https://doi.org/10.31288/oftalmolzh202354145

Clinical and pathomorphological changes in the rabbit retina after an injection of various doses of the cytostatic melphalan

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Background: *In recent years, there have been individual reports on intracameral chemotherapy (ICC) for aqueous seeding in retinoblastoma. The effect of melphalan on the structures of the ocular anterior segment (including the cornea, iris and anterior lens capsule) is however, still unknown, since no relevant experimental studies have been carried out so far.*

Purpose: *To experimentally assess the changes in the rabbit anterior segment induced by intracameral injection of various concentrations of the alkylating cytostatic melphalan.*

Material and Methods: *Twelve adult Chinchilla rabbits (22 eyes; age, 5–6 months; weight, 2.5–3 kg) were involved in this experimental study and maintained in the vivarium of the Filatov institute in separate cages under standard conditions.*

Results: *After a 5-µg melphalan injection, corneal and iris changes were reversible and the lens was still clear. With an increase in melphalan concentration in injection solution (to 10, 15 and 20 µg) and time point (to 1 month and 3 weeks) after injection, degenerative changes in some epithelial cells of the iris became irreversible, anterior capsular cataract developed, but the cornea and anterior chamber aqueous remained clear. After a single 20-µg intracameral injection of melphalan, there was depigmentation of the iris, posterior synechia and anterior capsular cataract.*

Conclusion: *Clinical and ultrastructural responses of ocular anterior segment tissue to intracameral melphalan injection depended on the injected dose and time point after injection. Most cells of examined tissues showed the capability to restore their ultrastructure following ceasing of the toxic effect of the drug.*

Keywords:

intracameral chemotherapy, pathomorphological changes, melphalan

Introduction

Aqueous seeding in an eye with retinoblastoma is not only associated with poor prognosis for eye salvage, but also is a risk factor for metastasis, and, therefore, retinoblastoma patients with aqueous seeding have absolute indication for enucleation and subsequent treatment [1-4, 5].

Recently, there have been individual reports on intracameral chemotherapy (ICC) for aqueous seeding in retinoblastoma.

Munier and colleagues [4, 6] were the first to report positive outcomes of ICC with the cytostatic melphalan for aqueous seeding in retinoblastoma in 2015-2018. The effect of melphalan on the structures of the ocular anterior segment (including the cornea, iris and anterior lens capsule) is however, still unknown, since no relevant experimental studies have been carried out so far.

The purpose of this experimental study was to assess the changes in the rabbit anterior segment induced by intracameral injections of various melphalan concentrations.

Material and Methods

Twelve adult Chinchilla rabbits (age, 5–6 months; weight, 2.5–3 kg) were involved in this experimental study and maintained in the vivarium of the Filatov institute in separate cages under standard conditions.

All animal experiments were performed in compliance with the Law of Ukraine on Protection of Animals from Cruel Treatment No. 1759-VI dated December 15, 2009 and approved by a local Bioethics Committee of the Filatov Institute (Bioethics Committee Meeting Minutes

No.1 of March 26, 2019). The research design did not include statistical analysis of outcome data.

Animals were divided into 5 groups depending on the concentration of intracamerally injected melphalan: Group 1 (3 rabbits) received melphalan at a concentration of 5 μg/0.1 mL; Group 2 (2 rabbits), 10 μg/0.1 mL; Group 3 (3 rabbits), 15 μg/0.1 mL; Group 4 (2 rabbits), 20 μg/0.1 mL; and Group 5 (2 rabbits) received saline solution only.

The intracameral melphalan injection procedure was performed using the operating microscope under general anesthesia. First, topical anesthetic was instilled into the conjunctival sac. Second, the operative field was cleansed with 0.5% chlorhexidine in alcohol. Third, a blepharostat was used to open the eyelids. Fourth, a 32G needle was used to make a long corneal limbal tunnel. Fifth, 0.25-0.3 mL aqueous was aspirated from the anterior chamber. Sixth, with the needle left in the corneal canal, the syringe was replaced with another syringe containing melphalan (at a concentration of 5 μg/0.1 mL, 10 μg/0.1 mL, 15 μg/0.1 mL, or 20 μg/0.1 mL), and the drug was injected into the anterior chamber. Finally, an antibiotic was instilled into the conjunctival sac.

