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Neuroprotective effects of uridine in a rat model of neonatal hypoxic–ischemic encephalopathy

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HIGHLIGHTS

- Uridine is the principal pyrimidine in humans and a membrane phospholipid precursor.
- Uridine treatment reduces brain infarction and apoptosis in rat neonatal HIE model.
- This is the first study to report cerebroprotection by uridine in rat neonatal HIE.

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ABSTRACT

Neonatal hypoxic–ischemic encephalopathy (HIE) is a major cause of neurological disability requiring newer therapeutic strategies. Uridine is the principal circulating pyrimidine in humans and a substrate for nucleotides and membrane phospholipids. The objective of this study was to investigate the effects of uridine in a neonatal rat model of HIE. Rat pups subjected to hypoxic–ischemic insult on postnatal day 7 were injected intraperitoneally with either saline or uridine (100, 300 or 500 mg/kg) for three consecutive days and brains were collected for evaluation of brain infarct volume and apoptosis. Compared with Control group, uridine at 300 and 500 mg/kg doses significantly reduced percent infarct volume, TUNEL(+) cell ratio and active Caspase-3 immunoreactivity in the cortex, as well as in CA1 and CA3 regions of the hippocampus. Uridine (300 and 500 mg/kg) also decreased active Caspase-3 expression in the ipsilateral hemisphere. These data indicate that uridine dose-dependently reduces brain injury in a rat model of neonatal HIE by decreasing apoptosis.

1. Introduction

Hypoxic–ischemic (HI) insult in newborns causes brain injury with significant consequences including motor and cognitive deficits and seizures [32]. Every 2 newborns per 1000 term births are affected by neonatal hypoxic–ischemic encephalopathy (HIE) [17] which causes death and neurological morbidities [29]. Although many strategies have been proposed in treatment of HIE, newer approaches are required to ameliorate the severity of brain pathology, and reduce mortality and morbidity.

Uridine is the principal circulating pyrimidine nucleoside in humans [4,35] and a constituent of breast milk [26]. It is also a precursor of brain membrane phosphatides via the Kennedy pathway [16] as a building block of uridine-5′-triphosphate (UTP). Uridine administration enhances cytidine-5′-diphosphocholine (CDP-choline) levels in PC12 cells [23], striatal slices [27] and rodent brains [8]. CDP-choline has been reported to exhibit beneficial effects in experimental and clinical studies of stroke and other neurodegenerative states [1] including HIE [13].

Uridine nucleotides (UTP and uridine-5′-diphosphate [UDP]) are ligands to P2Y receptors (P2Y2, P2Y4 and P2Y6), stimulation of which provides neuroprotection by reducing apoptosis [5,18]. Uridine administration leads to enhancement in UTP levels in PC12 cells [22] and rodent brains [8].

Since uridine is a precursor to uridine nucleotides and CDP-choline, we aimed to investigate the effects of uridine administration in a rat model of neonatal HIE. Our data show for the first time that intraperitoneal (i.p.) uridine administration for 3 consecutive days following HI brain insult reduces infarct volume,

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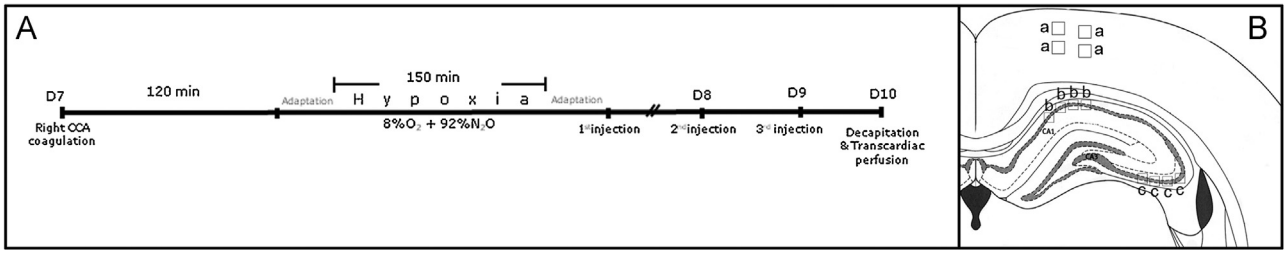

Fig. 1. Schematic diagrams of the experimental procedure (A) and the areas utilized for determining TUNEL(+) cell ratio and Caspase-3 immunoreactivity (B). "a", count area in cortex; "b" count area in CA1 region; "c" count area in CA3 region.

TUNEL(+) cell ratio and active Caspase-3 expression in the cortex as well as CA1 and CA3 regions of the hippocampus in newborn rats.

2. Materials and methods

Experimental protocols were approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (2009-07/4), and the experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996 and EC Directive 86/609/EEC.

