AN ANIMAL EXPERIMENTAL STUDY ON THE INTRAVITREAL INJECTION OF RANIBIZUMAB AS AN ADJUNCTIVE TREATMENT IN RETINOBLASTOMA

by

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2. DISCLAIMER

I hereby certify the work in this dissertation is my own except for the quotations and summaries which have been duly acknowledged.

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7. ABSTRAK

Objektif:

Untuk menentukan keberkesanan suntikan intravitreal ranibizumab ke atas arnab yang diransang ketumbuhan retinoblastoma, dan untuk menentukan kebarangkalian kesan sampingan ubat berkenaan.

Tatacara:

Ketumbuhan retinoblastoma telah dihidupkan ke dalam mata 6 ekor arnab *New Zealand White rabbit* dengan kaedah suntikan sel asas WERI-Rb ke dalam kawasan bawah retina mata kanan arnab tersebut. Selepas enam minggu, kumpulan A (n=3) telah diberi suntikan intravitreal ranibizumab (0.5mg) dan kumpulan B (n=3) sebagai kumpulan kawalan. Pemeriksaan klinikal asas dan secara berkala telah dijalankan pada hari pertama, ketiga, keenam, kedua belas, kelima belas, kelapan belas dan kedua puluh satu. Mata kanan telah dienukleasi pada hari kedua puluh satu dan dihantar ke makmal untuk ujian histopatologi.

Keputusan:

Ketumbuhan tisu telah dapat dikesan secara klinikal tumbuh pada bahagian bawah retina atau retina dan juga kawasan gel vitreous bermula satu minggu selepas suntikan sel ketumbuhan. Ketumbuhan yang dikesan tumbuh secara perlahan tanpa

tanda-tanda menyusut dengan sendiri. Selepas diberi suntikan intravitreal ranibizumab (0.5mg), terdapat kesan menyusut yang sedikit sementara ketumbuhan pada kumpulan kawalan terus bertumbuh secara perlahan. Ujian histopatologi mengesahkan ketumbuhan tersebut adalah sangat menyerupai sel retinoblastoma jenis tidak membahagi pada manusia. Ketumbuhan itu juga didapati lebih besar saiznya pada kumpulan kawalan berbanding kumpulan yang diberi rawatan. Walau bagaimanapun, kumpulan A yang diberi rawatan juga ada menunjukkan kesan pertumbuhan 'hyperplasia' pada retina. Tiada kesan sampingan merbahaya akibat suntikan ranibizumab melainkan kenaikan tekanan mata yang berlaku sejurus selepas suntikan. Kenaikan tekanan mata itu telah menurun seperti biasa setelah 'paracentesis' dijalankan.

Kesimpulan

Suntikan intravitreal ranibizumab (0.5mg) menyebabkan penyusutan ketumbuhan pada mata arnab. Walau bagaimanapun, keputusan ini didapati tidak signifikan secara statistik. Terdapat peningkatan tekanan mata sejurus selepas suntikan intravitreal ranibizumab yang menurun seperti biasa setelah 'paracentesis' dijalankan. Oleh itu, rawatan ini didapati selamat.

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8. ABSTRACT

Objective:

To determine the efficacy of intravitreal ranibizumab injection in rabbit induced with retinoblastoma and to determine the potential side effect of intravitreal ranibizumab injection in rabbit induced with retinoblastoma

Methodology:

Retinoblastoma was induced in 6 New Zealand White rabbit by subretinal injection of cultured WERI-Rb cell line into the right eye. After six week, Group A (n=3) was given intravitreal ranibizumab injection (0.5mg) and Group B (n=3) as control group. Baseline and serial clinical examination was performed at day 1, 3, 6, 12, 15, 18, 21. The right eyes were enucleated on day 21 and sent for histopathological examination.

