Ethanol Plus Caffeine (Caffeinol) for Treatment of Ischemic Stroke
Preclinical Experience

Jaroslaw Aronowski, PhD; Roger Strong, MS; Ali Shirzadi, BS; James C. Grotta, MD

Background and Purpose—Ethanol and caffeine are 2 common psychoactive dietary components. We have recently shown that low-dose ethanol plus caffeine results in a 70% to 80% reduction of infarct volume after reversible common carotid/middle cerebral artery (CCA/MCA) occlusion in rats. The combination (caffeinol) was effective after either oral pretreatment or intravenous administration starting up to 2 hours after stroke onset. Ethanol alone aggravated ischemic damage, while caffeine alone was without effect. Daily caffeinol for 2 weeks before ischemia eliminated the neuroprotection seen with acute treatment (tolerance). The purpose of our present study was to further characterize the properties of caffeinol as a possible treatment for ischemic stroke.

Methods—The transient CCA/MCA occlusion model was used in all experiments. Five sets of experiments were conducted (1) to test the effectiveness of various doses of ethanol (0.2 to 0.65 g/kg) and caffeine (3 to 10 mg/kg) in the caffeinol mixture; (2) to test whether the neuroprotective dose of caffeinol can improve behavioral dysfunction; (3) to test whether chronic ethanol or caffeine before ischemia will affect efficacy of caffeinol treatment; (4) to test whether the protective effect of caffeinol can be improved by pairing it with 35°C hypothermia; and (5) to test whether caffeinol affects frequency of hemorrhage after administration of recombinant tissue plasminogen activator (rtPA) in ischemic animals.

Results—Doses as low as 0.2 g/kg of ethanol and 6 mg/kg of caffeine in the caffeinol were effective in reducing cortical infarct volume and behavioral dysfunction after transient CCA/MCA occlusion. Daily exposure to ethanol but not caffeine before CCA/MCA occlusion eliminated the therapeutic efficacy of acute caffeinol treatment, similar to the tolerance observed after chronic exposure to caffeine. The therapeutic effect of caffeinol could be further improved by pairing it with mild intraischemic hypothermia, and caffeinol did not increase hemorrhagic infarction when given in combination with rtPA.

Conclusions—Low doses of caffeinol, equivalent to no more than 2 to 3 cups of strong coffee and 1 cocktail, are consistently and highly neuroprotective, are well tolerated, can be added to other therapies to increase the effect of each, and do not interfere with or complicate rtPA therapy. Caffeinol is an appropriate candidate for clinical trial in stroke patients, although it may be less effective in patients with regular alcohol intake. (Stroke. 2003;34:1246-1251.)

Key Words: caffeine ■ cerebral ischemia, focal ■ ethanol ■ intracerebral hemorrhage ■ neuroprotection

Despite extensive research over the last decade, the only therapy approved in humans for treatment of ischemic stroke is thrombolysis with recombinant tissue plasminogen activator (rtPA). The effectiveness of rtPA has significant limitations. Among these are the increased risk of hemorrhagic complications and incomplete efficacy. Even in patients treated within 3 hours who achieve successful recanalization of the occluded offending artery, excellent clinical recovery occurs in only 50%. The nature of brain damage that occurs despite timely recanalization is probably multifactorial but is a mixture of cellular necrosis and delayed cell death. This cytotoxicity is one target of so-called neuroprotective therapy. A neuroprotective strategy that has high efficacy, has low toxicity, has a long window of opportunity, can be easily and readily administered, and can be used in combination with thrombolysis may improve results presently achieved with intravenous rtPA.

We recently reported that a combination of a low dose of ethanol plus caffeine (caffeinol) administered to rats up to 2 to 3 hours after the onset of transient focal ischemia robustly reduces cortical infarct volume. The doses of ethanol and caffeine that showed neuroprotective effect were low and clinically relevant, suggesting that treatment based on this combination might potentially be used in the clinic to manage ischemic stroke.

