THE EFFECTS OF VARYING INTENSITIES OF BLUE LIGHT ON THE PROLIFERATION OF HUMAN UVEAL MELANOMA CELL LINES BY USING MULTIPLE BLUE LIGHT FILTERS

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English Abstract

Blue light (BL) exposure is considered a risk factor for the development of uveal melanoma (UM), and individuals with fair skin and light irises are at the greatest risk. Moreover, commercially available BL filtering intraocular lenses (IOLs; filter 50% of the BL spectrum) have been shown to protect against the development and progression of UM in multiple *in vitro* and *in vivo* studies. However, the angiogenic effect of BL on human UM cell lines has not yet been established. This is particularly important for UM because this tumor only metastasizes hematogenously. The purpose of this study is therefore to ascertain whether filtering lesser amounts of BL (less than 50%) will maintain the protective effect against UM development in order to customize each IOL based on individualized risk. Moreover, the effect of filtering less BL on the angiogenic properties of human UM cells will be evaluated.

One human UM cells line (92.1) and one transformed uveal melanocyte cell line (UW-1) were used for all experiments. The experimental setup included a light source of 10,000 lux, infrared and ultraviolet light cut-off filters and two different commercially available BL filters of different intensities (16% and 20%). Each experiment included a control group fully exposed to the light and a condition group covered with one of the two filters. The cells were then exposed to light for 3 hours daily over a total period of 4 days. Cell number was then determined using a proliferation assay (cell counting kit-8; CCK-8) at the end of the 4-day period. A similar experimental setup was used to quantify levels of different pro-angiogenic factors secreted by the two cell lines using a multiplex sandwich-ELISA-based quantitative array.

For the 92.1 cell line, filtering 20% of BL decreased the proliferation rate significantly compared to the control group (P <0.01). However, filtering 16% of BL was not sufficient to show this same effect. Conversely, for the UW-1 cell line, filtering 16% of BL decreased the proliferation rate significantly compared to the control group (P < 0.01). Filtering 20% of BL for 92.1 cell line and 16% for UW-1 cell line showed a non-significant decrease in the levels of various pro-angiogenic factors.

The protective effect against BL-induced proliferation of UM cell lines was demonstrated using the *in vitro* model described herein. Current commercially available IOLs filter 50% of BL. Based on ours results, the development of different BL filtering IOLs that can be prescribed according to each patient's risk of developing UM (personalized medicine) is recommended.

French Abstract

L'exposition répétée à la lumière bleue (LB) est considérée comme un facteur de risque pour le développement de mélanomes de l'uvée (MU). Ce risque est augmenté chez les individus au teint et aux iris clairs. De plus, un effet angiogénique de la LB fut établi pour des modèles cellulaires humains de MU. Ceci est d'une importance particulière étant donné que les MU métastasent par voie sanguine.

Il fut démontré dans plusieurs études *in vitro* et *in vivo* que certaines lentilles intraoculaires (LIO) commerciales ayant la capacité de filtrer 50% de la LB offrent une protection contre le développement et la progression des MU.

L'objectif de cette étude est de vérifier la possibilité de réduire le pouvoir de filtration des LIOs tout en maintenant leur effet protecteur dans un modèle cellulaire de MU. Par le fait même, la relation entre les propriétés angiogéniques de ces lignées cellulaires et la quantité de LB filtrée sera établie.

Cette étude vise à permettre le développement de lentilles ayant une capacité de filtration de LB ajustée selon les facteurs de risques de chaque individu, tel que proposé par la notion de médecine personnalisée.

Dans le cadre de ce projet, les lignées cellulaire 92.1 et UW-1, deux modèles de MU humains au pouvoir prolifératif et métastatique différents, ont été utilisées. Le design expérimental requiert une source lumineuse de 10 000 lux, des filtres infra-rouges et ultra-violets ainsi que deux filtres commerciaux ayant différents pouvoir de filtration de la LB (16% et 20%).

Pour chaque essai, un groupe contrôle fut complètement exposé à la lumière, tandis que les deux autres groupes étaient protégés par l'un des filtres à LB (16% et 20%). Les cellules furent quotidiennement exposées à la source lumineuse pour une période de trois heures, et cela pour quatre jours consécutifs. La prolifération cellulaire fut déterminée par un test cytotoxique (CCK-8, *Cell Counting Kit 8*) à la suite de chaque séance d'exposition à la lumière.

Le même design expérimental fût utilisé afin de quantifier les niveaux de facteurs d'angiogenèse sécrétés par chacune des lignées cellulaires. Pour ce faire, la méthode immuno-enzymatique ELISA en sandwich (*enzyme-linked Immunosorbent assay*) fût utilisée.

Les résultats suggèrent une diminution significative du taux de prolifération de la lignée 92.1 lorsque 20% de la LB est filtrée (P= <0.01). Par contre, la filtration de 16% de la LB n'est pas suffisante pour obtenir un effet semblable. Inversement, la filtration de 16% de la LB démontre un ralentissement significatif de la prolifération cellulaire pour la lignée UW-1 en comparaison au groupe contrôle complètement exposé à la source lumineuse (P= <0.01). D'un autre côté, la filtration de 20% et 26% de la LB pour la lignée 92.1 et UW-1 respectivement entraine une diminution non-significative du niveau de facteurs d'angiogenèse.

Le modèle d'étude *in vitro* ici-décrit vient appuyer l'hypothèse que la filtration de lumière bleue entraine un effet protecteur contre la prolifération de cellules humaine de mélanome de l'uvée. À ce jour, les seules LIOs commercialement disponibles filtrent 0% ou 50% de la lumière bleue. Suite à cette étude, le

développement de LIOs avec un pouvoir de filtration de la LB adaptée selon les facteurs de risques de chaque patient est souhaitable ; mettant ainsi de l'avant une approche de médecine personnalisée tout en conférant une protection optimale minimisant les risques de cancer de l'uvée.

Preface and Contribution of Authors

This is to certify that I have conducted all the experiments described in this thesis under supervision of Dr. Miguel Burnier and the guidance of Dr. Logan, Dr. Zoroquiain, Dr. Vila and Dr. Bravo-Filho. A manuscript originating from this thesis is under preparation for submission.

Papers and Presentations

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2. **Sultan Aldrees**, Pablo Zoroquiain, Patrick Logan, Vasco Bravo-Filho, Jacqueline Coblentz and Miguel Burnier Jr. The effect of varying intensities of blue light on the proliferation of human uveal melanoma cell lines. The Association of Research and Vision in Ophthalmology (ARVO). Seattle, Washington, May 1- 5; Juried Poster Presentation

3. **Sultan Aldrees**, Pablo Zoroquiain, Beatriz Nugent Da Cunha, Adel Helmi and Miguel N Burnier Jr. Clinicopathological findings in peri-ocular basal cell carcinoma: a 16-year experience. World Ophthalmology Congress 2016, Guadalajara, Mexico, Feb 5 – 9 2016; Juried poster presentation.

