Original Article

Intravitreal injection of methotrexate in an experimental rabbit model: Determination of ultrastructural changes

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Purpose: To investigate the ultrastructural changes of the rabbit retina induced by intravitreal methotrexate injection. **Materials and Methods:** Ten New Zealand white rabbits were enucleated bilaterally at different time periods after intravitreal methotrexate injection. One rabbit was used as control group and one rabbit was used as intact group. Histopathological examinations were performed under light and electron microscopy. Early (within first three days after injection) and long-term (one month after serial injections) effects of intravitreal methotrexate on the retina were investigated. **Results:** Retinal edema, vacuolization, and disintegration of mitochondria of the retinal cells were observed as early changes. The main long-term effects after serial injections were edema in the photoreceptor, inner nuclear, and ganglionic cell layers. Cellular disorganisation was seen on light microscopy. Electron microscopic examination revealed mitochondrial degeneration and vacuole formation in retinal cells, nuclear degeneration in outer nuclear layer, and membranous whorl formation in photoreceptor and nerve fiber layers. **Conclusions** High dose intravitreal methotrexate injection may cause significant ultrastructural changes in the rabbit retina in varying severity. This finding may highlight the potential side effects of methotrexate on human retina in higher doses.

Key words: Electron microscopy, intravitreal injection, light microscopy, methotrexate, rabbit, retina

The lymphoproliferative disorders including leukemia, reactive lymphoid hyperplasia, non-Hodgkin lymphoma, and primary central nervous system lymphoma may involve ocular structures. The main ocular manifestation of lymphoproliferative disorders is uveitis. Since the primary central nervous system lymphoma with isolated ocular involvement is radiosensitive, early central relapses requiring combination of radiotherapy and chemotherapy may occur.[1-3] Ocular manifestations that are resistant to conventional systemic treatment may require intratechal and intravitreal chemotherapy, which is often recommended as a first line treatment.[4] However, the effects of methotrexate on the retina are still under investigation. The aim of this study was to investigate the ultrastructural effects of intravitreally injected methotrexate on the rabbit retina. The dosage used in the study was higher compared to the routinely used dosage, because we aim to investigate the possible experimental retinotoxic concentrations of intravitreal methotrexate.

Materials and Methods

The early (within first three days after injection) and long-term effects (one month after serial injections) of the intravitreal injection of methotrexate on the retina were studied on a total of 20 eyes of 10 adult New Zealand white rabbits; male and female, weighting between 1500-2500 g, upon approval of the Ethical Board Committee. The adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research was confirmed.

The study protocol was summarized in Fig. 1. Two rabbits were enucleated at first day after methotrexate injection, 2 rabbits at second day, and 2 rabbits at third day, bilaterally. To investigate the long-term effects of serial intravitreal methotrexate injections, four separate 800 μg methotrexate injections were performed within seven days intervals. At one month after the last injection, four eyes of 2 rabbits were enucleated in the way as mentioned below. Both eyes of two other rabbits were used as intact group; to distinguish surgical trauma from drug-related damage, both eyes of one rabbit used as a control group and 0.1 cc saline solution was injected intravitreally instead of methotrexate at 3 mm posterior to the limbus at the upper temporal quadrant. The saline injection was repeated four times within seven days interval and at one month after the last injection, enucleation was performed.
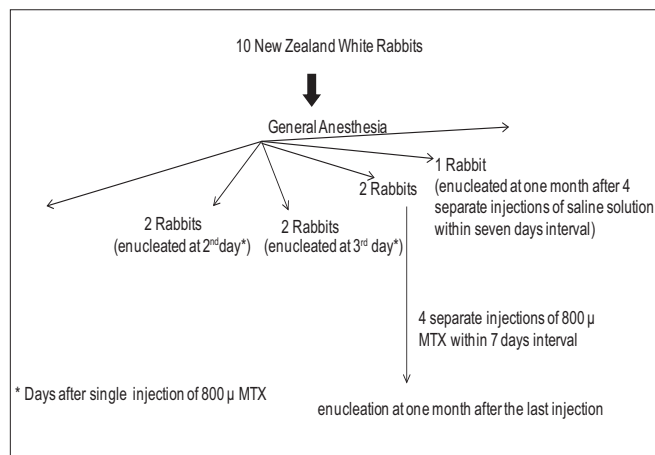
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Figure 1: Flowchart of study design