After each intravitreal injection of melphalan, animals received topical disinfectants and antibacterial therapy. At post-surgery days 1, 3, and 14, eyes were examined for slit-lamp biomicroscopy assessment of the anterior segment to check for inflammatory response and transparency of the ocular media. At day 14, month 1 and 1 month and 3 weeks, the rabbits were euthanized by air embolism, their eyes were enucleated, and material for histology and electron microscopy was taken in a routine manner. Corneal, iris, ciliary body, trabecular tissue and anterior lens samples were obtained 14 days (5, 10, 15, and 20 μg/0.1 mL), 1 month (5, 10, 15, and 20 μg/0.1 mL) and 1 month and 3 weeks (5 and 15 μg/0.1 mL) after a single injection of the above concentrations. Control animals received 0.1 mL saline solution into the anterior chamber.

Samples were obtained at day 14 in one rabbit (2 eyes), 1 month in one rabbit (2 eyes), and 1 month and 3 weeks in one rabbit (2 eyes) in group 1 (3 rabbits; 5 μg/0.1 mL); day 14 in one rabbit (2 eyes) and 1 month in one rabbit (2 eyes) in group 2 (2 rabbits; 10 μg/0.1 mL); day 14 in one rabbit (2 eyes), 1 month in one rabbit (2 eyes), and 1 month and 3 weeks in one rabbit (2 eyes) in group 3 (3 rabbits; 15 μg/0.1 mL); day 14 in one rabbit (2 eyes) and 1 month in one rabbit (2 eyes) in group 4 (2 rabbits; 20 μg/0.1 mL); and day 14 in one rabbit (2 eyes) and 1 month and 3 weeks in one rabbit (2 eyes) in group 5 (2 intact rabbits).

For electron microscopy studies, samples were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4), post-fixed with 1% osmium tetroxide in phosphate buffer (pH 7.4), dehydrated through an ascending ethanol series, and embedded in an Epon/Araldite mix. Thereafter, ultra-thin sections were cut, stained with lead citrate according to the procedure described by Reynolds [7], and observed with a PEM-100-01 Transmission Electron Microscope (Selmi, Sumy, Ukraine).

Results

At all time points, animal health and well being was normal in all groups irrespective of drug concentration, with no general toxic effects on experimental animals.

All experimental eyes demonstrated mild conjunctival injection on day 1 after surgery, which gradually subsided and ceased on day 5-7. In addition, all experimental eyes improved to moderate anterior chamber depth and showed normal intraocular pressure on palpation and a pink fundus reflex.

In groups 1 and 2 neither biomicroscopic evidence of changes in the anterior segment nor exudative inflammatory response was seen, and, at all time points, the cornea, anterior chamber aqueous and lens remained clear, and the iris pattern was normal. In group 3, mild iris depigmentation was seen at the sites of melphalan injection, while the cornea and lens remained clear. In group 4, the cornea and anterior chamber aqueous remained clear, a local opacification was seen in the anterior lens capsule, and local iris atrophy with depigmentation and posterior synechia was seen at the sites of melphalan injection. The iris color somewhat improved, synechia almost resolved, and there was no increase in the severity of the anterior capsular cataract over two weeks. No anterior chamber hemorrhage or exudative inflammatory response was observed in any melphalan injection group at any time points.

There were practically no histomorphological signs of pinocytosis in corneal endothelial cells (ECs) after injection of various concentrations of melphalan. There was an increase in the numbers of ECs showing signs of destructive changes and necrosis with an increase in the concentration of injected melphalan. In addition, there were insignificant numbers of ECs with almost normal morphology (Fig. 1 A, B).

At day 14, in group 1, degenerative changes (granular degeneration and mild hydropic degeneration) developed in the iris pigment epithelial cells (mostly posterior iris pigment epithelial cells). At day 14, in groups 2, 3 and 4, hydropic degeneration was seen in most pigment epithelial cells, and there was an increase in edema in pigment epithelial cells and stroma with an increase in the concentration of injected melphalan (Fig. 2). Therefore, at any time point (from day 14 to 1 month and 3 weeks) after a 5-μg melphalan injection, iris pigment epithelial cells exhibited similar mild changes manifested mostly by reversible granular degeneration and mild hydropic degeneration.

