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Local and Systemic Toxicity of Intravitreal Melphalan for Vitreous Seeding in Retinoblastoma

A Preclinical and Clinical Study

Jasmine H. Francis, MD,[1,*] Paula Schaiquevich, PhD,[2,3,*] Emiliano Buitrago, BSc,[2] María José Del Sole, PhD,[4] Gustavo Zapata, DVM,[5] J. Oscar Croxatto, MD, PhD,[6] Brian P. Marr, MD,[1] Scott E. Brodie, MD,[7] Alejandro Berra, PhD,[5] Guillermo L. Chantada, MD, PhD,[2,‡] David H. Abramson, MD[1,‡]

Purpose: Intravitreal melphalan is emerging as an effective treatment for refractory vitreous seeds in retinoblastoma, but there is limited understanding regarding its toxicity. This study evaluates the retinal and systemic toxicity of intravitreal melphalan in retinoblastoma patients, with preclinical validation in a rabbit model.

Design: Clinical and preclinical, prospective, cohort study.

Participants: In the clinical study, 16 patient eyes received 107 intravitreal injections of 30 μg melphalan given weekly, a median of 6.5 times (range, 5–8). In the animal study, 12 New Zealand/Dutch Belt pigmented rabbits were given 3 weekly injections of 15 μg of intravitreal melphalan or vehicle to the right eye.

Methods: Electroretinogram (ERG) responses were recorded in both humans and rabbits. For the clinical study, ERG responses were recorded at baseline, immediately before each injection, and at each follow-up visit; 82 of these studies were deemed evaluable. Median follow-up time was 5.2 months (range, 1–11). Complete blood counts (CBCs) were obtained on the day of injection at 46 patient visits. In the animal study, ERG responses were obtained along with fluorescein angiography, CBCs, and melphalan plasma concentration. After humane killing, the histopathology of the eyes was evaluated.

Main Outcome Measures: For the clinical study, we measured peak-to-peak ERG amplitudes in response to 30-Hz photopic flicker stimulation with comparisons between ERG studies before and after intravitreal melphalan. For the animal study, we collected ERG parameters before and after intravitreal melphalan injections with histopathologic findings.

Results: By linear regression analysis, over the course of weekly intravitreal injections in retinoblastoma patients, for every additional injection, the ERG amplitude decreased by approximately 5.8 μV. The ERG remained stable once the treatment course was completed. In retinoblastoma patients, there were no grade 3 or 4 hematologic events. One week after the second injection in rabbits, the a- and b-wave amplitude declined significantly in the melphalan treated eyes compared with vehicle-treated eyes ($P<0.05$). Histopathology revealed severely atrophic retina.

Conclusion: Weekly injections of 30 μg of melphalan can result in a decreased ERG response, which is indicative of retinal toxicity. These findings are confirmed at an equivalent dose in rabbit eyes by ERG measurements and by histopathologic evidence of severe retinal damage. Systemic toxicity with intravitreal melphalan at these doses in humans or rabbits was not detected. *Ophthalmology 2014;■:1–8 © 2014 by the American Academy of Ophthalmology.*

Intra-arterial chemosurgery has emerged as one of the frontline treatments for some retinoblastoma patients. Following work initiated by Kaneko et al,[1] we pioneered and began using this technique in 2006.[2] Despite great success and ocular survival rates surpassing other treatment modalities, vitreous seeding (spherical portions of tumor that float in the vitreous cavity) remains the primary reason for treatment failure and loss of the eye. However, intravitreal drug delivery provides a targeted approach that delivers the highest concentration of drug to the surrounding fluid that bathes vitreous seeds; it conceivably provides the best means of their treatment.

Munier et al[3] and other investigators[4–6] have recently reported on their experience with intravitreal melphalan and confirmed the efficacy of this treatment, with a 2-year Kaplan-Meier ocular survival estimate of >80%. Despite recent adoption of this technique by many physicians, there is limited knowledge of the local and systemic toxicity of intravitreal melphalan in humans. Now that treatment advancements in retinoblastoma show improved patient and ocular survival, the importance of saving vision places a greater emphasis on retinal toxicity. In addition to providing systemic toxicity data after providing 30 μg of intravitreal melphalan to retinoblastoma patients, this report describes

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the retinal toxicity as measured by electroretinogram (ERG). These findings are further validated in rabbit eyes, along with histopathologic assessment.

Methods

Melphalan (Alkeran; GlaxoSmithKline, Bretford, UK) was reconstituted with the commercial sterile diluent supplied by the manufacturer. Once reconstituted (50 mg of melphalan in 10 ml of diluent), serial dilution of melphalan with sterile saline was performed. In clinical studies, the final concentration was 417 μg/ml, and this was filtered through a 0.22-μ filter. Thus, 0.07 ml of the final solution of melphalan was injected to deliver 30 μg per eye. In animals, the final concentration was 150 μg/ml, so that 0.1 ml of the solution yielded 15 μg/ml (using a rabbit vitreous volume of 1.7 ml results in a human equivalent of 30 μg). To study the potential toxic effects of the commercial diluent in rabbits, melphalan's vehicle was diluted with sterile saline to the same dilution as the reconstituted melphalan. The clinical study was performed in New York and the preclinical animal study was conducted in Argentina.

