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AN IN VIVO STUDY OF INTRAVITREAL RANIBIZUMAB FOLLOWING SUBRETINAL INOCULATION OF RB CELLS IN RABBITS' EYES

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SUMMARY

Aim: This study aimed to determine the effects of a single intravitreal ranibizumab injection in rabbits induced with retinoblastoma (RB).
Material and Methods: RB was induced in six New Zealand white rabbits by subretinal injection of a cultured WERI-RBb-1 cell line into the right eye. After six weeks, Group A (n = 3) was given intravitreal ranibizumab injection (0.3mg in 0.03ml) and Group B (n = 3) was the control. Baseline and serial clinical examinations were performed on days 1, 3, 6, 12, 15, 18 and 21. The right eyes were enucleated for both groups on day 21 for histopathological examination.
Results: The rabbits in both groups developed intraocular lesions which was detectable clinically at one-week post-tumor inoculation. The tumor grew slowly without spontaneous regression. After the animals in Group A were given an intravitreal ranibizumab injection, regression of the tumor was detected clinically, while the tumor in Group B continued to grow slowly. Histopathological findings confirmed the presence of a tumor that closely resembled features of poorly differentiated human RB cells. At the end of 21 days, the size of the tumor was larger in Group B in comparison to Group A. However, the treated group also developed a focal area of retinal hyperplasia. There was no significant side effect of ranibizumab injection except temporary high intraocular pressure immediately post-injection, which was relieved after paracentesis.
Conclusions: Intravitreal ranibizumab is a potential treatment for RB. It is an effective therapy with a tolerable safety profile in this animal experimental study.
Keywords: retinoblastoma; intravitreal injection; anti-vascular endothelial growth factor; ranibizumab; animal experimental study

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INTRODUCTION

Retinoblastoma (RB) is the most common primary intraocular tumor in childhood [1], with an incidence of 1 in 15,000 to 20,000 live births [2]; it represents approximately 4% of paediatric malignancies [3]. There is no predilection in terms of gender, race, and socioeconomic status [4]. RB is a highly malignant intraocular tumor that occurs in childhood that requires accurate diagnosis and prompt treatment. The main aim of treatment is to save the patient's life [3]. Salvation of the globe and vision are the secondary and tertiary goals, respectively.

Enucleation is the most common surgical modality for advanced RB, especially when there is a concern about the potential spread to the optic nerve, choroid, or orbital cavity [5]. The management of RB has evolved significantly during the 20th century due to general developments in medicine, along with the specialised development of ophthalmic techniques, such as laser photocoagulation and cryosurgery. The most current method for RB treatment is chemoreduction followed by focal consolidation therapy [6]. It has largely replaced external-beam radiotherapy as the treatment of choice for bilateral RB.

The International Classification of Retinoblastoma is useful in guiding clinicians to select the most appropriate treatment methods and to assist in the predication of the success of chemoreduction and focal treatment [7]. Treatment options for local disease include laser, cryotherapy, plaque brachytherapy and more recently, intravitreal, and intra-arterial chemotherapy with Melphalan [8]. Intra-arterial chemotherapy has shown promising effect [9,10]. The most recent clinical trials including intravitreal chemotherapy appear to offer a safe and efficient salvage option [11,12]. A combination of intravitreal and intravenous chemotherapy has shown promising outcome in RB with vitreous seedling [13]. Due to the poor response of advanced RB tumors to conventional therapies and the inherent toxicities of current chemotherapeutic agents, development of local adjuvant therapies is imperative [14].

Previous studies have shown that vascular targeting therapies, such as 2-deoxyglucose (2-DG) [15] and the anti-vascular endothelial growth factor (VEGF) therapeutic antibody (bevacizumab) [16] have promising effects as adjuvant therapies in RB. There were very limited reported studies, if any, on the in vivo effects of anti-VEGF on RB tumor growth.

This pilot study aimed at analysing the changes of inoculated RB tumor cells in the eye following intravitreal ranibizumab treatment.

