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# Metabolic effects of silibinin in the rat liver

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ABSTRACT

The flavonolignan silibinin, which is a mixture of two diastereoisomers, silybin A and silybin B, is a component of the extract obtained from the fruit and seeds of the variegated milk thistle (*Silybum marianum* (L.) Gaertn. (Asteraceae)), known as silymarin. Among the therapeutic properties credited to silibinin, its antihyperglycaemic action has been extensively explored. Silibinin is structurally related to the flavonoids quercetin and fisetin, which have been previously demonstrated to be very active on liver metabolic processes related to glycaemic regulation. The aim of the present work was to investigate the effects of silibinin on metabolic pathways responsible for the maintenance of glycaemia, particularly glycogenolysis and gluconeogenesis, in the perfused rat liver. The activities of some key enzymes in these pathways and on parameters of energy metabolism in isolated mitochondria were also examined. At a concentration range of 50–300 μM, silibinin inhibited gluconeogenesis in the fasted condition and inhibited glycogenolysis and glycolysis in the fed condition. The mechanisms by which silibinin exerted these actions were multiple and complex. It inhibited the activity of glucose 6-phosphatase, inhibited the pyruvate carrier, and reduced the efficiency of mitochondrial energy transduction. It can also act by reducing the supply of NADH for gluconeogenesis and mitochondria through its pro-oxidative actions. In general, the effects and the potency of silibinin were similar to those of quercetin and fisetin. However, silibinin exerted some distinct effects such as the inhibitory effect on oxygen consumption in the fed condition and a change in the energy status of the perfused livers. It can be concluded that the effects of silibinin on liver glucose metabolism may explain its antihyperglycaemic property. However, this effect was, in part, secondary to impairment in cellular energy metabolism, a finding that should be considered in its therapeutic usage.



## 1. Introduction

The flavonolignan silibinin is a 1:1 mixture of two diastereoisomeric compounds, silybin A and silybin B (Fig. 1). Silibinin is a semipurified, commercially available fraction of silymarin that is extracted from the fruit and seeds of the variegated milk thistle (*Silybum marianum* (L.) Gaertn. (Asteraceae)). Silymarin is a standardised extract composed of at least seven flavonolignans (silybin A, silybin B, isosilybin A, isosilybin B, silychristin, isosilychristin, and silydianin) and one flavonoid (taxifolin) that comprise 65–80% of milk thistle extract. The two diastereoisomers that comprise the semipurified mixture, silibinin, are the most abundant compounds in silymarin, but not the unique compounds that present biological activities [1].

The most extensively studied and disseminated property of silymarin is its hepatoprotective activity [2–6]. Several clinical studies have been performed to evaluate the efficacy of silymarin/silibinin to treat a range of liver and gallbladder disorders such as acute and chronic hepatitis, cirrhosis and toxin-induced hepatitis. Doses ranging from 240 to 800 mg/day for a period between 7 days and 6 years have been employed [5]. For instance, patients with chronic alcoholic liver disease who received an oral dose of 420 mg/day of silymarin for 6 months, had normalised serum levels of transaminases, bilirubin and gamma glutamyl transferase [7]. There is also a reported improvement in the liver histology of patients with alcoholic cirrhosis who received oral doses of 140 mg silibinin three times a day for 4 years [5]. However, in another study with patients presenting symptoms of acute clinical hepatitis, treatment with 420 mg/day of silymarin for 4 weeks was able to reduce the symptoms related to biliary retention, including dark urine, jaundice and scleras icterus, but not modify

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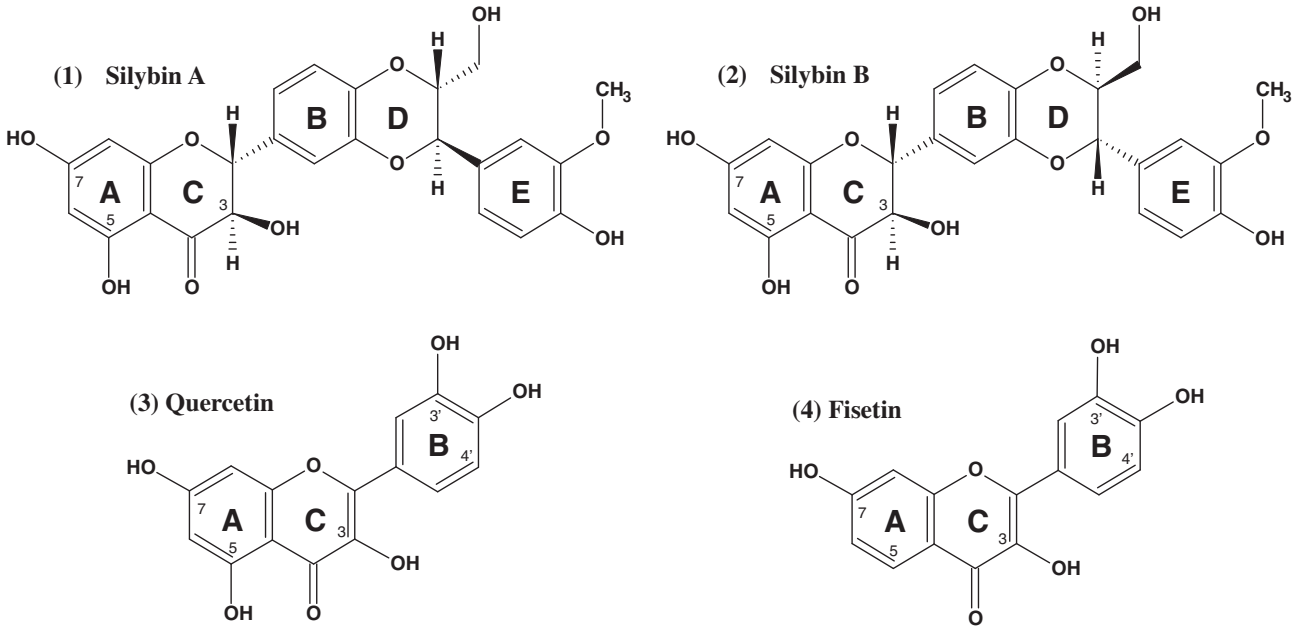


**Fig. 1.** Chemical structures of silibinin (1,2), quercetin (3) and fisetin (4). Silibinin is a mixture of two diastereoisomers: silybin A(1) and silybin B(2). In the figures, the rings of each compound are highlighted.

the levels of aminotransferases and direct bilirubin. In diabetic patients with cirrhosis [8] receiving an oral dose of 600 mg/day of silymarin, a decrease in fasting blood glucose and insulin levels was observed after 4 months of treatment.

The cellular mechanisms by which silymarin and silibinin exert their hepatoprotective effects have been suggested based on studies performed in cultures of hepatocytes and hepatomes or in animal experimental models [5]. They are believed to act as antioxidant, anti-inflammatory and anti-fibrotic agents. The anti-inflammatory effect seems to involve blocking the activation of intrahepatic Nuclear Factor kappa B (NF-kB), and consequent diminution of Tumour Necrosis Factor-alpha (TNF-α), Interferon (IFN-g), IL-2 and inducible Nitric Oxide Synthase (iNOS). The ability to act as cellular antioxidants, on the other hand, has been attributed to the many beneficial effects of silymarin and silibinin. Haddad et al. [9] for example, verified in an experimental model of NASH (non-alcoholic steatohepatitis) that treatment with 200 mg/kg of silibinin for 5 weeks caused an improvement of liver steatosis and inflammation and decreased the levels of plasma insulin and TNF-α. These effects were associated with decreased membrane lipid peroxidation, reduced free-radical release and restoration in the GSH levels.

Besides hepatoprotection for many disorders, the property of silymarin in reducing fasting glycaemia and insulin level have supported its use as an antihyperglycaemic compound [8,10–13]. A commercial preparation of silibinin, Legalon®, has been used in the treatment of diabetic patients, and there is evidence that it promotes a reduction in insulin resistance [8]. The potent hypoglycaemic and antihyperglycaemic activities of an aqueous extract of *S. marianum* have also been demonstrated in experimental animal models of diabetes [10,11].

Even so, the mechanisms by which silibinin exerts the antihyperglycaemic activity are not entirely understood. Besides a decrease in insulin resistance [8], there are suggestions that it may reduce the mitochondrial ROS generation that is implicated in the worsening of diabetes [13] and that it acts directly on liver metabolic pathways, inhibiting the activity of key enzymes in gluconeogenesis and glycolysis [12]. Actually, inhibition of glucose 6-phosphatase is an action shared among several other structurally related phenolic compounds, including the flavonoids quercetin [14] and fisetin [15].

We have already demonstrated that quercetin and fisetin are very active on liver metabolism, affecting several parameters that modulate glycaemia. Both compounds inhibit gluconeogenesis in livers from fasted rats. In livers from fed rats, quercetin stimulates glycogenolysis and inhibits glycolysis. Fisetin, conversely, inhibits glycogenolysis and glycolysis. Although, both quercetin and fisetin are very active on isolated mitochondria and affect various parameters of energy transduction [14–16], they do not cause significant changes in the concentrations of AMP, ADP and ATP in perfused livers. Another action common to both flavonoids is a pro-oxidative activity. They are able to oxidise NADH to shift the cellular conditions to a more oxidised state [17].

It must be stressed that quercetin and fisetin are classified as flavonoid compounds from the class of flavonols while silibinin is a flavonolignan, which is a dimer of phenylpropanoid. As shown in Fig. 1, flavonoids are usually characterised by a carbon skeleton $C_6$–$C_3$–$C_6$. While the first ring "A" is aromatic, the second ring "C" is a heterocyclic ring attached to oxygen and connected via a C–C bond to the third aromatic ring "B" [18]. The flavonolignan silibinin (mixture of silybin A and silybin B) has two additional rings, "D" and "E" (Fig. 1) [19]. Therefore, the question that arises is whether the structural differences between silibinin, which is a flavonolignan, and quercetin and fisetin, which are flavonols, exert important influences on their activities.

