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Research Report

Intraventricular ascorbic acid administration decreases hypoxic–ischemic brain injury in newborn rats

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Abbreviations:
AA, ascorbic acid
DHA, dehydroascorbic acid
FBDP, fodrin breakdown product
HI, hypoxic–ischemia
HIE, hypoxic–ischemic encephalopathy
NMDA, N-methyl-D-aspartate
PND, postnatal day
ROS, reactive oxygen species

ABSTRACT

Neuronal cell damage following hypoxic–ischemic (HI) brain injury is partly caused by production of free radicals and reactive oxygen species (ROS). Ascorbic acid (AA) is a potent antioxidant, which scavenges various types of ROS. Some studies have shown that it is neuroprotective, however, the issue is still controversial. In this study, we examined the effect of intraventricular AA administration on immature HI brain using the Rice–Vannucci model. After unilateral carotid artery ligation under isoflurane anesthesia, 7-day-old rat pups received varying concentrations of AA (0.04, 0.2, 1 and 5 mg/kg) by intraventricular injection and were exposed to 8% oxygen for 90 min. Vehicle controls received an equal volume of phosphate saline buffer. We assessed the neuroprotective effect of AA at 7 days post-HI. The percent brain damage measured by comparing the wet weight of the ligated side of hemisphere with that of contralateral one was reduced in both 1 and 5 mg/kg groups but not in either 0.04 or 0.2 mg/kg groups compared to vehicle controls (5 mg/kg 16.0 ± 4.3%, 1 mg/kg 10.9 ± 5.0%, vs. controls 36.7 ± 3.6%, $P < 0.05$). Macroscopic evaluation of brain injury revealed the neuroprotective effect of AA in both 1 and 5 mg/kg groups (5 mg/kg 1.1 ± 0.4, 1 mg/kg 0.4 ± 0.3, vs. controls 2.9 ± 0.3, $P < 0.05$). Western blots of fodrin on the ligated side also showed that AA significantly suppressed 150/145-kDa bands of fodrin breakdown products, which suggested that AA suppressed activation of calpain. Neuropathological quantitative analysis of cell death revealed that 1 mg/kg of AA injection significantly reduced the number of necrotic cells in cortex, caudate putamen, thalamus and hippocampus CA1, whereas that of apoptotic cells was only reduced in cortex. These findings show that intraventricular AA injection is neuroprotective after HI in immature rats.

1. Introduction

Neonatal hypoxic–ischemic encephalopathy (HIE) following birth asphyxia causes severe neurological disabilities. Excitatory amino acids, such as glutamate and aspartate, contribute to hypoxic–ischemic (HI) brain injury by activating N-methyl-D-aspartate (NMDA)-type glutamate receptors, flooding cells with Ca^{2+} and producing nitric oxide, free radicals and reactive oxygen species (ROS) (Lewen et al., 2000; Johnston et al., 2001; Gilgun-Sherki et al., 2002). The ROS initiate lipid

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peroxidation and other oxidative chain reactions that can lead to cell death. Although apoptosis is considered to be more prominent as a mode of cell death in neonates compared to the adult brain (Johnston et al., 2002), necrosis still predominates after HI in immature brain (Nakajima et al., 2000). Calpain, a calcium-activated cytosolic cysteine protease, mainly contributes to necrotic cell death in ischemic and excitotoxic neuronal injury. Calpain activation has been demonstrated in HI animal models in adult (Hong et al., 1994) and also in neonate (Blomgren et al., 1995).

Ascorbic acid (AA) is a water-soluble reducing agent and antioxidant that plays various physiological roles such as biosynthesis of collagen and catecholamine, and hematopoiesis. AA is widely distributed in the human body, and it is especially concentrated in adrenal gland and brain. Although human brain cells cannot synthesize AA de novo, it is highly concentrated in the central nervous system. AA concentrations are lowest in plasma (0.01–0.1 mM), intermediate in cerebrospinal and extracellular fluid (0.05–0.5 mM) and highest in neuropil of the brain (1–3 mM) (Grunewald, 1993). AA acts as an electron donor and free radical scavenger, and its neuroprotective effect has been demonstrated in several animal HI models (Ranjan et al., 1993; Stamford et al., 1999). Moreover, other studies have shown that administration of dehydroascorbic acid (DHA, oxidized form of AA) (Huang et al., 2001) or combined treatment of AA with alpha-tocopherol (Vitamin E) (Nakai et al., 2002) are neuroprotective in animal models. However, AA has also been shown to cause neurotoxicity in homogenates of rat cerebral cortex and cultured cortical neurons (Hisanaga et al., 1992; Song et al., 2001). Therefore, it has not been demonstrated that AA is neuroprotective following cerebral ischemia, and the effect of AA on neonatal HI brain has not been explored. In the newborn brain, excitotoxicity plays a greater role in neurodegeneration after HI than in adults (Johnston et al., 2001). Considering that AA plays a physiologic role in the human body, and might be safe for use in infants, we examined the effect of AA on HI brain injury in newborn rats.

