Neuroprotectants in Honghua: Glucose Attenuates Retinal Ischemic Damage

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Purpose. This study examined the neuroprotective properties of Honghua, an extract of safflower used as an herbal medicine in China, in several experimental models of retinal ischemia.

Methods. Honghua and other agents were tested (1) in the ex vivo chick embryo retina assay (CER) for anti-excitotoxin efficacy and against simulated ischemia (30 min glucose/oxygen deprivation); and (2) in the in vivo adult rat retina dye-photothrombosis assay. Active components of Honghua were purified by conventional chromatographic techniques.

Results. In the CER, Honghua protected against excitotoxicity of glutamate, N-methyl-D-aspartate, kainate, and quisqualate, and against neuronal degeneration caused by simulated ischemia. Honghua more potently protected against simulated ischemia than against the agonists. In the in vivo adult rat retina, ischemic damage was reduced greatly by intravitreal injection of Honghua. An approximately 100-fold purification of an active principle was achieved chromatographically. The purest fractions were rich in glucose, so the effects of glucose in the ischemia models were determined. Many neuroprotective effects of Honghua were mimicked by pure solutions of equivalent glucose concentration. Glucose (>3.2 mmol/l) in the CER-ischemia assay provided protection. Glucose did not protect against the lesions induced by direct application of the excitotoxic agonists. Intravitreal injection of glucose provided highly significant neuroprotection in the adult rat retina dye-photothrombosis model.

Conclusions. These results suggest that retinal excitotoxic damage in vivo can occur secondary to depletion of cellular energy reserves, and therefore may be prevented by simple procedures that maintain the availability of energy sources. Invest Ophthalmol Vis Sci. 1993;34:72–80.

The excitatory amino acid (EAA) transmitters glutamate and aspartate are likely involved in the pathophysiology of neuronal damage that occurs secondary to ischemia.1,2 These molecules interact with post-synaptic receptors to mediate normal excitatory synaptic transmission. When present in excessive amounts, however, they are excitotoxic, causing neuronal degeneration.3,4 Neuronal ischemia results in elevated concentrations of extracellular glutamate.5,6 This may be due to impaired operation of membrane transport systems secondary to the depletion of cellular energy reserves.7

Current models of the mechanisms involved in ischemic neuronal disease suggest that pharmacologic interventions can be devised to help manage these conditions. Antagonists of the EAA receptors are being developed for this purpose. Other agents that work pre-synaptically to lessen glutamate release,8 and those that act post-synaptically to alter the biochemical

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sequelae initiated by glutamate receptor activation also have been described.9

Many in vivo and in vitro models of ischemic and excitotoxic neuronal damage have been devised. Simple model systems offer the opportunity to discover new or previously unrecognized compounds with neuroprotective properties. We have been examining an extract prepared from the petals of the Chinese safflower (Carthamus tinctorius, Honghua)10 for neuroprotective activity. This material is used in traditional Chinese medicine for a wide variety of disorders, but most often for conditions expected to benefit from increased local or visceral blood flow (eg, applied topically for traumatic wound healing and taken systemically for amenorrhea, coronary heart disease, and cerebrovascular disease). We report that Honghua has neuroprotective activity in several in vitro and in vivo model systems. Some, but not all of these actions can be quantitatively accounted for by the glucose content of the extract.

MATERIALS AND METHODS

Preparation of the Honghua Extract

Dried safflower petals were obtained from several sources. All the experiments reported here were performed using materials obtained from pharmacies in Shanghai and believed to have been grown in Manchuria, or from Weng Li Sendiran Berhad (Malaysia) and believed to have been grown in Spain. Extracts derived from locally obtained safflower petals, or from petals obtained through several other Chinese sources, had much less neuroprotective activity. Petals that yielded potent extracts invariably were colored dark red streaked with yellow and were recognizable on sight. However, the data do not suggest that the neuroprotective activities of Honghua reside in the colored components.

