Antioxidative effects of uridine in a neonatal rat model of hyperoxic brain injury

Abstract

Background/aim: Premature birth is a major problem that results in increased risk of mortality and morbidity. Management of such infants consists of supraphysiological oxygen therapy, which affects brain development due, in part, to the deterioration caused by reactive oxygen species (ROS). We showed previously that exogenously-administered uridine provides neuroprotection in a neonatal rat model of hyperoxic brain injury. Hence, the aim of the present study was to investigate effects of uridine on ROS in the same setting.

Materials and methods: Hyperoxic brain injury was induced by subjecting total 53 six-day-old rat pups to 80% oxygen (Hyperoxia group) for a period of 48 hours. Pups in Normoxia group continued breathing room air (21% oxygen). Normoxia+saline or hyperoxia + saline or hyperoxia + uridine100 mg/kg or hyperoxia + uridine300 mg/kg or hyperoxia + uridine500 mg/kg was injected intraperitoneally (i. p.) 15 minutes prior to hyperoxia procedure. Pups were decapitated and brains were homogenized to analyze Superoxide Dismutase (SOD), Glutathione Peroxidase (GSH-Px), Myeloperoxidase (MPO) and Malondialdehyde (MDA) enzymes as well as DJ-1 (Protein deglycase DJ-1), an oxidative stress sensitive protein.

Results: Hyperoxia-induced may cause overproduction of oxygen radicals and the oxidant / antioxidant balance may be disturbed in the brain. Brain MPO and MDA levels were significantly increased in saline-receiving pups exposed to hyperoxia. Brain SOD and GSH-Px levels were significantly decreased in saline-receiving pups exposed to hyperoxia. Our results showed that uridine administration prevented the hyperoxia-induced decrease in SOD and GSH-Px while counteracting the hyperoxia-induced
increase in MPO and MDA in a dose-dependent manner. Uridine also increased DJ-1 levels in brains of rat pups subjected to hyperoxia.

**Conclusion:** These data suggest that uridine exhibits antioxidative properties which may mediate the protective effects of uridine in a neonatal rat model of hyperoxic brain injury.

**Key words:** Uridine, hyperoxia, antioxidative, neonatal rat, DJ-1

1. **Introduction**

Premature birth is a major problem all over the world and preterm infants have increased risk of mortality and morbidity despite recent advances in neonatal medicine [1]. Preterm infants are vulnerable to several complications including respiratory distress syndrome, bronchopulmonary dysplasia, intestinal injury, compromised immune system, cardiovascular disorders, as well as hearing/vision and neurological problems [2]. Most preterm infants are exposed to supraphysiological oxygen therapy during perinatal period in the neonatal intensive care unit (NICU). Supraphysiological oxygen therapy in the sensitive period of brain development affects the developmental processes as a result of hyperoxia [3-4]. Preterm neonates are especially susceptible to deterioration caused by reactive oxygen species (ROS) due to the fact that endogenous radical scavenging systems are not fully mature [3]. Although ROS is a function of signal molecules at low concentrations [5-6], its overproduction causes oxidative stress due to the imbalance between oxidative and antioxidative systems [7]. As a result, reactive oxygen radicals are formed that contribute to oxidative changes in proteins, lipids and nucleic acids [8] and negatively impacts survival of neuronal cells during development [4-9-10]. In the clinical setting, up to 50% of surviving preterm infants has been reported to exhibit cognitive deficits or behavioral problems during the later stages of development [11].
The level and activity of the most-relevant antioxidant enzymes, such as superoxide dismutase, catalase (CAT), and glutathione peroxidase (GPX), change dynamically during development and mature in the last weeks of gestation, preparing the fetus for lung respiration [12-13-14]. They are essential to preserving cells from exposure to oxidative damage [15]. On the other hand, brain levels of the oxidative stress markers myeloperoxidase and malondialdehyde are increased significantly after exposure to hyperoxia in animals and humans [16-17]. MPO is a highly potent oxidative enzyme that is capable of inducing both oxidative and nitrosative stress in vivo [18] and MDA is a lipid peroxidation product that is one of the most frequently used markers of oxidative stress [19]. Hence, treatments that raise levels of antioxidants and lower those of oxidative stress markers have been associated with neuroprotective effects [20].