Clinical Study

This institutional review board–approved study included all eyes that received 30 μg of intravitreal melphalan at Memorial Sloan-Kettering between September 2012 and September 2013. Informed consent was obtained for each patient from their guardian, caregiver, or parent. The study was compliant with the Health Insurance Portability and Accountability Act. Research adhered to the tenets of the Declaration of Helsinki. After administering inhaled sevoflurane, intraocular pressure was measured with a Tono-Pen (Reichert, Inc., Buffalo, NY) and if $\geq$10 mmHg was lowered to $<$10 mmHg with digital massage and confirmed by repeat Tono-Pen measurement. Intravitreal melphalan (30 μg) was injected 3.5 mm posterior to the limbus with a 33-gauge needle at a previously uninjected meridian to ensure that the injection sites were separated by at least one-quarter clock-hour to prevent thinning/weakening of the ocular wall from repetitive injections. The position of the needle within the eye was titrated to the burden of disease, proximity of tumor, and associated risk of seed tethering to injection site (publication in press): The needle shaft was inserted part way into the eye in instances of diffuse, dense disease, where there was concern for close tumor proximity, and inserted fully into the center of the eye for scant disease located far from the injection site. After needle withdrawal, the injection site was sealed and sterilized with cryotherapy and the eye was shaken in all directions during cryo-application, as previously described by Munier et al.[3] The ocular surface was submerged in irrigating sterile water for 3 minutes. Because of a fear that overmanipulation of the globe after injection may increase the risk of efflux, the intraocular pressure was not measured after injection.

Ocular Toxicity. Electroretinogram recordings were obtained during regularly scheduled examination under anesthesia, according to an International Society for Clinical Electrophysiology of Vision standard protocol that had been modified to limit anesthesia time, as previously described.[7] Reported herein are the response amplitudes to 30-Hz photopic flicker stimulation, which are representative of the full protocol.[8] In brief, ERGs were obtained using a hand-held ganzfeld stimulator (Espion ColorBurst; Diagnosys, LLC, Lowell, MA) and ERG-jet contact lens electrode. Light-adapted 3.0 single-flash and 30-Hz flicker responses were obtained singly and then averaged in groups of 10, with the averaged waveforms used for analysis.

The response to 30-Hz flicker stimulation was used to represent the complete set of ERG responses because photopic and scotopic responses were highly correlated, and the 30-Hz stimulus enabled signal detection in severely impaired retinas.[8] A change in 30-Hz response amplitude of 25 μV was considered clinically meaningful, based on statistical analysis of ERGs during examination of normal eyes under anesthesia (unpublished data). In the absence of scotopic data, photopic responses to single International Society for Clinical Electrophysiology of Vision standard light-adapted 3.0 flashes were also analyzed. The b-wave-to-a-wave ratio was compared before and after completing the injection course in an effort to localize the effect of ocular manipulation to the inner or the outer retina. Electroretinogram responses were measured at baseline, immediately before each injection, and at each follow-up visit. There were a total of 25 injections deemed to have non-evaluable ERG recordings: 3 patients (totaling 19 injections) in whom the ERG was undetectable at baseline and thereafter, and an additional 6 injections that did not have associated ERG testing (owing to the absence of an electrophysiologist). Thus, 82 injections had evaluable ERG measurements and were included in the analysis.

Systemic Toxicity. From November 7, 2012, to May 1, 2013, a total of 46 blood samples were collected from 11 patients for complete blood count (CBC) analysis. There were 16 instances where the intravitreal melphalan injection and CBC were performed within 21 days of prior ophthalmic artery chemosurgery (OAC), and 30 instances that were performed beyond this 21-day window.

Data Analysis. Associations were evaluated between number of 30-μg melphalan injections and (1) the weekly change in 30-Hz flicker response and (2) the weekly change in absolute neutrophil counts. Further associations were evaluated between months after completing the injection course and change in 30-Hz flicker response. Finally, associations were evaluated between patient/treatment variables (age and weight of patient at first injection, concomitant OAC, extent of salt-and-pepper retinopathy, and degree of eye pigment defined by iris color [blue, light brown, brown]) and the change from baseline in 30-Hz flicker response amplitude at the most recent follow-up. Salt-and-pepper retinopathy was defined as the retinal pigment epithelium changes that can be observed by indirect ophthalmoscopy, akin to the salt-and-pepper retinopathy described by Munier et al.[3] Patient 1 was excluded from age and weight comparisons because her variables (213 months and 67 kg, respectively) were outliers to the rest of the cohort and are not representative of a typical patient treated with intravitreal melphalan. Extent of salt-and-pepper retinopathy was measured by number of clock-hours of involved retina/retinal pigment epithelium (RPE; Fig 1). Differences in weekly 30-Hz flicker responses after intravitreal injections with concomitant OAC (defined as occurring within 2 days of each other) and injections without concomitant OAC were compared. Statistical analysis was performed with linear regression analysis, 2-tailed Student *t*-test, and analysis of variance using GraphPad software (www.graphpad.com, accessed February 10, 2014) and NCSS software (www.ncss.com; accessed February 10, 2014).

Animal Study

Institutional review board approval was granted from the Animal Care and Use Committee of the Faculty of Medicine, University of Buenos Aires, Argentina. We included 12 New Zealand/Dutch Belt pigmented rabbits, weighing between 1.8 and 2.2 kg. All experiments adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. The animals were fed standard laboratory food, given free access to water, and housed under 12-hour light–dark