Results:

All rabbits developed intraocular tumour in the subretinal/retinal and vitreous cavity detectable via fundus examination started after one week post tumour inoculation. The tumour grew slowly without evidence of spontaneous regression. After intravitreal ranibizumab injection in Group A (treated group), there was slight regression of the tumour detected clinically while the tumour in the group B (control group) continued to grow slowly. Histopathological finding confirmed presence of

tumour that closely resembled features of poorly differentiated human retinoblastoma cells. The tumour in the control group was larger compare to the treated group. The treated group however also developed focal area of retinal hyperplasia. There was no significant side effect of ranibizumab injection except temporary high intraocular pressure immediate post injection which relieved after paracentesis.

Conclusion:

Intravitreal injection ranibizumab (0.5mg) caused regression of tumour in this xenograft rabbit model clinically. However, the result was not statistically significant. There was temporary increase in intraocular pressure immediately post injection which relieve with paracentesis. Therefore, it was considered as a safe therapy.

Chapter 1

Introduction

1.1 Overview of retinoblastoma

Retinoblastoma is the most common primary intraocular tumour in childhood with an incidence of 1 in 15,000 to 20,000 live births (Bishop & Madsen, 1975). It represents approximately 4% of paediatric malignancies (Shields & Shields, 2004). The first enucleation for retinoblastoma was advocated by James Wadrop in 1809 (Abramson, 2005). Before the role of enucleation in the management of retinoblastoma was known, most cases of retinoblastoma proved fatal (Shields & Shields, 2004). As more patients survived and had their offspring, more evidence arose suggesting the hereditary nature of retinoblastoma (Abramson, 2005). There is no predilection in gender, race and socioeconomic status. However, patient from lower socioeconomic background has more advanced forms of disease at initial diagnosis and the median age at diagnosis tend to be substantially higher due to late presentation (Erwenne & Franco, 1989). With current chemotherapy and focal treatment modalities, survival in the United States and other developed countries has now climbed to almost 100% with children maintaining functional vision but in other continents such as Africa, survival rates drop significantly (Houston et al., 2012).

Retinoblastoma is generally classified into familial or sporadic, bilateral or unilateral, and heritable or non-heritable (Shield & Shields, 2002). The tumour is commonly unilateral (60%) with median age at presentation under 12 months in

heritable cases, and closer to 24 months in sporadic cases (Abramson & Servodidio, 1992). Retinoblastoma is initiated by mutations in both alleles of the RB1 tumour suppressor gene located on the long arm of chromosome 13 (13q14.2)(Sachdeva & Brien, 2012). Mutation of Rb1 allele results in defective formation of pRB protein leading to impaired cell cycle and uncontrolled cell proliferation (Bunin et al., 1989). Knudson (1971) proposed the 'two hit' hypothesis that the development of retinoblastoma is due to two mutational events. About 40% of retinoblastoma cases are heritable, with RB1 mutations occur ring in the germline while 60% are non-heritable (Knudson, 1971; Bunin et al., 1989). In heritable cases, the initial mutation of RB1 allele may be known to be present within the family (familial retinoblastoma, 25% of heritable cases) or may have occurred de novo in the parental gametes (sporadic heritable, 75% of heritable cases) (Sachdeva & Brien, 2012 ; Bunin et al., 1989). Subsequent mutation of the second allele occurs within the developing retina, resulting in the development of intraocular tumours. In non-heritable cases, both RB1 mutations occur locally within the affected retina (Knudson, 1971; Bunin et al. 1989; Mairal et al., 2000).

The clinical features of retinoblastoma vary with the stage of the disease at the time of the diagnosis. The earlier and the most frequent clinical manifestations of retinoblastoma are leukocoria and strabismus (Shields & Shields, 2004 ; Dimaras et al., 2012). Leukocoria is initially inconstant and visible only at certain angles and (Aerts et al., 2006). Strabismus, when present, becomes rapidly constant due to

impairment of the vision (Aerts et al. 2006). Other signs include rubeosis iridis, hypopyon, hyphaema, buphthalmos and orbital cellulitis (Aerts et al., 2006; Dimaras et al., 2 012). Some children with retinoblastoma may have no symptoms. Screening cases with positive family history or dysmorphic syndrome with a 13q14 deletion may lead to diagnosis of retinoblastoma (Baud et al., 1999).