In this report we present new data pertaining to dose response, functional outcome, tolerance, and interaction with hypothermia and rtPA that might be relevant to future clinical application of caffeinol.
Materials and Methods
Except for experiments with rtPA, all rats were adult male Long-Evans (Harland Long-Evans, Indianapolis, Ind) weighing 350 to 375 g allowed free access to water and food. In experiments with rtPA, male Long-Evans rats weighing 480 to 520 g were used. Caffeine (1,3,7-trimethylxanthine) was purchased from Acros Organics, ethyl alcohol 190 proof from Quantum Chemical Corporation, USI Division, and human rtPA from Genentech Inc.

Production of Ischemia
Focal ischemia was induced by temporary left common carotid/middle cerebral artery (CCA/MCA) occlusion as described previously. Briefly, animals fasted overnight were anesthetized with chloral hydrate (0.45 g/kg IP). The right femoral vein and artery were cannulated for arterial blood pressure recording and drug administration. Core body temperature was maintained at 36.5±0.5°C during ischemia and the first hour of reperfusion through the use of a feed-forward temperature controller. In a separate group of rats (data not included), we determined that intraischemic brain temperatures were similar in animals treated with saline (n=5) and caffeine (0.65 g/kg ethanol plus 10 mg/kg caffeine; n=4). The ipsilateral CCA was isolated and tagged through a ventral, cervical midline incision. A 0.005-inch-diameter stainless steel wire (Small Parts Inc) was placed underneath the left MCA rostral to the rhinal fissure, proximal to the major bifurcation of the MCA, and distal to the lenticulostriate arteries. The artery was then lifted, and the wire was rotated clockwise to ensure occlusion. The CCA was then occluded with an atrumatic aneurysm clip. Cerebral perfusion at the cortical surface, 3 mm distal to the locus of the MCA occlusion, was measured with a laser-Doppler flowmeter (LDF) (model BPM2, Vesamedic). Only those animals that displayed a cerebral perfusion reading < 10 (approximately 12% to 15% of the initial value) on the LDF scale (expressing relative values of cerebral perfusion) were included in the study. In our original studies with ethanol and caffeine we determined that caffeine did not affect blood pressure and other physiological variables including pH, PO2, and PCO2. After 180 minutes of MCA/CCA occlusion (120-minute MCA/CCA occlusion was used only in experiments with caffeine started after the onset of ischemia), reperfusion was established by reversing the occlusion procedure. After the indicated duration of reperfusion (depending on the study), animals were reanesthetized with chloral hydrate and had intracardiac perfusion with 50 mL of saline. Perfused isolated brains were transferred into ice-cold PBS for sectioning. With the use of a Jacobowitz brain slicer, 2-mm-thick coronal sections were cut before staining with 2% 2,3,5-triphenyl-tetrazolium chloride (TTC) in PBS for 30 minutes at room temperature for infarct volume discrimination. Stained sections were then transferred to 10% phosphate-buffered formalin for storage before infarct volume determination. Because of the rapidly developing nature of neuronal loss in our model of ischemia, in most of the studies infarct volume was determined 1 or 3 days after induction of ischemia. All procedures were in compliance with National Institutes of Health guidelines for the humane care of animals and were approved by the institutional animal welfare committee.

Infarct Volume Analysis
Morphometric determination of infarct volume was obtained with the help of a computer-based image analyzer operated by Brain software (Drexel University), as previously described. Infarcts produced by our protocol are restricted to cortical tissue. The infarct volume (mm3) was calculated from the difference between the volume of contralateral cortex and the volume of the TTC-stained (nonischemic) portion of ipsilateral cortex of each rat. This indirect measure of infarct volume, based on the assumption that the volumes of the ipsilateral and contralateral cortex are the same before ischemia, corrects the total infarct volume for the edema component.

Behavior
All sensorimotor tests were performed during the light cycle. Animals were pretested the day before the surgery and then tested on days 2, 4, 7, and 10 and just before animals were killed for infarct volume determination (day 14). Throughout the study we used 2 sensorimotor tests: the foot-fault test and the forelimb placing test. All scoring was blind as to the treatment group of the animal.

Measurement of Forelimb Placing
Animals were held by their torsos with forelimbs hanging freely. Contralateral and ipsilateral forelimb placing was induced by gently brushing the respective vibrissae on the edge of a tabletop once per trial for 10 trials. A score of 1 was given each time the rat placed its forelimb on the edge of the tabletop in response to vibrissae stimulation. The number of unsuccessful (missed) placements was determined. The proportion of unsuccessful placements with the affected forelimb was calculated and expressed as a percentage, indicating the amount of dysfunction.

