Ocular Effects of Sildenafil in Naïve Mice and a Mouse Model of Optic Nerve Crush

Alon Zahavi,[1,2] Shirel Weiss,[1,3] Mark Vieyra,[1] James D. Nicholson,[3] Orkun Muhsinoglu,[2] Orit Barinfeld,[1,3] David Zadok,[4,5] and Nitza Goldenberg-Cohen[1,3,6,7]

[1]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
[2]Department of Ophthalmology, Rabin Medical Center, Beilinson Hospital, Petach Tikva, Israel
[3]The Krieger Eye Research Laboratory, Felsenstein Medical Research Center, Petach Tikva, Israel
[4]Department of Ophthalmology, Shaare Zedek Medical Center, Jerusalem, Israel
[5]Faculty of Medicine, Hebrew University Medical Center, Jerusalem, Israel
[6]Department of Ophthalmology, Bnai Zion Medical Center, Haifa, Israel
[7]Ruth and Bruce Rappaport Faculty of Medicine, Technion - Israel Institute of Technology, Haifa, Israel

PURPOSE. To investigate the potential neuroprotective effect of sildenafil on the ocular circulation in mice with/without optic nerve crush (ONC).

METHODS. Male adult mice ($n = 63$) were treated with intravitreal (IVT) sildenafil 24 μg/3 μL, intraperitoneal (IP) sildenafil 24 μg/300 μL, or IP saline immediately before right ONC induction (ONC group). A second group ($n = 123$) received the same treatments without ONC induction (naïve group). Evaluations included fluorescein angiography (naïve group; day 0), molecular studies (days 1 and 3), and retinal and optic nerve histology (day 21).

RESULTS. Maximal retinal vessel dilatation and increased choroidal effusion were detected within 30 minutes of sildenafil injection. In the ONC group, moderate retinal ganglion cell (RGC) loss was noted at 21 days. However, molecular studies showed increased stress induced gene expression (IP superoxide dismutase [SOD]-1: 3.1-fold; heme oxygenase [HO]-1: 5.8-fold; IVT SOD-1: 1.47-fold), proapoptotic gene expression (IP BAX/B-cell lymphoma [BCL]-2 10.8-/2.3-fold), and glial gene expression (IP glial fibrillary acidic protein [GFAP]: 2.8- and myelin basic protein [MBP]: 2.5-fold). In the naïve group, IVT sildenafil was not associated with RGC loss or optic nerve stroke on histology, although in two samples, molecular parameters were compatible with stroke, showing increased gene expression of HO-1 (3.8-fold) and BCL-2 (2.5-fold). In the IP sildenafil subgroup, optic neuropathy was observed in 6/120 optic nerves, including 3 cyan fluorescence protein (CFP)-Thy-1 mice. Levels of antiapoptosis and anti-ischemia genes were decreased (<0.5-fold) except for three outliers.

CONCLUSIONS. Sildenafil affects retinal and choroidal perfusion in mice. When injected immediately before ONC, molecular parameters showed a preconditioning neuroprotective effect while histologic studies did not. In the absence of ONC, it is associated with neuropathy, possibly dose-dependent.

Keywords: optic neuropathy, mouse model, sildenafil, PDE-5 inhibitors, neuroprotection

Phosphodiesterase type 5 (PDE-5) inhibitors have been in widespread use for the safe and effective treatment of erectile dysfunction for over a decade.[1] Sildenafil (Viagra), vardenafil (Levitra), tadalafil (Cialis), and avanafil (Stendra) are currently approved by the Food and Drug Administration for this indication. Sildenafil is also used under another proprietary name (Revatio) for the treatment of pulmonary arterial hypertension.[2] Physiologically, penile erection is triggered by nitric oxide activation and increased levels of cyclic guanosine monophosphate (cGMP), which leads to arterial vasodilation and increased blood flow into the spongy tissue of the penis concomitant with smooth muscle relaxation. Sildenafil has a similar molecular structure to cGMP, and in patients with erectile dysfunction, it acts by binding competitively to cGMP-specific PDE-5, thereby inhibiting its degradation. This results in rising levels of cGMP and an increased duration of erections. The half-life of sildenafil is 3 to 5 hours.[3] Studies have shown that in the presence of low cGMP levels, activated PDE-5 has a higher sensitivity for sildenafil than nonactivated PDE-5.[4] Sildenafil also weakly inhibits PDE-6 with an efficacy of about one-tenth of that for PDE-5. PDE-6 is present in the photoreceptors of the retina and is an important component of the phototransduction cascade.[1]

PDE-5 is also involved also in the regulation of neuronal survival.[4,5] Accordingly, there is some experimental and clinical evidence that sildenafil, by enhancing angiogenesis and neurogenesis, may have a beneficial effect on several disorders[2] (e.g., stroke,[6–8] subarachnoid hemorrhage, dementia, various neurodegenerative disorders), and on learning.[9] It has also been found to favorably influence the nitric oxide-cGMP pathways that are involved in the pathogenesis of a number of neurologic diseases,[3,5,10,11] but this therapeutic effect is still under preclinical investigation.[7,12] Sildenafil is currently being used to treat neurologically related erectile dysfunction in patients

TABLE 1. Allocation of 186 Study Mice to ONC and Naïve Groups Treated by IVT or IP Sildenafil or Saline