Uridine is the principal circulating pyrimidine nucleoside in human circulation [21-22], a constituent of breast milk [23], and a precursor of brain membrane phospholipids via the Kennedy pathway [24]. We showed previously that exogenously-administered uridine provides neuroprotection in neonatal rat models of hypoxic-ischemic encephalopathy (HIE) [25-26-27] and hyperoxic brain injury [28]. The neuroprotection by uridine has been shown to be mediated by its anti-apoptotic [25] and epigenetic effects (i.e., reducing histone deacetylase activity) [27] in the neonatal HIE model.

Hence, for the first time, the present study aimed to investigate the mechanism by which uridine exhibited neuroprotection in the neonatal rat model of hyperoxic brain injury [28] with regard to hyperoxia-induced oxidative stress. Levels of most relevant oxidative/antioxidative enzymes and DJ-1 protein (Protein deglycase DJ-1, also known as Parkinson disease protein 7 [PARK7]) [29], an oxidative stress sensitive protein which
is known to maintain mitochondrial functions by scavenging reactive oxygen species (ROS) were analysed.[29]

2. Materials and Methods

2.1. Animals and experimental design

Seven dated pregnant Sprague-Dawley rats were housed in individual cages at 22°C with 12h light/dark cycle with free access to food and water. Dams (mothers of newborn rats) delivered 6 to 13 pups and the day of birth was considered postnatal day 1 (P1). A total of 53 pups were included in the study regardless of the gender and their weights were checked daily. Pups were pooled in wood shavings, divided randomly, and delivered back to nursing dams.

2.2. Induction of hyperoxic brain injury

The 6-day-old the pups were randomly divided into 5 groups as follows: Normoxia + Saline (n = 10), Hyperoxia + Saline (n = 11), Hyperoxia + Uridine 100mg/kg (n = 13), Hyperoxia + Uridine 300mg/kg (n = 9), Hyperoxia + Uridine 500mg/kg (n = 10).

From P6 to P8 (48 hours), pups in the Normoxia group were subjected to room air (21% oxygen) while those in Hyperoxia groups were subjected to 80% continuous oxygen, as has been described previously [9-17-20-29-30-31]. The preference for studying P6 pups depended on a precise calculation obtained at the web site [32] which showed that brain growth and neurogenesis at developing rat brain on post-conceptional (PC) 27 days corresponds to that on PC 204 days (29.1 weeks) of human brain.

Continuous oxygen was maintained in a Plexiglas chamber with 60 x 20 x 20 cm (width x depth x height) dimensions by a flow-through system. Saline (0.9 % NaCl; 0.1 ml/10 g body weight) or uridine (100 mg/kg, 300 mg/kg or 500 mg/kg; dissolved in saline) was administered intraperitoneally (i. p.) once 15 min before the onset of atmospheric air or
oxygen exposure. Doses of uridine administered in this study were selected from our previous studies reporting neuroprotective effects in neonatal rat models of hypoxic-ischemic encephalopathy [25-26-27] and hyperoxic brain injury [28]. The oxygen level inside the chamber was monitored continuously with a MiniOX 3000 oxygen analyzer (Ohio Medical Corporation, Gurnee, IL, USA) to maintain 80% oxygen saturation. Nursing dams were switched every 24h to prevent respiratory complications. On completion of 48-hour hyperoxic insult (at P8), pups were decapitated under deep anesthesia, brains were removed and chilled in liquid nitrogen immediately and analyzed immediately for biochemical parameters and DJ-1 protein by Western Blotting [33].

2.3. Biochemical Analyses

Brain tissues were homogenized in ice-cold phosphate-buffered saline (PBS, pH 7.4). The homogenates were utilized for analyzing levels of GSH-Px, SOD, MDA and MPO using commercially-available ELISA kits (Sunredbio Technology Co. Ltd., Shanghai, China) according to kit procedure.

