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Investigating short-term toxicity of melphalan in a model of an isolated and superfused bovine retina

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Abstract

Background Melphalan, as a treatment for retinoblastoma, has been applied intra-arterially by catheterisation of the ophthalmic artery or intravitreally, aiming to reduce systemic side effects of intravenous drug therapy. This study evaluates retinal toxicity of different melphalan concentrations measured by electroretinogram (ERG) in an isolated and perfused retinal whole mount culture.

Methods For functional testing, bovine retinas were prepared and perfused with an oxygen-saturated standard solution and the ERG was recorded until stable b-wave or a-wave amplitudes were reached. Thereafter, retinae were exposed to 80, 160 and 320 μg/ml of melphalan for 30 min. After exposure, a washout was performed thrice for 5 min each and the ERG amplitude recovery was monitored for 60 min. To investigate the effects on photoreceptor function, 1-mM asparate was added to suppress the b-wave and obtain isolated a-waves.

Results While no toxic effects for a concentration of 80 μg/ml were observed, both b- and a-waves were significantly reduced after application of 160 (b-wave 43.8 %, $p=0.03$; a-wave 28.2 %, $p=0.04$) and 320 μg/ml (b-wave 20.0 %, $p=0.04$; a-wave 35.8 %, $p=0.02$). For 320 μg/ml, this reduction remained significant at the end of the washout (b-wave 40.0 % $p=0.02$; a-wave 26.4 %, $p=0.02$).

Conclusions Epiretinal or intraretinal concentrations of 80-μg/ml melphalan do not cause toxic effects in this in vitro model. Concentrations higher than 160 μg/ml should be avoided.

Keywords Melphalan · Retinoblastoma · Retinal toxicity · Electroretinogram · Retina

Introduction

Retinoblastoma is the most common malignant intraocular tumor in children. The incidence is 1/15,000-20,000 live births [1]. Prognosis depends considerably on time of initial diagnosis and on the tumor stage [2]. To date, there are different treatment options depending on size and stage: radiotherapy, chemotherapy, laser photocoagulation, cryotherapy, or enucleation [3, 4]. Survival of the child and reducing side effects of systemic therapy are the main therapeutic goals. It is a therapeutic challenge to find a way to control tumor growth, preserve the eye and retain best possible visual acuity.

Radiotherapy was first established in the early 1950s for the treatment of retinoblastoma. Due to an increased risk for the development of secondary tumors, chemotherapy and focal laser treatment have replaced radiotherapy widely [5]. However, radiation therapy still is an option for some indications [6].

In 2004, Yamane et al. introduced the concept of highly selective intra-arterial chemotherapy where a catheter is placed in the ophthalmic artery allowing for selective drug infusion, e.g., melphalan [7]. The great advantage of this technique is the reduction of systemic side effects while achieving high tissue levels of the agent resulting in reduction of tumor size [8, 9].

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Other possibilities of drug application include periocular or intravitreal injection. Intravitreal treatment seems promising in selected cases and is mainly applied as adjunctive treatment, especially when there are persistent tumor infiltrations of the vitreous. Melphalan was first described in 1953 and has been mainly used in the treatment for multiple myeloma [10]. It is an alkylating agent that is not cell-cycle specific. Up to 90 % of melphalan is bound to plasma proteins and penetration of the blood brain barrier is rather low [10]. Due to hydrolysis, it has a short half-life of only 60 min [11]. While there is much data on systemic side effects, investigations regarding retinal toxicity are rare [12]. There are reports on occlusive vasculopathy and chorioretinal atrophies after intraarterial injection of melphalan, mostly due to intraoperative vasospasm [13]. Discussions about the optimal concentration of intraarterial or intravitreal injection are ongoing [14–16]. The goal of this study was to find the maximal, non-toxic epiretinal concentration of melphalan in an experimental setup, thus increasing the safety of future clinical applications..

Materials

Aspartate, glucose and other chemicals were obtained from Merck (Merck Pharma GmgH, Darmstadt, Germany) at pro analysis grade. Melphalan (Alkeran®) was purchased (Aspen Pharma Trading Limited, Dublin 24, Irland) and diluted in nutrient solution containing 120-mM NaCl, 2-mM KCl, 0.1-mM $MgCl_2$, 0.15-mM $CaCl_2$, 1.5-mM NaH_2PO_4, 13.5-mM Na_2HPO_4 and 5-mM glucose.

