Prooxidant Activity of Fisetin: Effects on Energy Metabolism in the Rat Liver

Rodrigo Polimeni Constantin, Jorgete Constantin, Clairce Luzia Salgueiro Pagadigorria, Emy Luiza Ishii-Iwamoto, Adelar Bracht, Cristiane Vizioli de Castro, and Nair Seiko Yamamoto

Laboratory of Liver Metabolism, Department of Biochemistry, University of Maringá, 87020900 Maringá, Brazil; E-mail: *nsyamamoto@uem.br*

Received 22 March 2010; revised 18 June 2010; accepted 7 July 2010

ABSTRACT: Flavonols, which possess the B-catechol ring, as quercetin, are capable of producing *o*-hemiquinones and to oxidize NADH in a variety of mammalian cells. The purpose of this study was to investigate whether fisetin affects the liver energy metabolism and the mitochondrial NADH to NAD^+ ratio. The action of fisetin on hepatic energy metabolism was investigated in the perfused rat liver and isolated mitochondria. In isolated mitochondria, fisetin decreased the respiratory control and ADP/O ratios with the substrates α-ketoglutarate and succinate. In the presence of ADP, respiration of isolated mitochondria was inhibited with both substrates, indicating an inhibitory action on the ATP-synthase. The stimulation of the ATPase activity of coupled mitochondria and the inhibition of NADH-oxidase activity pointed toward a possible uncoupling action and the interference of fisetin with mitochondrial energy transduction mechanisms. In livers from fasted rats, fisetin inhibited ketogenesis from endogenous sources. The β-hydroxybutyrate/acetoacetate ratio, which reflects the mitochondrial NADH/NAD^+ redox ratio, was also decreased. In addition, fisetin (200 μM) increased the production of $^{14}CO_2$ from exogenous oleate. 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. © 2010 Wiley Periodicals, Inc. J Biochem Mol Toxicol 25:117–126, 2011; View this article online at wileyonlinelibrary.com. DOI 10:1002/jbt.20367

KEYWORDS: **Fisetin; Liver; Prooxidant Activity; NADH oxidation, Mitochondria**

INTRODUCTION

Fisetin (3,3′,4′,7-tetrahydroxyflavone; Figure 1) and quercetin (3,5,7,3′,4′-pentahydroxyflavone; Figure 1) are flavonoids widely distributed in fruits and vegetables [1]. 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 the A-ring (Figure 1). The most known biological effect of the flavonoids, including fisetin and quercetin, is no doubt their antioxidant action, which represents protection of tissues against the action of free radicals and diminution of lipid peroxidation [2,3]. However, quercetin, fisetin, and other flavonoids are not exclusively antioxidants [4], since it has been shown that significant protective effects due to flavonoids would only be achieved upon the ingestion of high doses [3]. At high doses, the potentially toxic effects of quercetin and other flavonoids have to be taken into account [5], since these compounds can act as mutagens [6], prooxidants with the generation of free radicals [7–9], and as inhibitors of enzymes involved in energy metabolism and hormone actions [9–12].

It was demonstrated, in the perfused rat liver, that fisetin reduces the lactate to pyruvate ratio, which is an indicator of the cytosolic NADH to NAD^+ ratio [9]. Quercetin also reduced both the cytosolic and the mitochondrial NADH to NAD^+ ratio [13]. The origin of this phenomenon could be the oxidation of NADH in consequence of the interaction of these flavonoids with cellular enzymes such as peroxidases. In fact, it has been shown that peroxidases can generate *o*-hemiquinones from flavonoids and other catechol ring-containing polyphenolics with stoichiometric hydrogen peroxide reduction [6,7,9]. These *o*-hemiquinones can cause NADH oxidation, without oxygen consumption [7]. It is also well known that the liver possesses enzymatic systems capable of producing free radicals from phenolic or polyphenolic compounds [14–17].

Correspondence to: Nair Seiko Yamamoto.

Contract Grant Sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Contract Grant Sponsor: Programa Nacional de Núcleos de Excelência (PRONEX).

Contract Grant Sponsor: Fundação Araucária-Paraná.

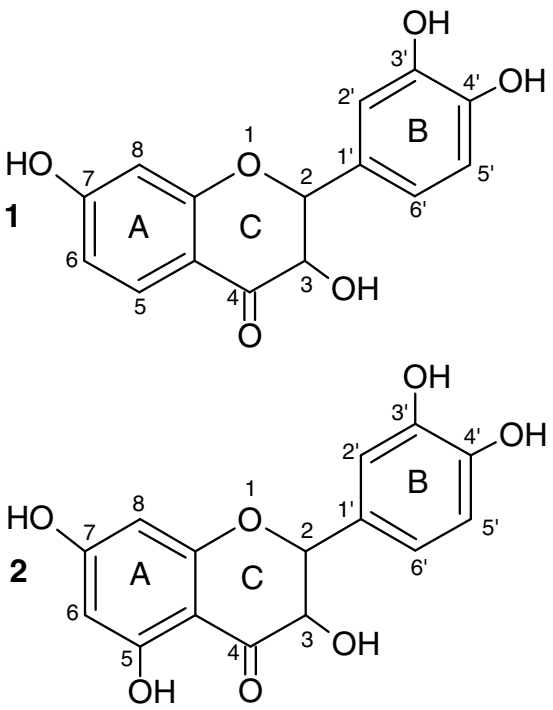


FIGURE 1. Structures of fisetin (**1**) and quercetin (**2**) with numbering scheme and ring labels.

If fisetin changes the cytosolic NADH to NAD^+ ratio because it causes the oxidation of NADH, then it should affect the mitochondrial NADH to NAD^+ ratio like quercetin [13]. The present work was planned to investigate whether fisetin shares with quercetin the ability to affect liver energy metabolism and the mitochondrial NADH to NAD^+ratio. Parameters of energy metabolism were evaluated in isolated mitochondria and also in the isolated perfused rat liver. Taking into account that fatty acids are the main substrate for liver energy metabolism, the oxidation of endogenous and exogenous fatty acid was also measured.

MATERIALS AND METHODS

Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá [18]. The equipment consists of a peristaltic pump (Miniplus 3, Gilson, France), a potentiometric recorder, and a polarograph. Fisetin was purchased from Sigma-Aldrich (St. Louis, MO). Oleate, enzymes, and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Oleate was from New England Nuclear (Boston, MA). All other chemicals were from the best available grade (98–99.8% purity).