MATERIAL AND METHODS

An animal experimental study was conducted using six New Zealand albino rabbits, weighing around 3 kg, at the Animal Research and Study Centre (ARASC), Universiti Sains Malaysia (USM), Health Campus, Kelantan, Malaysia. This experimental study received ethical approval from the Animal Ethics Committee, Health Campus, Universiti Sains Malaysia (USM/Animal Ethics Approval/2012/ (81) (429)) and was conducted according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmic and Vision Research. There were 4 phases to the study, namely: (1) WERI-RBb-1 cell culture and tumor induction, (2) intravitreal injection of ranibizumab, (3) serial clinical examination and (4) histopathological examination.

Six New Zealand albino rabbits free from any ocular diseases were kept, fed and cared for at ARASC, USM. The animals were divided into two groups: Group A (treated group n = 3) received intravitreal ranibizumab injection and Group B (control group n = 3) did not receive any treatment.

Phase 1: WERI-RBb-1 cell culture and tumor induction

Tumor cells culture originated from a human RB WERI-RBb-1 cell line [ATCC HTB-169; American Type Culture Collection (ATCC), Manassas, Virginia, USA] were cultured. Standard cell culture procedures were performed as described. Tumor cells lines were grown in a tissue culture flask using RPMI 1640 medium (ATCC, catalogue no. 30-2001, Manassas, Virginia, USA) supplemented with 10% foetal calf serum, 2 mM glutamine and two antibiotics, streptomycin (50 µg/ml, GIBCO, Life technologies, Waltham, Massachusetts, USA) and penicillin (50 U/ml, GIBCO, Life Technologies, Waltham, Massachusetts, USA), at 37 °C and 5% CO_2.

Prevention of tumor regression

To prevent spontaneous tumour regression, the rabbits received daily intramuscular injections of cyclosporin A (CsA) (Sandimmune 50 mg/ml; Novartis Pharmaceuticals, Cambridge, MA, USA) (15 mg/kg per day) 3 days prior to tumour cell inoculation. Following inoculation, intramuscular CsA administration were continued for 4 weeks at a reduced dose of 10 mg/kg per day. The CsA doses were adjusted weekly according to each animal's body weight. During the follow-up period, the animals were monitored weekly for signs of CsA toxicity, such as gingival hypertrophy, drooling, diarrhoea and weight loss, and no toxicity was observed.

Tumor inoculation

Baseline clinical examination, including general and ocular examinations, was conducted before the inoculation procedure. The WERI-RBb-1 cell inoculation was performed under sedation with intramuscular injection of ketamine (35 mg/kg body weight) and xylazine (5 mg/kg body weight). Tumour cells were inoculated by subretinal injection into the right eyes of each rabbit as follows: the right eye of each rabbit was dilated and anesthetized by application of 0.1% tropicamide and 2.5% phenylephrine, respectively. The procedure was performed using an aseptic technique.

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Hypromellose ointment (Gen Teal) was applied onto the right cornea, and a 22x40 mm microscope slide coverslip was then applied on the cornea for direct visualisation of the fundus using a surgical microscope. A 30-gauge needle attached to a 1 ml syringe containing a freshly prepared suspension (30 µl) of cultured human WERI-Rb-1 RB cells (1.5×106 cells) was used to inject the tumor cells at 11 o'clock and 2 mm from the limbus. The needle was introduced from the sclera through the site and advanced toward the retina, without breaching the retinal layer. Gentle pressure was applied, and the cells were injected into the subretinal space. Post-injection, the eye was examined for any signs of retinal detachment and vitreous haemorrhage. Topical moxifloxacin was instilled into the right eye post-procedure.

Phase 2: Intravitreal injection of ranibizumab

This procedure was conducted at the sixth week post-tumor injection under sedation using a sterile technique. The rabbit's pupils were dilated using 0.1% tropicamide and 2.5% phenylephrine, respectively. The rabbits were sedated with intramuscular injection of xylazine and ketamine. Group A received 0.3 mg (0.03 ml) of intravitreal ranibizumab injection; Group B was not given treatment. The injection site was 2 mm inferotemporal from the limbus. Topical moxifloxacin was given before and after the procedure.

Phase 3: Serial clinical examination

Serial clinical assessment to evaluate the progress of tumor growth, the adverse effects of CsA, such as gingival hypertrophy, drooling of saliva, diarrhoea and weight loss, and the effects of tumor induction, such as cachexia, concomitant infection, or any clinical evidence of distance metastases, such as a palpable abdominal mass was conducted. The examination was performed under sedation. Retinal growth of variable sizes was observed 6 weeks after the tumor injection in rabbits from both groups.