Evaluations	ONC $n = 63$			No ONC (Naïve) $n = 123$		
	IVT Sildenafil, $n = 27$	IP Sildenafil, $n = 10$	IP Saline Control, $n = 26$	IVT Sildenafil, $n = 31$	IP Sildenafil, $n = 82$	IP Saline Control, $n = 10$
Histologic studies						
Day 21	17	–	16	10	60*	–
Molecular studies						
Day 1	5	5	5	6	6	3
Day 3	5	5	5	5	6	3
Fluorescein angiography						
Day 0	–	–	–	10	10	4

All interventions were done in the right eyes. The left eyes were used as a control.
* Including 20 CFP-THY-1 mice.

with multiple sclerosis, Parkinson disease, multisystem atrophy, and spinal cord injury.[13] Conversely, sildenafil has been implicated in a number of neurologic pathologies, such as intracerebral hemorrhage, migraine, seizures, and transient global amnesia.[14]

A relatively small number of patients using erectile dysfunction drugs have been reported to experience adverse visual events such as nonarteritic anterior ischemic optic neuropathy (NAION)[15-22] and retinal artery or vein occlusion.[14,17,23] However, it remains unclear if these events were coincidental or associated with an effect of the PDE-5 inhibitor on the ocular circulation.[16] Concerns were raised by the close temporal relationship of the ocular events to sildenafil intake and the recurrent nature of the ocular symptoms. Furthermore, although most of the reported patients had vascular risk factors for NAION overlapping with erectile dysfunction, not all did so, and in some cases, unilateral NAION developed within minutes to hours of ingestion of sildenafil.[17,18,24–27] There is still a paucity of convincing epidemiologic evidence linking NAION with the use of erectile dysfunction drugs.[28–31] However, a recent study found that in patients using these drugs, the odds ratio for acquiring NAION increased 2-fold.[32]

The aim of the present study was to investigate the neuroprotective versus the toxic effect of sildenafil using a mouse model of optic neuropathy and naïve mice. This is the first study to use an animal model to examine the association of PDE-5 inhibition with visual disturbances.

METHODS

Experimental Design and Animals

A total of 186 mice were included in the study. C57/Bl6 male wild-type mice aged 6 to 8 weeks ($n = 166$) were obtained from Harlan Laboratories (Jerusalem) and CFP-Thy-1 transgenic mice ($n = 20$) were raised in a self-colony. All mice were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the National Institutes of Health guidelines. The animal protocols for the study were approved by the local institutional animal research committee.

The mice were divided into two groups for analysis: optic nerve crush (ONC) to evaluate the potential neuroprotective effect of sildenafil versus naïve (no ONC) to evaluate the potential toxic effect of sildenafil (Table 1; Fig. 1). The ONC group consisted of 63 mice divided into three subgroups: intravitreal (IVT) injection of sildenafil 0.24 µg/3 µL ($n = 27$); intraperitoneal (IP) injection of sildenafil 24 µg/300 µL ($n = 10$); and IP injection of saline (control, $n = 26$). All injections

were administered to the right eye; the left eyes served as an internal control. Immediately before injection of sildenafil, ONC was induced in the right eye by the method described by Dratviman-Storobinsky et al.[33] The naïve group consisted of 123 mice divided into three subgroups: IVT injection of sildenafil 0.24 µg/3 µL ($n = 31$); IP injection of sildenafil 24 µg/300 µL ($n = 82$); and IP injection of saline (control, $n = 10$). ONC induction was not performed in the naïve group.

Dose Calculation

The initial clinical oral dose of sildenafil recommended for erectile dysfunction is 50 mg. In an 80-kg man, a dose of 50 to 100 mg oral sildenafil is equivalent to 0.625 to 1.25 mg/kg.[34] In patients with pulmonary hypertension, the recommended dose is 10 mg (12.5 mL) three times daily administered as an intravenous bolus injection.[35] In a study of the pharmacokinetics and metabolism of sildenafil, Walker et al.[36] calculated that the volume of distribution is 1.2 L/kg in men and 1.0L/kg in mice, with a respective half-life of 3.7 hours and 1.3 hours and oral bioavailability, 38% and 17%. These values were used to calculate the doses of sildenafil (Revatio; Pfizer, NYC, USA) for the present study.

Intervention Procedure

Animals were placed under general anesthesia by intramuscular injection of combined ketamine/xylazine (80 and 4 mg/kg, respectively) supplemented with topical proparacaine hydrochloride 0.5%. Sildenafil or saline was then administered either to the right vitreous (0.24 µg/3 µL) as a single injection using a Hamilton syringe with a 30-gauge tip (Hamilton, Reno, NV, USA) or into the peritoneum (24 µg/300 µL), according to the group allocation. In the ONC group, immediately before sildenafil/sham injection, forceps were inserted ~2.5 to 3.0 mm posterior to the right globe and used to crush the right optic nerve and surrounding muscle and fascia. The procedure was performed 3 times for 7 seconds each, separated by a 3-second interval.[33]