Measurement of Foot-Fault Asymmetry
Animals were placed on an elevated grid, with square openings of 2.3 mm2, for 5 minutes. As the animals traversed the grid, a foot-fault was counted each time a paw slipped through an opening in the grid. The total number of foot-faults with ipsilateral and contralateral forelimbs per total of 50 steps was counted. The level of dysfunction was calculated as percentage of faulty steps with affected forelimb minus unaffected forelimb, per 50 steps.

Treatment Groups

Acute Caffeinol Treatment
The total doses of both ethanol and caffeine were diluted in normal saline to a total volume of 3 mL. A 0.5-mL bolus of the mixture was followed by 2.5 hours of continuous infusion at a constant rate of 1 mL per hour at doses ranging from 2 to 10 mg/kg for caffeine and from 0.2 to 0.65 g/kg for ethanol.

Use of Chronic Ethanol or Caffeine to Induce Tolerance
Ethanol (0.32 g/kg; 10% solution) or caffeine (10 mg/kg) was administered per gavage once a day for 14 days. Acute intravenous ethanol (0.32 g/kg) plus caffeine (10 mg/kg) was started 15 minutes after the onset of MCA/CCA occlusion.

Hypothermia and Caffeinol
We induced 35°C body core hypothermia by placing the anesthetized rat on crushed ice. Hypothermia was started 60 minutes after MCA/CCA occlusion and was sustained for 4 hours (2 hours of ischemia and 2 hours of reperfusion), followed by spontaneous rewarming. Intravenous caffeinol treatment was started 1 hour after onset of 180 minutes of ischemia and continued for 2.5 hours. The following 4 groups were compared: (1) 0.32 g/kg (5%) ethanol plus 10 mg/kg caffeine (E/C group); (2) 35°C hypothermia alone (4 hours' duration) (Hypothermia group); (3) combined caffeinol (same dose as in group 1 plus hypothermia (as in group 2) (Hypothermia+E/C group); and (4) normothermic saline-treated control.

Effect of Caffeinol on rtPA-Induced Hemorrhage
Ethanol (0.2 g/kg, 10% solution) plus caffeine (10 mg/kg) was started 15 minutes after the onset of ischemia as a continuous 2.5-hour-long infusion. rtPA treatment was initiated 15 minutes after reperfusion was established (confirmed by LDF) as an intravenous infusion of 5 mg/kg (2.5 mg/kg bolus followed by 30-minute infusion at 5 mg/kg per hour). At 24 hours, all animals were anesthetized with chloral hydrate and intracardiacally perfused with 150 mL of saline. After decapitation, brains were isolated and sliced into 2-mm-thick coronal sections for inspection of presence of intracerebral blood with the use of a surgical microscope (OpMi-1, Zeiss). Presence or absence of hemorrhage, as well as its morphological subtypes, was determined by 2 independent investigators blinded to treatment. To categorize the subtype of hemorrhagic transformation, we used criteria previously validated by Berger et al for use in human studies measuring the significance of hemorrhage after rtPA. Four subtypes of hemorrhagic transformation were recognized: (1) hemorrhagic infarction 1 (HI-1), with scattered,
Can Ethanol and Caffeine Be Administered Separately and Still Result in Neuroprotection?

We tested this question by separating the administration of these 2 agents by 2 hours. We first administered ethanol (0.65 g/kg and 10 mg/kg, respectively). Infarct volume was measured 24 hours later. In this experimental paradigm, caffeinol reduced infarct volume by 45% compared with saline controls (88.5 ± 16.0 mm³ [n=8] versus 161.2 ± 12.3 mm³ [n=9]).