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List of Abbreviations

UM	Uveal melanoma
BL	Blue light
PE	pigmented epithelium
AMD	Age related macular degeneration
PCNA	Proliferating cellular nuclear antigen
UV	Ultra-violet
RPE	Retinal pigmented epithelium
IOL	Intra-ocular lens
CCK-8	Cell-counting kit-8
OD	Optical density
PMMA	Polymethyle methacrylate
PET	Polyethylene terephthalate
PC	Polycarbonate
ELISA	Enzyme-linked immunosorbent assay
VEGF	Vascular endothelial growth factor
ANG-2	Angiopoietin-2
b-FGF	Basic fibroblastic growth factor
EGF	Epidermal growth factor
HB-EGF	Heparin binding EGF-like growth factor
HGF	hepatocyte growth factor
PDGF-BB	Platelet derived growth factor subtype BB
PIGF	placental growth factor

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1 Chapter One

1.1 Introduction

1.1.1 Anatomy and physiology of the eye

"The eyes are the window to the soul." Although the origin of this saying is not known, it clearly describes that our eyes have other important functions and are not merely responsible for the perception of vision. Through our eyes, we can navigate, avoid danger and see the beauty of the world. In addition, the eye has complex interactions with the brain and is an important part of our overall psychological status. For example, different personality traits, such as curiosity, can be predicted from eye movements (1). Moreover, people with visual impairment are associated with higher levels of depression (2). This elaborates the role of eyes and vision as an important part of the human body system.

Although the eye is relatively small in size, it is structurally complex and consists of many different layers and components that serve different functions. The eye is located in the anterior part of the orbit and is surrounded by muscles and connective tissue. The optic nerve connects the back of the eye to the brain and is responsible for transmitting visual information through electrical impulses to the brain. It is formed by the axons of the retinal ganglion cell layer and glial cells in the retina. Six extraocular muscles are attached to the eye: four recti and two oblique muscles. Through their different origins and insertion points, these extra-ocular muscles are responsible for complex eye movements. The eye itself is made mainly of three layers: an outer fibrous coating layer that is formed by two structures, the transparent cornea in the front and a whitish fibrous

sclera that connects with the cornea at the corneo-scleral limbus. The middle layer is a vascular layer that is comprised of the iris, ciliary body and the choroid. Finally, the innermost layer is the sensory part of the eye called the retina.

The cornea is an elliptical, transparent, multi-layer and avascular structure, which allows the passage of light. Attached to this transparent cornea at the corneo-scleral limbus is the whitish fibrous sclera. It is the whitish structure that we notice when we look at someone's eyes. The scleral fibers are oriented parallel to the surface of the eye and cross each other in all directions in order to provide maximum strength and to protect against minor traumatic injuries. Covering the anterior part of the sclera is the bulbar part of the conjunctiva. This is a semi-transparent, thin and vascularized structure made of stratified, non-keratinizing, columno-squamous epithelium resting on a fibrovascular substantia propria. This bulbar conjunctiva is reflected to form the palpebral conjunctiva that is tightly attached to the inner side of the eyelid. At the eyelid margin, the non-keratinized squamous epithelium turns into a keratinized squamous epithelium that is continuous with the skin of the eyelid. The function of the conjunctiva is to provide nutrients to the ocular structures and to fight infections through its blood supply. Moreover, it contains goblet cells that secrete mucin, which stabilizes the tear film and helps in lubricating the eye. The inner layer of the eye is formed by the retina and is responsible for converting visual inputs into electrical impulses that reach the brain through the optic nerve.

The uvea, or uveal tract, is located between the sclera and the retina. It is highly vascularized and consists of three main structures that are continuous with each other: the iris, the ciliary body and choroid. The iris is a muscular structure that lies behind the

cornea and is separated from it by a fluid filled space, the anterior chamber. It has a central hole called the pupil, which permits fluid exchange between the anterior chamber and the posterior chamber, which is located behind the iris. The pupil also contracts and dilates to control the amount of light entering to the back of the eye. The ciliary body is another muscular structure and is situated between the iris and the choroid. It is attached anteriorly to the root of the iris. Moreover, it has multiple projections on the inner surface behind the iris forming the ciliary processes that hold the lens in place by suspensory (zonular) ligaments. Covering these highly vascularized ciliary processes is the ciliary epithelium responsible for secreting aqueous humor, a fluid that fills both the anterior and posterior chambers. With its contractile ability, the ciliary body loosens the suspensory ligaments that are attached to the bi-convex lens, which will increase the curvature of its anterior surface and thereby altering its focusing power via a process called accommodation. Continuous with the ciliary body posteriorly is a highly vascularized connective tissue structure called the choroid. It consists of a thin layer of small vessels and is located between the sclera and the retina. Its major function is to provide nutrients to the outer part of the retina, mainly through the fine network of vessels in its inner part. Figure 1 shows the most important structures of the eye.