Fluorescein Angiography

Fluorescein angiography was performed in the naïve group after injection of IVT sildenafil ($n = 1 0$), IP sildenafil ($n = 10$), or saline ($n = 4$; Table 1). Digital photographs were taken during 30 minutes after IP injection of 0.04 mL of 25% sodium fluorescein (AK-Fluor 25% AMP; Akorn, Decatur, IL, USA) using a digital fundus camera (TRC ×50; Topcon, Farmingdale, NY, USA) and a customized plastic contact lens for mice.
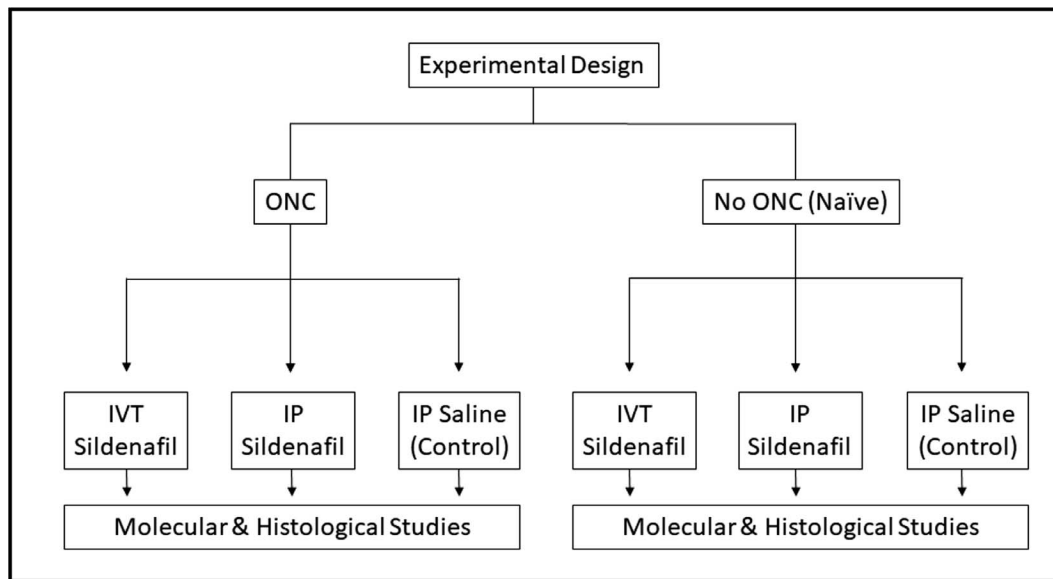
FIGURE 1. Experimental design. The mice were divided into two groups: the ONC group to evaluate the potential neuroprotective effect of sildenafil versus naïve (no ONC) group to evaluate the potential toxic effect of sildenafil. Each group was divided into three subgroups: IVT injection of sildenafil, IP injection of sildenafil, and IP injection of saline (control). All IVT injections were administered to the right eye while in those mice the left eyes served as an internal control.

Tissue Collection

Mice were euthanized by carbon dioxide asphyxiation at 1, 3, or 21 days after treatment. The eyes (globes and nerves) were enucleated for molecular and histological analysis (Table 1).

Molecular Analysis (Days 1 and 3)

In each group, retinas and optic nerves were dissected from both eyes on days 1 and 3 after the intervention (5–6 mice for each time point) and snap frozen in liquid nitrogen. Total RNA was isolated using a reagent (TRIzol; Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol and then reverse-transcribed into cDNA using random hexamers (Bioline, London, UK) and Moloney murine leukemia virus (M-MLV)-reverse transcriptase (Promega, Madison, WI, USA).

Two-stage real-time quantitative polymerase chain reaction (PCR; sequence detection system, Prism 7900; Applied Biosystems, Inc., Foster City, CA, USA) was applied to evaluate the mRNA expression levels of genes coding for proteins involved in apoptosis, ischemia, and oxidative stress: BAX, B-cell lymphoma (BCL-2), heme oxygenase 1 (HO-1), superoxide dismutase 1 (SOD-1), glial fibrillary acidic protein (GFAP), and myelin basic protein (MBP). Mouse β-actin was used to normalize the cDNA input levels. The primers are listed in Table 2. Reactions were performed in a 20-μL volume containing 4 μL cDNA, 0.5 μM each of forward and reverse primers, and buffer included in the master mix (SYBR Green I; Applied Biosystems, Inc.). Duplicate reactions were performed for each gene to minimize individual tube variability, and an average was taken for each time point. Threshold cycle efficiency corrections were calculated, and melting curves were obtained using cDNA for each individual gene PCR assay.

PCR cycling conditions consisted of an initial denaturation step of 95°C for 10 minutes followed by 40 cycles of 15 seconds of denaturation at 95°C and 1 minute of annealing and extension at 60°C. Standard curves were obtained using untreated mouse cDNA for each gene PCR assay. The results were quantified using a comparative threshold cycle (Ct) method, also known as the $2^{-\Delta\Delta Ct}$ method,[37] where: $\Delta\Delta Ct = \Delta Ct(sample) - \Delta Ct(reference\ gene)$.

Molecular analysis was performed on samples from 5 mice in the ONC subgroups on days 1 and 3 and similar numbers in the control subgroups (Table 1). In the IVT-sildenafil ONC group, findings on gene expression analysis were compared between the right (injected) and left (control) optic nerves. In the IP-sildenafil ONC group, the optic nerves of all 10 mice were analyzed and compared to the optic nerves from four IP saline-injected naïve mice.

