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Neuroprotection by flavonoids

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Abstract

The high morbidity, high socioeconomic costs and lack of specific treatments are key factors that define the relevance of brain pathology for human health and the importance of research on neuronal protective agents. Epidemiological studies have shown beneficial effects of flavonoids on arteriosclerosis-related pathology in general and neurodegeneration in particular. Flavonoids can protect the brain by their ability to modulate intracellular signals promoting cellular survival. Quercetin and structurally related flavonoids (myricetin, fisetin, luteolin) showed a marked cytoprotective capacity in *in vitro* experimental conditions in models of predominantly apoptotic death such as that induced by medium concentrations (200 μM) of H_2O_2 added to PC12 cells in culture. Nevertheless, quercetin did not protect substantia nigra neurons *in vivo* from an oxidative insult (6-hydroxydopamine), probably due to difficulties in crossing the blood-brain barrier. On the other hand, treatment of permanent focal ischemia with a lecithin/quercetin preparation decreased lesion volume, showing that preparations that help to cross the blood-brain barrier may be critical for the expression of the effects of flavonoids on the brain. The hypothesis is advanced that a group of quercetin-related flavonoids could become lead molecules for the development of neuroprotective compounds with multitarget anti-ischemic effects.

Key words
- Flavonoids
- Quercetin
- Focal ischemia
- Neuroprotection

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Brain vascular pathology and oxidative stress

It is known that brain pathology in the form of cerebrovascular and neurodegenerative disease is a leading cause of death all over the world, with an incidence of about 2/1000 and an 8% total death rate (1-3). Moreover, stroke and dementia are a source of high individual and family suffering mainly because of the lack of efficient therapeutic alternatives. The latter motivates research efforts to identify the mechanisms of neuronal death and to discover new compounds to control them.

Neuronal death in stroke is a complex event involving failure of metabolic processes, excitotoxicity, loss of calcium homeostasis and oxidative stress, among other factors (4). During ischemic stroke, a decrease in metabolic energy in the form of ATP affecting membrane ionic pumps leads to an increase in intracellular Ca^{2+} and Na^{+} concentrations and to increased glutamate

release (5). The massive Ca^{2+} entry activates enzymes such as proteases, oxidases, phospholipases and endonucleases (6) that can hydrolyze the DNA molecule and destroy the cytoskeleton (7). Phospholipase A_2 activation favors the metabolism of arachidonic acid through lipoxygenases and eicosanoids in turn activate lipid peroxidation. Increased intracellular Ca^{2+} also activates protein kinase C that can modify the function of many ion channels (8) (Figure 1).