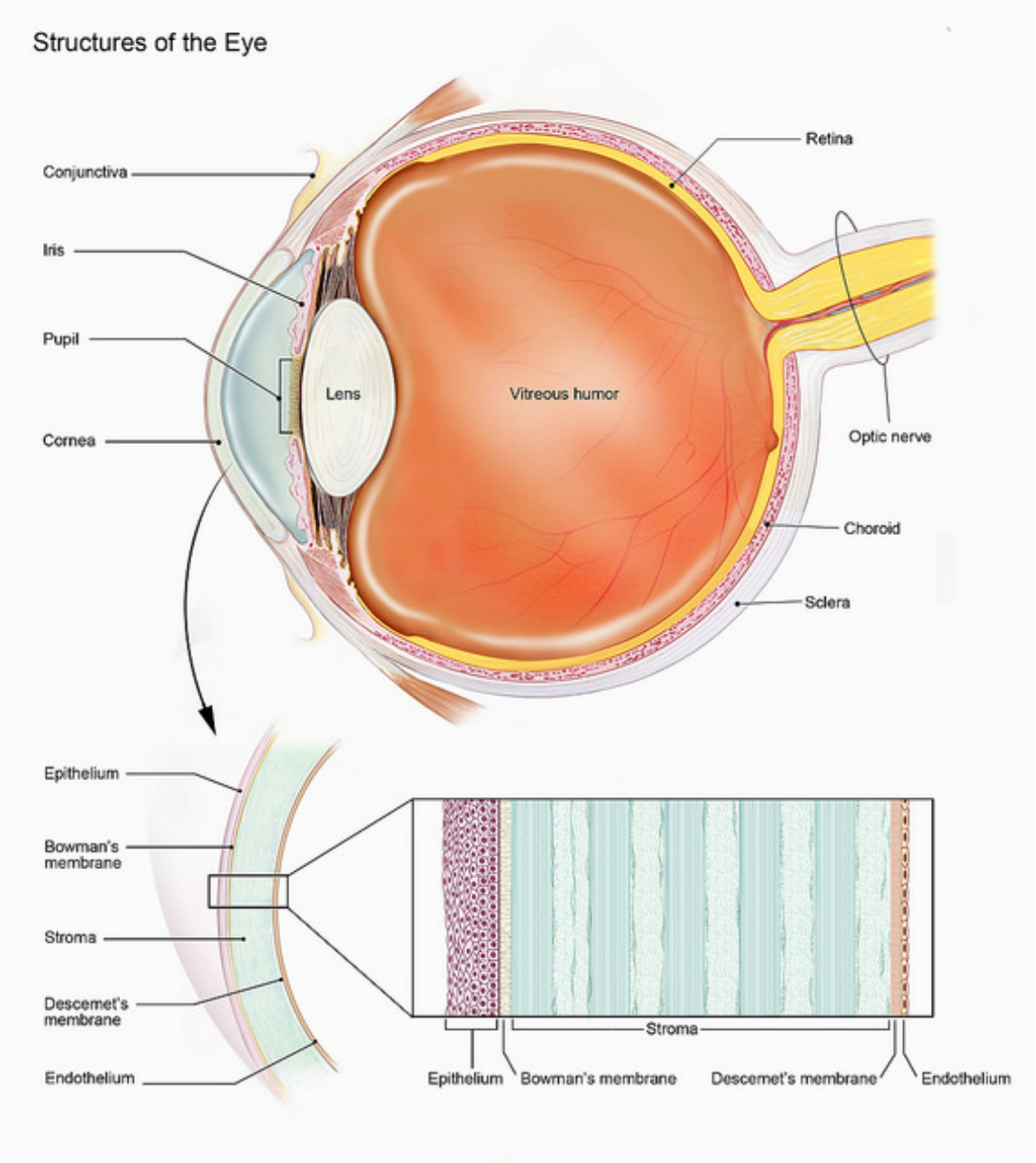

Figure 1. Anatomy of the human eye. A cross-section of the eye showing the most important internal and external structures (3).

1.1.2 Pigmented cells of the eye

There are two different types of pigmented cells in the eye, both of which contain melanin pigment in organelles called melanosomes. The first type is the melanocytes, which can be found mainly in the conjunctiva, iris, ciliary body and the choroid. They originate from the neural crest from melanoblasts. Melanoblasts migrate from the neural crest to their final destination in the eye before transforming into melanocytes (4-6). Melanomas, including uveal melanoma (UM), arise from these cells.

The other type of melanin-containing cells is the pigmented epithelium (PE), which can be found in the iris, ciliary body and the retina. These pigmented epithelial cells originate from the pigmented layer of the optic vesicle as early as 22 days of gestation (5-8). Both melanin containing cell types function mainly as photoprotective agents against light induced damage by absorbing light at all wavelengths, specifically at higher frequencies (4). Besides providing a protective effect via melanin, the PE serves multiple functions according to location. For instance, in the iris the PE can help dilate the pupil, while in the choroid it secretes the aqueous humor, which is aided by the underlying heavy vasculature (9). In the retina, the PE has important functions such as: ion transportation, phagocytosis of the outer segment membrane of the photoreceptors that undergo continuous destruction by photo-oxidative damage, isolating the inside of the eye from the immune system (immune privilege of the eye) and the secretion of various growth factors that play important role for the function of photoreceptors (10, 11).

Both cell types appear morphologically distinct: melanocytes have dendrites and contain melanin granules of varying sizes and numbers (12). On the other hand, PE cells are cuboidal in shape and contain large melanin granules (7). Another important difference between the two cellular types is the pigment distribution in the choroid versus the retinal-pigmented epithelium (RPE). In the choroid, melanin pigments in melanocytes increase in concentration from the periphery to the posterior pole of the eye. However, in the RPE melanin pigments distribution increase in concentration from the posterior pole towards the periphery of the eye (4). Interestingly, both cellular types show decreasing levels of melanin content with age (4, 13). This makes ocular pigmentation more important than skin pigmentation, as this process is not renewable in the eye (4).

1.1.3 Uveal Melanoma

UM is a malignant melanocytic tumor that develops in any of the three structures that constitute that uveal tract; however, it is far more common in the choroid (>86%) than the ciliary body or the iris (14). It is the most common primary intraocular malignancy in adults. Figure 2 shows a cross section of an enucleated eye having UM.

1.1.4 Epidemiology

UM represents around 3%–5% of all cases reported as melanoma (14-16). Most cases of UM arise in the choroid compared to the iris or the ciliary body (14-16). In Australia, the incidence of ocular melanoma appeared to be higher than reported in North America or Europe (17). This can be attributed to the fact that this study included all clinically diagnosed cases, which can artificially inflate this number as invariably there will be a small number of misdiagnoses, and likely not a result of any actual increased incidence (17).

UM occurs slightly more frequently in males with a median age at diagnosis of 62 years (14, 15). In 2001, Singh *el al*. reported a higher age adjusted incidence in males compared to females (5.8 versus 4.4 cases per million); however, the overall age adjusted incidence is reported to be 5.1 cases per million (15). Despite this relatively low incidence and despite the advances in the treatment of this type of cancer, the 5-year relative survival rate remained unchanged (81.6%) from 1973 to 2008 (15).

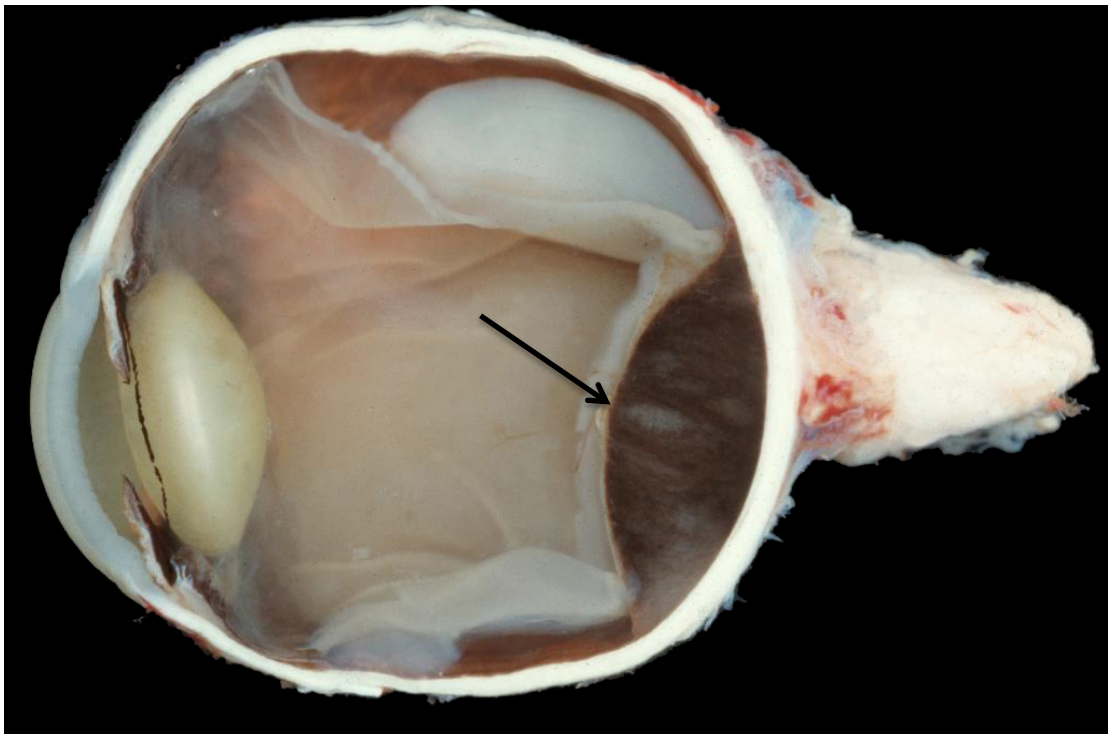

Figure 2. Uveal melanoma. Enucleation specimen showing a dome-shaped choroidal melanocytic tumor (arrow) underlying the retina.