Methods

The experimental setup is described in previous publications [17]. In brief, the electroretinogram (ERG) was recorded in the surrounding nutrient solution via two silver/silver-chloride electrodes on either side of the retina with a constant perfusion velocity of 1 ml/min. Temperature was kept constant at 30 °C (Fig. 1a). The nutrient solution was pre-equilibrated and saturated with oxygen. Retinas were dark-adapted and the ERG was elicited at intervals of 5 min using a single white xenon flash for stimulation (flash intensity was set to 6.3 millilux (mlx) at the retinal surface, Kodak Wratten filter, Fig. 1b).

The duration of light stimulation was 10 microseconds controlled by a timer (Photopic Stimulator PS33 Plus; Grass, Warwick, RI). The ERG was filtered and amplified (100-Hz high filter, 50-hz notch filter, 100,000× amplification) using a Grass RPS312RM amplifier. Data were processed and converted with an analog-to-digital data acquisition board (PCI-MIO-16XE-50; National Instruments, Austin, TX, Fig. 1c) on a desktop computer (PC-compatible, Fig. 1d).

Each retina was superfused with the serum-free nutrient solution and stimulated repeatedly until stable b-wave amplitudes were recorded. Retinae were exposed to melphalan at concentrations of 80, 160 and 320 µg/ml in an oxygen-saturated solution for 30 min. For security reasons, retinae were exposed to melphalan under an extractor hood protected from light. This was followed by a triple 5-min washout phase in nutrient medium. Then the ERG recording was continued for 60 min to monitor a possible recovery. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

To investigate the effect on the photoreceptor potential under scotopic conditions, the b-wave was suppressed by adding 1-mM aspartate to the nutrient solution. Aspartate is an inhibitor of synaptic transmission at the level of the first retinal synapse, thus unmasking the photoreceptor potential P III by abolishing the b-wave. Under these conditions, we were able to investigate the influence of different concentrations of melphalan on the photoreceptors. Five measurements each were taken for a- and b-waves.

