Evaluation of toxicity after periocular and intravitreal administration of carboplatin in rabbit eyes

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Abstract

The aim of this study was to characterize the extent of toxicity of focal carboplatin administration and to identify the dose limiting toxicity in rabbit eyes depending on administered concentrations. New Zealand white male rabbits (n = 18) were treated with 1 of 3 regimens: a single periocular injection of 15 mg of carboplatin (group I), a single periocular injection of 30 mg of carboplatin (group II) and a single transcorneal intravitreal injection of 0.05 mg of carboplatin (group III). Ophthalmologic examinations and vitreous samplings were performed under dissociative anaesthesia at regular intervals during next 2 (groups I and III) or 3 (group II) weeks. Carboplatin concentrations in vitreous samples were assessed by atomic absorption spectroscopy. At the end of experiments, all rabbit eyes were obtained for histopathologic examination. Clinical and histological evidence of toxicity was graded into four grades according to anatomical structures of the rabbit eye. The dose limiting toxicity was reached in group II after periocular injection of 30 mg of carboplatin and in group III after intravitreal injection of 0.05 mg of carboplatin. No systemic toxicity was observed in any group. Focal carboplatin administration may decrease systemic exposure to this cytotoxic drug in the retinoblastoma treatment. This moreover suggests that focal carboplatin administration is a promising approach and challenge for advanced retinoblastoma chemotherapy.

Carboplatin chemotherapy, New Zealand White Rabbits, intravitreal treatment, periocular toxicity, retinoblastoma

Carboplatin is now routinely administered in the treatment of intraocular retinoblastoma as a part of systemic chemotherapy. Although retinoblastoma is the most common primary intraocular malignancy in children, it has seldom been reported in animals. Two cases were reported in dogs (Syed et al. 1997; Jensen et al. 2003), one in a llama (Fugaro et al. 2005) and one in a gelding (Knottenbet al. 2007). Enucleation of the affected eye was used in these cases due to large tumours. To overcome this, systemic carboplatin chemotherapy may be used in large tumours with diffuse vitreous and subretinal seeds (Reese-Ellsworth group 4 and 5 tumours). This would lead to significant morbidity and potential mortality through drug-related toxicities including neutropenia and bacterial infections, anaemia, thrombocytopenia and organ toxicities including ototoxicity, nephrotoxicity and hepatotoxicity (Benz et al. 2000). Local administration of chemotherapeutics may be an option to minimize these side effects. Carboplatin is one of the few chemotherapeutic agents used as an alternative to systemic chemotherapy for retinoblastoma. *In vivo* studies in primates (*Macaca fascicularis*) and in New Zealand white rabbits treated by subconjunctivally administered injections of carboplatin at doses of 10 mg or lower indicated

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no evidence of toxic effects or changes of behaviour (Mendelsohn et al. 1998; Hayden et al. 2004). Rabbits treated with unilateral subconjunctival injection of 12.2 ± 1.0 mg/ml of carboplatin (Paraplatin) in saline solution or with 25.1 ± 7.7 mg/ml of subconjunctival carboplatin in fibrin sealant also induced no tissue damage and no abnormal findings on electroretinography after three weeks (Pardue et al. 2004). Nevertheless, most of these studies described short-term toxicity occurring up to 48 h after treatment. *In vivo* studies using LHβ-Tag transgenic mice showed that low-dose sealant-treated group (0.66 mg of carboplatin) with a single subconjunctival injection of carboplatin resulted in mild transient periorbital oedema but with no evidence of toxicity. High-dose sealant-treated eyes of transgenic mice (1.23 mg of carboplatin) induced significant local toxicities, including severe periorbital inflammation, enophthalmia and cataract (Van Quill et al. 2005). Another described modality was intravitreal transscleral injection of chemotherapeutic agents (Kaneko and Suzuki 2003). Transient increase in intraocular pressure without any serious toxic damages was observed.

