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Some Aspects of the *in vivo* Neuroprotective Capacity of Flavonoids: Bioavailability and Structure-Activity Relationship

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On the basis of previous work showing that flavonoids structurally related to quercetin are neuroprotective for cells in culture, this work was directed towards determining if several flavonoids (quercetin, fisetin and catechin) could acutely and by an intraperitoneal (IP) route reach significant cerebral concentrations and either prevent or facilitate recovery from a brain lesion induced by focal ischemia in rats.

Aqueous and liposomal preparations of quercetin, fisetin and catechin were administered IP in a single dose and assessed in the brain by HPLC at 30 min, 1 h, 2 h and 4 h. Ischemic damage from focal middle cerebral artery occlusion was assessed spectrophotometrically with 2,3,5,-triphenylltetrazolium chloride (TTC). Infarct volume was assessed by an image analysis system following perfusion with TTC. The status of the cerebral tissue was evaluated by hematoxylin-eosin.

Flavonoids administered in aqueous preparations were undetected in the brain. Cerebral concentrations of catechin (10.5 ng/g), fisetin (8.23 ng/g) and quercetin (509 ng/g) were detected in the brain only after IP injection of the liposomal preparations.

Spectrophotometric analysis of brain tissue with the TTC-technique showed that liposomal quercetin reduced ischemic damage and infarct volume after permanent occlusion of the middle cerebral artery (ischemic: 41.3 mm^3 *vs* liposomal quercetin: 17 mm^3). In liposomal quercetin-treated animals there was also recovery of the cytoarchitecture in ischemic areas of striatum and cortex. Although a liposomal preparation of fisetin had similar effects, catechin failed to protect brain tissue.

In conclusion, early administration of liposomal preparations of quercetin and structurally related flavonoids are beneficial and neuroprotective in experimental focal ischemia.

Keywords: Quercetin; Fisetin; Catechin; Neuroprotection; Permanent focal ischemia;

INTRODUCTION

Cerebral pathology in the form of cerebrovascular and neurodegenerative diseases is a leading cause of death throughout the world (Kolominsky-Rabas *et al.*, 1998; Leppala *et al.*, 1999). During cerebral ischemia there is a characteristic fall in blood flow to the brain, triggering a cascade of events that ends in nerve cell death. Oxidative stress appears to be a final common pathway for all those events, and reactive oxygen species have been implicated in many studies as important contributors to ischemic cell death (Liu *et al.* 1989; Kinouchi et al, 1991; Cao and Phillis, 1994; Yu *et al.*, 1998). Hence, interference with the oxidative process, using antioxidants, has always been considered an appropriate neuroprotective strategy.

In recent years there has been an increased interest in flavonoids, a widely distributed group of naturals antioxidants compounds. These compounds are ubiquitous plant molecules that are involved in the control of different aspects of the oxidative process, such as scavenging radical ions (Huk *et al.*, 1998; Ishige *et al.*, 2001) or as anti-lipoperoxidation compounds (Ratty and Das, 1988). The well known beneficial effects on cardiovascular risk of the Mediterranean diet have been correlated to its high content in polyphenols, mainly

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flavonoids (Bastianetto and Quiron, 2002; Esposito *et al.*, 2002). Also, flavonoids have positive effects on cardiovascular events in open clinical trials (Emmert and Kirchner, 1999).

Quercetin, the most common flavonoid, is an important dietary constituent present in tea, onions and apple skins (Hertog, 1996). The antioxidant activity of quercetin may result from direct scavenging of free radicals and other oxidizing intermediates, or from the chelation of iron or copper ions, and from inhibition of oxidases. Quercetin also activates glutathione peroxidase (Nagata *et al.*, 1999), prevents dehydroascorbic acid-induced glutathione depletion in rabbit red blood cells (Fiorani *et al.*, 2001), and inhibit hydrogen peroxide-induced oxidative damage (Aherne and O´Brien, 1999; Sanderson *et al.*, 1999). At the cellular level quercetin inhibits the activity of enzymes related to key intracellular oxidative and signaling processes: xanthine oxidase (Sanhueza *et al.*, 1992; Nagao *et al.*, 1999) protein kinase C (Ferriola *et al.*, 1989; Picq *et al.*, 1989) and phosphatidylinositol 3- kinase (Prajda *et al.*, 1995; Agullo *et al.*, 1997) and reverses the decreased levels of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, catalase and superoxide dismutase (SOD) (Erden Inal *et al.*, 2001).

We recently demonstrated that after an oxidative insult to cells and neurons in culture, quercetin showed marked neuroprotective activity, and this was shared by a group of structurally related flavonoids such as fisetin and myricetin (Dajas *et al.*, 2003). In the same study, preliminary results also showed that quercetin could be acutely neuroprotective *in vivo* if provided with an adequate carrier (Dajas *et al.*, 2003a,b).

