Sildenafil Improves Functional and Structural Outcome of Retinal Injury Following Term Neonatal Hypoxia-Ischemia

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PURPOSE. The purpose of this study was to investigate the effects of sildenafil on retinal injury following neonatal hypoxia-ischemia (HI) at term-equivalent age in rat pups.

METHODS. Hypoxia-ischemia was induced in male Long-Evans rat pups at postnatal day 10 (P10) by a left common carotid ligation followed by a 2-hour exposure to 8% oxygen. Sham-operated rats served as the control group. Both groups were administered vehicle or 2, 10, or 50 mg/kg sildenafil, twice daily for 7 consecutive days. Retinal function was assessed by flash electroretinograms (ERGs) at P29, and retinal structure was assessed by retinal histology at P30.

RESULTS. Hypoxia-ischemia caused significant functional (i.e., attenuation of the ERG a-wave and b-wave amplitudes and photopic negative response) and structural (i.e., thinning of the total retina, especially the inner retinal layers) retinal damage in the left eyes (i.e., ipsilateral to the carotid ligation). Treatment with the different doses of sildenafil led to a dose-dependent increase in the amplitudes of the ERG a- and b-waves and of the photopic negative response in HI animals, with higher doses associated with greater effect sizes. Similarly, a dose response was observed in terms of improvements in the retinal layer thicknesses.

CONCLUSIONS. Hypoxia-ischemia at term-equivalent age induced functional and structural damage mainly to the inner retina. Treatment with sildenafil provided a dose-dependent recovery of retinal function and structure.

Key words: hypoxia-ischemia, neonatal encephalopathy, newborn, retina, sildenafil

Neonatal encephalopathy associated with birth asphyxia is one of the most important causes of pediatric visual impairments in developed countries.1 Although visual impairments occurring in these newborns have been thought to be due primarily to brain injury, recent evidence suggests that retinal injury may also play a role.2–6 In a rat model of premature neonatal hypoxic-ischemic encephalopathy (HIE), the function and structure of the inner retina were found to be damaged, whereas those of the outer retina relatively were spared2; recently, similar results were demonstrated in a rat model of term neonatal encephalopathy.5 In both studies, no correlation was found between the degree of cerebral injury and retinal injury, which suggests that retinal injury may occur independently of cerebral injury as a result of neonatal hypoxia-ischemia (HI).2,5

Sildenafil is a vasodilator that acts by inhibiting the phosphodiesterase type-5 (PDE5) enzyme, which breaks down cyclic guanosine monophosphate (cGMP). Sildenafil has been used widely to treat erectile dysfunction in adults7 and pulmonary hypertension in both adults and newborns.8,9 More recently, the potential therapeutic role of sildenafil has been expanded beyond vasodilation, because an accumulating body of literature has demonstrated the neuroprotective and/or neurorestorative roles of sildenafil in animal models of neurologic diseases, such as ischemic stroke, multiple sclerosis, and Alzheimer’s disease.10–15

Surprisingly, neuroprotective and/or neurorestorative potential of sildenafil on retinal diseases have not been explored. Most available studies regarding the effect of sildenafil on the retina have investigated the risks of the side effects of sildenafil on retinal function or structure.16–25 Recent studies have suggested that sildenafil may have direct effects on inner retinal cells,17,20,27 because PDE5 is expressed in the inner nuclear layer and the ganglion cell layer in the human retina.28 PDE5 also is expressed in the choroidal and retinal blood vessels.28 Furthermore, sildenafil also inhibits, but with a reduced efficiency, the PDE6 that is expressed in the outer segment of photoreceptors and is involved in phototransduction16,19,21,29 and the survival of photoreceptors.30 With respect to newborns, a concern has been raised over the possible link between the use of sildenafil and the exacerbation of retinopathy of prematurity in a case report based on one newborn22; however, the described newborn also was ventilated and septic and presented with a severe problem of oxygenation before the use of sildenafil, so the causal link between sildenafil and retinopathy was not clear. A later study with larger number of newborns reported no adverse ocular findings in association with sildenafil.20 In fact, one study suggested that vaso-obliteration and neovascularization, which
are the hallmarks of retinopathy of prematurity, were decreased after sildenafil administration in a mouse model of retinopathy of prematurity.21

Thus, we hypothesized that sildenafil may be a therapeutic candidate to treat retinal injury induced by neonatal HI at term-equivalent age. In this study, we investigated the effects of neonatal HI on retinal function and structure and whether different doses of sildenafil could alleviate the retinal anomalies induced by neonatal HI in a rat model of term neonatal encephalopathy.

