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

Suna Jung,1,2 Aaron Johnstone,1 Zehra Khoja,1 Emmanouil Rampakakis,3 Pierre Lachapelle,2 and Pia Wintermark1

1Division of Newborn Medicine, Department of Pediatrics, Montreal Children’s Hospital, Montreal, Québec, Canada
2Department of Ophthalmology/Neurology-Neurosurgery, Montreal Children’s Hospital Research Institute, McGill University, Montreal, Québec, Canada
3JSS Medical Research, St-Laurent, Québec, Canada

Correspondence: Pia Wintermark, Montreal Children’s Hospital, Division of Newborn Medicine, Research Institute of the McGill University Health Centre, 1001 Boul. Décarie, Site Glen Block E, EM0.3244, Montreal, QC H4A 3J1, Canada; pia.wintermark@bluemail.ch.

PL and PW contributed equally to the supervision of this work.

Submitted: February 18, 2016
Accepted: July 5, 2016

Citation: Jung S, Johnstone A, Khoja Z, Rampakakis E, Lachapelle P, Wintermark P. Sildenafil improves functional and structural outcome of retinal injury following term neonatal hypoxia-ischemia. Invest Ophthalmol Vis Sci. 2016;57:4306-4314. DOI:10.1167/iovs.16-19385

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 spared;2 recently, similar results were demonstrated in a rat model of term neonatal encephalopathy.3 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).2,3

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.26 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.30 In fact, one study suggested that vaso-obliteration and neovascularization, which
Sildenafil to Treat Neonatal HI Retina

are the hallmarks of retinopathy of prematurity, were decreased after sildenafil administration in a mouse model of retinopathy of prematurity. 3,4

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 newborns 32-35 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 electroretinograms (ERGs; LKC Technologies, Inc., Gaithersburg, MD, USA) were recorded binocularly following a previously described protocol. 3 The maximum mixed rod-cone a-wave amplitude 36 was measured from the prestimulus baseline to the trough of the a-wave, and the maximum mixed rod-cone b-wave amplitude 37,38 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.

Retinal Structure

At P30, the animals were euthanized, and the eyes were enucleated. Retinal histology was performed as per a previously described protocol. 3 Using AxioVision software (Version 4.8.2.0; Carl Zeiss Microscopy GmbH, Jena, Germany), the thicknesses of the different retinal layers were measured at approximately 1000 µm inferior from the optic nerve head, a region that showed the most prominent HI-induced damage. 3 For retinal reconstruction, retinal segments of 75 µm in width—taken every 340 µm along the entire length of the superior and inferior retinas—were assembled side by side (Adobe Photoshop, Adobe Systems, Inc., San Jose, CA, USA) to yield a pan-retinal view. Then, the retinal layer thickness was plotted against eccentricity to obtain the spider graphs (see Fig. 3B).

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.
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 eccentricities, 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, 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
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 thicknesses of all the affected layers. Our study is the first to explore the therapeutic role of sildenafil on retinal injury induced by neonatal HI at term-equivalent age.

The underlying mechanisms, however, remain to be elucidated and most probably involve multiple pathways. One possible mechanism is through a vascular effect. The endothelial cells and the smooth muscles of the retinal vasculature, the choroidal vasculature, and the ophthalmic artery express PDE5. Sildenafil has been shown to increase the diameter of some of these blood vessels and consequently increase the ocular blood flow in porcine eyes, as well as in healthy human subjects. Thus, in the short term, sildenafil may help restore the blood flow to the affected retina. In fact, Charriaut-Marlangue et al. demonstrated a blood flow increase in the common carotid artery contralateral to the ligated side after a single administration of intraperitoneal sildenafil immediately following an HI insult in a premature rat model of neonatal HIE. However, in our study, sildenafil was administered with a 12-hour delay and continued for 7 days; thus, a process other than acute vasodilation is likely involved. Another possible vascular effect of sildenafil is through the mediation of retinal angiogenesis. Fawzi et al. reported that sildenafil administration in a neonatal mouse model of oxygen-induced retinopathy prevented the hyperoxia-induced retinal vaso-obliteration through hypoxia-inducible factor 1-α (HIF-1α) stabilization, which in turn prevented the retinal neovascularization known to take place when the pups are returned to normoxia.

The beneficial effects of sildenafil may also arise from nonvascular mechanisms. Sildenafil has been shown to up-regulate neurotrophic factors in adult rodent models of

**Table 1. Electoretinogram Amplitudes**

<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, 14.16)</td>
<td>53.09 (9.24, 96.94)</td>
<td>53.25 (10.02, 96.47)</td>
<td></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>8.03 ± 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></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>27.87 ± 2.09</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></td>
<td></td>
</tr>
<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>14.43 ± 1.15</td>
<td>14.16 ± 0.25</td>
</tr>
<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></td>
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</tr>
<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 ± 0.76</td>
<td>52.81 ± 1.06</td>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>0.71 (–13.16, 12.79)</td>
<td>–19.22 (–27.79, –10.64)</td>
<td>–17.36 (–27.51, –7.21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPL thickness, μm</td>
<td>10.55 ± 0.52</td>
<td>0.81 ± 0.81</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>
</tr>
<tr>
<td>Effect size (95% CI)</td>
<td>5.72 ± 3.05</td>
<td>9.48 ± 0.38</td>
<td>9.76 ± 0.84</td>
<td></td>
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</tr>
<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>
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<td></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>44.25 ± 11.86</td>
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<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></td>
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</tr>
<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>17.50 ± 4.07</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>
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</tbody>
</table>

