ACUTE ETHANOL ADMINISTRATION AND TRANSIENT ISCHEMIA: A BEHAVIORAL AND NEUROPATHOLOGICAL STUDY

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Summary

A pressing clinical question is how acute ethanol exposure might alter the outcome of a simultaneous transient ischemic attack (TIA), since ethanol is known to dysregulate key intermediary metabolites post-ischemia. Mongolian gerbils were administered ethanol (1 or 4g/kg, sc) 1 hour before induction of transient ischemia, via bilateral carotid occlusions of 5 minutes duration. A control group was administered isotonic saline and rendered ischemic. All animals were maintained normothermic during the ischemic procedure. Subjects underwent behavioral assay of acquisition to the water maze 7 days after recovery from the surgery, and neuropathological examination 1-month after the ischemic brain insult. There were no behavioral or neuropathological between-group differences suggesting that mechanisms other than adverse ethanol-induced perturbations of ischemic processes predominate in mediating epidemiological findings of elevated stroke morbidity with high ethanol consumption.

Key Words: ethanol, transient ischemia, neuropathology, Morris water maze, place learning, stroke

Acute ethanol ingestion is known to contribute to stroke by induction of cardiac arrhythmia, hypertension, alteration of platelet aggregation processes and reduction of regional cerebral blood flow (1). Epidemiological studies have noted that stroke prognosis is worse in binge and heavy drinkers (2,3,4) and that this effect is still detectable even when adjusted for the high proportion of hypertensive patients in these groups (5,6). High levels of ethanol consumption is a significant risk factor for stroke recurrence (7) and cerebral infarction is more common at younger ages in heavy drinkers compared with social drinkers (8). However there is some contradictory evidence which suggests that light drinking may confer small protective effects against stroke (4). It is unclear what proportion of stroke patients are admitted to hospital under the influence of alcohol since there is a paucity of well-controlled studies examining stroke-ethanol interactions (9), however anecdotal evidence from the neurology ward suggests

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that this is likely a common emergency room scenario.

Neuropharmacologically acute ethanol promotes rapid depletion of intracellular magnesium ion (10), which catalyzes cellular and subcellular disturbances in cytoplasmic and mitochondrial bioenergetic pathways leading to calcium ion overload (11). Since calcium ion accumulation in-vivo plays a central role in the pathogenesis of ischemia-induced neuronal injury (12), these alterations associated with ethanol consumption could aggravate stroke outcome. In addition ethanol significantly alters the course of metabolism of amino acid neurotransmitters involved in both heightened neuroprotective inhibitory and diminished neurotoxic excitatory responses post-ischemia. Ethanol exerts beneficial effects on γ-aminobutyric acid (GABA) transmission, a potent inhibitory amino acid neurotransmitter (13) and collateral evidence suggests that GABA agonists such as muscimol and clomethiazole are neuroprotective to gerbil hippocampal neurons when administered immediately pre- and post-ischemia (14,15). Furthermore acute ethanol administration attenuates N-methyl-d-aspartate (NMDA) mediated excitation, an essential excitatory amino acid neurotransmitter (13) and NMDA receptor antagonists such as CGS-19755 are neuroprotective when administered immediately post-ischemia (16). Similarly chronic pre-administration of ethanol has also been found to attenuate glutamate release during ischemia consistent with an ethanol-induced long-lasting sensitivity of NMDA receptors (17).