TABLE 2. List of Primers for Molecular Studies of the Retina and Nerves in Mice Treated With Sildenafil

Gene	Forward	Reverse
BAX	CTGAGCTGACCTTGGAGC	GACTCCAGCCACAAAGATG
HO-1	CAGGTGTCCAGAGAAGGCTTT	TCTTCCAGGGCCGTGTAGAT
SOD-1	GCCCGGCGGATGAAGA	CGTCCTTTCAGCAGTCACA
BCL-2	GCATCGTGGCCTTTTTCTCC	CGACTGAAGAGTGAGCCCAG
GFAP	CCAGCTTCGAGCCAAGGA	GAAGCTCCGCCTGGTAGACA
MBP	TGATGGCATCACAGAAGAGAC	GCCCAGGACGGCTGCGGGCAT
ACTB*	TAGGCACCAGGGTGTGATGGT	CATGTCGTCCCAGTTGGTAACA

* Control.

Histologic Analysis

Hematoxylin and Eosin (Day 21). Enucleated eyes were fixed in 4% formaldehyde, placed overnight in 30% sucrose dissolved in phosphate-buffered saline (1X; Beit HaEmek, Israel) at 4°C, and embedded in optimum cutting temperature compound (Sakura Tissue-Tek Tokyo, Japan). Cryosections of the globes and optic nerve (6 μm) were mounted on slides and stained with hematoxylin and eosin for light microscope assessment, three consecutive sections on each slide.

Luxol Fast Blue Staining (Optic Nerves, Days 1 and 3). Staining for Luxol fast blue (LFB) was performed for optic nerve sections in the same manner as for hematoxylin and eosin. Five slides of each optic nerve taken from mice euthanized on days 1 and 3 after sildenafil injection were stained by combined LFB and neutral red for detection of myelin damage and cell loss. Slides were photographed in 10x magnification (Fluoview X; Olympus Corp., Tokyo, Japan).

LFB stains myelinated axons in a bright blue color. Therefore, areas with pale staining suggest myelin damage or loss, enabling a semiquantitative evaluation. The degree of myelin damage was classified as follows: 0, normal intensity of LFB stain; 1, decreased intensity of LFB stain compared with controls (mild damage); 2, decreased intensity of LFB stain and vacuole formation (moderate damage).

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Staining for triphenyltetrazolium chloride (TTC) was performed to evaluate infarct size in optic nerve samples using the same procedure as for hematoxylin and eosin. TTC is chemically reduced by most dehydrogenase enzymes, precipitating into a bright red water-insoluble compound. Therefore, ischemic tissue lacking dehydrogenase enzymes will not stain and contrast sharply with the surrounding viable tissue stained in red. The demarcation line seen between irreversibly damaged tissue and normal tissue with TTC staining is sharper than with other staining techniques used for cerebral ischemia. We applied a previously described technique with slight modifications.[38,39] The optic nerves were removed and embedded in TTC at 37°C for 10 minutes. Cryosections of the stained optic nerves were obtained and photographed at ×10 magnification (Olympus Corp.).

CFP-Thy-1 Mice (Day 21). The 20 transgenic adult CFP-Thy-1 adult mice expressing enhanced cyan fluorescent protein in RGCs under Thy1 promoter control [B6.Cg-Tg(Thy1-CFP)23Jrs/J 003710] were allocated to the naïve IP sildenafil group and euthanized at 21 days after injection by carbon dioxide asphyxiation (Table 1). The eyes were enucleated, including >4 mm of the optic nerve, and the globes were fixed for 2 days in 10% normal buffered formalin. The optic nerves were then dissected from the globes, embedded in gelatin, placed in 30% sucrose in PBS overnight, and either cryosectioned longitudinally into 40-μm thick sections or divided into two pieces, one of which was longitudinally sectioned and the other transversely sectioned to allow for both views of a single nerve. The optic nerve sections were mounted in aqueous mounting media (Fluor-glo, Valley Scientific, Mayville, NY, USA) and imaged by fluorescence and/or confocal microscopy (Zeiss LSM 510, Carl Zeiss Microscopes, Germany) using either a cyan fluorescence protein (CFP) or a green fluorescence protein (GFP) filter set.

Flatmount Retinas

Retinas were extracted from the enucleated globes of the CFP-Thy mice, flatmounted as previously described and imaged
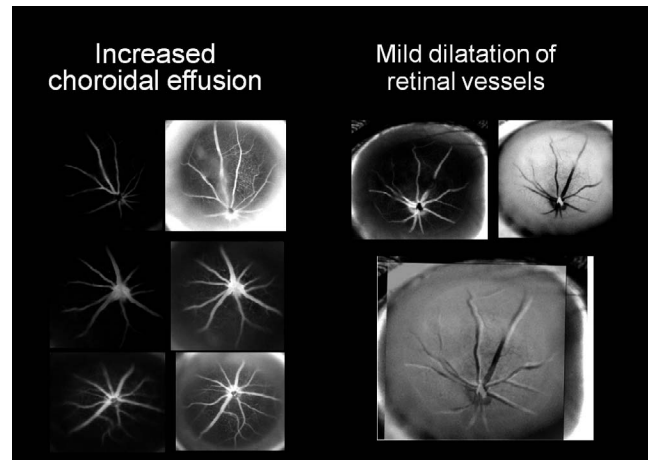

FIGURE 2. Vasodilatation of retinal vessels after IVT and IP sildenafil injection. Digital photograph showing increased choroidal effusion and mild dilatation of retinal vessels.

using a similar procedure as for the optic nerves. A microscopic analysis of RGC count was performed (Olympus Corp.).

Statistical Analysis

Differences between groups were analyzed using an unpaired Student's *t*-test and ANOVA. Significance was set at $P < 0.05$.