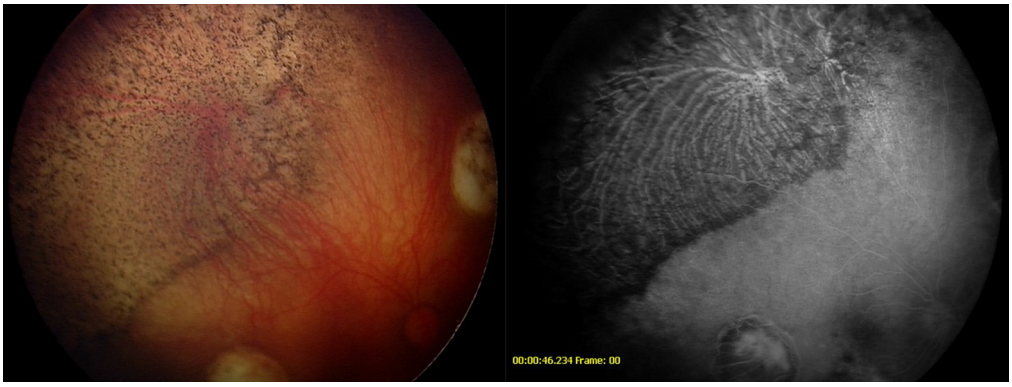

Figure 1. Representative case (patient 4) of salt-and-pepper retinopathy after intravitreal melphalan. **Left**, Color fundus photograph of right eye, Reese-Ellsworth Group VB (IC D), demonstrating salt-and-pepper retinopathy after 5 weekly injections of 30 μg of melphalan. Note the speckled retinal pigment epithelial (RPE) changes extending from 9 to 1 o'clock. **Right**, Fluorescein angiography at 46 seconds of the same quadrant demonstrating speckled RPE, an apparent window defect due to RPE atrophy, and prominent hyperfluorescence of the vortex vein with intact choroid in the region of the salt-and-pepper retinopathy.

cycles. All eyes underwent mydriasis and topical anesthesia. Animals were assigned to 3 groups (n = 4 in each group). Animals in groups A and B received an intravitreal injection of 15 μg of melphalan into the right eye using a 31-G 5/16″ gauge needle (BD insulin syringe, catalog no. 328440). The needle was inserted 2 mm posterior to the limbus and directed toward the center of the globe until the position was visualized. Group A (acute toxicity) was humanely killed 1 week after the last intravitreal injection; group B (subacute toxicity) underwent ERG assessment at 1 month after the last dose and then were humanely killed. Last, the right eye of animals in group C (vehicle control group) received 0.1 ml of the vehicle solution diluted in sterile saline only. The left eye of each animal served as double control because no treatment was performed. The same procedure was performed every week for a total of 3 administrations in each eye. Anterior chamber paracentesis was not performed. At experiment completion, the rabbits were humanely killed by intravenous injection of pentobarbital sodium and their eyes enucleated immediately.

Systemic and Ocular Toxicity. All animals were examined weekly, including weight control, hair loss, and general condition. Complete blood counts were determined by an automated flow cytometer as previously described (Coulter Counter VCS; Beckman Coulter, Brea, CA). A small blood sample (50 μl) was treated with 55 μl of cold acid methanol to precipitate the proteins and stabilize melphalan. Methanolic supernatant extracts were stored at −20°C pending melphalan analysis by high-performance liquid chromatography coupled with fluorometric detection.[9]

Indirect ophthalmoscopy was performed at baseline (before melphalan injection on day 1) and before each melphalan or vehicle administration in all eyes. Intraocular pressure was measured with a tonometer (Tono-Pen, AVIA, Vet) before every intravitreal administration and in control eyes. We undertook ERG

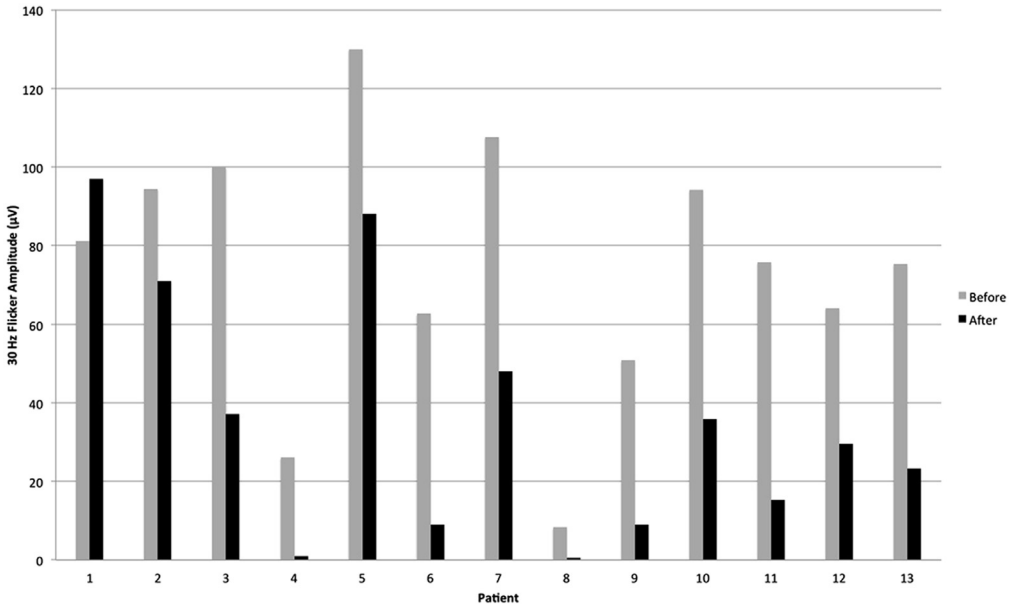

Figure 2. Bar graph depicting the 30-Hz flicker amplitude for each patient before and after (at most recent follow-up) melphalan injections. Note how the electroretinogram response is reduced after the injection course.

measurements in both eyes of each anesthetized animal (ketamine hydrochloride, 37.5 mg/kg intramuscular and xylazine 5 mg/kg intramuscular) at baseline, 3 hours after the first injection, before the third dose, and before humane killing. The rabbits adapted to the dark for 20 minutes. The ERG was performed as previously described,[10] and ERG parameters (a- and b-wave amplitude and implicit time) were recorded. Fundus photography and fluorescein angiography were done 2 weeks after the last intravitreal injection. After enucleation, each eye was fixed in 4% paraformaldehyde in 0.1 mol/L of phosphate buffer (pH 7.4) and processed for routine histopathology.

