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A Novel Approach to Screening for New Neuroprotective Compounds for the Treatment of Stroke

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Abstract

Despite the significant advances that have been made in understanding the pathophysiology of cerebral ischemia on the cellular and molecular level, only one drug, the thrombolytic tissue plasminogen activator (rt-PA), is approved by the FDA for use in patients with acute ischemic stroke. Therefore, there is a critical need for additional safe and effective treatments for stroke. In order to identify novel compounds that might be effective, we have developed a cell culture-based assay with death being an endpoint as a screening tool. We have performed an initial screening for potential neuroprotective drugs among a group of flavonoids by using the mouse hippocampal cell line, HT22, in combination with chemical ischemia. Further screens were provided by biochemical assays for ATP and glutathione, the major intracellular antioxidant, as well as for long-term induction of antioxidant proteins. Based upon the results of these screens, we tested the best flavonoid, fisetin, in the small clot embolism model of cerebral ischemia in rabbits. Fisetin significantly reduced the behavioral deficits following a stroke, providing proof of principle for this novel approach to identifying new compounds for the treatment of stroke.

Keywords

ischemia; flavonoids; ATP; Nrf2; heme oxygenase 1; glutathione

Introduction

Stroke is the leading cause of adult disability and the third leading cause of death in the US. Worldwide, approximately 6 million people died of stroke in 2005, with a projected increase over the next decade of 12% (Ingall, 2004). Ischemic stroke occurs when the normal blood supply to the brain is disrupted, usually due to artery blockage by a blood clot, thereby depriving the brain of oxygen and metabolic substrates and hindering the removal of waste products (for review see Lapchak and Araujo, 2007). The nerve cell damage caused by cerebral ischemia results in functional impairment and/or death. Despite the significant advances that have been made in understanding the pathophysiology of cerebral ischemia on the cellular and molecular

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level, only one drug, recombinant tissue-type plasminogen activator (rt-PA), is approved by the FDA for use in patients with ischemic stroke (Green and Shuaib, 2006). Unfortunately, the utilization of rt-PA is limited by its short time window of efficacy and its potential to cause intracerebral hemorrhage (NINDS rt-PA trial) (Lapchak, 2002a; Lapchak, 2002b; Lyden and Zivin, 1993). Thus, there is a critical need for additional safe and effective treatments for stroke.

To date, most of the compounds that have been tested in clinical trials for the treatment of ischemic stroke were chosen based on mechanisms proposed to underlie nerve cell death in stroke (Green and Shuaib, 2006). In order to identify novel compounds that might be effective for the treatment of stroke, a new approach is needed. Rather than taking a mechanism-based approach, we have chosen to utilize a cell culture-based assay with death being an endpoint as a screening tool. In this way we can identify potential neuroprotective compounds that act at multiple sites in the cell death pathway and therefore could provide a better chance at protecting the cells.

Most cell culture models of stroke utilize primary cortical cultures that are exposed to some form of hypoxia/glucose deprivation (e.g. Arthur et al., 2004; Munns et al., 2003). While these assays are clearly closer to the *in vivo* condition than assays utilizing neuronal cell lines, they have a number of problems which make them difficult to use for the routine screening of potential neuroprotective compounds. The most serious problem is that the conditions needed to kill a fixed percentage of cells are highly variable from experiment to experiment. In order to circumvent these problems, we used the mouse hippocampal cell line, HT22, in combination with chemical ischemia as an initial screen for potential neuroprotective drugs for the treatment of stroke. Further screens were provided by biochemical assays for ATP and glutathione, the major intracellular antioxidant, as well as for the long-term induction of antioxidant proteins.