1.1.4.1 Risk factors for developing UM

The pathogenesis of UM is complex and involves sophisticated interactions between host and environmental factors. Nevertheless, host factors are the most important (18). Individual studies may sometimes report contradicting results regarding the relationship between certain factors and the development of UM. However, more powerful studies using meta-analysis pool data from these individual studies to reach a more powerful conclusion. These risk factors may include: congenital ocular and oculodermal melanocytosis (known as nevus of Ota), choroidal nevi, iris nevi, light irises, fair skin, inability to tan, atypical cutaneous nevi, common cutaneous nevi and cutaneous freckles (19, 20).

In general, as is the case with cutaneous melanoma, UM is more common in Caucasians compared to black people (21).

Choroidal nevi are an interesting risk factor for UM as they are very common, occurring in 5% to 6% of the general population (22, 23). They are usually found in the posterior pole and they may appear flat or as a raised gray area; amelanosis in some cases makes the diagnosis even more difficult (7). Their importance relies on their potential to transform into choroidal melanoma. It is estimated that 1 in 8,000 choroidal nevi will transform into a melanoma, and this risk increases with age (22, 24, 25). Moreover, it is more difficult to clinically differentiate between choroidal nevi and small uveal melanomas because of their overlapping features, including tumor size, color, location and associated changes in the retina (26). Several factors indicative of malignant transformation of choroidal nevi have been combined into the useful mnemonic “to find

small ocular melanoma using helpful hints" (TFSOMUHH), and includes: **t**hickness greater than 2 mm, subretinal **f**luid, **s**ymptoms, **o**range pigment, tumor **m**argin within 3 mm of the optic disk, **u**ltrasonographic **h**ollowness of the tumor and the presence of **h**alo around the tumor (26-28). Interestingly, the risk of malignant transformation of choroidal nevi can reach up to 69% with a specific combination of these factors (28). This is particularly important for patients undergoing cataract surgery and thereby exposing pre-existing nevi to the harmful effect of high-energy wavelengths of light.

1.1.4.2 Clinical presentation and diagnosis of UM

At the time of diagnosis, most UM patients are symptomatic; however, up to 30% can be asymptomatic (29). Based on the size and location of the tumor and its associated features, patients may report decreased vision, flashes, floaters or pain. For choroidal melanoma, indirect fundoscopy allows visualization of the tumor and assessing the shape and degree of pigmentation. Ultrasonographic examination using either A or B scans will help in the diagnosis. B scans will show acoustic hollowness and choroidal excavation. On the other hand, A mode sonography will show low to medium internal reflectivity with individual spikes. Ultrasonography not only determines these features but also helps measuring tumor height, which is an important prognostic factor (30). Computed tomography and magnetic resonance imaging can be used to assess extraocular extension of the tumor, while optical coherence tomography can be used to assess the associated retinal changes (26). By using fundoscopic examination with the help of the aforementioned ancillary studies, a correct diagnosis can be reached 99% of the time in most cases (31).

1.1.4.3 Histopathology and UM cytopathology

In 1931, Callender (32) proposed a system to classify UM based on histopathological and cytological characteristics. He identified two different cellular compositions: spindle and epithelioid types. Spindle cells are further divided into spindle A and spindle B cells. Both spindle cells are classified based on their nuclear features. Spindle A cells have fine and longitudinal nucleus with faint nucleolus, while spindle B cells have a coarse and well-defined nucleus and a dense nucleolus. On the other hand, epithelioid cells are usually large with well-defined nucleus and nucleolus. They also have a clear cytoplasm and a definite cell border. Moreover, they tend not to attach to each other (they lack cohesiveness). Therefore, UM was classified based on these cytological features (spindle A, spindle B, epithelioid, and mixed type) in addition to two histological features of having either a fascicular pattern growth or being predominantly necrotic. The importance of this classification is its prediction of survival where spindle A, spindle B and fascicular tumor have less metastasis and better survival than necrotic, epithelioid and mixed types. In 1983, McLean et al. (33, 34) developed a modified classification system, which is the main classification system used currently, with two groups (spindle and mixed cell types only) and found that it correlates better with survival. Figure 3 shows the most common histopathological pictures of UM with the characteristic cytological features.

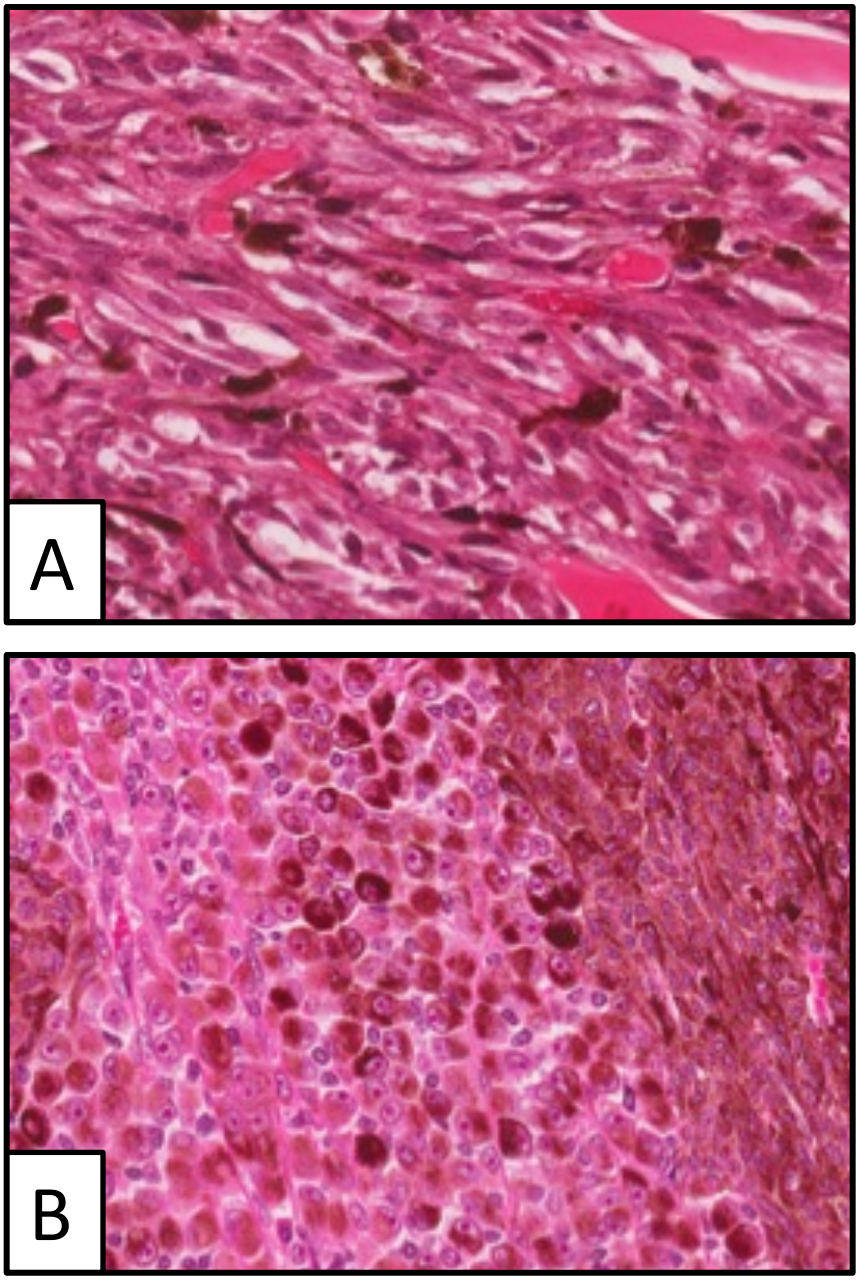

Figure 3. Histopathological pictures of UM. A) Spindle-cell-type tumor. B) Mixed-type-cell tumor. (Hematoxylin and eosin; 40X)