Each examination and surgical procedure of all rabbits was carried out under general anesthesia after an intramuscular injection of ketamine hydrochloride 35 mg/kg and Xylazine hydrochloridine 5 mg/kg. Fifteen minutes after one drop of phenylephrine HCl 2.5% (Mydfrin®, Alcon Lab.) and tropicamide 1% (Tropamid Forte®, Bilim Lab); anterior segment, vitreous and retina were evaluated.

A 50 mg/2 ml methotrexate without preservative (Methotrexate "Ebewe"®, Liba Lab) was diluted by 4.25 ml distilled water to obtain methotrexate 8 mg/ml. A volume of 0.1 ml of the preparation (methotrexate 800 μg, 1.76 μmol) was injected into the vitreous of both eyes of the rabbits by an insulin injector with a 26 gauge needle under general anesthesia. Intravitreal injection was performed 3 mm posterior to the limbus at the upper temporal quadrant. Measurement of the intraocular pressure and retinal examination of the rabbits were repeated following the injection. There was no significant difference of intraocular pressure measured before and after injection. Intraocular 5% glutaraldehyde injection was done at 3 mm posterior to the limbus at 3 or 9 o'clock position in order to achieve complete stiffness of the ocular tissues. Euthanasia was performed via intravenous administration of a high-dose anesthetic agent to the rabbits after surgical procedures.

The tissue samples were fixed in 10% neutral formalin solution for 48-72 hours. The samples were dehydrated using alcohol and xylol series and were embedded in paraffin. Histological sections were taken and processed for light microscopy using conventional methods.

The tissue samples for electron microscopy were fixed for 4 hours with 5% glutaraldehyde in Millioning phosphate buffer at pH 7.4 and then fixed with 1% osmium tetroxide in the same phosphate buffer for 2 hours at 4°C. Tissues were dehydrated with ethanol and embedded in araldite. Semi-thin sections were taken with Reichert Ultracut S® ultramicrotome, stained with toluidine blue, and appropriate areas for electron microscopic observation were determined thereafter. Thin sections were taken from selected areas and stained with uranyl acetate and lead citrate. They were examined with Jeol JEM 1400® transmission electron microscope. The process of preparation for microscopic evaluation was demonstrated in detail in Fig. 2.

Results

The retina pigment epithelium, outer nuclear, outer plexiform, inner nuclear, inner plexiform, ganglion, and nerve fiber layers were clearly normal by light and electron microscopy in the native rabbit retina [Fig. 3].

The light microscopic examination of the retinal layers of the rabbit with intravitreally injected saline solution was similar to the native rabbit's retina, although there was slight vacuolization representing intercellular edema in the retina pigment epithelium, the photoreceptor, outer nuclear, outer plexiform, inner nuclear, inner plexiform layers. The external limiting membrane was normal on ultrastructural examination [Fig. 4].