With an increase in melphalan concentration in injection solution (to 10, 15 and 20 μg) and time point (to 1 month and 3 weeks) after melphalan injection, degenerative changes in some epithelial cells became irreversible, which resulted in organelle destruction, gelation of the hyaloplasm, an increase in the contact area between cells and an impaired structure of the epithelial layer, leading to reduced metabolism and function of the iris. Nonpigmented ciliary epithelial cells

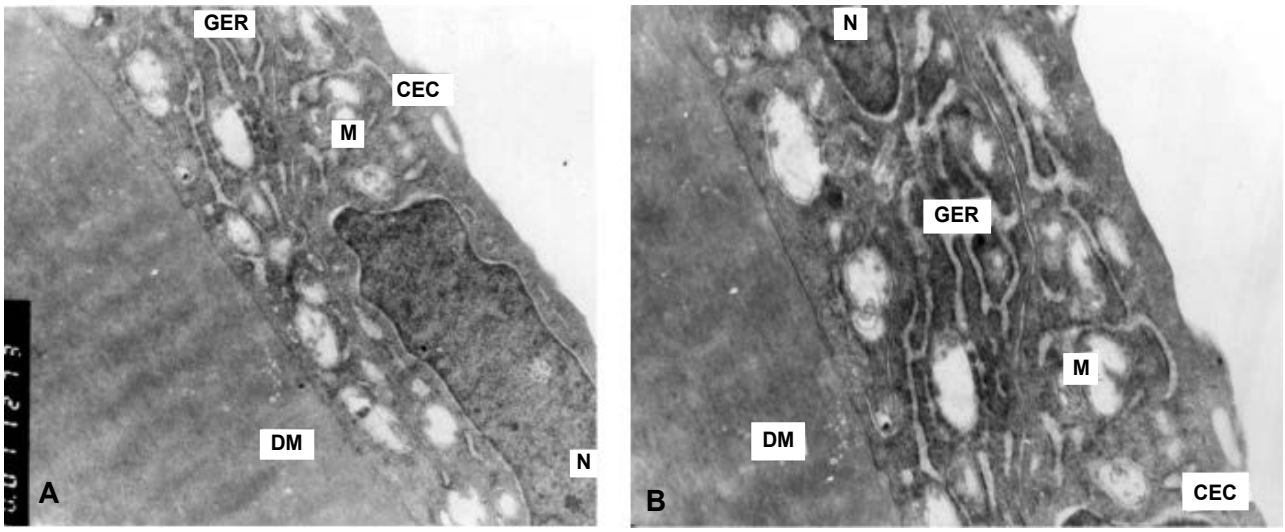

Fig. 1. Electron micrographs. Ultrastructure of rabbit cornea at 1 month after an intracameral injection of 10 μg melphalan. (A) Mytochondrial vacuolization and atrophy of the granular endoplasmic reticulum in a corneal endothelial cell (original magnification, ×10000). (B) Mytochondrial vacuolization and atrophy of the granular endoplasmic reticulum in a corneal endothelial cell (original magnification, ×6000). Note: CEC, corneal endothelial cell; DM, Descemet membrane; GER, granular endoplasmic reticulum; N, nucleus; M, mytochondria.

developed degenerative changes, with their severity increasing with an increase in melphalan concentration in injection solution. After a 5-μg melphalan injection, this degeneration was initially manifested by mild, reversible hydropic changes in the nonpigmented layer of epithelial cells (mostly, in the processes of these cells). With an increase in melphalan concentration in injection solution and time point after melphalan injection, degeneration in the cells of this layer increased in severity, and spread over the pigmented epithelial cell layer. In addition, stromal edema increased, collagen fibers became more loosely aggregated, and connective tissue cells showed signs of hydropic degeneration. It should be, however, noted that the severity of ultrastructural damage in ciliary epithelial cells was less than in iris epithelial cells.