Data Analysis. Individual animal weight, hematologic values, and ERG data (a- and b-waves and implicit times) were obtained for all animals. Two-way repeated measures analysis of variance was used to test for differences between the animal groups (treatment vs. vehicle) with time as the second dependent variable. In all cases, significance was set at 0.05.

Results

Clinical Study

We included 107 injections in 16 eyes (all Reese-Ellsworth Group VB and International Classification D) in this study. The median follow-up was 5.2 months (range, 1−11 months), median age was 43 months (range, 13−213 months), median weight was 16 kg (range, 8−67 kg), and median number of weekly injections administered was 6.5 injections (range, 5−8 injections).

Retinal Toxicity. Linear regression analysis revealed that during the course of weekly injections, for every 30-μg melphalan injection, the 30-Hz flicker response decreased by 5.8 μV ($P = 0.0001$; Fig 2). The mean response reduction over the injection course (from initial to final injection) was calculated as 34 μV ($P = 0.00$). After the injection course was complete, the 30-Hz flicker response remained constant ($P = 0.6$). Age ($P = 0.3$), weight ($P = 0.5$), previous systemic chemotherapy ($P = 0.4$), previous external beam radiation ($P = 0.2$), and concomitant OAC ($P = 0.4$) were not associated with changes in weekly ERG responses over the injection course. Extent of salt-and-pepper retinopathy and degree of eye pigmentation were significantly associated with increased reduction of ERG response from baseline to last follow-up ($P = 0.005$ and $P = 0.04$, respectively). Patient and treatment variables (including prior and concomitant treatment) and their respective change in ERG responses and complications/outcome are given in Table 1. The b-wave-to-a-wave ratios for responses before and after injection course were unchanged ($P = 0.43$).

Systemic Toxicity. Neither the group with CBC analysis and intravitreal injection within 21 days of prior OAC nor the group beyond 21 days of prior OAC had any grade 3 or 4 neutropenia. By linear regression analysis, an increasing number of intravitreal melphalan injections does not seem to significantly effect changes in the absolute neutrophil count ($P = 0.07$)

Animal Study

Ocular and Systemic Toxicity. No significant changes in body weight or hematologic values were detected in animal groups A, B, or C. Furthermore, in these animals, the plasma melphalan concentration was almost nondetectable (<7 ng/ml) or below the limit of quantitation. In all eyes, the cornea, lens, vitreous, and anterior chamber remained clear without evidence of inflammation. However, fundus changes were observed 1 week after the second dose and were clearly attributable to melphalan toxicity because no change was observed in fellow eyes or in vehicle-treated eyes. Figure 3 depicts a representative animal with the melphalan-treated (right) eye and the fellow control (left) eye 1 week after the third dose. A clear decrease in the number and quality of vessels was observed in melphalan-treated eyes and was further confirmed by fluorescein angiography. The intraocular pressures remained in the normotensive range for all eyes.

There were no changes in the ERG parameters among the control (left) eyes of groups A, B, and C and there was no difference between control and vehicle-treated (group C) eyes ($P>0.05$). One week after the second injection, the a- and b-wave amplitude declined significantly in the melphalan-treated eyes (groups A and B) compared with vehicle-treated eyes (group C;

Table 1. Characteristics of Patients, Eyes, Treatment, Electroretinogram Responses, and Outcome

Patient	Age (mos)	Prior Tx*	Concomitant Tx	No. of Injections	Iris Pigment	RPE Changes Distribution†	Change in ERG Response (%)	Complications/Outcome
1	213	c, ebr	OAC × 1 (T0.5,C50)	8	Blue	None	19.5	
2	92	c, sc	OAC × 1 (M6,T2,C60)	8	Light brown	NA	NA	
3	15	c, l, sc	OAC × 1 (T2 C60)	6	Light brown	10 to 2	−39.3	
4	16			5	Blue	9 to 1	−69.5	
5	21		OAC × 1 (M4, C50)	6	Brown	Diffuse	−96.2	Intraret heme
6	63	sc, ebr, l		6	Brown	NA	NA	Recurrence → enucleated
7	29	sc, i	OAC × 1 (M2,C30)	6	Light brown	11 to 1	−32.2	
8	40	sc, ebr	OAC × 1 (M6,T2,C60)	8	Brown	Diffuse	−85.8	
9	54	l		8	Blue	None	−29.5	
10	45	sc	OAC × 1 (M7.5,T1,C60)	7	Light brown	NA	−94.0	Phthisis
11	45	l	OAC × 1 (M4,T2,C50)	7	Brown	NA	−82.5	
12	17	sc		8	Brown	NA	−79.1	Vitreous heme
13	14	sc	OAC × 1 (M4,T2,C60)	8	Light brown	9 to 2	−82.7	
14	92	sc, l, c		6	Brown	8 to 12	−68.0	
15	33	p, l		5	Light brown	11 to 3	−52.1	
16	65	sc, c, l	OAC × 1 (M7.5,T2,C50)	5	Light brown	11 to 3	NA	

c = cryo; Diffuse = all clock hours; ebr = external beam radiation; ERG = electroretinogram; heme = hemorrhage; l = laser; NA = not able to evaluate; p = plaque brachytherapy; RPE = retinal pigment epithelium; sc = systemic chemotherapy; Tx = treatment.
*Treatment before institution of injections besides ophthalmic artery chemosurgery (OAC), which all eyes received.
†Distribution measured by clock-hours.