Petals (5–50 g) were boiled in >10 volumes of water for at least 30 min. The spent petals were removed by centrifugation, particulate matter was removed by 0.4 μm filtration, and the volume was reduced by further boiling until the final concentration was 1 or 2 g initial weight per milliliter concentrated extract. Concentrations of Honghua used in experiments are referred to as milligrams initial weight per milliliter assay volume. For example, 100 μl of an extract that was boiled to 1 g initial weight/ml, when diluted to 1 ml in assay buffer, provides a final concentration of 100 mg/ml. The undiluted extracts were dark brown and viscous, and the A257 was ≈2000. Precipitate continuously accumulated with time, so experiments were performed with freshly prepared material, or the extracts were clarified by centrifugation and filtration before use. Although not rigorously investi-gated, removal of precipitate from the crude concentrated extract did not appear to diminish potency or efficacy.

Assays for Neuroprotective Activities

All investigations involving animals conformed to the ARVO Statement for the Use of Animals in Ophthal-mic and Vision Research.

Chick Embryo Retina. Fifteen-day-old chick embryos were decapitated and their retinas were rapidly removed and cut into thirds. Each retinal segment was incubated for 30 min at 37°C in basic salt solution containing (in mmol/l) 123 Na+, 5 K+, 1.2 Ca2+, 0.9 Mg2+, 133 Cl−, 24 HCO3−, 0.44 PO43−, and 5 (3-[N-morpholino]propane sulfonic acid) at pH 7.4. Various concentrations of Honghua, glucose, pyruvate, or 2-deoxyglucose (0–250 mmol/l) were included in the incubation media. Some vials also contained toxic amounts of excitotoxins (40 μmol/l NMDA, 25 μmol/l KA, 15 μmol/l Quis, or 600 μmol/l Glu), as indicated. Simulated ischemic conditions were generated by excluding glucose from the incubation media and subjecting the retinal segments to a 100% nitrogen atmosphere. After incubation, retinal segments were fixed by immersion in phosphate buffered solution that contained 1.5% glutaraldehyde and 1% paraformaldehyde. They then were fixed in 1% osmium tetroxide and embedded in araldite. One micrometer sections were cut on ultramicrotomes, mounted, and stained with methylene blue/azure II for evaluation by light microscopy.11

To quantitate the potency of Honghua and other potential neuroprotectants, retinal neuronal damage was assessed using a three point scale. Concentrations that provided little or no protection from the effects of excitotoxins or simulated ischemia were assigned a score of zero, and concentrations that provided full or substantial protection were assigned a score of two. Because of the difficulty of distinguishing different degrees of intermediate protection, concentrations that provided partial protection were assigned a score of 1. Each concentration of protectant was examined in two to six retinal segments in individual experiments, in two to seven independent experiments. EC90 values were estimated by fitting the pooled data to the Hill equation (curve fitting done using IGOR data analysis software; WaveMetrics, Lake Oswego, OR).

Dye/Photothrombosis Ischemia Model. Adult female Sprague Dawley rats (200–300 g) were anesthetized with chloral hydrate and placed in a stereotactic holder.12 Their pupils were dilated by topical administration of phenylephrine HCl and tropicamide. Seven microliters of Honghua, or saline containing 1–100 μmol/l glucose, pyruvate, or 2-deoxyglucose were injected via a microsyringe guided by a micromanipula-tor through the scleral wall into the vitreal chamber of
the experimental eye. The control eye received 7 μl saline. Plastic contact lenses were positioned on each cornea and held in place with a drop of Goniosol (Iolab Pharmaceuticals, Claremont, CA) to protect the cornea from drying and also to uniformly distribute the light across the entire retina. Rose bengal (80 mg/kg) was injected into a femoral vein and both eyes were immediately subjected to strong white light (500 W) filtered (550 nm) to optimize the wavelength for dye sensitivity. Animals were killed 1 hr later by chloral hydrate overdose. Retinas were immediately removed and processed as described for chick embryo retinas above. The severity of retinal damage in this model was evaluated and quantitated as described in detail previously.

Purification of Neuroprotective Activity

Conventional gel-filtration, anion exchange and cation exchange chromatography was performed using BioGel P6, AG1X8, and DOWEX 50 resins (BioRad, Richmond, CA). Hydrophobic adsorption used C18 SepPaks (Waters, Milford, MA).