The response of trabecular cells to melphalan injection was similar to the response of other tissues of the anterior chamber angle. In the trabecular tissue region, however, there were severe edema of the connective tissue substance, loosely arranged trabeculae, and pathological structural

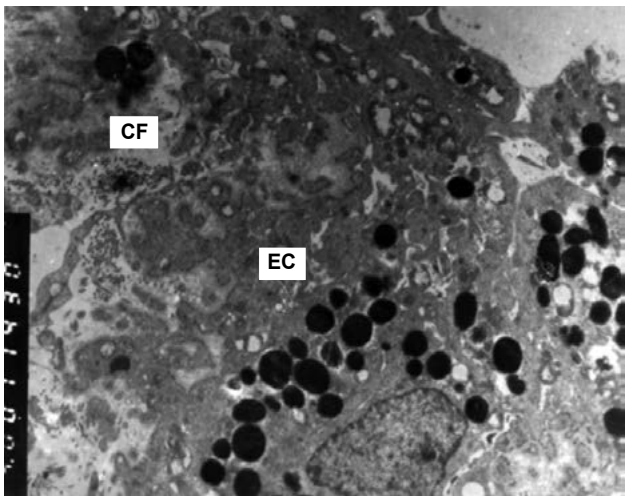

Fig. 2. Electron micrograph (original magnification, ×4000). Ultrastructure of rabbit iris at 1 month and 3 weeks after an intracameral injection of 15 μg melphalan. Hydropic degeneration of cells in the two pigment epithelial cell layers aas well as an increase in the contact area between cells is observed. Note: CF, collagen fibrils; EC, epithelial cell; IR, iris.

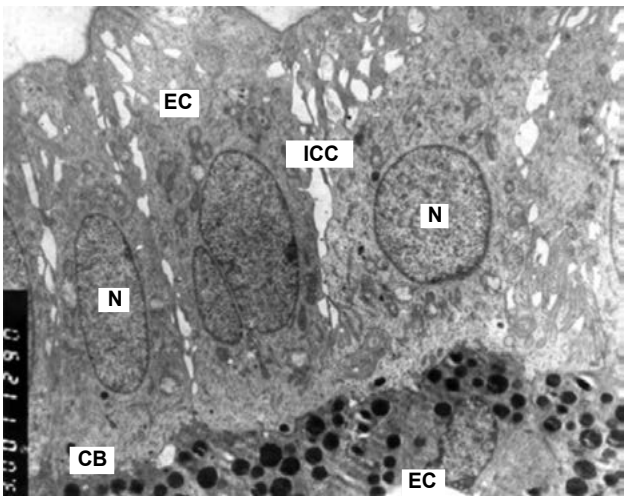

Fig. 3. Electron micrograph (original magnification, ×3000). Ultrastructure of rabbit ciliary body at 1 month after an intracameral injection of 10 μg melphalan. An increase in the contact area between epithelial cells of the non-pigmented layer of granular endoplasmic reticulum cisterns (in the processes of these cells) is observed. Note: CB, ciliary body; EC, epithelial cell; N, nucleus; ICC, intercellular contact