Given the therapeutic potential of silibinin and its current use by human patients, it is highly desirable to more extensively characterise its mode of action in biological systems. Thus, the present work was planned to examine the effects of silibinin on liver metabolic pathways, particularly glycogenolysis and gluconeogenesis, and its effects on related metabolic parameters, including the activities of some key enzymes of these pathways and on parameters of energy metabolism in isolated mitochondria. Besides the elucidation of the role of the liver in the silibinin-induced changes in glycaemia, this work will allow for a comparison of the effects of silibinin with other structurally related phenolic compounds, quercetin and fisetin, and thus contribute to the understanding of this class of natural substances with therapeutic properties. It must

be stressed that although the silibinin used in this work is described as a simple compound, it is in fact the mixture of two diastereoisomeric compounds, silybin A and silybin B.

## 2. Material and methods

### 2.1. Materials

Silibinin that was purchased from Sigma Chemical Co. (St. Louis, USA) is a mixture of two diastereoisomeric compounds (silybin A and silybin B). All enzymes and coenzymes used in the enzymatic assays were from Sigma Chemical Co. (St. Louis, USA). Sodium [$^{14}$C] bicarbonate (specific activity of 58 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals were of the best available grade.

### 2.2. Animals

Male Wistar rats weighing 180–220 g and fed *ad libitum* with a standard laboratory diet (Nuvilab CR-1®) were used in all experiments. In some experimental protocols, the rats were fasted for 24 h before the surgical removal of the liver. All experiments were performed in accordance with the internationally accepted recommendations for the care and use of animals.

### 2.3. Liver perfusion

The liver perfusion apparatus was built in the workshops of the University of Maringá. For the surgical procedure, the animals were anaesthetised by an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The haemoglobin-free, non-recirculating perfusion was performed according to the technique described by Scholz and Bücher [20]. After cannulation of the portal and cava veins, the liver was positioned in a plexiglass chamber. Flow was maintained constant by a peristaltic pump (Miniplus 3®, Gilson, France) and was adjusted to between 30 and 35 mL/min, depending on the liver weight. The perfusion fluid was a Krebs/Henseleit-bicarbonate buffer (pH 7.4) that was saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with a simultaneous temperature adjustment for 37 °C. The composition of the Krebs/Henseleit-bicarbonate buffer is the following: 115 mM NaCl, 25 mM $NaHCO_3$, 5.8 mM KCl, 1.2 mM $Na_2SO_4$, 1.18 mM $MgCl_2$, 1.2 mM $NaH_2PO_4$, and 2.5 mM $CaCl_2$. Livers from rats that were fasted for 24 h were used in the measurements for gluconeogenesis, and the livers of fed rats were used in the measurements for glycogen breakdown. Silibinin was dissolved in the perfusion fluid, and its solubilisation was achieved by the simultaneous addition of an equivalent amount of 1.0 M NaOH.

### 2.4. Analytical assays

Samples of the effluent perfusion fluid were collected at 2-min intervals and analysed for their metabolite content. Lactate and pyruvate were assayed by means of standard enzymatic procedures using lactate dehydrogenase [21,22]. Interference by silibinin (absorbance at 340 nm) was excluded by running blanks. Glucose was also assayed by means of standard enzymatic procedures [23]. The oxygen concentration in the outflowing perfusate was continuously monitored by employing a Teflon-shielded platinum electrode adequately positioned in a plexiglass chamber where the perfusate exited [24]. Metabolic rates were calculated from the differences between the input and output and the total flow rates and were analysed in reference to the wet weights of the livers. The hepatic contents of the adenine nucleotides were measured after freeze-clamping the perfused liver with liquid nitrogen. The freeze-clamped livers were extracted with perchloric acid. The extract was neutralised with $K_2CO_3$ and AMP, ADP, and ATP were assayed by means of high-performance liquid chromatography (HPLC). The HPLC system (Shimadzu, Japan) consisted of a system controller (SCL-10AVP), two pumps (model LC10ADVP), a column oven (model CTO-10AVP) and a UV–Vis detector (model SPD-10AV). A reversed-phase C18 HRC-ODS column (5 μm, 150 × 6 mm i.d., Shimadzu) protected with a GHRC-ODS precolumn (5 μm, 10 × 4 mm i.d., Shimadzu) was used with a gradient from reversed-phase 0.044 mol/L phosphate buffer solution, pH 6.0, to 0.044 mol/L phosphate buffer solution plus methanol (1.1), pH 7.0. In percent methanol, the gradient was the following: at 0 min, 0%; at 2.5 min, 0.5%; at 5 min, 3%; at 7 min, 5%; at 8 min, 12%; at 10 min, 15%; at 12 min, 20%; at 20 min, 28%; and at 30 min, 0%. The temperature was kept at 35 °C, and the injection volume was 20 μL with a flow rate of 0.8 mL/min. Monitoring was performed spectrophotometrically at 254 nm. Identification of the peaks of the investigated compounds was carried out by a comparison of their retention times with those obtained by injecting standards under the same conditions. The concentrations of the compounds were calculated by means of the regression parameters obtained from the calibration curves. The calibration curves were constructed by separating chromatographically standard solutions of the compounds. Linear relationships were obtained between the concentrations and the areas under the elution curves.

### 2.5. Experiments with microsomes

Microsomes from livers of 24 h-fasted rats were isolated by differential centrifugation according to Mihara and Sato [25]. Rats were decapitated, and their livers were cut into small pieces with scissors and suspended in cold isolation medium (4 °C) containing 150 mM KCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM Tris–HCl (pH 7.4). After the suspension in 10 volumes of the isolation medium, the tissue was homogenised with a Dounce homogeniser. The homogenate was filtered through gauze and centrifuged at 2550*g* for 10 min in a refrigerated centrifuge. The supernatant was again centrifuged in two steps at 7100 and 12400*g* for 10 min. The supernatant of the last centrifugation was collected and centrifuged at 105,000*g* for 1 h. The pellet containing the microsomal fraction was suspended in cold isolation medium at a final protein concentration of 20 mg/mL. The protein content of the microsomes was measured according to the method of Lowry et al. [26] using bovine serum albumin as a standard. The glucose 6-phosphatase activity was measured in a medium containing 100 mM KCl, 20 mM Tris–HCl (pH 6.5), 15 mM glucose 6-phosphate and 0.2 mg protein/mL. The D-fructose 1,6-bisphosphatase activity was assayed using the supernatant of the 105,000*g* centrifugation according to the procedure described by Mendicino et al. [27]. The reaction mixture contained 100 mM Tris–HCl (pH 8.0), 12 mM $MgCl_2$, 5 mM cysteine, 1 mM D-fructose 1,6-bisphosphate and 1 mg protein/mL. Silibinin (100–300 μM) was added to the incubation medium as a solution in dimethylformamide (0.1 M). After a 20 min incubation at 37 °C, the reaction was stopped by the addition of one volume of 5% trichloroacetic acid and phosphate release was measured according to the method of Fiske and Subbarow [28].

### 2.6. Mitochondria isolation

Fed rats were decapitated, and their livers were removed immediately and cut into small pieces. These fragments were suspended in a medium containing 0.2 M mannitol, 75 mM sucrose, 2.0 mM Tris-HCl (pH 7.4), 0.2 mM EGTA (ethylene glycol-bis

(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid), 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 50 mg% (w/v) fatty acid-free bovine serum albumin. Homogenisation was carried out in the same medium by means of a Dounce homogeniser. After homogenisation, the mitochondria were isolated by differential centrifugation according Voss et al. [29] using a sucrose-mannitol isolation medium and suspended in the same medium, which was kept at 0–4 °C.

### 2.7. Determination of mitochondrial oxygen consumption, ADP/O ratio and respiratory control ratio (RC)

Oxygen consumption by coupled isolated mitochondria was measured polarographically using a Teflon-shielded platinum electrode [24]. Intact mitochondria (2 mg protein/mL) were incubated in the closed Oxygraph chamber in a medium containing 250 mM mannitol, 10 mM KCl, 10 mM Tris–HCl (pH 7.4), 0.2 mM EGTA, 5 mM potassium phosphate, and 50 mg% fatty acid free bovine serum albumin. Silibinin (100−300 μM) was added to the incubation medium as a solution in dimethylformamide (0.1 M). Succinate (10 mM) and α-ketoglutarate (10 mM) were used as substrates. ADP, at a final concentration of 125 μM, was added at the appropriate times. Rates of oxygen consumption were computed from the slopes of the recorded tracings and expressed as nmol $min^{-1}$ mg $protein^{-1}$. The respiratory control ratio (RC) and the ADP/O ratio were calculated according to Chance and Williams [30]. The protein content of the mitochondrial suspensions was measured by means of the method described by Lowry et al. [26] using bovine serum albumin as a standard.

### 2.8. Pyruvate carboxylase assay

The pyruvate carboxylase activity of intact mitochondria was assayed by measuring the incorporation of $^{14}C$ from [$^{14}C$]$NaHCO_3$ into components of the tricarboxylic acid cycle. The incubation medium contained 5 mM sodium pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 120 mM KCl, 10 mM HEPES (pH 7.5), and 3 mg protein/mL [31]. The reaction was initiated by introducing 15 mM [$^{14}C$]$NaHCO_3$ (0.25 μCi). After 10 min of incubation at 37 °C, the reaction was arrested by the addition of 0.5 volume of 2 M perchloric acid. After a 5-min expulsion of the remaining [$^{14}C$]$NaHCO_3$, aliquots were taken for counting the acid-stable incorporation of radioactivity. The pyruvate carboxylase of disrupted mitochondria was measured using a medium able to generate steady-state concentrations of acetyl-CoA, as originally described by Henning et al. [32]. Rat liver mitochondria, isolated as described above, were disrupted by successive freezing and thawing procedures using liquid nitrogen. The incubation medium contained 3 mg protein/mL of disrupted mitochondria, 5 mM sodium pyruvate, 12.5 mM $MgCl_2$, 2.5 mM potassium phosphate, 0.3 M sucrose, 1 mM EDTA (ethylenediaminetetraacetate), 5 mM tris(hydroxymethyl) aminomethane (TRIS, pH 7.5), 0.5 mM lithium coenzyme A, 5 mM adenosine triphosphate, 1.1 mM acetyl phosphate, 1 unit/mL phosphotransacetylase and 1 unit/mL citrate synthase. The reaction was initiated by introducing 15 mM [$^{14}C$]$NaHCO_3$ (0.25 μCi). After a 10 min incubation at 37 °C, the reaction was arrested by the addition of 0.5 volume of 2 M perchloric acid. After expulsion of the remaining [$^{14}C$]$NaHCO_3$ for 5 min, aliquots were taken for counting the acid-stable incorporation of radioactivity. The incorporated radioactivity in both incubations, intact and disrupted mitochondria, was expressed as nmol $min^{-1}$ mg $protein^{-1}$. The scintillation solution for counting $^{14}C$ was composed of toluene:Triton X-100® (1.5:0.5), 10 g/L 1.5-diphenyloxazole with 0.4 g/L 2,2-*p*-phenyl-bis-5-pheny-leneoxazole. The protein content of all experiments was measured by means of the method described by Lowry et al. [26] using bovine serum albumin as a standard.

