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Safety of intracerebroventricular copper histidine in adult rats

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Abstract

Classical Menkes disease is an X-linked recessive neurodegenerative disorder caused by mutations in a P-type ATPase (ATP7A) that normally delivers copper to the developing central nervous system. Infants with large deletions, or other mutations in ATP7A that incapacitate copper transport to the brain, show poor clinical outcomes and subnormal brain copper despite early subcutaneous copper histidine (CuHis) injections. These findings suggest a need for direct central nervous system approaches in such patients. To begin to evaluate an aggressive but potentially useful new strategy for metabolic improvement of this disorder, we studied the acute and chronic effects of CuHis administered by intracerebroventricular (ICV) injection in healthy adult rats. Magnetic resonance imaging (MRI) after ICV CuHis showed diffuse T_1-signal enhancement, indicating wide brain distribution of copper after ICV administration, and implying the utility of this paramagnetic metal as a MRI contrast agent. The maximum tolerated dose (MTD) of CuHis, defined as the highest dose that did not induce overt toxicity, growth retardation, or reduce lifespan, was 0.5 mcg. Animals receiving multiple infusions of this MTD showed increased brain copper concentrations, but no significant differences in activity, behavior, and somatic growth, or brain histology compared to saline-injected controls. Based on estimates of the brain copper deficit in Menkes disease patients, CuHis doses 10-fold lower than the MTD found in this study may restore proper brain copper concentration. Our results suggest that ICV CuHis administration have potential as a novel treatment approach in Menkes disease infants with severe mutations. Future trials of direct CNS copper administration in mouse models of Menkes disease will be informative.
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Keywords: Copper histidine; Intracerebral administration; Maximum tolerated dose; Menkes disease; Copper transport

Introduction

Menkes disease (MD) is an X-linked recessive disorder of copper transport caused by defects in a gene that encodes an evolutionarily conserved copper-transporting ATPase [1–3]. In mammals, this gene product functions as an intracellular pump to transport copper into the *trans*-Golgi network for incorporation by copper-dependent enzymes and also mediates copper exodus from cells [4]. The disorder presents in infancy with delayed development, failure to thrive, neurodegeneration, and premature death (typically by 3 years of age) [5].

Daily subcutaneous injections of copper histidine (CuHis) can significantly modify this dismal natural history when commenced in early infancy and in the context of certain ATP7A mutations that allow partial copper transport to the developing brain [6–8]. However, the blood-brain barrier poses a challenging obstacle in MD patients whose mutations do not permit any residual copper transport [9,10]. Consequently, we are exploring alternative therapeutic

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approaches—including intracerebroventricular (ICV) CuHis administration—to bypass the blood-brain barrier.

There are several important caveats related to the potential efficacy of this approach. First, copper uptake by neuronal cells via high affinity copper uptake genes (e.g., hCTR1) and expression of copper chaperones (e.g., Cox17 and CCS1) in human neuronal cells are necessary, both of which have strong experimental support [11–14]. A second requirement is that the distribution and cerebrospinal fluid clearance of copper administered by ICV injection be adequate for generalized neuronal uptake. ICV injection of other trace metals, e.g., zinc chloride ($^{65}ZnCl_2$), zinc histidine (^{65}Zn-His) and manganese chloride ($MnCl_2$), results in wide distribution and sustained presence within brain as documented by autoradiography or magnetic resonance imaging [15–19]. These latter results augur well for neuronal delivery of ICV CuHis. As small molecules, (e.g., atomic mass 55 Da for manganese, 63.5 Da for copper, and 65.4 Da for zinc), the distribution and brain penetration of trace metal ions, especially paramagnetic ions such as Mn and Cu administered by ICV injection, are superior compared to larger drug molecules [20].

To begin to evaluate the safety of this aggressive treatment approach and to determine a maximum tolerated dose, we performed acute and chronic toxicology assessments of ICV CuHis in adult male rats.

Materials and methods

The study was approved by the Animal Care and Use Committee of the National Institute of Child Health and Human Development.

ICV catheter placement

Thirty-six Sprague–Dawley (Taconic, Germantown, NY) rats were used. Rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital (Abbott Laboratories, Chicago, IL) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) with the incisor bar 3.1 mm below the interaural line. The skin was incised and a 0.9 mm diameter hole was drilled through the skull for placement of a 33 g cannula (Plastics One, Roanoke, VA) into the left lateral ventricle of the brain via stereotaxic guidance. Stereotaxic coordinates were 1.6 mm left lateral and 0.8 mm posterior to bregma, and catheter insertion from the skull surface was to a depth of 3.2 mm [21]. Aseptic technique was used throughout the procedure. A 26 Ga guide cannula was secured with acrylic dental cement and two bone screws. Between infusions, the internal cannula was replaced with a 0.2 mm diameter dummy cannula.