It is generally accepted that the accumulation of flavonoids in brain and their potential bioactivity may be restricted by the blood-brain barrier (BBB), and that their potential for permeation depends on their bioavailability and lipophilicity (Mandel, 2003; Youdim *et al.*, 2003). Chronic treatments appear to be necessary to obtain therapeutic concentrations in the brain after oral administration of flavonoids. Thus, the oral administration of catechin during 2 weeks protected against ischemia-reperfusion-induced neuronal death in the gerbil (Inanami *et al.*, 1998), and the flavonoid crataegus administrated also orally to gerbils, showed neuroprotective effects (Zhang *et al.*, 2004). In contrast, when the flavonoid rutin was intravenously administrated to mice before and after ischemia/reperfusion, rutin was able to prevent the cerebral damage caused by the ischemic process (Gupta *et al.*, 2003). Reports on the neuroprotective effects of quercetin have also utilized chronic administration for some weeks and by an oral route. Hence, quercetin treatment for 30 days reverses cognitive deficits in aged and ethanol-intoxicated mice (Singh *et al.*, 2003), and quercetin for a period of 4 weeks significantly and dose-dependently reduced reserpine-induced vacuous chewing movements and tongue protrusions in reserpine-treated animals (Naidu *et al.*, 2004). After 21 days quercetin also proved to be a useful therapeutic agent in neuroleptic-induced orofacial dyskinesia (Naidu *et al.*, 2003).

Assessing preliminarily the potential of the *in vitro* neuroprotective actions of quercetin for the acute treatment of stroke, we observed that aqueous quercetin was ineffective and that a liposomal-quercetin preparation appeared to confer protection against experimental ischemia (Dajas *et al.*, 2003). Liposomes are recognized as an effective tool utilized in drug-carrier technology, enhancing the therapeutic efficacy of given molecules (Gregoriadis, 1995; Kanno *et al.*, 1999; Ueoka *et al.*, 2000). It has been demonstrated that liposomes are effective carriers of active molecules in the blood and into the brain (Azuma *et al.* 2002), delaying metabolism of encapsulated molecules, facilitating their crossing the BBB and prolonging the contact of carried molecules with their targets. For these reasons, we utilized liposomes of lecithin to study the effectiveness of flavonoid liposomal preparations to increase flavonoid bioavailability and to protect the brain in experimental ischemia in acute conditions, shortly after ischemic insult. Additionally, because we observed a structure-activity relationship of flavonoids for neuroprotection *in vitro* (Dajas *et al.*, 2003a,b), it appeared meaningful to test whether this relationship occurs also *in vivo*.

The permanent focal ischemia produced by middle cerebral artery occlusion (pMCAo) in the rat, a widely accepted model of human ischemic pathology (Sydserff *et al.*, 1995) appeared to be a suitable model to assess flavonoid effects. For the assessment of the brain lesion produced by experimental ischemia we incubated coronal slices of brain in TTC, assessing spectrophotometrically the produced formazan (Preston and Webster, 2000). Tetrazolium salts have been widely used in assays of cell survival (Denizot and Lang, 1986; Hussain *et al.*, 1993; Varming *et al.*, 1996) and also in the evaluation of the cerebral tissue damage after 24 h of pMCAo (Chung *et al.*, 2003; Shichinohe *et al.*, 2004; Wen *et al.*, 2004; Yulug *et al.*, 2004). This same salt was used to calculate the infarct volume by an image analysis system (Swanson *et al.*, 1990). To assess the status of cerebral tissue we used a histological hematoxylin-eosin technique (Corbett and Nurse, 1998).

MATERIAL AND METHODS

Chemicals and Reagents

Halothane (Fluothane, Astra-Zeneca), TTC (2,3,5,-triphenylltetrazolium chloride), lecithin and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Quercetin was obtained from Great Dragón Enterprises Co. All others chemicals were of highest commercially available purity and were purchased from Baker (Phillipsburg, PA, USA). Phosphate acid and phosphoric acid were purchased from Baker Analyzed. Methanol was purchased from Mallinckrodt Chemical Co.

Aqueous and Liposomal Preparations of Quercetin, Fisetin and Catechin

Quercetin, fisetin and catechin (FIG. 1) were dissolved in distilled water and sonicated in the dark for 2 min. For lecithin-flavonoid preparations, 50 mg of each flavonoid and 120 mg lecithin were dissolved in 20 ml chloroform, in the case of quercetin, and in 20 ml of methanol, in the case of fisetin and catechin. This mixture was rotoevaporated, suspended in distilled water and sonicated in the dark for 4 h. Aliquots (1ml) were kept at -20ºC until the time of use.

Animals

Experiments were conducted on male Sprague-Dawley rats (280-350 g). Animals were housed in groups of six in a temperature-controlled environment on a 12 h/12 h light/dark cycle and had access to food and water *ad libitum*. Experiments were approved by the Ethical Committee of the Clemente Estable Institute.