Fig. 1 Schematic of the experimental set-up: The nutrient solution (a) superfuses the retina in an isolated box, where the retina is stimulated with an epiretinal flash with an intensity of 6.3 mlx (b). After filtering and amplification with the Grass RPS312RM amplifier (c), data is recorded and analyzed (d)
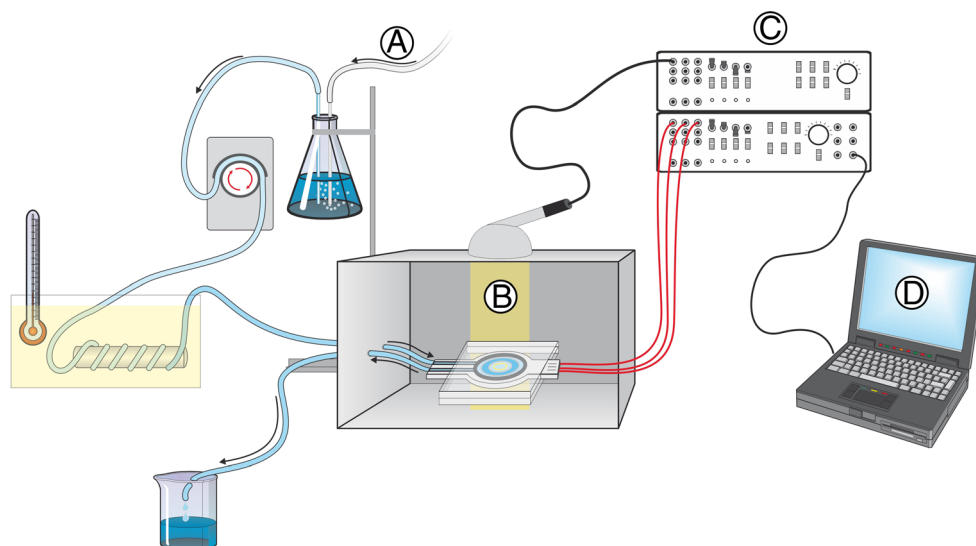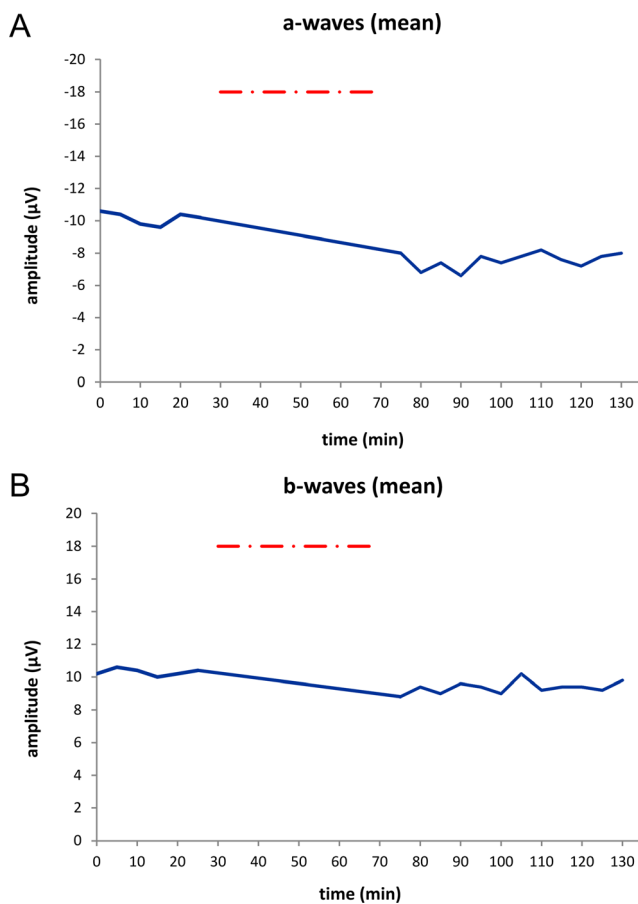
After recording a stable photoreceptor potential for 30 min, we proceeded as described before using the same exposure times. The changes of the a-wave amplitude were recorded; recovery was followed up for 60 min. We calculated the percentage reduction of the a- and b-wave amplitudes after the different applied concentrations.

Data analysis

Normal distribution was ensured for all data. The reduction of the ERG amplitude at the end of the 30-min exposure period was compared to the last amplitude measured before adding melphalan. The a- and b-wave amplitudes at the end of the experiment were compared to the corresponding amplitudes before exposure.

For statistical analysis, data was calculated throughout as the mean±standard deviation (SD). Significance was estimated by the student's paired t-test, and $p \leq 0.05$ was considered to indicate a statistically significant difference.
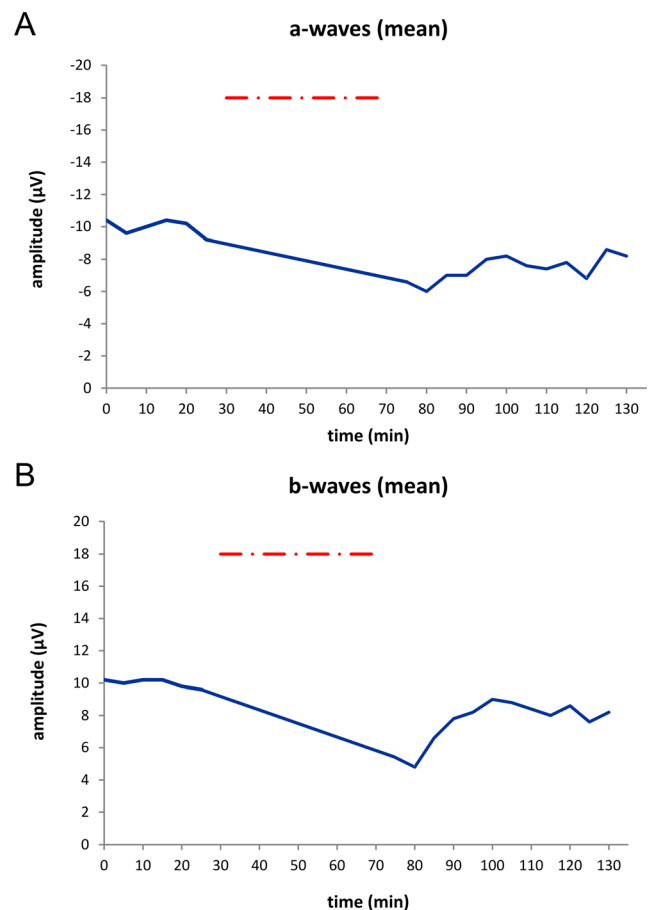
Results

