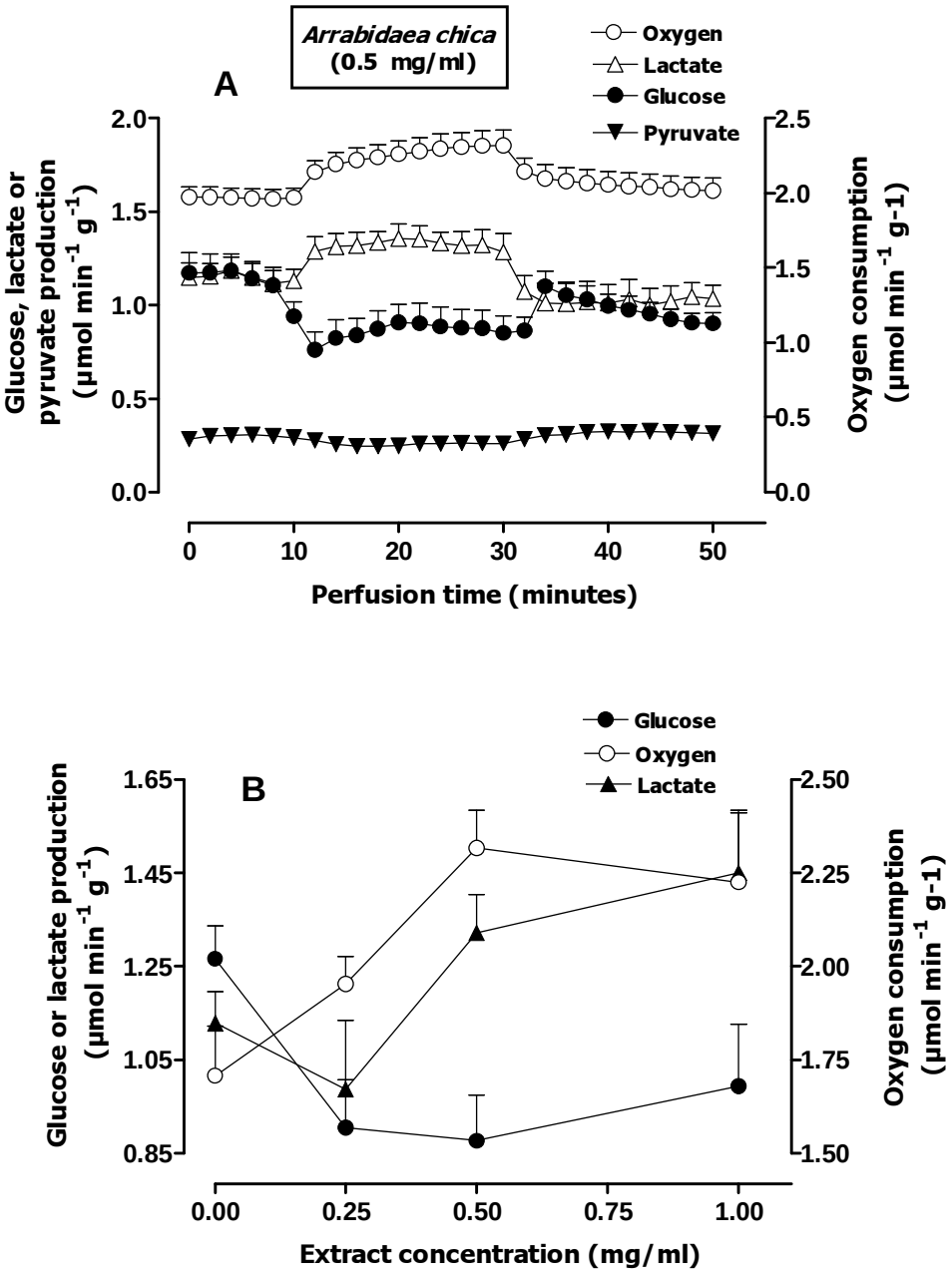

Fig. 6. Effects of the extract of *Arrabidaea chica* on metabolic fluxes in perfused livers isolated from fed rats. Panel A: Time course of the changes caused by the extract in glycogen catabolism and oxygen uptake. Livers from fed rats were perfused as described in Materials and methods. The extract (0.5 mg/ml) was infused at 10-30 min, as indicated by the horizontal bar. Oxygen consumption was followed polarographically. Panel B: Concentration dependence of the effects of the *A. chica* extract on glucose and lactate release and oxygen consumption. The experimental protocol was the same described for panel A. Each data point is the mean ± SEM of five experiments.