### 2.9. Membrane-bound enzymatic activities

Rat liver mitochondria, isolated as described above, were disrupted by successive freezing and thawing procedures using liquid nitrogen and used as an 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. A polarographic assay was also run with TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) with ascorbate as substrates. The reactions were started by the addition of 1.0 mM NADH, 10 mM succinate, or 0.2 mM TMPD with 5 mM ascorbate [33].

### 2.10. ATPase activity

The mitochondrial ATPase activity was assayed by measuring the phosphate release according to Pullman et al. [34]. When intact mitochondria were used as an enzyme source, the reaction medium contained 200 mM sucrose, 10 mM Tris–HCl (pH 7.4), 50 mM KCl, 0.2 mM EGTA, and, when required, 100 μM 2,4-dinitrophenol. For incubation with the disrupted mitochondria, the medium contained 20 mM Tris–HCl (pH 7.4). The reaction was started by the addition of 5 mM ATP, incubated for 20 min at 37 °C and stopped by the addition of ice-cold 5% trichloroacetic acid. Phosphate was measured as described by Fiske and Subbarow [28]. When coupled mitochondria were assayed, around 1 mg/mL of mitochondrial protein was used as an enzyme source, and when uncoupled or disrupted mitochondria were assayed, around 0.5 mg/mL of mitochondrial protein was used. The protein content of all experiments was measured by the method described by Lowry et al. [26] using bovine serum albumin as a standard.

### 2.11. Measurement of NADH oxidation in a cell-free system

The action of silibinin on NADH oxidation in a cell-free system was measured according to the method of Chan et al. [35]. The reaction mixtures contained 0.1 M Tris–HCl/1.0 mM EDTA buffer (pH 7.4), 1.0–4.0 μM silibinin, 25 μM $H_2O_2$, 200 μM NADH, and 0.1 μM horseradish peroxidase (HRP) type VI-A. Reactions were started by the addition of 25 μM $H_2O_2$ and the oxidation of NADH was monitored at 340 nm using a spectrophotometer.

### 2.12. Treatment of data

The statistical significance of the differences between parameters obtained in the experiments was evaluated by means of Student's *t*-test or by a Newman–Keuls test after submitting the data to variance analysis according to context. The results are discussed in the text using $p$ values where $p < 0.05$ was the criterion used for significance.

## 3. Results

### 3.1. The effects of silibinin on gluconeogenesis from lactate and pyruvate and oxygen consumption in the livers of fasted rats

The first set of experiments was planned to evaluate the action of silibinin on the gluconeogenesis pathway. Livers from rats that were fasted 24 h were perfused with gluconeogenic substrates and with different concentrations of silibinin. The experimental protocol is illustrated in Fig. 2A. In this series, the substrates were a mixture of 2.0 mM lactate plus 0.2 mM pyruvate, which is close

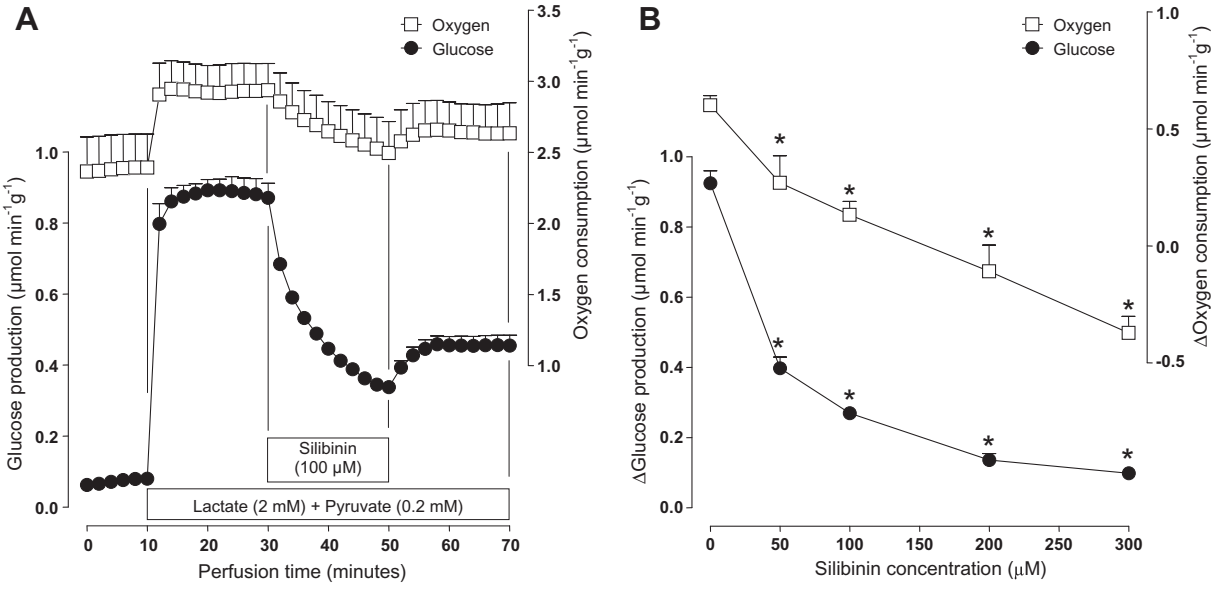


**Fig. 2.** The effects of silibinin on the metabolic fluxes from lactate and pyruvate in perfused livers isolated from fasted rats. Livers from 24 h fasted rats were perfused as described in the Section 2. Panel A: time courses of the changes caused by 100 μM silibinin in glucose production and oxygen consumption. Lactate and pyruvate were infused at 10–70 min and silibinin at 30–50 min, as indicated by the horizontal bars. Panel B: concentration-dependent effects of silibinin on glucose production and oxygen consumption. The control values (zero silibinin) correspond to the rates found in the presence of lactate plus pyruvate just before the onset of silibinin infusion (30 min of perfusion) minus the basal rates (i.e., before the onset of lactate and pyruvate infusion). Rates in the presence of lactate with pyruvate and silibinin were evaluated after 50 min of perfusion and also subtracted from the same basal rates. Each experimental point is the mean ± SEM of 3–5 experiments. Asterisks indicate a statistical significance in comparison to the control condition as revealed by variance analysis with *post-hoc* Newman–Keuls testing ($^*p < 0.05$).

to the physiological condition (Fig. 2A). Samples were collected in 2-min intervals for a total of 70 min of perfusion to measure glucose release. The oxygen consumption was monitored during the full experimental time. In the first 10 min, only Krebs-Henseleit buffer was infused, and the amounts of glucose released were very small due to a low content of hepatic glycogen. Under this condition, the oxygen consumption was maintained by endogenous fatty acid oxidation. When 2 mM lactate with 0.2 mM pyruvate were infused, there was a substantial increase in both glucose production and oxygen consumption. The infusion of 100 μM silibinin at the 30 min time period caused a progressive decrease in glucose production and in oxygen consumption, as shown in Fig. 2A. When silibinin infusion was stopped at 50 min of perfusion, there was only a partial recovery of the glucose production and oxygen consumption and reached values well below those found before the silibinin infusion.

Fig. 2B shows the results of four sets of experiments like those illustrated in Fig. 2A, in which the concentration of silibinin was varied (50, 100, 200 and 300 μM). The curves represent the concentration-dependent effects of silibinin on glucose release and on oxygen consumption. The control values correspond to the average rates found in the presence of 2 mM lactate with 0.2 mM pyruvate just before the silibinin infusion and minus the basal rates (first 10 min of perfusion time). The values for each concentration of silibinin were calculated as the differences between the control values (before silibinin infusion) and the values at the end of the silibinin infusion period. The glucose release was inhibited 89% by 300 μM silibinin, but the inhibition was already significant at the 50 μM concentration (57% inhibition). Oxygen consumption was also significantly inhibited; a 55% and 78% inhibition was found in the presence of 50 μM and 100 μM silibinin, respectively. At higher concentrations, the oxygen consumption was reduced to values below the basal ones (before the gluconeogenic substrates infusion). At 300 μM concentration, for example, oxygen consumption was 62% lower than the basal rates.

### *3.2. The effects of silibinin on oxygen consumption and gluconeogenesis from lactate or pyruvate in the livers of fasted rats*

The inhibition of silibinin on the gluconeogenesis pathway from 2 mM lactate with 0.2 mM pyruvate could be explain by at least three mechanisms: (1) a direct action on the enzyme lactate dehydrogenase which interconverts lactate to pyruvate; (2) a change in the cytosolic redox potential or (3) an interference at a metabolic step that is common for lactate and pyruvate conversion to glucose. To investigate these possibilities, two series of experiments similar to the ones illustrated in Fig. 2 were performed in which 2 mM lactate (Fig. 3) or 0.2 mM pyruvate (Fig. 4) was infused separately.

As shown in Fig. 3, substantial amounts of glucose and pyruvate were released by the livers after the infusion of 2.0 mM lactate. A parallel increase in oxygen consumption was also observed. The infusion of 100 μM silibinin promoted a progressive decrease in both oxygen consumption and glucose production. There was, in contrast, a small increment in pyruvate production (Fig. 3). When the silibinin infusion was terminated, there was only a partial recovery of the glucose production and oxygen consumption, except for the pyruvate rates that tended to be higher than before the silibinin infusion.

With 0.2 mM pyruvate as a substrate (Fig. 4A), the response profile was similar to those shown in Fig. 2A and Fig. 3 for glucose and oxygen consumption, i.e., increases in both parameters with 0.2 mM pyruvate infusion and an inhibition with the 100 μM silibinin infusion. There was a small increment in lactate production with 0.2 mM pyruvate infusion and a higher increment with 100 μM silibinin infusion (Fig. 4). The effects promoted by silibinin were also partially reversed.