In the first test of this approach we decided to focus on flavonoids, polyphenolic compounds widely distributed in fruits and vegetables and therefore regularly consumed in the human diet (for reviews see Heim et al., 2002; Middleton et al., 2000; Ross and Kasum, 2002). Flavonoids were historically characterized on the basis of their antioxidant and free radical scavenging effects. However, more recent studies have shown that flavonoids have a wide range of activities that could make them particularly effective as neuroprotective agents for the treatment of stroke. First, flavonoids can protect nerve cells from oxidative stress-induced death by both acting as a direct antioxidant and by maintaining high levels of glutathione (GSH), the major intracellular antioxidant (Ishige et al., 2001). Second, multiple studies have shown that certain flavonoids can induce the activity and expression of phase II detoxification proteins (Hanneken et al., 2006; Hou et al., 2001; Maher and Hanneken, 2005b; Maher, 2006; Myhrstad et al., 2002; Valerio et al., 2001). The phase II detoxification proteins include enzymes associated with glutathione biosynthesis and metabolism and redox sensitive proteins such as heme oxygenase 1 (HO-1) (Hayes and McLellan, 1999). By inducing the expression of antioxidant defense enzymes, these flavonoids have the potential to have long lasting effects on cellular function. Finally, flavonoids have been shown to induce neurite outgrowth (Sagara et al., 2004), reduce inflammation (Read, 1995), improve cerebral blood flow (Curin et al., 2006), prevent platelet aggregation (Curin et al., 2006) and enhance cognition (Maher et al., 2006), all properties that could have additional benefits for the treatment of stroke. Indeed, the phenylpropanoid chlorogenic acid was recently shown to improve behavioral performance in a rabbit embolic stroke model (Lapchak, 2007). Finally, in animal studies, flavonoids have generally shown low levels of toxicity over a wide range of doses.

In order to test the hypothesis that *in vitro* assays can be used to identify compounds that might be useful for the treatment of stroke, we used our cell culture-based assays to identify flavonoids with neuroprotective activities. Based on the results of these screens, we then tested the best flavonoid, fisetin, in the small clot embolism model of cerebral ischemia in rabbits.

Results

In order to induce chemical ischemia, we used the compound iodoacetic acid (IAA), a well known, irreversible inhibitor of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (Winkler et al., 2003) in combination with the HT22 mouse hippocampal cell line. IAA has been used in a number of other studies to induce ischemia in nerve cells (Rego et al., 1999; Reiner et al., 1990; Reshef et al., 1997; Sigalov et al., 2000; Sperling et al., 2003) but not previously as a screen for neuroprotective compounds. As shown in Figure 1, a 2 h treatment of the HT22 cells with IAA shows a dose-dependent increase in cell death 20 h later with <5% survival at 20 μ M. We chose to use 20 μ M IAA for further studies in order to ensure that any neuroprotection that was seen would be robust. This toxic dose of IAA is highly reproducible from assay to assay. Since only one dose of IAA needs to be used, it makes it quite easy to screen potential neuroprotective compounds over a wide range of concentrations.

We began by testing flavonoids that had previously been shown to be neuroprotective in a different toxicity paradigm, oxidative glutamate toxicity (Ishige et al., 2001). Surprisingly, all of these flavonoids were also protective against IAA toxicity (Fig. 2 and Table 1) although they work through distinct mechanisms (Ishige et al., 2001). Similarly, flavonoids such as epigallocatechin gallate (EGCG) that were not effective against oxidative glutamate toxicity also failed to protect against IAA toxicity (Table 1).

We next looked at when the flavonoids were required to be present with respect to the IAA treatment in order to provide protection. We were particularly interested in determining if they could be added after the removal of the IAA and still provide protection. In the basic assay, the flavonoids were present both during the treatment with IAA and they were added to the fresh medium following IAA removal. To determine when the flavonoids needed to be present in order to prevent IAA-induced cell death, the protection seen when flavonoids were present only during the IAA treatment or only following the IAA treatment (Fig. 2, R only) was compared to that seen when the flavonoids were present both during and after the IAA treatment (Fig. 2, I/R). When the flavonoids were present only during the 2 h treatment with IAA, no protection was seen (data not shown). Importantly, when the flavonoids were added only after the removal of the IAA, they still provided significant levels of protection (Fig. 2, R only and Table 1).

Although flavonoids are classically described as antioxidants, they have a number of other actions that contribute to their neuroprotective activity. This idea was substantiated when a variety of classical antioxidants, including vitamin E, Trolox and vitamin C were compared with the best flavonoids against IAA toxicity. As shown in Table 1, the flavonoids are significantly more effective than any of these antioxidants at inhibiting IAA toxicity.