**MATERIALS AND METHODS**

**Animals**

All experiments were conducted in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research and were approved by the local animal care committee. Adult female Long-Evans rats with their male-only litters (Harlan Laboratories, Indianapolis, IN, USA) were received in our animal facility, housed under standard environment, and allowed food and water ad libitum. Rat pups remained with their mother until weaning at postnatal day 21 (P21).

**Induction of Term Neonatal HIE**

A well-established rat model of term neonatal HIE (Vannucci model),32–35 combining a left common carotid artery ligation and a 2-hour exposure to 8% oxygen, was used with 10-day-old rat pups as previously described,3 because this model mimics the patterns of brain injury observed in human term asphyxiated newborns32–35 and produces concomitant retinal injury.3 Rats undergoing both the ligation and hypoxia were considered the HI group. Sham-operated rats (identical procedure as the HI group, but without ligation and hypoxia) served as the control group.

**Sildenafil Administration**

HI and sham rat pups were weighed daily and then randomized to sildenafil (Viagra; Pfizer Canada, Inc., Kirkland, QC, Canada) or vehicle (Ora-Blend suspension media; Perrigo Company PLC, Minneapolis, MN, USA) twice daily by oral gavage, starting from 12 hours after HI for 7 consecutive days. Different doses of sildenafil (i.e., low [2 mg/kg], medium [10 mg/kg], and high [50 mg/kg]) were used in the HI and sham rat pups (n = 4–7 animals/group).

**Retinal Function**

At P29, full-field flash electrotoretinograms (ERGs; LKC Technologies, Inc., Gaithersburg, MD, USA) were recorded binocularly following a previously described protocol.3 The maximum mixed rod-cone a-wave amplitude36 was measured from the prestimulus baseline to the trough of the a-wave, and the maximum mixed rod-cone b-wave amplitude37,38 was measured from the trough of the a-wave to the peak of the b-wave. The photopic b-wave amplitude was measured from the baseline to the b-wave peak, and the photopic negative response (PhNR)39,40 was measured from the baseline to the most negative trough following the photopic b-wave. Measurements were performed using EM for Windows software (LKC Technologies, Inc.). When the peak of the b-wave could not be determined, the amplitude of the b-wave was measured at the time when the b-wave peaked in the control animals.

**Statistical Analysis**

The HI and sham rat pups were subdivided into the following groups: vehicle (0 mg/kg) or sildenafil 2 mg/kg, 10 mg/kg, or 50 mg/kg. Differences in the ERG amplitudes and retinal thicknesses between the different doses of sildenafil and the vehicle group were assessed with the respective effect sizes (nonstandardized difference of the means) and corresponding 95% confidence intervals (CIs).

**RESULTS**

**Sildenafil Improved the Retinal Function Outcome in the HI Rat Pups at P29**

Hypoxia-ischemia caused impairment in the retinal function of the left eye (i.e., ipsilateral to the carotid ligation) of the rat pups treated with vehicle alone. Hypoxia-ischemia induced an attenuation in the amplitude of the ERG mixed rod-cone b-wave, photopic b-wave, and PhNR, and to a lesser extent, of the mixed rod-cone a-wave, compared to the sham vehicle rat pups (Table 1; Fig. 1). Hypoxia-ischemia did not affect the ERGs recorded from the right eyes (i.e., contralateral to the carotid ligation).

The left eyes of the HI animals showed a dose-dependent improvement in all ERG parameters with treatment with different doses of sildenafil, where higher doses were associated with greater effect sizes (Table 1; Fig. 1). The 50-mg/kg dose of sildenafil induced significantly improved response in terms of all ERG parameters, whereas 10 mg/kg sildenafil induced significantly improved response in terms of the mixed rod-cone b-wave, the photopic b-wave, and the PhNR, but not the a-wave.