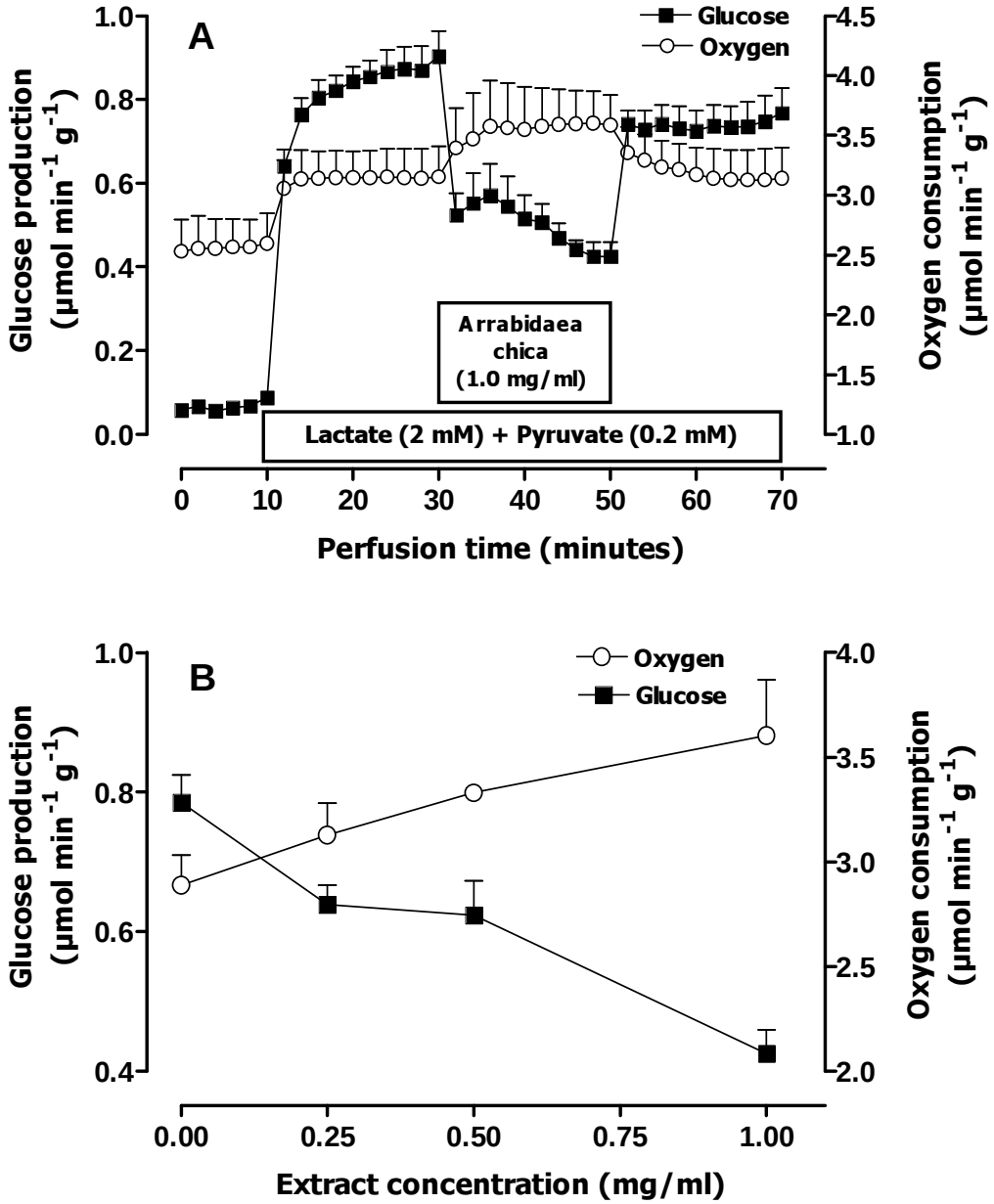

Fig. 7. The effects of the extract of *Arrabidaea chica* on metabolic fluxes in perfused livers isolated from fasted rats. Panel A: Time course of the changes caused by 1.0 mg/ml of extract in glucose production and oxygen uptake. Livers from fasted rats were perfused as described in Materials and methods. Lactate (2 mM) and pyruvate (0.2 mM) were infused at 10-70 min and the extract at 30-50 min as indicated by the horizontal bars. Panel B: Concentration dependence of the effects of the *A. chica* extract on oxygen uptake and gluconeogenesis. The experimental protocol was the same described for panel A. Each data point is the mean ± SEM of four experiments.

Livros Grátis

(http://www.livrosgratis.com.br)

Milhares de Livros para Download:

Baixar livros de Literatura
Baixar livros de Literatura de Cordel
Baixar livros de Literatura Infantil
Baixar livros de Matemática
Baixar livros de Medicina
Baixar livros de Medicina Veterinária
Baixar livros de Meio Ambiente
Baixar livros de Meteorologia
Baixar Monografias e TCC
Baixar livros Multidisciplinar
Baixar livros de Música
Baixar livros de Psicologia
Baixar livros de Química
Baixar livros de Saúde Coletiva
Baixar livros de Serviço Social
Baixar livros de Sociologia
Baixar livros de Teologia
Baixar livros de Trabalho
Baixar livros de Turismo