In order to further enhance our screen for potential neuroprotective compounds for the treatment of stroke, we tested the flavonoids in several additional assays, examining their ability to maintain ATP and GSH levels in the presence of IAA and their ability to induce the expression of phase II detoxification proteins. ATP loss is one of the hallmarks of ischemia (Lipton, 1999) and is rapidly induced in retinal cells following treatment with IAA (Winkler et al., 2003). Similarly, treatment of the HT22 cells with 20 μ M IAA for 2 hr results in a 30% loss of ATP which continues to decrease further to 50–60% over the following 2 h (Fig. 3A). Treatment with several of the flavonoids reduces or prevents the loss of ATP in response to IAA treatment (Fig. 3A and Table 1). Of the flavonoids tested, fisetin is one of the best at maintaining ATP levels.

GSH is the major antioxidant in the brain. It scavenges free radicals, reduces peroxides and can be conjugated with electrophilic compounds, thereby providing cells with multiple defenses against both reactive oxygen species (ROS) and their toxic by-products (Maher,

2005). Both total (Koroshetz and Moskowitz, 1996) and mitochondrial (Anderson and Sims, 2002) GSH levels fall during ischemia and increased amounts of both GSH disulfide and oxidative stress are detected in ischemic brain tissue (Baek et al., 2000). Similar to the *in vivo* situation, IAA treatment of the HT22 cells leads to a substantial loss of GSH (Fig. 3). This loss can be prevented by the same subset of neuroprotective flavonoids that maintain ATP levels and, among these, fisetin was the best (Fig. 3B and Table 1).

Induction of the genes encoding phase II detoxification proteins through the activation of the antioxidant response element (ARE) in the promoter region can provide long-term protection of cells against oxidative stress (Dickinson et al., 2004; Hayes and McLellan, 1999; Nguyen et al., 2003). Transcriptional activation of the ARE is dependent on the transcription factor Nrf2, a member of the Cap'n'Collar family of bZip proteins (Nguyen et al., 2003). To determine whether any of the flavonoids that protected against IAA toxicity can activate the ARE and induce the synthesis of phase II detoxification proteins, we first treated the HT22 cells for 30 min to 4 h with the optimal effective concentrations of the different protective flavonoids or resveratrol and looked for an increase in the level of the transcription factor Nrf2 in the nuclei of cells. Among the group of neuroprotective flavonoids, only fisetin, quercetin and kaempferol increased Nrf2 levels (Fig. 4). To determine whether the increases in Nrf2 levels translated into an increase in phase II detoxification proteins, we treated the HT22 cells for 24 h with the same flavonoids and looked for an increase in HO-1 levels. We chose HO-1 because its synthesis is generally dependent on the ARE and robust antibodies are available. As shown in Figure 4, two out of three of the flavonoids that increase nuclear Nrf2 also induce HO-1 synthesis. Of the two active flavonoids, fisetin is the most robust.

To determine if the combination of the *in vitro* ischemia assay and the biochemical assays provides a good screen for novel neuroprotective compounds for the treatment of stroke, we decided to test one of the flavonoids in a rigorous animal stroke model, the rabbit SC/M. Based on its performance in the *in vitro* ischemia assay, in combination with its excellent activity in the additional biochemical assays, and coupled with its previously shown abilities to promote neuronal differentiation (Sagara et al., 2004) and enhance memory (Maher et al., 2006), we chose to test fisetin. In this experiment, we administered either vehicle (90% β -hydroxypropyl cyclodextrin in PBS containing 10% HPLC-grade dimethylsulfoxide) or bolus injections of fisetin (50 mg/kg) over 1 minute starting 5 minutes following embolization. This dose and treatment schedule were chosen based on earlier studies with the phenylpropanoid chlorogenic acid (Lapchak, 2007) and the free radical trapping spin trap NXY-059 (Lapchak et al., 2004b) using this stroke model. Behavioral analysis was conducted 24 h following treatment, which allowed for the construction of quantal analysis curves. Figure 5 shows a graphical representation of the raw data that is superimposed on the theoretical quantal analysis curves. For the superimposed graphs, normal animals are plotted on the y-axis at 0 and abnormal animals are plotted at 100. The figure shows that there is positive correlation between the data (circles or triangles) and the statistically fitted quantal curve. Fisetin significantly ($p < 0.05$) reduced stroke-induced behavioral deficits and increased the P_{50} value to 2.53 ± 0.55 mg. The fisetin-induced improvement in behavior was directly correlated with an increase in the number of animals that were behaviorally "normal" as shown on the y-axis plotted at 0. The P_{50} value for the vehicle-treated control group was 1.06 ± 0.15 mg.