Interestingly, sildenafil had no significant effect on the ERG amplitudes of the right eyes of the HI rat pups (i.e., contralateral to the carotid ligation) and both eyes of the sham rat pups treated with the different doses of sildenafil.

**Sildenafil Improved the Retinal Structure Outcome in the HI Animals at P30**

Considering the ERG results, retinal histology was performed only on the left eyes of the sham vehicle group and the HI groups treated with the different doses of sildenafil.

Hypoxia-ischemia induced damage to the retinal structure in the left eyes (i.e., ipsilateral to the carotid ligation) of the rat pups treated with vehicle (Fig. 2). The total retinal thickness was reduced in the HI vehicle rat pups compared with the sham vehicle rat pups (Table 2; Fig. 2). Specifically, the HI vehicle rat pups showed a thinning of the inner retinal layers.
Sildenafil to Treat Neonatal HI Retina

Sildenafil to Treat Neonatal HI Retina

(i.e., inner nuclear layer [INL], inner plexiform layer [IPL], and retinal ganglion cell/fiber layer [RGC/FL]) and the outer plexiform layer (OPL), compared with the sham vehicle rat pups. In contrast, the thickness of the outer nuclear layer (ONL) was greater in the HI rat pups treated with the vehicle compared with the sham vehicle rat pups. No significant difference was found in the thickness of the retinal pigment epithelium (RPE), the outer segment (OS), and the inner segment (IS) between groups.

The thicknesses of the affected layers (total retina, ONL, OPL, INL, inner plexiform layer [IPL], and RGC/FL) in the HI animals showed a dose-dependent improvement with treatment with different doses of sildenafil. Again, higher doses were associated with greater effect sizes (Table 2; Fig. 2). The 10- and 50-mg/kg doses induced significantly improved response in terms of thicknesses of all the affected layers.

The pattern of retinal injury was not uniform along the superior–inferior axis of the retina (Fig. 3A). The spider graph revealed that the HI rat pups treated with vehicle had thinner INL, IPL, and RGC/FL, which spanned almost all retinal eccentricity, whereas the increase in ONL and the decrease in OPL thicknesses were detected mostly in the central retina (Fig. 3B). Treatment with the low dose of sildenafil did not seem to reverse any of the HI-induced changes in retinal thickness, except in the inferior retina, where the areas showing a thinning of the OPL were limited to a smaller portion of the central region compared with the HI rat pups treated with the vehicle. In contrast, the HI rat pups treated with the medium and high doses of sildenafil disclosed a nearly normal inner retina at almost all retinal eccentricities along the superior–inferior axis.

Discussion

Neonatal HI induced significant retinal damage, including a reduction in the ERG amplitudes and a thinning of the retina. As previously reported,^2,3^ HI affected mostly the inner retina, both functionally (i.e., attenuation of the ERG b-wave and PhNR) and structurally (i.e., destruction of the INL, IPL, and RGC/FL). The photoreceptor function (attenuation of the ERG a-wave) also was affected, but to a lesser extent. Treatment

FIGURE 1.  Flash electroretinograms of the sham and HI rat pups treated with different doses of sildenafil. Veh, vehicle; 2 mg/kg, sildenafil 2 mg/kg; 10 mg/kg, sildenafil 10 mg/kg; 50 mg/kg, sildenafil 50 mg/kg. (A, B) Representative scotopic (A) and photopic (B) ERG waveforms obtained from the left (i.e., ipsilateral to the carotid ligation) (solid line) and right (i.e., contralateral to the carotid ligation) (dashed line) eyes. Vertical arrows denote the stimulus onset. Double-headed arrows display how the amplitudes were measured. The units of horizontal and vertical scale bars are, respectively, in milliseconds and microvolts. (C–F) Amplitude measurements of the ERG waves. Mean ± SE. (C) Mixed rod-cone a-wave amplitude. (D) Mixed rod-cone b-wave amplitude. (E) Photopic b-wave amplitude. (F) Photopic negative response (PhNR) amplitude.

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with the different doses of sildenafil led to a dose-dependent improvement in the ERG amplitudes and in the retinal layer thicknesses, with higher doses associated with greater effect sizes. The 50 mg/kg dose of sildenafil induced significantly improved response in terms of all ERG parameters, whereas the 10 mg/kg of sildenafil induced significantly improved response in terms of the mixed rod-cone b-wave, the photopic b-wave, and the PhNR, but not the a-wave. The 10- and the 50-mg/kg doses induced significantly improved response in terms of all ERG parameters, whereas the 50 mg/kg dose of sildenafil induced significantly.