Measurements of Glucose Concentration

Glucose was measured using a portable monitor (One-Touch; Johnson and Johnson, Arlington, TX) or spectrophotometrically on an AU 5000 (Olympus, Lake Success, NY; using hexokinase coupled to NADP+ reduction by glucose-6 phosphate dehydrogenase) at the Clinical Chemistry Laboratory of Barnes Hospital, St. Louis.

RESULTS

Neuroprotectant Properties of Honghua

The isolated chick embryo retina (CER) exhibits characteristic patterns of histopathologic change when exposed to the selective EAA agonists NMDA, quisqualate, and kainate (illustrated for NMDA in Fig. 1). Honghua fully protected against the lesions induced by each of these agonists (illustrated for NMDA in Fig. 1). Dose-response studies indicated that Honghua more potently protected against NMDA-induced lesions than those resulting from kainate or quisqualate (Fig. 2).

When the isolated CER is subjected to simulated ischemic conditions (oxygen/glucose deprivation) for 30 min, marked histopathologic changes occur that morphologically resemble an excitotoxic lesion caused by glutamate (Fig. 1). These changes can be fully prevented by a combination of non-NMDA and NMDA antagonists, but not by individual receptor-selective antagonists. Honghua provided full protection against the lesion induced by simulated ischemia (Fig. 1). The extract was markedly more potent at protecting against simulated ischemia than against the excitotox-
FIGURE 2. Concentration-response relationships of neuroprotective effects of Honghua against excitotoxins and simulated ischemia. Retinal damage assessed on a three-point scale of no (0), partial (1), or full (2) protection. Each point is the mean of 2–7 independent experiments. NMDA, 40 μmol/l N-methyl D-aspartate; QA, 15 μmol/l quisqualic acid; KA, 25 μmol/l kainic acid; GLU, 600 μmol/l glutamic acid. EC50 values determined by curve-fitting are 10 mg/ml versus simulated ischemia; 43 mg/ml versus NMDA; 99 mg/ml versus QA; 120 mg/ml versus KA; and 94 mg/ml versus GLU.

Ischemia + Saline

Ischemia + Honghua

FIGURE 3. Honghua protects against the lesion induced by dye-photothrombosis in the adult rat retina. Eyes were injected intravitreally with saline (7 μl) or with Honghua extract (7 μl of 1/gm/ml), then subjected to dye-photothrombosis and processed as described in "Materials and Methods."
Identification of the Active Component

The potency of Honghua as a neuroprotectant in the ischemia models prompted our efforts to purify the components of the extract responsible for this activity. Ultraviolet-visible spectroscopy of the crude extract showed maxima at 257, 320, and 435 nm (data not shown). To monitor the course of the purification, fractions were assayed in the CER-simulated ischemia model for neuroprotective potency, and the A257 of each fraction was measured. An example of the enrichment obtained in a typical preparation is illustrated in Fig. 4. An index of neuroprotection versus optical density at 257 nm is plotted for the starting material, the active peak after anion-exchange chromatography, and the active fraction after this fraction is subjected to hydrophobic adsorption. These two steps lead to an approximately 100-fold increase in activity per absorbance unit. Further purification was achieved by combining these steps with cation-exchange and gel filtration chromatography.

The chemical properties of the active component of the extract were revealed by its behavior during purification. It was a small molecule, eluting in the salt volume of BioGel P6. It was heat stable. It appeared to be uncharged, because the activity would not adsorb to anion or cation exchange resins over a wide range of pHs. It also was hydrophilic, because it would not adsorb to SepPak cartridges. A considerable purification was obtained by applying these adsorptive techniques, because inactive components of the extract were removed.

As the chemical properties just described indicated the active principle may be a neutral monosaccharide, the glucose concentration of the crude and purified extracts were determined. Crude Honghua extract (1 gm/ml) contained 149-163 mmol/l glucose, and the purest material contained 179 mmol/l. Although unlikely to explain all the neuroprotectant activities observed, the effects of glucose in the several assays of neurotoxicity were examined.