Assessment of Flavonoids in Cerebral Tissue

The flavonoids were evaluated according to a standard procedure with minor modifications (Azuma *et al.*, 2002). After IP administration of aqueous or liposomal preparations of the flavonoids at different times (30 min, 1, 2, and 4 hours), the animals were anaesthetized with urethane (1.2 g/ ml), intracardially perfused with 0.9% NaCl, and brains were quickly removed. Brain tissues were vortexed in methanol (5 ml) and centrifuged (15000*g*) for 15 min at 4ºC. The supernatant (50 µl) was injected onto the HPLC. The quercetin dose was selected on the basis of a dose-response curve performed for quercetin in our laboratory.

Chromatographic Conditions for Flavonoids

The sample was loaded in an injection valve (Rheodyne Incorporated, loop 200 µl). The C-18 column (Biophase; 22 cm x 4.6 mm; 5 µm) was protected with a precolumn (Biophase; 3 cm x 4.6 mm; 5 µm). A bomb was used (LKB Bromma) maintaining the flow at a constant rate of 1.0 µl/min. A UV detector (UV-Vis Wilson 118) set at 260 nm was used to increase the specificity. For the detection of flavonoids, an electrochemichal detector (BAS LC-4C) with oxidation potential at + 0.65 V (glassy working carbon electrode *vs* an Ag/AgCl reference electrode) was used. The mobile phase was composed of a mixture of 25 mM phosphate buffer (pH 2.4) and methanol (1:1).

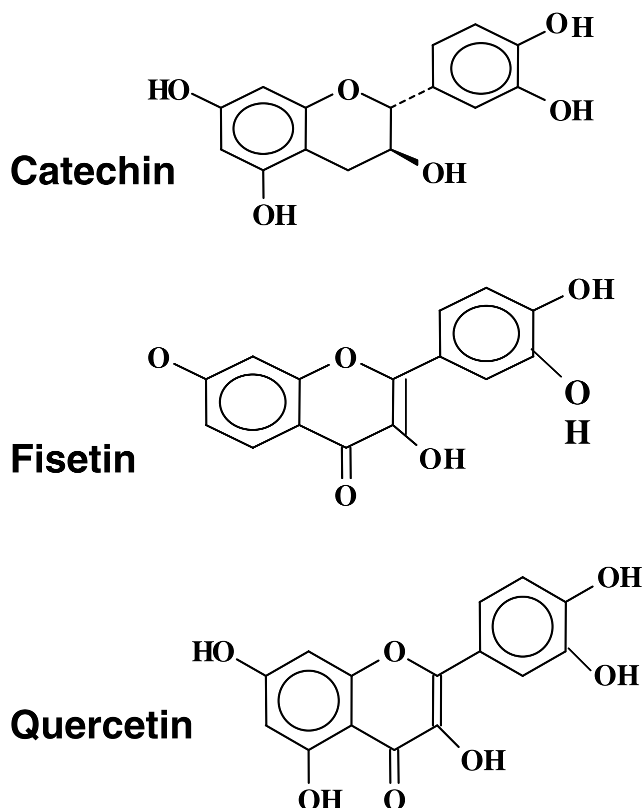

FIGURE 1 Structure of the flavonoids.

Permanent Focal Middle Cerebral Artery Occlusion

Animals were anaesthetized by inhalation of halothane, in a mixture of oxygen and air, through a facemask. During surgery, body temperature of the animals was continuously monitored with a rectal thermometer, and maintained at 37.5ºC with a heating pad. Permanent focal cerebral ischemia was induced, as described by Sydserff and co-workers (1995) with minor modifications. In brief, a surgical midline incision was made to expose the left common, internal and external carotid arteries. The external carotid and the common carotid arteries were closed by a ligature, the occipital artery

was cut by diathermy using a coagulator, and the internal carotid artery was temporarily occluded using a micro-aneurysm clip. A small incision was then made in the common carotid artery, and a 19-mm length of 4-0 monofilament nylon suture, its tip rounded by heating, was introduced into the internal carotid artery. The occluder filament was advanced to close the origin of the middle cerebral artery. The anesthesia was then discontinued, and the animals were returned to their cages after the surgery.

TTC Incubation and Measurement of Tissue Formazan

For the incubation with TTC and measurement of tissue formazan, rats were anaesthetized with urethane (1.2 g/ ml) and then decapitated. The head was chilled in an ice bath for 30 s; the brain was removed and placed in ice bath. Samples of striatum and parietal cortex were dissected and two samples of each cerebral hemisphere were obtained and weighed. A bathing solution containing (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 10 HEPES, and 3 glucose was used to freshly dissolve TTC (2% solution) and 5 ml was added to each tissue vial. The vials were laid on their side in a covered water bath shaker set at 37ºC and allowed to incubate for 90 min. Afterwards the TCC was removed and the tissues rinsed twice in saline. A 50:50 mixture of ethanol/dimethylsulfoxide was then added to solubilize the formazan. The vials were tightly capped and placed in a dark cupboard for a 24 h period, to dissolve and redistribute the tissue formazan throughout the contents of the vial. For analysis, each vial was briefly shaken, and four 100 µl aliquots of the red solvent extract were placed in cuvettes and diluted with 1900 µl of fresh ethanol/DMSO solvent. Average absorbance of the cuvettes was read at 485λ and absorbance per g tissue was calculated for each sample (Preston and Webster, 2000). Results were expressed in percentage of ipsilateral side *vs* contralateral side in control, sham, and ischemic animals treated with saline, lecithin and liposomal preparations of catechin (LC), fisetin (LF) and quercetin (LQ).