TABLE 2. Thicknesses of the Retinal Layers

<table>
<thead>
<tr>
<th>Retinal Layers</th>
<th>Sham–Veh</th>
<th>HI–Veh</th>
<th>HI–Sild 2 mg/kg</th>
<th>HI–Sild 10 mg/kg</th>
<th>HI–Sild 50 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total thickness, μm</td>
<td>244.14 ± 16.56</td>
<td>152.55 ± 10.48</td>
<td>167.28 ± 2.63</td>
<td>205.64 ± 14.20</td>
<td>205.80 ± 14.22</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>14.72 (–11.71, 41.16)</td>
<td>53.09 (9.24, 96.94)</td>
<td>53.25 (10.02, 96.47)</td>
<td>8.03 (–0.27, 0.27)</td>
<td></td>
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<tr>
<td>RPE thickness, μm</td>
<td>8.38 ± 0.32</td>
<td>8.62 ± 0.23</td>
<td>8.88 ± 0.24</td>
<td>7.89 ± 0.35</td>
<td>7.61 ± 0.27</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>0.25 (–0.51, 1.02)</td>
<td>–0.73 (–1.69, 0.24)</td>
<td>–0.59 (–1.43, 0.25)</td>
<td>27.87 ± 2.09</td>
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</tr>
<tr>
<td>OS thickness, μm</td>
<td>32.45 ± 4.70</td>
<td>37.74 ± 7.28</td>
<td>38.14 ± 3.66</td>
<td>29.18 ± 2.14</td>
<td>29.18 ± 2.14</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>0.40 (–19.53, 20.33)</td>
<td>–8.55 (–24.72, 7.62)</td>
<td>–9.86 (–28.39, 8.66)</td>
<td>14.16 ± 0.25</td>
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<tr>
<td>IS thickness, μm</td>
<td>16.50 ± 1.04</td>
<td>21.67 ± 3.61</td>
<td>23.83 ± 3.12</td>
<td>14.35 ± 1.15</td>
<td>14.35 ± 1.15</td>
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<tr>
<td>Effect size (95% CI)</td>
<td>2.16 (–9.52, 13.84)</td>
<td>–7.24 (–15.35, 0.88)</td>
<td>–7.50 (–16.37, 1.36)</td>
<td>8.59 ± 0.27</td>
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<tr>
<td>ONL thickness, μm</td>
<td>57.08 ± 4.02</td>
<td>70.17 ± 4.01</td>
<td>70.88 ± 2.88</td>
<td>50.95 ± 2.76</td>
<td>50.95 ± 2.76</td>
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<tr>
<td>Effect size (95% CI)</td>
<td>0.71 (–11.36, 12.79)</td>
<td>–19.22 (–27.79, 10.64)</td>
<td>–17.36 (–27.51, –7.21)</td>
<td>28.11 ± 1.06</td>
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<tr>
<td>OPL thickness, μm</td>
<td>10.55 ± 0.52</td>
<td>0.81 ± 0.81</td>
<td>9.48 ± 0.38</td>
<td>9.48 ± 0.38</td>
<td>9.48 ± 0.38</td>
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<tr>
<td>Effect size (95% CI)</td>
<td>4.91 (–2.81, 12.63)</td>
<td>8.67 (6.70, 10.63)</td>
<td>8.95 (6.11, 11.79)</td>
<td>9.76 ± 0.84</td>
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<tr>
<td>INL thickness, μm</td>
<td>37.03 ± 3.02</td>
<td>9.76 ± 0.83</td>
<td>14.71 ± 2.57</td>
<td>29.50 ± 1.70</td>
<td>31.42 ± 1.66</td>
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<tr>
<td>Effect size (95% CI)</td>
<td>4.94 (–1.68, 11.57)</td>
<td>19.74 (14.85, 24.62)</td>
<td>21.65 (17.11, 26.19)</td>
<td>34.25 ± 1.18</td>
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<tr>
<td>IPL thickness, μm</td>
<td>59.79 ± 3.08</td>
<td>2.85 ± 2.31</td>
<td>2.95 ± 2.60</td>
<td>44.93 ± 7.35</td>
<td>44.93 ± 7.35</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>0.11 (–8.40, 8.61)</td>
<td>42.08 (21.79, 62.37)</td>
<td>41.40 (11.85, 70.96)</td>
<td>17.50 ± 4.07</td>
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<tr>
<td>RGC/FL thickness, μm</td>
<td>22.37 ± 1.09</td>
<td>0.91 ± 0.91</td>
<td>2.28 ± 2.28</td>
<td>19.28 ± 3.36</td>
<td>16.59 (6.38, 26.80)</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>1.37 (–4.63, 7.37)</td>
<td>18.37 (9.16, 27.58)</td>
<td>16.59 (6.38, 26.80)</td>
<td>16.59 (6.38, 26.80)</td>
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</table>