The concentration-dependent effect of silibinin on glucose and lactate production and on oxygen consumption in the presence of 0.2 mM pyruvate as substrate is shown in Fig. 4B. The data were obtained in the same way as those presented in Fig. 2B. There were

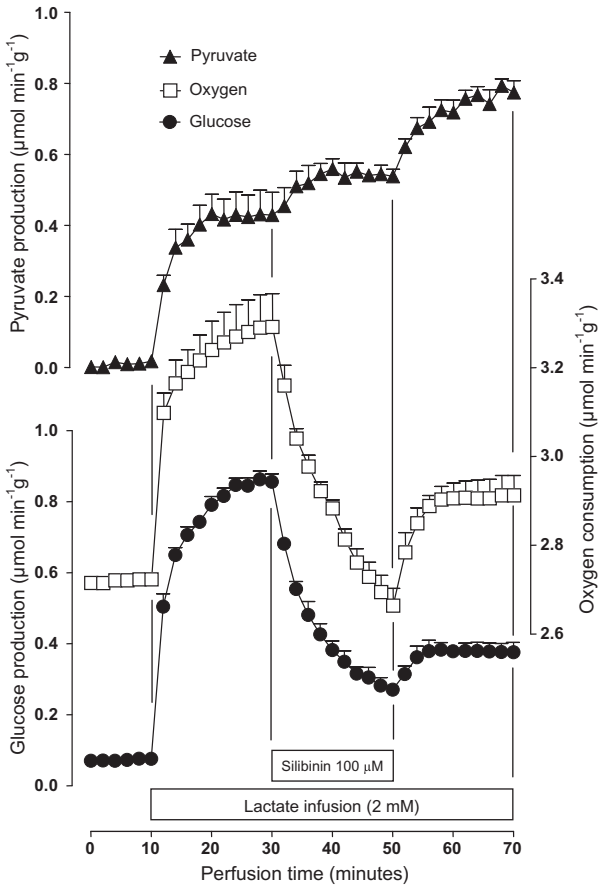


**Fig. 3.** The effects of silibinin on the metabolic fluxes from lactate in perfused livers isolated from fasted rats. Livers from 24 h fasted rats were perfused as described in the Section 2. Lactate was infused at 10–70 min and 100 μM silibinin from 30–50 min, as indicated by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analysed for glucose and pyruvate productions. Oxygen consumption was followed polarographically. Each experimental point is the mean ± SEM of 3 liver perfusion experiments.

approximately 50% and 100% inhibitions in glucose production with 50 μM and 200 μM silibinin infusion, respectively. The effects on oxygen consumption were more pronounced; 50% inhibition was caused by 50 μM silibinin, and at higher concentrations, the rates of oxygen consumption decreased to values below the basal levels. The inhibition in the presence of 100, 200 and 300 μM of silibinin was 123%, 177% and 320%, respectively. The lactate production was increased by nearly 53% with all of the concentrations of silibinin used.

### 3.3. The effects of silibinin on the activities of key enzymes of the gluconeogenic pathway

The effects of silibinin on the activities of key enzymes of gluconeogenesis pathway were evaluated. The enzymes examined were glucose 6-phosphatase, D-fructose 1,6-bisphosphatase, and pyruvate carboxylase. As shown in Fig. 5A, silibinin had no effect on D-fructose 1,6-bisphosphatase activity, but it caused a strong inhibition on glucose 6-phosphatase activity. At the 100 μM concentration, silibinin decreased the enzyme activity by 50%, and an almost total inhibition was observed at higher concentrations.

Silibinin was also able to inhibit the pyruvate carboxylation catalysed by pyruvate carboxylase in preparations of isolated mitochondria (Fig. 5B). In intact mitochondria, a 50% inhibition was found at the 175 μM silibinin concentration (data obtained by interpolation). At the higher concentration assayed (300 μM), the pyruvate carboxylation decreased by 75%. However, in freeze–thaw-disrupted mitochondria, silibinin had no significant effect on pyruvate carboxylation.

### 3.4. The effects of silibinin on glycogenolysis and glycolysis

In the fed conditions, liver glycogen catabolism contributes to the maintenance of glucose levels in circulation. When livers from fed rats are perfused in the absence of exogenous substrate, the release of glucose, lactate, and pyruvate are derived from glycogen catabolism, and the sum of lactate and pyruvate represents a fraction of the glucose oxidised in glycolysis. Fig. 6 illustrates a typical protocol in which silibinin was infused in these livers. In the first 10 min of perfusion, there was a substantial release of glucose, lactate, and pyruvate. The infusion of 200 μM silibinin between 10 and 40 min caused a significant decrease in all of the metabolic fluxes. The stronger reduction in lactate production relative to the reduction in pyruvate production indicated a decrease in the cytosolic NADH–$NAD^+$ redox potential, i.e., a change in the cytosolic potential to a more oxidised condition. After the cessation of the silibinin infusion, there was only a partial recovery of the oxygen consumption and lactate production toward the basal values (before the silibinin infusion). A distinct phenomenon was observed in the glucose release. There was a rapid release of glucose, reaching values 25% higher than the basal values 6 min after the cessation of silibinin infusion. After this time, there was a progressive decrease, but at the end of the experimental time period, the values were still higher than the basal ones.

The concentration-dependent effects of silibinin on glucose release, glycolysis (the sum of the lactate and pyruvate production) and oxygen consumption were shown in Fig. 6B. The data were calculated from experiments similar to those illustrated in Fig. 6A, but with different concentrations of silibinin (50, 100, 200 and 300 μM). The control values correspond to the rates found in the absence of silibinin (basal rates during the first 10 min) and the values of other points correspond to the values obtained during the last time point of silibinin infusion. The inhibition obtained in each parameter was concentration-dependent. The degree of inhibition in the presence of 300 μM of silibinin was 36%, 41% and 21% for glucose release, glycolysis and oxygen consumption, respectively. However, significant inhibitions of glucose release and oxygen consumption were already reached with 50 μM of silibinin. With 100 μM of silibinin, all parameters were significantly inhibited.

### 3.5. The effects of silibinin on parameters of energy transduction in isolated mitochondria

To evaluate whether the metabolic changes caused by silibinin in perfused livers could be a consequence of disturbances in the energy metabolism, the direct effects of silibinin on several parameters of energy transduction were evaluated in isolated mitochondria. The effects of silibinin, at a concentration range of 100–300 μM, on respiration driven by succinate ($FAD^+$-dependent) and α-ketoglutarate ($NAD^+$-dependent) are shown in Table 1. Silibinin inhibited the state III respiration driven by both succinate and α-ketoglutarate. Conversely, the state IV respiration was significantly stimulated when the respiration was driven by succinate, but not when the substrate was α-ketoglutarate. Accordingly, there was an activation of the initial basal respiration only with succinate. As a consequence of the effects of silibinin on states III and IV of respiration, there was a progressive decrease in the respiratory control ratio (RC) with both substrates (Table 1).

A distinct effect caused by silibinin on the state IV and on the basal respirations when succinate and α-ketoglutarate were

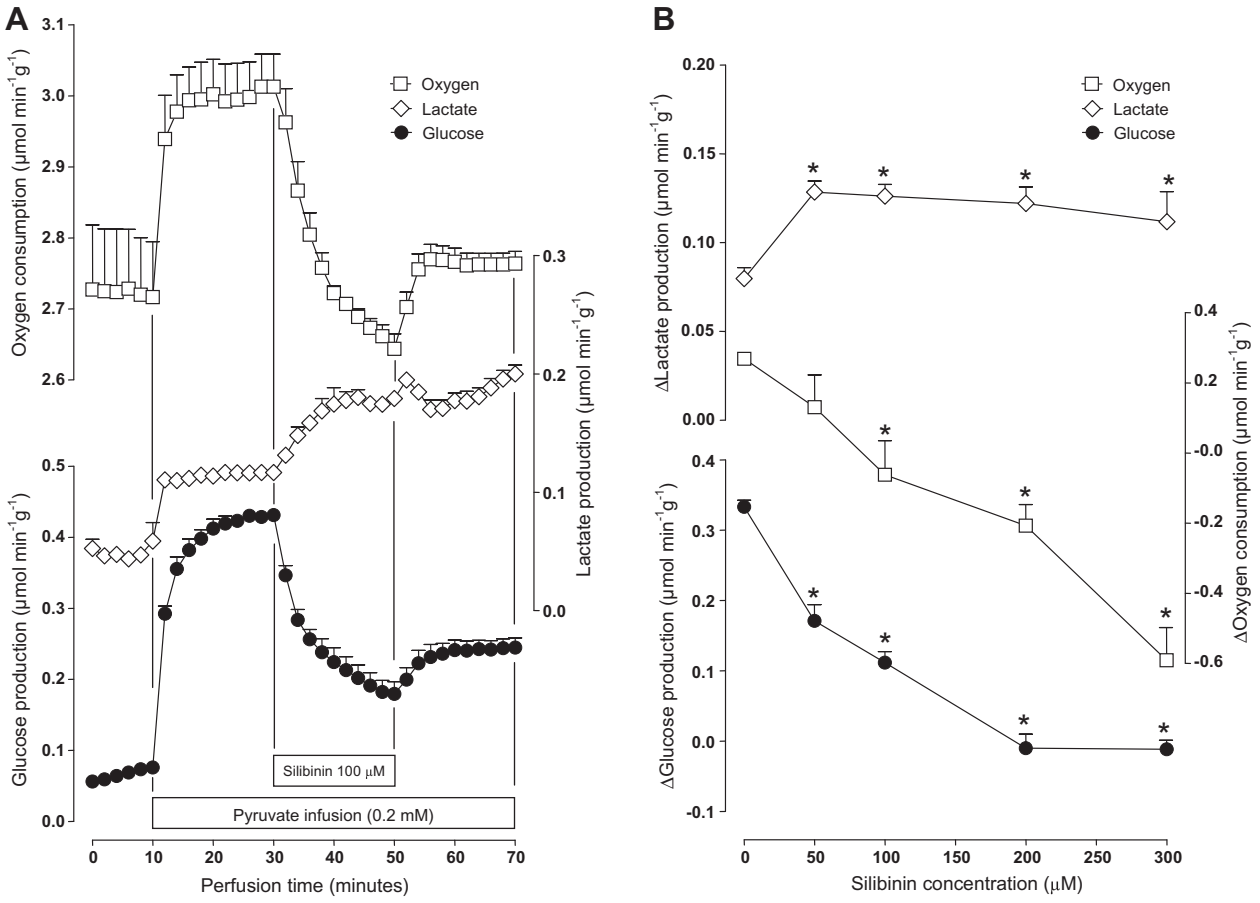


**Fig. 4.** The effects of silibinin on the metabolic fluxes from pyruvate in perfused livers isolated from fasted rats. Livers from 24 h fasted rats were perfused as described in the Section 2. Panel A: time courses of the changes caused by 100 μM silibinin in glucose or lactate productions and oxygen consumption. Pyruvate was infused at 10–70 min and silibinin at 30–50 min, as indicated by the horizontal bars. Panel B: concentration-dependent effects of silibinin on parameters measured. The control values (zero silibinin) correspond to the rates found in the presence of pyruvate just before the onset of silibinin infusion (30 min of perfusion) minus the basal rates (i.e., before the onset of pyruvate infusion). Rates in the presence of pyruvate and silibinin were evaluated after 50 min of perfusion and also subtracted from the same basal rates. The data points represented the mean ± SEM of 3–5 liver perfusion experiments. Asterisks indicate a statistical significance in comparison to the control condition as revealed by variance analysis with *post-hoc* Newman–Keuls testing (*$p < 0.05$).