Animals

Male Wistar rats (*Rattus novergicus*) weighing 200–280 g, fed with a standard laboratory diet (Nuvital–Nuvilab CR-1) were used in all experiments. Food was withdrawn 24 h prior to the perfusion experiments when necessary. All experiments were conducted in strict adherence to the guidelines of the Ethics Committee for Animal Experiments of the University of Maringá.

Liver Perfusion

For the surgical procedure, the animals were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Hemoglobin-free, nonrecirculating perfusion was undertaken according to the technique described by Scholz and Bücher [19]. After cannulation of the portal and cava veins, the liver was positioned in a Plexiglas chamber. Flow was maintained constant by a peristaltic pump and was adjusted to between 30 and 35 mL min^{-1}, depending on the liver weight. The perfusion fluid was the 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 $NaHCO_3$, 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 $CaCl_2$. Fisetin was dissolved in the perfusion fluid, and its solubilization was achieved by the simultaneous addition of an equivalent amount of 1.0 M NaOH. After this procedure, the pH of the perfusion fluid containing fisetin was adjusted again to 7.4 before its use.

Analytical

When [1-^{14}C]oleate was infused, the effluent perfusion fluid was fractionated at 2 min intervals and collected in Erlenmeyer flasks, which were immediately closed for $^{14}CO_2$ 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 Plexiglass chamber at the exit of the perfusate [20]. Acetoacetate and β-hydroxybutyrate in the outflowing perfusate were measured enzymatically using β-hydroxybutyrate dehydrogenase [21]. Interference by fisetin (absorbance at 340 nm) was excluded by running blanks. The β-hydroxybutyrate to acetoacetate ratio in the liver of fasted rats was utilized as an indicator for the mitochondrial $NADH/NAD^+$ ratio [22,23]. This is

possible because the enzyme β-hydroxybutyrate dehydrogenase is present solely in the mitochondria and also because it operates under near-equilibrium conditions [22]. Direct measurements of the mitochondrial NADH/NAD^+ ratio are very difficult to perform. This highly dynamic parameter is strictly dependent on the cellular conditions, which are changed immediately as a result of organelle isolation. The carbon dioxide production from [1-^{14}C]oleate was measured by trapping $^{14}CO_2$ in phenylethylamine [24]. Radioactivity was measured by liquid scintillation spectroscopy. The following scintillation solution was used: toluene/ethanol (2/1) containing 5 g/L 2.5-diphenyloxazole and 0.15 g/L 2.2-*p*-phenylenebis(5-phenyloxazole).

Mitochondria Isolation and Measurement of the Respiratory Activity

Fed rats, weighing between 200 and 280 g, 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, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mg% fatty acid-free bovine serum albumin. Homogenization was carried out in the same medium by means of a Dounce homogenizer. After homogenization, the mitochondria were isolated by differential centrifugation [25], using a sucrose–mannitol isolation medium and suspended in the same medium, which was kept at 0–4°C. Oxygen consumption by coupled isolated mitochondria was measured polarographically using a Teflon-shielded platinum electrode [20,25]. Mitochondria were added for a final protein concentration around 2 mg mL^{-1} and were incubated in the closed oxygraph chamber in a medium containing 250 mM mannitol, 10 mM KCl, 10 mM tris(hydroxymethyl) aminomethane–HCl (Tris–HCl, pH 7.4), 0.2 mM ethylene glycol tetraacetic acid (EGTA), 5 mM potassium phosphate, and 50 mg% fatty acid free bovine serum albumin. Fisetin was added to the incubation medium as a solution in dimethylformamide (100–600 μM). The substrates were succinate (10 mM) and α-ketoglutarate (10 mM). Appropriate control experiments were performed to exclude solvent effects. ADP, at a final concentration of 125 μM, was added at appropriate times to evaluate the action of fisetin on oxidative phosphorylation. Rates of oxygen consumption were computed from the slopes of the recorder tracings. The ADP/O ratios and the respiratory control ratios (RC) were computed from the recorder tracings according to Chance and Williams [26].

Membrane-Bound Enzymatic Activities

Rat liver mitochondria, isolated as described above [25], were disrupted by successive freeze and thawing procedures using liquid nitrogen and 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. 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 1.0 mM NADH, 10 mM succinate, or 0.2 mM TMPD plus 5 mM ascorbate [27].

ATPase Activity

The ATPase activity was assayed by measuring phosphate release according to Pullman et al. [28]. When intact mitochondria were used as 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. When disrupted mitochondria were incubated, 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 [29]. When coupled mitochondria were assayed, around 1 mg mL^{-1} of mitochondrial protein was used as enzyme source, and when uncoupled or disrupted mitochondria were assayed, around 0.5 mg mL^{-1} of mitochondrial protein was used as enzyme source. Protein contents of all experiments with mitochondria were measured using the method of Lowry et al. [30].

Treatment of Data

The statistical significance of the differences between parameters was evaluated by mean 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.

RESULTS

Effects of Fisetin on Fatty Acids Metabolism and Oxygen Consumption

The results of the experiments in which the actions of fisetin on ketogenesis (β-hydroxybutyrate and acetoacetate productions), oxygen consumption,

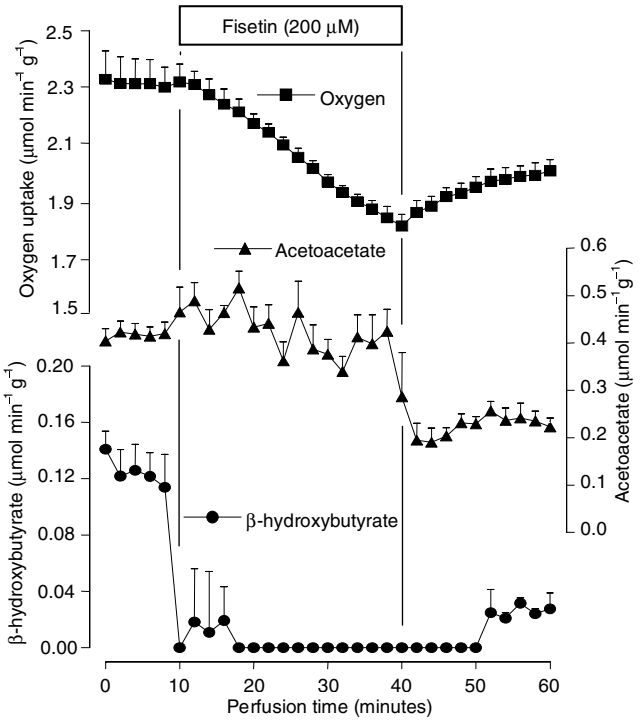


FIGURE 2. Time course of the changes caused by 200 μM fisetin on ketogenesis and oxygen uptake in the isolated perfused liver from fasted rats. Fisetin 200 μM was infused at 10–40 min as indicated by the horizontal bar. The effluent perfusate was sampled in 2-min intervals and analyzed for acetoacetate (▲) and betahydroxybutyrate (●). Oxygen consumption (■) was followed polarographically. Each experimental point is the mean ± SEM of four experiments with identical protocol.