Effect size: calculated compared with the HI vehicle rat pups.
Underlying the beneficial effects of sildenafil on retinal function, investigations are needed to determine the exact mechanisms of phototransduction. Selective inhibition of PDE6 has been shown to increase intracellular cGMP in the photoreceptors and lead to photoreceptor degeneration. Sildenafil can be used as a novel therapy to treat term asphyxiated newborns with retinal injury to significantly improve the potential future outcomes of these newborns.

In our study protocol, we chose to reproduce as much as possible what would be a feasible therapeutic approach for human term asphyxiated newborns. We administered sildenafil by oral route instead of the intraperitoneal or subcutaneous route described in most of the previous animal experiments, because oral sildenafil is the most commonly used route with human newborns. It is safe to assume that oral sildenafil reaches the retina, as it has been demonstrated to improve the potential future outcomes of these newborns.

The fact that sildenafil had a beneficial effect even when used route with human newborns. It is safe to assume that oral sildenafil reaches the retina, as it has been demonstrated to improve the potential future outcomes of these newborns.

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treatment window compared to neuroprotective strategies. Other neuroprotective therapies (such as N-methyl-d-aspartate (NMDA) blocker, antioxidants, and anti-inflammatory agents) were found effective only when given before or during the HI insult in adult rat models of ischemic retinopathy. Hypothermia treatment during HI in P0 rats prevented the development of retinopathy, however, the effect of delayed hypothermia treatment was not tested. With respect to the brain, hypothermia treatment started 12 hours after HI in a P7 Vannucci model offered no benefit to animals with moderate HI injury and was even deleterious to animals with severe HI injury. Our results strongly suggest that sildenafil holds promise for the treatment of retinal injury following HI at term-equivalent age.

**Figure 2.** Retinal structure in the left eyes of the sham vehicle rat pups and the HI rat pups treated with different doses of sildenafil. Veh, vehicle; 2 mg/kg, sildenafil 2 mg/kg; 10 mg/kg, sildenafil 10 mg/kg; 50 mg/kg, sildenafil 50 mg/kg. (A) Representative toluidine blue–stained retinal cross sections (magnification: 40x). Images were taken at 1000 μm inferior to the optic nerve head. (B) Thicknesses of the different retinal layers. Solid horizontal line represents the mean of the sham vehicle group; dashed horizontal lines represent the SEM of the sham vehicle group. Mean ± SE.
blue could not be used for immunohistochemistry, explaining why we only investigated the impact of retinal function and structure in the present study. Additional animal experiments for immunohistochemistry and Western blot are needed to further understand the mechanism involved with these beneficial effects. It will also be important to further test the potential sex difference in response to treatment, as the experiments described here were only performed in male rat pups. Further studies also need to assess whether the beneficial effects of sildenafil on the retina persist at long term as the rats mature.

In conclusion, HI at term-equivalent age induced functional and structural damages mainly in the inner retina in rats. Treatment with oral sildenafil provided a dose-dependent beneficial effect on the function and structure of the retina in a rat model of term neonatal encephalopathy. In addition, treatment with sildenafil had no adverse effect on normal retinal function. These results highlight the potential therapeutic role of sildenafil for retinal injury induced by neonatal HI at term-equivalent age. Neurorestorative mechanisms of sildenafil on the retina remain to be elucidated.

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