Stable ERG amplitudes were obtained approximately 2 h after perfusion of the retinal whole mounts with standard solution. Environmental parameters such as pH, osmotic pressure, temperature and pO_2 remained unchanged during all tests.
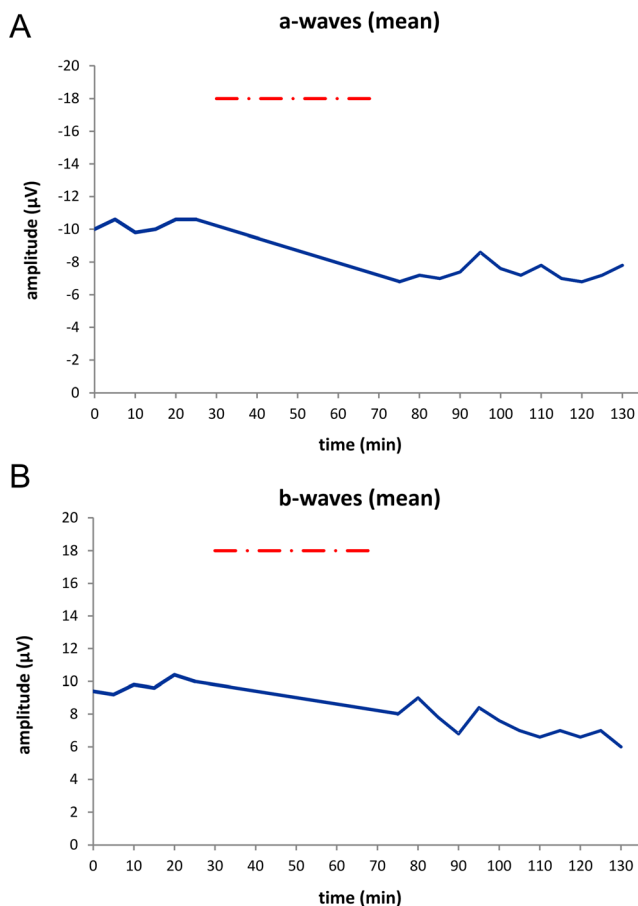
After exposure to 80-g/ml melphalan, an initial decrease of the b-wave amplitudes of 15.4 % was noted, which was not statistically significant ($p>0.05$). At the end of the washout, a recovery was noted, but b-waves remained reduced by 5.8 % compared to the baseline value. This change was not statistically relevant ($p>0.05$, Fig. 2b). Exposure to a concentration of 160-µg/ml melphalan resulted in a statistically significant reduction of 43.8 % ($p=0.03$) directly after exposure. At the end of the washout phase, the amplitude remained reduced by 14.6 %. However, this reduction was not significant ($p>0.05$, Fig. 3b). For the highest concentration of 320-µg/ml melphalan, there was only a slight initial decrease of 20.0 % directly after exposure, which was significant ($p=0.04$). This was followed by another decrease of 40.0 % compared to the amplitudes before exposure. For the decrease of 40 %, statistical

Fig. 2 Effects of 80-µg/ml melphalan applied for 30 min on the a-wave (**a**) and b-wave (**b**) amplitude of the ERG of an isolated perfused bovine retina, average of representative drug series ($n=5$). The horizontal bar above the curve marks the application. Three representative standard deviations for each drug series are given directly before and after application as well as at the end of the trial

Fig. 3 Effects of 160-µg/ml melphalan applied for 30 min on the a-wave (**a**) and b-wave (**b**) amplitude of the ERG of an isolated perfused bovine retina, average of representative drug series ($n=5$)

significance was reached ($p=0.02$, Fig. 4b). An overview of the results is given in Table 1.