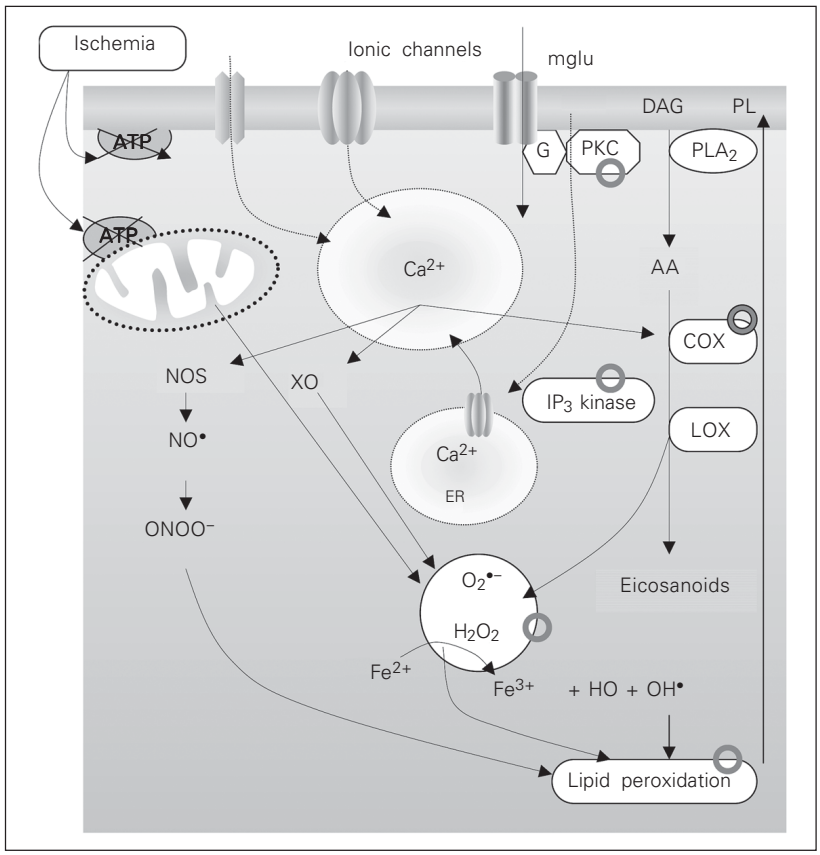

Figure 1. Schematic drawing of the ischemic process at the cellular level. The initial mitochondrial metabolic failure (ATP) leads to disruption of ionic pump functioning at the membrane level and massive neurotransmitter and glutamate release which in turn increase the Ca^{2+} entry. There is an activation of enzymes (XO, NOS, COX, LOX kinases). Reactive oxygen and nitrogen species production (such as $O_2^{\bullet -}$, $NO^{\bullet}$ and H_2O_2) is part of this process, generating lipid peroxidation and nuclear DNA damage. Flavonoids act by inhibiting several steps in this proces: XO, lipid peroxidation, IP_3 kinase, LOX, PKC and scavenging free radicals (marked with circles in the figure). AA = arachidonic acid; COX = cyclooxygenase; DAG = diacylglycerol; ER = endoplasmic reticulum; G = G protein; IP_3 = phosphatidylinositol-3; LOX = lipoxygenase; mglu = metabotropic glutamate receptors; NO = nitric oxide; NOS = nitric oxide synthase; PKC = protein kinase C; PL = phospholipids; PLA_2 = phospholipase A_2; XO = xanthine oxidase.

Several of these activated intracellular metabolic events lead to the generation of oxygen free radicals, which overcome the antioxidant defenses and provoke oxidative stress (9). Oxidative stress, in turn, provokes changes in macromolecules and in lipid membranes, generating a vicious cycle of more oxidation and more oxidative damage. Self-maintained oxidative reactions have been identified in arteriosclerosis, the main pathological condition leading to stroke (10,11).

Oxidative stress and natural antioxidants

In spite of the many lines of evidence linking oxidative stress to clinical symptoms related to arteriosclerosis in myocardial infarction and stroke (10,11), treatment with external antioxidants to regain oxidative equilibrium and to control the evolution of disease has provided controversial results. Some clinical trials with a recognized antioxidant like vitamin E did not show beneficial effects on the treatment of cardiovascular pathology risk (12) and doses higher than 500 mg of ascorbic acid as well as ß-carotene seem to have negative effects (13,14).

On the other hand, several studies have shown that fruits, red wine, vegetables and some plants increase the total antioxidant capacity of blood (15,16) and the antioxidant actions of polyphenol metabolites have been suggested to explain these beneficial effects, particularly for the Mediterranean diet (17-19). The most important polyphenol compounds are the flavonoids, which are abundant components of the human diet (8,20,21). Quercetin, a key representative flavonoid molecule of the group, is present widely in vegetables and fruits, with a daily intake of up to 25 mg/day in a normal human diet (22). Other effects such as antitumoral, antithrombotic, anti-inflammatory and antiapoptotic ones, as well as effects inhibiting platelet aggregation and the growth of certain types

of cancer, have been described for quercetin and other flavonoids (23-28).

Quercetin actively participates in intracellular signaling, inhibiting phosphatidylinositol-3 kinase, protein kinase C, xanthine oxidase and NADPH diaphorase (8,29-31). Nevertheless, in spite of this multiplicity of actions, the cardiovascular and/or neuroprotective effects of flavonoids and quercetin are mainly explained by their antioxidant capacity and their ability to scavenge free radicals (18).

Natural antioxidants and cytoprotection

Nature has been a continuous source of pharmacologically active molecules and medicinal herbs have been used by countless human generations. Nevertheless, surprisingly few plant extracts have been demonstrated to be neuroactive. Kava and *Ginkgo biloba* extracts have been shown to have neuroprotective actions based on experimental evidence (17,32-35). However, adverse effects have also been reported for kava and *Ginkgo biloba* (36,37).

Among the very few investigators of the effects of isolated flavonoids on the brain *in vivo*, Shutenko et al. (38) characterized changes in brain nitric oxide levels in a model of global ischemia with reperfusion in the presence of quercetin, attributing the observed changes to the scavenger action of the flavonoid. Another report has described the beneficial effects of quercetin on endotoxic shock (39) and the authors explained these effects by lipoperoxidation inhibition and increases in glutathione peroxidase activity.