Caution in interpreting these studies is warranted though since ethanol is known to affect almost every major neurotransmitter system in the brain including dopaminergic and serotonergic pathways in addition to important neuromodulatory systems such as nitric oxide synthase (1,9,11,13,17-25). A common ground is that both ischemic processes (12) and the neuropharmacological effects of ethanol on the CNS yield reliable perturbations in intracellular calcium homeostasis, resulting in alterations in subsequent gene expression post-ischemia ultimately leading to neuronal cell death (9). Recent in situ mRNA hybridization studies have sought to elaborate these findings at the intracellular level. Two identified important mediators controlling cytosolic free calcium concentrations are sarcoplasmic or endoplasmic reticulum CA2+-ATPase or (SERCA) and inositol 1,4,5-triphosphate receptor or IP3R (9). Chronic ethanol exposure decreases SERCA mRNA selectively in cerebellar as opposed to hippocampal neurons, suggesting a causal role between findings of regional alterations in calcium homeostasis and prominent cerebellar degeneration often associated with chronic alcoholism (9), since cerebellar Purkinje cells are rich in SERCA (25).

Although the gerbil is not commonly used for alcohol research, preliminary studies indicate the feasibility of the administration of ethanol to this species via intraperitoneal injection (21) or intragastric intubation methods (9). Prior studies examining ethanol-ischemia interactions have found neither neuroprotective nor neurotoxic effects of ethanol administration post-ischemia at doses of 5 g/kg and 2.5 g/kg in the rat four-vessel occlusion model; respectively (22,23). The gerbil model might prove to be a useful alternative to examine ethanol-stroke interactions given its more predictable range of neuropathological outcomes (14), since the Mongolian gerbil’s incomplete Circle of Willis restricts collateral blood flow during ischemia. Moreover ethanol appears to exhibit biphasic effects on catecholamines (18) and on key homeostatic mediators such as SERCA such that at low doses it functions to increase CA+ uptake, while high doses inhibit CA+ uptake (24).

Prior studies found that a dosage of 1g/kg ethanol administered 30 minutes before 5 minutes of ischemia yielded significantly reduced CA1 pyramidal cell loss as compared to control saline injected gerbils (21). However the gerbil’s brains were extracted before 7 days in that study, thereby potentially overestimating the efficacy of the neuroprotective effects of
ethanol, since delayed neuronal death processes may continue for up to 7 days (16). Moreover these small neuropathological savings as a consequence of ethanolic pre-treatment prior to stroke were not associated with any significant functional savings as determined by a locomotor activity assay in this study. In contrast in another study using the gerbil it was found that chronic intragastric intubation with a liquid diet of 4 g/kg ethanol for a period of 35 days prior to a 5 minute ischemic insult was not found to alter the sensitivity of CA1 neurons to ischemia (9).

Until recently studies relating the effects of alcohol consumption to stroke have been conducted almost exclusively at the epidemiological level and few studies using animals with controlled conditions have been reported. The purpose of this study was to determine whether acute ethanol pre-treatment might alter the neuropathological and functional outcome in an animal model of TIA.

Method

Injection Schedule. Following institutional ethics approval, male Mongolian gerbils (Tumblebrook Farms, USA), weighing between 60-80 g were obtained for this study (N=30). Gerbils were housed in groups of five with free access to food and water, and maintained on a 12 hour day/night cycle. One hour prior to ischemia, one group (N = 10) received saline (control condition), a second group (N= 10) 1 g/kg ethanol, (acute low dose), and a third group (N= 10) 4 g/kg at 1 hour (acute high dose). All doses were made up to 1 ml in isotonic saline and injected subcutaneously with a 30-gauge needle in order to minimize discomfort to the animal. The lower dosage of ethanol was calibrated to parallel the levels of human intoxication commonly encountered in emergency room settings, while the higher dosage was utilized since prior studies have found that ethanol has biphasic effects upon the release of catecholamines and cellular metabolites involved in calcium ion homeostasis (18,24). Behaviorally a loss of the righting reflex was noted in the high dose group for a period of approximately 4-6 hours post-ischemia; while low dose subjects were rendered ataxic for a period of 1-3 hours.