1.1.4.4 UM treatment

The main treatment modalities for UM include brachytherapy (plaque radiotherapy), enucleation and local resection. Plaque radiotherapy (using 125-iodine radioactive plaque) is the most commonly used treatment modality for UM because of its effectiveness in treating the primary tumor while preserving the vision in some cases. It is done through fixing the radioactive plaque to the sclera overlying the tumor. The main side effects of plaque radiotherapy include radiation-induced retinopathy and optic neuropathy (35). In rare cases where the tumor is large and radiation therapy is associated with high morbidity, enucleation can be performed. In terms of survival, the Collaborative Ocular Melanoma Study (COMS) found that for medium sized tumors, patients treated with brachytherapy or enucleation have similar survival rates (36). Local resection can be done in small number of cases where the tumor is small and not invading other structures. Other treatment modalities include: proton-beam radiotherapy, transpupillary thermotherapy, and laser photocoagulation.

1.1.5 Electromagnetic radiation and the eye

The electromagnetic spectrum extends to include low frequency wavelengths used in radio communications to ionizing radiation, such as gamma rays with the highest frequencies. Part of this spectrum is the visible spectrum, which ranges between 400 nm and 700 nm wavelengths. Ultraviolet (UV) rays are characterized by high-energy wavelengths shorter than 400 nm. On the other hand, infrared (IR) radiation is characterized by wavelengths longer than 700 nm.

The different ocular structures have different transmission spectra for electromagnetic radiation (37). For example, the cornea absorbs most of the UV-B (280–315 nm) light, allowing UV-A (315–400 nm) to enter the eye before most of it gets absorbed by the natural crystalline lens (38-42). The ozone layer blocks UV-C; however, this type of electromagnetic radiation is becoming a health concern because of holes in the ozone layer (43).

The retina is highly oxygenated and constantly exposed to light, and this combination puts the retina under oxidative stress most of the time (44, 45). Moreover, as we increase in age, the defense mechanisms that are key components of the retina decline thereby exposing the retina to more oxidative stress leading to cellular death and a disease called age related macular degeneration (AMD) (46-48). UV rays are an essential component of our solar system; however, as mentioned previously, the cornea and the lens filter most of them (38-42). In addition to cataract formation, as we age, our natural crystalline lens develops a yellow chromophore that is capable of filtering some of short wavelengths in the visible spectrum in addition to most of the UV-A (BL, 400 nm to 500 nm) (40, 42, 49-52). This natural yellowing is caused by the interactions between lens proteins (crystallins) with the compound 3-hydroxykynurenine glucoside (53). This natural process of BL filtering continues at a rate of 0.7%–0.8%/year, amounting to 50% of BL filtering at the age of 52 and 80% by the age of 70 (54, 55). Therefore, removing the natural crystalline lens with its protective filtering properties during cataract surgery will expose the back of the eye to the harmful effect of both UV rays and violet-blue light. Thus, filtering these high-energy wavelengths is an important characteristic of the implanted intraocular lens (IOL) to avoid the associated damage to the retina (56, 57).

The association between UV retinal damage and transparent non-UV blocking IOLs was reported as early as 1978 and UV filtering IOLs were first used in the early 1980s (58, 59).

1.1.6 Ultraviolet exposure and UM

Both cutaneous and uveal melanocytes arise from melanoblasts, which come from the neural crest during embryogenesis (60). Therefore, both share a common histopathologic and immunogenic profile (60, 61). As a result, a common phenotypic pattern exists (fair skin, light iris and inability to tan) that increases the risk of both malignant cutaneous melanoma and UM (60). Moreover, there is strong evidence suggesting the role of UV (10–400 nm wavelength) exposure and the pathogenesis of cutaneous melanoma (19, 20, 62, 63). This risk is higher in patients with a history of early, high and intermittent UV exposure during childhood (64, 65). Interestingly, despite the strong association between UV and cutaneous melanoma, the role of UV in the pathogenesis of UM is inconclusive (19, 20, 66).

Li and colleagues (67) studied tumor initiation related to retinal topography and found that it is not uniformly distributed: there are higher rates of UM in the macular area. The rate decreased as they moved away from the macular area (high light exposure area) towards the ciliary body (low light exposure area). Accordingly, this distribution was consistent with the dose distribution of solar light perceived by the retina; thus, the authors stated that solar radiation in the form of both UV and visible light played a role in the pathogenesis of UM. Moreover, in Australia, ocular sun exposure was found to be an independent risk factor for both choroidal and ciliary body melanomas (68). Although

this association was strongest with early age exposure, the authors recommended that UV filtering through close-fitting sunglasses should also be considered for older people. Interestingly, this association was not found in iris or conjunctival melanoma, which are the sites of highest UV exposure. Similarly, in the United States, people who were born in the southern part of the country have a higher incidence of UM than people born in the northern areas (69). The authors hence attributed latitude difference and UV exposure as a risk factor for developing UM.

On the other hand, Schwartz and co-workers (70) in 1997 demonstrated a lack of association between UV exposure and the development of choroidal melanoma. They commented that UV-B and UV-C are not transmitted through the eye and that UV-A is mostly filtered by the cornea and the lens, leading to only a small proportion reaching the posterior pole of the eye. Moreover, if UV-A is the only factor that promotes UM development, one would expect more tumors to arise in the posterior pole of the eye than in any other choroidal location. However, this is the exact opposite result to what they found through geographical mapping of UM in 92 eyes; tumors were equally distributed throughout all quadrants of the choroid.

Other studies have found that although the incidence of non-melanoma skin cancer increases in patients diagnosed with cutaneous melanoma, this association is not found in patients with UM, which contradicts the theory of UV exposure as a risk factor for UM (71). Moreover, Shah and colleagues (66) in 2005 performed a meta-analysis and concluded that latitude of birth or frequency of outdoor leisure activities are non-significant factors for UM development. Based on the summary presented herein, it is not clear whether there is an association between UV exposure and UM; however, it is

apparent that if indeed such a link exists, it is much more complex than the relationship between cutaneous melanoma and UV.

Several genetic studies also support the lack of association between UV exposure and UM development. For example, BRAF is a proto-oncogene responsible for the formation of B-Raf molecule involved in cell signaling that, when mutated, contributes to the development of cancer (72). Mutations in BRAF gene, especially in the BRAF V600 codon, are usually detected in skin melanomas and are believed to be caused by UV exposure in addition to other mutations in low susceptibility genes (73-75). Moreover, mucosal melanomas, such as those in the mouth and vulva, regions that are not exposed to UV, do not show BRAF mutations (75). This supports the role of UV in the pathogenesis of cutaneous melanoma in sun-exposed areas but not in mucosal melanomas. Interestingly, these BRAF mutations are found in large proportion of anterior UM (iris), but to a lesser extent in choroidal melanomas (76, 77). Moreover, this mutation, if present in choroidal melanomas, will be expressed heterogeneously throughout the tumor (76). This indicates that UV exposure may have a role in anterior UM where exposure to UV light is higher, but not in choroidal melanomas, which are the most common and carry the worst prognosis.