The rabbits were examined before the intraocular injection (day 0) to determine the baseline size of the tumor. This was followed by serial examinations on days 1, 3, 6, 12, 15, 18 and 21 to evaluate the animals for signs of tumor regression, such as reduction in the tumor size and changes in the pattern of tumor regression. Ocular examination was performed using binocular indirect ophthalmoscopy, and fundus videography using a smartphone digital camera (Procam v2) and a 30 D lens. Signs of complications related to the procedure, such as ocular infection, and ocular-related toxicity were documented. General examination was also performed for signs of systemic infection and drug-related adverse effects. Image J software was applied to available images retrospectively. Tumor size was defined as the largest diameter obtained from Image J software.

Phase 4: Enucleation and histopathological examination

Three weeks post-injection of ranibizumab, the rabbits were sacrificed with sodium pentobarbital (65 mg/kg). The right eyes were enucleated and fixed immediately in 10% formalin solution. The samples were transported to the Pathology Laboratory, USM for paraffin embedding. The eyes were serially sectioned and stained with haematoxylin-eosin. Light microscopic examination was performed on all the histopathologic sections to evalu-

Table 1. Detailed comparison of tumor growth based on clinical observations

	Group A (Treated)			Group B (control)		
	1	2	3	4	5	6
Tumor type Endophytic (+) Exophytic (++) Mixed (+++)	+++	+++	+++	+++	+++	+++
Vitreous involvement Localized (+) Diffuse (++)	+	+	+	+	+	+
Optic disc infiltration No (-) Yes (+)	-	-	-	-	-	-
Lens Normal (+) Retrolental adhesion (++) Cataract (+++)	+	+	+	++	+	++
Anterior Chamber involvement No (-) Yes (+)	-	-	-	-	-	-
Contralateral eye involvement No (-) Yes (+)	-	-	-	-	-	-

Note: The clinical data are expressed as positive (+) and negative (-) symbols

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ate the presence and extent of the tumor and to observe evidence of extra-scleral extension and optic nerve involvement of the tumor.

Statistical Analysis

For categorical data, frequency and percentage were measured. Fisher's exact test was used to compare the clinical features of the tumors induced in the treatment group (Group A), the presence of tumor regression and to compare the side effects of ranibizumab (SPSS Statistics v21; IBM, Armonk, NY, USA).

RESULTS

A detailed comparison of clinical observations is listed in Table 1. All the rabbits in both groups developed multiple round greyish intraocular tumour-like lesions in the right subretinal spaces, and/or retinal and vitreous cavities which were detectable through fundus examination 1 week after tumour inoculation (Figure 1). Tumor size was evaluated qualitatively and quantitatively in Table 2 and Table 3. Mixed tumor type (presence of both endophytic and exophytic lesions) with a localised tumor in the vitreous adjacent to the lesion was observed in all six rabbits, but no sign of disc infiltration was seen (Table 1). No obvious tumor vasculature or neovascularisation was noted clinically. Two rabbits in Group B (control) were observed to have retrolental adhesion on the posterior part of the lens (Table 1). No anterior chamber or contralateral eye involvement was observed in Group A or Group B. The tumor continued to gradually increase in size without spontaneous regression as shown in a representative rabbit (Figure 1). Clinically, all the tumors were found to be less than half the size of the disc size.

Table 2. Growth of tumor from week 1 to 6 after tumor inoculation in rabbit 1

Time	Tumor diameter (μm)
Week 1	180
Week 2	250
Week 3	670
Week 4	1020
Week 5	1410
Week 6	1700

