



## THE EFFECTS OF Fisetin ON KETOGENESIS AND CITRIC ACID CYCLE IN THE RAT LIVER

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**ABSTRACT:** The most known biological effects of the flavonoids, including quercetin and fisetin, is no doubt their antioxidant action, which represents protection of tissues against the action of free-radicals and diminution of lipid peroxidation. However, the antioxidant and anticancer effects of quercetin and other flavonoids are not an unanimity, since it has been shown that significant protective effects due to quercetin would only be achieved upon the ingestion of high doses. At high doses, however, the potentially toxic effects of quercetin and other flavonoids have to be taken into account. The present work was planned to investigate the effects of fisetin on ketogenesis and citric acid cycle in the rat liver. Male Wistar rats, weighing 200 to 280 g, fed with a standard laboratory diet were utilized. But in this case, livers from 24 h fasted rats were used in all experiments. The isolated liver was perfused in the non-recirculating system. The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4), saturated with a mixture of O<sub>2</sub> and CO<sub>2</sub> (95:5) by means of a membrane oxygenator and simultaneously heated to 37°C. Ketogenesis ( $\beta$ -hydroxybutyrate and acetoacetoacetate production) from endogenous sources was inhibited by fisetin in the whole concentration range (50-200  $\mu$ M). The  $\beta$ -hydroxybutyrate to acetoacetoacetate ratio was also reduced, and <sup>14</sup>CO<sub>2</sub> production from exogenous [1-<sup>14</sup>C]-oleate was increased by fisetin. The results of this investigation suggest that fisetin causes a shift in the mitochondrial redox potential toward a more oxidized state with a clear predominance of its prooxidant activity.

**KEY WORDS:** Fisetin; Flavonoids; Ketogenesis; Liver.

### 1 INTRODUCTION

Fisetin (3,7,3',4'-tetrahydroxiflavone) is a flavonoid dietary ingredient found in the smoke tree (*Cotinus coggyria*) and is also found in fruits and vegetables such as strawberry, apple, persimmon, grape, onion, and cucumber at concentrations of 2 to 160  $\mu$ g/g (ARAI et al., 2000). Flavonoids have been proposed to exert beneficial effects in a multitude of disease states, including cancer, cardiovascular disease, and neurodegenerative disorders. The most known biological effects of the flavonoids, including quercetin and fisetin, is no doubt their antioxidant action, which represents protection of tissues against the action of free-radicals and diminution of lipid peroxidation. However, the antioxidant and anticancer effects of quercetin and other flavonoids are not an unanimity, since it has been shown that significant protective effects due to quercetin

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would only be achieved upon the ingestion of high doses. At high doses, however, the potentially toxic effects of quercetin and other flavonoids have to be taken into account.

It has been reported that flavonoids can act as mutagens, prooxidants with the generation of free radicals and as inhibitors of enzymes involved in energy metabolism and hormone actions). The property of quercetin to induce the oxidation of NADH in mammalian cells, shifting the cellular conditions to a more oxidized state (prooxidant activity) is suggested in some works. It was demonstrated that, at low concentrations, quercetin reduces the mitochondrial and cytosolic NADH to NAD<sup>+</sup> ratios in the perfused rat liver. Fisetin and quercetin, possess the 3'- and 4'-OH groups in the B-ring (catechol ring), but fisetin differs structurally from quercetin by the absence of the hydroxyl in the 5' position of A-ring.

The present work was planned to investigate if fisetin shares with quercetin the ability to affect liver energy metabolism and the NADH-NAD<sup>+</sup> redox potential.

## 2 MATERIALS AND METHODS

The liver perfusion apparatus was built in the workshops of the University of Maringá. Fisetin was purchased from Sigma-Aldrich (ST Louis, MO, USA). Enzymes and coenzymes used in the enzymatic assays were purchased from Sigma-Aldrich (St Louis, MO, USA). Male Wistar rats (weighing 200-280 g) fed with a standard laboratory diet (Nuvital - Nuvilab CR-1<sup>®</sup>) were used in all experiments. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá. For the surgical procedure, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg<sup>-1</sup>). Haemoglobin-free, non-recirculating perfusion was undertaken. After cannulation of the portal and cava veins, the liver was positioned in a plexiglas chamber. Flow was maintained constant by a peristaltic pump (Miniplus 3, Gilson, France) and was adjusted to between 30 and 35 ml min<sup>-1</sup>, depending on the liver weight.