1.1.7 The role of blue light in UM pathogenesis

As mentioned previously, it is not clear whether UV exposure has a role in the development of UM due to the fact that most of it being filtered by the anterior structures of the eye (38-42). However, other visible high-energy wavelengths, such as in the BL spectrum (400 – 500 nm), can reach the posterior of the eye and are important in this

context. Visible BL can be found everywhere including sunlight, which is characterized by a very effective radiance, making it hazardous to the retina and its underlying structures (78).

The effect of BL on the mitotic activity of UM cells has been studied both *in vitro* and *in vivo*. For instance, Marshall and colleagues in 2006 tested the effect of BL on the proliferation rate of four different human UM cell lines (92.1, MKT-BR, OCM-1 and SP6.5) (79). They concluded that BL exposure resulted in an increase in the proliferation rate of these cell lines compared to their controls, which had no light exposure. Moreover, filtering BL using a commercially available BL filtering intra-ocular lens (SN60AT, Alcon Laboratories Inc., Fort Worth, TX, USA) protected the cells and resulted in a growth rate similar to the control groups. In order to replicate human disease, Di Cesare and colleagues further confirmed these results using human UM cells (92.1) in a rabbit model (80). At the end of the experiment, the cells from the BL exposed group showed a significant higher rate of proliferation. These results illustrated the ability of BL to penetrate the eye and to reach the posterior ocular structures. All these observations link BL as a factor for UM progression and proliferation.

Although the previous two studies used human UM cells, Manning and colleagues (81) also reported a large dark mass protruding from the globe of a Long Evan's rat following long-term exposure to BL. The mass was confirmed later to be a melanoma by histopathology. This confirms the involvement of BL in the malignant transformation of normal melanocytes in rats. Moreover, meta-analyses indicate that two occupations are associated with higher incidence of UM: welding and occupational cooking. Interestingly, both studies hypothesized that high frequency visible light exposure is the

possible factor underlying this increased risk (66, 82). There is also strong evidence implicating BL-induced changes in normal melanocytes of the skin. For example, neonates with congenital problems leading to hyperbilirubinemia are usually treated with phototherapy using the BL spectrum (83, 84). Interestingly, those neonates treated with BL therapy were found to have a higher number of moles (>100) and significantly higher number of atypical nevi than normal controls (85, 86). Because atypical cutaneous nevi are well known risk factor for skin melanoma, it is recommended that children who undergo phototherapy, especially with BL, should be screened frequently (85, 86).

The exact mechanism by which BL causes an increase in the mitotic activity of UM cell lines is not fully understood. However, evidence suggests that visible light with short wavelengths may lead to an increase in mitochondrial reactive oxygen species (ROS) production by retinal pigmented epithelial cells (RPE) in culture (87). Moreover, in another *in vitro* model, an RPE cell line (ARPE-19) showed a decrease in the metabolic activity and increased levels of intracellular stress proteins and reactive oxygen species when exposed to small doses of BL stimuli (88). The mitochondria of these cells became enlarged and showed other ultra-structural changes (88). Other models have also shown that BL exposure can lead to apoptotic cell death in the RPE and photoreceptor layers (89-91). It is believed that the oxidative stress in these cells causes apoptosis (90-92). Moreover, this oxidative stress is found to be higher in the presence of intracellular photoinducible pigments, such as lipofuscin and melanin, which are key components for both RPE cells and uveal melanocytes; respectively (91, 93-95). Although further studies are needed, it seems that ROS production is an influencing factor for various cellular

processes, such as proliferation, apoptosis, and tumor genesis and progression in uveal melanocytes (96, 97).

1.1.8 Current concepts regarding blue light filtering intra-ocular lenses

As mentioned previously, BL has been found to affect RPE cells *in vitro* leading to oxidative stress and eventually their death, which is manifested clinically as AMD. As a result, BL-filtering IOLs were first introduced in the 1990s (88-91, 93, 98-102). AMD is the most common cause of blindness in developed countries, affecting approximately 12.7 million individuals in Europe and USA (103-106). Worldwide, it is estimated that more that 196 million people will have AMD by 2020 (107).

Despite all of the studies that indicate BL is a risk factor for RPE death, BL filtering IOLs and AMD remain a source of continuous arguments in the literature with respect to the potential benefits of these lenses versus their side effects (98, 108-110). Although filtering UV alone blocks the harmful effects of this spectrum on the retina, large amounts of high energy BL still be able to enter the eye and may result in a condition called cyanopsia, where the patient sees more blue than normal (111, 112). Yellow tinted lenses usually filter both UV and the violet-blue spectrum and are manufactured to have a transmission spectrum similar to the lens of a 52 year-old human (37, 108, 113, 114). Therefore, replacing the natural lens during cataract surgery with an IOL with both UV and BL-filtering properties is more physiological than implanting only a UV filtering lens.

There are different types of BL filtering IOLs currently available on the market, and all demonstrate different transmission spectra for the BL spectrum (400 nm – 500

nm) (113). For instance, AcrySoft IOLs (Alcon Laboratories, Inc.) filter around 50% of BL at the middle of the spectrum (450 nm) (108, 113, 114). On the other hand, the orange-tinted PC 440Y IOLs (Ophtec, Inc. Groningen, the Netherlands) filter around 90% of BL at 450 nm (114). Although filtering BL by these lenses confer protection, it is important to note that they may also affect photoreception leading to poor dark adaptation, circadian rhythm disturbances and abnormal color perception (113).

Under mesopic (dim but not dark conditions) situations, our spectral sensitivity curve moves toward the blue spectrum and we become more sensitive to BL, which is known as the Purkinje effect. Although clinical studies are lacking, theoretical evidence suggests filtering BL under these conditions may affect this physiological mechanism of dark adaptation (113, 115-117). Moreover, as we increase in age, our rod cells, which are responsible for dark adaptation, decrease in both number and sensitivity. This further complicates dark adaptation for the elderly who undergo cataract surgery and yellow IOL implantation (109, 118).

In addition to dark adaptation, BL is an important stimulus for melanopsin, a photopigment present in the photoreceptive retinal ganglion cells (119). Melanopsin plays an important role in photoentrainment of the circadian rhythm and thus, BL filtering may affect the sleep-wake cycle (120).

Another problem that can be encountered when filtering BL is abnormal color perception manifested clinically as “yellow vision” (121). This effect maybe exacerbated when a transparent lens is implanted in one eye and a yellow tinted lens in the other eye (121).

1.1.9 Personalized IOLs

Personalized medicine is a large and rapidly advancing approach to medicine that describes treatments or diagnostic procedures conducted based on the unique clinical, environmental and genomic characteristics of each individual patient. It is an area of medicine that looks into the patient as a whole, taking into consideration all aspect of patient health including the psychological status. It uses information from the patient's medical history, clinical features and diagnostic procedures and even involves molecular studies to characterize each patient. Therefore, personalized medicine enables disease prevention, early diagnosis and timely treatment for the patient. Despite being a new approach to healthcare, personalized medicine has proven to be safe, effective, and contributes to a lower economic burden (122).

