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Sex-specific effects of N-acetylcysteine in neonatal rats treated with hypothermia after severe hypoxia-ischemia

Xingju Nie[a], Danielle W. Lowe[b], Laura Grace Rollins[c], Jessica Bentzley[b], Jamie L. Fraser[d], Renee Martin[e], Inderjit Singh[b], Dorothea Jenkins[b,*]

[a] Center for Biomedical Imaging, Medical University of South Carolina, 165 Ashley Ave, Charleston, SC 29425, United States
[b] Department of Pediatrics, Medical University of South Carolina, 165 Ashley Ave, Charleston, SC 29425, United States
[c] Department of Psychology, University of Massachusetts, 100 Morrissey Blvd, Boston, MA 02125, United States
[d] Medical Genetics Training Program, National Human Genome Research Institute, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892-2152, United States
[e] Department of Biostatistics and Epidemiology, Medical University of South Carolina, 165 Ashley Ave, Charleston, SC 29425, United States

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ABSTRACT

Approximately half of moderate to severely hypoxic-ischemic (HI) newborns do not respond to hypothermia, the only proven neuroprotective treatment. N-acetylcysteine (NAC), an antioxidant and glutathione precursor, shows promise for neuroprotection in combination with hypothermia, mitigating post-HI neuroinflammation due to oxidative stress. As mechanisms of HI injury and cell death differ in males and females, sex differences must be considered in translational research of neuroprotection. We assessed the potential toxicity and efficacy of NAC in combination with hypothermia, in male and female neonatal rats after severe HI injury. NAC 50 mg/kg/d administered 1 h after initiation of hypothermia significantly decreased iNOS expression and caspase 3 activation in the injured hemisphere versus hypothermia alone. However, only females treated with hypothermia +NAC 50 mg/kg showed improvement in short-term infarct volumes compared with saline treated animals. Hypothermia alone had no effect in this severe model. When NAC was continued for 6 weeks, significant improvement in long-term neuromotor outcomes over hypothermia treatment alone was observed, controlling for sex. Antioxidants may provide insufficient neuroprotection after HI for neonatal males in the short term, while long-term therapy may benefit both sexes.

1. Introduction

Hypothermia treatment for hypoxic-ischemic (HI) neonates decreases the incidence of severe neurodevelopmental outcomes at 12–24 months of age to approximately 45–55%, without apparent differences in efficacy between sexes (Eicher et al., 2005; Shankaran et al., 2008; Azzopardi et al., 2009; Simbruner et al., 2010). With hypothermia now recognized as the clinical standard of care, the addition of other neuroprotective agents to hypothermia treatment are being investigated to improve outcomes further (Barks et al., 2010; Liu et al., 2012; Hobbs et al., 2008; Fan et al., 2013). N-acetylcysteine (NAC) is a promising antioxidant therapy that impacts many pathways of injury and has established neuroprotective effects in animal models of HI and neuroinflammation (Jatana et al., 2006; Paintlia et al., 2004; Liu et al., 2010). Immune and inflammatory responses to HI are modulated by cellular redox status, and increased apoptotic signaling pathways are present in cells with low anti-oxidant reserves (Lu, 2009; Cook et al., 2004; Circu and Aw, 2010; Wang and Kaufman, 2012; Ten and Starkov, 2012; Jager et al., 2012).

In our previous work, hypothermia plus NAC 50 mg/kg/day improved infarct volumes and negative geotaxis performance in neonatal animals subjected to right common carotid artery

Abbreviations: CXCL-1/GROα, chemokine C-X-C ligand 1/growth-regulated oncogene alpha; CXCL-2/GROβ, chemokine C-X-C ligand 2/macrophage inflammatory protein 2-alpha/growth-regulated protein beta; HI, hypoxia ischemia; HNAC, hypothermia with N-acetylcysteine treatment; HYPO, hypothermia treatment; ICAM, intercellular adhesion molecule; IL, interleukin; iNOS, nNOS, inducible or neuronal nitric oxide synthase; MBP, myelin basic protein; MMP, matrix metalloproteinase; NAC, N-acetylcysteine; NO, nitric oxide; PND, postnatal day; TTC, 2,3,5-Triphenyl-tetrazolium chloride; VEH, vehicle, saline treatment.