Dose-Response Curve

To construct a dose response curve for quercetin, doses of 30, 60 and 90 mg/kg of aqueous and liposomal preparations were used. The ischemic damage after pMCAo and the effects of the different doses in striatum and cortex were assessed spectrophotometrically using TTC.

Morphometric Determination of Infarct Volume

Animals of all experimental groups were re-anaesthetized with urethane (1.2 g/ml) and intracardially perfused with 100 ml of 0.9% NaCl and 60 ml of 2% TTC solution (Bederson *et al.*, 1986; Preston and Webster, 2000). Brains were quickly removed and placed in 4% paraformaldehyde and 2% glutaraldehyde for 48 h. Twenty serial coronal sections from each brain were cut in a vibratome (500 µm). Digital images were captured from the stained coronal sections using a flatbed color scanner. The zones of infarction (which were clearly demarcated) and brain total area were outlined (Belayev *et al.*, 1996; 2001). These areas were quantified by the image processing program JAVA (Jandel Scientific Software) and the total infarct volume was calculated by integrating the infarcted area of all 20 sections (area of infarct in mm^2 x section thickness). To compensate for swelling, the following formula was applied: corrected infarct size = infarct size x contralateral hemisphere size / ipsilateral hemisphere size, according to Chan *et al.* (2002). Measurements were performed someone blinded to the treatment of the processed animal tissue. Results were expressed as infarct volume in mm^3.

Histological Evaluation

Rats belonging to control and ischemic groups treated with saline and liposomal quercetin groups (n = 3 per group) were evaluated by histological procedures 24 h after pMCAo. Animals were anaesthetized with urethane (1.2 g/ml) and intracardially perfused with 100 ml of 0.9% NaCl and 150 ml of 4% paraformaldehyde - 2% glutaraldehyde. The brains were quickly removed and placed in 30% Sucrose for at least 48 h. Afterwards, brains were frozen in liquid nitrogen and coronal serial sections were cut in a cryostat (6 µm). Sections were stained with hematoxylin and eosin, and morphological cellular damage (nuclear hyperchromasia with nuclear retraction and fragmentation), decrease of cell population, and loss of the tissue architecture as evidence of brain edema, were chosen as indicators of ischemic tissue damage and were assessed separately in striatum and cortex.

Statistical Analysis

All data are presented as mean ± S.D. Data were statistically analyzed and the differences between control, sham and ischemic treated with saline, lecitin and liposomal preparation groups were compared by one way analysis of variance (ANOVA) followed by Tukey´s-Kramer test. Statistical significance was accepted at $p < 0.05$.

RESULTS

Dose-Response Curve for Quercetin

The dose of 30 mg/kg of the liposomal preparation of quercetin was the most effective, producing a 69.9% increase of the formazan precipitate in the striatum (FIG. 2) and a 134.4% increase in cortex (data not shown). The improvement of the brain lesion showed by the increase in the colored formazan was significantly higher *vs* saline (striatum 9.6% - cortex 9.3%), lecithin (striatum: 10% - cortex: 13.5%) and the 60 (striatum: 44% - cortex: 60%) and 90 (striatum: 21.9% - cortex: 11.1%) mg/kg doses (FIG. 2). The aqueous preparations did not show protection (data not shown).

Quantification of the Flavonoids in Cerebral Tissue

After IP administration of the aqueous preparations none of the flavonoids studied was detectable in the brain by HPLC techniques. Utilizing liposomal preparations, LC reached its highest cerebral concentrations (10.5 ng/g) 1 h after IP administration (FIG. 3) while LF showed the highest levels (8.23 ng/g) 2 h after administration (FIG. 3). LQ reached its peak concentration (509 ng/g) 1 h after injection, followed by a quick drop (FIG. 4). LQ concentrations were higher than LC and LF concentraions.

Spectrophotometric Evaluation

The spectrophotometric evaluation of the ischemic damage expressed in % left /right hemisphere (O.D. / g of cerebral tissue) showed with LC and LF a significantly increase in the color density of the formazan as an indicator of mitochondria survival and protection against ischemic lesion (FIG. 5A, 5B). In LQ there was a 69.9% increase in the striatum and 134.4% increase in cortex. Assessment of LF homogenates showed 62.6% increase in the striatum and 108.7% increase in cortex. Values after liposomal catechin were 8.8 5% and 19.9% for striatum and cortex, respectively, non statistically different. Lecithin (striatum: 10% - cortex: 13.5%) values were low, in a range similar to the ischemic animals (striatum: 9.6% - cortex: 9.3%) (FIG. 5A, 5B).