The perfusion fluid was Krebs/Henseleit bicarbonate buffer (pH 7.4), saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment at 37°C. The composition of the Krebs/Henseleit bicarbonate buffer is as follows: 115 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5.8 mM KCl, 1.2 mM Na<sub>2</sub>SO<sub>4</sub>, 1.18 mM MgCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub> and 2.5 mM CaCl<sub>2</sub>. Fisetin was dissolved in the perfusion fluid. Solubilization was achieved by the simultaneous addition of an equivalent amount of NaOH. Livers from 24 h fasted rats were used in all experiments.

When [1-<sup>14</sup>C]oleate was infused, the effluent perfusion fluid was fractionated in 2 minute intervals and collected in Erlenmeyer flasks which were immediately closed for <sup>14</sup>CO<sub>2</sub> measurement. Small samples (2 ml) were collected for the measurement of β-hydroxybutyrate and acetoacetate. The oxygen concentration in the effluent perfusate was monitored polarographically, employing a teflon-shielded platinum electrode adequately positioned in a plexiglas chamber at the exit of the perfusate (CLARK, 1956). Acetoacetate and β-hydroxybutyrate in the outflowing perfusate were measured enzymatically using β-hydroxybutyrate dehydrogenase. Interference by fisetin (absorbance at 340 nm) was excluded by running blanks. The carbon dioxide production from [1-<sup>14</sup>C]oleate was measured by trapping <sup>14</sup>CO<sub>2</sub> in phenylethylamine. Radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5g/l 2,5-diphenyloxazole and 0.15g/l 2,2-*p*-phenylenebis(5-phenyloxazole). NADH oxidation was measured and the reaction mixtures contained 0.1 M Tris-HCl/1.0 mM EDTA buffer (pH 7.4), fisetin (0.5-4.0 μM), H<sub>2</sub>O<sub>2</sub> (25 μM), NADH (200 μM) and horseradish peroxidase (HRP) type VI-A (0.1 μM). Reactions were started by the addition of H<sub>2</sub>O<sub>2</sub> (25 μM) and the oxidation of NADH was followed at 340 nm using a spectrophotometer.

The statistical significance of the differences between parameters was evaluated by means of Student's t test or 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.

### 3 RESULTS AND DISCUSSION

The experimental protocols adopted for investigating the effects of fisetin on ketogenesis from endogenous sources and from an exogenous fatty acid in livers from 24 h fasted rats are shown in Figures 1A and 2. The data of the experiments in which the effects of fisetin 200  $\mu$ M on ketogenesis and oxygen uptake from endogenous fatty acids were evaluated are summarized in figure 1A.

After stabilization of oxygen consumption, samples for the enzymatic measurement of  $\beta$ -hydroxybutyrate and acetoacetate production were collected. The infusion of fisetin at 10 minutes of perfusion rapidly decreased the production of  $\beta$ -hydroxybutyrate to non-detectable levels. Acetoacetate production was not altered by fisetin. Oxygen uptake decreased continuously during the whole infusion period until the fisetin infusion was interrupted. All metabolic fluxes showed little tendency toward recovery even after 20 minutes. A series of experiments was also accomplished with 50 and 100  $\mu$ M fisetin in order to evaluate the concentration dependence of the effects at the end of the infusion period on ketogenesis in liver perfusion experiments similar to those shown in figure 1A. The results shown in figure 1B allow to correlate the actions of several concentrations of fisetin on oxygen uptake, ketogenesis and the  $\beta$ -hydroxybutyrate/acetoacetate ratio. The total production of ketone bodies was reduced 36.6%, 29.6% and 31.4%, respectively, with 50, 100 and 200  $\mu$ M fisetin. The most pronounced effect was, that, on the  $\beta$ -hydroxybutyrate to acetoacetate ratio which was reduced in a concentration dependent manner, decreasing from  $0.35 \pm 0.05$  to non-measurable value, at 200  $\mu$ M concentration.

Figure 2 illustrates the action of fisetin 200  $\mu$ M on the oxidation of exogenous oleate. Fisetin was introduced after stabilization of the metabolic changes caused by the long-chain fatty acid. The introduction of the perfusion fluid containing 0.2 mM oleate and [1- $^{14}$ C]oleate resulted in [ $^{14}$ C]CO<sub>2</sub> production and an accentuated enhancement in oxygen consumption and ketone body production. The subsequent introduction of fisetin caused decreases in  $\beta$ -hydroxybutyrate production and increased acetoacetate production, but ketogenesis was not inhibited by fisetin. There was a slight tendency of stimulation in oxygen consumption at the beginning of fisetin infusion. The production of  $^{14}$ CO<sub>2</sub> was also increased by fisetin by about 25% (*p*<0.05). The effects of fisetin on  $\beta$ -hydroxybutyrate and acetoacetate production were only partially reversed after the infusion was stopped. Fisetin clearly decreased the  $\beta$ -hydroxybutyrate/acetoacetate ratio.