ICV infusions of CuHis and normal saline

CuHis (prepared by the National Institute of Health Pharmaceutical Development Service) was diluted in normal saline to deliver the desired dose in a volume of 20 μL. Controls received 20 μL of normal saline alone. Doses were administered at a rate of 2 μL per minute for 10 min. For animals receiving subsequent infusions, the protocol was repeated (without surgery) with each respective dose.

Brain MRI

Rodent brain MRI was performed as previously described [18,19]. Following injection of Cu contrast agent, or saline, the animals were transferred to a custom made holder for imaging and anesthesia was maintained with 1–3% isoflurane in a medical air/oxygen mix (60/40) via a mask. Body core temperature was monitored and maintained at 35 °C with a recirculating hot water pad. During scanning, the breath rate was monitored and the anesthetic gas adjusted to maintain a rate of 30–40 breaths per minute.

All MR Imaging was performed at 4.7T using a Bruker Avance MRI system (Bruker-Biospin, Billerica, MA). A 4 cm surface coil was placed over the head and the animal was positioned in the isocenter of the magnet. After initial setup and locator scans, a 3D data set was acquired with a gradient echo pulse sequence. The imaging parameters were: TR/TE = 150/6 ms, FOV = 40 × 30 × 30 mm, acquisition matrix = 256 × 128 × 64, and the flip angle was Ernst-optimized. The images were reconstructed to a 256 × 128 × 128 matrix size.

CuHis dosing

To assess acute toxicologic effects, rats were administered single doses of either 100, 20, 10, 5, or 1 mcg CuHis and were euthanized after 6 h. To assess chronic effects, rats were administered single doses of 7.5 or 2.5 mcg CuHis, or two to four doses of 0.5 mcg CuHis at 1 week intervals and were euthanized within 1 or 2 weeks of the final infusion. In acute and chronic studies, an equivalent number of normal saline ICV infusions were administered to control rats.

Animal monitoring

Daily health checks were conducted for all animals to assess eating, drinking, as well as general activity and behavior. When indicated, animals with clinical evidence of untoward effects from the experimental treatment were euthanized immediately.

Histopathology

The animals were euthanized with CO_2 and their brains harvested by the NIH Division of Veterinary Resources. After fixation in 10% buffered formalin, brains were sectioned, trimmed, and embedded in paraffin. Five micron sections were mounted on slides and stained with hematoxylin and eosin (Histoserv, Inc., Germantown, MD). Slides from animals receiving CuHis doses of 5 or 0.5 μg, and saline-treated controls were scored by one of the authors (LRB) in five categories of brain histopathology: spongiosis, ventricular dilation, cavitation, encephalitis, and meningitis. Scoring was from 0 to 4 using the following scale: 0 = no change or within normal limits; 1 = minimal; 2 = mild; 3 = moderate; and 4 = marked.

Brain copper levels

Brain copper levels were determined from paraffin-embedded tissues. Paraffin surrounding the tissue was dissolved with *n*-hexane and the tissue dried overnight (at 50–55 °C). Brain and control (e.g., National Institute of Standards and Technology, Gaithersburg, MD; liver specimen SRM#1577b) tissues were microwave digested (CEM Corporation, North Carolina; model MARS-CEM at 600 W power) in 1 mL nitric acid (HNO_3), 1 mL of 30% hydrogen peroxide (H_2O_2), and 1 mL distilled water. Digestion was performed using a closed vessel system at a controlled temperature and pressure using with the following program: 20 psi for 10 min; 40 psi for 10 min; 60 psi for 10 min; 90 psi for 10 min; and 120 psi for 30 min. Digested samples were brought to a final volume of 4 mL using distilled deionized water (Millipore Corporation System). The tissue extracts were then analyzed, employing an inductively coupled plasma optical emission spectrometer, ICP-OES, (Perkin-Elmer Corporation, New Jersey) instrument. To control for matrix interferences and changes in rate of ionization, we used an internal standard based on a 2 ppm gallium (Ga) solution. The instrument was calibrated with a five-point calibration curve including blank standard, 0.050, 0.2, 1, 2 and 5 ppm Cu standards. All standards, samples, blanks, and controls contained 2 ppm gallium. Samples were analyzed in duplicates and each measurement is the result of three replicates. The relative standard deviation (%RSD) between