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Retinal Toxicity of Intravitreal Melphalan in Albino Rabbits

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Abstract

Background: Intravitreal melphalan injections have been used for treating retinoblastoma with vitreous seeds. The aim of this study was to evaluate the safety margins for intravitreal melphalan, using clinical observations, electrophysiological testing and morphological observations.

Methods: In this experimental study, 18 albino rabbits, were treated with intravitreal injection of 0.1 ml melphalan solution to the right, experimental eye, and were divided into 4 dose groups: 5 µg (N=4); 15 µg (N=4); 30 µg (N=5); 60 µg (N=5). The left, control eye, of each rabbit was injected with 0.1 ml saline. Clinical examination, electroretinography (ERG) and visual evoked potentials (VEP) were conducted at baseline and periodically throughout the 4-week follow-up. The eyes were then enucleated and the retinas were prepared for histology and glial fibrillary acidic protein (GFAP) immunocytochemistry.

Results: No clinical, ERG, or histologic damage were found in rabbits treated with 5 µg melphalan. However, expression of glial fibrillary acidic protein was detected in retinal Müller cells of the experimental eyes and not in the control eyes. With all other doses of melphalan, dose-dependent funduscopic changes, ERG amplitude, histological damage and GFAP expression were found. VEP responses were similar between the experimental eyes and control eyes of all rabbits regardless of melphalan dose injected into the eye, indicating no change in retinal output.

Conclusions: These findings indicate that intravitreal melphalan dose of 5 µg in rabbits, approximately equivalent to 10 µg in human, appears to be safe, but induce a mild stress to the retina. However, higher doses are toxic, and their utilization should be executed with caution, particularly if visual potential exists.

Keywords: Intravitreal injection; Melphalan; Retina; Toxicity; Rabbit; Electroretinogram; Visual evoked potential

Introduction

Retinoblastoma is the most common primary intraocular malignancy of children, representing 3% of all childhood malignancies with an incidence rate of 1 in 14000-18000 live births. Two-thirds of the children are diagnosed before 2 years of age, [1] although it may also present after 5 years of age [2]. It is unilateral and unifocal in 75% of cases while bilateral or multifocal in the remaining 25% [2].

Retinoblastoma is a fatal disease unless the affected eye is enucleated when the tumor is confined to the eye. The management of retinoblastoma has undergone significant changes in recent decades, and while the focus continues to be on improving survival, there has been a shift in emphasis towards eye salvage and the retaining of residual vision. This is particularly important when the patient with bilateral retinoblastoma has only one functioning eye or when there is residual visual potential.

Intravenous chemotherapy and intra-arterial chemotherapy are currently the most commonly used approaches for treating retinoblastoma by reducing the tumor and saving the globe. However, while solid tumors show excellent response to both treatment approaches, control of vitreous seeds can be more challenging. These are viable tumor cells floating in the vitreous cavity, and they may be refractory to repeated cycles of systemic chemotherapy, since its effectiveness depends on diffusion of the drug into the vitreous cavity [3]. Therefore, the presence of vitreous seeds is considered a poor prognostic sign for tumor control and salvage of the globe, whereupon enucleation may be a matter of saving life.

Intravitreal chemotherapy was first considered as a means to provide high tumoricidal drug concentration locally for retinoblastoma with vitreous seeds [3]. However, that approach was strongly rejected by most of the ophthalmic oncologists due to the risk of metastasis by malignant cells spreading through the needle track [4]. However, refinement of the intravitreal injection technique has established it as a safe procedure and it has been advocated by the ophthalmic community since 2012 [5].

Melphalan is a chemotherapy drug that belongs to the class of nitrogen mustard alkylating agents. Its cytotoxic effects arise from interference with DNA replication and transcription events, thus slowing the growth and spread of cancer cells [6]. *In vitro* testing demonstrated the superior sensitivity of melphalan compared to other chemotherapeutic agents in retinoblastoma [7], and several case series

evaluated the safety and efficacy of intravitreal melphalan injections for retinoblastoma complicated with vitreous seeds. A low dose of melphalan (8-10 μg) showed insufficient control of vitreous seed with minimal side effects, an intermediate dose (20-40 μg) showed moderate efficacy and some toxicity, while a high dose (50 μg) was highly effective but highly toxic [8]. The drug's toxic effects included severe retinopathy, optic neuropathy and even hypotony and phthisis that may lead to loss of vision and the need for enucleation [8].

Only two preclinical studies evaluated melphalan toxicity *in vivo* in rabbits. The effects of a single intravitreal melphalan injection on the electroretinogram (ERG) responses and retinal structure of rabbits indicated no damage with a dose of 10 μg, moderate changes after an injection of 20 μg, and severe damage with an injection of 90 μg [9]. The outcome of 3 weekly intravitreal injections of 15 μg melphalan each was evaluated: there was a reduction in the ERG amplitudes after 2nd and 3rd injections and a significant histopathological injury to the retina and optic nerve after 3 injections [10]. Those studies did not systematically test the dose-response relationship for retinal toxicity by intravitreal melphalan in order to determine safety margins for treatment, nor did they evaluate the potential effects of melphalan on retinal output.

The major goal of the current study was to define the safety margins of intravitreally injected melphalan in albino rabbits, and to describe its effects on retinal structure.

Material and Methods

Animals

Eighteen adult New Zealand White (NZW) rabbits weighing 2 to 3 kg each were used in this study. The rabbits were housed under 12/12 hour light/dark cycle, and were allowed free access to water and food. We chose the albino rabbits as our animal model, and not pigmented ones, which are more similar to humans, because melanin was shown to bind to melphalan [11], and thus it can reduce melphalan free concentration in the retina. Under these circumstances, the exact free concentration of the drug in the retina cannot be assessed and would hamper our goal of defining safety margins for intravitreal melphalan dose.

Prior to intravitreal injection and electrophysiological recordings, the rabbits were anesthetized by an intramuscular injection (0.5 ml/kg body weight) of a mixture [12,13] containing ketamine hydrochloride, acepromazine maleate solution, and xylazine. Topical anesthesia (benoxinate hydrogen chloride 0.4%) was administered to the eyes to reduce any potential discomfort, and pupils were fully dilated with cyclopentolate hydrochloride 1%.

Research plan

The rabbits underwent clinical inspection and ERG recordings before intravitreal injection (baseline), and at 3-days, 1-week, 2-weeks and 4-weeks after injection. The VEP was recorded at baseline and at 4 weeks post injection. A follow-up period of 4 weeks was chosen based on a previous report, showing that at 2 weeks after injection, drug effects stabilized and did not change during additional 6 weeks of follow-up [12].

At the end of the follow-up period, the rabbits were euthanized by intravenous injection of sodium pentobarbital (80 mg/kg body weight), the eyes were enucleated and the retinas were prepared for histologic and immunocytochemical observations.

All experimental procedures complied with the ARRIVE guidelines and were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978, and with institutional guidelines).

Drug administration

The freeze-dried melphalan powder (Alkeran; Aspen Pharmacare Australia Pty Ltd, St Leonards, NSW, Australia) was dissolved in the provided sterile solution, and then diluted in saline (BSS; Alcon, Fort Worth, Tx, USA) for the desired concentration according to the manufacturer instructions, and injected without filtration [5].

The rabbits were divided into four groups based on the dosage of a single intravitreal melphalan injection: group 1: 5 μg, N=4; group 2: 15 μg, N=4; group 3: 30 μg, N=5; group 4: 60 μg, N=5. In all rabbits, melphalan solution (0.1 ml) was injected into the vitreous of the right eye (experimental eye), and an identical volume (0.1 ml) of saline was injected into the vitreous of the left eye (control eye).