Effect size: calculated compared with the vehicle rat pups.
neurologic diseases, reduce neuroinflammation, and activate prosurvival signaling pathways, which have resulted in reduced neuronal loss and synaptic damage, and enhanced neurogenesis. In a premature rat model of brain injury, sildenafil has been shown to decrease neuronal apoptosis and microglial activation. Because neuronal apoptosis has been shown to peak at 24 hours after HI in the neonatal retina, the sildenafil administered 12 hours after HI may have limited apoptosis and further degeneration by its possible antiapoptotic and anti-inflammatory effects, which have been observed in the adult rat brain. Further investigations are needed to determine the exact mechanisms underlying the beneficial effects of sildenafil on retinal function and structure.

Interestingly, the outer segment of photoreceptors expresses PDE6, which is involved in photoreceptor survival and phototransduction. Selective inhibition of PDE6 has been shown to increase intracellular cGMP in the photoreceptors and lead to photoreceptor degeneration. Sildenafil also inhibits PDE6, but with a reduced efficiency. In our study, sildenafil did not induce photoreceptor degeneration. In fact, treatment with sildenafil restored the function of the photoreceptors, which had been impaired following HI at term-equivalent age. Of note, a decrease in a-wave amplitude in HI vehicle animals was accompanied by an increase in ONL thickness. This discrepancy between function and structure is perplexing; however, on a closer observation, the increase in ONL thickness appeared to be a result of cells taking up more space (increased space between rows of cells) in the now absent inner retina (resulting from the absence of the structural support that the inner retina normally provides to the outer retina), as the number of rows in ONL remained similar across the different groups (13–14 rows). The stacking of the cells also appeared more disorganized in the HI vehicle rats compared with the sham vehicle rats. Hence, a decrease in a-wave amplitude can be a sign of functional anomaly of the photoreceptors that may precede the degeneration of the cells. Although there was no obvious loss of photoreceptors at this time point, it is possible that there were already some ultrastructural changes that affected the ERG a-wave. Secondly, a decrease in the a-wave amplitude could have resulted partly from the loss of inner retina. There is evidence that the off-bipolar cells contribute to the ERG a-wave in Long-Evans rats. Our own data lend some support toward this possibility as the HI animals treated with the low-dose sildenafil showed a trend toward improved OPL and INL thicknesses in some parts of the retina, along with a trend toward improved ERG a-wave amplitudes.

Sildenafil is already safely used in newborns with persistent pulmonary hypertension. Studies of the neonatal population have shown no adverse ocular findings in association with sildenafil. Our results from the sham groups confirm that sildenafil does not affect normal retinal function, as is demonstrated by the absence of a difference in the ERG amplitudes between the sham rat pups treated with different doses of sildenafil and the sham vehicle rat pups. Most importantly, our results from the HI animals suggest that sildenafil limited or repaired the retinal injury resulting from neonatal HI, with a substantial improvement of retinal function and structure compared with the untreated rat pups. Further research with larger sample size and additional screening for side effects is therefore warranted to determine whether 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 transiently affect the ERG in healthy human subjects 1 hour after intake. Oral sildenafil displays an adequate bioavailability (23% in male rats and 38% in humans). Sildenafil is metabolized faster in male rats compared with humans, with the elimination half-life of, respectively, 0.4 and 3.7 hours after intake. Dosages in the present study were chosen so that they correspond somewhat to the equivalent recommended amount for human newborns ranging from low doses that are usually initially used to maximal described doses. In addition, we started the sildenafil administration 12 hours after the HI event, taking into consideration the delay that it would take to identify human newborns at risk of developing injury despite the hypothermia treatment. Finally, we repeated the treatment for 7 days so that it would potentially continue acting on the cascades of chemical reactions triggered following HI.

The fact that sildenafil had a beneficial effect even when started 12 hours after the HI insult highlights the probable neurorestorative role of sildenafil in the retina, with a wider...
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: 40×). 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.

Acknowledgments
The authors thank Wayne Ross Egers for his professional English correction of the manuscript and Laura Dale and Laurel Stephens for technical support.
Supported by studentship support from the Canadian Institutes of Health Research (CIHR) (Frederick Banting and Charles Best Canada Graduate Scholarship), the Research Institute of the McGill University Health Centre Foundation of Stars, and the McGill University Integrated Program in Neuroscience (all to SJ). This work was funded through an operating grant (MOP-133707) awarded by CIHR to PW and PL, as well as a CIHR grant (MOP 126082) to PL and a Clinical Research Scholar Career Award Junior 1 and 2 awarded by the Fonds de recherche en Santé Québec to PW. The authors alone are responsible for the content and writing of the paper.

Disclosure: S. Jung, None; A. Johnstone, None; Z. Khoja, None; E. Rampakakis, None; P. Lachapelle, None; P. Wintermark, None.

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