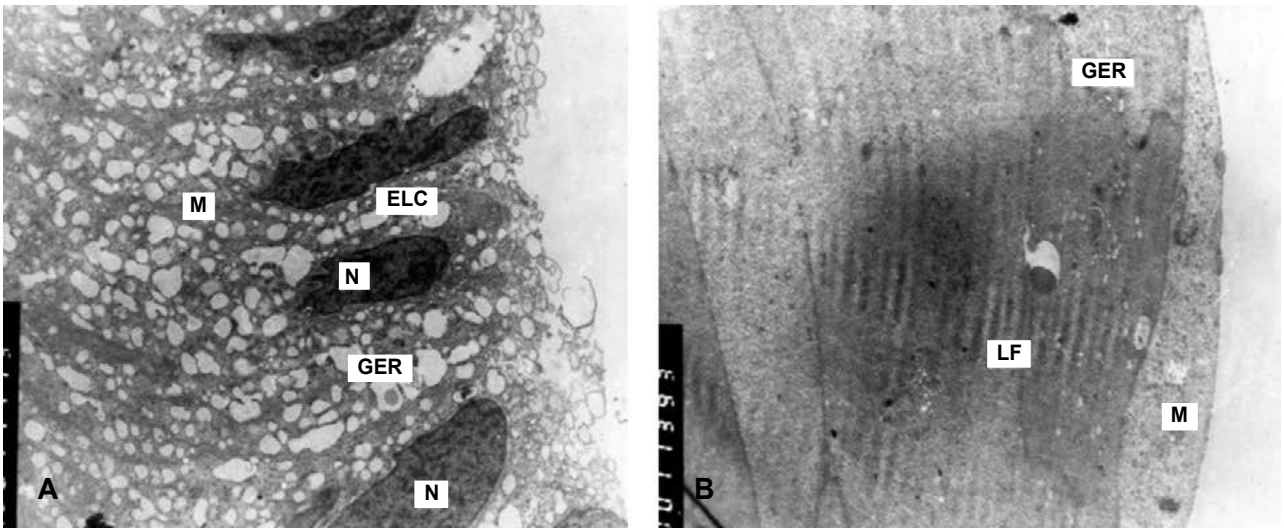

Fig. 4. Electron micrographs. Ultrastructure of rabbit lens at 1 month and 3 weeks after an intracameral injection of 15 μg melphalan. (A) Soapy cytoplasm of epithelial cells with plasmalemma breakage (original magnification, ×4000). (B) Granular degeneration of lens fibers (original magnification, ×3000). Note: ELC, epithelial lens cells; GER, granular endoplasmic reticulum; LF, lens fibers; M, mytochondria; N, nucleus.

changes in epithelial cells. With an increase in melphalan concentration in intracameral injection solution, the degree of trabecular disintegration increased, leading to the breakage of the endothelial wall of Schlemm's canal.

Endothelial cells of the anterior lens capsule responded to a 5-μg melphalan injection by manifestations of granular degeneration, and to an increased concentration of intracamerally injected melphalan, by increased hydropic degeneration. Moreover, with an increase in melphalan concentration in injection solution and time point after melphalan injection, there was an increase in the severity of edematous changes in cells leading to the breakage of their plasmalemma. Degenerative changes were observed also in lens capsule fibers (Fig. 4 A, B).

It should be also noted that the corneal endothelium, lens epithelium and trabecular tissue were more susceptible to the toxic effect of intracamerally injected melphalan. Most cells of examined tissues showed the capability to restore their ultrastructure following ceasing of the toxic effect of the drug.

Discussion

Reports are scarce on the application of ICC. Munier and colleagues [4, 6] reported on the success of ICC for retinoblastoma with primary or secondary anterior chamber invasion. The largest case series by Munier and colleagues [6] included 11 eyes with secondary anterior chamber seeding (ACS) treated with intracameral melphalan injection. Complete aqueous seeding regression was initially obtained in all eyes after a mean of 0.7 months (range, 0.2-1.3) as assessed clinically and by negative cytopathology and cell culture, after a mean number of 4.3 injections (range, 2-7) with a mean dose of 6.3 mg (range, 2.9-15) per injection. Eye preservation was achieved in 6/11 cases. Munier and colleagues [6] noted that a disrupted anterior hyaloid of iatrogenic origin adversely effects the prognosis for eye preservation due to an insufficient dose intensity of melphalan chemotherapy for retinoblastoma progression into the anterior segment. Toxic cataract and iris heterochromia have been noted as complications [6, 8]. In total, enucleation for progressive disease was performed in 5 cases after a mean retention time of 8.8 months (range, 1.0-20.7) after the first ICC [6]. Pavlidou and colleagues [9] believe that (1) clinical indications for secondary enucleation are tumor resistance to melphalan and tumor seeding in the anterior segment, and (2) treatment with chemotherapy may result in partial tumor necrosis and may cause seeds to disperse and travel anteriorly.

Conclusion

Our rabbit study on the clinical and ultrastructural anterior-segment tissue response to intracameral melphalan injection demonstrated that, after a 5-μg melphalan injection, corneal and iris changes were reversible and the lens was still clear. With an increase in melphalan concentration in injection solution (to 10, 15 and 20 μg) and time point after injection, degenerative changes in some epithelial cells of the iris became irreversible, anterior capsular cataract developed, but the cornea and anterior chamber aqueous remained clear. In addition, after a single 20-μg intracameral injection of melphalan, there was depigmentation of the iris, posterior synechia and anterior capsular cataract, and our ultrastructural study found degenerative changes in the epithelial cells of the lens, iris and trabecular, with these changes increasing in severity and becoming irreversible with time.

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Disclosures

Received 11.08.2023

Accepted 28.09.2023

Author contributions. *All authors participated in data collection, analysis of results, writing the manuscript, and approved the final version of the manuscript.*

Conflict of interest. *The authors declare that they have no conflict of interest that could influence their opinions regarding the subject matter or materials described and discussed in this manuscript.*

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