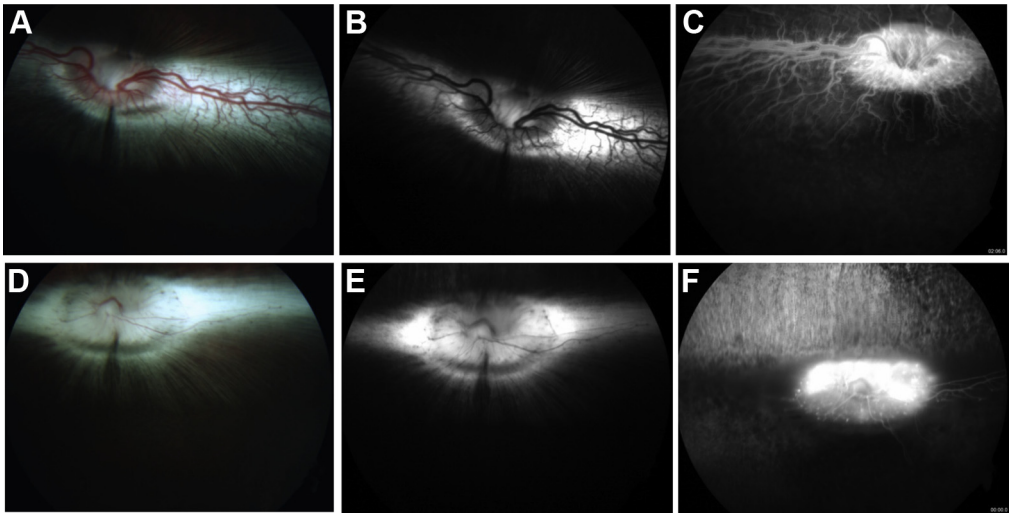

Figure 3. Representative rabbit eye depicting fundoscopic appearance at 2 weeks after the third intravitreal injection of melphalan. Compared with the normal untreated eye (**A**, **B**, **C**), the color fundus photograph (**D**) and red-free image (**E**) of the treated eye demonstrate sclerotic vessels and retinal whitening. The fluorescein angiograph of the treated eye (**F**) reveals impaired filling of the sclerotic vessels.

$P<0.05$; Fig 4). No recovery in a- or b-wave amplitude could be recorded at 1 week (group A, acute toxicity) or 1 month (group B, subacute toxicity) after the last intravitreal injection ($P<0.05$; Fig 4).

Data Analysis. In the treated eyes (groups A and B), light microscopy revealed histologic evidence of retinal, vascular, and optic nerve damage, as demonstrated in the representative micrographs (Fig 5). The control (fellow, left) eyes and vehicle-treated eyes (group C) seemed to be histologically normal.

Discussion

Our knowledge of retinal toxicity associated with intravitreal melphalan is established from limited clinical reports and 2 preclinical animal models. Ghassemi et al[11] demonstrated that 50-μg injections in human eyes can result in vitreous and subretinal hemorrhage, hypotonia, and phthsis. Salt-and-pepper retinopathy occurred in 43% of human eyes in the cohort studied by Munier et al[3], although it is unclear whether this was more predominant with the 20- or 30-μg dose. In rabbit eyes, Ueda et al[12] reported that the approximate human equivalent dose of 23 μg induced no ERG changes, but double this dose did produce a blunted response. Although of limited applicability owing to the use of melphalan-containing infusion during pars plana vitrectomy, Shimoda et al[13] suggest that an approximate human-equivalent dose as low as 35 μg can cause ERG degradation. From this work, it

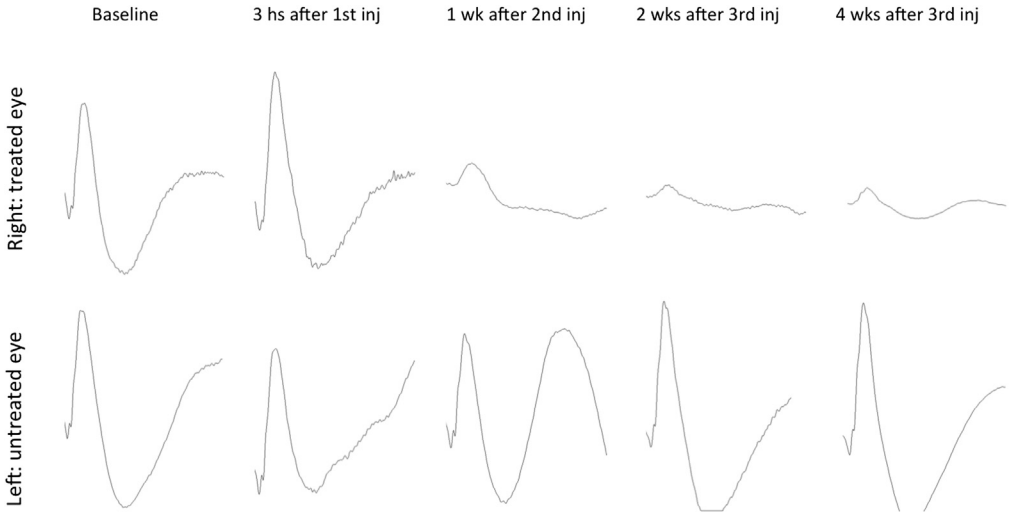

Figure 4. Representative case of electroretinogram response after intravitreal melphalan injection in rabbit eye. Responses in the treated right eye (top row) decrease by 1 week after the second intravitreal injection, continue to decrease after the third injection, and then remain stable. Conversely, responses in the untreated left eye (bottom row) remain normal. Hs = hours; inj = injury.