Effects of Glucose in the Model Systems

CER-Simulated Ischemia. The effects of Honghua in the CER-simulated ischemia model could be quantitatively accounted for by the glucose concentration of the extract (Fig. 5). Glucose concentrations of 3.2 mmol/l provided measurable protection, and higher concentrations provided full protection from damage in the simulated ischemia assay, despite the absence of oxygen. A nonmetabolizable analog of glucose, 2-deoxyglucose, in concentrations up to 32 mmol/l, did not protect, indicating that a metabolic, not a purely physical mechanism was responsible for the effect. Pyruvate, an energy source that can be used only oxidatively, did not provide protection when tested in concentrations up to 32 mmol/l. These results suggest that anaerobic, glycolytic metabolism of the added glucose occurred and was sufficient to maintain tissue energy needs.

CER-Direct Excitotoxicity. Glucose did not protect the CER from excitotoxic damage induced by direct application of EAA agonists. Glucose at concentr-
Glucose/Honghua in Ischemic Retina

tions of up to 36 mmol/l provided no protection against NMDA-induced damage. In single experiments, glucose at high concentrations (>20 mmol/l) provided partial protection against kainate and quisqualate-induced damage. However, this protection against the non-NMDA agonists was neither reproducible nor consistent. These results indicate that the neuroprotective effects of Honghua against damage induced by the excitotoxins are not the result of the glucose content of the extract.

Dye-Photothrombosis. In the in vivo adult rat retina model, glucose (1.05 or 10 μmol) provided substantial attenuation of the lesion observed 60 min after dye-photothrombosis (Table 1).

The mechanism of action of glucose was explored in several control experiments. If glucose attenuated the lesion by serving as an energy source, the nonmetabolizable sugar 2-deoxyglucose should not substitute for it. If some other chemical or physical property of glucose was responsible (eg, an osmotic or redox effect), 2-deoxyglucose should effectively substitute. The former result was obtained: No protection was provided by intravitreal 2-deoxyglucose (Table 1). If glucose was being metabolized by glycolysis, an energy source that depended upon oxidative metabolism (ie, pyruvate) should not protect. This result was obtained: Intravitreal pyruvate did not attenuate the lesion induced by dye-photothrombosis (Table 1).

DISCUSSION

Our findings help clarify the mechanism or mechanisms by which ischemia causes cytopathologic processes that lead to cell death. In investigating what appeared to be a pharmacologic curiosity, we found a straightforward intervention that forestalls ischemic neuronal damage in an in vivo system and focuses attention on the relationship between fundamental metabolic processes and excitotoxic neuronal degeneration.

Regarding the use of Honghua as a medicine, the results of these studies indicate that an aqueous extract of safflower petals contains neuroprotectant activities. However, this does not mean we identified the particular components of this material that may be responsible for its traditional use in any disorder. For example, Honghua is thought to have vascular effects, but our studies have not addressed this question. The experiments described here provide insights only into the components of Honghua responsible for its neuroprotectant activities as expressed in the particular model systems we have employed.

Honghua blocked the histopathologic changes in the in vivo adult rat retina caused by exposure of the preparation to excitotoxins or simulated ischemia. We previously established that the histopathology observed in the CER-simulated ischemia assay has an excitotoxic mechanism, because these changes could be prevented by using a combination of NMDA and non-NMDA receptor antagonists.15 However, the extract more potently protected against simulated ischemia than against the agonist excitotoxins. This raised the interesting possibility that the active principle may not be a receptor antagonist, but might be working by a novel mechanism. Honghua also protected the in vivo adult rat retina from developing a lesion during the 60 min immediately after dye-photothrombosis. This lesion also has been shown to have an excitotoxic mechanism, because it is prevented by using a combination of NMDA and non-NMDA receptor antagonists.15 The purification of the active components of Honghua were monitored with the CER-simulated ischemia assay because of the analogy to the in vivo model, the possibly novel mechanism of action, and the relative simplicity of the experiments.