the substrates suggested that silibinin affects components of the respiratory chain that were not common to the oxidation of both substrates. A way to evaluate this possibility is the measurement of the respiratory activity in freeze–thaw-disrupted mitochondria using NADH (NADH oxidase activity), succinate (succinate oxidase activity) or TMPD-ascorbate as substrates for complex I, II and IV, respectively. The results are shown in Fig. 7. Silibinin inhibited the respiration driven by succinate and by NADH. However, the inhibition was stronger with NADH. Whereas 300 μM silibinin promoted a 50% inhibition of respiration driven by NADH, the respiration driven by succinate was inhibited by only 15%. Silibinin did not affect the respiration driven by TMPD with ascorbate that reduces the cytochrome c-oxidase (Fig. 7).

The inhibition caused by silibinin on state III respiration could be a consequence of a direct effect on the $F_1F_0$–ATP synthase complex. The evaluation of the ATPase activity in three different mitochondrial preparations (intact-coupled, intact-uncoupled and freeze–thaw-disrupted mitochondria) may help in the understanding of the mode of action of silibinin. As shown in Fig. 8, the effects of silibinin were dependent on its concentration and the kind of mitochondrial preparation. In intact-coupled mitochondria, silibinin stimulated ATPase activity within the concentration range of 100–300 μM.

In 2,4-dinitrophenol-uncoupled mitochondria, the ATPase activity was not significantly inhibited by silibinin, but an inhibition was apparent in the freeze–thaw-disrupted mitochondria. At 300 μM silibinin, 27% inhibition was found.

### *3.6. The effects of silibinin on the adenine nucleotide contents in perfused livers from fasted and fed rats*

The observation that silibinin was active on several parameters of energy metabolism in isolated mitochondria raised the question of whether these effects also manifest in the intact livers and change the energy status of the hepatocytes. To examine this possibility, the hepatic contents of ATP, ADP and AMP were measured in livers from rats submitted to the same conditions as the experiments performed in the fasted condition, in which gluconeogenesis was measured with 2 mM lactate and 0.2 mM pyruvate as substrates (Fig. 2), and also in livers from fed rats as those shown in the experimental series of Fig. 6A. In both series, the measurements were made in control livers (without silibinin) and in livers perfused with 300 μM silibinin. The results are shown in Table 2 (fasted rats) and Table 3 (fed rats). In the fasted rats, silibinin decreased the contents of ATP and AMP in comparison with the control values (absence of silibinin). The ADP content was not modified. As a consequence, there was a significant change in the total amount of nucleotides and in the ratios of ATP/ADP and ATP/AMP.

In the fed livers (Table 3), no significant changes were found in the ATP and AMP contents. Only the content of ADP was increased

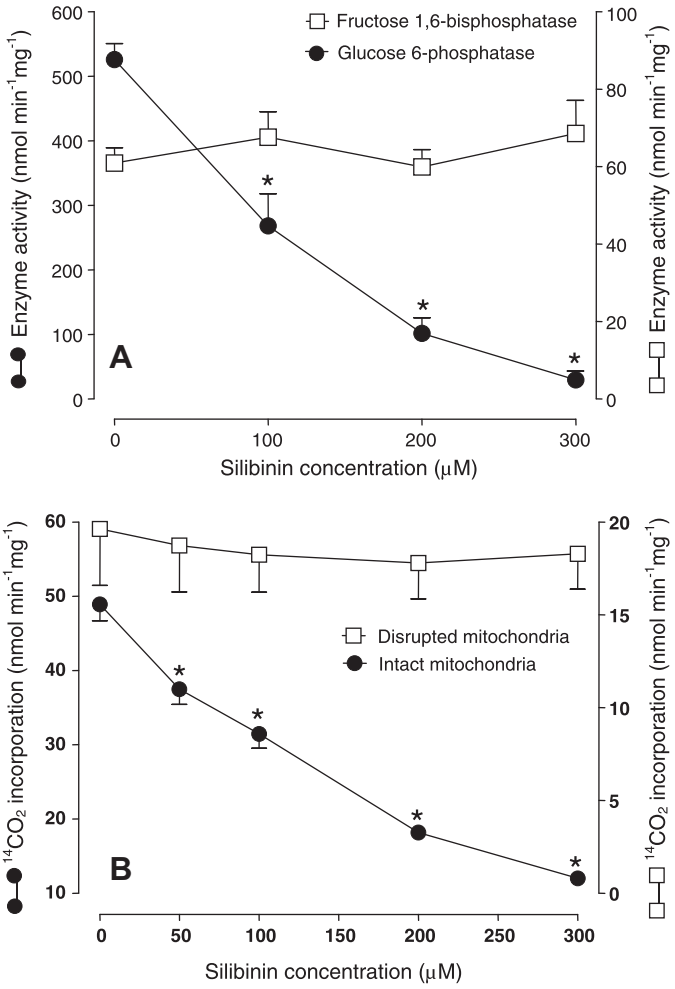


**Fig. 5.** The effects of silibinin on several enzymatic activities. Panel A: glucose 6-phosphatase and fructose 1,6-bisphosphatase activities at various silibinin concentrations. Livers from fasted rats were homogenised and subjected to differential centrifugation as described in the Section 2. The microsomal fraction was used for a glucose 6-phosphatase assay and the supernatant was used for the fructose 1,6-bisphosphatase assay. Panel B: pyruvate carboxylase activity of intact and disrupted rat liver mitochondria at various silibinin concentrations. The $^{14}CO_2$ incorporation into non-volatile components of the citric acid cycle was measured as described in the Section 2. Each data point is the mean ± SEM of 3–4 independent experiments. $^{*}p < 0.05$, ANOVA with *post-hoc* Newman–Keuls testing.

by silibinin when compared with the control values. The total amounts of nucleotides were not modified, but significant decreases were detected in the ratios of ATP/ADP and ATP/AMP.

### 3.7. The effects of silibinin on the catalytic oxidation of NADH

The capacity of silibinin to induce the catalytic oxidation of NADH, which is dependent on the action of peroxidases in the presence of $H_2O_2$, was also investigated. The results are shown in Fig. 9. Silibinin was able to promote a strong and significant oxidation of NADH even when very low concentrations were used (1–4 μM).

## 4. Discussion

The results of the present study revealed that 50–300 μM of silibinin exerts pronounced effects on liver carbohydrate metabolism. The metabolic pathways that contribute to glycaemia maintenance, i.e., gluconeogenesis in the fasted condition and glycogenolysis and glycolysis in the fed condition were both reduced by silibinin corroborating the role of the liver in the antihyperglycaemic effect of silibinin. The mechanisms by which silibinin exerts these actions are multiple and complex as summarised in the illustration in Fig. 10. Silibinin has at least four different sites of action: (a) glucose 6-phosphatase activity; (b) pyruvate carrier (monocarboxylate carrier, which operates in the plasma and mitochondrial membranes); (c) mitochondrial respiratory chain at complex I; (d) deviation of NADH supply for gluconeogenesis and mitochondria due to a pro-oxidative action.

The inhibitory effects of silibinin on gluconeogenesis occurred independently of the substrate that was infused. The degree of inhibition was very similar whether the substrate was lactate, pyruvate or a combination of both substrates. This finding excluded a change in the reaction catalysed by lactate dehydrogenase as the mechanism of gluconeogenesis inhibition. Glucose production from lactate needs the lactate dehydrogenase-catalysed reaction of lactate oxidation to pyruvate with $NAD^+$. This enzyme acts near the equilibrium of the reaction, that is, the reaction direction is determined essentially by the $NADH/NAD^+$ ratio. Therefore, an increase in the cytosolic $NADH/NAD^+$ ratio is expected to decrease the conversion of lactate to pyruvate and then to glucose. However, the conversion of pyruvate to glucose would not be affected. However, silibinin, at the 100 μM concentration, reduced glucose production by approximately 70% irrespective of the substrate infused, which was lactate with pyruvate, lactate, or pyruvate.

The possible inhibition of an enzymatic step common for both substrates was examined, and among the key enzymes of gluconeogenesis, the decreased activity of two enzymes, glucose 6-phosphatase and pyruvate carboxylase, was found. The inhibitory action on the activity of glucose 6-phosphatase confirms previous reports from Guigas et al. [12] and Detaille et al. [13]. The authors measured the enzyme activity in permeabilised and impermeabilised microsomes and concluded that the site of silibinin action was on the carrier unit of glucose 6-phosphatase.

Glucose 6-phosphatase catalyses the last step of gluconeogenesis, and its inhibition could explain only partially the reduction of gluconeogenesis. As already demonstrated by Ishii and Bracht [36], a strong inhibition of glucose 6-phosphatase provokes only a transitory modification in the rates of glucose release from the liver, as a new steady-state condition of a high intracellular glucose 6-phosphate is reached. After the removal of inhibition, a rapid increase of glucose release occurs frequently, a phenomenon that was observed in the livers of fed rats (Fig. 6A) but not in the gluconeogenesis experiments (fasted rats) where the rates of glucose release remained inhibited. So, it is unlikely that the effect on glucose 6-phosphatase is the major mechanism of silibinin-induced gluconeogenesis inhibition.