Discussion

The above data show that an *in vitro* neuroprotection screen using a neuronal cell line and chemical ischemia in combination with biochemical assays targeting key metabolic changes associated with ischemic stroke *in vivo* can be used to identify novel neuroprotective compounds that are effective against stroke in an animal model. Although in this study only a limited screen of potential neuroprotective compounds was carried out for the purpose of

providing proof of principle for this approach, in the future it could be used to screen a large number of compounds in order to provide potential new therapeutic compounds for the treatment of stroke.

Ischemic stroke causes a decrease in the availability of both oxygen and glucose to nerve cells (Lipton, 1999). Based on its known mode of action, treatment with IAA would be expected to impact glycolysis while having little effect on mitochondrial function. However, the changes observed following IAA treatment of neural cells are very similar to changes which have been seen in animal models of ischemic stroke (Lipton, 1999) and include alterations in membrane potential (Reiner et al., 1990), breakdown of phospholipids (Taylor et al., 1996), loss of ATP (Sperling et al., 2003; Winkler et al., 2003) and an increase in reactive oxygen species (ROS) (Sperling et al., 2003; Taylor et al., 1996). These changes are greater than those seen with either oxygen depletion or inhibition of mitochondrial function alone and are more similar to the effects seen with a combination of mitochondrial inhibitors and glucose uptake inhibitors. Thus, IAA treatment appears to more closely mimic the intracellular changes seen in stroke than other *in vitro* approaches to modeling ischemia.

Similar to stroke *in vivo*, IAA treatment also induces oxidative stress as measured by decreases in GSH (Fig. 3B), increases in ROS (Sperling et al., 2003; Taylor et al., 1996) and enhancement of lipid peroxidation (Taylor et al., 1996). However, classical antioxidants such as vitamin E are only weakly protective against IAA toxicity (Table 1) which is similar to the *in vivo* situation where classical antioxidants have not proven effective for the treatment of stroke (Green and Shuaib, 2006). The reasons for this are not entirely clear, although several possibilities can be postulated. First, classical antioxidants may not reach the sites of ROS production in cells. Second, classical antioxidants may not be particularly effective against the specific types of ROS that are produced in stroke. Third, classical antioxidants may not maintain GSH levels. Indeed, Bains and Shaw (Bains and Shaw, 1997) have suggested that an impairment of GSH status is the precipitating event in a wide range of neurodegenerative disorders, including stroke. Furthermore, treatment with GSH analogues (Anderson et al., 2004; Margail et al., 2005), pharmacological stimulation of GSH synthesis by tert-butylhydroquinone (Shih et al., 2005) or transgenic overexpression of GSH peroxidase (Weisbrot-Lefkowitz et al., 1998) can reduce infarct size in animal models of ischemic stroke. However, regardless of the reason, our results suggest that non-classical antioxidants such as flavonoids that also have additional biological activities, such as maintenance of ATP and GSH levels as well as induction of Nrf2 and phase II detoxification proteins, may be much more effective for the treatment of stroke.

One of the more important observations to emerge from our study is that flavonoids can be added even after the initial ischemic insult and still provide a significant level of protection. Since treatment of stroke often does not begin until several hours after the initial insult, drugs which have long time windows of efficacy are likely to be the most useful. Indeed, the failure of many clinical trials is likely due to the short treatment window in which the compounds can be administered. Our data suggest that a focus on compounds that are particularly effective in the *in vitro* assay when added after the initial insult could provide a more direct way of identifying drugs which might be useful over a longer time window.