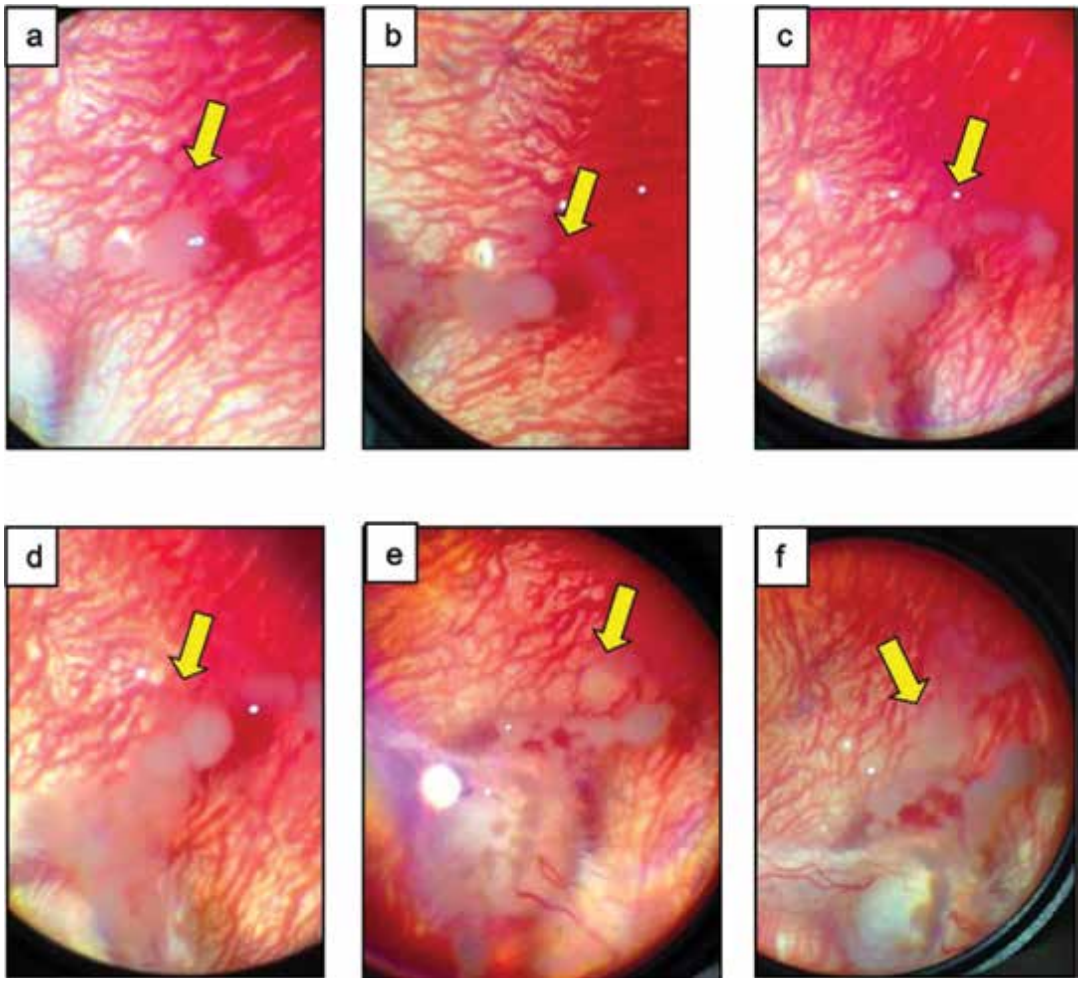

Figure 1. Weekly fundus examination of rabbit 1 after tumor inoculation showing progression of tumor from week 1 to 6 (a–f)