The inhibition of pyruvate carboxylase pointed to an alternative mechanism. It is known that pyruvate transport across the mitochondrial membrane is rate-limiting for pyruvate carboxylation [37,38]. The finding that inhibition of pyruvate carboxylation was observed in intact mitochondria, but not in disrupted mitochondria, indicated that silibinin does not inhibit the catalytic activity of pyruvate carboxylase but rather the transport of pyruvate into the mitochondria. This effect is similar to that of α-cyano-4-hydroxycinnamic acid, a classic inhibitor of pyruvate transport [39] and it was also described for two other phenolic compounds, *p*-coumaric acid [40] and fisetin [15].

It seems highly probable that the inhibitory effect of silibinin on pyruvate carboxylation contributed to the inhibition of gluconeogenesis from lactate and pyruvate. However, the effects of silibinin on the parameters of energy metabolism indicated that it is not the only mechanism. The strong inhibition of oxygen consumption exerted by silibinin in the liver perfusion experiments is certainly an important mechanism to be considered because it indicates an

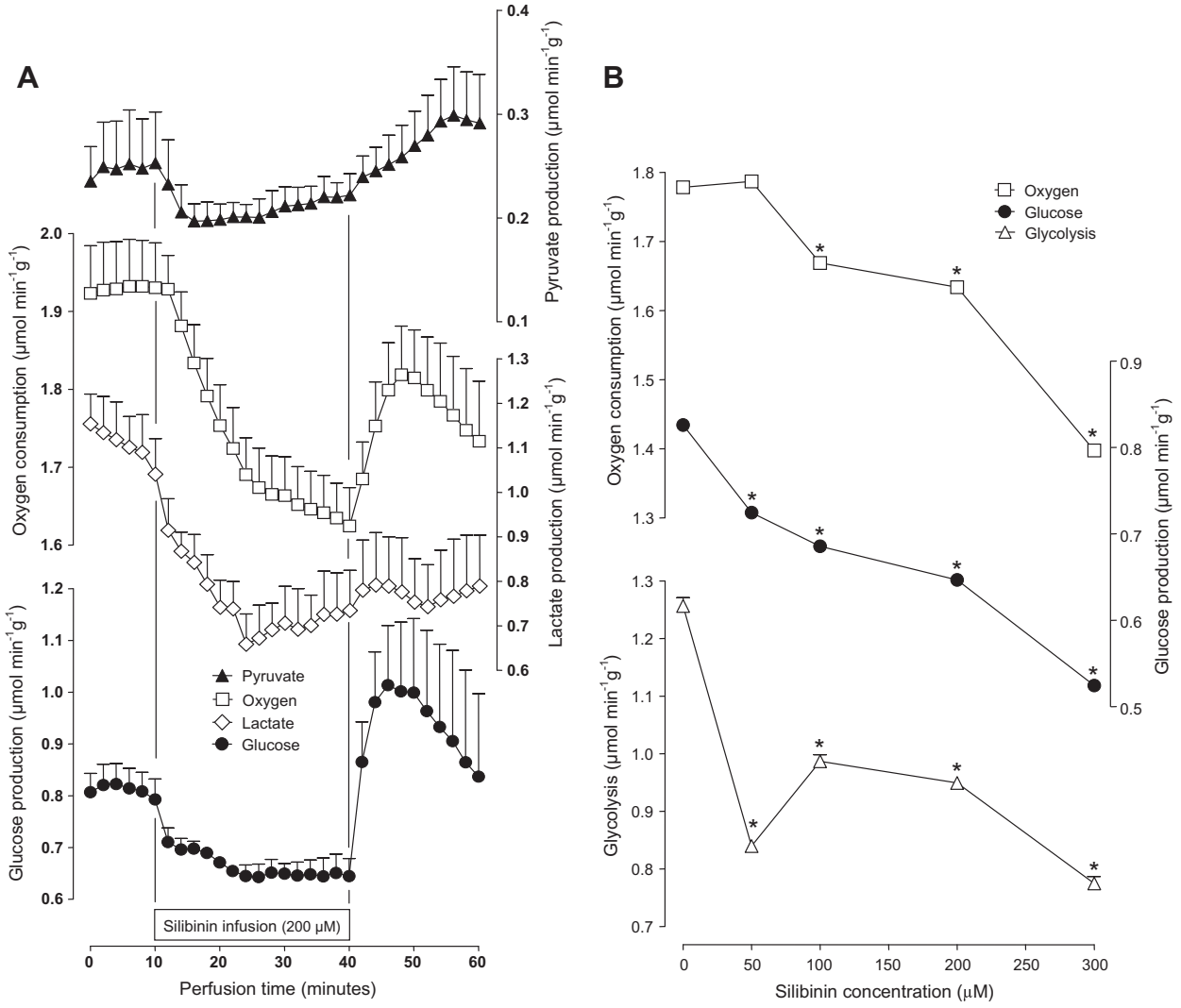


**Fig. 6.** The effects of silibinin on the metabolic fluxes in perfused livers isolated from fed rats. Panel A: time courses of the changes caused by silibinin in glycogen catabolism and oxygen consumption. Silibinin was infused as indicated by the horizontal bar. Samples of the effluent perfusate were collected for the metabolites assays. Oxygen consumption was followed polarographically. Panel B: The livers were perfused using different silibinin concentrations (50–300 μM). Rates of glycolysis were calculated from lactate plus pyruvate production. The control values (zero silibinin) correspond to the basal rates found just before the onset of silibinin infusion. Rates in the presence of silibinin were evaluated after 40 min of perfusion. Each data point represents the means of 3–5 liver perfusion experiments. Bars are standard errors of the mean. Asterisks indicate a statistical significance in comparison to the control condition as revealed by variance analysis with *post-hoc* Newman–Keuls testing (*$p < 0.05$).

**Table 1**
The action of silibinin on mitochondrial respiration driven by α-ketoglutarate and succinate in the presence or absence of exogenously added ADP. Mitochondria were isolated and assayed as described in the Section 2. Incubations were performed in the presence of the substrates α-ketoglutarate and succinate, as indicated. The respiratory control ratio (RC) and the ADP/O ratio were calculated according to the method by Chance and Williams (1955).

| Substrate | Silibinin (μM) | Substrate respiration | State III respiration | State IV respiration | Respiratory control ratio | ADP/O ratio |
|---|---|---|---|---|---|---|
| | | (nmol $O_2$ $min^{-1}$ mg $protein^{-1}$) | | | | |
| α-Ketoglutarate | 0 | 4.77 ± 0.34 | 23.31 ± 1.71 | 4.97 ± 0.58 | 4.87 ± 0.38 | 2.96 ± 0.20 |
| ($n$ = 6) | 100 | 4.70 ± 0.51 | 21.29 ± 2.34 | 5.67 ± 0.75 | 3.98 ± 0.60 | 2.49 ± 0.21 |
| | 200 | 4.76 ± 0.30 | 15.28 ± 2.03* | 5.18 ± 0.31 | 2.94 ± 0.35* | 2.64 ± 0.18 |
| | 300 | 4.53 ± 0.26 | 10.79 ± 0.91* | 5.82 ± 0.93 | 2.04 ± 0.32* | 2.28 ± 0.32 |
| Succinate | 0 | 14.26 ± 0.89 | 75.28 ± 3.91 | 10.66 ± 1.13 | 7.45 ± 0.92 | 2.05 ± 0.07 |
| ($n$ = 6) | 100 | 16.39 ± 1.21 | 78.13 ± 2.87 | 13.18 ± 1.29 | 6.11 ± 0.40* | 2,08 ± 0.05 |
| | 200 | 19.17 ± 1.32* | 69.53 ± 2.91 | 16.18 ± 1.85* | 4,47 ± 0.32* | 1.91 ± 0.06 |
| | 300 | 21.48 ± 1.48* | 48.27 ± 4.40* | 17.60 ± 2.07* | 2.79 ± 0.16* | 1.84 ± 0.08 |

Data are the mean ± standard errors of six experiments performed by identical protocols. The statistical significance relative to the controls is indicated by asterisks.
* $p < 0.05$, ANOVA with *post-hoc* Newman–Keuls testing.

impairment of the mitochondrial ATP generation, which is essential for gluconeogenesis.

That silibinin actually interfered with the efficiency of mitochondrial oxidative phosphorylation was clearly evident in the

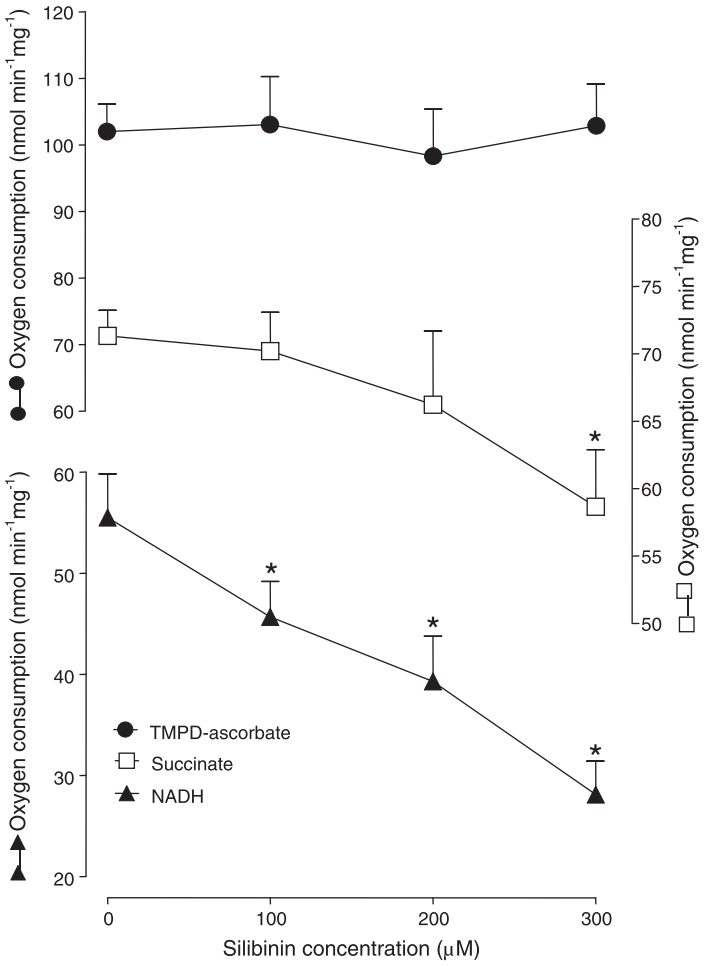


**Fig. 7.** The effects of silibinin on several membrane-bound enzymatic activities in rat liver mitochondria. NADH-oxidase and succinate-oxidase activities and TMPD-ascorbate oxidation were measured in freeze–thaw-disrupted mitochondria that were incubated at 37 °C in reaction medium as described in the Section 2. Each data point is the mean ± SEM of six independent experiments. $^{*}p < 0.05$, ANOVA with *post-hoc* Newman–Keuls testing.

experiments performed in isolated mitochondria. The most pronounced effect of silibinin on respiratory activity was during the state III respiration, which combines experimental conditions that are more similar to the conditions that exist within the cell, i.e., a continuous requirement for the rapid regeneration of $NAD^+$ to replace the electrochemical gradient expended during the phosphorylation of ADP [30]. The decrease in the RC (respiratory control ratio) in the presence of crescent concentrations of silibinin indicated a decrease in the efficiency of ADP phosphorylation. This action may be a consequence of the following: (a) a decrease in the rate of electron transfer through the respiratory chain; (b) an effect on the adenine nucleotide translocator and/or ATP synthase complex; and (c) an uncoupling of oxidative phosphorylation.