Different patients have different risk factors for developing UM, with higher risk patients presenting with fair skin, light irises and pre-existing choroidal nevi (19, 20). On the other hand, patients with dark skin and dark irises have the lowest risk of developing UM (19, 20). Therefore, not all patients will require the 50% filtration of BL provided in the commercially available IOLs. Approaching each patient using the concept of personalized medicine is something that should be adopted in the context of preventing UM when using BL filtering IOLs. Patients at high risk of developing UM can be offered an IOL with greater BL-filtering capacity during cataract surgery to provide high protection against UM development. On the other hand, people with a low risk profile can be offered an IOL with less BL filtering to provide some protection, while avoiding the aforementioned side effects.

1.1.10 The effect of BL on the angiogenic properties of UM

Cancer cells can spread locally within an organ or metastasize to remote organs. Distant metastasis requires the formation of new blood vessels or lymph vessels leading to hematogenous or lymphatic spread, respectively. Moreover, angiogenesis is important for cancer stability, growth and to provide nutrients, oxygen and even to remove waste from the rapidly dividing cancer cells. In fact, cancer cells may undergo necrosis in the absence of vasculature (123).

Angiogenesis is very important in UM, as this type of tumor develops in a highly vascularized structure. Due to the lack of lymphatics in the eye, UM can only metastasize through the blood, and the liver is the most commons site for metastasis (124, 125). Unfortunately, up to 40% of patients diagnosed with UM will die from liver metastasis within 10 years of diagnosis (15, 126). Moreover, the mean survival rate after liver metastasis diagnosis is a paltry 2.2 months (124, 125). Despite advancements in primary tumor diagnosis and treatment, the incidence of metastasis and survival rates have remained unchanged (15, 127).

Primary UM can be eradicated using surgery or radiation; however, metastatic UM represents a challenge. If isolated hepatic metastases are found, surgical resection can double the survival rate compared to patients with residual disease (128). However, if metastatic tumor cells are distributed throughout the liver, surgical excision is impossible (129). This makes therapeutic treatments, such anti-angiogenic drugs, possible alternatives in order to improve patient survival.

In 2002, a study showed that eyes with UM had significantly higher levels of vascular endothelial growth factor-A (VEGF-A; up to a 20-fold increase) in both vitreous and/or aqueous humor samples compared with controls that underwent routine cataract surgery (130). An *in vitro* model also showed that different UM cell lines synthesized and secreted VEGF-A and basic fibroblastic growth factor (b-FGF) in culture (131, 132). Other *in vitro* models also confirmed that vascular remodeling is an important step for UM metastasis, as evidenced by the synthesis and excretion of various pro-angiogenic factors, including VEGF-1 and angiopoietin-2 (ANG-2), and down regulation of anti-angiogenic compounds, such as angiopietin-1 (133, 134). Moreover, using non-immune suppressed mice, serum levels of VEGF correlated with the number and location of hepatic micrometastases (135).

The BL component of the visible spectrum is involved in UM progression and metastasis in which angiogenesis plays an important role (79, 80). However, the relationship between BL exposure and BL filtering on the synthesis and excretion of the different pro-angiogenic factors by UM cells in culture has not been studied. Nevertheless, exposing ARPE-19 cells to light has been found to increase production of VEGF and when the BL spectrum was filtered by a yellow IOL, VEGF levels decreased significantly (136-138). Therefore, studying the effects of BL on the angiogenic properties of UM cell lines might provide insight into the role of BL in the metastatic process.

2 Chapter Two

2.1 Purpose

The purpose of this study is to test the effect of filtering less than 50% of BL (at 450 nm) on the proliferation rates of one human UM cell line (92.1) and one human transformed uveal melanocyte cell line (UW-1). The secondary objective involves testing the effect of BL on the levels of the different pro-angiogenic factors excreted by these cells.

3 Chapter Three

3.1 Methods

3.1.1 Cell culture

One human UM cell line (92.1) and one transformed human uveal melanocyte cell line (UW-1) were used to test the effects of filtering different intensities of BL. These cell lines were established by Dr. Jager (University Hospital Leiden, the Netherlands) and Dr. Albert (University of Wisconsin-Madison, WI, USA), respectively. Initially, low passage cells were seeded in a 6-well plate and then incubated at 37ºC in a humidified environment supplemented with 5% CO_2. They were cultured in an RPMI-1640 medium with Glutamax^{TM} and a phenol red indicator (Invitrogen, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (Invitrogen), 1% penicillin/ streptomycin (Invitrogen) and 1% fungizone (Invitrogen). The cells were then passaged routinely using trypsin-EDTA (Invitrogen) when approaching 70% –80% confluency.

3.1.2 Experimental setup

On the first day of the experiment, the cells were passaged as usual. The cells were then seeded in 24 wells of a 96-well plate (Corning Inc, Corning, NY, USA; Cat no. 3603) at a concentration of 2500 cells in 200 µl media/well using the same culture medium described above. Twelve biological replicates represented the control group and the other 12 represented the filter group. The 96-well plate was black with a clear bottom in order to allow the passage of light in one direction (up and down) without scattering to other wells. The entire plate was then placed inside the incubator overnight in order to

allow the cells to adhere to the bottom. On the second day, the media was removed and the wells were washed with Hanks' balanced salt solution (HBSS; Invitrogen). After washing, each cell containing well was filled with 200 µl of a serum-free RPMI-1640 medium with Glutamax^{TM} **without** phenol red indicator (Invitrogen) and supplemented with 1% penicillin/streptomycin (Invitrogen) and 1% fungizone (Invitrogen). The phenol-red-free RPMI will provide a colorless medium that allows the passage of the full spectrum of light without potential blocking of any wavelength. The filters were then applied (to be discussed in the next section) and the cells were then exposed to light for 3 hours daily for a total of 4 days inside a dark and clean room. After exposure, the cells were visualized through an inverted microscope in order to confirm their viability and then put back into the incubator until the next day. A thermometer was used to ensure temperature stability during the experiment. The same experimental setup was used for each filter and a cellular proliferation assay was performed after the experimental period at the end of the fourth day. Each experiment was performed in duplicates to ensure the accuracy of the results. Figure 5 shows the basic experimental setup.

3.1.3 Light filters

Three different filters were purchased from Rosco Laboratories (Rosco Laboratories Incorporation. Stamford, CT, USA). The first filter is transparent (Cat no. #00 Clear) and was used to cover the control group in each experiment to ensure that equal lux reached the cells in the control and experimental groups. The other two BL filters were used to cover the experimental group during each experiment. Each filter was selected based on its BL-filtering properties at the middle of the BL spectrum (450 nm wavelength) according to the transmission curve published by the company, filtering

either 16% or 20% of BL. Filters that filter less than 16% of BL or between 16% and 20% of BL were not commercially available. These filters were made of two different materials: polyethylene terephthalate (PET) or polycarbonate (PC). The different types of filters used in our experiments and their BL filtering properties are shown in Table 1, and the transmission curves for each filter are shown in Figure 4.