DJ-1 Protein Analyses:

Homogenates were boiled in Laemmli buffer [34] and loaded onto Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE; Mini Protean II, Bio-Rad, Hercules, CA, USA) at equal protein content analyzed by Lowry method [35]. Each sample was run electrophoretically and protein bands were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk dissolved in tris buffered saline (TBST) and incubated overnight with the primary antibody, DJ-1 (1:1000, Cell Signaling Technology, Danvers, MA, USA). The following day, the membranes were incubated with HRP (Horse radish peroxidase) bound rabbit anti IgG secondary antibody (1:5000, Cell Signaling
Technology, Danvers, MA, USA) for 1 hour and then visualized by incubation with enhanced chemiluminescence solution (Millipore, Billerica, MA, USA). Protein bands were scanned by Licor CDigit scanner (LI-COR Biotechnology, Lincoln, NE, USA) and the density of the bands was compared using the software of Licor CDigit system. Following this procedure the membranes were cleared with stripping buffer (Thermo Fisher Scientific, Rockford IL, USA) and then incubated with mouse- anti-β-III-tubulin antibody (Millipore, Temecula, CA, USA) used as a house-keeping protein and the procedure was repeated with the appropriate secondary antibody.

2.4. Statistical Analysis

Statistical analyses were performed using Sigma Plot 12.0 software. Data were expressed as mean ± standard error of means (SEM). The Shapiro-Wilk test was used for determining normality of data. When the data showed a normal distribution and passed normality test, difference between groups was determined by the parametric One-Way ANOVA followed by post hoc Tukey test. On the other hand, when the data failed normality test, difference between groups was determined by the non-parametric Kruskal-Wallis H test followed by post hoc Dunn’s test with Bonferroni correction. p < 0.05 was considered statistically significant.

3. RESULTS

3.1. Biochemical Analyses

Compared with the Normoxia+Saline group, brain MDA levels of pups in Hyperoxia+Saline group were significantly increased (p < 0.001) (Figure 1A, Table 1). Uridine treatment at 300 mg/kg and 500 mg/kg in pups exposed to hyperoxia reduced brain MDA levels significantly (p < 0.01 and p < 0.001, respectively), while uridine at 100 mg/kg did not.
Similarly, brain MPO levels were increased in saline-receiving pups exposed to hyperoxia significantly (p < 0.001) (Figure 1B, Table 1) compared with the normoxic group. The enhanced brain MPO levels were reduced significantly in hyperoxic pups treated with uridine at 300 mg/kg and 500 mg/kg (p < 0.0 and p < 0.001, respectively) doses, while no change was observed with 100 mg/kg uridine.

On the contrary, brain SOD levels of pups in Hyperoxia + Saline group were significantly decreased (p < 0.001) (Figure 1C, Table 1) compared to those in Normoxia + Saline group. Treatment with uridine at 300 mg/kg and 500 mg/kg in pups exposed to hyperoxia increased brain SOD levels significantly (p < 0.01 and p < 0.001, respectively), but uridine at 100 mg/kg was not effective.

In good accord with data derived in SOD analyses, levels of GSH-Px in brains of pups in Hyperoxia + Saline group were significantly decreased (p < 0.001) (Figure 1D, Table 1) compared to those in Normoxia + Saline group, and uridine increased brain GSH-Px levels at 300 mg/kg and 500 mg/kg in pups exposed to hyperoxia significantly (p < 0.05 and p < 0.01, respectively). Uridine administered at 100 mg/kg dose was again not effective.

**3.2. Levels of DJ-1 Protein**

Brain DJ-1 protein levels (expressed as % change in DJ-1/ β-tubulin ratio) of pups in Hyperoxia + Saline group were significantly (p < 0.001) reduced by about 33% compared to those in Normoxia + Saline group (Figure 2, Table 2). Uridine treatment at 300 mg/kg and 500 mg/kg in pups exposed to hyperoxia prevented the decrease in brain DJ-1 protein levels significantly (p < 0.01 and p < 0.001, respectively), but not at 100 mg/kg dose (Figure 2, Table 2).
4. DISCUSSION

These data show that uridine exhibits antioxidative properties in a neonatal rat model of hyperoxic brain damage suggesting one mechanism by which uridine provides neuroprotection in such model [28]. We showed, for the first time, that a single dose of uridine injected to P6 pups prevented the hyperoxia-induced decreases in SOD and GSH-Px levels as well as the hyperoxia-induced increases in MDA and MPO levels, in a dose-dependent manner. In addition, uridine treatment also enhanced the decreased levels of DJ-1, an oxidative stress sensitive protein.