One day after injection

The large intercellular spaces were observed especially at inner nuclear and nerve fiber layers by light microscopy. Furthermore, electron microscopic examinations revealed mild cytoplasmic vacuolization at the apical region of the cells. The
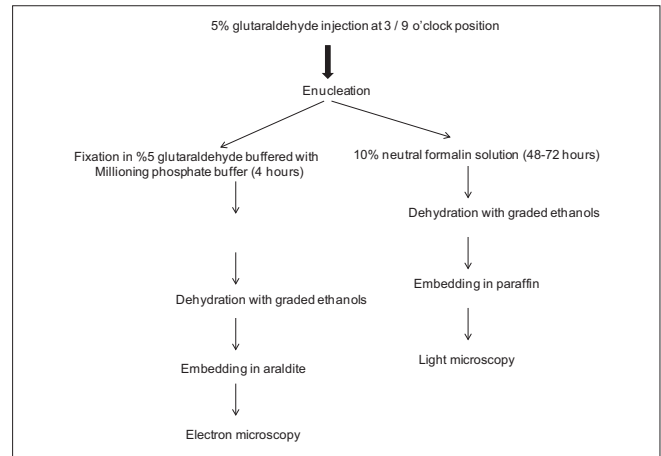
Figure 2: Flowchart about preparation of the tissue before microscopic evaluation
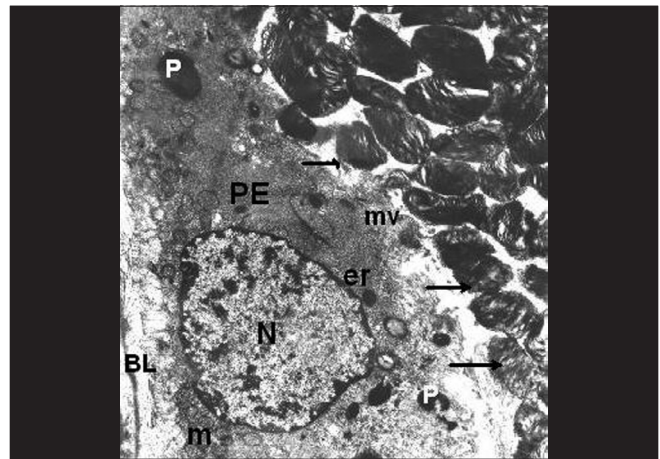
Figure 3: Intact group. The ultrastructure of the retina pigment epithelium (PE) was normal. (Nucleus (N), endoplasmic reticulum (er), mitochondrion (m), pigment granules (P), microvilli (mv), basal lamina (BL), arrows for photoreceptor cells of the retina, original magnification, ×12400)
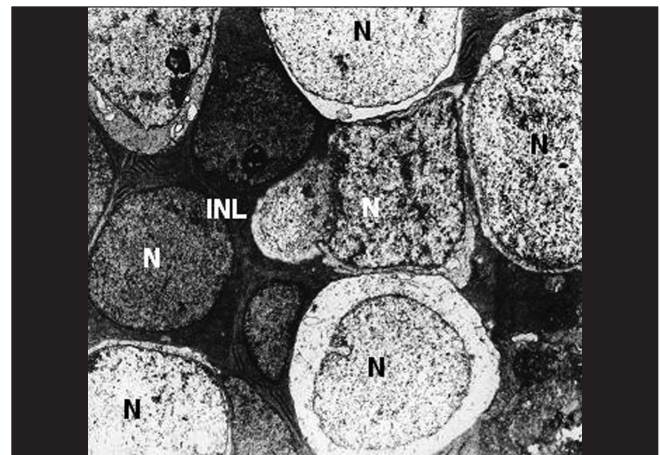
Figure 4: Control group. Electron microscopy of the control group was similar to the intact group. In this micrograph, the inner nuclear layers (INL) exhibited normal structures. (Nucleus (N), original magnification, ×10100)

external limiting membrane was normal. There was an increase of chromatin in nuclei. Some of the cells had empty vacuoles and slightly enlarged mitochondria with disintegration of their cristae. Most of the cells of the inner nuclear layer were normal. The inner plexiform layer exhibited various sized vacuoles. Additionally, most of the cells of the ganglion cell layer disclosed normal nuclear pattern with enlargement of the mitochondria, electron dens bodies, and cytoplasmic vacuoles. However, the nerve fiber layer and the inner limiting membrane were normal [Fig. 5].