Cerebral Infarct Volume

When the cerebral infarct volume was determined by computer-assisted imaging methods in ischemic animals treated with saline, L, and with the liposomal preparation of all flavonoids, a significant reduction of the infarct volume was observed with LF (19.3 ± 1.2 mm^3) and LQ (17.0 ± 5.7 mm^3) when they were compared with ischemic (41.3 ± 10 mm^3); L (29.8 ± 10.5

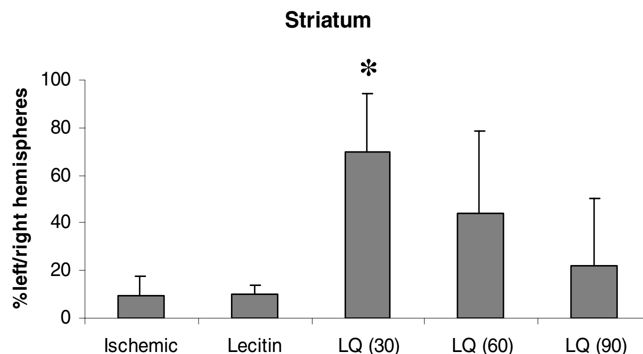

FIGURE 2 Dose response curve of the anti-ischemic effects of a liposomal preparation of quercetin (30, 60 and 90 mg/kg), evaluated by the spectrophotometric method using TTC. The values were expressed as % left / right hemisphere (O.D./ gram of cerebral tissue) in striatum of ischemic rats. LQ(30), 30 mg/kg; LQ(60), 60 mg/kg ; and LQ(90), 90 mg/kg. The statistical analysis was by one way ANOVA followed by a multiple range test. *$p < 0.005$.

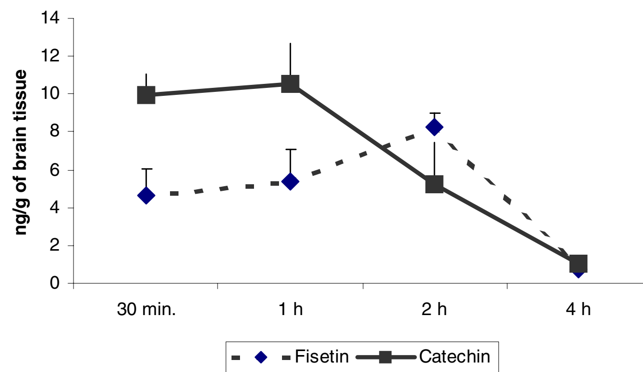

FIGURE 3 Concentration curves (ng/g) of catechin and fisetin in cerebral tissue of rats injected IP with a single dose (30 mg/kg) of the liposomal flavonoids. Brain tissue was studied 30 min, 1 h, 2 h and 4 h after administration of the flavonoids.

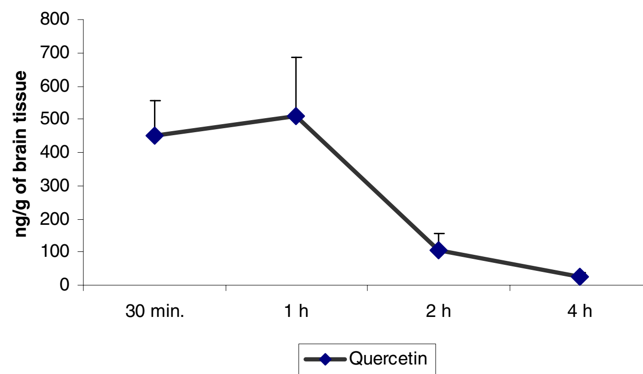

FIGURE 4 Concentrations of liposomal quercetin in ng/g of cerebral tissue of rats injected IP with a single dose of quercetin (30 mg/kg). Brains were studied at 30 min, 1 h, 2 h and 4 h after administration.