A decrease in the rate of electron transfer through the respiratory chain was supported by the inhibition caused by silibinin on the oxidation of NADH and succinate in disrupted mitochondria. The findings that the inhibition was much more pronounced with NADH as substrate in comparison with succinate, and that the oxidation of TMPD-ascorbate was not modified, indicated that silibinin acted on at least in two points in the respiratory chain; the first one was in the segment between complex II and complex III, and the second one was in a segment preceding the transfer of the electrons from complex I to complex III, probably at complex I, being the action in the last step much more pronounced.

Another alternative explanation should also be considered for the inhibition of NADH or $NAD^+$-dependent substrate oxidation in isolated mitochondria. Silibinin has been shown to be a strong pro-oxidative agent, i.e., it was able to oxidise NADH *in vitro* in the presence of peroxidase and $H_2O_2$ (Fig. 9). This pro-oxidative action results from the production of free-radical derivatives, such as *o*-hemiquinones and *o*-quinones and subsequent NADH oxidation [41–44]. It has been suggested that these reactions may also occur *in vivo* with the participation of catalase, which functions as a peroxidase at low $H_2O_2$ concentrations [15,17,45]. In this case, a competition for NADH could occur between the respiratory chain and the silibinin metabolites, which anticipate the NADH oxidation. This hypothesis is also consistent with the observation that an uncoupling action was evident when succinate was the substrate but not with α-ketoglutarate (a $NAD^+$-dependent substrate).

This uncoupling action was indicated by the stimulation of the respiration driven by succinate (basal and state IV respiration) associated with a decrease in the respiratory control (RC) irrespective of the substrate (succinate or α-ketoglutarate). The stimulation of the ATPase activity in intact and coupled mitochondria also corroborated the uncoupling action of silibinin. It is known that the ATPase activity in intact and coupled mitochondria is retroactively controlled by the electrochemical proton gradient generated by proton extrusion. Any agent that causes a dissipation of this gradient will lead to a stimulation of the ATPase activity along with a stimulation of respiratory activity in intact mitochondria [52].

It should be remembered, that in intact mitochondria, the transport of adenine nucleotides is a necessary and rate-limiting step of the ATPase activity. It was demonstrated that the activity of the

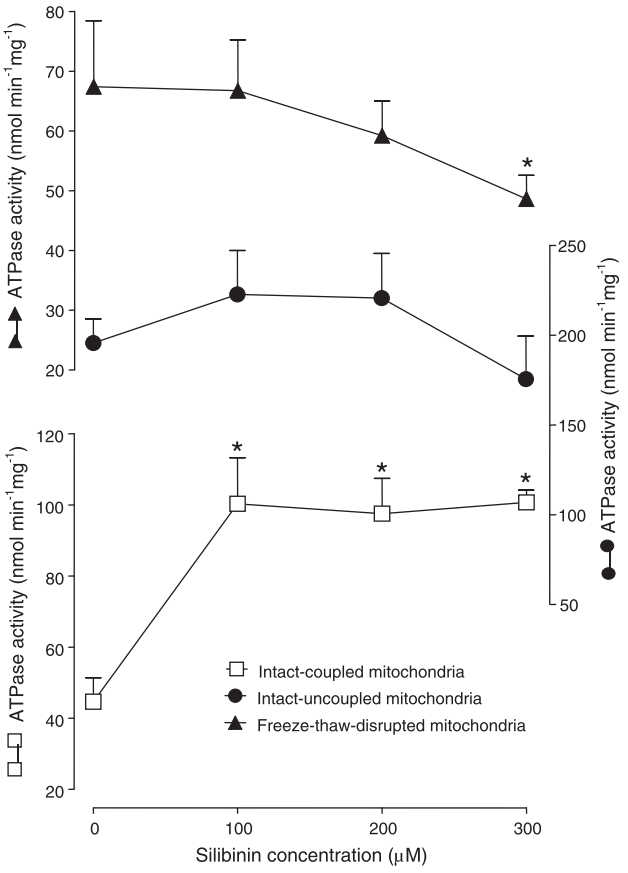


**Fig. 8.** The effects of silibinin on the ATPase activity of coupled, uncoupled and disrupted mitochondria. The mitochondria were incubated at 37 °C in reaction medium as described in the Section 2. Each assay point represents the mean of six independent experiments and the bars are the SEM. *$p < 0.05$, ANOVA with *post-hoc* Newman–Keuls testing.

ATPase was not significantly reduced by silibinin (Fig. 8) in intact-uncoupled mitochondria but there was an inhibition of the enzyme

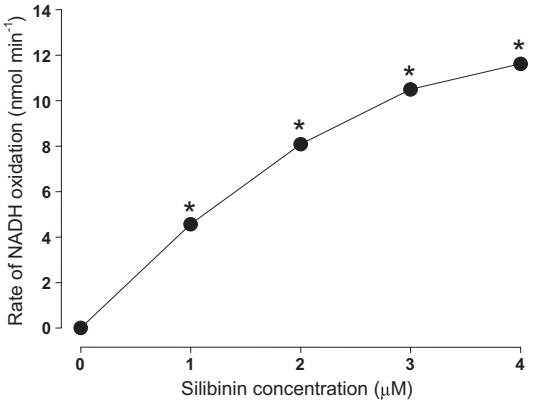


**Fig. 9.** The dependence of the rate of peroxidase catalysed NADH oxidation on the silibinin concentration. The rate of NADH oxidation was determined at 340 nm in a reaction mixture containing 0.1 M Tris–HCl/1.0 mM EDTA buffer pH 7.4, 0.1 μM horseradish peroxidase (HRP), 200 μM NADH, 25 μM $H_2O_2$ and different silibinin concentrations. Values represent mean ± SEM of three separate experiments. *$p < 0.05$, ANOVA with *post-hoc* Newman–Keuls testing.

in disrupted mitochondria at 300 μM silibinin concentration. Under the last condition, ATP has free access to the ATPase complex. It seems plausible to suggest, thus, that silibinin exerted an additional inhibitory effect on the ATPase complex, an effect that could contribute to a reduction of the state III respiration observed during the oxidation of α-ketoglutarate and succinate (Table 1).

Most of the effects of silibinin observed in isolated mitochondria were clearly manifested in intact livers. The inhibition of oxygen consumption observed during the infusion of silibinin in perfused liver (Figs. 2–4 and 6) is consistent with the inhibitory action on respiration linked to ADP phosphorylation (state III respiration). However, the most prominent indicator of impairment of mitochondrial function in the intact liver was the decreased levels of cellular ATP and the changes in the ATP/ADP and ATP/AMP ratios. The pro-oxidative property of silibinin could also be contributing to these metabolic disturbances. A reduction in the levels of NADH not only could diminish the supply of reducing equivalents to the mitochondrial respiratory chain but also to the gluconeogenic

**Table 2**
The influence of silibinin on hepatic contents of adenine nucleotides in substrate-perfused livers of fasted rats. Livers from fasted rats were perfused in an open system as described in the Section 2. Lactate (2.0 mM) and pyruvate (0.2 mM) were infused at 10 min and 300 μM silibinin was infused 20 min after the beginning of the lactate and pyruvate infusion. The livers were freeze-clamped in liquid nitrogen and extracted with perchloric acid. The neutralised extracts were used for the determination of the adenine nucleotides by HPLC. Control determinations were performed with livers that were freeze-clamped at the same perfusion time but without silibinin infusion.

| Condition | ATP | ADP | AMP | AMP+ADP+ATP | ATP/ADP ratio | ATP/AMP ratio |
|---|---|---|---|---|---|---|
| | μmol × (g liver wet weight)$^{-1}$ | | | | | |
| Control ($n = 5$) | 1.75 ± 0.06 | 0.86 ± 0.07 | 0.28 ± 0.03 | 2.90 ± 0.12 | 2.07 ± 0.19 | 6.44 ± 0.78 |
| Silibinin (300 μM) ($n = 7$) | 1.27 ± 0.08* | 0.92 ± 0.03 | 0.11 ± 0.01* | 2.31 ± 0.09* | 1.37 ± 0.07* | 13.02 ± 2.38* |

Values are means ± standard errors of the mean.
* Statistically different from the corresponding control values according to Student's *t*-test ($p < 0.05$).

**Table 3**
The influence of silibinin on the hepatic contents of adenine nucleotides in substrate-free perfused livers of fed rats. Livers were perfused in an open system as described in the Section 2. Silibinin (300 μM) was infused at 30 min after the start of the liver infusion. The livers were freeze-clamped in liquid nitrogen and extracted with perchloric acid. The neutralised extracts were used for the determination of the adenine nucleotides by HPLC. Control determinations were performed with livers that were freeze-clamped at the same perfusion time but without silibinin infusion.

| Condition | ATP | ADP | AMP | AMP + ADP + ATP | ATP/ADP ratio | ATP/AMP ratio |
|---|---|---|---|---|---|---|
| | μmol × (g liver wet weight)$^{-1}$ | | | | | |
| Control ($n = 3$) | 2.22 ± 0.04 | 0.81 ± 0.02 | 0.12 ± 0.01 | 3.16 ± 0.05 | 2.73 ± 0.11 | 17.71 ± 1.66 |
| Silibinin (300 μM) ($n = 3$) | 1.85 ± 0.15 | 1.07 ± 0.04* | 0.20 ± 0.03 | 3.13 ± 0.22 | 1.71 ± 0.08* | 9.64 ± 1.34* |

Values are means ± standard errors of the mean.
* Statistically different from the corresponding control values according to Student's *t*-test ($p < 0.05$).