While there is some limited epidemiological (Graf et al., 2005) and experimental evidence in animals (Rivera et al., 2004; van Leyen et al., 2006) that flavonoids could be useful for the treatment of stroke, there have been no systematic evaluations of what flavonoids might be most effective. For this study, we tested flavonoids that had already been shown to be effective in another model of oxidative stress-induced nerve cell death, oxidative glutamate toxicity. Since this toxic insult has several features in common with ischemic stroke, including GSH loss and mitochondrial dysfunction (Tan et al., 2001), it is not surprising that compounds that were effective in this assay could also protect in the chemical ischemia model. However, what

is surprising, is that even flavonoids such as hydroxyflavone, 3,6 dihydroxyflavone and 3,7 dihydroxyflavone that have been shown to protect against oxidative glutamate toxicity via mechanisms unrelated to antioxidant activity were protective against *in vitro* ischemia. Furthermore, given the success of this screening approach, it should now be worthwhile to screen a much wider range of flavonoids in order to identify ones which might be significantly more potent than those that we tested.

In the present study, we tested the flavonoid fisetin in the rabbit SCCEM. The improvement in behavioral outcome relative to the control rabbits that we observed was better than that seen with the antioxidant ebselen (Lapchak and Zivin, 2003) and similar to that seen with several other compounds including the phenylpropanoid chlorogenic acid (Lapchak, 2007) and the spin trap NXY-059 (Lapchak et al., 2004b). This result further supports the use of the *in vitro* chemical ischemia assay in combination with the biochemical assays as an initial screen for potential neuroprotective compounds for the treatment of stroke.

In summary, using a chemical ischemia survival assay in combination with several biochemical assays, we have characterized a new approach to identifying novel compounds for the treatment of ischemic stroke. The validity of this screening method is supported by the *in vivo* neuroprotective activity seen in the rabbit SCCEM with fisetin, thereby setting the stage for the identification of even more potent neuroprotective compounds.

Experimental Procedure

Chemicals

Flavonoids were from Alexis (San Diego, CA), Sigma/Aldrich (St. Louis, MO) or Indofine Chemical Co. (Hillsborough, NJ). All other chemicals were from Sigma.

Cell culture

Fetal calf serum (FCS) and dialyzed FCS (DFCS) were from Hyclone (Logan, UT). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). HT-22 cells (Davis and Maher, 1994; Maher and Davis, 1996) were grown in DMEM supplemented with 10% FCS and antibiotics.

Cytotoxicity assay

Cell viability was determined by a modified version of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay based on the standard procedure (Hansen et al., 1989). Briefly, cells were seeded onto 96-well microtiter plates at a density of 5×10^3 cells per well. The next day, the medium was replaced with DMEM supplemented with 7.5% DFCS and the cells were treated with 20 μ M iodoacetic acid (IAA) alone or in the presence of the different flavonoids or antioxidants. After 2 h the medium in each well was aspirated and replaced with fresh medium without IAA but containing the flavonoids or antioxidants. 20 h later, the medium in each well was aspirated and replaced with fresh medium containing 2.5 μ g/ml MTT. After 4 h of incubation at 37°C, cells were solubilized with 100 μ l of a solution containing 50% dimethylformamide and 20% SDS (pH 4.7). The absorbance at 570 nm was measured on the following day with a microplate reader (Molecular Devices). Results obtained from the MTT assay correlated directly with the extent of cell death as confirmed visually. Controls included compound alone to test for toxicity and compound with no cells to test for interference with the assay chemistry.