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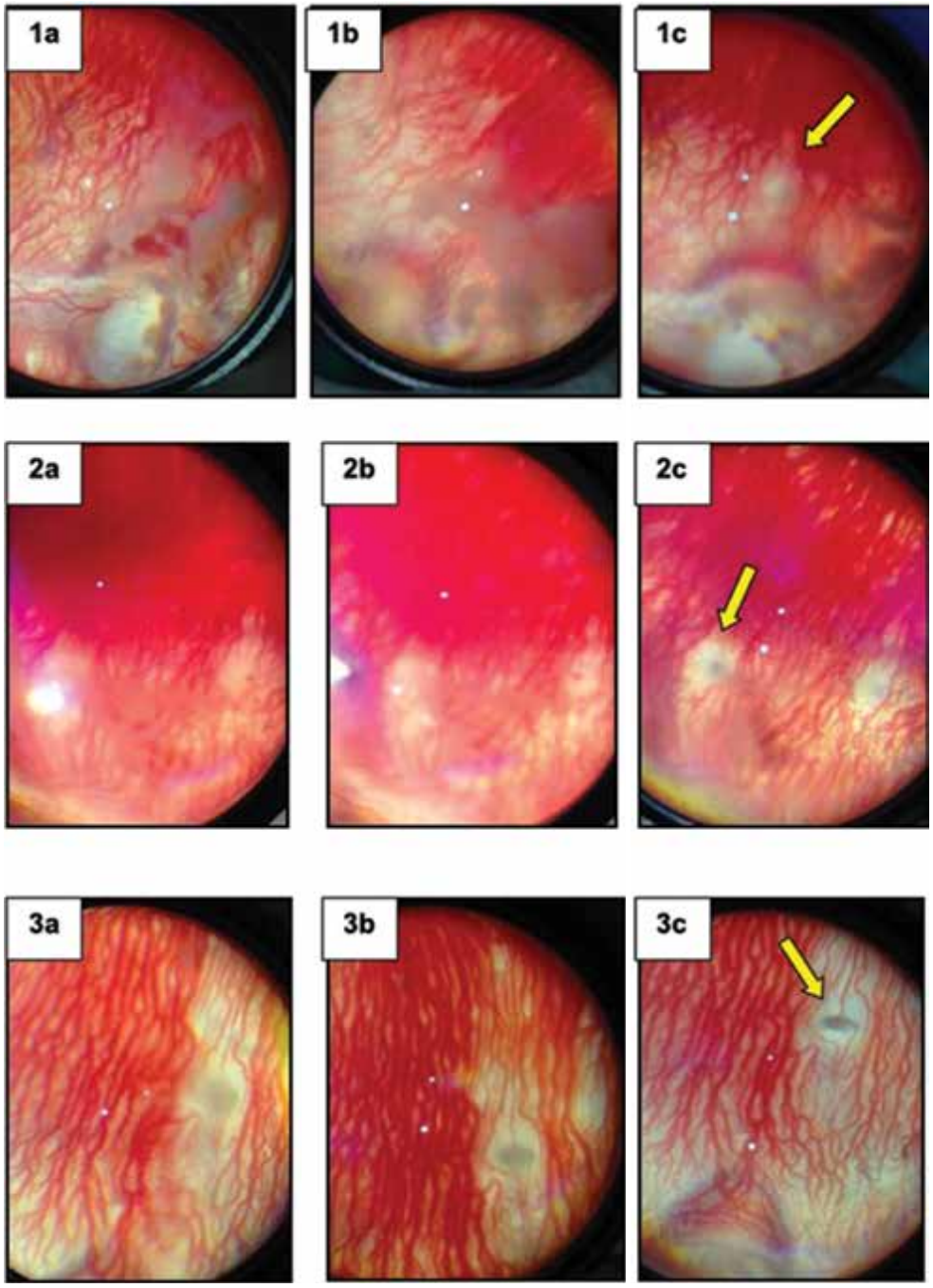

Figure 2. Fundus photographs of three subjects from group A
1a, 2a & 3a: Before ranibizumab injection (rabbit 1–3)
1b, 2b & 3b: Post ranibizumab injection 1 week (rabbit 1–3)
1c, 2c & 3c: Post ranibizumab injection 3 weeks (rabbit 1–3)
Arrow: Qualitative assessment of retinal lesion showing clearer margin and less dense

Table 3. Tumor diameter at week 6, 7 and 9 according to each rabbit in both groups

Time*	Tumor diameter (µm)					
	Group A (Treated)			Group B (Control)		
	Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	Rabbit 6
Week 6	810	150	90	1020	730	920
Week 7	710	140	40	1130	790	1080
Week 9	500	50	20	1350	960	1270

**Week 6 – post tumor inoculation, pre-intravitreal ranibizumab, Week 7 & 9 – post tumor inoculation, post-intravitreal ranibizumab*

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Tumor size changes

The margin of the lesions became well demarcated and less dense 1 week after intravitreal ranibizumab injection (Figure 2). Table 3 showed the largest diameter of the retinal lesion of all 6 rabbits. In fact, one of the rabbits started to show reduction in size of the lesion at day 3 and remarkable changes were seen on day 6 post-treatment (Table 4). However, there was no sign of lesion regression in the vitreous cavity. The lesions in Group B (control) showed a slow increase in size until the end of the study without any sign of improvement.