Diagnosis establishment in a child with suspected retinoblastoma is accomplished by thorough history and physical evaluation including external ocular examination, slit lamp biomicroscopy, and binocular indirect ophthalmoscopy with indentation. The diagnosis is established by the classic appearance of the retinal tumours by an experienced examiner. In general, retinoblastoma appears as a whitish retinal mass that may be solitary or multifocal (Houston et al., 2012).

At early clinical stage, a small retinoblastoma less than 2 mm in basal dimension, appears as a subtle, slightly translucent lesion in the sensory retina (Shield & Shield, 1992). Larger tumours lead to dilated retinal blood vessels and may show foci of chalk-like calcification that resemble cottage cheese. Retinoblastoma of any size can produce leukocoria particularly larger tumour. The white pupillary reflex is a result of reflection of light from the white mass in the retrolental area (Shields & Shields, 2004).

Ancillary diagnostic studies such as fundus fluorescein angiography and ultrasonography can be helpful in confirming the diagnosis of retinoblastoma (Shields & Shields, 2004). Fluorescein angiography shows early vascularity and late hyperfluorescence of the tumour. Ultrasonography and computed tomography can demonstrate the intraocular tumour and possibly the calcification area. Approximately 5% to 10% of retinoblastomas show no intrinsic calcification. Magnetic resonance imaging does not usually detect calcium but may be of value in the assessment of the optic nerve, orbit, and brain. Optic coherence tomography has been found useful in the detection of cystic retinoblastoma that might show less dramatic response to chemotherapy, and it is also helpful in the follow-up of patients to assess macular anatomy.

Retinoblastoma is a highly malignant intraocular tumour of childhood that requires accurate diagnosis and prompt treatment. When left untreated, it is almost always fatal. The most important objective in the management of retinoblastoma is to save life (Shield & Shield, 2004). Salvation of the globe and vision are the secondary and tertiary goals respectively. Enucleation is the most commonly employed surgical modality for advanced retinoblastoma, especially when there is a concern of the spread to the optic nerve, choroid, or orbital cavity.

Two classifications are currently used for prognosis or survival of retinoblastoma. The Reese Ellsworth Classification was originally developed to predict globe salvage after external beam radiotherapy (Table 1) (Ellsworth, 1969). The International Classification of Retinoblastoma (ICRB) is the new classification system which simplifies the grouping scheme and allows a more practical approach in judging results of chemoreduction (Shield et al., 2004). This classification is based on tumour size, location, and associated seeding (Table 2). Shield & Shield (2006) suggested that this classification can reliably predict chemoreduction outcome, as success was achieved in 100% of group A, 93% of group B, 90% of group C, and 47% of group D cases. In advanced retinoblastoma, TNM classification is used for the staging.

Table 1.1: Reese-Ellsworth Classification for Conservative Treatment of Retinoblastoma

Group	Likelihood of Globe Salvage	Features
I	Very favourable	a. Solitary tumour, <4 disc diameters, at or behind equator
		b. Multiple tumours, none <4 disc diameters, all at or behind equator
II	Favourable	a. Solitary tumour, 4-10 disc diameters, at or behind equator
		b. Multiple tumours, 4-10 disc diameters, behind
III	Doubtful	a. Any lesion anterior to equator
		b. Solitary tumours <10 disc diameters behind equator
IV	Unfavourable	a. Multiple tumours, some >10 disc diameters
		b. Any lesion extending anteriorly to ora serrata
V	Very unfavourable	a. Massive tumours involving more than half of retina
		b. Vitreous seeding

*Refers to chances of salvaging the affected eye and not systemic prognosis.