Blood Alcohol Determinations. A probe group of gerbils (N=30) were used for blood ethanol determinations. Blood alcohol levels were determined with probe subjects (n=5 per group) at 1, 6, 12 and 18 hours post-injection for the high dose group. For the low dose ethanol group blood alcohol determinations were made only at 1 and 6 hours post-injections. At criterion times subjects were anaesthetized with an overdose of sodium pentobarbitol and the chest cavity was exposed. The left ventricle was pierced with a heparinized phlebotomy needle with attached vial and collection of 1 ml of venous blood commenced. The whole blood was centrifuged at 2500 g for 15 minutes and blood plasma aliquot were exposed to commercially available alcohol dehydrogenase Ektachem slide strips (Kodak Co., Rochester, NY). The enzyme strip reaction was allowed to proceed to its endpoint and was measured colorimetrically with a Ektachem colorimeter at 340 nm yielding a reading in millimoles of ethanol per liter of blood plasma.

Ischemia Surgery. At the pre-specified time after the ethanol injection each gerbil underwent bilateral common carotid artery occlusion. A single ischemic insult of 5 minutes duration was utilized since such durations result in small but detectable deficits both structurally and functionally (26). Prior studies of ethanol-ischemia interaction in the gerbil have used an insult of identical duration (9,21) and ischemia was produced by methods described in detail elsewhere (26).

Briefly the gerbils were anaesthetized with 1.5% halothane and a mixture nitrous oxide (70%) and oxygen (30%). Core body temperatures were estimated via a temperature probe held in place directly adjacent to the cranium under the temporalis muscle and additionally with a
rectal probe. These two measurements were combined to yield a reliable extrapolated ‘true score’ tabled value of estimated brain temperature based upon prior pilot studies simultaneously employing monitoring of in-vivo brain, temporalis and rectal probe temperatures (14,15). A warming blanket and a heating lamp was used in combination to maintain the cerebral temperature between 36.5 and 37.5 °C during the ischemic procedure since hypothermia can have neuroprotective effects (16). The gerbils were kept in a warmed enclosure for half a day post-ischemia to prevent any drop in core body temperature. The two carotid arteries were isolated with threads before the actual occlusion so that they could be easily manipulated and care was taken to ensure that the threads did not hamper blood flow to the brain in any way before the ischemic insult. The arteries were occluded by aneurysm clips under visual inspection. Behavioral indicators were used to verify successful occlusion. Gerbils not exhibiting all three indicators of bilateral ptosis, failure of respond to whisker stimulation and hunched posture were excluded from the analysis. After visual verification of reperfusion following removal of the clips, the incision was sutured, the nitrous oxide was discontinued and the animals observed until mobile.

**Behavioral Assay.** Behavioral testing in the Morris water maze (27) began 7 days after surgery and continued for 7 days. The Morris water maze is a neurobehavorial assay that has been found to be especially sensitive to deficits in acquisition of place learning in rodents as a consequence of lesions of the hippocampus (17,26-28). The water maze was 1.3 m in diameter and 0.3 m high. It was filled with 27 °C water rendered opaque by the addition of milk powder. A 22 cm diameter circular platform 1 cm below the water surface was maintained in the same position for all tests. Testing was consistently completed at the same time each day. Each gerbil was given 5 trials per day at 30 minute intervals by being gently lowered into the pool at one of three locations randomly chosen for each trial. Maximum swim time was 90 seconds. Following the location of the platform (or after sixty seconds of swimming the animal was placed on the platform), the animal remained on the platform for 30 seconds. The latency to find and climb on the hidden platform was recorded for each trial per animal.

**Histopathology.** One month post-ischemia each animal was anaesthetized, perfused, and the brain extracted and cryoprotected according to methods described previously (29). The brains were then cut with a microtome into 40-micron thickness coronal slices, and stained with a modification of the silver impregnation method (30,31). Representative sections were cut through the cortex including structures such as the striatum, hippocampus, thalamus, and distally through the substantia nigra and the medial geniculate nucleus according to the gerbil atlas (32). Neuronal damage was assessed with light microscopy by first randomizing the coded slides and having them rated by a technician blind to the experimental hypothesis. The following scoring system was used: 0 = no damage; 1 = less than 25% damage; 2 = 25-75% damage; 3 = >75% damage and 4 = total infarction (29).