Histopathological examination

Gross examination revealed the presence of whitish tumor clumps in the vitreous cavity, with an area of retrolental

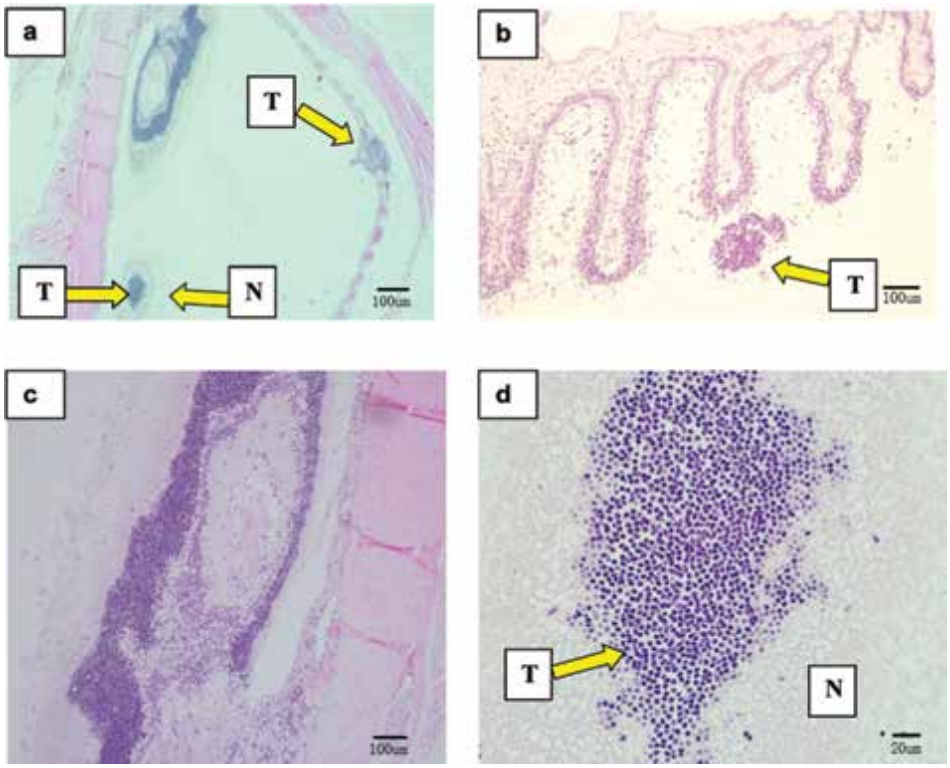

Figure 3. Tumor in the group A
a) Small area of tumor cells (T) with necrosis (N) (H&E x10)
b) Presence of tumor in the vitreous
c) Marked retinal hyperplasia
d) Appearance of tumor cells in treated group with surrounding area of necrosis (H&E x40)

Table 4. Tumour regression based on clinical observations

Time	Tumour regression	Group A (Treated) n = 3/group	Group B (Control) n = 3/group	p-value[a]
Day 1	Yes No	0 3	0 3	1.000
Day 3	Yes No	0 3	0 3	1.000
Day 6	Yes No	1 2	0 3	1.000
Day 12	Yes No	1 2	0 3	1.000
Day 15	Yes No	3 0	0 3	0.100
Day 18	Yes No	3 0	0 3	0.100
Day 21	Yes No	3 0	0 3	0.100

[a]*Fisher exact test, all assumptions are met*

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adhesion in all the rabbits from both groups. Grossly, the retinal lesion was not prominent with no sign of vascularisation in all samples from the right eye and the left eyes appeared normal. Microscopic examination revealed the presence of poorly differentiated tumor cells with scanty cytoplasm in the subretinal, retina and vitreous cavity, resembling human RB cells in all the rabbits from both groups. There were no typical rosettes or fleurettes with no sign of vascularisation or calcification in the tumor area. The size of the tumor was larger in the Group B (control) rabbits than the Group A (treated group) rabbits. The tumor cell area was small and mainly found in the vitreous cavity (Figure 3) and (Figure 4). Two of the animals from Group A also exhibited a focal area of retinal hyperplasia. No anterior segment involvement was observed in any of the rabbits.