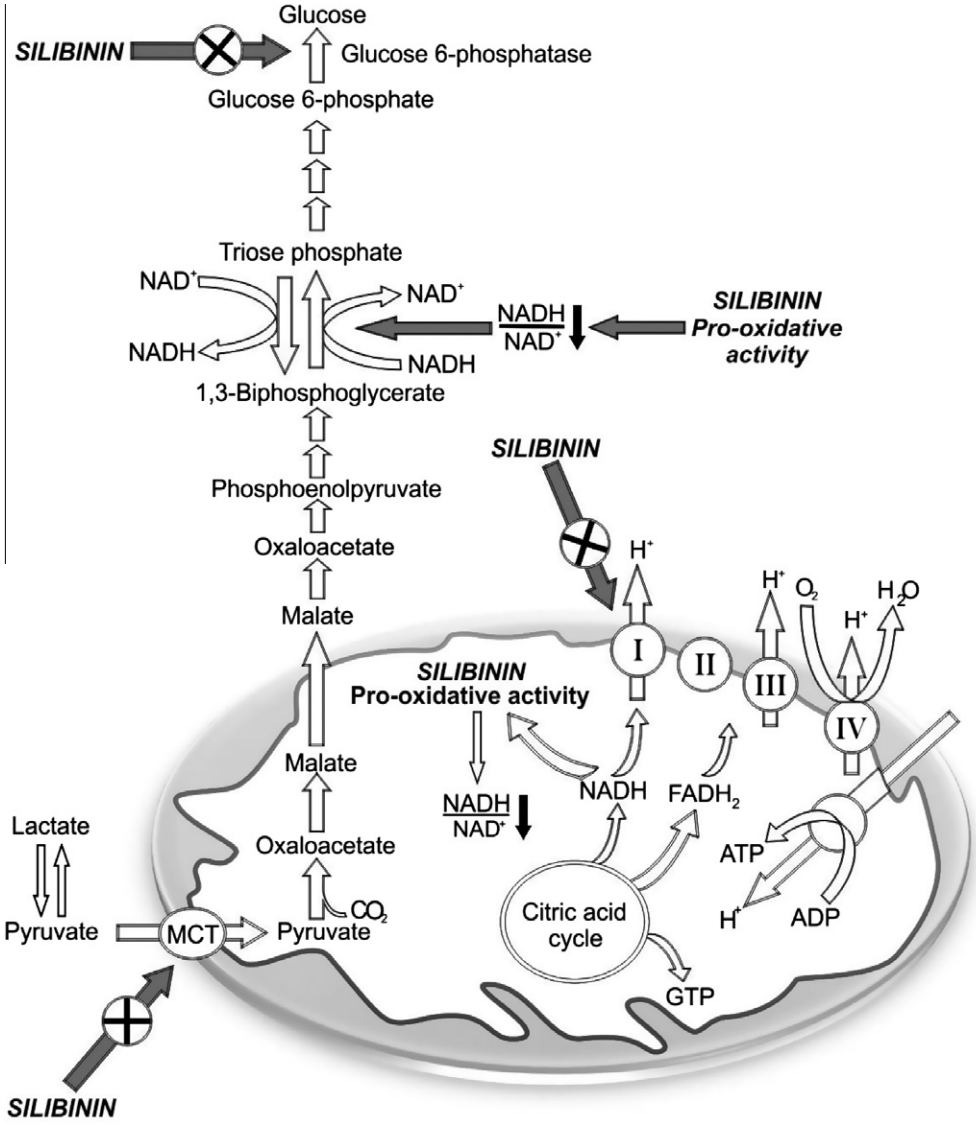


**Fig. 10.** Schematic representation of the effects of silibinin on metabolic pathways. The largest and grey arrows indicate the points of action of silibinin: inhibits the activity of glucose 6-phosphatase, inhibits the pyruvate carrier (MCT monocarboxylate carrier), inhibits the respiratory chain at complex I and also acts by reducing the supply of NADH for gluconeogenesis and mitochondria through a pro-oxidative action.

pathway at the reaction step catalysed by glyceraldehyde 3-phosphate dehydrogenase.

It is well known that the levels of adenine nucleotides regulate not only the biosynthetic pathways dependent on ATP, such as gluconeogenesis, but they are also activators of glycogenolysis and glycolysis. A stimulation of both processes is an expected compensatory result of a decrease in the mitochondrial ATP production, as it occurs classically for drugs that alter the mitochondrial energy efficiency [34,36,46,47].

Silibinin, however, did not cause such effects. In contrast, it caused a reduction of both processes in livers from fed rats. The inhibitory effects of silibinin on the pathways of glycolysis and glycogenolysis had been already described by Detaille et al. [13]. The mechanism for this action is not clear. The inhibition of the activity of glucose 6-phosphatase cannot be the major mechanism, as we have discussed above. However, an intracellular accumulation of glucose 6-phosphate in the hepatocytes is highly probable [12]. Although we have not directly measured the intracellular concentration of glucose 6-phosphate in our experiments, we have an indicator that this phenomenon also happens, as there was an increased release of glucose after the withdrawal of the infusion of silibinin, upon which we have already commented.

Glucose 6-phosphate, in addition to being an intermediate of several metabolic pathways, is also an important cell-signalling intermediate. For instance, an increase in its levels can cause a decrease in glycogen degradation by stimulating the activity of glycogen synthase b and of glycogen synthase phosphatase that leads to an increase in glycogen synthesis [48]. A repartitioning of glucose 6-phosphate into glycogen synthesis at high cellular glucose 6-phosphate concentrations has been reported, possibly as a cellular response aimed at maintaining cellular glucose 6-phosphate homeostasis [49]. We cannot discard this phenomenon in our experiments as an explanation for the reduction of glycogenolysis pathway with a subsequent reduction in glucose release.

In our liver perfusion system (Fig. 6), the theoretically accumulated glucose 6-phosphate was not completely released in free glucose when the silibinin infusion was stopped, suggesting that it was consumed in another metabolic pathways. A stimulation of the pentose-phosphate pathway could be a possible way to

regenerate the NADPH that may be necessary for silibinin metabolisation and also to counteract the pro-oxidative action of silibinin.

The possibility of a direct action of silibinin on the activity of other enzymes involved in glycogen catabolism and glycolysis can also be considered, as was reported for other structurally-related phenolic compounds [50,51]. The inhibition of pyruvate kinase by silibinin, with a resultant accumulation of phosphoenolpyruvate inside the cell, was reported by Detaille et al. [13].

Our data allow the conclusion that the inhibitory effects of silibinin on gluconeogenesis in the fasted condition and on glycogen metabolism in the fed condition may contribute to the antihyperglycaemic action of silibinin, corroborating, thus, its clinical utilisation for diabetes treatment. However, we have also demonstrated that the mechanisms of these actions are complex, including a pro-oxidative effect and an impairment of the mitochondrial energy transduction. Impairment of ATP production and the cellular redox state could lead, potentially at least, to metabolic disorders, a finding that should be considered in its therapeutic usage.

The comparison of the effects of silibinin revealed in the present work and those reported for quercetin and fisetin, under the same experimental conditions, allow some considerations about the relationship between their structures and biological activities. The inhibition of gluconeogenesis in the fasted condition and the inhibition of glycolysis in the fed condition are effects shared by all of them [14,15]. Quercetin activated glycogenolysis at concentrations near 300 μM [16], but an inhibitory effect was caused by fisetin [15] and silibinin. The most prominent differences between the three compounds in the perfused rat liver were their effects on oxygen consumption in fed rats. Whereas silibinin caused an inhibition of oxygen consumption, quercetin, at similar concentration, induced a stimulation of oxygen consumption [16] and fisetin did not induce a significant effect [15]. Furthermore, silibinin was the unique compound that significantly reduced the adenine nucleotide contents in the liver.

The actions on mitochondria were also very similar for all compounds. They acted as uncouplers of oxidative phosphorylation and inhibitors of the respiratory chain and as inhibitors of ATPase activity. There was also a similarity in their effects on key enzymes of gluconeogenesis [14–16,52].

It has not yet been established exactly what region of the flavonoid molecules is responsible for their different and numerous biological effects. There are some well-established correlations for the antioxidant capacity: (a) the presence of 2,3 unsaturation in conjugation with the 4-oxo group in ring C; (b) the dihydroxylated B-ring (catechol), which allows prompt hydrogen donation (electrons); (c) a high degree of hydroxylation; (d) the presence of the 3- and 5-hydroxyls [53,54]. Quercetin has all the related characteristics (a, b, c and d) and fisetin has the features listed in items a, b and c. Silibinin possesses only the last two related characteristics (items c and d) and thus could have a lower antioxidative property. It should be remembered that silibinin also possesses an additional chemical group (a dimer of phenylpropanoid, Fig. 1).

Our results indicated that these structural differences did not exert a great influence on the metabolic effects of silibinin in the liver when compared with the effects of quercetin and fisetin [14–17,52]. However, silibinin exerted some distinct effects such as the inhibitory effect on oxygen consumption in the livers of fed rats and a decrease in the ratio of adenine nucleotides in the livers of fed and fasted rats; this could be attributed to some of these structural differences, i.e., the absence of the 2,3 unsaturation in conjugation with the 4-oxo group, or the absence of catechol-ring or the presence of the phenylpropanoid dimer. Thus, it seems that regarding its effects on energy metabolism, silibinin exerts additional undesirable effects when compared with quercetin and fisetin.

Finally, we relate the concentration range of silibinin used in the present study with therapeutic doses commonly administrated. At therapeutic doses from 100 to 300 mg, the plasma concentration reaches levels between 200 and 1400 μg/L [55,56]. It is known also that the portal concentration can achieve levels much higher than in the systemic circulation and also that the bile concentration can reach values 100 times higher than the plasma [57,58]. Thus, it is highly probable that the portal concentrations reach values that are similar to the range of silibinin used in this work.

## Conflict of interest statement

The authors declare that they do not have any conflict of interest.

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