The aim of this study was to use a non-tumour-bearing rabbit's model to determine the extent of long-term toxicity of focal carboplatin administration and to identify the dose-limiting toxicity depending on used carboplatin concentrations.

We hypothesized that a very low dose of carboplatin administered intravitreally may result in higher concentrations of carboplatin in the vitreous humour with no extraocular toxicity than periocular administration of a higher dose.

Materials and Methods

Animals and treatment

Male New Zealand white rabbits (Anlab, Prague, Czech Republic) were handled according to the regulation Nr. 207/2004 of the Ministry of Agriculture of the Czech Republic regarding the use of animals in experiments. *In vivo* experiment was approved by the ethics committee for animal welfare of Charles University, 2nd Faculty of Medicine. The animals were anaesthetized intramuscularly with a mixture of ketamine hydrochloride (30-50 mg/kg, Narketan 10 a.u.v. inj., Vétoquinol, Lure Cedex, France) and xylazine hydrochloride (5 mg/kg, Rometar 2% a.u.v. inj., Spofa, Prague, Czech Republic) throughout the experiment. Before the treatment and sampling, conjunctival sacks were disinfected by 3 ml of 1% Povidone-Iodine solution (Betadine, EGIS Pharmaceuticals Ltd., Budapest, Hungary) and anaesthetized by topical oxybuprocaine eye drops (0.4%, Benoxi gtt., Unimed Pharma, Bratislava, Slovakia).

For the *in vivo* studies, 18 rabbits were included and divided into six animals per group. Groups I and II received a single periocular injection of carboplatin (15 mg in 1.5 ml solution and 30 mg in 1.5 ml solution, respectively) with a 25-gauge needle in the superior temporal quadrant of the right eye. Group III received a single transcorneal intravitreal injection of carboplatin (0.05 mg in 0.1 ml solution). Absence of leakage from the injection sites was verified. Left eyes were not injected as being control eyes.

Commercial Carboplatin-Teva (Pharmachemie B.V, Haarlem, Netherlands) is a colourless fluid containing the additional substances mannitol and sterile water for injection. Glucose 5% was added to prepare concentrations of 10 mg/ml, 20 mg/ml and 0.5 mg/ml.

Sampling schedule

Vitreous and blood samples were taken at 0, 1, 2 and 6 h, in 1, 2 and 7 days and in 2 (groups I and III) or 3 (group II) weeks after the injection. We have extended the period of observation in group II due to evaluating the occurrence of retinal toxicity that developed gradually. After 2 weeks, no histopathological evidence of retinal toxicity was found in group I. At each withdrawal, 150 µl to 400 µl of vitreous humour were aspirated from the vitreous chamber with a 25-gauge needle inserted transcorneally by the limbus through the anterior chamber and the base of the iris. Samples of vitreous humour were placed in plastic tubes and frozen at -80 °C until analysis. Blood samples were drawn from the peripheral auricular vein and placed in tubes with ammonium heparinate. The blood was centrifuged at 10 868 *g* for 10 min (Centrifugal Machine Eppendorf 5810R) to isolate blood plasma.

At each sampling time, the rabbit's eyes underwent microscopic and indirect fundoscopic examinations to observe any signs of inflammation, media opacification and toxicity. Intraocular pressure was measured by TONO-PEN XL Applanation Tonometer.

Electrothermal atomic absorption spectroscopy

Carboplatin, platinum metabolites and protein conjugates were determined in ocular fluids and plasma as total soluble platinum (Pt) by electrothermal atomic absorption spectrometry as described previously (Kukacka et al. 2008; Pochop et al. 2010).

Histological examination

After euthanasia, performed by exsanguination from the carotid arteries, eyes were enucleated together with eyelids and retrobulbar tissues and fixed in 10% formalin. The specimens were dehydrated by a graded series of ethanol and embedded in paraffin. Sections (5 μm in thickness) were stained with haematoxyline and eosin and examined by an experienced pathologist.