Model for perinatal HI brain injury was adapted from Vanucci and Vanucci [30]. A total of 51 pups were born to 5 dated pregnant Sprague-Dawley rats which were housed in individual cages with free access to food and water with 12 h light/dark cycle. The day of birth was considered postnatal day 0 (PND0) and experiments were initiated on day 7 (PND7), at which day the brain maturation of rats coincides with that of 32- to 34-week-old human [31].

2.1. Induction of HI brain injury

Out of the initial 51 pups, 8 pups were excluded due to low weight on PND7 and 3 pups were excluded due to bleeding after surgery. Forty rat pups of either sex (55% males [$n = 22$] and 45% females [$n = 18$]) weighing 12–16 g on PND7 were used in the experiments. Under isoflurane anesthesia, right common carotid arteries of pups were ligated and coagulated. Following a 2-h period of nesting, three pups from each dam were placed into chambers partially submerged in a 37 °C water bath and exposed to hypoxia (8% oxygen balanced with nitrogen mixture) for 150 min (Fig. 1A). Hypoxic chambers were attached to an anesthesia device (AMS Minor 612, GMS Generra Medikal, Ankara, Turkey) and the gas flow was monitored using a gas flow analyzer (VT-PLUS HF, Fluke Biomedical, Everett, WA) before each experiment.

2.2. Treatment groups

PND7 rats ($n = 32$) were divided randomly, ensuring that all treatment groups were represented by pups from each dam, into 4 groups and injected i.p. with the following: saline (0.9% NaCl, 0.1 ml [Control group]), uridine (100 mg/kg [U100 group]), uridine (300 mg/kg [U300 group]), and uridine (500 mg/kg [U500 group]) (dissolved in saline and injected 0.1 ml) (Fig. 1A). No behavioral changes were observed after HI insult or uridine treatments. Sham operation by exposing the common carotid artery without ligation and hypoxia was performed on another set of rat pups ($n = 8$). The dose range of uridine used in the present study has been selected in the light of a previous report which tested CDP-choline [13] in a neonatal rat model of HIE, as well as our preliminary observations with 300 mg/kg uridine with three consecutive injections.

2.3. Brain tissue procurement

Following completion of experiments, pups were divided into 2 groups on PND10; under deep anesthesia, pups were either decapitated and brain tissues were removed ($n = 20$) or sacrificed following transcardiac perfusion with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) (pH 7.4) ($n = 20$) for western blotting or immunohistochemistry, respectively.

2.4. Measurement of brain infarct volume

One in every five 20 μm-thick brain sections selected with a defined range was stained with 0.5% cresyl violet (Acros Organics, Geel, Belgium) in order to evaluate brain infarct volume for each pup using images captured at a magnification of 1.25× (Fig. 2). Volume fraction of the infarct (corresponding to percent infarct volume [12]) in ipsilateral hemisphere was estimated by Cavalieri principle by a blinded investigator using a combined point-counting grid as described previously [9,12,14].

2.5. In situ cell death detection

DNA fragmentation was detected using an in situ cell death detection POD kit (Roche Molecular Biochemicals, 11684817910, Germany) for TUNEL (terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end-labeling) technique in cryosections. Slides were counterstained with Harris's hematoxylin (Sigma–Aldrich, St. Louis, MO).

TUNEL(+) cell count was performed on sections from four different levels in rostrocaudal plane of cortex as well as CA1 and CA3 areas of the hippocampus that corresponded approximately to panels 11–14 according to the Atlas of Prenatal Rat Brain Development [2].

Images with size of 4080 × 3072 square pixels were captured with CCD camera (Olympus DP71 CCD color camera, 1.5 million pixel) attached to a light microscope at 100× magnification (Olympus BX50). Four areas in cortex and regions of the hippocampus were hemilaterally analyzed (Fig. 1B). After top of the section of the selected microscopic field was found, eight consecutive focal planes with a distance of 2 μm were scanned between the top and the bottom surfaces. Images of focal planes were superimposed prior to counting the nuclei and percent TUNEL(+) cells over all cells were calculated for each pup and data were presented as TUNEL(+) cell ratio (Fig. 3).

In addition, four different coronal sections adjacent to those utilized for TUNEL staining were chosen for active Caspase-3 immunohistochemistry. Sections were labeled with rabbit anti-cleaved Caspase-3 (1:200; Asp-175, Cell Signaling Technology Inc., Danvers, MA) and incubated with an appropriate secondary antibody (biotinylated anti-rabbit IgG, Jackson Immuno Research Laboratories Inc., West Grove, PA) before treatment with avidine biotin complex (ABC Vectastain Elite ABC Kit, Vector Laboratories Inc., Burlingame, CA) and visualization using DAB substrate.