Preterm birth is a major health problem for neonates and their caregivers due to the lack of maturity of organs and systems, including the brain. Survival rates of preterm neonates have been increasing with efforts in modern Neonatal Intensive Care Units (NICUs) while the same is not true for morbidity rates [36-37]. In addition to the complications observed due to prematurity itself, the fact that most premature infants receive supraphysiological oxygen therapy for conditions like cardiac resuscitation and respiratory distress during their NICU stay [38] contributes to the morbidities associated especially with tissues like the intestines [39], lungs [40], retina [41-42] and the brain [3-10-43].

The fact that extraterine environment (PaO$_2$ 100 mm Hg) is richer in oxygen compared to the intrauterine environment (PaO$_2$ 20–25 mm Hg) renders birth an oxidative challenge for the newborn. Hence, the fetal to neonatal transition exposes the newborn to an oxygen-rich world [3] creating a considerably high risk for oxidative stress at birth. This risk is additionally exacerbated by the low efficiency of natural antioxidant systems in preterm newborns [44-45] and the supraphysiological oxygen therapy that they receive. Pathological hallmarks of supraphysiological oxygen treatment in the brain include enhanced inflammation, oxidative stress and matrix metalloproteinase activity.
accompanied by autophagy, increased apoptotic cell death and reduced neuro-glial
development in the brain [17-20-31-46-47]. Hyperoxic insult to the brain is associated
with increased levels of IL-1 b and IL-18 mRNA, oxidized glutathione (GSSG), MDA
[17-47], thiobarbituric acid reactive substances and hydrogen peroxide [20]. In addition,
hyperoxia changes the balance of the reactive oxygen species (ROS)-dependent
thioredoxin/peroxiredoxin system and reduces the level of DJ-1, a hydroperoxide-
response protein [29].

Various neuroprotective strategies have been investigated experimentally to reduce
hyperoxic brain damage in preterm neonates including caffeine [20], erythropoietin [17-
47-48], dexmedetomidine [31], topiramate [49], acetylcholinesterase (AChE) inhibitors
[50], and, recently, mesenchymal stem cells [51] and fingolimod [52].

Uridine is the principal circulating pyrimidine nucleoside in humans [21-22] and a
precursor of membrane phospholipids [24].

We showed previously that exogenously-administered uridine provides neuroprotection
and improves long-term cognitive deficits [26] by reducing apoptotic cell death [25] and
inhibiting histone deacetylase activity [27] in a rat model of HIE. In addition, our previous
findings showed, in the hyperoxia model, that uridine administration for five consecutive
days during continuous hyperoxic insult reduced apoptosis in pups’ brains and protected
against long-term cognitive deficits [28]. Although uridine has been shown to exhibit
anti-inflammatory effects in models of pulmonary diseases [53-54] or colitis [55], the
effect of uridine on oxidative stress has not yet been studied.

Hence the aim of the present study was to investigate whether uridine prevents oxidative
injury as a mechanism of action in the benefit it provides in neonatal hyperoxic brain
damage. We showed, for the first time, that uridine exhibits antioxidative properties by
preventing the hyperoxia-induced decreases in SOD and GSH-Px levels and the increases in MDA and MPO levels, as well as by enhancing the decreased levels of an oxidative stress sensitive protein, DJ-1. These data provide the first evidence that the neuroprotective effect of uridine in a neonatal rat model of hyperoxic brain injury depends, at least in part, on uridine’s action on oxidative stress parameters. Our data on SOD, GSH-Px, MDA and MPO are in good accord with previous studies reporting that antioxidative strategies provide neuroprotection in the hyperoxia-induced brain injury in neonatal animals [17-20-31-47]. In addition, we found, in accordance with previous studies [29], that levels of DJ-1, an oxidative stress sensitive protein, were reduced following supraphysiological oxygen treatment. DJ-1 functions as a redox-sensitive chaperone to protect tissues against oxidative stress and cell death [56-57-58-59]. Hence, in cases where DJ-1 levels are decreased, oxidative stress is triggered, which in turn contributes to cell death, as shown in a neuronal cell line [60], or in the immature brain following hyperoxic insult [29]. Therefore, treatments that raise the expression of DJ-1 protein may prevent oxidative stress and provide benefit in terms of neuroprotection. In the present study, we showed that uridine treatment significantly restored DJ-1 levels in a dose-dependent manner and this effect may comprise one mechanism by which uridine prevents brain cell death in the hyperoxic setting [28]. In conclusion, our data shows, for the first time, that uridine prevents oxidative damage by preventing the hyperoxia-induced decreases in SOD and GSH-Px levels and the increases in MDA and MPO levels, as well as by enhancing the decreased levels of DJ-1 protein. These data provide a deeper mechanistic insight into the neuroprotective effect of uridine in neonatal hyperoxic brain injury.