* Corresponding author at: Department of Pediatrics, MSC 917, Medical University of South Carolina, 165 Ashley Ave., Charleston, SC 29425, United States. Tel.: +1 843 792 4341.
E-mail addresses: niexi@musc.edu (X. Nie), clarkdw@musc.edu (D.W. Lowe), lgrollins@gmail.com (L.G. Rollins), perkel@musc.edu (J. Bentzley), jamie.fraser@nih.gov (J.L. Fraser), hebertrl@musc.edu (R. Martin), singhi@musc.edu (I. Singh), jenkd@musc.edu (D. Jenkins).

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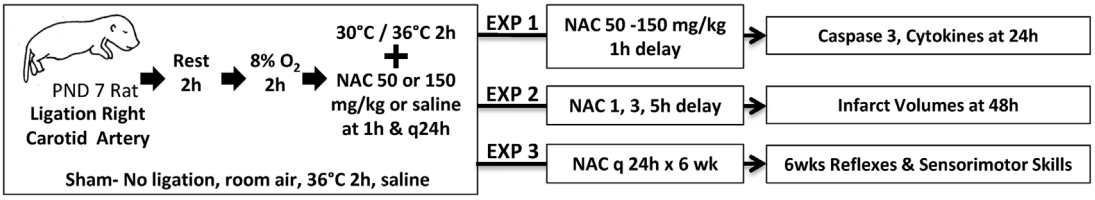

Fig. 1. Outline of experimental design.

ligation and 2 h of hypoxia, although sex effects were not evaluated (Jatana et al., 2006). For translation to therapeutic trials, these findings need to be replicated with robust statistical power, and optimal timing and dosing regimens determined. Furthermore, increasing evidence from in vitro and in vivo studies point to different mechanisms of injury, cell death, neuroinflammation and possibly repair between males and females in neonatal animals, even though both sexes are exposed to significant maternal estrogen (Zhang et al., 2010; Offner et al., 2009; Liesz et al., 2009; Nijboer et al., 2007; Zhu et al., 2006; Wen et al., 2006; Park et al., 2006; Weis et al., 2012). These different injury mechanisms warrant analytical consideration of sex-specific treatment effects. We performed 3 experiments with NAC plus hypothermia to determine sex differences in mechanisms of neuroprotection, to choose dose and timing of NAC administration, and to determine long-term outcomes in a severe HI model.

2. Materials and methods

2.1. Animal

Postnatal day (PND) 7 Sprague-Dawley rats were used for all experiments (Harlan, Indianapolis, IN). Animals were housed in the animal care facility of the Medical University of South Carolina (MUSC) with a 12/12 h light/dark cycle, and given standard chow and water ad libitum. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the National Institutes of Health and approved by the MUSC Animal Care and Use Committee.

2.2. Reagents

2,3,5-Triphenyl-tetrazolium chloride (TTC) and paraformaldehyde (Sigma Chemical Co., St. Louis, MO); Novaplus™ (Isoflurane, USP) (Abbot Laboratories, North Chicago, IL); VECTASHIELD Hard Set™ Mounting Medium with DAPI (H-1500, Vector Laboratories, Burlingame, CA); anti-Activated Caspase 3 (Abcam, Cambridge, MA); N-acetylcysteine (Acetadote, Cumberland Pharmaceuticals, Nashville, TN).