Caffeinol Reduces Initial Severity and Speeds Improvement of Behavioral Dysfunction

We previously established 2 simple behavioral tests sensitive to ischemic damage produced by our model of ischemia.7,8 These tests involve quantitative forelimb placing and footfaults. As illustrated in Figure 1, 180 minutes of CCA/MCA occlusion produced significant behavioral dysfunction, increased the number of foot-faults with the affected forelimb, and increased the number of unsuccessful placements with the affected forelimb lasting at least 4 days, compared with the saline controls (88.5 ± 16.0 mm³ [n=8] versus 161.2 ± 12.3 mm³ [n=9]).
Tolerance to Neuroprotective Effect: Chronic Ethanol Intake Alone or With Caffeine, but Not Caffeine Alone, Abrogates the Neuroprotective Effect Of Acute Caffeinol

Our working hypothesis was that chronic exposure to ethanol and caffeine results in adaptive changes. To test this hypothesis, we orally treated animals with either ethanol (0.325 g/kg) or caffeine (10 mg/kg) daily for 14 days before assessing the neuroprotective efficacy of acute (15 minutes postischemic) intravenous caffeinol (ethanol 0.325 g/kg plus caffeine 10 mg/kg). The rats chronically administered 10 mg/kg caffeine alone showed significantly reduced infarct volume (48.1±14.5 mm³; n=7) compared with the group treated chronically with water (137.0±19.5 mm³; n=10), with 65% neuroprotection (P<0.002), suggesting that the adaptive changes induced by chronic caffeine alone are not sufficient to inactivate the protective effect of caffeinol. In contrast, chronic ethanol before treatment with the combination resulted in elimination of the therapeutic effect of caffeinol. Infarct volume was 104.3±20.1 mm³ (n=9) in ethanol-treated rats compared with animals chronically treated with water (94.1±22.6 mm³; n=6).

Caffeinol+Hypothermia: Can the Neuroprotective Effect of Ethanol and Caffeine Be Improved?

We subjected 4 groups of animals to 180 minutes of reversible ischemia and 3 days of reperfusion. All anti-ischemic interventions (caffeinol and hypothermia) were delayed 60 minutes after the initiation of MCA/CCA occlusion. The following 4 groups were evaluated: (1) caffeinol (ethanol 0.32 g/kg plus caffeine 10 mg/kg) (E/C group); (2) 35°C hypothermia (Hypothermia group), (3) combined caffeinol+hypothermia (Hypothermia+E/C group); and (4) saline-treated control (delivered as in the caffeinol group). The infarct volumes of all treated groups were significantly different from those of the saline control group (Hypothermia, 73.8±11.5 mm³; n=15; E/C, 81.7±15.2 mm³, n=16; and Hypothermia+E/C, 33.4±7.3 mm³, n=18; versus control, 142.1±7.52 mm³, n=36; P<0.05). There was a statistically significant improvement in protection of ischemic brain in the Hypothermia+E/C group compared with all other treatment groups (P<0.05) (Figure 2). Importantly, the effect of Hypothermia+E/C was significantly more than that of either E/C or Hypothermia alone.

Caffeinol Does Not Increase the Incidence of Hemorrhagic Transformation After rtPA Treatment

Twenty-nine rats were subjected to 180 minutes of MCA/CCA occlusion. Twelve of these rats received caffeinol, 10 mg/kg, and then 2 hours later administered caffeinol (10 mg/kg); in another group we administered caffeinol first and then ethanol. All treatments were oral and were started 3 hours before the onset of ischemia (n=8 per group). Animals treated with ethanol first and then caffeine displayed large infarction (121.4±32.6 mm³). In contrast, animals treated with caffeine first and then with ethanol showed significantly reduced infarct volumes (41.8±15.8 mm³), indicating that the order of administration is important to ensure pharmacological benefit.
Protective Effect of Caffeinol Is Not Dependent on the Anesthetic Used
Chloral hydrate, the anesthetic used during our work, has complex pharmacology and may contribute to polypharmacological interactions. The amount of neuroprotection with caffeinol was as robust with the use of isoflurane as it was with chloral hydrate anesthesia. Under isoflurane anesthesia, oral pretreatment with 0.65 g/kg ethanol plus 10 mg/kg caffeine versus saline resulted in 82% (n=8 per group; P<0.02) reduction of infarct volume.

Discussion
There is a pressing need for a stroke treatment that will be effective, has low toxicity, has a long window of opportunity, can be easily and readily administered, and can be used in combination with thrombolysis. In this report we demonstrated that one such candidate for stroke treatment is a mixture of ethanol and caffeine (caffeinol). Furthermore, we demonstrate that even greater protection might occur by combining caffeinol with 35° hypothermia.