Side effects of ranibizumab

All the rabbits developed high intraocular pressure (IOP) immediately post-intravitreal ranibizumab injection based on digital pressure estimation. Digital pressure returned to normal after paracentesis was conducted, and this clinical estimation was done by simultaneously comparing it with the contralateral eye. There was no other side effect, such as eye redness, retinal detachment and endophthalmitis, in all the rabbits in both groups. However, all the rabbits developed a reduction of appetite based on a decrease in the number of pallets consumed per day; they also experienced weight reduction. However, the weight loss did not exceed more than 10% from the animal's initial weight. The rabbits in both groups developed mild gingival hypertrophy and drooling of saliva due to CsA toxicity, which resolved after reducing the dose to 10 mg/kg/day.

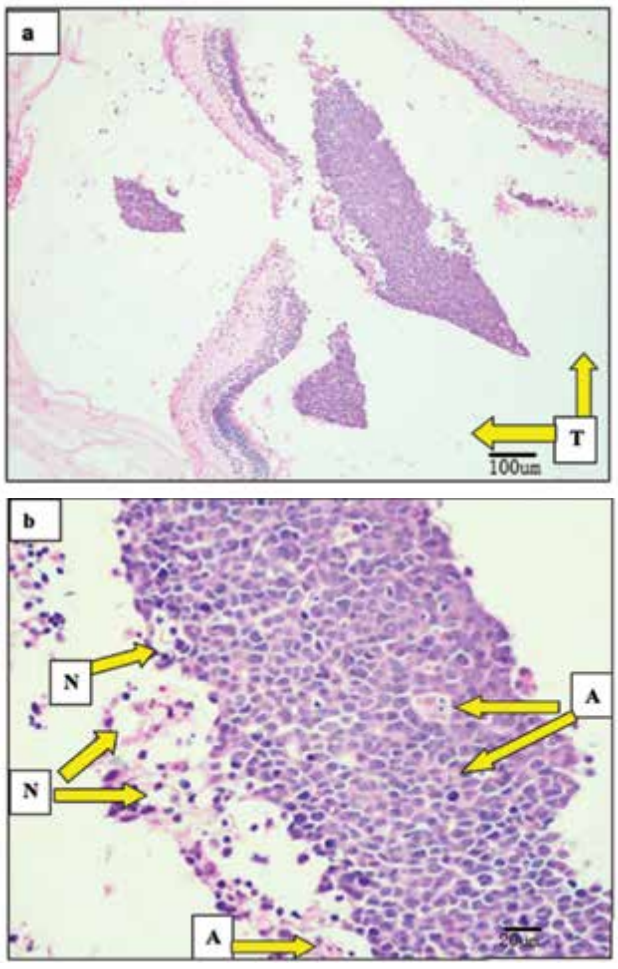

Figure 4. Tumor cells in control group which occupied subretinal area and vitreous. (Haematoxyline & Eosin **a)** x10, **b)** x40)
T– tumor cells, A – apoptotic area characterized by fragmented nuclei, N – necrosis

DISCUSSION

In general, a tumor requires an adequate blood supply to grow. Tumor angiogenesis is induced by various growth factors, including VEGF [15,16]. Thus, arresting VEGF is the basis of arresting the tumor growth. Intravitreal anti-VEGF has been proven to be effective in treating active lesions with vessel formations, including wet age-related macular degeneration, choroidal neovascularisation and neovascular glaucoma [17]. Systemic anti-VEGF has been shown to facilitate a promising outcome for RB [16]. Therefore, treating RB with intravitreal anti-VEGF may provide an effective adjunctive treatment for globe salvation even in the advanced stage of the disease.

Based on this pilot experimental study, we found that a single intravitreal anti-VEGF reduced the size of the RB-like tumor in the induced rabbits. We used the WERI-Rb-1 cell line, a commercially available RB cell line, to develop an animal model of RB. We modified the techniques developed by Kang and Grossniklaus [18] and Johnson et al. [19]. All the rabbits developed a slow growing tumor in the subretinal layer and vitreous without spontaneous regression, which was detected clinically 1-week post-injection. The tumor appeared as small whitish-greyish lesions in the retina, resembling a small intraocular human RB, similar to previous studies [18,20]. Hence, using rabbits as an animal model for human RB cells is ideal for targeted drug delivery experiments.