Table 1.2: International Classification of Retinoblastoma (ICRB)

Group	Features
A	Small tumour: ≤ 3 mm
B	Large tumour: >3 mm Macular: ≤ 3 mm to foveola Juxtapapillary: ≤ 3 mm to disc Subretinal fluid: ≤ 3 mm from the tumour margin
C C1 C2 C3	Focal seeds Subretinal seeds: ≤ 3mm from tumour Vitreous seeds: ≤ 3 mm from tumour Both subretinal and vitreous seeds: ≤ 3 mm from tumour
D D1 D2 D3	Diffused seeds Subretinal seeds: >3 mm Vitreous seeds: >3 mm Both subretinal and vitreous seeds: >3 mm
E	Extensive retinoblastoma occupying more than 50% or neovascular glaucoma or opaque media from hemorrhage in anterior chamber, vitreous or subretinal space Invasion of postlaminar optic nerve, > 2mm of choroid, sclera, orbit, or anterior chamber

1.2 Treatment modalities of retinoblastoma

The management of retinoblastoma has evolved tremendously for the past century. The success in managing retinoblastoma is attributable to general developments in medicine, along with the specialised development of ophthalmic techniques such as laser photocoagulation and cryosurgery. In 1921 the first eye with retinoblastoma was salvaged and in 1926 the first case was reported of successful treatment of retinoblastoma with retention of sight (Abramson, 2005). External beam radiotherapy is a method of delivering whole eye irradiation to treat advanced retinoblastoma, particularly when there is diffuse vitreous seeding (Shields & Shields 2004). Various treatment plans have been employed including the whole eye technique and lens-sparing technique. Recurrence of retinoblastoma after external beam radiotherapy continues to be a problem that can develop within the first 1 to 4 years post treatment. Tumour recurrence has also been found to be related to the stage of the disease and largest tumour size at the time of treatment (Shields & Shields 2004).

The most current method for retinoblastoma treatment is chemoreduction followed by focal consolidation therapy (Shield & Shield, 2008). It has largely replaced the external-beam radiotherapy (ERBT) as the treatment of choice for bilateral retinoblastoma. EBRT which has been used for treating advanced retinoblastoma currently has lost its favour due to increased risk of secondary tumour and also effect of radiation causing damage to surrounding intraocular structure (Weiss et al., 1994).

The risk of secondary malignancies is greater than 50% by the age of fifty years if the retinoblastoma is treated with EBRT (Wong et. al., 1997).

The International Classification of Retinoblastoma (ICRB) is very useful in guiding the clinician to choose the most appropriate treatment methods and assist the predication of the success of chemoreduction and focal treatment. In general, eyes classified as group A is managed by primary focal therapy such as cryotherapy, laser photocoagulation or transpuppillary thermotherapy (TTT). Group B is managed by six cycles of chemotherapy, especially if only two drugs are used followed by focal consolidation while Group C with six cycles of chemotherapy plus focal consolidation therapy. By using the ICRB, the bilateral cases of group D or E are managed by chemoreduction, thermotherapy or low dose of external beam radiotherapy, but group E often require enucleation.

Chemoreduction involves intravenous chemotherapy to reduce the tumour size, followed by focal consolidation to permanently devitalize each retinoblastoma (Shields et al., 2004). The benefits of chemoreduction are not only tumour control within the eye, but may also prevent or delay the onset associated pinealoblastoma and other intracranial neuroblastic malignancies (trilateral retinoblastoma). Chemoreduction has been most successful for eyes with tumour confined to the retina and least successful for eyes with additional vitreous and subretinal seeds and those eyes with subretinal fluid at initial examination (Shields et al., 2002). Eyes that failed chemoreduction is generally treated with external beam radiotherapy or enucleation (Shields et al., 2002).