Direct scavenging of reactive oxygen species is one of the many antioxidant actions required to restore oxidative equilibrium once it is lost in different pathologies. The hypothesis that restoring redox equilibrium through activation of intracellular signals is also an important step of the antioxidation process is gaining increasing support (40). It is likely that the trapping of free radical excess could restore redox equilibrium in the initial states of cellular oxidative stress. In massive cellular insults like ischemia, involving metabolic failure, loss of Ca^{2+} homeostasis and excitotoxicity, scavenger activity or one-target antioxidant mechanisms (NMDA receptor blockers, chain-breaking vitamin E or pure scavenger molecules such as boldine) may fail to protect cells from free radical damage.

Accordingly, in experimental conditions, the capacity of a given molecule to block the multiple sources of oxidative signals in situations like ischemia would be better assessed by its effectiveness in increasing cell survival. Hence, it could be hypothesized that the cytoprotective capacity of a given antioxidant would be critical to define a putative neuroprotective therapeutic activity. Protection of cells in culture against diverse insults (glutamate, AB peptide, and others) has proved to be a useful approach (18).

The oxidative insult with hydrogen peroxide (H_2O_2) has been widely used to assess cytoprotection, mainly in PC12 cells in culture (41-43). H_2O_2 offers the unique possibility of a graded action regulating the extent and severity of cell death by the selection of particular points of the H_2O_2-cell interaction. Thus, it has been shown that exposure of PC12 cells to 200 μM H_2O_2 for 30 min resulted in 50% cell viability. Cell death was accompanied by DNA damage without lactic dehydrogenase release, suggesting a "non-necrotic" type of cell death (44). Under closely similar experimental conditions, H_2O_2-induced cell death in PC12 was characterized as apoptotic (41). When various potent antioxidants (vitamin E, trolox, boldine, quercetin) were studied for their capacity to increase cell survival in the H_2O_2-induced PC12 cell death, only quercetin or structurally similar flavonoids protected PC12 cells from the oxidative insult. Of several flavonoids structurally related to quercetin - myricetin,

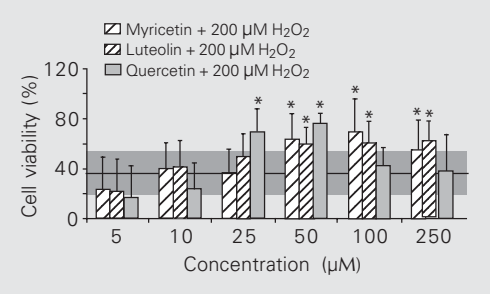

Figure 2. PC12 cell viability after a 30-min H_2O_2 insult in the presence of diverse flavonoids. *P < 0.01 for H_2O_2 plus flavonoid treatment compared to only H_2O_2 treatment (ANOVA and multiple comparison Tukey test). The horizontal line behind the bars represents the control's mean and the gray area the standard deviation of control experiments.

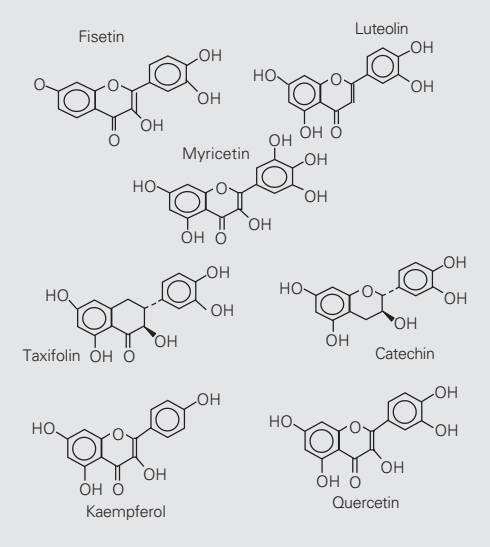

Figure 3. Structures of the flavonoids studied.

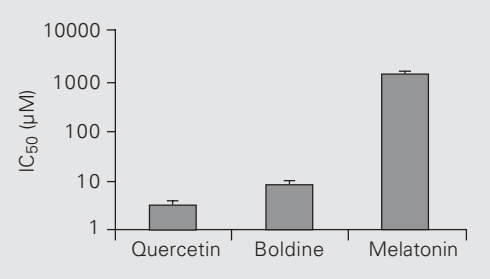

Figure 4. Inhibition of spontaneous lipoperoxidation in isolated rat brain membranes by different antioxidants. Data are reported as IC_{50} (concentrations required to obtain 50% inhibition).