Toxicity grading

Clinical evidence of toxicity was classified into four grades according to the anatomical structure of the rabbit eye. Grade 1 included signs of periocular toxicity, regarding to eyelids and periocular orbital contents. We searched for periorbital and lid oedema, proptosis, any inflammatory and fibrotic changes and loss of lashes. Grade 2 was designed as scleral toxicity. Carboplatin toxicity affecting the conjunctiva, the sclera and the cornea was grouped in this grade. The signs of toxic conjunctivitis or widespread inflammation or necrosis involving any part of sclera presented also this grade of toxicity. Further, we searched for any depressed epithelial defects which stain with fluorescein, any swollen epithelial cells seen unstained, any corneal infiltrates, loss of normal corneal lustre and subepithelial ingrowth of fibrovascular tissue from the limbus. Remaining substructures of the rabbit's eye with clinical evidence of carboplatin toxicity formed grade 3. We searched for any inflammatory changes, hyphema and retinal detachment. Grade 4, systemic toxicity, included all clinical and histological evidence of systemic carboplatin toxicity on rabbits. We searched for any loss of the rabbit's weight, changes of the rabbit's behaviour and if necessary we examined the whole body histopathologically. Grades 1 and 2 were usually not considered for dose limiting toxicity. Local carboplatin treatment should not be broken in these cases because of the minimal effect on visual function. Grades 3 and 4 were designed as dose limiting toxicity.

Results

The maximum achieved vitreous and plasma concentrations of carboplatin in each group are listed in Tables 1 and 2. Plasma concentrations of carboplatin in group III were not measurable. Assessment of carboplatin concentrations in plasma and vitreous humour by electrothermal atomic absorption spectroscopy revealed higher concentrations of carboplatin in the vitreous humour after the transcorneal intravitreal injection of carboplatin compared to the periocular administrations. In case of the periocular carboplatin administrations, achieved concentrations in the vitreous humour were lower than the achieved concentrations in plasma.

Table 1. Maximal achieved platinum concentration and time of its achievement in the vitreous humour of rabbits after periocular and intravitreal administration of carboplatin

Treatment modality	Time (h)	Vitreous platinum (μg/l)
Periocular group I	2	539.5 ± 278.0
Periocular group II	6	368.3 ± 109.0
Intravitreal group III	1	40285.3 ± 7436.0

Data are mean ± SD

Table 2. Maximal achieved platinum concentration in plasma of rabbits after periocular and intravitreal administration of carboplatin

Treatment modality	Time (h)	Plasma platinum (μg/l)
Periocular group I	1	3668.3 ± 120.0
Periocular group II	1	5833.3 ± 134.5
Intravitreal group III	non-measurable	

Data are mean ± SD

A healthy New Zealand white rabbit's eye is covered by a clear conjunctiva consisting of epithelial cells and underlying basement membrane that overlays the sclera (white part of the eye). In albino rabbits, the conjunctival injection is visible, mainly along the limbus. The cornea is the transparent, lustrous and smooth front part of the eye. The iris colour is pinkish-white due to lack of pigmentation. The lens is a transparent, biconvex structure in the rabbit eye similar to the human lens. The retina lacks pigment and contains conspicuous choroidal vessels and large, oval optic nerve papilla in its nasal part. Clinical findings are summarized in Table 3. Grade 1 periocular toxicity was observed after periocular carboplatin injection. All rabbits in group I developed mild, transient periorbital oedema that disappeared spontaneously in 2 h. Besides, only three rabbit's eyes demonstrated

Table 3. Occurrence and type of observed ocular toxicity in rabbits after periocular and intravitreal administration of carboplatin

	Group I (n = 6)	Group II (n = 6)	Group III (n = 6)
Grade 1 - periocular toxicity			
Periorbital edema	6	3	0
Eyelid necrosis	0	2	0
Grade 2 - scleral toxicity			
Corneal vascularisation	1	2	0
Subconjunctival haemorrhage	2	2	0
Grade 3 - ocular toxicity			
Hyphema	4	3	3
Traumatic cataract	5	1	1
Haemophthalmus	3	3	5
Focal retinal atrophy	0	1	5

periorbital oedema in group II, but this kind of oedema was marked and disappeared within 48 h after injection.