RESULTS

Effect of Sildenafil on Retinal Vessels

Maximal retinal vessel dilatation and increased choroidal effusion were detected by fluorescein angiography immediately after IVT injection of sildenafil, and 30 minutes after IP injection of sildenafil (Fig. 2). This effect was temporary and reverted to normal after 20 minutes.

Effect of IVT Sildenafil on RGCs Following ONC Induction

Histologic study at 21 days following ONC and IVT sildenafil injection showed moderate RGC loss.

Molecular analysis of the optic nerves at 1 and 3 days following ONC and IVT sildenafil injection showed an initial increased expression of BAX (1.32 ± 0.68-fold), but not as high as compared to the ONC control group injected with IP saline in which day 1 levels were statistically significantly higher (6.37 ± 0.78, $P < 0.01$). Both groups decreased toward baseline on day 3 (Table 3).

The initial day 1 increase in BCL-2 (2.51 ± 3.49-fold) was similar to the control group (1.94 ± 0.14), while the increased HO-1 (1.44 ± 1.42-fold) was not seen in the control group (0.8 ± 0.25). The day 3 decreased levels in both gene expression levels were statistically significantly different between the groups ($P = 0.002$ and $P = 0.01$, respectively).

Day 1 GFAP levels (1.27 ± 0.48-fold) decreased toward baseline by day 3. However, SOD-1 and MBP showed initial decreased expression (0.74- ± 0.52-fold, 0.91- ± 0.59-fold, respectively), and increase by day 3 (Table 3).

Specifically, of the 5 mice tested, BAX expression increased in one mouse on day 1 and in a second mouse on day 3; BCL-2 expression increased in 3 mice on day 1 and one mouse on day 3; HO-1 expression increased in 2 mice on day 1 and one

TABLE 3. Changes in Optic Nerve Levels of Apoptosis, Stress-Related and Myelin Gene Expression at 1 and 3 Days After ONC and Sildenafil Treatment

Gene	IVT Sildenafil (Mean ± SD)		IP Sildenafil (Mean ± SD)		IP Saline (Mean ± SD)	
	Day 1 (5 Mice, 10 Eyes)	Day 3 (5 Mice, 10 Eyes)	Day 1 (6 Mice, 10 Eyes)	Day 3 (5 Mice, 10 Eyes)	Day 1 (5 Mice, 10 Eyes)	Day 3 (5 Mice, 10 Eyes)
BAX	1.32 ± 0.68	0.86 ± 0.76	6.5	10.8	6.37 ± 0.78	0.58 ± 0.07
BCL-2	2.51 ± 3.49	1.13 ± 0.71	1.8	2.3	1.94 ± 0.14	0.29 ± 0.01
SOD-1	0.74 ± 0.52	1.47 ± 1.01	0.03	3.1	0.9 ± 0.23	0.87 ± 0.2
HO-1	1.44 ± 1.42	0.84 ± 0.42	2.42	5.8	0.8 ± 0.25	0.15 ± 0.01
GFAP	1.27 ± 0.48	0.92 ± 0.62	1.63	2.8	NA	NA
MBP	0.91 ± 0.59	1.29 ± 0.87	1.9	2.5	NA	NA

The comparison is between the right eye ONC nerve to the left eye healthy internal control.

mouse on day 3; and SOD-1 expression increased in 1 mouse (out of 4) on day 1 and 3 mice on day 3. The increase in BCl-2 on day 1 suggested an early protective effect of sildenafil (Table 3).

Effect of IP Sildenafil on RGCs Following ONC Induction

On days 1 and 3, molecular analysis of the optic nerves following IP injection revealed major increases in BAX expression (6.5-fold on day 1, 10.8-fold on day 3). Day 1 levels were similar to the control group, while day 3 levels significantly higher than the control ($P < 0.05$; Table 3). A decrease in SOD-1 expression on day 1 (0.03-fold) followed by an increase on day 3 (3.1-fold) was noted, while the control group levels remained stable throughout with a statistically significant difference on day 3 ($P = 0.01$). Increases in HO-1 expression (2.42-fold on day 1, 5.8-fold on day 3) were not seen in the control group ($P < 0.05$). Values of the other genes were as follows: BCL-2 expression increased 1.8-fold on day 1, 2.3-fold on day 3; GFAP, 1.63-fold on day 1, 2.8-fold on day 3; MBP, 1.9-fold on day 1, 2.5-fold on day 3 (Table 3).

Effect of IVT Sildenafil on RGCs Without ONC Induction

In the naïve mice injected with IVT sildenafil, no RGC loss was apparent on histologic studies and there was no optic nerve damage on day 21 ($nx = \times 10$). However, molecular studies had a wide variability, and revealed stroke matching molecular parameters in 2 nerves (out of 11). Suspected stroke was found in 1 sample on day 1, with elevated expression of HO-1, and in

a second sample on day 3, with increased expression of BCL-2, SOD-1, and MPB (Figs. 3A, 3B). No statistically significant differences existed when compared with the IP saline injected control group.

Effect of IP Sildenafil on RGCs Without ONC Induction

In the naïve mice injected with IP sildenafil, molecular studies ($n = 12$) revealed a decrease in genes relating to apoptosis and oxidative stress (<0.5-fold) on days 1 and 3 (Figs. 2D, 3C; Table 4). All were statistically significantly lower when compared to the IP saline-injected control group ($P < 0.05$; Table 4). Histology of the optic nerve on day 21 ($n = 60$) revealed RGC loss and associated optic nerve damage in 6 of the total 120 optic nerves evaluated (5%). Additionally, 3 of the 20 CFP THY-1 mice showed optic nerve damage accompanied with detectable RGC loss in the paired retina (Fig. 4).