On the basis of our dose optimization study, it emerged that caffeinol may reduce ischemic damage at low, toxicologically safe, doses. The dose of ethanol sufficient to support neuroprotection can be as low as 0.2 g/kg when given in combination with caffeine. This dose contrasts with the doses of ethanol used to study ethanol intoxication in rats ranging from 2 to 5 g/kg. For humans, 0.2 g/kg ethanol reflects approximately 30 to 35 mL of hard liquor or 12 to 14 mL of pure ethanol. Additionally, the effective dose of caffeine supporting neuroprotection is rather low. We found that 6 mg/kg of caffeine was sufficient to provide neuroprotection. This dose is equivalent to the caffeine present in approximately 2 to 3 cups of strong coffee. In other studies, far larger doses of caffeine, 50 to 100 mg/kg, were required to produce generalized stress response, such as activation of immediate early genes in the brain. We believe that effective doses of caffeinol are low, are nontoxic, and should be easy to achieve without significant side effects in humans.

Our results show that it is very important to apply ethanol and caffeine at the same time to achieve optimal neuroprotection. Animals that were treated first with ethanol and then 2 hours later with caffeine showed no benefit. The reason for the loss of the benefit following this paradigm of treatment is unknown. One possible explanation is that, to exert neuroprotection, both caffeine and ethanol must be present together at effective concentrations at their biological target site. Because of its fast metabolism, administration of ethanol 2 hours before administration of caffeine might result in insufficient ethanol levels to support ethanol/caffeine interaction at the time of caffeine administration. In support of this scenario, we found that administration of caffeine, which has a significantly longer plasma half-life than ethanol, 2 hours before ethanol yields significant neuroprotection. In summary, we believe that to ensure the best results, ethanol and caffeine should be administered at the same time.

Another intriguing aspect of caffeinol pharmacology is the development of tolerance to its neuroprotective effect. Adenosine receptors are known to desensitize rapidly in response to repetitive (as opposed to acute) activation. Analogously, repetitive treatment with ethanol upregulates N-methyl-D-aspartate (NMDA) receptor activity, downregulates GABA<sub>A</sub> receptor function, and potentiates susceptibility to glutamate excitotoxicity. We have reported in the past that daily (2 to 3 weeks) administration of caffeinol before stroke builds up a tolerance to its neuroprotective effect. Now we are reporting that this tolerance is likely due to the adaptive response to ethanol since daily treatment with ethanol alone also entirely eliminates the neuroprotective effect of acute caffeinol. In contrast, daily caffeine does not produce tolerance. Animals treated chronically with caffeine before ischemia benefited from the acute caffeinol treatment equally well as naive animals. Since we do not know the exact neuroprotective mechanism(s) of the ethanol in the caffeinol combination, it is difficult to point out any specific cellular changes triggered by chronic ethanol that may be responsible for the tolerance. These mechanistic studies are the subject of ongoing experiments in our laboratory. Through upregulation of NMDA receptors and downregulation of GABA<sub>A</sub> receptors, chronic treatment with ethanol could make ischemic brain tissue more excitable and therefore more prone to damage and less prone to rescue by caffeinol. Tolerance to chronic ethanol could affect the utility of caffeinol in treatment of stroke. It might be important to identify patients who, because of chronic alcohol intake, could develop tolerance. Chronic ethanol consumption in humans results in a variety of adaptive changes that can be measured and utilized as a surrogate marker of previous alcohol consumption. One of the most predictable biological markers of alcohol consumption is serum level of γ-glutamyl transferase (γGT). Measurement of γGT was recently shown to be a valuable and predictable marker in assessing history of alcohol drinking. Measurement of γGT in stroke patients before the treatment with ethanol plus caffeine may allow for preselection of patients who could benefit from the treatment (ie, those with normal γGT).

In these experiments we have demonstrated that caffeinol is not only effective in reducing infarct volume but is also very effective in reducing initial behavioral dysfunction measured by tests sensitive to changes in proprioception and motor and sensorimotor processing and integration, which correspond to the area of cortex damaged after unilateral CCA/MCA occlusion. These findings are reassuring since the important outcome in humans is functional rather than anatomic.