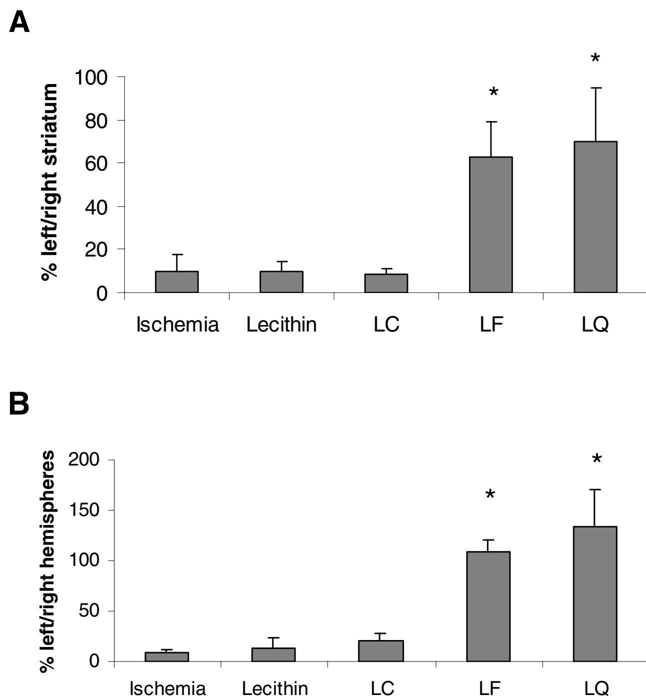

FIGURE 5 Spectrophotometric assessment of the color intensity of formazan precipitates expressed in % of left / righ hemisphere of the O.D./ gram of cerebral tissue in the striatum (**A**) and parietal cortex (**B**) of rats submitted to the pMCAo. Bars represent values of ischemic brain (Ischemia) or treatments with liposomal preparations of the different flavonoids (LC: catechin; LF: fisetin; LQ: quercetin). Lecithin represents treatment with lecithin alone. Data were analyzed by one way ANOVA and *post hoc* multiple range test). $*p < 0.005$.

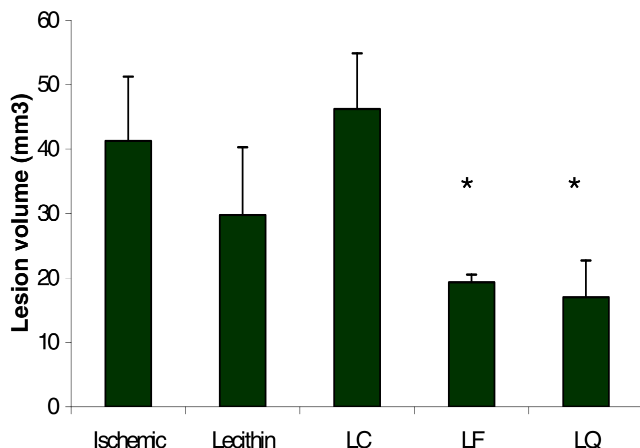

FIGURE 6 Cerebral lesion volume of rats (mm^3) 24 h after permanent middle cerebral artery occlusion. Thirty minutes after arterial occlusion ischemic rats received an IP injection of saline (Ischemic), lecithin (Lecithin) and the liposomal preparations of Catechin (LC), Fisetin (LF) and Quercetin (LQ). Values are compensated for the oedema and expressed as mean ± S.D. *, $p < 0.05$: different from saline group by ANOVA followed by multiple range test.

mm^3) and LC (46.2 ± 8.7 mm^3) groups. No lesion was observed in sham operated animals (data not shown) (FIG. 6).

Status of Tissue Stained with Hematoxylin and Eosin

In the striatal preparations of ischemic animals, a loss of the characteristic architecture of the striatum was observed. As an index of the importance of the brain edema the delimitation between white and gray substance was lost. Isolated nuclei or cells with pyknotic nuclei and retracted cytoplasm were observed. Similar damage was observed in the ischemic cortex preparations. In the animals treated with LQ, a recovery of the striatal and cortex architecture was observed, indicating a decrease in cerebral edema. An increase in the number of cells conserving their morphological features was observed (FIG. 7).

DISCUSSION

The spectrophotometric increase in the activity of TTC formazan after LQ and LF and the decrease in brain lesion volume by morphometric analysis with the same flavonoids administered after permanent ischemia represent strong evidence that these flavonoids protected brain tissue when administered acutely - if they were provided with an adequate carrier. Besides, the LQ treatment contributes to restore the general structure of the brain parenchyma, likely reducing edema formation and apparently protecting the cellular population. Preliminary evidence of a structure-activity relationship is shown by failure of catechin to protect under the same conditions.

It is important to consider that these results were obtained with the permanent variant of focal ischemia by the MCAo model and that the left common carotid artery was also occluded. The model is thus different from global ischemia in two very important ways. First, even at the core of the lesion, the blood flow is almost always higher than during global ischemia, so that longer insults are required to produce damage. Secondly, there is a significant gradation of ischemia from the core of the lesion to its outermost boundary, and hence there are different metabolic conditions within the affected site (Lipton, 1999). Because of its duration and heterogeneity, the focal insult is much more complex than global ischemia, but it is an invaluable model for ischemia and is thus widely studied (Sydserff *et al.*, 1995; Lipton, 1999). In contrast to our results, some of the flavonoids studied for their neuroprotective capacity, like rutin (Gupta *et al.*, 2003) and