Intravitreal injections were performed as described previously [12,13], using a 30-gauge needle attached to a 1 ml tuberculin syringe. The needle was inserted into the eye approximately 1 mm posterior to the limbus and was advanced under visual control with an indirect ophthalmoscope (Neitz Instruments, Tokyo, Japan) towards the middle of the vitreous cavity, above the optic disc, and then a volume of 0.1 ml was slowly injected. We did not employ anterior chamber paracentesis to prevent increase in intraocular pressure (IOP) because in previous studies [12,13], including in the present one, there were no short-term or long-term effects of the intravitreal injection of 0.1 ml saline in the control eye. We also did not use cryoapplications after injection, as often used for patients [5], because our goal was to test toxicity of melphalan alone, and not the effects of the injection procedure.

Clinical observation

Rabbits underwent clinical inspection using an indirect ophthalmoscope searching for signs of ocular inflammation, cataract formation, and retinal damage.

Electroretinogram

Flash ERG responses were recorded simultaneously from the experimental and control eyes using corneal electrodes (Medical Workshop, Groningen, Netherlands). The reference and ground electrodes, made of stainless steel surgical needles, were inserted into the ears. Flash ERG responses were recorded with UTAS 3000 electrophysiology system (LKC Technologies, Gaithersburg, MD, USA).

The ERG responses were recorded in the dark-adapted state (at least 30 min in darkness) for assessment of rod system function, and then in the light-adapted state (background illumination of 30 cd/m^2) for evaluation of the cone system. The ERG signals were amplified and filtered (1-500 Hz) by the recording system. In order to improve signal/noise ratio, consecutive stimuli of identical strength were delivered, and the elicited responses were averaged on-line. In the dark-adapted state, we used for averaging different number of repeated responses delivered at variable time intervals depending upon stimulus strength using the ISCEV standard [14] as guidelines. As an example, with stimulus strength of 0.0025 cd-s/m^2, 10 responses elicited at 2 s

interval were recorded and averaged, while with stimulus strength of 2.5 cd-s/m^2 only 5 responses, elicited at 10 s intervals, were averaged. In the light-adapted state, 15 responses elicited at 1 s interval were averaged.

ERG analysis was based on amplitude and implicit time measurements. The a-wave amplitude was measured from the pre-stimulus baseline to the trough of the negative a-wave, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. The b-wave implicit time was defined by the duration from stimulus onset to the peak of the b-wave. The amplitudes of the b-wave were plotted as a function of log stimulus strength, and fitted to a hyperbolic function [15,16].

$$V/Vmax = I/(I+ \sigma) \quad (1)$$

Where V is the amplitude of the ERG wave elicited by a stimulus of strength I and Vmax is the maximal response amplitude. The semi-saturation constant, σ, is the light stimulus strength needed to elicit a response of half maximum amplitude (1/2 Vmax).

Functional damage of the rod system in the experimental eye was assessed from the dark-adapted (DA) b-wave Vmax ratio (experimental eye/control eye) and log σ difference (experimental eye-control eye). Light-adapted (LA) b-wave amplitude ratio (experimental eye/control eye) and implicit time difference (experimental eye-control eye) was derived from the ERG responses elicited by bright (2.5 cd-s/m^2) light stimuli under background illumination (30 cd/m^2), and was used to assess the effects of melphalan upon the cone system. This type of analysis circumvents technical factors, contributing to ERG variability between consecutive tests of the same animals [13].

Visual evoked potentials

Flash VEP signals were recorded using a stainless-steel needle as the active electrode that was inserted under the skin in the region of the visual cortex mid-way between the two ears. The reference and ground electrodes were inserted in the ears. Twenty-five consecutive (0.5 Hz) VEP signals, elicited by identical white light stimuli (I=2.5 cd-s/m^2), were averaged after amplification and filtration (1-100 Hz) by the UTAS-3000 electrophysiological system (LKC Technologies, Gaithersburg, MD, USA). To assure monocular recording of VEP, the non-recorded eye was tightly covered with half of a black rubber ball of 5 cm diameter.

VEP analysis consisted of amplitude and implicit time measurements. The implicit time was defined as the duration from stimulus onset to the trough of the first negative wave, appearing 40-60 ms after stimulus onset. VEP amplitude was measured from the trough of the first negative wave to the peak of the following positive wave. Functional damage to retinal output of the experimental eye was assessed from the amplitude ratio (experimental eye/control eye), and the implicit time difference (experimental eye–control eye).

GFAP immunohistochemistry

Glial fibrillary acidic protein (GFAP) is an intermediate filament that is normally expressed in astrocytes, but not in retinal Müller cells. GFAP expression is up-regulated in activated Müller cells following a variety of retinal injuries, and is used as a sensitive cellular marker for retinal stress [17-19].

The enucleated eye was soaked for 10 min in 4% paraformaldehyde solution (in PBS buffer; 0.1 M; pH 7.4) for fixation. The eyeball was opened 2 mm posterior to the limbus, and fixed in the same solution for 1 h. Then, the anterior segment of the eye (cornea and lens) was removed by a circumferential incision, and the vitreous was cleared. The posterior eyecup was bisected at the optic disc along the vertical meridian. One half of the eyecup was washed in a 0.1 M PBS solution, cryo-protected in 15% sucrose (in 0.1 M PBS pH 7.4) for 1 h and in 20% sucrose for another hour, and in 30% sucrose overnight. Then, it was embedded in OCT, and cut into 16-μm thick sections along the vertical meridian on a cryostat (Leica CM1900, Germany).

Cryostat sections were soaked in 0.1 M PBS (pH 7.4) solution, and then incubated in normal non-immune serum (3% serum+0.1% TritonX-100+PBS 0.1 M). The sections were soaked overnight at 4℃ in a moist chamber with primary monoclonal antibody to GFAP (Chemicon, CA, USA), at 1:400 dilution in PBS 0.1 M+3% serum +0.1% TritonX-100 at 1:100 dilution. For immunofluorescence visualization, the slides were rinsed three times in PBS, and then incubated for 1 h in donkey anti mouse Alexafluor 594 labeled antibody (Molecular Probes, Eugene, Oregon, USA) at 1:100 dilution in the above solution. The slides were also stained with DAPI (1:1000) to allow visualization of cells' nuclei for easy identifications of retinal layers. The stained retinal sections were examined by an Olympus Flouview laser scanning confocal microscope.

Retinal histology

The second half of the eyecup was rinsed in 0.1 M PBS, and dehydrated in ethanol (twice in 70%, twice in 96%; 1.5 h each), and then soaked in a solution of the resin (JB-4, Bio-Rad, Watford city, UK) and catalyst without the hardener overnight in 4℃. Then, the eye was embedded in the resin solution containing the hardener. Tissue sections of 2 μm were cut by a microtome (Reichert-Jung, Nussloch, Germany) along the vertical meridian, and mounted on slides. Retinal sections were stained with Richardson's solution and photographed at the light microscope level (Olympus BH2 and DP70, Germany).