The evidence suggests that the component of Honghua purified—based on its protective effects in the CER-simulated ischemia assay—is glucose. First, the active component shares many chemical properties with glucose (hydrophilicity, heat stability, neutrality over a wide pH range, molecular weight < 1000). Also, glucose is present in the crude extract and co-purifies with the active component. Most importantly, glucose substitutes for Honghua in the CER-simulated ischemia assay, and the glucose content of the crude extract quantitatively accounts for the neuroprotective activity there.

The evidence indicates that the most likely mechanism of action of glucose involves its use as an energy source under anaerobic conditions, because neither 2-deoxyglucose nor pyruvate would protect in the CER-ischemia assay. It is not clear which metabolic processes supported by glucose are the critical ones that prevent excitotoxic damage. Two nonexclusive hypotheses have been advanced, the first concerning extracellular concentrations of glutamate, the second regarding post-synaptic sensitivity to glutamate.

### TABLE 1. Effects of Intravitreal Glucose and Other Agents on Histopathology Resulting From Dye-Photothrombosis In Vivo

<table>
<thead>
<tr>
<th>Agent</th>
<th>n</th>
<th>Percent Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, 1.05 μmol</td>
<td>14</td>
<td>67</td>
</tr>
<tr>
<td>Glucose, 10 μmol</td>
<td>10</td>
<td>81</td>
</tr>
<tr>
<td>2-deoxyglucose, 10 μmol</td>
<td>7</td>
<td>No protection</td>
</tr>
<tr>
<td>Pyruvate, 10 μmol</td>
<td>6</td>
<td>No protection</td>
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Severity of retinal damage was assessed on a four-point scale, as described previously.13 Percent protection was determined by comparison to lesion in the saline-treated contralateral eye.
concentration gradients of Na\(^+\), K\(^+\), and glutamate across the cell membrane are maintained by energy-dependent mechanisms. As adenosine triphosphate (ATP) levels decline after oxygen/glucose deprivation, these gradients cannot be maintained. It has been proposed that under anoxic/ischemic conditions the electrogenic glutamate uptake system in neurons and glia operates in reverse to transport glutamate out of cells.\(^7\) Extracellular glutamate does become elevated in some models of central nervous system ischemia.\(^5,6\) This pathologically high concentration of extracellular glutamate then interacts with post-synaptic receptors to trigger excitotoxic cell death.

The second hypothesis invokes an increased post-synaptic sensitivity to glutamate under metabolically compromised conditions. This second mechanism may be important in retina, because Zeevak and Nicklas\(^6\) have been unable to detect elevated extracellular glutamate in the CER-simulated ischemia assay despite an extensive excitotoxic lesion. Depletion of ATP will lead to loss of ionic gradients and hence to a gradually developing depolarization. Extracellular concentrations of glutamate that would otherwise be benign may be excitotoxic under these conditions, because the voltage-dependent Mg\(^{2+}\) blockade of NMDA receptors is lost in depolarized cells.\(^17\) In addition, Non-NMDA receptors may more powerfully mediate excitotoxic damage in compromised cells, but this possibility has not been carefully examined. An important avenue for future work will be studying in detail the mechanisms by which energy depletion leads to a lowering of the thresholds for initiation of excitotoxic neurodegeneration.

Notwithstanding our ignorance regarding the precise cellular locus of action, glucose may forestall neurodegenerative events by permitting ATP levels to remain high in the pre- and post-synaptic cells. Without oxygen, the glucose is used relatively inefficiently, and pyruvate and lactate are expected to increase. However, glycolytic metabolism apparently provides sufficient energy to prevent neurotoxicity in the experimental paradigm employed here.

Glucose provided no protection against the neurodegenerative changes seen upon direct application of the excitotoxic amino acids glutamate, NMDA, kainate, or quisqualate to the isolated CER. These results indicate that the activity of Honghua to protect against these neurotoxins must reside in other components of the extract. The chemical nature and mechanisms of action of these other components are being investigated.