SDS-PAGE and immunoblotting

For immunoblotting of HO-1, untreated and flavonoid-treated HT22 cells from the same density cultures as used for the cell death assays were washed twice in cold phosphate-buffered

saline (PBS) then scraped into lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄ and 1/100 volume Sigma protease inhibitor mix. Lysates were incubated at 4°C for 30 min, then cleared by centrifugation at 16,000 x g for 10 min. For immunoblotting of Nrf2, nuclear extracts were prepared as described (Schreiber et al., 1989) from untreated and flavonoid-treated cells. For each flavonoid, the concentration which was most effective at preventing cell death was used. Protein concentrations in the cell extracts were determined using the BCA protein assay (Pierce). Equal amounts of protein were solubilized in 2.5X SDS-sample buffer, separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. The primary antibodies used were: anti-Nrf2 (#SC13032; 1/1000) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-heme oxygenase-1 (HO-1) (#SPA-896; 1/5000) from Stressgen (Victoria, BC Canada) and anti-actin (#A5441; 1/40,000) from Sigma.

Total intracellular glutathione (GSH)

Total intracellular GSH was determined using whole cell lysates from untreated, IAA treated, flavonoid or antioxidant treated and IAA plus flavonoid or antioxidant treated cells as described (Maher and Hanneken, 2005a) and normalized to total cellular protein. For each flavonoid or antioxidant tested, the concentration which was most effective at preventing cell death as determined by a dose response experiment was used.

Intracellular ATP

Intracellular ATP was determined using lysates from untreated, IAA treated, flavonoid or antioxidant treated and IAA plus flavonoid or antioxidant treated cells. HT22 cells from the same density cultures as used for the cell death assays were washed twice in cold phosphate-buffered saline (PBS) then scraped into lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM sodium pyrophosphate, 1 mM Na₃VO₄ and 1% Triton X-100. Lysates were incubated at 4°C for 30 min, then cleared by centrifugation at 14,000 x g for 10 min. ATP levels were determined using a chemiluminescent kit from Molecular Probes and normalized to total cellular protein. For each flavonoid or antioxidant tested, the concentration which was most effective at preventing cell death as determined by a dose response experiment was used.

Statistical analysis

The *in vitro* cell death assays and the biochemical assays were repeated at least three times. The data are presented as the mean ± SEM. ANOVA followed by Bonferroni's test was used to compare the data obtained. P < 0.05 was considered significant.

Rabbit Small Clot Embolism Model (SCEM)

Using the rabbit SCEM as described previously (Lapchak et al., 2002; Lapchak et al., 2004a; Lapchak et al., 2004b; Lapchak et al., 2004c) male New Zealand white rabbits weighing 2.2–2.6 kg were anesthetized and a catheter was inserted into the common carotid artery through which microclots were injected. Briefly, the bifurcation of one carotid artery is exposed and the external carotid is ligated just distal to the bifurcation. A catheter is inserted into the common carotid and secured with ligatures. The incision is closed around the catheter so that the distal end is accessible outside the animal's neck. The catheter is then filled with heparinized saline and plugged with an injection cap. Rabbits are allowed to recover from anesthesia until they are awake and behaving normally. The Department of Veterans Affairs approved the procedures used in this study.

For the SCEM, blood is drawn from one or more donor rabbits and allowed to clot for 3 h at 37°C. The large blood clots are then suspended in PBS with 0.1% bovine serum albumin and

Polytron-generated fragments are sequentially passed through metal screens and nylon filters (Lapchak et al., 2002; Lapchak et al., 2004a; Lapchak et al., 2004b; Lapchak et al., 2004c). The resulting small clot suspension is then labeled with ^{57}Co containing NEN-Trac microspheres. The addition of microspheres allows us to calculate the dose of clots that becomes lodged in the brain following embolization. For embolization, rabbits are placed in Plexi-glass restrainers, the catheter injection cap is removed and the heparinized saline is cleared from the carotid catheter system. Then 1 ml of a clot particle suspension containing small sized blood clots and ^{57}Co NEN-Trac microspheres is rapidly injected through the catheter into the brain, which is then flushed with 5 ml of normal sterile saline. Rabbits are fully awake during the embolization procedure and they are self-maintaining (i.e. they do not require artificial respiration or other external support). This allows for immediate observation of the effects of embolization on behavior at the time of clot injection and thereafter.