The focal treatment modalities include laser photocoagulation, cryotherapy, transpupillary thermotherapy (TTT), chemothermotherapy and custom design plaque radiotherapy. These focal therapies are employed for small tumours, especially those that have been reduced by chemoreduction (Shields & Shields, 2004). Laser photocoagulation is performed using the indirect ophthalmoscopic argon or green diode laser with two rows of photocoagulation surrounding the tumour base and avoiding direct treatment to the tumour as it could lead to vitreous seeding. Laser photocoagulation is usually employed for small retinoblastomas posterior to the equator of the eye. In this era of chemoreduction, laser photocoagulation is rarely employed as its success depends on vascular coagulation and tumour ischaemia, whereas the opposite applies to chemoreduction. Thus, it is not employed in eyes receiving chemoreduction.

Cryotherapy was introduced by Harvey Lincoff et al. in the 1960s and has proven to be an important adjunct in the treatment of peripheral, small retinoblastomas. Cryotherapy remains an important method for tumour consolidation following chemoreduction. The therapy is useful in the treatment of equatorial and peripheral small retinoblastomas. It is a critical modality for management of recurrent subretinal seeds near the ora serrata (Shields & Shields, 2004).

Enucleation is still an important management in advanced disease with no hope for useful vision in the affected eye or if there is a concern of invasion of the tumour into the optic nerve, choroid, or orbit (Shields & Shields 2004). Eyes with secondary

glaucoma, pars plana seeding, or anterior chamber invasion are also generally best managed with enucleation

1.3 Animal model of retinoblastoma

Animal model of retinoblastoma is important particularly to study the efficacy of new therapies. It has been established previously mostly in murine models which includes the transgenic murine model and knockout mice. The majority of the animal models are xenograft models that have been created by injecting human retinoblastoma tumour cells into either the anterior chamber or the subretinal space of the eyes of immunodeficient mice or rats (Chevez-Barrios et al., 2000). The first animal model of retinoblastoma was introduced by injecting human retinoblastoma cells (Y79) into immunocompromised mice by McFall et al., (1974). However, it was not an ideal model due to obvious anatomic difference between the adult flank and the eye (White et al., 1989).

Chevez-Barrios et al., (2000) developed another animal model by injecting human retinoblastoma cell lines in mice. The WERI-Rb cell line and Y79 retinoblastoma cell line were used, where both derived from undifferentiated human retinoblastoma. These tumour cell lines were cultured and the tumour cells were injected intravitreally in mice. From the study, the former cell line produce more closely

resembled non-metastatic human retinoblastoma whereas the latter demonstrated specific tumour evolution similar to that seen in invasive and metastatic disease.

Rabbit model of retinoblastoma has recently been established by (Kang & Grossniklaus, 2011) where the WERI-Rb cell line was injected in the cyclosporin-induced immunodeficient rabbit. The tumour was detectable starting at week one post injection in all rabbit, then became vascularised starting at week five and continue to increase in size up to eight week. The advantage of using this rabbit model is their larger eye size which is similar to human infant's eye that permits better visualization for clinical assessment, administration of tumour cells and intraocular drug delivery.

The WERI-Rb cell line is one of two retinoblastoma cell line that has been established in 1974. (Mcfall et al., 1977) It shows similar morphology as compared to the earlier Y79 cell line in vitro. WERI-Rb cell line grew as a suspension of small round cells in grape-like clusters in 3 dimensions. Each exhibited growth of cells in rosettes, as well as unusual chain formations. The population-doubling times for WERI-Rb were 96 hour.