2.3. Hypoxia-ischemia animal model

PND 7 rat pups were randomized to experimental groups within litters. Liters were limited to 10 pups to ensure equal maternal access. We used the modified Levine model of HI injury with unilateral ligation of the right common carotid artery and 2 h exposure to 8% oxygen atmosphere for all experiments as previously described (Jatana et al., 2006; Geddes et al., 2001). At the end of hypoxia, pups were exposed to systemic hypothermia ($30 \pm 0.5\,^{\circ}C$) or normothermia ($36.3 \pm 0.5\,^{\circ}C$) in temperature controlled chambers for 2 h (Jatana et al., 2006). Rectal temperatures were in the target range of 33.5–34.5 °C for the hypothermic rats, and 36.5–37.5 °C for the normothermic group, similar to that used in clinical trials of therapeutic hypothermia. Sham operated animals underwent anesthesia and a neck incision without ligation or hypoxia and received normothermia and intraperitoneal saline. All treatment groups were administered NAC or vehicle (saline) intraperitoneal 1 h after hypoxia (1 h after initiation of hypothermia). Animals were removed from respective temperature chambers briefly for the injection and replaced. NAC or saline injections were repeated daily until sacrifice: Sham, Vehicle (VEH) and hypothermia alone (HYPO) groups received saline; Hypothermic NAC rats received 50 or 150 mg/kg/day of NAC (HNAC 50, HNAC 150). Control groups were limited to sham surgery, untreated HI injury (VEH), and the clinical standard of care hypothermia after HI (HYPO), as prospective therapies would be used only in addition to hypothermia clinically. Daily weights were recorded from PND 7 until sacrifice.

2.4. Experimental design

Experiment 1: For caspase 3 activation by paraffin-embedded immunohistochemistry, 31 rats were randomized to Sham ($n = 2$), VEH ($n = 8$), HYPO ($n = 8$), HNAC 50 ($n = 7$), HNAC 150 ($n = 4$ survived, $n = 2$ expired) with sacrifice at 24 h. For cytokine and inflammatory mediator expression by quantitative RT-PCR in flash frozen brain tissue, an additional 44 rats were randomized to the 5 groups (4–5 rats per sex per treatment) with sacrifice at 24 h. *Experiment 2*: PND 7 rats ($n = 184$) were randomized into 9 groups to evaluate for timing of NAC administration based on hours after onset of hypothermia: Sham ($n = 12$), VEH ($n = 20$), HYPO ($n = 28$), HNAC 50 1 h ($n = 20$), HNAC 50 3 h ($n = 21$), HNAC 50 5 h ($n = 20$), HNAC 150 ($n = 21$), HNAC 150 3 h ($n = 21$), and HNAC 150 5 h ($n = 21$) for 48 h infarct volumes. Infarct volumes were measured at 48 h, comparing volumes *within* NAC doses of 50 and 150 mg/kg/d, to select the most effective timing for each dose for further study, considering possible sex effects. *Experiment 3*: PND 7 rats were randomized to Sham, VEH, HYPO, HNAC 50 1 h, HNAC 150 1 h, HNAC 150 3 h ($n = 33$, 5–8 rats per treatment group), and saline or NAC were administered once daily for 6 weeks. Mortality, physical characteristics, neurological reflexes, and strength and coordination testing were measured until sacrifice at 6 weeks after HI (Fig. 1).

2.5. Randomization, power analyses and outcome measures

Each litter was randomized to include pups of each sex and treatment group. For Experiment 1, a minimum of $n = 4$ per sex to observe a 50% difference by Wilcoxon signed rank test (80% power, $a = 0.05$) was based on previous work looking at iNOS mRNA levels after HI (Park et al., 2006). For Experiment 2, effect sizes for differences in infarct volume stratified by sex were estimated from a report by Bona et al. (1998), which reported differential sex effects of hypothermia and 50% reduction in gross morphology score in female PND 7 HI rats. For median infarct volumes by sex with 50% effect size by Wilcoxon signed rank test (80% power, $a = 0.05$), a required sample size of 18 rats per treatment group, (9 per sex). This number was verified by an independent investigator

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