Clinical evidence of more significant local toxicity was observed among rabbits treated with 30 mg of carboplatin periocularly. Two in this group II developed eyelid necrosis 7 days after injection, which was designated as dose limiting toxicity (Plate XY, Fig 1a,b). The right eyes of these two rabbits also developed periorbital oedema during the first 48 h. Two eyes in group I and one eye in group II manifested grade 2 scleral toxicity in corneal vascularization that was observed mostly in the inferior half of the cornea and not only in the region of the puncture. We observed subconjunctival haemorrhage in two of 6 eyes of both groups I and II. This is in compliance with periocular administration, which exposes the conjunctiva and the cornea to a high concentration of carboplatin.

Hyphaema, traumatic cataract (Plate IV, Fig. 1c) and haemophthalmus occurred in all groups after repeated sampling. Repeated sampling of 150 µl to 400 µl of vitreous humour from the vitreous chamber can cause hypotonia of the rabbit's eyes. Normal intraocular pressure of the rabbit's eye is 8–10 torr, measured by TONO-PEN XL Applanation Tonometer. After 6 h, intraocular pressure was non-measurable in rabbits.

There was no evidence of grade 4 systemic toxicity in any group as measured by weight loss. Mean body weight loss among rabbits was manifested during the first 48 h of the experiment but we observed gradual body weight gain in all rabbits until 2 weeks (group I and III) and until 3 weeks (group II). No rabbit lost more than 10% of body weight, with the exception of one in each group which lost less than 15% of body weight over the first 48 h.

No evidence of toxicity was verified on histopathological examination of group I (Table 3). In contrast to group I, one of 6 eyes in group II and five of 6 eyes in group III manifested histopathological evidence of toxicity. We observed focal retinal atrophy in these cases (Plate IV, Fig. 1d).

Disscusion

To date, there are no reports of retinal toxicity in an animal model induced by periocular carboplatin therapy. Focal retinal atrophy in group II probably accords with the anatomy of the rabbit retina. Rabbit retinal vasculature emerges from the large, oval disc into nasal and temporal branches, extending until a short distance behind the equator. Outside this area the retina is avascular (Davis 1929). Lesser amount of blood vessels could induce higher sensitivity of metabolic active retinal cells to carboplatin. Repeated sampling caused short-term hypotony of rabbit's eyes in this study. This intraocular pressure fluctuation could

contribute to reduce the ganglion cell layer and to develop the focal retinal atrophy. A limitation of this study is the fact that we did not perform a functional examination of the retina.

Higher carboplatin concentrations achieved in plasma compared to vitreous humour could be related with anatomical-physiological differences between rabbit and primate eye and orbit. The orbital contents in rabbits are constituted of the venous sinus, surrounding the alkaline Harder's gland, that serves to lubricate the third eyelid, and of the extra-ocular muscles (Davis 1929). Periocular carboplatin injected in aqueous solution could diffuse quickly into the surrounding orbital space and penetrate the venous sinus rather than the sclera. Differences in the regional choroidal blood flow in rabbits and monkeys contribute to this hypothesis. Peripheral choroidal blood flow is greater in rabbits than in monkeys, by which central choroidal blood flow is preferred, conversely (Nork 2006). Greater peripheral choroidal blood flow could induce a bias toward systemic absorption in the rabbit model. Although we used high concentrations of periocular injected carboplatin, we observed no systemic toxicity.