Statistical analysis

ERG, VEP and histological data were analyzed using SPSS software package version 21 (SPSS Inc., Chicago, IL, USA). One-way Analysis of variance (ANOVA) was performed on baseline ERG and VEP data, VEP data that was obtained at the end of follow-up period, and on the histological data that were derived from retinal section that were measured by light microscope. ANOVA with repeated measures was performed on post-injection ERG data. Regression line analysis was conducted to compare statistically the b-wave to a-wave relationships of the experimental eyes to those of the control eyes for each melphalan dose group for the ERG recording session prior to injection and at termination of follow-up period. A p value of <0.05 was defined as the threshold for statistical significance.

Results

Clinical observation

Sclerotic retinal vessels and central retinal whitening were observed at 3 days after injection and throughout the follow-up period in all the experimental eyes, but not in the control eyes of the rabbits belonging to the 60 μg, 30 μg and 15 μg groups. No differences were seen between the retinas of the experimental eyes and the control eyes in the 5 μg group. The cornea, the lens, and the vitreous appeared clear, and no evidence of inflammation was observed.

Retinal function (ERG)

Figure 1 demonstrates the effects of intravitreal melphalan upon the dark-adapted ERG responses of 4 rabbits, each belonging to one of the 4 treatment groups. Representative ERG responses, elicited by bright light stimuli (2.5 cd-s/m^2) during the last ERG recording session (4 weeks after injection), from the experimental and control eyes (upper and lower traces respectively), clearly demonstrate a dose-dependent melphalan effect (Figure 1A). The larger was the melphalan injected dose, the larger was the ERG deficit. In order to assess quantitatively the melphalan effect upon retinal function, ERG responses were elicited by light stimuli of different strengths, and the amplitude-log stimulus strength relationships were constructed (Figure 1B), and fitted to the hyperbolic function (Equation 1). While no effect was seen in the experimental eye injected with 5 µg (1st column), eyes injected with 15 µg, 30 µg or 60 µg melphalan exhibited dose-dependent increase in ERG deficit.

Similar ERG measurements were conducted in all rabbits treated with different melphalan dosage. For each rabbit, at each ERG recording session, we derived the maximal response amplitude (Vmax) and the semi-saturation constant (σ) of the dark-adapted ERG b-wave. The mean ± standard deviation (SD) of the dark-adapted b-wave Vmax ratio (experimental eye/control eye), and of log σ difference (experimental eye–control eye) for the 4 dose groups are shown in figure 1C (left and right panels respectively) for the entire 4-weeks of follow-up. The dark-adapted Vmax ratios for the rabbits treated with 5 µg melphalan remained relatively stable, throughout the entire follow-up period. In contrast, the mean Vmax ratio of rabbits in the 15 µg group, 30 µg group, and 60 µg decreased at a dose-dependent manner indicating permanent loss in the dark-adapted ERG b-wave of 23%, 58%, and 100% respectively (Table 1).

Statistical analysis for the pre-injection dark-adapted b-wave Vmax ratio revealed similar (p=0.59) baseline values for the b-wave Vmax between the two eyes of rabbits belonging to the same melphalan dose. On the other hand, significant difference was found in the post-injection dark-adapted b-wave Vmax ratios between the different melphalan dose groups (p<0.0001). Post-hoc analysis showed that increasing melphalan dose between each of the 4 dose groups (e.g. 5 µg to 15 µg; 15 µg to 30 µg; 30 µg to 60 µg) resulted in a significant decrease (p=0.003) in the post-injection Vmax ratio, indicating a significant dose-dependent decrease in the Vmax ratio. However, there was no significant interaction between time and melphalan dose (p=0.14), indicating that, for all dose groups, the melphalan effect, measured 3-days after injection, remained constant throughout the 4-week follow-up period.

Time-depended changes in log σ difference for the dark-adapted ERG b-wave for the 3 dose (5 µg, 15 µg, 30 µg) groups (Figure 1C, right panel), fluctuated around zero value with high variability throughout the follow-up period. Consequently, statistical analyses for baseline and post-injection log σ differences revealed similar values in the 3 dose groups (p=0.24 and p=0.14, respectively). Moreover, there was no significant interaction between time and melphalan dose (p=0.51), indicating no change in dark-adapted Log σ value since day 3 after injection till termination of the follow-up period.

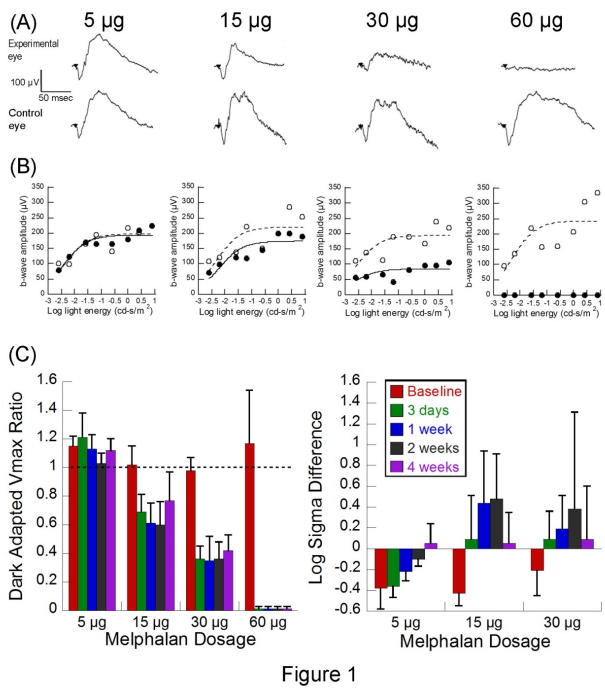

Figure 1

Figure 1: The effects of melphalan on the dark-adapted ERG responses of albino rabbits. (A) Representative ERG responses, recorded 4 weeks after melphalan injection from 4 rabbits, each injected intravitreally with a different melphalan dose, as denoted above each column. These responses were elicited by a bright white stimulus (I=2.5 cd-s/m^2). Each pair of traces compares the experimental eye to the control eye (upper and lower traces respectively). The onset of light stimuli is indicated by arrowheads. (B) Response-log stimulus strength relationships for the 4 rabbits were constructed from ERG responses elicited by light stimuli of different strength. The ERG data were fit to a hyperbolic function (eq. 1) in order to derive the values of Vmax and log σ for the dark-adapted ERG b-wave. (C) The effects of melphalan dose were assessed from the Vmax ratio (experimental eye/control eye) and log σ difference (experimental eye-control eye) of the dark-adapted ERG b-wave. Average ± SD of Vmax ratios (left part) and of Log σ differences (right part) are shown, for each melphalan dose, as a function of time after melphalan injection during the 4-weeks follow-up.

Figure 2A shows representative ERG responses that were elicited by bright light stimuli (I=2.5 cd-s/m^2) under background illumination (30 cd/m^2), each belonging to one of the 4 melphalan dose groups. These ERG responses were recorded at the end of the follow-up period. The ERG responses of the experimental eyes are clearly smaller in amplitude compared to those of the control eyes (upper and lower traces respectively), in a dose-dependent manner, for the rabbits treated with 15 µg, 30 µg and 60 µg melphalan, while no effect is evident in the ERG of the rabbit that was treated with 5 µg melphalan (Figure 2A).