2. Results

2.1. General observations

We used 50 rat pups in total in this study. Eight out of 50 pups died (2 pups died in 0.04 mg/kg group, 1 in 0.2 mg/kg, 2 in 1 mg/kg, 1 in 5 mg/kg, and 2 in vehicle controls) in the middle of exposure to hypoxia or malnutrition before postnatal day (PND) 14. Pups that died were excluded from the study.

The rectal temperature just before hypoxia did not significantly differ among AA groups and vehicle controls; 37.3 ± 0.3 °C (AA 0.04 mg/kg), 37.7 ± 0.1 °C (0.2 mg/kg), 37.7 ± 0.1 °C (1 mg/kg), 37.6 ± 0.1 °C (5 mg/kg) and 37.7 ± 0.1 °C (vehicle control). The rectal temperature 30 min after hypoxia did not significantly differ either; 36.8 ± 0.2 °C (AA 0.04 mg/kg), 36.6 ± 0.2 °C (0.2 mg/kg), 36.9 ± 0.1 °C (1 mg/kg), 36.5 ± 0.1 °C (5 mg/kg) and 36.6 ± 0.2 °C (vehicle control). This confirmed that intraventricular injection of AA did not bring non-specific drug-induced hypothermia. The body weight at PND 7 did not significantly differ among AA groups, vehicle controls and normal ones. The weight gain in 1 mg/kg of AA group at 7 days post-HI was significantly larger compared with vehicle controls (9.0 ± 0.7 g vs. 5.1 ± 0.8 g), whereas it did not significantly differ among the other 3 AA groups and vehicle controls (Table 1).

2.2. Macroscopic evaluation of brain injury

All brains in 0.04 and 0.2 mg/kg of AA groups, 1 out of 8 in 1 mg/kg, 3 out of 9 in 5 mg/kg, and 10 out of 12 in vehicle controls had infarcts. The percent brain damage in both 1 and 5 mg/kg groups was significantly lower compared with vehicle controls, but there was no difference compared to 0.04 and 0.2 mg/kg groups (Fig. 1). The scores for macroscopic brain injury in both 1 and 5 mg/kg groups were significantly lower than that in vehicle controls, but there was no difference compared to 0.04 and 0.2 mg/kg groups (Fig. 2).

2.3. Western blots of fodrin

Fodrin (also called non-erythroid spectrin) is an actin-binding, fibrous protein. This protein is widely distributed in vertebrates, and it forms part of the sub-membranous cytoskeleton within many cell types including neurons. Alpha-fodrin is broken down by caspases and calpains during cell death (Wang, 2000), and cleavage by either of these enzymes yields different sized products. When it is cleaved by caspase-3, the products are 150- and 120-kDa peptides, but when cleaved by calpain, the products are 150- and 145-kDa (Wang, 2000). Therefore, fodrin breakdown products (FBDPs) can be served as dual molecular markers for both calpain and caspase-3 activation. The antibody against alpha-fodrin detects several bands, including the intact 240-kDa protein, the 150/145- and 120-kDa FBDPs.

For Western blotting, we used right (ligated side) hemispheres of both 1 and 5 mg/kg of AA groups and vehicle controls (Fig. 3). We used all 4 samples in 1 mg/kg group and selected 4 samples at random in both 5 mg/kg group and

Table 1 – The body weight gain at 7 days post-hypoxic–ischemia

Group	Body weight (PND7)	Weight gain (PND7–14)
5 mg/kg (n = 5)	10.2 ± 0.5 (g)	5.1 ± 2.0 (g)
1 mg/kg (n = 4)	11.4 ± 0.3	9.0 ± 0.7*
0.2 mg/kg (n = 5)	11.5 ± 0.4	5.0 ± 1.1
0.04 mg/kg (n = 4)	10.7 ± 0.2	6.2 ± 0.4
Vehicle control (n = 8)	10.7 ± 0.2	5.1 ± 0.8
Normal control (n = 4)	11.4 ± 0.3	10.5 ± 0.4

The weight gain in 1 mg/kg group at postnatal day (PND) 14 (7 days post-HI) was significantly larger compared with vehicle controls (*$P < 0.05$ versus control group). There was no significant difference between 1 mg/kg group and normal controls. Data are presented as mean ± SEM. Pups that died were excluded from the study. Vehicle control: pups underwent carotid ligation and hypoxia, and received intraventricular injections of phosphate buffered saline. Normal control: pups underwent neither carotid ligation nor hypoxia, and received no injections.