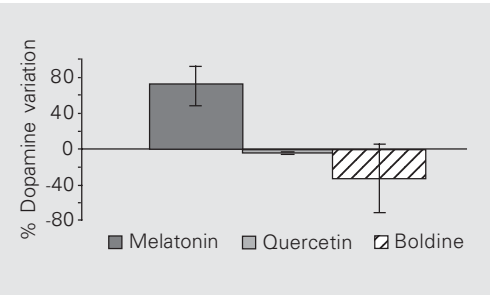

Figure 5. Dopamine tissue levels in the corpus striatum of rats injected with 6-hydroxydopamine (6-OHDA) into the substantia nigra. Data are reported as percent of the contralateral, nonlesioned side. Rats were treated *ip* with 5 mg/kg melatonin, 30 mg/kg quercetin, and 15 mg/kg boldine 30 min before the 6-OHDA lesion.

kaempferol, taxifolin, catechin, luteolin and fisetin - only fisetin, luteolin and myricetin increased cell survival (45; Figure 2).

The common structural features of the molecules that increased survival were a 3',4'-dihydroxy catechol structure in the B ring, coplanarity of the molecule and the presence of 2,3 unsaturation together with an oxo function at position 4 in the C ring. This structural profile is the one recognized as important also for antioxidant scavenger activity (19) (Figure 3).

The fact that quercetin and structurally related molecules increased cell survival in an oxidative stress model where scavenging antioxidants (vitamin E, boldine) failed to protect cells from the oxidative insult suggests that these molecules have a specific cell survival-increasing activity in addition to their scavenger activity. This cytoprotective capacity of flavonoids may be linked to their ability to activate intracellular molecules (kinases, phosphatases, gene promoters), which in turn activate components of intracellular cascades, promoting the expression of survival signals. This would be especially true for cellular protection in cases in which apoptosis is an important component of cell death.

Flavonoids in experimental brain pathology

The antioxidant profile of flavonoids would be a strong basis for a neuron-protective activity in the brain. Their general bioavailability and particularly their presence in the brain *in vivo* appear to play an important role in the expression of the neuroprotective capacity of flavonoids. It is accepted that metabolic transformations (glucuronidation, methylation, etc.) are the rule and that a very small amount of a given intake of flavonoids are free as aglycones in blood (46,47). For the specific aim of neuroprotection, the blood-brain barrier appears to be an added obstacle to flavonoids reaching the brain.

Dajas and co-workers (48) administered

recognized antioxidants, including quercetin, in an experimental model of Parkinson to test the ability of flavonoids to cross the blood-brain barrier and act on the brain. Microinjection of 6-hydroxydopamine (6-OHDA) induces death of dopaminergic neurons in the substantia nigra with a concomitant loss of terminals in the corpus striatum. Assessment of dopamine in the latter after a 6-OHDA insult gives an idea of the extent of the lesion. In agreement with the prevalent hypothesis regarding the cause and/or progression of Parkinson's disease, 6-OHDA lesion is produced by oxidative stress (49). In the study of Dajas et al. (48), natural compounds such as boldine, quercetin and melatonin, with marked antioxidant potency and different mechanisms of action, were tested by being injected intraperitoneally in saline solution before 6-OHDA lesion. An interesting aspect was the comparison of the *in vitro* antioxidant capacity of these natural antioxidants with their neuroprotective activity in the experimental model of Parkinson's disease: boldine and quercetin had the highest antioxidant potency in the antilipoperoxidation assay, followed by melatonin (Figure 4). When the capacity to increase neuronal survival was tested, melatonin, the weaker antioxidant, reversed the dopamine levels in the striatum while quercetin and boldine did not (Figure 5). Accordingly, and in spite of its antioxidant potency *in vitro* and cytoprotective actions in cell cultures, quercetin did not protect substantia nigra neurons *in vivo* (50). These results would show that flavonoids like quercetin cross the blood-brain barrier poorly. The surprisingly few papers reporting effects of flavonoids in the brain *in vivo*, cited above, appear to confirm this fact. Most of the reports of neuroprotection by natural compounds from plants refer to complex extracts like those of *Ginkgo biloba* and not to single compounds.