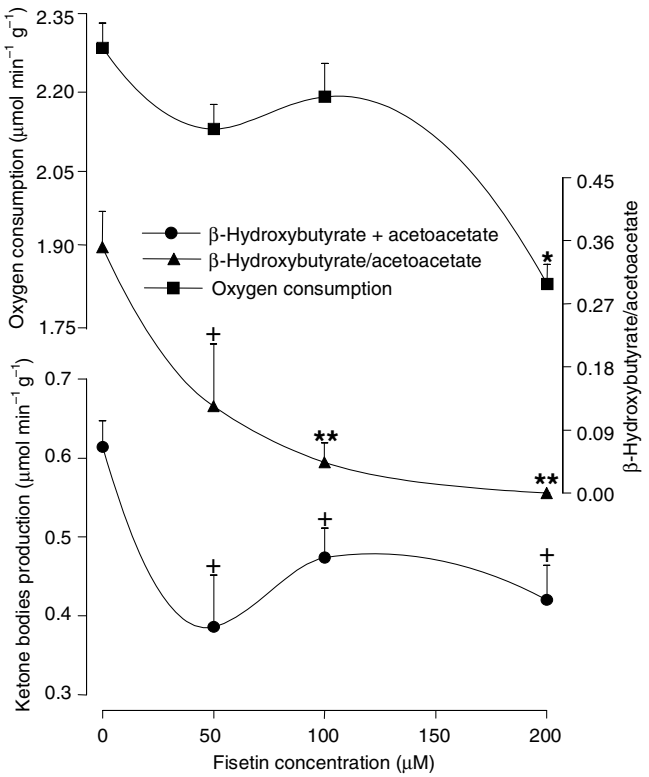


FIGURE 3. Concentration dependences of the effects of fisetin on ketogenesis, oxygen uptake, and β-hydroxybutyrate to acetoacetate ratio (NADH to NAD^+ ratio). Data were obtained from experiments similar to that one illustrated in Figure 2. Values in the absence of fisetin (control values) are the mean 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 and crosses indicate statistical significance in comparison with the control condition as revealed by variance analysis with post hoc Newman–Keuls testing ($^+p < 0.05$, $^{**}p < 0.01$ $^*p < 0.001$).

and $^{14}CO_2$ production were measured are shown in Figures 2–4. Figure 2 illustrates the experimental protocol and shows the time courses of the changes caused by 200 μM fisetin on ketogenesis and oxygen uptake from endogenous fatty acids. Zero time corresponds to the instant at which sampling of the outflowing perfusate was initiated. Ten minutes after initiation of perfusate sampling, the fisetin infusion was started and continued for the next 30 min. The infusion of fisetin at 10 min of perfusion rapidly decreased the production of β-hydroxybutyrate to nondetectable levels. Acetoacetate production apparently was not altered by fisetin. Oxygen consumption was stimulated only slightly and transiently just after starting the infusion. Following this increase, it decreased continuously during the whole infusion period until the fisetin infusion was interrupted. When the infusion of fisetin was interrupted, β-hydroxybutyrate and oxygen consumption showed little tendency toward recovery even after 20 min, but the production of acetoacetate was decreased.

The experimental protocol with 200 μM fisetin illustrated in Figure 2 was repeated with 50 and 100 μM fisetin to evaluate the concentration dependence of the effects. The mean results are summarized in Figure 3. The metabolic rates that were measured at 30 min after starting fisetin infusion were plotted against the perfusate fisetin infusion. The values at zero fisetin concentration correspond to the mean basal rates of all experiments. Figure 3 reveals that oxygen consumption was significantly inhibited only at the concentration of 200 μM fisetin (21%, $p < 0.001$). The total production of ketone bodies (β-hydroxybutyrate + acetoacetate) was reduced 36.6%, 29.6%, and 31.4%, respectively, with 50, 100, and 200 μM fisetin ($p < 0.05$). The most pronounced effect was that on the β-hydroxybutyrate to acetoacetate ratio, which was reduced in a concentration-dependent manner, decreasing from 0.35 ± 0.05 to a nonmeasurable value, at 200 μM. Half-maximal reduction can be expected at a concentration of 37.01 ± 4.47 μM, as revealed by numerical interpolation.