Glucose, in concentrations equal to that present in Honghua, provided substantial protection against the damage caused by ischemia that resulted from dye-phothotrombosis in vivo. Neither 2-deoxyglucose, a nonmetabolizable analogue, nor pyruvate, a substrate for oxidative metabolism, provided protection. Therefore, it is likely that glycolytic metabolism of the glucose was the mechanism responsible for the protection obtained in vivo as well as that observed in the in vitro assay.

Our results suggest that when glucose is not provided to the retina by blood flow, local energy stores such as vitreal glucose are used. This interpretation is supported by other studies. Weiss\(^18\) demonstrated that under ischemic conditions (induced by elevated intraocular pressure or enucleation), endogenous glucose in the vitreous of the rabbit is used anaerobically by the retina. The other local source of energy, retinal glycogen, also is used during ischemia.\(^18,19\) Hayreh and Weingeist\(^20\) demonstrated that rhesus monkey retinas tolerated up to 98 min of ischemia (induced by central retinal artery occlusion) without irreversible functional or histopathologic changes. These authors believed the availability of vitreal glucose and retinal glycogen was essential for retinal tolerance to lengthy periods of ischemia.

These studies suggest that local energy stores do support metabolism in the ischemic retina, and they predict that supplementing these stores may forestall ischemic damage. This prediction is borne out by the data reported here and by another recent study. Büchi et al\(^21\) cannulated the anterior chamber of intact rat eyes and studied retinal degeneration after periods of pressure-induced ischemia. When 5% dextrose replaced saline in the reservoir, the retinal damage was significantly less severe. Our results indicate that substantial protection can be observed with a single injection of glucose. Blair et al\(^22\) using a considerably more complex paradigm, also reported results consistent with ours. They surgically removed the lens and vitreous from cat eyes, and 1 mo later induced experimental ischemia by raising the intraocular pressure to 170 mm Hg for up to 4 hr. Severe retinal degeneration was noted 1 wk later. When the vitreal cavity was perfused at 4 ml/min with an oxygenated, nutrient-rich medium during the period of ischemia, the retinal degeneration was prevented. Our findings, and those of Büchi et al\(^23\) indicate that similar results may be obtained by less invasive procedures that use perfusates of much simpler composition.

Regarding the practical implications of our findings, an important caveat should be mentioned. The results from the in vitro and in vivo ischemia preparations pertain to mechanisms that trigger an excitotoxic process that initiates a cascade of pathologic events culminating in the death of many neurons. We have shown that total blockade of EAA receptors or maintenance of energy sources at the tissue level—if implemented from the very beginning of the ischemic epi-
sode—can prevent ischemic neuronal degeneration within the time frame of the experiment. In the in vitro model, we delayed onset of ischemic degeneration for 30 min. In the in vivo model, onset was delayed 1 hr. This signifies that if an energy source can be delivered directly to the affected tissue very early in the course of ischemia, the onset of degeneration could be delayed. This finding sheds light on the dual mechanism involved (EAA release and lowering of EAA excitotoxicity threshold). However, it does not signify that similar protective effects could be achieved by delivering the energy source into an occluded circulatory system that is unable to reach the ischemic tissue, nor does it imply that delivering energy after the excitotoxic cascade has run its course would be beneficial. In global ischemia/reperfusion models and in human studies, it has been shown that hyperglycemia during the course of ischemia or reperfusion can be detrimental. To understand how glucose can be both beneficial and detrimental to ischemic neural tissue, it would be valuable to have a reperfusion model in which glucose can be delivered directly to the ischemic tissue at various times pre- and post-reperfusion. This is a goal of our retinal research.

Although much work is required, our results support the notion that depletion of cellular energy reserves can trigger an acute neurodegenerative reaction with an excitotoxic basis, and therefore can be prevented by EAA receptor antagonists or procedures that maintain the availability of energy sources, as long as treatment is instituted before extensive excitotoxic changes have occurred. As a result, intravitreal delivery of glucose in conditions such as acute central retinal artery occlusion may be therapeutically rational under these circumstances. Maintaining energy stores in this manner may provide additional time for establishing reperfusion before irreversible changes occur.

Key Words

excitotoxicity, glucose, glutamate, ischemia, retina.

References