Dark-adapted				
Melphalan Dose	**Dark-adapted ERG b-wave Vmax (mV)**		**Dark-adapted ERG b-wave log σ (cd-s/m^2)**	
	Control eye	Experimental eye	Control eye	Experimental eye
60 µg	-2.2 ± 0.3	0 ± 0	259 ± 47	
30 µg	210 ± 35	-2.1 ± 0.4	-2.2 ± 0.3	88 ± 35
15 µg	-2.3 ± 0.1	-2.4 ± 0.4	155 ± 54	200 ± 33
5 µg*	-2.4 ± 0.2	-2.5 ± 0.3	219 ± 29	195 ± 18
Light-Adapted				
Melphalan Dose	**Light-adapted ERG b-wave Vmax (mV)**		**Light-adapted ERG b-wave log σ (cd-s/m2)**	
	Control eye	Experimental eye	Control eye	Experimental eye
60 µg	86 ± 16	0 ± 0	28.7 ± 2.0	
30 µg	96 ± 32	41 ± 25	29.5 ± 1.1	30.6 ± 4.1
15 µg	81 ± 18	57 ± 25	29.0 ± 2.2	32.9 ± 3.4
5 µg*	74 ± 11	101 ± 6	30.3 ± 1.9	31.9 ± 1.0

Table 1: Average (± SD) of ERG parameters derived from ERG recordings at 4-weeks after melphalan injection in the dark-adapted state (A) and in the light-adapted state (B). (*Technical factors caused light-adapted ERG recordings in the right (experimental) eye channel to be consistently higher by ~15% compared to the recording in the left (control) eye channel).

For quantitative assessment of melphalan effect upon the light-adapted ERG, we calculated the b-wave amplitude ratio (experimental eye/control eye) from the b-wave amplitude of the ERG responses elicited by bright light stimuli (2.5 cd-s/m^2). Figure 2B presents the mean ± SD values of light-adapted b-wave amplitude ratios during the entire 4-weeks of follow-up, and Table 1 summarizes the values obtained at the end of follow-up. Figure 2B clearly shows that the light-adapted amplitude ratio remained approximately unchanged throughout the follow-up period in the rabbits belonging to the 5 µg group. In contrast, rabbits treated with 15 µg, 30 µg or 60 µg melphalan, exhibited a dose-dependent melphalan effect on the light-adapted ERG; 30%, 57% and 100% for the 15 µg, 30 µg and 60 µg groups, respectively (Table 1). Statistical analysis of the light-adapted b-wave amplitude ratio for the pre-injection ERG recording revealed no difference (p=0.54) between the 2 eyes of all rabbits in the different melphalan dose groups. In contrast, a significant difference in the post-injection values was found between the different melphalan dose groups (p<0.0001). Post-hoc analysis indicated that increasing of melphalan dose from 5 µg to 15 µg; from 15 µg to 30 µg; and from 30 µg to 60 µg resulted in a significant decrease (p=0.019) in the post-injection amplitude ratio, indicating a significant melphalan-induced dose related decrease in the amplitude ratio. No significant (p=0.56) interaction was found between time and melphalan dose, indicating no change in the post-injection values since ERG measurement at day 3 post injection throughout the follow-up period.

Time-depended changes in implicit time difference for the light-adapted b-wave for the 3 dose groups (Figure 2C, right) fluctuates in the range 1-4 ms with high variability throughout the follow-up period. Accordingly, statistical analysis for pre- and post-injection implicit time differences revealed similar values in the 3 different melphalan dose groups (p=0.74 and p=0.13, respectively). Moreover, there was no significant interaction between time and melphalan dose (p=0.58), indicating no change in the post-injection implicit times values of the light-adapted b-wave throughout the follow-up period between the 3 dose groups.

The melphalan effect upon the dark-adapted ERG b-wave (Figure 1) can reflect direct action on the photoreceptors, on signal transmission from the photoreceptors to the bipolar cells, and/or on the ON-center bipolar cells. Analysis of the b-wave to a-wave relationship can assist in determining the site/s of melphalan action [20,21]. Figure 3 compares the dark-adapted b-wave to a-wave relationships for 3 melphalan dose groups; 5 µg, 15 µg and 30 µg that were obtained at baseline, before melphalan injection (left column), and at termination (4-weeks) of the follow-up period (right column). Rabbits injected with 60 µg were not included in this analysis because there were no measurable ERG responses in the experimental eyes at termination of the follow-up period (Figure 1). For the construction of Figure 3, we used all the ERG responses of all rabbits in each melphalan dose group for the experimental eyes and control eyes (filled and open symbols respectively) that were characterized by measurable a-waves. Each set of b-wave to a-wave data was fitted to a linear regression line. Under baseline condition (Figure 3, left column), rabbits in each melphalan dose group show similar b-wave to a-wave relationships for the eye to be treated and for the control eye (filled and open squares respectively).

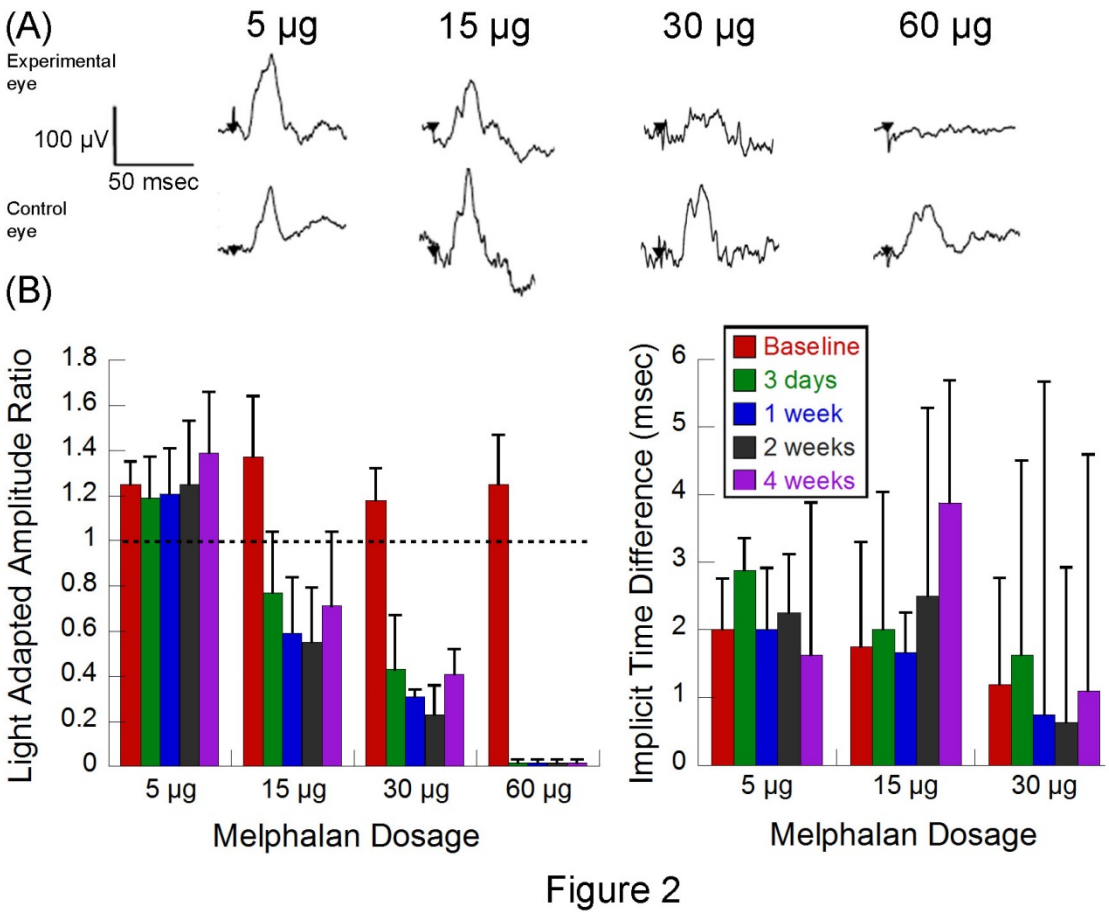

Figure 2

Figure 2: The effects of melphalan on the light-adapted ERG responses of albino rabbits. (A) Representative ERG responses, recorded 4 weeks after melphalan injection from 4 rabbits, each injected intravitreally with a different melphalan dose. These responses were elicited by a bright white stimulus (I=2.5 cd-s/m^2). Each pair of traces compares the experimental eye to the control eye (upper and lower traces respectively). (B) Melphalan effects on the light-adapted ERGs were assessed from the b-wave amplitudes and the b-wave implicit times. Average ± SD of the b-wave amplitude ratios (experimental eye/control eye), and of the b-wave implicit time differences (experimental eye-control eye) for the 4 melphalan dose groups are shown for the entire follow-up period (left and right parts respectively).