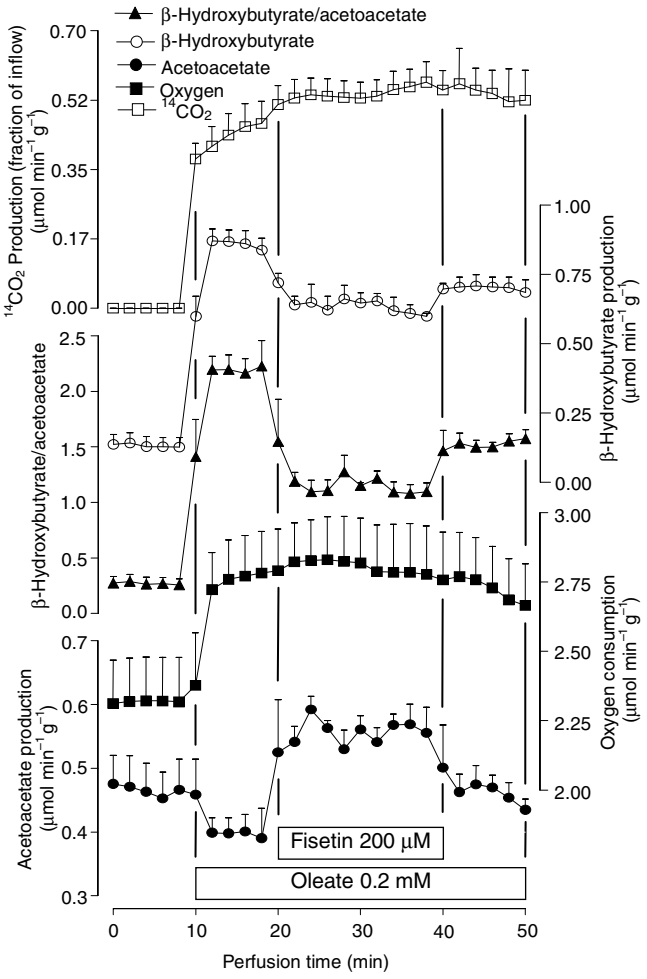


FIGURE 4. Time course of the changes caused by 200 μM fisetin on ketogenesis, oxygen uptake, β-hydroxybutyrate to acetoacetate ratio (NADH to NAD^+ ratio), and $^{14}CO_2$ production in the perfused rat liver. Livers from fasted rats were perfused with Krebs/Henseleit-bicarbonate buffer (pH 7.4), as described in the Experimental section. [^{14}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 $^{14}CO_2$. Oxygen consumption was followed polarographically. Each experimental point is the mean ± SEM of four experiments with identical protocol.

Ketogenesis from endogenous sources was partly inhibited by fisetin at concentrations up to 200 μM, but ketogenesis from exogenous oleate was not modified by fisetin. Figure 4 illustrates the action of 200 μM fisetin on the oxidation of exogenous oleate. Oleate (0.2 mM) infusion was started after a short preperfusion of 10 min and continued during all over the experiment. Fisetin was introduced after stabilization of the metabolic changes caused by the long-chain fatty acid at 10 min. The introduction of the perfusion fluid containing 0.2 mM oleate and [1-^{14}C]oleate resulted in [^{14}C]CO_2 production and an accentuated enhancement in oxygen consumption and ketone bodies production. The subsequent introduction of fisetin decreased the β-hydroxybutyrate production (26%, $p < 0.01$) and increased the acetoacetate production (37%, $p < 0.05$), but the total ketone bodies production was not inhibited. There were no significant effects on oxygen consumption. The production of $^{14}CO_2$ was also increased by fisetin by about 25% ($p < 0.05$), and the β-hydroxybutyrate/acetoacetate ratio was decreased from 2.196 ± 0.018 to 1.088 ± 0.004, which corresponds to a reduction of approximately 50% ($p < 0.001$).

Effects of Fisetin on Isolated Mitochondria

If the action of fisetin on hepatic oxygen consumption is of mitochondrial origin, it should be possible, in principle at least, to reproduce these effects in isolated mitochondria. With this purpose in mind, the influence of fisetin on respiration of isolated rat liver mitochondria was measured using two different substrates as electron donors. As shown in Table 1, these substrates were succinate (FAD dependent) and α-ketoglutarate (NAD^+ dependent). The mitochondrial respiration driven by the oxidation of these substrates was measured in isolated rat liver mitochondria incubated in the absence of exogenous ADP (substrate respiration), the presence of exogenous ADP (state III respiration), and after the exhaustion of the exogenously added ADP (state IV respiration) in the absence and presence of several fisetin concentration.

The data shown in Table 1 reveal that mitochondrial respiration before ADP addition (substrate respiration) and after exhaustion of ADP (state IV respiration) was increased by fisetin in a dose-dependent manner only with the FAD-dependent substrate (succinate). The mitochondrial respiration driven by both substrates in the presence of ADP (state III respiration) was clearly decreased in a concentration-dependent manner by fisetin. The inhibitory effect was more pronounced with the NAD^+-dependent substrate, and it reached 42% ($p < 0.001$) at the concentration of 300 μM; whereas with succinate the inhibition was about 23.5% ($p < 0.05$). Furthermore, the respiratory control was abolished at high concentrations using the NAD^+-dependent substrate, and almost abolished when the FAD-dependent substrate was utilized, as can be judged from the state IV respiratory rates and from the respiratory control ratios (state III/state IV). Table 1 also shows the effects of fisetin on the ADP/O ratios. With the NAD^+-dependent substrate, no respiratory control was found at the concentration of 500 μM. At this concentration, evidently, no ADP/O ratio could be evaluated, but with succinate the ADP/O ratio was significantly decreased only at higher drug

TABLE 1. Action of Fisetin on Mitochondrial Respiration Driven by α-Ketoglutarate (α-KG) and Succinate in the Presence and Absence of Exogenously Added ADP

	Fisetin (μM)	*Substrate Respiration*	*State III Respiration*			
Substrate		*(μmol O_2 min^{-1} mg $protein^{-1}$)*		*State IV Respiration*	*Respiratory Control Ratio*	*ADP/O Ratio*
α-KG ($n = 6$)	0	4.58 ± 0.29	17.27 ± 1.68	4.95 ± 0.31	3.53 ± 0.27	2.48 ± 0.06
	100	4.12 ± 0.32	13.84 ± 1.25^{+}	5.57 ± 0.54	2.50 ± 0.11*	2.16 ± 0.09^{+}
	200	3.51 ± 0.63	10.51 ± 0.80*	5.29 ± 0.31	1.98 ± 0.09*	1.82 ± 0.10*
	300	4.17 ± 0.54	10.02 ± 1.44*	6.28 ± 0.66	1.57 ± 0.07*	1.57 ± 0.08*
	400	4.50 ± 0.42	8.47 ± 0.93*	6.91 ± 0.59	1.22 ± 0.06*	1.00 ± 0.21*
	500	3.76 ± 0.70	7.31 ± 0.90*	7.31 ± 0.90	1.00 ± 0.00*	–
	600	2.04 ± 0.23	5.04 ± 0.68*	5.04 ± 0.68	1.00 ± 0.00*	–
Succinate ($n = 6$)	0	13.94 ± 1.46	62.95 ± 6.35	12.61 ± 1.09	5.10 ± 0.18	1.77 ± 0.08
	100	16.88 ± 1.87	64.24 ± 5.45	17.50 ± 1.68^{+}	3.69 ± 0.09*	1.73 ± 0.09
	200	19.30 ± 1.08^{+}	57.11 ± 4.19	19.88 ± 1.40**	2.87 ± 0.07*	1.59 ± 0.09
	300	18.44 ± 1.34	48.10 ± 2.99^{+}	20.88 ± 1.19**	2.31 ± 0.10*	1.50 ± 0.08
	400	21.57 ± 0.64**	43.49 ± 1.52**	22.91 ± 1.52*	1.93 ± 0.12*	1.41 ± 0.09
	500	24.62 ± 1.04*	40.88 ± 1.25**	27.82 ± 0.89*	1.47 ± 0.04*	1.07 ± 0.07*
	600	26.01 ± 1.43*	34.96 ± 2.55*	26.62 ± 1.93*	1.31 ± 0.03*	1.06 ± 0.11*

Data are the mean ± standard errors of six experiments with identical protocol. Statistical significance relative to the controls is indicated by asterisks and crosses. $^{+}p < 0.05$, $^{**}p < 0.01$, $^{*}p < 0.001$, ANOVA with the Newman–Keuls test.