Kang and Grossniklaus [18] successfully induced a subretinal tumor, which was confined to the retina posterior to the equator, without evidence of invasion into the anterior compartment or extrascleral extension. Chévez-Barrios et al. [20] established an animal model of RB using intravitreal injection of Y79 and WERI-Rb-1 cultured cells in transgenic mice. They successfully established a tumor in the vitreous cavity at the posterior and mid-equatorial regions starting at 2-weeks post-injection. WERI-Rb-1 tumors exhibit characteristics of localised, non-metastatic human RB, whereas Y79 tumors exhibit the histopathological characteristics of aggressive human RB with invasion of the optic nerve and brain. Perhaps, for a future study, injecting a fast-growing type of cell line, such as Y79, may help accelerate the tumor growth and shorten the study period [21,22].

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Clinically, the intravitreal anti-VEGF group (Group A) demonstrated a reduction in the size of the RB lesions with well demarcated margins in comparison to the control (Group B). However, clinically, there was no sign of regression of the lesion in the vitreous cavity. In the non-treated group (Group B), the tumor continued to grow without any sign of regression. The present study found a positive clinical response towards single ranibizumab injection, which was in line with a previous study using anti-VEGF antibodies [16]. Anti-VEGF antibodies have been found to have a remarkable antiangiogenic effect on RB in vitro and in vivo. Hence, intravitreal ranibizumab should be advocated as an adjunctive therapy. However, the present study did not include group of rabbits exposed to other existing treatment, which include external beam radiation, systemic, intra-arterial, and intravitreal chemotherapy.

In our study, there was no significant side effect of the intravitreal ranibizumab injection except a temporarily high IOP, which was detected through digital palpation. All the rabbits developed a tense eyeball post-injection, which was relieved by paracentesis. Reduced appetite and weight reduction was observed in all the rabbits. However, the weight loss did not exceed more than 10% from the animal's initial weight, and all the rabbits survived until the end of the study period. The rabbits in both groups developed mild gingival hypertrophy and drooling of saliva, similar to symptoms of long standing CsA therapy. These side effects were likely due to CsA toxicity [23]. Complete resolution of the symptoms was observed after reducing the CsA dose to 10 mg/kg/day.

The histopathology findings showed that the tumor closely resembled human RB [24], indicating successful tumor induction. Poorly differentiated cells with hyperchromatic nuclei and scanty cytoplasm with some areas of necrosis, but no vascularisation or calcification, were seen in the subretinal area. There was a minimal tumor area in the retina with focal areas of marked retinal hyperplasia and minimal tumor cells in the vitreous cavity. Due to the relatively shorter duration of induction prior to intervention, the histopathological finding may represent retinal hyperplasia in some areas. However, in Group B (control), in which the sample was harvested at week nine after tumor inoculation, the histopathological finding typically revealed features of RB.

A serial cut section at each stage of the observation is the best, but this requires a larger sample size. Major limitation in this present study is small sample size. Clinical and histopathological findings from six rabbits may not provide strong evidence for conclusive finding. Gross sectioning and histopathology processing may affect the findings [25-27]. This may explain the absent of a lesion in the vitreous cavity. This process may also induce retinal detachment and fragmentation, causing difficulty in detecting the site of the lesion [28]. The effectiveness of intravitreal anti-VEGF was based on qualitative clinical and histopathological evaluations. Quantitative parameters, such as biochemical markers and immunohistochemistry staining [29-32], were not included in the present study. Nevertheless, the current finding as a pilot study provides potential basis for larger randomized control trial in the future.

CONCLUSION

A single intravitreal injection of 0.3 mg in 0.03 ml ranibizumab was found to have a potential effect as treatment in RB. Tumor size appeared subjectively smaller, with more demarcated edges and the treatment is safe in an animal model. Multiple injection may accelerate tumor regression further.

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Ethics approval

This study received approval from Animal Ethics Committee, Health Campus, Universiti Sains Malaysia on 30th January 2013 with reference number USM/Animal Ethics Approval/2012/(81)(429). This study was conducted in accordance to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

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