1.4 Tumour angiogenesis and vascular endothelial growth factor (VEGF)

Angiogenesis, the recruitment of new blood vessels is an essential component in tumour growth and metastasis (Zetter, 1998). Angiogenesis enhances entry of tumour cells into the circulation by providing an increased density of immature, highly permeable blood vessels that have little basement membrane and fewer intercellular junctional complexes than normal mature vessels (Dvorak et al., 1995). Folkman et al., (1971) made initial efforts aimed to isolate a tumour angiogenesis factor from human and animal tumours. Subsequently, the angiogenic effects of various factors, including TGF-α TGFβ , TNF-α, and angiogenin were reported. Although these factors promoted angiogenesis in various bioassays, initial attempts to directly link them to tumour angiogenesis yielded largely negative results (Ferrara, 2002). However, recent studies had demonstrated the expression of VEGF in tumours. In situ hybridization studies demonstrated that the VEGF mRNA is expressed in the vast majority of human tumours. Many tumour cell lines secrete VEGF in vitro suggesting that the possibility of this molecule may be a mediator of tumour angiogenesis (Ferrara, 2004).

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen in vitro and an angiogenic inducer in various in vivo models. Hypoxia has been shown to be a major inducer of VEGF gene transcription (Ferrara, 2004). The history of VEGF began in 1983 when Senger et al. discovered the partial purification from the

conditioned medium of a guinea-pig tumour cell line of a protein able to induce vascular leakage in the skin, which was termed "tumour vascular permeability factor" (VPF). VPF had been suggested to be a mediator of the high permeability of tumour blood vessels; unfortunately it was not isolated and sequenced. Hence, this factor remained molecularly unknown at that time. VEGF was formally identified in 1989 by Ferrara & Henzel when they isolate a diffusible endothelial cell-specific mitogen from medium conditioned by bovine pituitary follicular cells. The role of VEGF in developmental angiogenesis is further emphasized when loss of a single VEGF allele results in defective vascularisation and early embryonic lethality (Ferrara, 2004). VEGF is currently established as a major regulator of physiological and pathological angiogenesis.

1.4.1 Tumour angiogenesis in retinoblastoma

Retinoblastoma is a well vascularised tumour that is dependent on its vascular supply (Burnier et al., 1990). Study by Kvanta et al., (1996) and Arean et.al., (2010) showed that VEGF is highly expressed in patients with retinoblastoma. Piña et al., (2009) found a heterogeneous vessel population in retinoblastoma containing both neovessels and mature vessels in advanced disease. Another study done by Rossler et al. (2004) on 107 retinoblastomas collected between 1980 and 1990, demonstrated that vessel density in retinoblastoma correlate with local invasive growth and the presence of metastasis at the time of diagnosis. Therefore, angiogenesis seems to

play an important role for invasive growth in retinoblastoma both locally and systemically. Hence, anti-angiogenesis therapy may play an important (adjuvant) role in the regression of tumour growth and prevention of metastases of retinoblastoma.

1.4.2 Anti vascular endothelial growth factor (antiVEGF)

Various clinical trials regarding the intravitreal injection of anti-VEGF agents including ranibizumab, bevacizumab, pegaptanib, and aflibercept have shown excellent results in the treatment of angiogenic pathologies including choroidal neovascularisation, macular edema, proliferative diabetic retinopathy and neovascular glaucoma (Kimoto & Kubota, 2012). Bevacizumab and ranibizumab target all VEGF isoforms while pegaptanib specifically targets the 165 isoform of VEGF (Christoforidis et al., 2012). Ranibizumab contains a fragment of a humanized monoclonal antibody, is proven beneficial in the treatment of choroidal neovascularization secondary to age-related macular degeneration. Bevacizumab is a humanized recombinant monoclonal IgG antibody that has been approved as an adjuvant agent for the treatment of colorectal carcinoma and has also been increasingly used as an off-label therapy in the field of ophthalmology.

1.4.3 The role of antiVEGF as adjuvant therapy of tumour

VEGF-blocking therapy appears very promising in the treatment of cancer as it is directed only against migrating and proliferating capillary endothelial cells at a site of angiogenesis (Hoeben et al., 2004). Combination therapy seems more effective, because both the endothelial cell and tumour cell compartments of a tumour are targeted and blocking angiogenesis may decrease the interstitial pressure in tumours, leading to a greater penetration of the cytotoxic drugs.