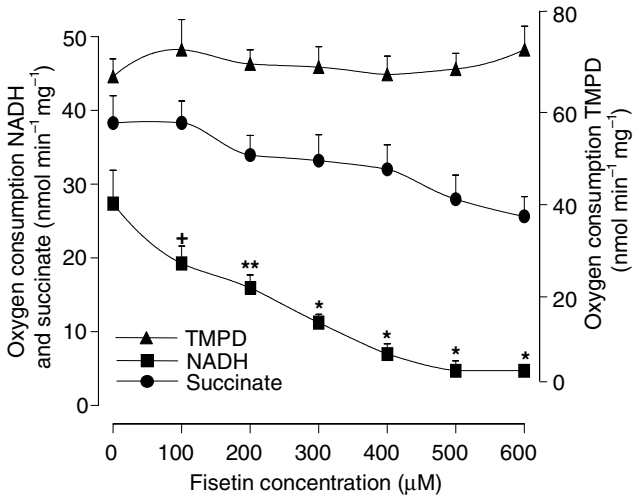


FIGURE 5. Effects of fisetin on several membrane-bound enzymatic activities in rat liver mitochondria. NADH-oxidase, succinate-oxidase activities, and TMPD-ascorbate oxidation were measured with freeze–thawing disrupted mitochondria, incubated at 37°C in reaction medium as described in the Experimental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

concentrations. On the other hand, with succinate, significant effects were seen only at the highest fisetin concentrations (500 and 600 μM).

Effects of Fisetin on Membrane-Bound Enzymatic Activities

The effects observed in isolated mitochondria indicate that fisetin is able to affect mitochondrial energy metabolism acting through different mechanisms. To evaluate whether these effects are related to the action of the flavonoid on the electron flow, the effects of several concentrations of fisetin on the NADH-oxidase activity, succinate-oxidase activity, and TMPD-ascorbate oxidation were measured in disrupted mitochondria and the mean values are shown in Figure 5. Neither succinate-oxidase activity nor TMPD-ascorbate oxidation was significantly affected; however, the NADH-oxidase activity was inhibited in a dose-dependent manner. The mean concentration producing 50% inhibition (ID_{50}) of the NADH-oxidase activity was 242.80 ± 20.32 μM, and the maximal inhibition of the NADH-oxidase activity exceeded 82% ($p < 0.001$).

Effects of Fisetin on the ATPase Activity

The effects of fisetin 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) and in freeze–thawed disrupted mitochondria, as shown in Figure 6. The actions of fisetin were different in each preparation. The ATPase activity of coupled mitochondria was increased almost over the whole concentration range of fisetin, reaching a stimulation of about 120% with concentrations ranging from 200 to 600 μM ($p < 0.05$). The ATPase activity of uncoupled and disrupted mitochondria, on the other hand, was inhibited. This inhibition was significant within the concentration range of 300–600 μM for both mitochondrial preparations. When disrupted or uncoupled mitochondria were used as the enzyme source, the ATPase activity was inhibited

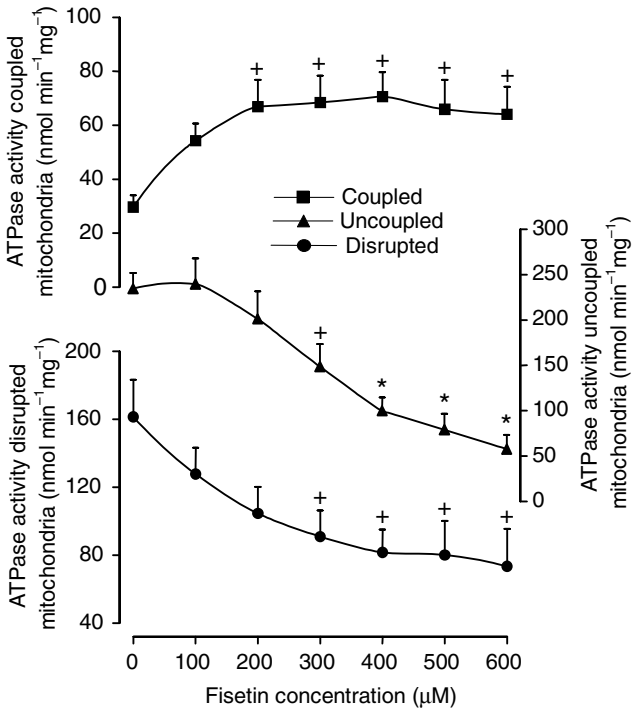


FIGURE 6. Effects of fisetin on the ATPase activity of coupled, uncoupled, and disrupted mitochondria. The mitochondria were incubated at 37°C in reaction medium as described in the Experimental section. Each assay point represents the mean of six (coupled mitochondria and uncoupled mitochondria) and five (disrupted mitochondria) independent experiments. $^{+}p < 0.05$, $^{*}p < 0.001$, ANOVA with Newman–Keuls test.

43.6% ($p < 0.05$) and 36.7% ($p < 0.05$), respectively, by 300 μM fisetin.