CFP-Thy-1 Subgroup

Three of the 20 CFP-Thy-1 mice (total 40 optic nerves; 7.5%) showed axonal degeneration when viewed with fluorescence microscopy (Fig. 4). Longitudinal and cross-section studies showed axonal dropout and ubiquitous focal swelling with a beads-on-a-string appearance. Analysis of a single optic nerve slice of the thick (40 μm) sections by confocal microscopy supported these findings (see Fig. 4 for CFP alone and CFP with transmitted light). The corresponding retinal axons did not show pronounced swelling, but all had an uneven distribution of CFP. The damage distribution was not uniform throughout the retina, and some portions of the axons appeared to have a normal morphology. By contrast, control (saline-injected) naïve
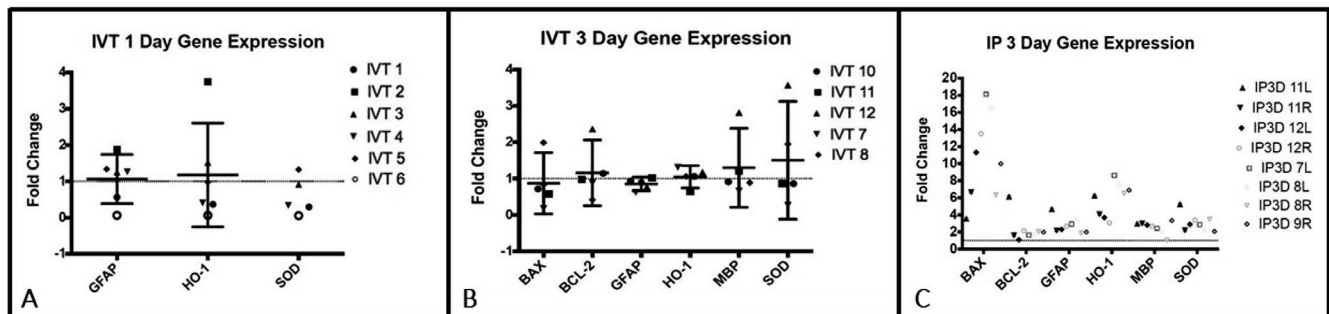
FIGURE 3. Molecular analysis of gene expression following IVT or IP injection of sildenafil without ONC induction, days 1 and 3. **(A)** IVT day 1. Note the variability in molecular studies of the IVT in the individual mice. Suspected stroke was found in mouse number IVT2 on day 1, with elevated expression of HO-1. **(B)** IVT day 3. Suspected stroke was found in mouse number IVT12 on day 3, with increased expression of BCL-2, SOD-1, and MPB. **(C)** IP day 3. Eight optic nerve samples of five different mice. All samples showed elevation in BAX and HO-1, and to a lesser extent in the other genes. All samples were compared to normal optic nerves without intervention.

TABLE 4. Changes in Optic Nerve Levels of Prosurvival Gene Expression at 1 and 3 Days After IP Sildenafil Treatment in Naïve Mice

| Gene | IP Sildenafil (Mean ± SD) | | IP Saline (Mean ± SD) | |
	Day 1 (6 Mice, 12 Optic Nerves)	Day 3 (6 Mice, 12 Optic Nerves)	Day 1 (3 Mice, 6 Eyes)	Day 3 (6 Mice, 6 Eyes)
BAX	0.027 ± 0.014	0.071 ± 0.050	1.01 ± 0.23	0.99 ± 0.01
HO-1	0.386 ± 0.119	0.499 ± 0.202	0.86 ± 0.06	0.84 ± 0.18
SOD-1	0.273 ± −0.065	0.523 ± 0.266	0.7 ± 0.12	0.87 ± 0.16
GFAP	0.149 ± −0.072	0.352 ± 0.048*	0.78 ± 0.02	1.02 ± 0.15
MBP	0.161 ± −0.042	0.367 ± 0.154	1.01 ± 0.13	0.99 ± 0.01

Note the reduction in expression of all genes to less than 0.5-fold.
* $n = 5$.

retinas were almost completely uniform without axonal swelling.

Retinas from the three optic nerves with sildenafil-induced axonal disruption were fluorescently imaged en face. Small but obvious patches of RGC loss were seen in all of them. RGC density was compared with six naïve control retinas using fluorescence microscopy and automated cell counting. One retina showed a significant reduction in average RGC density, whereas the other two showed no significant difference despite the small patches of axonal dropout.

DISCUSSION

An association of sildenafil with NAION has long been suspected, but a direct causative relationship is difficult to evaluate clinically owing to vascular risk factors and underlying disc-at-risk. In contrast, a neuroprotective property of sildenafil has been suggested in several clinical scenarios. The aim of this study was to test the toxic versus neuroprotective effect of sildenafil for the first time in a mouse model with and without ONC.