To evaluate the quantitative relationship between clot dose and behavioral deficits, logistic S-shaped quantal analysis curves are fitted to the dose-response data as originally described by Waud (Waud, 1972) and thereafter (Lapchak et al., 2002; Lapchak et al., 2004a; Lapchak et al., 2004b; Lapchak et al., 2004c; Zivin and Waud, 1992). A wide range of clot doses is used resulting in behaviorally normal and abnormal animals. In the absence of a neuroprotective treatment regimen, small numbers of microclots cause no grossly apparent neurologic dysfunction and large numbers of microclots invariably caused encephalopathy or death. Using a simple dichotomous rating system, with a reproducible composite result and low inter-rater variability (<5%), each animal was rated by a naïve-observer as either behaviorally normal or abnormal 24 h post-embolization. Abnormal rabbits include those with one or more of the following symptoms: ataxia, leaning, circling, lethargy, nystagmus, loss of balance, loss of limb/facial sensation and occasionally, paraplegia. Using quantal analysis, we are to detect behavioral changes following pharmacological intervention. With this simple rating system, the composite result for a group of animals is quite reproducible. A separate curve is generated for each treatment condition and a statistically significant increase in the P_{50} value or the amount of microclots that produce neurologic dysfunction in 50% of a group of animals (Lapchak et al., 2002; Lapchak et al., 2004a; Lapchak et al., 2004b; Lapchak et al., 2004c; Zivin and Waud, 1992) compared to control is indicative of a behavioral improvement.

The behavioral data are presented as P_{50} (mean $\pm$ SEM) in mg clots for the number of rabbits in each group (n). P_{50} values were analyzed using the Student's t-test. Drug-treated groups are directly compared to respective control groups from each study. For all experiments in this study, rabbits were randomly allocated into treatment groups before the embolization procedure, with concealment of the randomization guaranteed by using an independent third party. The randomization sequence was not revealed until all post-mortem analyses were complete.

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Abbreviations

ARE	antioxidant response element
DMEM	Dulbecco's modified Eagle's medium
EGCG	epigallocatechin gallate
FCS	fetal calf serum
GSH	glutathione
HO-1	heme oxygenase 1
IAA	iodoacetic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Nrf2	

NF-E2-related factor 2

ROS

reactive oxygen species

rt-PA

recombinant tissue-type plasminogen activator

SCEM

small clot embolism model

Figure 1. Dose Dependence of IAA Toxicity

HT22 cells were treated with increasing doses of IAA for 2 hr. % survival was confirmed by microscopy and measured after 24 hr by the MTT assay. Similar results were obtained in four independent experiments. * indicates a significant difference between control and IAA-treated cells ($p < 0.001$; ANOVA followed by Bonferroni's test)

Figure 2. Flavonoids Protect from IAA Toxicity in HT22 Cells

HT22 cells were treated with 20 μ M IAA (HT22) for 2 hr alone (Ct) or in the presence of 10 μ M 3 hydroxyflavone (HF), 10 μ M 3,6 dihydroxyflavone (3,6 DHF), 10 μ M 3,7 dihydroxyflavone (3,7 DHF), 10 μ M galangin, 10 μ M baicalein, 10 μ M kaempferol, 10 μ M luteolin, 10 μ M fisetin or 10 μ M quercetin. The flavonoids were also included in the fresh medium added after the 2 hr treatment with IAA (I/R). In other studies, the flavonoids at the same doses were added only to the fresh medium added after the 2 hr IAA treatment (R only). % survival was confirmed by microscopy and measured after 24 hr by the MTT assay. Similar results were obtained in 3–5 independent experiments. All flavonoid treatments provided significant protection ($p < 0.001$; ANOVA followed by Bonferroni's test) from IAA toxicity.