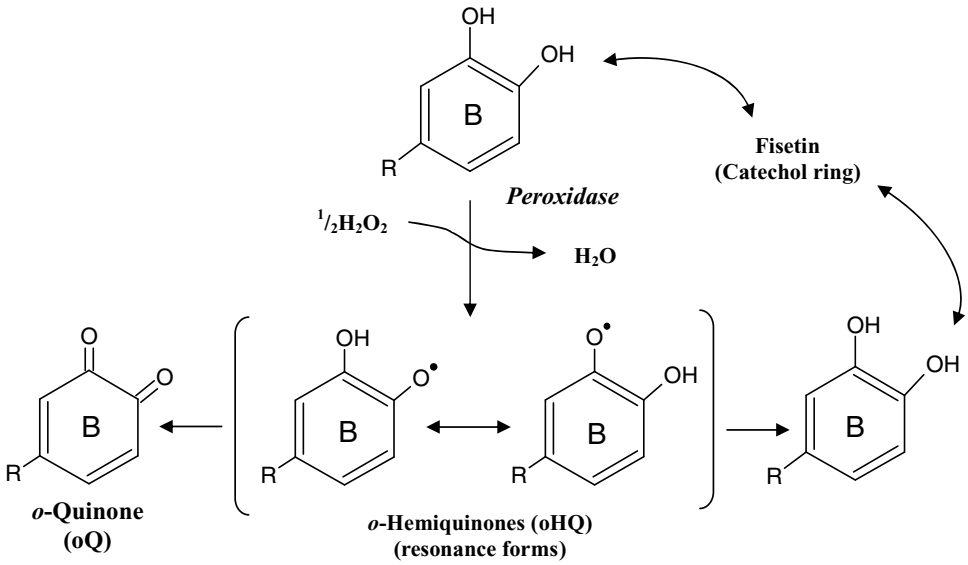
DISCUSSION

The results of the present work reveal that fisetin decreases the mitochondrial NADH to NAD^+ ratio, as indicated by the reduced β-hydroxybutyrate to acetoacetate ratio in the perfused rat liver. This action was already pronounced at relatively low fisetin concentrations (half-maximal action at 37.01 μM), and it is consistent with previous reports about a similar reduction of the cytosolic NADH/NAD^+ ratio provoked by fisetin [9].

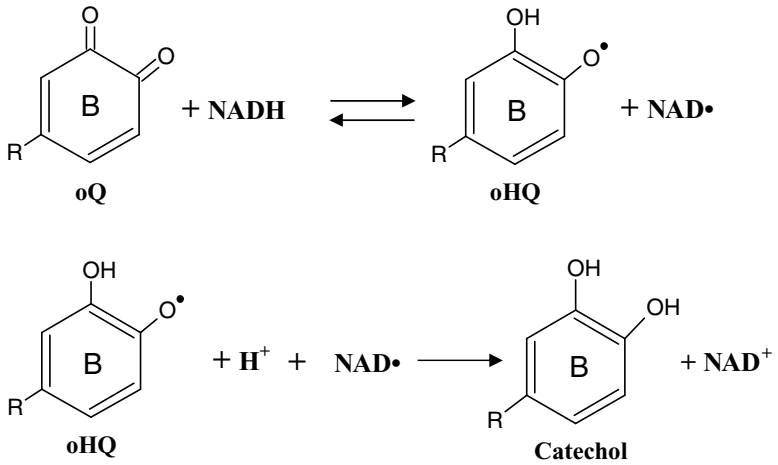
It was also recently demonstrated that flavonoids such as quercetin and fisetin are able to oxidize NADH in the presence of horseradish peroxidase (HRP) and stoichiometric amounts of hydrogen peroxide [7,9]. This phenomenon was not accompanied by oxygen consumption, suggesting that two electrons are transferred to the flavonoid metabolite [7]. This enzymatic action results in products as *o*-hemiquinones (free radicals) and *o*-quinones in the presence of hydrogen peroxide as illustrated in Figure 7 [6,31–35]. The participation of hepatic catalase, which functions as a peroxidase at low H_2O_2 concentrations, has been suggested [13,15]. It has also been proposed that oxidation of quercetin by the enzyme lipoxygenase is equally able to produce prooxidant species in the presence of hydrogen peroxide [36]. Evidence that this phenomenon may occur in the living cell was obtained in perfused rat liver, here in this work, and in previous studies [9,10,13].

The action of fisetin on the NADH-NAD^+ redox potential was accompanied by a diversity of effects. The change in the NADH/NAD^+ ratio was probably the main mechanism by which fisetin reduced ketone bodies production from endogenous fatty acids. As shown previously by experiments with isolated mitochondria [37], ketogenesis decreases with lower NADH/NAD^+ ratios, because the near equilibrium of the 3-hydroxyacyl-CoA dehydrogenase is shifted toward acetoacetyl-CoA, which inhibits acetyl-CoA acetyltransferase. The same phenomenon favors the citric acid cycle because a less reduced state of the NADH/NAD^+ couple shifts the near-equilibrium catalyzed by L-malate dehydrogenase in the direction of oxaloacetate, the acceptor of acetyl CoA in the reaction of citrate synthase [37,38]. In agreement to this hypothesis, it was found that 200 μM fisetin increased $^{14}CO_2$ production from [1-^{14}C]oleate, a finding that could be the result of the increased activity of the tricarboxylic acid cycle [37]. On the other hand, oxygen consumption was inhibited by 200 μM fisetin in the absence of exogenous fatty acids. This may have two causes: the diminished NADH concentration in the mitochondria and the inhibition of ADP phosphorylation. The slight tendency of stimulation of respiration, which occurred in the presence of exogenous oleate, is probably the consequence of the uncoupling action that superimposes on the inhibition of coupled respiration [10]. It is important to report that there are some differences between the effects of fisetin on ketone bodies production from endogenous and exogenous fatty acids. Despite the prooxidant effects of fisetin that appear in both cases, as shown by the reduction of the mitochondrial NADH/NAD^+ratios, the effect was more pronounced on the ketone bodies production from endogenous fatty acids. This phenomenon can be explained by the higher reducing power established by the introduction of 0.2 mM oleate.

The data obtained with isolated mitochondria indicate that the direct actions of fisetin on mitochondrial energy metabolism were not the primary cause of the alterations observed in liver fatty acid metabolism. The uncoupling effect of fisetin on oxidative phosphorylation was indicated by its effects on mitochondrial respiration and ATPase activity, namely stimulation of



I. Formation of ortoquinones



II. NADH oxidation

FIGURE 7. Possible mechanisms for ortoquinones and *o*-hemiquinone formation and subsequent NADH oxidation (Chan et al., 2003).

state IV respiration with succinate, a decrease in the respiratory control ratio, and an increase in ATP hydrolysis in intact and coupled mitochondria [39,40]. The decrease in ADP/O ratios with both substrates (FAD and NAD^+ dependent) and the effects of fisetin on the ATPase activity of uncoupled and disrupted mitochondrial preparations suggest that, in addition to the uncoupling action, the flavonoid could also act as a direct inhibitor of ATP-synthase [10].