The study was not designed to determine the site of action of sildenafil-induced pathology, but given the known the ability of sildenafil to modify ophthalmic artery flux in a time-dependent manner,[38] we speculated that its administration in naïve mice may produce a transient ischemic event. Accordingly, we found that a single injection of sildenafil directly induced optic nerve stroke in relatively high numbers. Molecular studies using previously described markers[40] yielded an 18% rate of presumed stroke following IVT sildenafil injection and histologic analysis confirmed an at least 5% rate of optic nerve stroke following IP sildenafil injection. These findings are strengthened by the large IP sildenafil group of 82 mice (164 optic nerves) of which 60 (120 optic nerves) were evaluated 21 days after injection.

An association between sildenafil and optic neuropathy was seen in paired retinas and nerves in 3 of the 20 CFP-ThY-1 mice in the IP sildenafil naïve group (7.5%). This rate is too high to correspond to the apparent clinical sildenafil-associated NAION seen clinically. The discrepancy might be explained by sildenafil-induced changes in optic nerve autoregulation that are not well tolerated by patients at risk, including decreased optic nerve head perfusion secondary to systemic arterial hypotension and alterations in nitric-oxide-mediated blood flow parameters. The mechanism resembles the pharmacologically induced nocturnal blood pressure reduction in hypertensive patients which is thought to contribute to the early morning acute NAION phenomenon.[41] Several studies have reported a different sensitivity of retinal vessels to sildenafil in healthy individuals and patients at risk.[42,43] The concentration-dependent influence of sildenafil on reactive oxygen species production[44] might have exacerbated the observed optic nerve damage.[44] Our findings are in line with a few case reports in which NAION was temporally associated with sildenafil intake.[45,46]

In the study group, ONC was induced before sildenafil injection to simulate an ischemic insult to the optic nerve head. Based on the reported clinical effect of sildenafil on vascular flow, mainly in patients at risk,[38,40] we hypothesized that by improving reperfusion or causing changes in autoregulation, sildenafil might protect the nerve from damage induced by optic nerve crush injury. Although the histological results did not show a protective effect, the molecular studies demonstrated a statistically significant elevation in the expression of anti-apoptotic and pro-survival genes (BLC-2, SOD-1, and HO-1). This is in line with improved blood flow in other systemic conditions, such as Raynaud phenomenon.[47] A cause for the mild neuroprotective effect of sildenafil may be supported by our fluorescein angiography findings showing dilatation of the retinal vessels in the naïve group (Fig. 2). PDE-5 is known to be present in the retinal vessels[48] and PDE-5 inhibitors have been used to measure dilatation in porcine eyes.[49] The dilatation of the retinal arterioles is thought to be mediated via ERK signaling, leading to activation of nitric oxide synthase and nitric oxide production with subsequent guanylyl cyclase activation and K(ATP) channel opening. Moreover, owing to the elevation in cGMP due to endogenous or exogenous nitric oxide, sildenafil may inhibit the PDE-5 pathway independently of ERK signaling, with a vasodilatory effect.[49]

Studies of the effect of sildenafil on the ocular circulation in healthy adults reported varying results.[42,43,50–52] Patients with erectile dysfunction, however, were found to have a significant increase in peak systolic velocity, end diastolic velocity, and mean velocity in the ophthalmic and short posterior ciliary arteries 1 hour after intake of 100 mg sildenafil compared to placebo,[53,54] with no changes in central retinal artery velocities. The optic nerve head vasculature, which is largely dependent on the posterior ciliary arteries, is highly sensitive to minor changes and may therefore suffer an ischemic insult under such circumstances. The increased flow velocity in the ophthalmic artery was apparently attributable to the vasodilator effect of sildenafil,[55] as placebo caused no significant change in optic nerve rim or foveolar choroidal blood flow.[54] This suggests that nitrate compounds and sildenafil may differentially affect the ocular circulation. Furthermore, sildenafil had no significant effect on intraocular pressure, systemic blood pressure, or ocular perfusion pressure[50] despite a concomitant significant increase in pulsatile ocular blood flow velocity,[42] suggesting that sildenafil affects the choroidal circulation and has a lesser effect on the retinal vasculature.[4,42]