Confirming previous observation, fisetin promoted NADH oxidation in the presence of peroxidase and catalytic amounts of H<sub>2</sub>O<sub>2</sub> (figure 3), indicating that fisetin is able to form prooxidant metabolites.

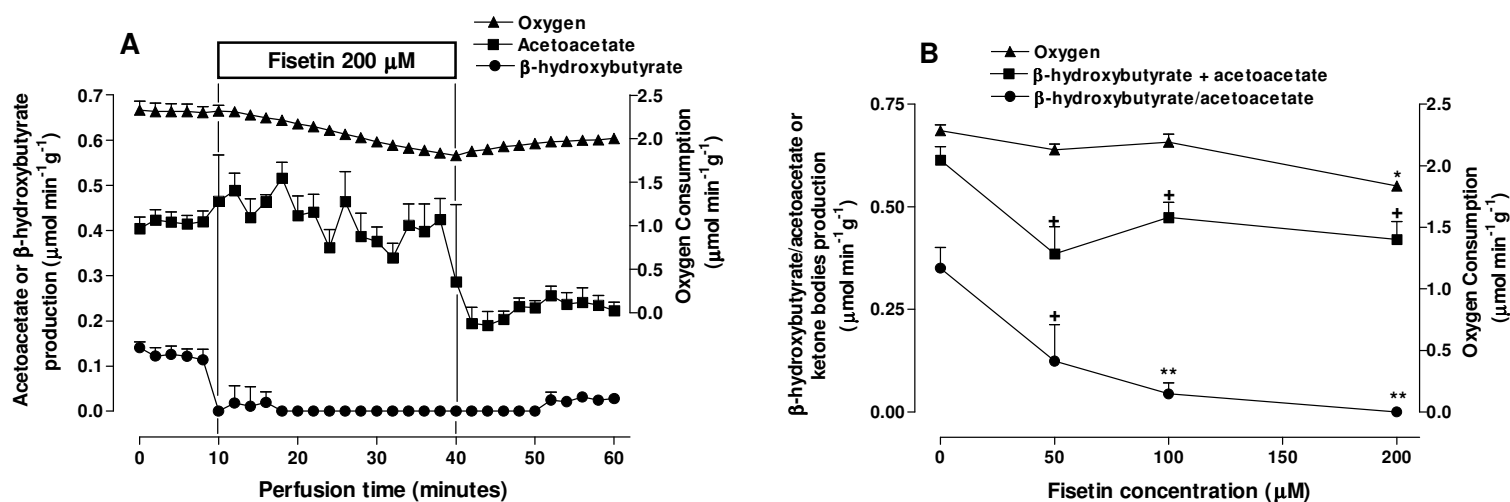


Figure 1. Panel A: Time course of the changes caused by 200 μM fisetin on ketogenesis and oxygen uptake in the isolated perfused liver from fasted rats. Panel B: Concentration dependencies of the effects of fisetin on ketogenesis, NADH to NAD<sup>+</sup> ratio and oxygen consumption. Fisetin 200 μM was infused at 10-40 min as indicated by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analyzed for acetoacetate and β-hydroxybutyrate. Oxygen consumption was followed polarographically. Values in the absence of fisetin (control values) are the mean values before the set of fisetin infusion. Values in the presence of fisetin were computed at the end of the infusion. Each experimental point is the mean ± SEM of three to four experiments with identical protocol. Asterisks indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman-Keuls testing (\**p*<0.05, \*\**p*<0.01, \**p*<0.001).