In order to investigate the effects of 80-, 160- and 320-μg/ml melphalan on the isolated photoreceptor function, 1-mM aspartate was added to the standard solution. Having reached steady a-wave amplitudes, we proceeded as with the recording of the b-wave amplitudes. After exposure to 80-μg/ml melphalan, an initial insignificant decrease of 21.6 % ($p>0.05$) was measured. This continued throughout the washout phase. At the end of the experiments, the reduction of a-wave amplitudes remained unchanged (reduction of 21.6 %, $p>0.05$, Fig. 2a). After exposure to 160-μg/ml melphalan, an initial decrease of 28.2 % was observed. This decrease was statistically significant ($p=0.04$), followed by a slight recovery. At the end of the washout, only a reduction of 10.9 % was recorded, which was not statistically significant ($p>0.05$, Fig. 3a). For a concentration of 320-μg/ml melphalan, we found significantly reduced amplitudes of 35.8 % directly after exposure ($p=0.02$). The a-wave amplitudes remained reduced until the end of the washout (reduction of 26.4 %, $p=0.02$, Figs. 4a). An overview is given in Table 2.

Table 1 Mean and standard deviation (SD) of the ERG b-wave amplitudes.

Time (min)	80 μg/ml (μV)	SD	160 μg/ml (μV)	SD	320 μg/ml (μV)	SD
0	10.2	0.45	10.2	0.84	9.4	0.55
5	10.6	0.55	10.0	0.71	9.2	0.45
10	10.4	0.55	10.2	0.84	9.8	0.84
15	10.0	0.71	10.2	0.45	9.6	0.89
20	10.2	0.45	9.8	0.45	10.4	0.89
25	10.4	0.55	9.6	0.89	10.0	1.00
75	8.8	2.39	5.4	3.65	7.8	2.05
80	9.4	1.67	4.8	2.77	9.0	2.55
85	9.0	1.58	6.6	4.62	7.8	2.95
90	9.6	1.14	7.8	4.55	6.8	2.95
95	9.4	1.52	8.2	4.76	8.4	2.88
100	9.0	2.00	9.0	4.80	7.6	2.70
105	10.2	1.48	8.8	3.90	7.0	3.39
110	9.2	1.64	8.4	4.51	6.6	2.70
115	9.4	2.61	8.0	4.36	7.0	4.06
120	9.4	1.95	8.6	4.04	6.6	3.65
125	9.2	2.05	7.6	3.65	7.0	2.83
130	9.8	1.64	8.2	2.68	6.0	3.08

Exposure to melphalan was performed after 25 min

In order to investigate whether the 5 min-washout conducted thrice could possibly bias the obtained results, we waited until stable ERG amplitudes were recorded after perfusion of the retinal whole mounts with standard solution. Retinae were then exposed only to standard solution without melphalan, followed by a washout phase of 60 min to observe b-wave recovery. During those tests, no statistically significant changes could be observed (b-wave reduction of 7.7 %, $p=0.294$, a-wave reduction 12.0 %, $p=0.208$).

Discussion

The catheterisation of the ophthalmic artery and administering melphalan or injecting melphalan directly into the vitreous cavity are two possibilities to decrease systemic side effects, such as bone marrow suppression, bearing the risk of infections, renal and ototoxicity and the risk of secondary cancers [18]. This is especially significant for young patients. Local toxicity to the sensitive neurosensory retina via diffusion or a direct effect is possible and worth investigating. Although a concentration of 4 μg/ml is considered to be effective against retinoblastoma [19], these results derive from experiments in cell cultures. Therefore, finding an upper limit for the concentration of a, e.g., single intravitreal melphalan injection, is important to the clinician, being even more important than accumulated toxicity because of the very short half-life of

Fig. 4 Effects of 320-μg/ml melphalan applied for 30 min on the a-wave (**a**) and b-wave (**b**) amplitude of the ERG of an isolated perfused bovine retina, average of representative drug series ($n=5$)