At termination of the follow-up period (Figure 3, right column), rabbits in the 5 µg group were characterized by similar b-wave to a-wave relationship, with slightly smaller a-waves between the experimental eyes and control eyes. Rabbits in the 15 µg group were characterized by smaller amplitudes of the b-wave and a-waves of the experimental eyes compared to the corresponding values in the control eyes, but similar slope between the b-wave to a-wave relationships between the two eyes. Thus, for a given a-wave, the amplitude of the b-wave in the experimental eyes and control eyes are similar. This finding is indicative to melphalan-induced photoreceptor damage [20,21]. In the 30 µg group, the b-wave to a-wave relationship of the experimental eyes differs from that of the control eyes. The b-wave amplitudes in the experimental eyes are considerably smaller from those of the control eyes for the same a-wave amplitudes. This finding indicates additional damage by 30 µg melphalan to synaptic transmission from the photoreceptors to ON-center bipolar cells and/or to the ON-center bipolar cells themselves [20,21].

Retinal function (VEP)

Melphalan was injected into the vitreous, and therefore as it diffused towards the retina it first encountered the nerve fiber layer and the ganglion cells. Damage to these retinal structures can cause blindness even if the outer retinal is not affected. In order to test for potential toxic effects of melphalan upon inner retinal neurons and retinal output, the VEP responses were recorded at 4-weeks post-injection.

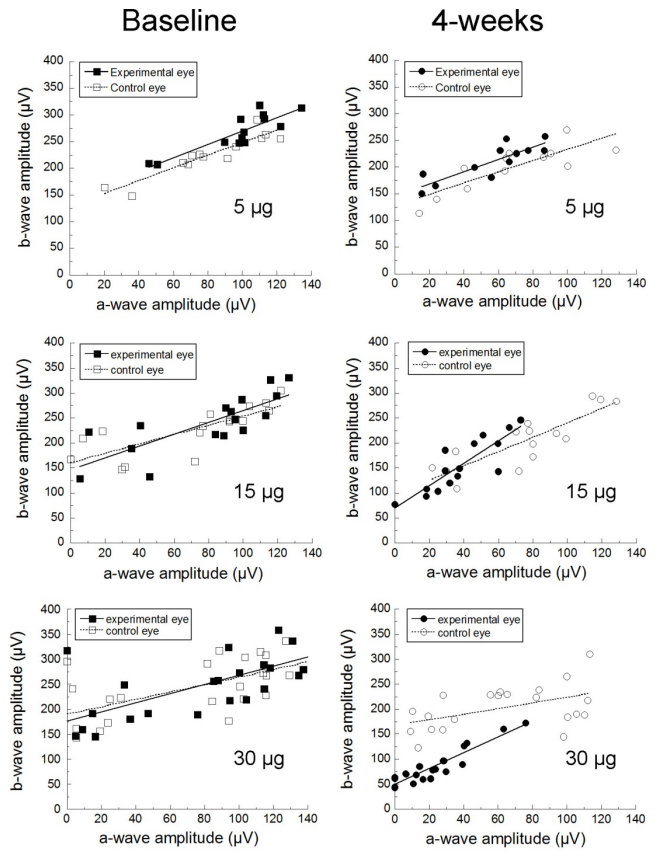

Figure 3

Figure 3: The effects of melphalan upon the dark-adapted b-wave to a-wave relationships. ERG data from all rabbits that were treated with a given melphalan dose (5 μg, 15 μg, 30 μg), were used to compare the experimental eye (filled symbols) to the control eye (open symbols) before melphalan injection (left column) and at termination of the follow-up period (right column). Linear regression procedure was applied to the data points (experimental eye–continuous line; control eye–dotted line).

Figure 4 compares representative VEP responses evoked by monocular stimulations of the experimental eye and the control eye (left and right columns respectively) in 4 rabbits, each belonging to one of the 4-melphalan dose groups. The typical pattern of a negative wave (arrow) appearing 40 to 60 ms after light stimulus onset (arrowhead), followed by a positive wave, is evident in all the VEPs. Similar VEPs were recorded from all the rabbits belonging to the 4 melphalan dose groups. We measured the implicit time and the amplitude, as defined in the Methods section, and the results (mean ± SD) are listed in Table 2. There was no reduction in the mean VEP amplitude in eyes injected with 5 μg and 15 μg, and a small reduction of only 10% and 18% in eyes injected with 30 μg and 60 μg respectively. One-way ANOVA of the baseline and the 4-weeks post-injection VEP measurements showed no significant differences between the 4 melphalan dose groups with regards to VEP amplitude (p=0.46 and 0.63, respectively) and VEP implicit time (p=0.66 and p=0.40, respectively), indicating no melphalan effects on retinal output.

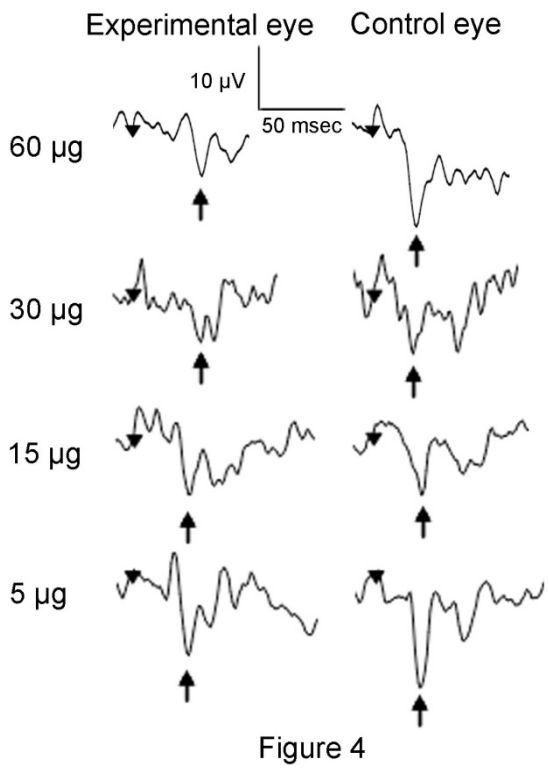

Figure 4

Figure 4: Representative flash VEP traces, recorded 4 weeks after melphalan injection, in 4 rabbits, each injected with a different dose of melphalan in the experimental eye, as denoted to the left of each row. The VEP recordings, elicited by monocular photic stimulation of the experimental eye or the control eye are compared (left and right columns respectively). The onset of the light stimuli is indicated by an arrowhead, and the first negative wave by an arrow.