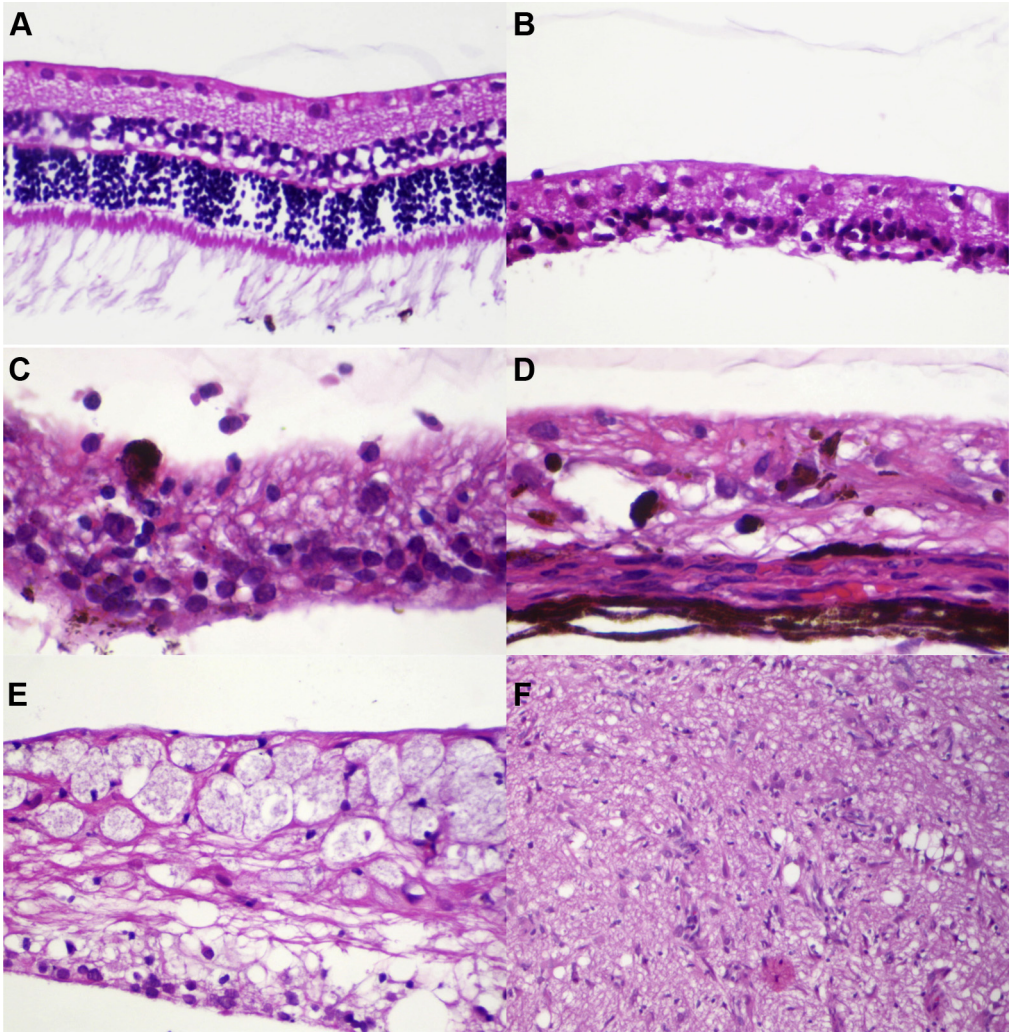

Figure 5. Hematoxylin and eosin–stained representative rabbit eyes depicting histopathologic appearance 1 month after the third intravitreal injection of melphalan. Compared with the normal untreated retina (**A**; original magnification, ×100), the treated eye demonstrates a severely atrophic retina with loss of photoreceptors and outer nuclear layers (**B**; original magnification, ×100), vitreous cells and pigment migration from the retinal pigment epithelium (**C**; original magnification, ×200), diffuse retinochoroidal adherence devoid of retinal pigment epithelium (**D**; original magnification, ×200), myelinated nerve fibers with histocytic-like cells containing granular material (**E**; original magnification, ×200), and treated optic nerve depicting disorganized arrangement of nerve fibers and glial cells with scant blood vessels (**F**; original magnification, ×100).

can be postulated that in humans, the toxic threshold for intravitreal melphalan lies between 23 and 35 μg.

This study has the advantage of treating all patients, irrespective of age, weight, or response, with 30 μg of intravitreal melphalan, thereby providing an understanding of the toxicity at this defined dose. By linear regression analysis, we demonstrate that for every weekly 30-μg injection of melphalan, the ERG response decreases by about 6 μV (with mean degradation of 35 μV throughout the average treatment course of 6.5 injections), but after completing the treatment course the ERG remains stable, neither worsening nor improving. In this small cohort, these changes were unrelated to concomitant OAC, age, or weight at initial injection. The toxicity from 30 μg intravitreal melphalan seems to impact retinal function in the immediate time course (within 1 week of injection), and although apparently permanent, the effect is not progressive once the treatment course is complete.