Periocular administration of carboplatin exposed particulary extraocular tissues to high transient concentrations of carboplatin. The absence of a large mass of fat around the globe was the reason for observing no evidence of fat necrosis in rabbit model as it was observed and published by Abramson (1999) and by Mulvihill (2003). Although the rabbit orbital space is made up largely of the extra-ocular muscles (Davis 1929), no decreased ocular motility was noted during the period of 2 and 3 weeks. Contrary to described findings in an animal model, periocular carboplatin injection induced orbital fibrosis, fat necrosis and atrophy of the optic nerve in children with retinoblastoma (Schmack et al. 2006; Kim et al. 2010). Different anatomical and physiological findings in children orbit and eye may lead to higher depot of periocular injected carboplatin, inducing toxic side-effects.

In group III, 0.05 mg of carboplatin developed histopathological evidence of retinal toxicity. This is equivalent to vitreous concentrations of 36 μg/ml related to 1.4 ml of rabbit vitreous volume (Kane et al. 1981). We used previously proven approach through the limbus, anterior chamber and iris root (Pochop et al. 2010) that seems to be safer than the traditionally used injection through the posterior part of the ciliary body. There may be a smaller risk of clusters of tumour cells implanted through the eye wall extraocularly.

Other observed findings like corneal vascularization, hyphema, cataractous changes and vitreous haemorrhage may be the result of repeated mechanical irritation and incautious puncture than carboplatin toxicity and cannot be identified as grade 3 ocular toxicity.

The dose limiting toxicity was reached in groups II and III. In group I no evidence of local or fundoscopic toxicity was found 2 weeks after carboplatin injection. Local carboplatin administration led to no evidence of systemic side effects in rabbits. Carboplatin administered intravitreally resulted in higher concentrations of carboplatin in the vitreous humour compared to periocular administration, which may be promising in the treatment of "seeding retinoblastoma". We proved higher sensitivity of the retina compared to other periocular tissues to the toxic effects of carboplatin.

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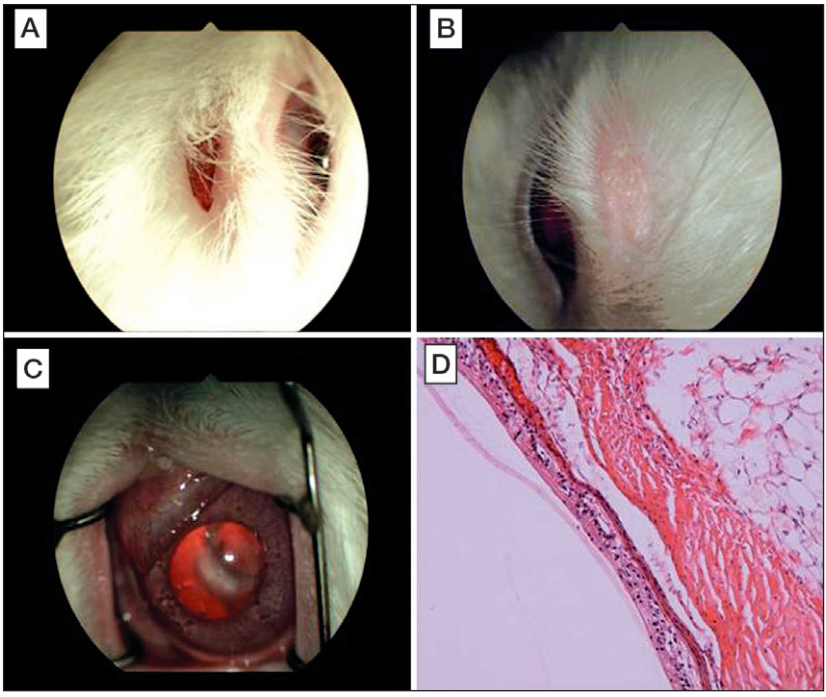

Fig. 1. Carboplatin side effects on the rabbit eye after intravitreal and periocular administration

A, B - eyelid necrosis after periocular administration of 30 mg of carboplatin, C - traumatic cataract after repeated sampling (rabbit after intravitreal injection of carboplatin) , D - reduction of ganglion cells with small cystoid spaces in the inner nuclear layer of the retina after intravitreal injection of carboplatin