Melphalan Dose	VEP amplitude (μV)		VEP Implicit time (ms)	
	Control eye	Experimental eye	Control eye	Experimental eye
60 μg	10.1 ± 3.1	8.3 ± 2.3	58.4 ± 7.5	54.6 ± 8.6
30 μg	8.9 ± 2.6	8.0 ± 2.4	56.6 ± 4.3	47.8 ± 7.2
15 μg	7.4 ± 2.5	7.5 ± 2.6	47.3 ± 6.2	47.8 ± 4.0
5 μg	12.1 ± 7.6	11.3 ± 5.2	47.3 ± 6.4	49.8 ± 13.4

Table 2: Average (± SD) of VEP parameters derived from recordings at 4 weeks after melphalan injection.

Retinal morphology

Since the ERG data clearly demonstrated a dose-dependent melphalan-induced functional damage to the rabbit retina, we tested the retinas of all the studied rabbits for the extent of structural damage and its localization. Figure 5 shows representative retinal micrographs of one rabbit from each of the 4 dose groups. Two micrographs are

shown for each eye (experimental and control) of each rabbit; one of a region close to the site of drug injection (Figure 5A), and the other of a peripheral region, remote from the injection site (Figure 5B).

Figure 5A suggests that the retinas of the experimental eyes, treated with melphalan dose of 5 μg (4th row), 15 μg (3rd row), and 30 μg (2nd row), retain a layered structure, but exhibit a dose-dependent thinning of the Inner Nuclear Layer (INL) and of the Outer Nuclear Layer (ONL). In contrast, the retina from the experimental eye injected with 60 μg (1st row), completely lost its layered organization indicating severe structural damage.

Peripheral retinal loci, remote from the injection site, suffered considerably lesser degree of melphalan-induced damage (Figure 5B). The retinal micrographs of the experimental eyes from the rabbits treated with 5 μg (4th row) and 15 μg (3rd row) are very similar to those of the control eyes. The peripheral retina of the experimental eye injected with 30 μg (2nd row) and of the eye injected with 60 μg suffered a small dose-dependent damage, but the layered organization of the retina was maintained.

Histologic findings, similar to those presented in Figure 5, were found in all studied rabbits, but the magnitude of retinal structural damage differed between rabbits. In order to assess quantitatively the degree of melphalan-induced retinal structural damage, we measured the thickness of the INL and the ONL for each of 16 rabbits, 4 in each dose group. The mean ± SD of the thickness of the INL and ONL in the central retina, close to the site of injection, and in the peripheral retina, remote from the site of injection, are presented for the 4-melphalan dose groups in Figures 6A and 6B, respectively), and listed in Table 3.

In retinal regions close to the injection site, a mild thinning of 20% was found for the ONL but not for the INL of the smallest dose (5 μg) group. More apparent damage was found in the retinas from the experimental eyes of rabbits in the 15 μg and 30 μg groups that are expressed in thinning of the INL (21% and 44%, respectively), and of the ONL (50% and 64%, respectively). The retinas from the experimental eyes injected with 60 μg melphalan were completely disorganized and the thickness of the INL and ONL in the regions close to the injection site could not be measured reliably, indicating 100% damage to both nuclear layers. One-way ANOVA for thickness measurements in regions close to the injection site revealed a significant difference in INL and ONL thickness between the melphalan dose groups (p=0.001 and p=0.005, respectively). Post-hoc analysis revealed a significant reduction in INL thickness ratio in the 60 μg group, compared with the 5 μg, 15 μg and 30 μg groups (p=0.001, p=0.005 and p=0.039, respectively), and a significant reduction in ONL thickness in the 60 μg group, compared with the 5 μg (p=0.003). The differences in INL and ONL thickness between the other groups were not significant owing to high variability and relatively small sample size. Nevertheless, the trend is obvious for sections close to the injection site: increasing melphalan dose resulted in decreasing INL and ONL thickness ratios, indicating melphalan-induced dose dependent structural damage. It should be noted that large variability in the degree of melphalan-induced structural damage was expected because the intravitreal location of the injected drug could not be identical in different animals.

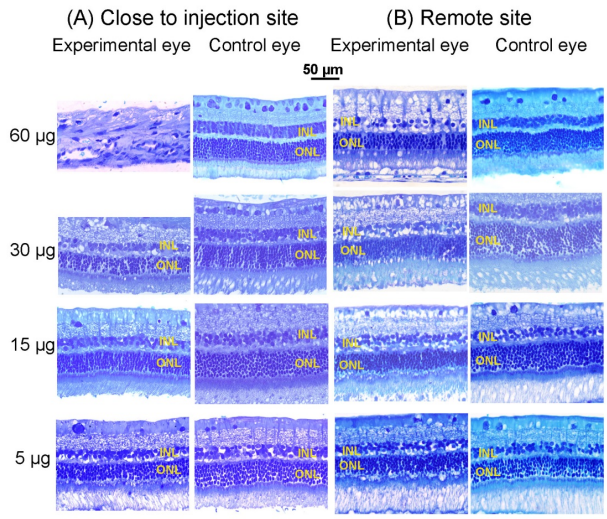

Figure 5

Figure 5: Melphalan effects on retinal histology. Representative micrographs of retinal sections from 4 rabbits, each treated with a different dose of melphalan as denoted to the left of each row of micrographs, are shown. For each rabbit, retinal sections from the experimental eye and the control eye that were obtained from an area close to the injection site in the central retina (A) and from a remote site in the peripheral retina (B) are compared. INL: inner nuclear layer; ONL: outer nuclear layer. The calibration bar applies to all micrographs.

The retinas from the experimental eyes injected with 60 μg melphalan were completely disorganized and the thickness of the INL and ONL in the regions close to the injection site could not be measured reliably, indicating 100% damage to both nuclear layers. One-way ANOVA for thickness measurements in regions close to the injection site revealed a significant difference in INL and ONL thickness between the melphalan dose groups (p=0.001 and p=0.005, respectively). Post-hoc analysis revealed a significant reduction in INL thickness ratio in the 60 μg group, compared with the 5 μg, 15 μg and 30 μg groups (p=0.001, p=0.005 and p=0.039, respectively), and a significant reduction in ONL thickness in the 60 μg group, compared with the 5 μg (p=0.003).

The differences in INL and ONL thickness between the other groups were not significant owing to high variability and relatively small sample size. Nevertheless, the trend is obvious for sections close to the injection site: increasing melphalan dose resulted in decreasing INL and ONL thickness ratios, indicating melphalan-induced dose dependent structural damage. It should be noted that large variability in the degree of melphalan-induced structural damage was expected because the intravitreal location of the injected drug could not be identical in different animals.