Acknowledgements
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References


![Figure 1: Brain levels of (A) MDA, (B) MPO, (C) SOD and (D) GSH-Px in neonatal rats subjected to hyperoxic brain damage. Hyperoxia+Saline group, *p<0.05, **p<0.01 and ***p<0.001 compared to Normoxia+Saline group. Normoxia+Saline group, #p<0.05, ##p<0.01 and ###p<0.001 compared to Hyperoxia+Saline group.]
**Figure 2:** Levels of DJ-1 protein in brains of neonatal rats subjected to hyperoxic damage.

Hyperoxia+Saline group, ***p<0.001 compared to Normoxia+Saline group.

Normoxia+Saline group, ##p<0.01 and ###p<0.001 compared to Hyperoxia+Saline group.

**Table 1.** Brain levels of MDA, MPO, SOD and GSH-Px in neonatal rats subjected to hyperoxic brain damage.

<table>
<thead>
<tr>
<th></th>
<th>Normoxia + Saline</th>
<th>Hyperoxia + Saline</th>
<th>Hyperoxia + U100</th>
<th>Hyperoxia + U300</th>
<th>Hyperoxia + U500</th>
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<tbody>
<tr>
<td>MDA (nmol/g protein)</td>
<td>4.35 ± 0.1</td>
<td>5.75 ± 0.2***</td>
<td>5.45 ± 0.2***</td>
<td>4.85 ± 0.1**</td>
<td>4.47 ± 0.1***</td>
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<td>MPO (ng/g protein)</td>
<td>3.19 ± 0.1</td>
<td>4.83 ± 0.1***</td>
<td>4.57 ± 0.2***</td>
<td>4.04 ± 0.1**</td>
<td>3.70 ± 0.1***</td>
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<td>SOD (ng/g protein)</td>
<td>7.51 ± 0.2</td>
<td>5.54 ± 0.3***</td>
<td>6.30 ± 0.2</td>
<td>7.09 ± 0.3**</td>
<td>7.39 ± 0.3***</td>
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<td>GSH-Px (ng/g protein)</td>
<td>17.66 ± 0.6</td>
<td>13.23 ± 0.5***</td>
<td>14.67 ± 0.7*</td>
<td>16.28 ± 0.5#</td>
<td>17.06 ± 0.7**</td>
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Hyperoxia+Saline group, *p<0.05, **p<0.01 and ***p<0.001 compared to Normoxia+Saline group. Normoxia+Saline group, #p<0.05, ##p<0.01 and ###p<0.001 compared to Hyperoxia+Saline group.
**Table 2.** Levels of DJ-1 protein in brains of neonatal rats subjected to hyperoxic damage.

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<th>% Change in DJ-1/β-tubulin ratio</th>
<th>Normoxia + Saline</th>
<th>Hyperoxia + Saline</th>
<th>Hyperoxia + U100</th>
<th>Hyperoxia + U300</th>
<th>Hyperoxia + U500</th>
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<tr>
<td>Normoxia + Saline</td>
<td>100 ± 2</td>
<td>67.2 ± 2.6***</td>
<td>75.4 ± 4.8***</td>
<td>88.2 ± 5.5**</td>
<td>98.7 ± 3.2***</td>
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<td>Hyperoxia + Saline</td>
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Hyperoxia+Saline group, ***p<0.001 compared to Normoxia+Saline group.

Normoxia+Saline group, ##p<0.01 and ###p<0.001 compared to Hyperoxia+Saline group.