We validated these findings in a preclinical model using rabbit eyes. Estimating the equivalent rabbit dose as approximately 15 μg ([30 μg melphalan/3.5 ml of pediatric human vitreous volume] × 1.7 ml rabbit vitreous volume = 15 μg), we found reduction in ERG response amplitudes after 2 and 3 injections. Normal ERG and histopathology in the vehicle-treated eyes suggest that the observed toxicity was not due to elevated intraocular pressure from increased intraocular volume but implicates melphalan as the toxic agent. Intravitreal injections of melphalan have been used extensively in Japan[12] where the cultural belief obliges salvage of an eye at any cost, including retinal toxicity and loss of vision. Although intravitreal melphalan may effectively save eyes that were previously refractory to

treatment, the temptation to use this method must be balanced with its toxicity profile and the high probability of it rendering the retina less functional.

As in our animal study, previous work in rabbit eyes suggests both the a- and b-wave are affected by intravitreal melphalan.[12,13] Similarly, our attempt to localize the effects of intravitreal melphalan within the human retina was unsuccessful. In particular, there was no reduction in the b-to-a-wave ratio, which would have signaled selective impairment of inner retinal function. However, this methodology cannot distinguish between selective dysfunction of the outer retina and diffuse retinal injury and suggests either scenario could be implicated. Based on rabbit histopathology, the injury is diffuse and involves all layers of the retina (Fig 5). Furthermore, histopathologically the retinal damage is not limited to vascular toxicity and ischemic necrosis but is also attributable to a direct toxic effect from melphalan. Areas devoid of RPE with pigment migration histopathologically demonstrate the salt-and-pepper retinopathy correlate in rabbit eyes. Interestingly, as suggested by the clinical fluorescein angiography results (Fig 4), the choroid seems to be spared and demonstrates no damage.

In humans, an attempt was made to correlate the extent of salt-and-pepper retinopathy with changes in ERG response from baseline to most recent follow-up. Of 16 eyes, 6 were deemed nonevaluable because of baseline ERG being nondetectable or previous treatment having already resulted in diffuse RPE changes (making subsequent progression difficult to recognize). By univariate regression analysis, increased salt-and-pepper retinopathy was significantly associated with more ERG reduction from baseline to most recent follow-up ($P = 0.005$). There was also a suggestion that more darkly pigmented eyes (established by iris pigmentation) suffered greater toxicity ($P = 0.04$). However, both these conclusions are drawn from a small number of eyes and warrant confirmation with a higher-volume study. We may speculate why eyes with more pigment are associated with greater retinal toxicity. After OAC in pigs, we have previously demonstrated a higher concentration of melphalan in the RPE-choroid compared with the retina and suggested that melphalan may be preferentially taken up by pigmented tissues.[14] Therefore, more deeply pigmented eyes may absorb increased levels of melphalan and experience the consequences of more RPE and, by extension, retinal and choroidal toxicity (with perhaps more enhanced efficacy). Accordingly, we chose a pigmented animal model for our evaluations, and this may also account for the high toxicity we found.

Because the extent of salt-and-pepper retinopathy was correlated with further ERG degradation, we can extrapolate techniques to limit salt-and-pepper retinopathy and possibly preserve more retinal function. However, these techniques may come at an expense. We separate our injection sites by at least one-quarter clock-hour to prevent ocular wall weakening/thinning from repetitive injections and cryotherapy. Conversely, it is conceivable that injecting in the same location may only expose a limited area of retina to melphalan and thereby place a smaller portion at risk for retinopathy and ERG changes. Furthermore, inserting the full shaft of the needle into the eye may position the melphalan further from the retinal surface, thereby reducing the likelihood of salt-and-pepper retinopathy. However, in many cases this is not possible because of the heavy burden of disease, proximity of tumor to the injection site, and concerns for extraocular extension. As proposed by Munier et al[3] and performed herein, careful movement of the globe after the injection may allow for an even distribution of drug throughout the vitreous cavity and reduce the risks of pooled melphalan exposure to the retina. Finally, it is conceivable that a higher injected volume of drug may result in more optimal drug diffusion across the vitreous humor; however, a higher injected volume would likely necessitate paracentesis as an antireflux safety technique.[15] We prefer to inject a smaller volume (0.07 ml is approximately one half of previously reported volumes) to obviate the need for paracentesis, which creates an additional breach in the ocular surface and generates another outlet for potential disease reflux. As a final thought, 1 patient's ERG response diminished by 30 μV despite there being no evidence of salt-and-pepper retinopathy; therefore, although a technique that limits salt-and-pepper retinopathy may help to preserve ERG function,[16] in many cases it may not be the full explanation for retinal function preservation.

With melphalan delivered via OAC, approximately one tenth of patients experience significant neutropenia (grade 3 or 4). We have previously demonstrated that intra-arterial doses >0.4 mg/kg increase this risk of myelosupression.[17] With the 30 μg of intravitreal melphalan used in this study, the weight-adjusted doses ranged from 0.0004 to 0.002 mg/kg, thereby conferring a low risk for myelosupression.[14] As predicted, in the 46 instances that were studied, there was no grade 3 or 4 neutropenia. This was true of both groups (the group that received CBC analysis and injection within 21 days of prior OAC and the group that had OAC beyond this 21-day window), suggesting concomitant OAC does not increase the risk of myelosuppression associated with intravitreal injection of 30 μg of melphalan. This was further confirmed in the animal study, which revealed clinically insignificant plasma concentrations of melphalan and no change in the CBC values. Further analysis shows that increasing the number of intravitreal melphalan injections does not impact significantly changes in the absolute neutrophil count. Therefore, intravitreal delivery of melphalan has a more acceptable systemic toxicity profile than OAC, and systemic toxicity would not be a limiting factor to eyes conceivably receiving 30 μg of intravitreal melphalan in the context of concomitant OAC melphalan exceeding 0.4 mg/kg.