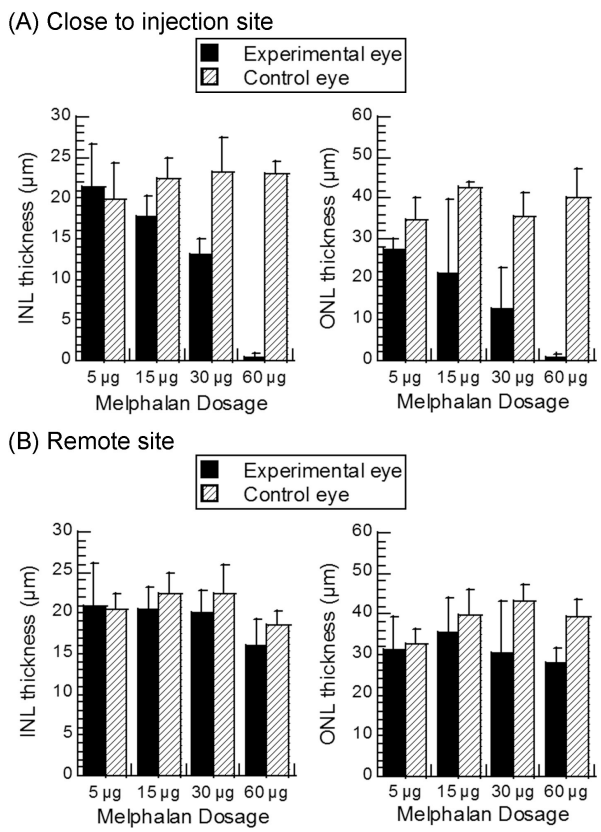

Figure 6

Figure 6: Effects of melphalan dose upon retinal structure of albino rabbits. The average thickness ± SD of the inner nuclear layer (INL) and the outer nuclear layer (ONL) in the experimental retina and control retina are compared for the central retina, close to the injection site (A), and for the peripheral retina, remote from the injection site (B).

The peripheral retina in all rabbits, including those treated with 60 µg melphalan, melphalan, retained the layered structure (Figure 5B). However, mild thinning of the INL was observed for the 5 µg, 15 µg, 30 µg and 60 µg (0%, 9%, 11%, 14%, respectively, Table 3) and for the ONL (5%, 10%, 30%, 30%, respectively, Table 3). Statistical analysis revealed a non-significant difference in the INL and ONL thickness ratio between the 4-melphalan dose groups (p=0.76 and p=0.19, respectively). Hence, only mild melphalan-induced structural damage was found in retinal loci remote from the injection site.

Permanent melphalan toxicity was demonstrated by electrophysiological measurements of retinal function (ERG responses), and by histological observations in the central retina, close to the region of melphalan injection. In order to test for functional-structural correlation, we compared in Figure 7 for each rabbit (N=16), regardless of the melphalan dose used, the relationship between the degree of functional damage, as assessed from the dark-adapted b-wave Vmax ratio (experimental eye/control eye), and the structural damage, expressed by the combined thickness of the ONL and INL in the experimental eye relative to the control eye; (ONL+INL) thickness ratio (experimental eye/control eye). The data show some variability, as expected from *in vivo* studies, but demonstrate a clear linear relationship; the larger the degree of structural damage (lower ONL +INL thickness ratio), the larger the degree of ERG damage (smaller dark-adapted b-wave Vmax ratio). The data were fitted to a linear function with r=0.95.

Central retina				
Melphalan Dose	**ONL thickness (mm)**		**INL thickness (mm)**	
	Control eye	Experimental eye	Control eye	Experimental eye
60 µg	0.0 ± 0.0	40.4 ± 7.1	0.0 ± 0.0	22.9 ± 1.5
30 µg	12.7 ± 10.3	35.6 ± 5.9	13.0 ± 2.1	23.3 ± 4.2
15 µg	21.5 ± 18.0	42.8 ± 1.1	17.8 ± 2.5	22.5 ± 2.5
5 µg	27.5 ± 2.7	34.6 ± 5.3	21.3 ± 5.3	20.0 ± 4.4
Peripheral retina				
Melphalan Dose	**ONL thickness (mm)**		**INL thickness (mm)**	
	Control eye	Experimental eye	Control eye	Experimental eye
60 µg	27.7 ± 4.1	39.4 ± 4.3	16.0 ± 3.3	18.5 ± 1.8
30 µg	30.3 ± 12.6	43.2 ± 4.2	20.0 ± 2.8	22.4 ± 3.5
15 µg	35.6 ± 8.3	39.7 ± 6.5	20.5 ± 2.7	22.5 ± 2.4
5 µg	31.1 ± 8.0	32.7 ± 3.7	20.9 ± 5.3	20.6 ± 1.8

Table 3: Average (± SD) of histological data measured from retinal sections of 4 rabbits from each melphalan dose group.

GFAP expression in Müller cells is used as a sensitive molecular marker for retinal stress [17-19]. Figure 8 compares retinal micrographs from peripheral retinal regions of the experimental eyes and control eyes (left and right columns respectively) from 4 rabbits, each treated with a different dose of melphalan. We chose peripheral retinal regions for 2 reasons. First, the peripheral rabbit retina lacks retinal blood vessels that are engulfed by astrocytes that express GFAP under normal conditions, and may obscure GFAP expression in Müller cells. Second, peripheral retinal regions showed lesser degree of structural damage, and therefore GFAP expression in Müller cells would be more apparent.

GFAP expression was upregulated in Müller cells of the experimental retinas (Figure 8 left column), but not in Müller cells of the control retinas (Figure 8, right column) in all 4 rabbits. Similar GFAP expression was found in the retinas of 10 rabbits; 4 in the 5 µg group, and 2 in each of the other three dose groups.

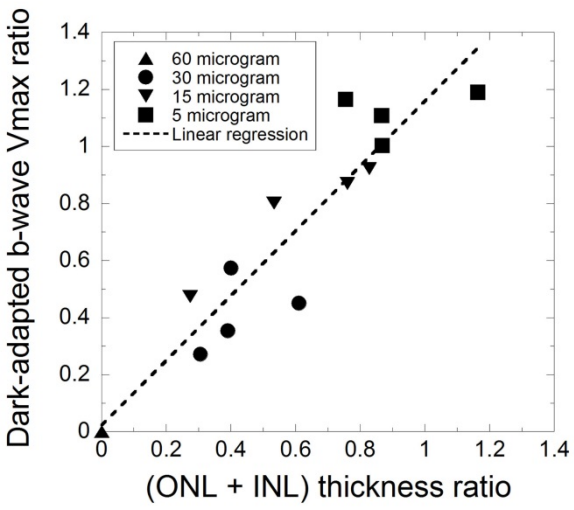

Figure 7

Figure 7: Correlation between functional and structural melphalan-induced retinal damage. Functional damage, assessed from the Vmax ratio of the dark-adapted ERG b-wave, is compared to the structural damage, assessed from the ONL+INL thickness ratio in the central retina, for each rabbit regardless of melphalan dose. The regression line (dashed line) through the data points indicates linear correlation between the two parameters of melphalan toxicity to the rabbit retina.

Discussion

Our results show that intravitreal melphalan exerts dose-dependent permanent injury to the albino rabbit retina, which is expressed as structural damage and reduced distal retina function. The smallest intravitreal melphalan dose that we used, 5 µg, had neither transient nor permanent effects upon the rabbit distal retinal function, as indicated by similar ERG responses for the experimental eye and the control eye (Figures 1 and 2) (Table 1) throughout the entire 4-weeks of follow-up. However, higher melphalan doses caused significant ERG deficits that were evident as early as 3 days after intravitreal injection. Those deficits remained relatively unchanged throughout the entire follow-up period, indicating that the drug had caused permanent functional damage to the outer retina. Rabbits in the groups treated with doses of 15 µg, 30 µg and 60 µg suffered a permanent and significant dose-dependent deficits in the ERG b-wave of 23%, 58%, and 100%, respectively, in the dark-adapted state and of 30%, 57% and 100%, respectively, in the light-adapted state (Figures 1 and 2) (Table 1).