Two days after injection;

Large edematous areas were seen by light microscopy in the inner nuclear and nerve fiber layers. Furthermore, slight to moderate vacuolization was found in pigment epithelium layer, in outer segments of some of the photoreceptors and in outer nuclear layer. Although various sized vacuoles were seen in ganglion cell layer, most of the cells exhibited normal structures. Additionally, the inner plexiform layer and the internal limiting membrane were also normal [Fig. 6].
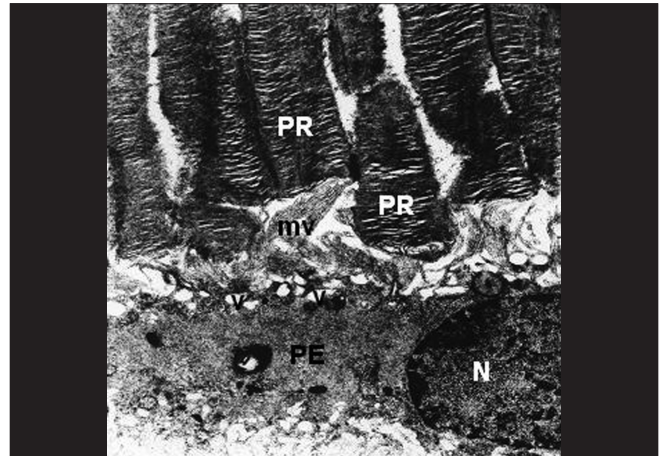
Three days after injection;

Light microscopic examination of the retina showed enlargement of the intercellular spaces in outer and inner nuclear, and nerve fiber layers. On the other hand, there was mitochondrial disintegration and slight-to-moderate vacuolization in the pigment epithelium, photoreceptor, and ganglion cell layers [Fig. 7].

### Thirty days after serial injections;

Although retina pigment epithelium and outer nuclear layer were normal in structure, prominent edematous changes were seen in photoreceptor, inner and outer nuclear layers by light microscopy. Some cellular irregularities in the inner nuclear layer were also seen. The ultrastructural alterations were more prominent in this group. Nuclear degeneration in outer nuclear layer, vacuolization in apical cytoplasm of the pigment epithelial cells, membranous structures in photoreceptor, outer nuclear and nerve fiber layers were all essential findings [Figs. 8 and 9].

Discussion

Primary central nervous system lymphomas have different treatment options. Radiotherapy is one of the most commonly preferred treatment option for intraocular lymphoma to control the disease, but the frequency of complications and the increased mortality rate are the main disadvantages of the therapy. The survival rate is found to be higher for patients who receive combined radiotherapy-chemotherapy compared to radiotherapy alone.[5] The chemotherapy protocoles including methotrexate were found to be more successful when compared to others.[6,7] Intratechal methotrexate, cytarabine, and dexamethasone injections cause neurotoxicity and chemical meningitis. Mason *et al.*, found that intratechal methotrexate-cytosine arabinoside combination provided 5-year remission with preserving visual acuity. The intratechal methotrexate was not effective for parenchymal lesions but intratechally administered araniboside-C had good penetration within parenchyme.[8] However, the main problem of the chemotherapy protocol is the weak intraocular penetration of the agents. De Smet *et al.*, demonstrated that intratechal methotrexate could not achieve therapeutic levels in intraocular fluids. The