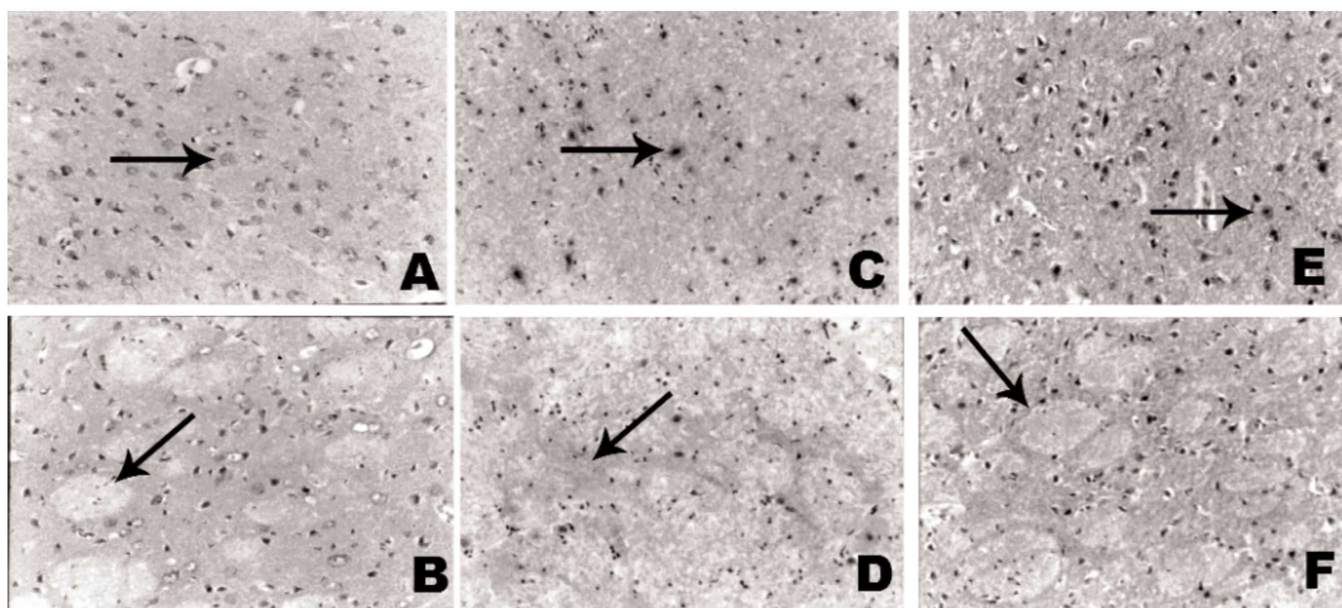

FIGURE 7 Hematoxylin-eosin stain of cryostat slices of rat brain controls (**A, B**) or submitted to pMCAo and injected IP 30 min afterwards with saline (**C, D**) and LQ (**E, F**). In cortex (**A, C, E**) arrows show : intact cells (**A**), pyknotic nuclei (**C**) and intact cells (**E**). In striatum (**B, D, F**) arrows show: well defined white matter fibre tracts (**B**) that are lost in **D** as evidence of oedema and recovered in **F** after treatment with LQ. (Magnification: 20X).

crataegus (Zhang *et al.*, 2004) have been evaluated in the global ischemic model and in mice and gerbils, respectively.

Despite the differences between permanent and temporary focal ischemic models, maximal lesion sizes are comparable in the two cases, and the progression of damage, in terms of the numbers of damaged neurons and the extent of that damage over 6 - 72 h, is remarkably similar (Zhang *et al.*, 1994). The permanent focal ischemic model has been shown to be highly sensitive to pharmacological intervention with various neuroprotective drugs, including NMDA receptor antagonists (Steinberg *et al.*, 1995; Williams *et al.*, 2000), antioxidants (Ahlemeyer *et al.*, 2001), antilipoperoxidation (Huh *et al.*, 2000) and anti-inflammatory compounds (Welton *et al.*, 1986).

Brain ischemia triggers a variety of cellular processes, such as release of neurotransmitters, particularly glutamate, with NMDA receptor activation and massive entry of calcium into the cells (Nicotera and Lipton, 1999; Aoki *et al.*, 2001). There is general activation of intracellular enzymes, and free radicals are generated at several points (Nicotera and Lipton, 1999), producing lipoperoxidation, DNA damage, cell membrane disruption, and ultimate neuronal death. Therapeutic strategies to control the ischemic process have focused on one or another of these factors, and neuroprotective effects have been observed after *e.g.*, the utilization of NMDA receptor blockers (Williams *et al.*, 2000) or calcium antagonists (Aoki *et al.*, 2001).The widespread generation of free radicals has also prompted the development and application of a variety of antioxidant approaches to attenuate neuronal injury (Clemens and Panetta, 1994; Clark *et al.*, 2001)