Accordingly, in a previous study of male rats, chronic use of sildenafil caused dilatation and congestion of the choroidal vasculature,[56] and in our model, increased choroidal flow was observed in the sildenafil-treated mice. A histopathologic study
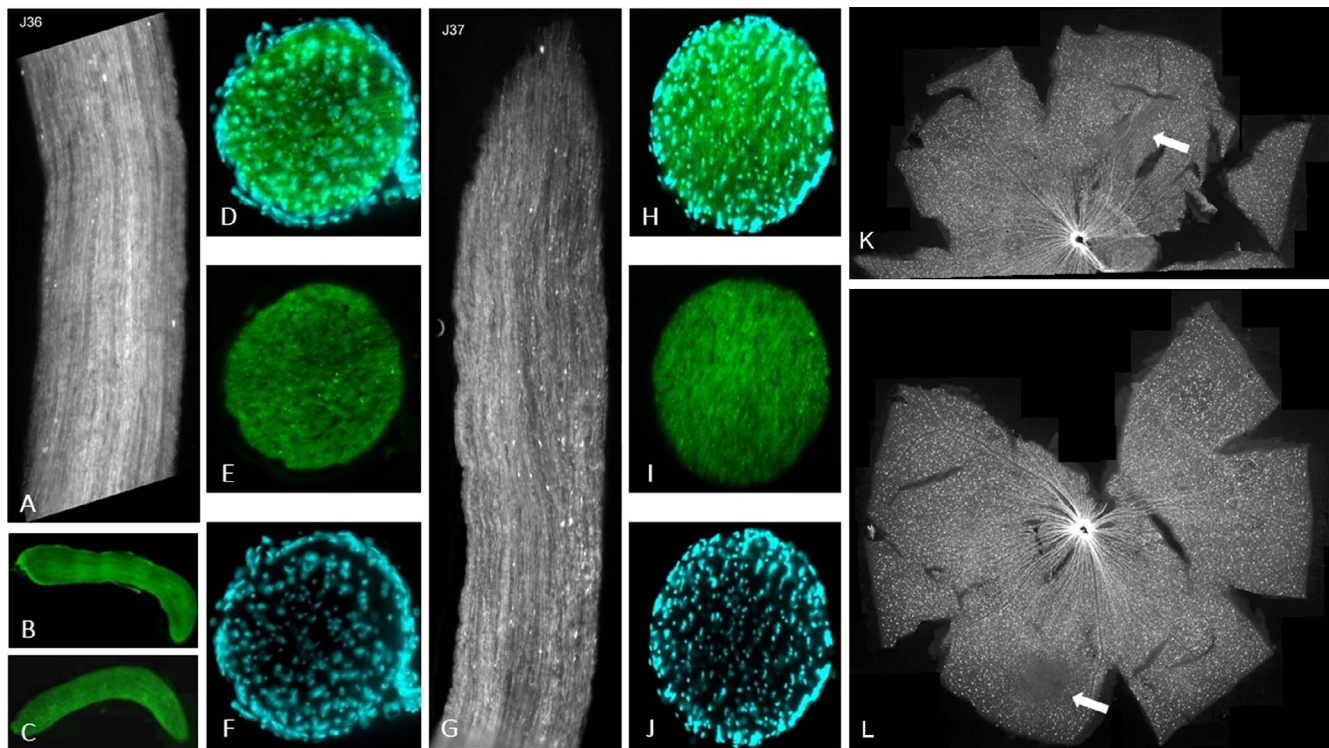
FIGURE 4. Longitudinal and cross-sections of the optic nerves showing axonal degeneration with corresponding loss of RGCs in the paired retina after IP injection of sildenafil without ONC induction. (**A**, **G**) Longitudinal section of CFP-THY1 optic nerves following IP injection of sildenafil. No damage in (**A**) and stroke affected optic nerve following IP sildenafil in (**G**). Note the fluorescence dropout in axons and focal swelling with a beads-on-a-string appearance in the damaged nerve (**G**). (**B**, **C**) Same nerves under fluorescence microscopy showing the normal (**B**) and reduced fluorescence due to stroke following IP sildenafil (**C**). Cross sectioned optic nerves of CFP-THY1 mice presenting with stroke following IP sildenafil and control, respectively. (**D**, **H**) Merging of the nuclei staining (DAPI, **F**, **J**) and CFP fluorescence (**E**, **I**). (**K**, **L**) The corresponding flatmount retinae showing florescence RGCs in CFP-THY1IP injected sildenafil. Note stroke area with patchy dropout and diffuse RGCs loss (*white arrows*).

reported that male rats chronically treated with sildenafil showed greater dilatation of the choroidal capillaries than controls.[56] There was no change in the retinal layers or their configuration in either group. However, the authors failed to investigate possible optic nerve damage resulting from sildenafil treatment. Additional studies have investigated other possible neuroprotective effects of sildenafil, such as modulating inflammatory mediators.[57]

Sildenafil was recently shown to affect human sperm mitochondrial function, producing complex concentration-dependent effects.[44] In view of the multiple focal axonal swellings on the otherwise intact axons observed here, it is possible that a decrease in ATP levels and an increase in mitochondria-generated reactive oxygen species may lead to optic neuropathy.[58] In healthy subjects, sildenafil appears to produce primarily long-term axonal damage with occasional loss of RGCs.

Our study has several limitations. A relatively high sildenafil dose was administered compared to standard human dosing (10-fold). This might have affected both the stroke-inducing effect of sildenafil seen in the naïve group and its protective effect seen in the ONC group. However, it should be noted that sildenafil has a shorter half-life in mice than in humans.[36]

After IVT sildenafil injection to the ONC groups, neuroprotection was not demonstrated histologically but only assumed from the gene expression levels. Molecular studies are not as conclusive as histological studies. Even if a neuroprotective effect is proven in mice, it will be difficult to recruit patients with acute NAION for a clinical trial owing to the rarity of the condition.

A higher-than-expected rate of suspected optic nerve stroke was observed. We continuously increased the size of the cohort, but the rate did not change. This finding is in line with a recent publication by Campbell et al.[32]

The mechanisms underlying the stroke-inducing effect of sildenafil in healthy mice and its neuroprotective effect in ONC mice are unclear. Several possibilities were suggested, but further studies are required to elucidate the exact pathophysiological process.

We did not measure retinal or optic nerve blood flow. Vessel dilation was interpreted from fluorescein angiography, and blood flow changes were expected as a result.

To conclude, this is the first mouse model study of the possible association of PDE-5 inhibitors and optic nerve stroke versus neuroprotective properties. Our findings suggest that sildenafil may induce ischemic retinal changes in naïve animals and may have some neuroprotective benefit in the presence of poststroke ocular damage. This conclusion is supported by molecular changes in antiapoptotic genes, although we could not show histologic evidence of the preservation of RGCs after ONC. Further studies of the mechanism underlying the action of sildenafil are needed in other neurologic conditions.

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