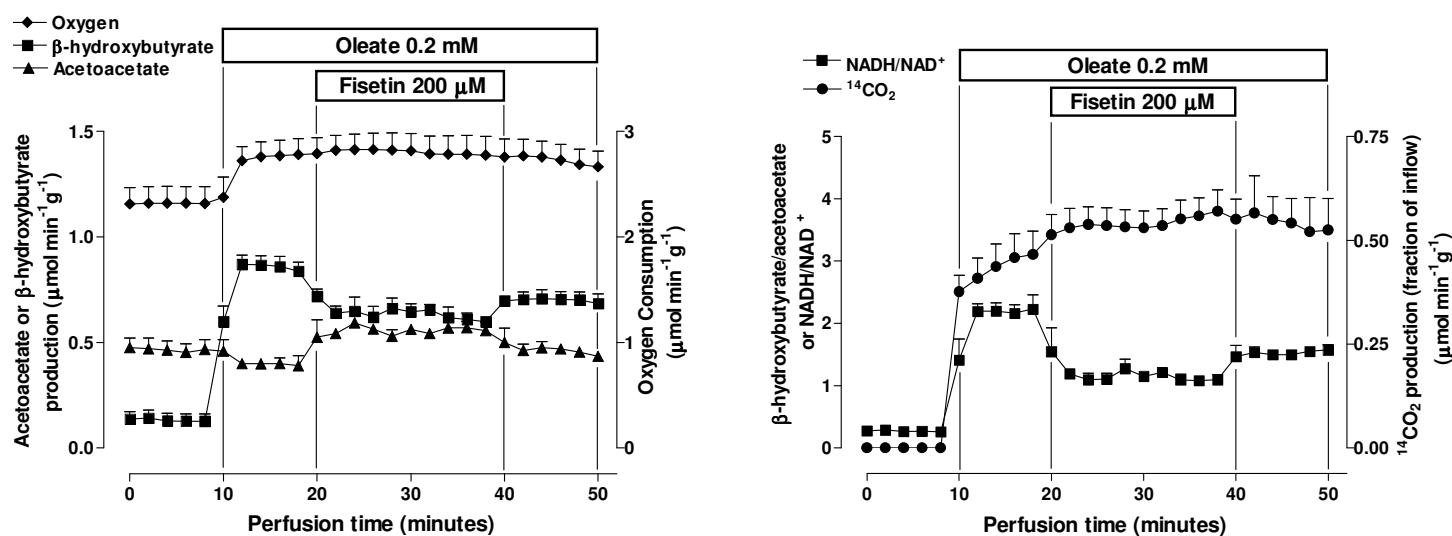


Figure 2. Time course of the changes caused by 200 μM fisetin on ketogenesis, oxygen uptake, NADH to NAD<sup>+</sup> ratio and <sup>14</sup>CO<sub>2</sub> production in the perfused rat liver. Livers from fasted rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4), as described in Materials and methods. [<sup>14</sup>C]oleate (0.2 mM) was infused at 10-50 min and fisetin 200 μM at 20-40 min as indicated by the horizontal bars. The effluent perfusate was sampled in 2-min intervals and analyzed for acetoacetate, β-hydroxybutyrate and <sup>14</sup>CO<sub>2</sub>. Oxygen consumption was followed polarographically. Each experimental point is the mean ± SEM of four experiments with identical protocol.

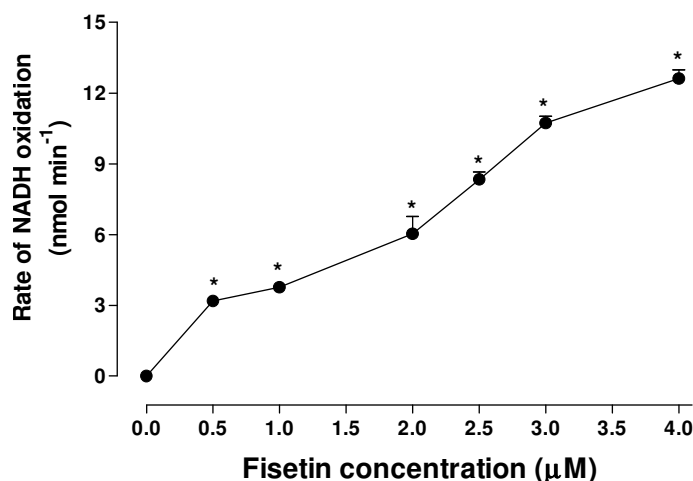


Figure 3. Dependence of the rate of peroxidase catalysed NADH oxidation on fisetin concentration. Reaction conditions: the rate of NADH oxidation was determined at 340 nm in a reaction mixture containing 0.1 M Tris-HCl/1.0 mM EDTA buffer pH 7.4, 0.1 µM horseradish peroxidase (HRP), 200 µM NADH, 25 µM H<sub>2</sub>O<sub>2</sub> and different fisetin concentrations. Values represent mean ± SEM of three to four separate experiments. p refers to the ANOVA with Newman-Keuls test. \**p* < 0.001.

#### 4 CONCLUSION

Fisetin reduced the β-hydroxybutyrate/acetoacetate ratio which is considered as the indicator of the mitochondrial NADH/NAD<sup>+</sup> redox potential. This change in the NADH/NAD<sup>+</sup> ratios was probably the main mechanism by which fisetin reduced ketone bodies production from endogenous fatty acids. It was found that fisetin increased <sup>14</sup>CO<sub>2</sub> production from [1-<sup>14</sup>C]oleate, a finding that could be the result of the increased activity of the tricarboxylic acid cycle. It can be concluded from the results of the present work that the effects of fisetin on fatty acid oxidation in perfused livers from fasted rats were not consequence of its ability to impair mitochondrial oxidative phosphorylation, but was due to its prooxidant activity.

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