We wanted to determine whether we might amplify the robust neuroprotective effect of caffeinol by combining it with another clinically relevant neuroprotective strategy that might have a complementary mechanism of action. Hypothermia represents a very promising neuroprotective modality. Together with numerous preclinical studies, recent data indicate that use of hypothermia is not only feasible but also effective in protecting brain from ischemic damage after cardiac arrest. In our study the combination of hypothermia and caffeinol augmented the neuroprotective effect of caffeinol or hypothermia alone by >50%. Not all neuroprotectants combined with hypothermia show such augmenting benefit. In a parallel study investigating the efficacy of FK-506 combined with hypothermia in a similar paradigm, we observed no augmentation of neuroprotection beyond that provided by each strategy alone (J. Aronowski, PhD, unpublished data, 2001).

At high (>1.0 g/kg) doses, ethanol may produce vascular damage and microhemorrhages. Since one clinical application...
of caffeinol might be to try to amplify the benefit of rtPA by using the 2 in combination, it is important to assess whether the combination of rtPA with caffeinol will interact in a fashion that might produce more intracerebral bleeding than that occurring after rtPA alone. To test this safety issue, we first developed an ischemic model that produces reproducible bleeding in response to rtPA. Since risk of bleeding increases with age, we used aged, 10-month-old, Long-Evans rats. On the basis of the doses of rtPA reported to produce hemorrhages in rats, we first used a dose of 10 mg/kg intravenous rtPA started 5 minutes after 180 minutes of unilateral MCA/CCA occlusion. Unfortunately, this dose was very toxic and resulted in severe bleeding and high mortality in all treated animals (J. Aronowski, PhD, et al, unpublished data, 2000). We conducted a dose-response study and found that 5 mg/kg rtPA produced bleeding in approximately 85% to 90% of animals and caused limited mortality. The clinically relevant dose of caffeinol did not augment the prohemorrhagic effect of rtPA at this dose. In fact, we observed slight reduction in mortality and hemorrhage rate in animals treated with rtPA and caffeinol. In addition, concentrations of caffeine and ethanol found in plasma of animals treated with a neuroprotective dose of caffeinol had no effect on rtPA clot lysis in vitro (J. Aronowski, PhD, et al, unpublished data), further suggesting that caffeinol may be safe and have no effect on the rate of clot lysis when used in conjunction with rtPA.

Finally, we found that caffeinol was effective even if treatment was delayed until after reperfusion occurs following temporary CCA/MCA occlusion. In human clinical studies monitoring arterial status by transcranial Doppler ultrasonography, we found that it usually takes 30 to 60 minutes for arteries to recanalize after the intravenous rtPA bolus is given. In a clinical trial combining caffeinol with rtPA, it is likely that caffeinol would be started during the 1-hour rtPA infusion after the bolus. Therefore, it is likely that therapeutic blood levels of caffeinol would be achieved either immediately before, at the time of, or immediately after recanalization if it occurs. Our studies support the efficacy of this combination in all 3 possible situations.

Our studies are limited by the usual concerns about the applicability of animal models to human stroke. Our studies have addressed most of these concerns except for 2. First, as mentioned, our rats were young and naive to exposure to ethanol and caffeine. Caffeinol may not be effective in older humans who have chronically ingested these substances over their lifetime. In addition, our model is characterized by purely cortical damage. Most human stroke includes at least some subcortical injury. It will be important to determine in future studies whether caffeinol is also effective in reducing white matter damage. However, in the typical patient with MCA occlusion being treated within the first few hours after stroke onset, the majority of the irreversibly damaged ischemic core is subcortical, while penumbral regions, the target of neuroprotective therapies such as caffeinol, are more frequently located in surrounding cortex.

In summary, while we remain cautious about extrapolating our results to humans, caffeinol appears to be an attractive candidate for clinical testing in acute stroke patients. It is inexpensive and widely available, and our studies suggest that it is highly effective at low doses that should be well tolerated. Importantly, it can be linked to other therapies such as hypothermia and rtPA, which may result in heightened efficacy.

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