**Results**

Blood ethanol concentrations quickly reached equilibrium levels approximately 1 hour after the subcutaneous injections. The peak blood ethanol concentrations and the time-course of the metabolism of ethanol attained in this study are quite similar to that observed in rats (18,19) and gerbils (9,21).
Table 1: Blood alcohol concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>1 hr</th>
<th>6 hrs</th>
<th>12 hrs</th>
<th>18 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/g/kg</td>
<td>76.8±8.1</td>
<td>83.0±2.5</td>
<td>23.6±2.4</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>1g/kg</td>
<td>27.1±2.4</td>
<td>0.0±0.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean ± standard error of the mean expressed in millimoles of ethanol per liter of blood plasma, n = 5 per group, (mmol L⁻¹).

One subject died in the low dose group during the ischemic procedure. A two-way ANOVA (3 groups x 7 trials) with repeated measures on trial blocks was carried out on the swim latency data. All groups required approximately 60 seconds on average to locate the platform during the first five trials. By the 35th trial, the groups averaged approximately 10 seconds to perform the same task. The ANOVA revealed that the only significant main effect was of trials [F(6,24) = 81.3, p < 0.001], suggesting that all animals learned the position of the submerged platform at the same rate. All 3 ischemia group's rate of acquisition to the water maze was impaired compared to sham-operation ischemia control gerbils without hippocampal damage tested in prior studies (17,26). The results obtained from rates of water maze acquisition in ischemic and sham-operation ischemia gerbils were similar to the patterns of acquisition to the water maze typically observed in rats with and without hippocampal damage (27,28).

Brain damage was limited to the CA1 subfield of the hippocampus in all gerbils and damage ratings ranged from 2 to 3, in contrast to the absence of brain damage consistently found with sham-operation ischemia control groups in prior studies (17,26). The summary brain damage ratings were analyzed with Kruskal-Wallis one-way ANOVA and no between-group differences were found on any of the regional brain structures.

In an effort to gauge if the brain damage scores mapped with the water maze acquisition data a Spearman's rank correlation was performed due to the ordinal nature of the neuropathological ratings. The mean latency to find the platform during trial blocks 5,6,7, (the late phase of acquisition), was utilized to prevent the considerable non-specific instrumental learning that occurs early in training (e.g., learning to swim away from the side wall) from masking impairments in true place learning (28). A significant correlation was found between water maze latency of acquisition and the damage ratings in the CA1 subfield of the hippocampus (r = 0.40, p = 0.03).

Discussion

Gerbils injected with 1 or 4 g/kg ethanol and then subjected to 5 minutes of bilateral carotid artery occlusion were indistinguishable from gerbils injected with saline and then given ischemia on the neuropathological rating scale. There were no between-group differences on rates of acquisition to the water maze. This further supports the conclusion that acute ethanol administration does not alter the structural/functional outcome of a simultaneous ischemic episode in the gerbil, confirming previous study findings using similar paradigms in rats (22,23), and gerbils (9) but contradicting one similar study using gerbils (21).

Significant associations have been found at the epidemiological level between binge/heavy drinking and subsequent stroke onset (2-8). It is unclear whether alterations in system-level cerebrovascular parameters, imbalances between excitotoxic/inhibitory amino acid neurotransmitters, or ethanol-induced perturbations in calcium-ion homeostasis mediate epidemiological findings of exacerbated outcome with ethanol-stroke interactions. Currently the
literature suggests that the link between binge drinking and hemorrhagic stroke is stronger than the link between ethanol consumption and ischemic stroke (9). However more accurate information is needed using animal models under which both ethanol consumption, species-specific cerebral blood flow and ischemic metabolic parameters can be experimentally covaried both in-vivo and in-vitro.

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References