The uncoupling effect of fisetin did not appear when the substrate NAD^+ dependent was utilized, probably due to the competition for NADH established between the respiratory chain and the fisetin metabolites, anticipating the NADH oxidation. This possibly occurs because mitochondrial preparations obtained by differential centrifugation contain peroxisomes and microsomes, with peroxidases activities. Thus, *o*-hemiquinones can be produced in the respiratory chain. This effect was corroborated by the effects of fisetin on membrane-bound enzymatic activities, where fisetin did not affect succinate-oxidase activity or TMPD-ascorbate oxidation but inhibited NADH-oxidase activity in a dose-dependent manner. The flavonoids, including quercetin, also revealed capacity of autooxidation, generating O_2^- and H_2O_2 in isolated mitochondria [8]. However, the data found in

rat liver perfusion provided evidence that the energy metabolism of intact cells was not affected to the same extent. It should be remarked that the intact liver differs from the system containing isolated mitochondria. While in an incubation system with isolated mitochondria the drug 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 or binding to intracellular components. The inhibition of oxygen consumption along with the decrease in ketogenesis and the increase in ^{14}CO production were actions consistent with the prooxidant actions of fisetin reported above [6].

It is important to remark that the concentrations employed here were those used in most studies. Such concentrations, however, are impossible to reach when fisetin is ingested in the normal diet [41]. The plasma concentrations of quercetin and other flavonoids are relatively low upon oral administration, and it has been speculated that for humans a daily dose of 1500 mg is necessary to attain plasma concentrations above 10 μM [42]. The action of fisetin on the mitochondrial NADH/NAD^+ ratio was already pronounced at low fisetin concentrations (half-maximal action at 37.01 μM). It has been claimed that the use of fisetin in cancer treatment requires plasma concentrations between 10 and 120 μM because this is the range for which the strongest tumor growth inhibitory effects of the compound have been reported [43–46]. There is a good overlap of the fisetin concentrations, which can reduce the hepatic NADH/NAD^+ ratio, and those which have antitumor effects. In humans, such concentrations can only be achieved by means of endovenous administration [41,47,48].

In conclusion, it is likely that fisetin is able to induce a more oxidized state in the liver cells and affect energy metabolism, acting through mechanisms similar to the demonstrated for quercetin [13]. At least under the conditions of the isolated perfused rat liver, the prooxidant effects of fisetin seem to predominate over its antioxidant effects. Furthermore, especially in the liver, the prooxidant effects are possibly combined with other effects such as inhibition of gluconeogenesis [9]. The prooxidant activity would be advantageous when the anticancer and apoptosis induction properties of flavonoids are considered, since reactive oxygen species can mediate apoptotic DNA fragmentation [49,50]. Prooxidant phenoxyl radicals could also cause mitochondrial toxicity by collapsing the mitochondrial membrane potential [51]. However, the net effects of fisetin could be different in different tissues, and only an overall analysis based on in vivo studies can determine the physiological significance of the hepatotoxic effects of this flavonoid for the whole organism.

REFERENCES

1. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr 2000;130:2243–2250.
2. Bors W, Heller W, Michel C, Saran M. Flavonoids as antioxidants: determination of radical-scavenging efficiencies. Methods Enzymol 1990;186:343–355.
3. Breinholt V, Lauridsen ST, Dragsted LO. Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes in female rats. Xenobiotica 1999;29:1227–1240.
4. Kawanishi S, Oikawa S, Murata M. Evaluation for safety of antioxidant chemopreventive agents. Antioxid Redox Signal 2005;7:1728–1739.
5. Skibola CF, Smith MT. Potential health impacts of excessive flavonoid intake. Free Radic Biol Med 2000;29:375–383.
6. Metodiewa D, Jaiswal AK, Cenas N, Dickancaite E, Segura-Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 1999;26:107–116.
7. Chan T, Galati G, O'Brien PJ. Oxygen activation during peroxidase catalysed metabolism of flavones or flavanones. Chem Biol Interact 1999;122:15–25.
8. Constantin J, Bracht A. Quercetin, mechanisms of anti- and prooxidant activities. In: Sing K, Govil JN, editors. Recent progress in medicinal plants, volume 21: Phytopharmacology and therapeutic values III. Houston, TX: Studium Press; 2008. pp 39–61.
9. Constantin RP, Constantin J, Salgueiro-Pagadigorria CL, Ishii-Iwamoto EL, Bracht A, Ono MKC, Yamamoto NS. The actions of fisetin on glucose metabolism in the rat liver. Cell Biochem Funct 2010;28:149–158.
10. Gasparin FR, Salgueiro-Pagadigorria CL, Bracht L, Ishii-Iwamoto EL, Bracht A, Constantin J. Action of quercetin on glycogen catabolism in the rat liver. Xenobiotica 2003;33:587–602.
11. Gasparin FR, Spitzner FL, Ishii-Iwamoto EL, Bracht A, Constantin J. Action of quercetin on gluconeogenesis and glycolysis in the rat liver. Xenobiotica 2003;33:903–911.
12. Kato A, Nasu N, Takebayashi K, Adachi I, Minami Y, Sanae F, Asano N, Watson, AA, Nash RJ. Structure–activity relationships of flavonoids as potential inhibitors of glycogen phosphorylase. J Agric Food Chem 2008;56:4469–4473.
13. Buss GD, Constantin J, Lima LC, Teodoro GR, Comar JF, Ishii-Iwamoto EL, Bracht A. The action of quercetin on the mitochondrial NADH to NAD^+ ratio in the isolated perfused rat liver. Planta Med 2005;71:1118–1122.
14. Aebi H. Catalase. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press; 1974. pp 673–684.
15. Püter J. Peroxidases. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press; 1974. pp 685–690.
16. Mason RP, Fischer V. Free radicals of acetaminophen: their subsequent reactions and toxicological significance. Fed Proc 1986;45:2493–2499.
17. Komatso H, Koo A, Ghadishah E, Zeng H, Kuhlenkamp JF, Inoue M. Neutrophil accumulation in ischemic reperfused rat liver: evidence for a role for superoxide free radicals. Am J Physiol 1992;262:G669–676.