Figure 3. Flavonoids Maintain ATP and GSH Levels in Response to Chemical Ischemia

HT22 cells were treated with 20 μ M IAA for 2 hr followed by 2 hr in fresh medium alone (Ct) or in the presence of 10 μ M 3 hydroxyflavone (HF), 10 μ M 3,6 dihydroxyflavone (3,6 DHF), 10 μ M 3,7 dihydroxyflavone (3,7 DHF), 10 μ M galangin, 10 μ M baicalein, 10 μ M kaempferol, 10 μ M luteolin, 10 μ M fisetin, 10 μ M quercetin or 10 μ M resveratrol. Total ATP (A) and GSH (B) levels were measured by chemical and chemiluminescent assays, respectively as described in Methods. Similar results were obtained in 3–5 independent experiments. * indicates a significant difference between IAA-treated cells (Ct) and cells treated with IAA + flavonoids ($p < 0.001$; ANOVA followed by Bonferroni's test) ** indicates a significant difference between IAA + fisetin and IAA + other flavonoids ($p < 0.001$; ANOVA followed by Bonferroni's test)

Figure 4. Flavonoids Induce the Expression of Nrf2, the ARE-specific transcription factor, and HO-1, a phase II detoxification protein

HT22 cells were untreated (Ct) or treated with 10 μ M fisetin, 10 μ M 3 hydroxyflavone (HF), 10 μ M 3,6 dihydroxyflavone (3,6 DHF), 10 μ M 3,7 dihydroxyflavone (3,7 DHF), 10 μ M galangin, 10 μ M baicalein, 10 μ M quercetin, 10 μ M luteolin, 10 μ M kaempferol or 10 μ M resveratrol for 2 hr (Nrf2) or 24 hr (HO-1). Nuclei were prepared (Nrf2) and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with Nrf2 antibodies or cell lysates were prepared and equal amounts of protein were analyzed by SDS-PAGE and immunoblotting with HO-1 antibodies. Immunoblotting with anti-actin is shown as a loading control. Similar results were obtained in 3 independent experiments.

Figure 5. Behavioral Improvements in the Rabbit SCEM Following Fisetin Treatment

The control curve (dotted line) has a P₅₀ value of 1.06 ± 0.15 mg (n = 19). Fisetin treatment (50 mg/kg IV) initiated 5 minutes following embolization increased the P₅₀ value to 2.53 ± 0.55 mg (n = 19, *P < 0.05) (dark solid line). The dark circles ● represent the raw data from the control group and the triangles ▲ represent the raw data for the fisetin-treated group. A normal animal for a specific clot weight is represented by a symbol plotted at 0% on the y-axis, whereas an abnormal animal for a specific clot weight is represented by a symbol plotted at 100% on the y-axis.

Table 1
Protection of HT22 Cells from IAA Toxicity by Flavonoids and Antioxidants

Compound	Free hydroxyl positions	IAA EC ₅₀	Post-IAA EC ₅₀	ATP	GSH	ARE
Flavonol	3	6.6 ± 1.5	14.7 ± 1.4	no	no	no
	3,6	5.2 ± 0.6	7.4 ± 0.6	no	no	no
	3,7	3.3 ± 0.1	8.2 ± 0.5	no	no	no
galangin	3,5,7	5.0 ± 0.6	4.6 ± 0.6	no	no	no
baicalein	5,6,7	1.4 ± 0.2	1.9 ± 0.1	no	no	no
kaempferol	3,4',5,7	3.7 ± 0.3	7.1 ± 0.8	yes	yes	no
luteolin	3',4',5,7	2.9 ± 0.2	4.2 ± 0.3	yes	yes	no
fisetin	3,3',4',7	2.8 ± 0.5	4.8 ± 0.4	yes	yes	yes
quercetin	3,3',4,5,7	6.4 ± 0.3	7.8 ± 0.9	yes	yes	yes
EGCG		>50	>50	ND	ND	ND
resveratrol		16.4 ± 0.4	15.4 ± 0.7	no	no	no
vitamin E		>100	>100	ND	ND	no
Trolox		>50	>50	ND	ND	ND
vitamin C		>100	>100	ND	ND	ND

Half maximal effective concentrations (EC₅₀ ± SEM) for protection were determined by exposing HT22 cells to different doses of each flavonoid or antioxidant in the presence of 20 μM IAA for 2 h. Cell viability was determined after 24 h by the MTT assay. Total GSH and ATP levels were measured by chemical and chemiluminescent assays, respectively (see Fig. 3). ARE activation was determined by measuring the levels of both nuclear Nrf2 and cellular HO-1 (see Fig. 4). EGCG, epigallocatechin gallate. ND-not done