Figure 5: One day after injection. The retina pigment epithelium (PE) revealed slight cytoplasmic vacuolization (v). (Nucleus (N), microvilli (mv), photoreceptor cells, original magnification, ×16100)
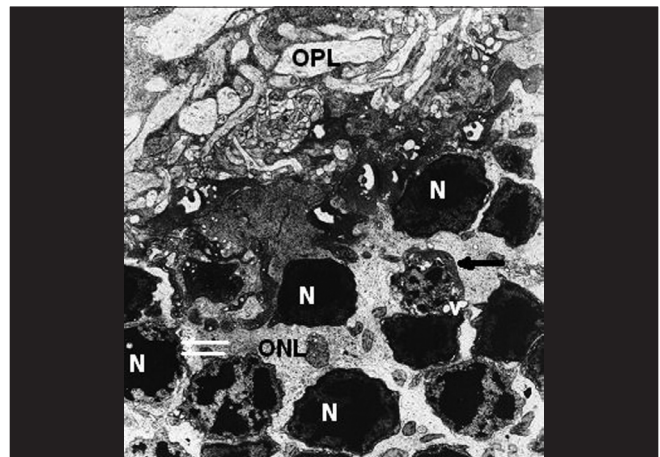
Figure 6: Two days after injection. Although some of the outer nuclear layer cells (ONL) exhibited normal structure, some of them showed nuclear heterochromatin condensation (white arrows) and cytoplasmic vacuolization (v) (black arrow). (Nucleus (N) of the cells, outer plexiform (OPL) layer, original magnification, ×8100)
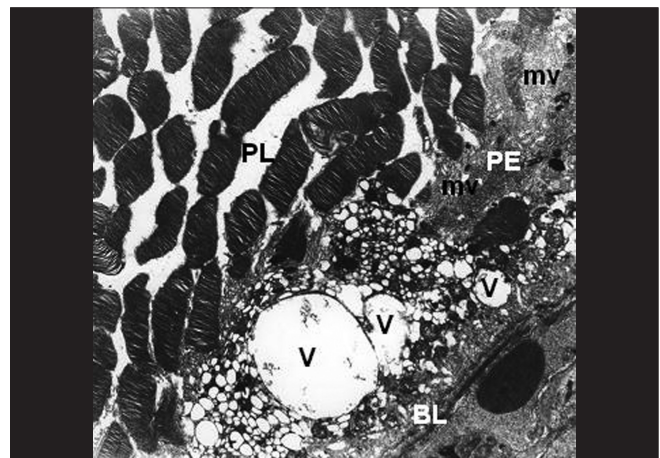

Figure 7: Three days after injection. The retina pigment epithelium (PE) showed increase in cytoplasmic vacuolization (v). (Microvilli (mv), basal lamina (BL), photoreceptor cells layer (PL), original magnification, ×10100)
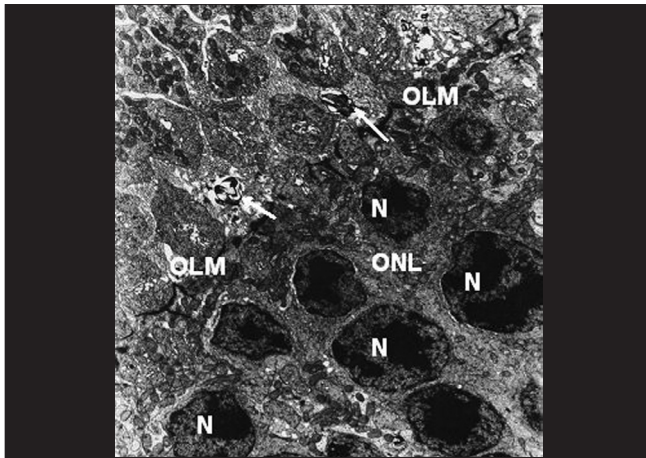
Figure 8: Thirty days after injection. The outer nuclear layer (ONL) cells revealed normal nuclear (N) pattern and cytoplasmic structure, but some of them also showed intracytoplasmic membranous whorl-lik structures (arrows). (OLM: outer limiting membrane, Original magnification, ×10100)

Figure 9: Thirty days after injection. Although most of the outer nuclear layer cells revealed normal ultrastructure, some of them (arrows) showed nuclear (N) degeneration. (Original magnification, ×12400)