The era of antiangiogenic treatment for cancer began in 2004, with the approval of bevacizumab for the treatment of metastatic colorectal cancer. Lee et al., (2008) conducted a study on inhibitory effect of bevacizumab on the angiogenesis and growth of retinoblastoma. In this study, systemic administration of bevacizumab was injected into nude mice induced with retinoblastoma. There was 75% reduction in the growth of retinoblastoma without producing significant systemic toxicity. Another study done by Houston et al., (2011) on vascular targeting therapies with angiogenic inhibitors alone or in combination with chemotherapeutic agents have been shown to effectively control tumour burden in the knockout mouse model for retinoblastoma.

Based on the promising outcome from the previous studies, intravitreal administration of antiVEGF is potentially advantageous and might have the same inhibitory effect as the one given via systemic route. (Lee et al. 2008; Houston et al. 2011). Ranibizumab is a monoclonal antibody fragment have a higher binding affinity for VEGF possibly has similar efficacy as bevacizumab as adjuvant therapy in tumour. To date, there was no study done on intravitreal antiVEGF therapy on retinoblastoma. Intravitreal ranibizumab injection may have possible advantageous effect as it directly targeting the source of lesion. It also confers less antigenicity and greater retinal penetration because of the smaller molecule size. Administration of intravitreal ranibizumab will reduce the VEGF load intraocularly. Therefore, reduce the inflammatory process, risk of new vessel formation and further reduce the aggressiveness and growth of retinoblastoma.

Chapter 2

Objectives

2. STUDY OBJECTIVE

2.2 General Objective

To evaluate the efficacy and potential side effect of intravitreal ranibizumab injection as adjunctive therapy for retinoblastoma in rabbits

2.3 Specific Objectives

i. To determine the regression of tumour size after 0.5mg intravitreal ranibizumab injection in rabbit induced with retinoblastoma

ii. To determine the potential side effect of 0.5mg intravitreal ranibizumab injection in rabbit induced with retinoblastoma

Chapter 3

Material & Method

3. MATERALS AND METHOD

3.1 Research design

Animal experimental study

3.2 Population and setting time

Six healthy New Zealand albino rabbits obtained from Animal Research and Study Centre (ARASC), Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan

3.3 Ethical approval and financial support

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) and was supported by short term grant (304/PPSP/61313066).

3 .4 Sampling method

3.4.1 Sampling method

Six New Zealand albino rabbit, weighing around 3kg, were recruited in this study. All rabbits were free from any ocular disease. They are kept, fed and cared in ARASC. The animals were divided into two group; Group A (treated group) and Group B (control group).

3.4.2 Sample size

The sample size was determined based on the two proportion formula using PS software version 3.0.

i. Power of study (1-β) = 80%

ii. Significant level (α) = 5%

iii. Ratio between control group and the treated group (m) = 1

iv. P_0 = 0.95 (Lee et. al., 2008)

v. P_1 = 0.01

vi. Detectable difference: 0.9

i. Sample size calculated = 3 per group

ii. Sample size of this study is 3 rabbits per group

3.5 Selection criteria

3.5.1 Inclusion criteria

All rabbits that were successfully induced with retinoblastoma

3.5.2 Exclusion criteria

i. Evidence of early cachexia before commencing treatment
ii. The presence of concomitant ocular infection.
iii. The development of severe infection post intraocular injection, retinal detachment or vitreous haemorrhage.

3.6 Definition of Terms

3.6.1 Successful induction of tumour:

i. Single tumour at least 1disc diameter size

ii. Multiple tumour less than one disc diameter

3.6.2 Regression of tumour

Tumour regression pattern were initially described following radiotherapy (ERBT) and include:

Type 0: no remnant (tumour completely disappears, leaving no retinal