Table 2 Mean and standard deviation (SD) of the ERG a-wave amplitudes

Time (min)	80 μg/ml (μV)	SD	160 μg/ml (μV)	SD	320 μg/ml (μV)	SD
0	−10.6	0.55	−10.4	0.55	−10.0	0.71
5	−10.4	0.89	−9.6	0.55	−10.6	0.55
10	−9.8	0.84	−10.0	1.00	−9.8	0.45
15	−9.6	0.55	−10.4	0.89	−10.0	0.71
20	−10.4	0.89	−10.2	0.84	−10.6	0.55
25	−10.2	0.84	−9.2	0.45	−10.6	0.55
75	−8.0	2.35	−6.6	2.61	−6.8	2.28
80	−6.8	3.11	−6.0	2.83	−7.2	2.17
85	−7.4	1.82	−7.0	1.58	−7.0	1.58
90	−6.6	1.95	−7.0	3.08	−7.4	1.52
95	−7.8	2.17	−8.0	3.08	−8.6	1.52
100	−7.4	0.89	−8.2	2.28	−7.6	1.34
105	−7.8	2.77	−7.6	2.30	−7.2	1.79
110	−8.2	2.17	−7.4	2.88	−7.8	1.30
115	−7.6	2.07	−7.8	2.39	−7.0	1.87
120	−7.2	3.56	−6.8	2.77	−6.8	1.92
125	−7.8	1.10	−8.6	2.19	−7.2	1.92
130	−8.0	2.45	−8.2	2.95	−7.8	1.48

Exposure to melphalan was performed after 25 min

melphalan [11]. The isolated retina model is an excellent preclinical tool for determining toxic limits.

We could detect a sustained neurotoxic effect of 320-μg/ml melphalan applied for 30 min on the inner retina, represented by the b-wave amplitude, and on the photoreceptors, represented by the a-wave amplitude. A reversible toxicity starts at 160 μg/ml. Our data is supported by other publications that could detect a toxicity in vivo [20] and in vitro, as well as clinical data [15, 20]. The isolated superfused retina model is a highly standardized model [17] that show effects comparable to the human situation [21]. However, the data should be considered experimental data from an in-vitro model. Therefore, our data can be only carefully transferred to the clinical situation.

There is a slight contradiction to other studies regarding the safety profile of melphalan: Shimoda et al. performed pars plana vitrectomy in albino rabbits and irrigated the eye with different concentrations of melphalan for 1 h. They monitored the ERG and found significantly decreased a- and b-wave amplitudes for concentrations of 10 μg/ml and 20 μg/ml [16]. Ueda et al. on the other hand showed a deterioration of the ERG with a single intravitreal dose of 90 μg on albino rabbits [14]. The rather significant difference in the findings between Shimoda et al. and Ueda et al. can be explained by the nature of their setup: Ueda et al. irrigated the rabbit eye after vitrectomy for 1 h; The surgical procedure alone, endoillumination and the flow of an intraocular solution are all influencing factors and explain the different results [22].

Our data, on the other hand, suggest that concentrations of 80 μg/ml can be used without any toxicity. The difference might be explained by the fact that we used a shorter exposure time, a different animal model and an in vitro approach in our setup. The bovine eye is more similar to the human eye. In our experimental setup, variables such as depth of anesthesia, positioning of electrodes, surgical trauma even caused by a simple intravitreal injection are all eliminated [17]; therefore, our results seem to reflect the human situation better. This hypothesis is supported by recent literature:

Francis et al. showed a reduced ERG in a study on patients that received 6.5 intravitreal injections of 30 μg of melphalan weekly [20]. An intravitreal dose of 22 μg was extrapolated to be optimal dose, and a cumulative dose of 14 mg was seen as rather problematic. The authors cautioned treating physicians to be careful [15]. Our data suggest that a single dose of 640 μg already shows a significant decrease in ERG, but 320 μg did not. Since the half-life of melphalan is 60 min [11] and we only tested an exposure time of 30 min, we would suggest that the extrapolated dose of 22 μg is safe and that even higher doses can be used for intravitreal injections.

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