blood-aqueous barrier limits the intraocular penetration.[9] The posterior uveal capillaries and pigmented ciliary body capillary are fenestrated and permits the passage of the drugs. However, the tight junctions located on the vascular endothelium of the iris and non-pigmented ciliary body epithelium prevents the increase of the concentration in the aqueous and in the vitreous, and hence local administration is preferred for ophthalmic chemotherapy. Therefore, Chan and Wallace recommended intravitreal chemotherapy for initial and recurrent intraocular lymphoma.[10] Intravitreal methotrexate is able to maintain therapeutical levels in vitreous for 5 days and avoids systemic side effects. A single dose of intravitreal injection can lead prolonged tumoricidal concentration.[11] Furthermore, vitreoretinal involvement can be controlled effectively with intravitreal methotrexate.[4] However, intravitreal methotrexate has several limitations and side effects. Ericson *et al.*, showed the occurence of vitreous membranes after 0,3 ml injection of 10 mg/ml of methotrexate. There was no abnormality in electrophysiological studies.[12] Vernot *et al.*, demonstrated localized retinal thinning, peripheral retinal fold formation, occurence of vacuoles within inner nuclear layer, and focal loss of photoreceptor layer after intravitreal injection of 5- fluorouracil, doxorubicine, bleomycin, thiothepa, ethoposide, and methotrexate.[13] In the present study, we observed similar changes including vacuole formation and disintegration of organelles in the rabbit retina. On the other hand, Velez *et al.*, found neither electrophysiological nor pathological changes.[14] Smith *et al.*, demonstrated that the most common complications of intravitreal methotrexate were cataract, corneal epitheliopathy and maculopathy and rarely limbal stem cell damage, optic atrophy and sterile endophthalmitis.[15,16] However, intravitreal methotrexate was found to be safe for other ocular diseases such as uveitis and diabetic retinopathy despite its side effects.[17]

In the present study, we aimed to investigate the early and long-term ultrastructural changes induced by high dose intravitreal methotrexate. We have preferred a different dose of methotrexate to evaluate its potential retinotoxicity, and we
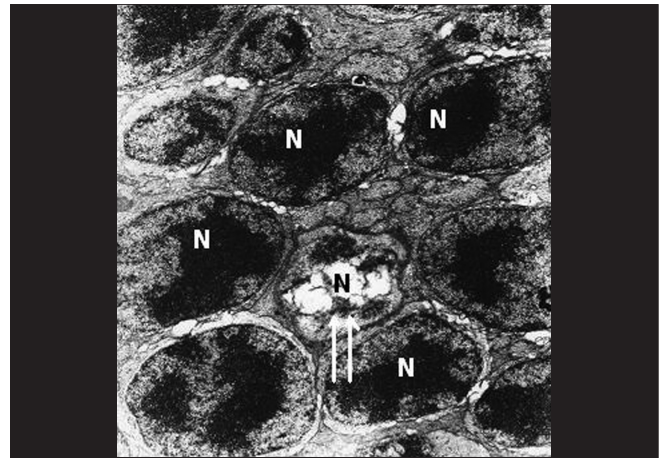
aimed to shed light on its potential side effects on human retina. The intact native rabbit retina was also studied to distinguish the structural alterations due to ischemia from changes secondary to surgical intervention and antineoplastic agents. The intact rabbit retina showed normal cellular structure supporting that the method of preparation was suitable and had caused no artifacts. The retinal damage in the control group could be occurred as a result of inflammatory response or acute increase of intraocular pressure after recurrent injections. As a matter of fact, in the study of Velez *et al.*, the absence of retinal damage in the control group was thought to be explained with previous aspiration of the anterior chamber fluid to control intraocular pressure before injection.[14] However, in the present study there was no difference of intraocular pressure measured before and after the injection.

Microscopic examinations showed intercellular edema and swelling of mitochondria in the study group from the first day after intravitreal injection. These changes were thought to be related to methotrexate. The thinning, disruption, or disorganisation of the cellular layers seemed to be reversible. However, microscopy also revealed permanent changes, which may be classified as retinotoxicity.

Methotrexate is one of the most currently and commonly used antineoplastic agent for the treatment of lymphomas. Its effects to the human retina are not clearly demonstrated and understood. The effect of histopathological changes on visual function also remains unknown. The major limitation of the study was the lack of electrophysiological investigation. The electrophysiological answer of the retina to the intravitreally administered drugs would give better information about ultrastructural damage.

To the best of our knowledge, this is the first study in the literature studying the histopathological changes secondary to the intravitreally administrated high dose methotrexate.