In a later study, Dajas et al. (51) increased the possibility of quercetin crossing the blood-brain barrier by mixing it with lecithin, generating a liposomal preparation. This preparation is a recognized way of transporting molecules in the body, increasing the time of interaction of a given molecule with its target (46). The authors utilized the middle cerebral artery occlusion model in the rat (52). A single intraperitoneal dose of a lecithin/quercetin preparation (30 mg/kg) was administered 30 min after artery occlusion and rats were sacrificed 24 h after occlusion. Assessed by means of a computer (53), the volume of the ischemic lesion decreased 56% after treatment (Figures 6 and 7).

While the ischemic area extended over the striatum and parietal cortex (Figure 6), the recovery was more marked in the striatum. The decrease of the lesion area and volume corresponded to decreased edema and to neuronal survival as assessed by histology.

The decrease in lesion volume obtained with lecithin/quercetin was similar to the lesion improvement observed after excitotoxicity antagonism (54) or the use of cal-

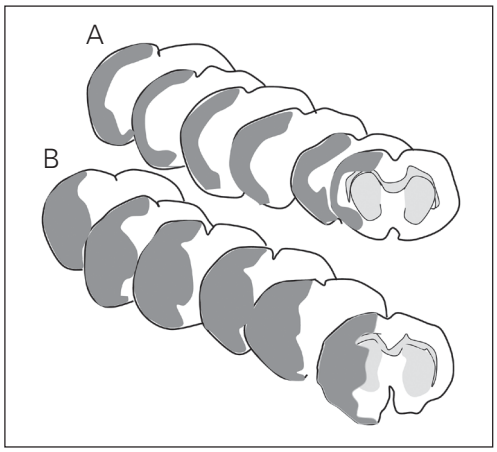

Figure 6. Computer-generated schematic drawings of representative frontal slices of 2,3,5 triphenyl tetrazolium chloride-stained coronal brain sections obtained 24 h after permanent middle cerebral artery occlusion in rats receiving an *ip* injection of either lecithin/quercetin preparation (A) or saline (B), 30 min after occlusion. The recovery of the ischemic areas is shown.

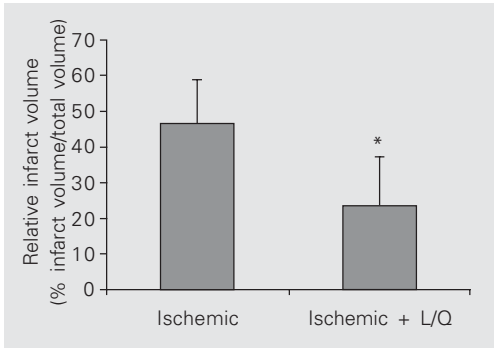

Figure 7. Relative infarct volume 24 h after permanent middle cerebral artery occlusion. Rats received an *ip* injection of saline (N = 5, ischemic in figure) or lecithin/quercetin (N = 7, ischemic + L/Q in figure) 30 min after middle cerebral artery occlusion. Values are reported as means ± SD. *P < 0.05 compared to the saline group (ANOVA and Kruskal-Wallis test).

cium channel blockers (55). Accordingly, in contrast with the lack of effects in the oxidative lesion of experimental parkinsonism, the work on permanent ischemia showed a neuroprotective action of lecithin/quercetin in the brain, probably demonstrating the importance of the way of administering quercetin to assure the crossing of the blood-brain barrier in sufficient quantities to be beneficial against the oxidative damage. These preliminary results with quercetin would be showing a putative relationship, in a group of related flavonoids, between the cytoprotective potency in apoptotic models of cell death and central neuroprotective activity *in vivo*.

Up to now, neuroprotective strategies in ischemia that have focused on the development of molecules targeting one mechanism at a time have not proven to be successful in clinical trials. The multiple cell effects of flavonoid indicate that several targets could be reached with only one molecule by administering the flavonoid in a preparation that could cross the blood-brain barrier.

The capacity of flavonoids to inhibit the action of several enzymes should activate survival signals exerting a net indirect antioxidant effect in addition to the direct scavenging of reactive oxygen species. Additionally, it is important to mention that the effects of quercetin and flavonoids are also exerted at the level of glia and vessels in the brain. At the microvessel level, antioxidation and anti-inflammation would be added to vasodilatation, improving blood flow and counteracting the ischemic process (56).

Although the exact explanation of the mechanisms of action of flavonoids on the brain has just started to be addressed and a wide diversity of questions remain open, flavonoids are likely to become leading compounds for the development of a new generation of molecules clinically effective in human brain ischemia.

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