In conclusion, 30 μg of intravitreal melphalan has a satisfactory systemic toxicity profile. However, in human eyes 30 μg of intravitreal melphalan causes abrupt, permanent retinal dysfunction that seems to be nonprogressive once the treatment course is complete. An equivalent dose in rabbit eyes results in severely damaged retina on histopathology with a retinal response undetectable by ERG. Future studies are needed to determine the influence of injection technique on retinal damage or the impact of retinal toxicity on central visual acuity or function. Although intravitreal injections of melphalan may salvage eyes from enucleation, this may come at the price of compromised

retinal function. Our findings suggest caution in the use of intravitreal melphalan at doses of ≥30 μg, particularly in cases with vitreous seeds, which retain significant visual potential.

References

1. Kaneko A, Suzuki S. Eye-preservation treatment of retinoblastoma with vitreous seeding. Jpn J Clin Oncol 2003;33:601–7.
2. Abramson DH, Dunkel IJ, Brodie SE, et al. A phase I/II study of direct intraarterial (ophthalmic artery) chemotherapy with melphalan for intraocular retinoblastoma: initial results. Ophthalmology 2008;115:1398–404.
3. Munier FL, Gaillard MC, Balmer A, et al. Intravitreal chemotherapy for vitreous disease in retinoblastoma revisited: from prohibition to conditional indications. Br J Ophthalmol 2012;96:1078–83.
4. Ghassemi F, Shields CL. Intravitreal melphalan for refractory or recurrent vitreous seeding from retinoblastoma. Arch Ophthalmol 2012;130:1268–71.
5. Sun YB, Hui P, Punyara K, et al. Intravitreal injection of melphalan in the treatment of retinoblastoma with vitreous cavity seeding. Chin Med J (Engl) 2013;126:1587.
6. Smith SJ, Smith BD. Evaluating the risk of extraocular tumour spread following intravitreal injection therapy for retinoblastoma: a systematic review. Br J Ophthalmol 2013;97:1231–6.
7. Brodie SE, Paulus YM, Patel M, et al. ERG monitoring of retinal function during systemic chemotherapy for retinoblastoma. Br J Ophthalmol 2012;96:877–80.
8. Liu CY, Jonna G, Francis JH, et al. Non-selectivity of ERG reductions in eyes treated for retinoblastoma. Doc Ophthalmol 2014;128:13–23.
9. Schaiquevich P, Ceciliano A, Millan N, et al. Intra-arterial chemotherapy is more effective than sequential periocular and intravenous chemotherapy as salvage treatment for relapsed retinoblastoma. Pediatr Blood Cancer 2013;60:766–70.
10. Buitrago E, Del Sole MJ, Torbidoni A, et al. Ocular and systemic toxicity of intravitreal topotecan in rabbits for potential treatment of retinoblastoma. Exp Eye Res 2013;108:103–9.
11. Ghassemi F, Ghadimi H, Amoli FA, et al. Diffuse infiltrating retinoblastoma coexisting with ocular toxoplasmosis. Int Ophthalmol 2014;34:137–40.
12. Ueda M, Tanabe J, Inomata M, et al. Study on conservative treatment of retinoblastoma—effect of intravitreal injection of melphalan on the rabbit retina [in Japanese]. Nippon Ganka Gakkai Zasshi 1995;99:1230–5.
13. Shimoda Y, Hamano R, Ishihara K, et al. Effects of intraocular irrigation with melphalan on rabbit retinas during vitrectomy. Graefes Arch Clin Exp Ophthalmol 2008;246:501–8.
14. Schaiquevich P, Buitrago E, Taich P, et al. Pharmacokinetic analysis of melphalan after superselective ophthalmic artery infusion in preclinical models and retinoblastoma patients. Invest Ophthalmol Vis Sci 2012;53:4205–12.
15. Munier FL, Soliman S, Moulin AP, et al. Profiling safety of intravitreal injections for retinoblastoma using an anti-reflux procedure and sterilisation of the needle track. Br J Ophthalmol 2012;96:1084–7.
16. Brodie SE, Munier FL, Francis JH, et al. Persistence of retinal function after intravitreal melphalan injection for retinoblastoma. Doc Ophthalmol 2013;126:79–84.
17. Gobin YP, Dunkel IJ, Marr BP, et al. Intra-arterial chemotherapy for the management of retinoblastoma: four-year experience. Arch Ophthalmol 2011;129:732–7.

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[1] Ophthalmic Oncology Service, Memorial Sloan-Kettering Cancer Center, New York, New York.
[2] Unidad de Farmacocinética Clínica, Hospital de Pediatría JP Garrahan, Buenos Aires, Argentina.
[3] CONICET.
[4] Laboratorio de Farmacologia, CIVETAN-CONICET, Facultad de Veterinaria, Universidad Nacional del Centro de Buenos Aires, Tandil, Argentina.
[5] Laboratorio de Investigaciones Oculares, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina.
[6] Fundación Oftalmológica Argentina Jorge Malbrán, Buenos Aires, Argentina.
[7] Ophthalmology, Mount Sinai School of Medicine, New York, New York.
[8] Servicio de Hematología-Oncología, Hospital de Pediatría JP Garrahan, Buenos Aires, Argentina.

*Both authors (Dr. Francis and Dr. Schaiquevich) contributed equally as first authors.
‡Both authors (Dr. Chantada and Dr. Abramson) contributed equally as last authors.

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Abbreviations and Acronyms:
ERG = electroretinogram; **RPE** = retinal pigment epithelium.

Correspondence:
Jasmine H. Francis, MD, 70 East 66th Street, New York, NY 10065. E-mail: francij1@mskcc.org.