As a conclusion, the present study demonstrated early and long-term significant ultrastructural changes including mitochondrial disintegration, vacuolization, prominent edematous changes, nuclear disintegration, of all were in varying severity in almost all levels of the rabbit retina.

The results of the present study may give a basic idea about the effects of methotrexate on the ultrastructure of the retina, and the potential risk of increased dosage. However, further studies are needed in order to determine the histopathological effects of intravitreal methotrexate on human retina and on visual functions, and to make the interpretation of the laboratory results to the current clinical practice.

References

1. Peterson K, Gordon KB, Heinemann MH, DeAngelis LM. The clinical spectrum of ocular lymphoma. Cancer 1993;72:843-9.

2. Nelson DF. Radiotherapy in the treatment of primary central nervous system lymphoma. J Neurooncol 1999;43:241-7.

3. DeAngelis LM, Yalahom J, Thaler HT. Combined modality therapy for primary CNS lymphoma. J Clin Oncol 1992;10:635-43.

4. Frenkel S, Hendler K, Siegal T, Shalom E, Péer J. Intravitreal methotrexate for treating vitreoretinal lymphoma: 10 years of experience. Br J Ophthalmol 2008;92:383-8.

5. Strauchen JA, Dalton J, Friedman HA. Chemotherapy in the management of intraocular lymphoma. Cancer 1989;63:1918-21.

6. Blay JY, Conroy T, Chevreau C, Thyss A, Quesnel N, Eghbali H, *et al.* High dose methotrexate for the treatment for primary cerebral lymphomas: Analysis of survival and late neurologic toxicity in a retrospective series. J Clin Oncol 1998;16:864-71.

7. Ferreri AJ, Dell'oro S, Reni M, Foppoli M, Anzalone N, Govi S, *et al.* Questions and answers in the management of primary central nervous system and ocular ymphomas. J Haematol 2003;88:1063-8.

8. Mason JO, Fisher DH. Intrathecal chemotherapy for recurrent central nervous system intraocular lymphoma. Ophthalmology 2003;110:1241-4.

9. de Smet MD, Stark-Vancs V, Kohler DR, Smith J, Wittes R, Nussenblatt RB. Intraocular levels of methotrexate after intravenous administration. Am J Ophthalmol 1996;121:442-4.

10. Chan CC, Wallace DJ. Intraocular lymphoma: Update on diagnosis and management. Cancer Control 2004;11:285-95.

11. de Smet MD, Vancs VS, Kohler D, Solomon D, Chan CC. Intravitreal chemotherapy for the treatment of recurrent intraocular lymphoma. Br J Ophthalmol 1999;83:448-51.

12. Ericson L, Karlberg B, Rosengren BH. Trials of intravitreal injections of chemotherapeutic agents in rabbits. Acta Ophthalmol 1964;42:721-6.

13. Vernot J, Peyman GA, Gailitis R, Fiscella R, Woodhouse M, Weinberg D. Effects of selected repeated intravitreal chemotherapeutic agents. Int Ophthalmol 1985;8:193-8.

14. Velez G, Yuan P, Sung C, Tansey G, Reed GF, Chan CC, *et al.* Pharmacokinetics and toxicity of intravitreal chemotherapy for primary intraocular lymphoma. Arch Ophthalmol 2001;119:1518-24.

15. Smith JR, Rosenbaum JT, Wilson DJ, Doolittle ND, Siegal T, Neuwelt EA, *et al.* Role of intravitreal methotrexate in the management of primary central nervous system lymphoma with ocular involvement. Ophthalmology 2002;109:1709-16.

16. Vicuna-Kojchen J, Frenkel S, Siegal T, Shalom E, Chowers I, Péer J. Maculopathy in patients with primary CNS lymphoma treated with chemotherapy in conjunction with blood-brain barrier disruption. Br J Ophthalmol 2008;92:231-5.

17. Hardwig PW, Pulido JS, Erie JC, Baratz KH, Buettner H. Intraocular methotrexate in ocular diseases other than primary central nervous system lymphoma. Am J Ophthalmol 2006;142:883-5.