In this context, the well known antioxidant activity of flavonoids (Huk *et al.*, 1998; Ishige *et al.*, 2001) could be considered the most immediate explanation of our results. Common biological properties such as scavenging radical ions (Huk *et al.*, 1998; Ishige *et al.*, 2001; Cho *et al.*, 2003), acting as antilipoperoxidative compounds (Ratty and Das, 1988; Miura *et al.*, 2003; Nardi *et al.*, 2003) as well as anti-inflammatory and platelet ant aggregation actions (Ferriola *et al.*, 1989; Nardi *et al.*, 2003), with attenuation of nitric oxide production (Soliman and Mazzio, 1998) have been attributed to quercetin, fisetin and catechin flavonoids. Nevertheless, and in spite of their structural and biological functional similarity they differ in effects in some experimental paradigms. While catechin and quercetin are effective antioxidants that increase the activity of SOD, fisetin does not (Chan *et al.*, 2002). Conversely, while quercetin and fisetin were the most active in inhibiting phosphatidylinositol 3-kinase (Agullo *et al.*, 1997), protein kinase C (Ferriola *et al.*, 1989) and glucose uptake in U937 cells (Park, 1999), there is no experimental evidence of the participation of catechin in these effects. Quercetin but not catechin had also strong inhibition of xanthine oxidase (XO)

(Chang *et al.*, 1993), and fisetin effects have not been reported on XO activity. Beyond these point to point similarities and differences, quercetin and fisetin bear the common structural features that have been recognized as important for antioxidant scavenger activity: a 3′, 4′ -dihydroxycatechol structure in the B ring, and the presence of 2,3 unsaturation together with an oxo function in 4 position in the C ring (Rice-Evans, 2001).

To obtain measurable cerebral concentrations of flavonoids after single administration it was necessary to use liposomal preparations. It is very likely that the interaction of flavonoids with lecithin increases the possibilities to provide protection, since aqueous preparations did not show any protective effect after single IP injections. Liposomes appear to be effective carriers into the brain, delaying metabolism of encapsulated molecules (Azuma *et al.*, 2002), a reasonable explanation of the concentrations of flavonoids found in the brain after IP administratrion. Our results are in agreement with studies showing that preparations using lecithin (SB-tPS and Pluronic F-68) reduced cerebral ischemic damage (Sakas *et al.*, 1994; Suzuki *et al.*, 1999).

The liposomal quercetin dose utilized in this study (30 mg/kg) was the most effective dose obtained after an exploratory dose response curve. This dose coincides with the ones utilized by other authors to study the neuroprotective effects of quercetin. In this sense, the chronic quercetin treatment for 30 days in doses of 10, 25 and 50 mg/kg, reverses cognitive deficits in aged and ethanol-intoxicated mice (Singh *et al.*, 2003). Quercetin in doses of 50 and 100 mg/kg significantly and dose-dependently reduced the reserpine-induced vacuous chewing movements and tongue protrusions in rats (Naidu *et al.*, 2004). Besides, this flavonoid in doses of 25-100 mg/kg proved to be a useful agent in neuroleptic-induced orofacial dyskinesia (Naidu *et al.*, 2003). Also, synthetic compounds like SB 239063, a p38 inhibitor, attenuates early neuronal injury following pMCAo in dose of 15, 30 and 60 mg/kg (Legos *et al.*, 2001).

Ours results showed that although LC was detected and measured in the brain, it could not offer cerebral protection during permanent ischemia, likely due to the quick metabolic transformation of this flavonoid. Voltammograms of catechin and quercetin showed also that the electron-donating ability of catechin was lower than that of quercetin, though antioxidative effects of catechin were comparable to that of quercetin in the AAPH initiated peroxidation (Terao *et al.*, 1994). Referring also to the structure-activity profile mentioned above, catechin possesses different basic structures but the same hydroxylation pattern (3,5,7,3′4′-OH) - structural features that are important for antioxidant scavenger activity (FIG. 1) (Rice-Evans, 2001).

Undoubtedly, the strong antioxidant capacity of quercetin and fisetin that contribute to regain cellular redox equilibrium after ischemia partly explains their neuroprotective potency. Given the myriad effects of flavonoids, actions on other targets like intracellular kinases can no longer be discounted. Though the brain concentrations of LF were lower than those of LQ, LF remained high for longer periods, thus making any tentative explanation of different mechanisms of action among these flavonoids purely speculative at this stage of our research.

The lesion volume decrease observed by us with LF and LQ was higher than the neuroprotection obtained after NMDA receptor blockade with the MK 801 antagonist (Margail, *et al.*, 1996; Bertorelli *et al.*, 1998) or with hypothermia (Zausinger *et al.*, 2000). In agreement with these results, the observed histological changes in the LQ groups were readily apparent and corresponded precisely to the areas delineated by the TTC stain. Areas that remained non-reactive exhibited clear histological signs of infarction, confirming the selectivity of the TTC technique (Park *et al.*, 1988; Davies *et al.*, 1998). Besides, our experiments clearly demonstrated that the decrease in lesion volume was accompanied by a recovery of the cerebral architecture, indicating a decrease in the edema and an increase in cellular survival.

The correlation between acute *in vitro* and *in vivo* neuroprotective capacity of flavonoids structurally related to quercetin, when provided with an adequate carrier, are the most important findings in our study, and these represent a reliable basis for continuing the search for flavonoids as leading and active therapeutic molecules for protecting the brain in stroke.

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