18. Kelmer-Bracht AM, Ishii-Iwamoto EL, Andrade PV, Bracht A. Construction of a liver perfusion apparatus for studies on metabolic regulation and mechanisms of drug action. Braz Arch Biol Technol 1984;27:419–438.
19. Scholz R, Bücher T. Hemoglobin-free perfusion of rat liver. In: Chance B, Estabrook RW, Williamson JR, editors. Control of energy metabolism. New York: Academic Press; 1965. pp 393–414.
20. Clark LM. Monitoring and control of blood O_2 tension. Trans Am Soc Artif Intern Organs 1956;2:41–49.
21. Mellanby J, Williamson, DH. Acetoacetate. In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York: Academic Press; 1974. pp 1840–1843.
22. Sies H. Nicotinamide nucleotide compartmentation. In: Sies S, editor. Metabolic compartmentation. New York: Academic Press; 1982. pp 205–231.
23. Kimura RE, Warshaw JB. Control of fatty acid oxidation by intra-mitochondrial [NADH]/[NAD^+] in developing rat small intestine. Pediatr Res 1988;23:262–265.
24. Scholz R, Olson MS, Schwab AJ, Schwabe U, Noell CH, Braun W. The effect of fatty acids on the regulation of pyruvate dehydrogenase in perfused rat liver. Eur J Biochem 1978;86:519–530.
25. Voss DO, Campello SP, Bacila M. The respiratory chain and the oxidative phosphorylation of rat brain mitochondria. Biochem Biophys Res Commun 1961;4: 48–51.
26. Chance B, Williams GR. A simple and rapid assay of oxidative phosphorylation. Nature 1955;175:1120–1121.
27. Singer TP. Determination of activity of succinate, NADH, choline and alpha-glycerophosphate dehydrogenases. Methods Biochem Anal 1974;22:123–175.
28. Pullman ME, Penefski HS, Datta A, Racker E. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphatase. J Biol Chem 1960;235:3322–3329.
29. Fiske CH, Subbarow YJ. The colorimetric determination of phosphorus. Biol Chem 1925;66:375–400.
30. Lowry OH, Rosebrough NJ, Farr AC, Randall RJJ. Protein measurement with the Folin phenol reagent. Biol Chem 1951;193:265–275.
31. Laughton MJ, Halliwell B, Evans PJ, Hoult JR. Antioxidant and prooxidant actions of the plant phenolics quercetin, gossypol and myrecetin. Effects on lipid peroxidation, hydroxyl radical generation and bleoycin-dependent damage to DNA. Biochem Pharmacol 1989;38:2859–2865.
32. Chan TS, Galati G, Pannala AS, Rice-Evans C, O'Brien PJ. Simultaneous detection of the antioxidant and prooxidant activity of dietary polyphenolics in a peroxidase system. Free Radic Res 2003;37:787–794.
33. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J, Rietjens IMCM. Structure–activity study on the quinone/quinone methide chemistry of flavonoids. Chem Res Toxicol 2001;14:398–408.
34. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J, Rietjens IMCM. The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent. Chem Res Toxicol 2002;15:343–351.
35. Awad HM, Boersma MG, Boeren S, van der Woude H, van Zanden J, van Bladeren PJ, Vervoort J, Rietjens IMCM. Identification of *o*-quinone/quinone methide metabolites of quercetin in a cellular in vitro system. FEBS Lett 2002;520:30–34.
36. Pinto MC, Macias P. Oxidation of dietary polyphenolics by hydroperoxidase activity of lipoxygenase. J Agric Food Chem 1995;53:9225–9230.
37. Stermann WH, Holze G, Seubert W. Regulation of ketogenesis at the site of acetyl-CoA acetyl transferase. In: Söling HD, Seufert CD, editors. Biochemical and Clinical Aspects of Ketone Body Metabolism. Stuttgard: Geor Thieme Publishers; 1978. pp 11–23.
38. Bücher T, Sies H. Metabolic interactions of mitochondrial and cytosolic systems in rat liver. In: Nover L, Lynen F, Mothes K, editors. Cell Compartmentation and Metabolic Channeling. Jena-Amsterdam: Gustav Fischer Verlag; 1980. pp 279–301.
39. Itinose AM, Doi-Sakuno ML, Bracht A. Metabolic effects of acetaminophen. Studies in the isolated perfused rat liver. Cell Biochem Funct 1989;7:263–273.
40. Acco A, Comar JF, Bracht A. Metabolic effects of propofol in the isolated perfused rat liver. Basic Clin Pharmacol Toxicol 2004;95:166–174.
41. Shia CS, Tsai SY, Kuo SC, Hou YC, Chao PD. Metabolism and pharmacokinetics of 3,3′,4′,7-tetrahydroxyflavone (fisetin), 5-hydroxyflavone, and 7-hydroxyflavone and antihemolysis effects of fisetin and its serum metabolites. J Agric Food Chem 2009;57:83–89.
42. Lamson DW, Brignall MS. Antioxidants and cancer, part 3: quercetin. Altern Med Rev 2000;5:196–208.
43. Lu X, Jung J, Cho HJ, Lim DY, Lee HS, Chun HS, Kwon DY, Park JH. Fisetin inhibits the activities of cyclin-dependent kinases leading to cell cycle arrest in HT-29 human colon cancer cells. J Nutr 2005;135:2884–2890.
44. Khan N, Afaq F, Syed DN, Mukhtar H. Fisetin, a novel dietary flavonoid, causes apoptosis and cell cycle arrest in human prostate cancer LNCaP cells. Carcinogenesis 2008;29:1049–1056.
45. Suh Y, Afaq F, Johnson JJ, Mukhta H. A plant flavonoid fisetin induces apoptosis in colon cancer cells by inhibition of COX2 and Wnt/EGFR/NF-κB-signaling pathways. Carcinogenesis 2009;30:300–307.
46. Lee WR, Shen SC, Lin HY, Hou WC, Yang LL, Chen YC. Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase 3 and Ca^{2+}-dependent endonuclease. Biochem Pharmacol 2002;63:225–236.
47. Ferry DR, Smith A, Malkhandi J, Fyfe DW, de Takats PG, Anderson D. Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosinase kinase inhibition. Clin Cancer Res 1996;2:659–668.
48. Gugler R, Leschik M, Dengler HJ. Dispositon of quercetin in man after single oral and intravenous doses. Eur J Clin Pharmacol 1975;9:229–234.
49. Rahman A, Shahabuddin, Hadi SM, Parish JH. Complexes involving quercetin, DNA and Cu(II). Carcinogenesis 1990;11:2001–2003.
50. Bhat KP, Pezzuto JM. Cancer chemopreventive activity of resveratrol. Ann N Y Acad Sci 2002;957:210–229.
51. Cenas NK, Kanapieniene JJ, Kulys JJ. NADH oxidation by quinone electron acceptors. Biochim Biophys Acta 1984;767:108–112.