The ERG findings were supported by clinical observations and histological examinations of the retinal structure: the eyes injected with 5 µg melphalan appeared normal, while the eyes treated with higher melphalan doses showed evidence of sclerotic retinal vessels and retinal whitening. Dose-dependent retinal structural damage was most apparent in the central retina close to the optic disc where the melphalan had been injected. In contrast, peripheral retinal loci, remote from the site of injection, suffered only mild structural damage (Figures 5 and 6). These findings are consistent with previous ones that showed an uneven distribution of drug-induced structural retinal toxicity in rabbits, specifically, larger in retinal regions close to the site of injection compared to remote retinal loci [12,13,22]. The degree of functional damage to the outer retina varied in rabbits treated with the same dose, probably reflecting variability in the injection procedure that could not be technically identical for each rabbit. This is supported by the linear relationship between the degree of ERG deficit and the degree of structural damage (Figure 7), similar to previous observations [23].

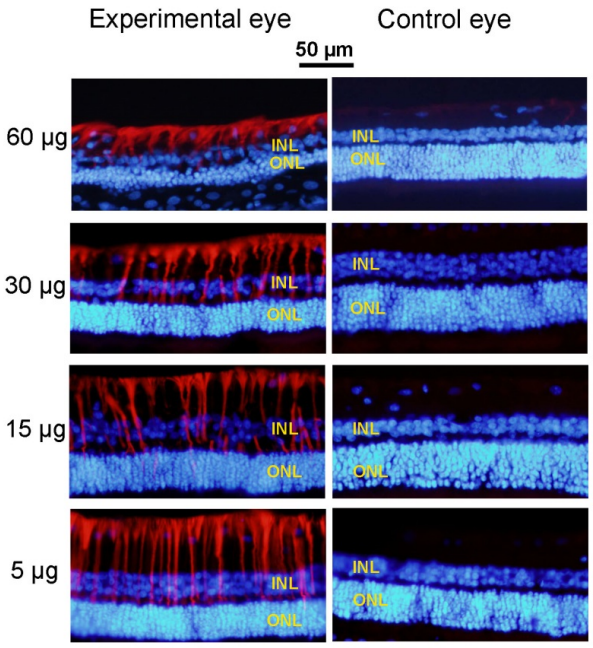

Figure 8

Figure 8: GFAP immunoreactivity of retinal sections from 4 rabbits, each treated with a different dose of melphalan, as indicated to the left of each row. Representative micrographs of peripheral retinal regions of the experimental eye (left column) and control eye (right column) are compared. Müller cells immunoreactive for GFAP are seen in the retina of all the experimental eyes, but not in the control eyes. Cell nuclei were stained with DAPI (blue) to visualize the retinal layers. INL, inner nuclear layer; ONL, outer nuclear layer. Calibration bar applies to all micrographs.

The relative melphalan-induced structural damage, expressed by per cent thinning of retinal layers was larger to the ONL compared to INL (Table 3), indicating a higher susceptibility of the photoreceptors to melphalan compared to inner retinal neurons. This is also supported by the b-wave to a-wave relationships of the dark-adapted ERG response. The 15 µg dose significantly reduced the amplitudes of the a-wave and the b-wave without altering the b-wave to a-wave relationship (Figure 3), suggesting a selective damage to photoreceptors [20-22]. The 30 µg dose significantly reduced the amplitudes of the b-wave and a-wave, and the b-wave to a-wave relationship changed, with the b-wave becoming smaller than expected from a given a-wave (Figure 3). It is reasonable to consider that synaptic transmission in the OPL and/or the function of the ON-

center bipolar cells were probably also affected by the drug at a dose of 30 μg [20-22].

Melphalan-induced functional and structural retinal damage was also evident in up-regulation of GFAP expression in retinal Müller cells in all the experimental eyes but none of the control eyes (Figure 8). Surprisingly the retinas from the rabbits treated with the lowest melphalan dose of 5 μg showed GFAP immunoreactivity in the Müller cells (Figure 8). Since GFAP immunoreactivity in Müller cells serves as a sensitive molecular marker for retinal stress of varying etiologies [17-19], we suggest that 5 μg dose of melphalan, injected into the vitreous of albino rabbits induced sufficient retinal stress to cause activation of retinal Müller cells, but that it was too mild to produce a measurable ERG deficit and structural changes as viewed at the light microscopy level.

In contrast to the considerable melphalan-induced dose-dependent ERG deficit and structural damage, we found no significant melphalan-induced changes in the flash visual evoked potential in all dose groups, including 60 μg melphalan (Figure 4) (Table 2). There was no effect of any melphalan dose on the implicit time of the flash-VEP (Table 2). These results probably reflect the major difference between the ERG and the flash VEP signals. While the ERG reflects light-induced electrical activity of the entire outer retina, and is severely reduced when a major portion of the retina is damaged, the flash VEP reflects light-induced activity in ganglion cells. Ganglion cells are not evenly distributed across the albino rabbit retina, but rather highest in the visual streak region and lowest in the peripheral retina [24,25]. However, because the area of the peripheral retina is larger than that of central retina, where the visual streak is located, the total number of ganglion cells is similar for both [24,25]. We therefore suggest that sufficient peripheral retina was spared (Figures 5 and 6) to contribute to almost normal flash VEP, while the ERG was too small to be measured reliably, in rabbits that were treated with the 60 μg dose.

There are only two *in vivo* studies that evaluated melphalan induced toxicity after intravitreal injections in albino rabbits. In one of those reports, consistent with our results, the ERG responses and the retinal structure were not affected by a dose of 10 μg melphalan, moderate effects were caused by a dose of 20 μg, and significant deterioration was caused by a dose of 90 μg [9]. In the other report, the consequences of 3 repeated weekly injections of 15 μg melphalan each were tested and, again, consistent with our results, there was a significant decline in the ERG amplitude after cumulative doses of 30 μg, and severe structural damage to the retina after cumulative doses of 45 μg [10]. Furthermore, those latter authors supported our observations of retinal whitening and sclerotic vessels after treatment with cumulative doses of both 30 μg and 45 μg.

Drug concentration in the vitreous must be taken into account when extrapolating the results from studies in rabbits for applications in humans. The volume of the distribution of intravitreally injected drugs in humans is about twice that of rabbits [26], making the human dose equivalent to twice that of rabbits [10]. The reported findings on the use of intravitreal melphalan injections in humans are inconsistent. Several case series reported the results of intravitreal melphalan injections for retinoblastoma and reported mostly minor ocular complications, including cataract and retinal pigment epithelium mottling [8]. Others, however, have described more significant side effects, including retinal vasculitis, vitreous hemorrhage, pre- and subretinal hemorrhage, retinal detachment, optic neuropathy, hypotony and phthisis [8]. Moreover, ERG recordings in 16 patients after 5-8 weekly melphalan injections of 30 μg each showed a reduction of 19.5%-94% in the ERG amplitude that was associated with salt-and-pepper retinopathy [10]. It is difficult to relate these results to our findings in rabbits because melphalan in the human reports was injected into sick eyes in which the effects of the medication could be exacerbated.

Intravitreal melphalan has been used for treating retinoblastoma complicated with vitreous seeds that was not responsive to other globe-preserving treatment modalities. Therefore, any adverse effect of intravitreal melphalan should be weighed against the alternative of enucleation that is required for tumor control. Our results show that a dose of 5 μg in a rabbit model that is closely equivalent to a dose of 10 μg in humans causes no clinical, histological or electrophysiological damage, and that appears to be safe, despite mild retinal stress that is expressed by GFAP immunoreactivity in the Müller cells. Any larger melphalan dose will exert dose-dependent functional and structural damage, and caution should be exercised when it is used to treat retinoblastoma, especially when there is visual potential.

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