Filter number	**Percentage of BL filtering**
#373 Theatre Booster 3	16 %
#4307 Calcolor 7.5 Cyan	20 %

Table 1. The identification numbers of the different filters used in our experiments with their filtering properties for BL at 450 nm wavelength.

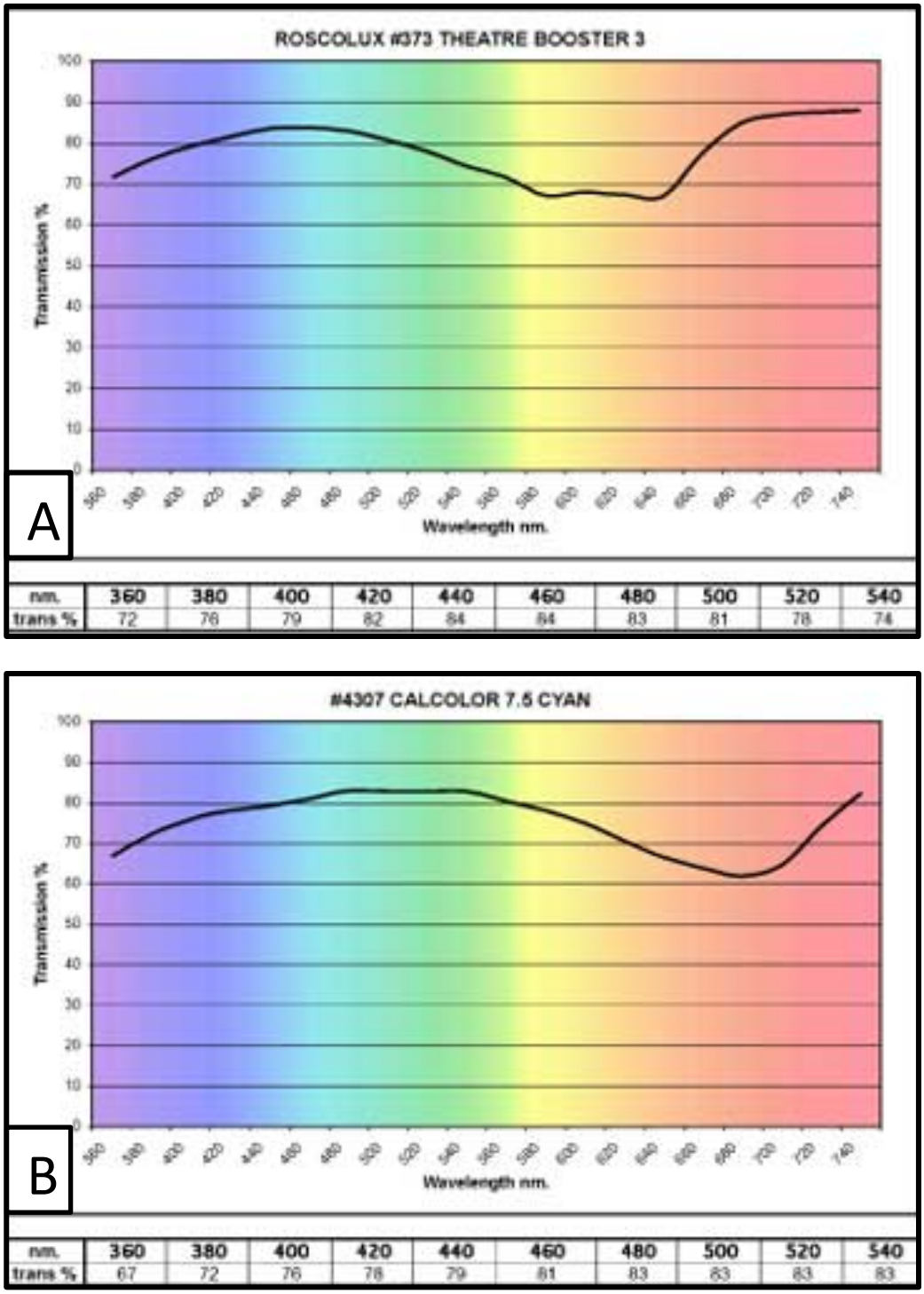

Figure 4. The transmission curves of the BL filters used during our experiments. (A) The transmission curve for filter #373, filters 16% of BL at 450 nm. (B) The transmission curve for filter #4307, filters 20% of BL at 450 nm. Adopted from: www.rosco.com

3.1.4 Blue light source

The cells were exposed to light using a 150-watt fiber-optic illuminator (Model: 21AC, Edmond Industrial Optics, Barrington, NJ, USA) connected to a fiber-optic light guide. Using the control panel, the level of illuminance was set at 10,000 lux, which represents the actual lux we are exposed to normally (at noon of a sunny day with scattered clouds). This illuminance was confirmed using a lux meter (PLMT12; Pyle Audio Inc., NY, USA). In addition to the previously mentioned (experimental) filters, two other filters were used during each experiment to ensure the passage of the visible spectrum only: an infrared (IR) cutoff filter (heat absorbing filter) and UV cutoff filter. The IR cut-off filter was used to prevent the heating effect of IR radiation on the cells, while the UV cut-off filter prevented any confounding effects of the non-visible short wavelength on cellular proliferation.

3.1.5 Cell proliferation assay

At the end of the fourth day, a cell proliferation assay was performed using cell-counting kit-8 (CCK-8; Dojindo Molecular Technologies Inc, Kamimashiki, Japan) according to the protocol provided with the kit. Briefly, the CCK-8 reagent was added into each of the cells containing wells in an amount equal to 10% of the medium (20 µl) with special attention not to create bubbles, as they may interfere with the reading using the plate reader. Two more wells with only media—without cells—were used as blanks. Next, the entire culture plate was placed in the incubator for 2 hours. After the designated incubation period, the number of cells in each well was determined using a multifunctional microplate reader (Infinite ® 200pro; Tecan, Männnedorf, Switzerland)

with the following settings: 450 nm wavelength, bandwidth of 9 nm; number of flashes 25. The absorbance of each well was represented by optical density (OD), which is highly dependent on the amount of cells present in each well. The average OD of the blank wells was deducted from the OD of each well to remove the background effect. This assay has been to shown to have higher detection sensitivity than other assays using other tetrazolium salts. This cell proliferation assay consists of a highly water soluble tetrazolium salt (WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)- 5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) that is reduced by intra-cellular dehydrogenases leading to the formation of an orange pigment, thereby indirectly reflecting the number of viable cells.

3.1.6 Human angiogenesis array

3.1.6.1 Experimental setup:

Based on the results of the proliferation assay, we decided to test the effect of BL filtering on the angiogenic properties of the tested cells using 20% filtration for the 92.1 cell line and 16% filtration for the UW-1 cell line. We used the same culture methods described previously with some modifications. For instance, on the first day of the experiment, we seeded 7,500 cells in each of the 24 wells of a 96-well plate using serum-containing media. We increased the number of seeded cells because these pro-angiogenic factors are excreted in low quantities, which can fall below the array's level of detection. The 24 wells were divided into 12 replicates for the control group and 12 for the filter group. The cells were then left inside the incubator overnight to allow attachment to the bottom of the wells. On the second day of the experiment, we removed the serum-

containing media and washed the wells twice using HBSS. Next, each well was filled with 100 µl of serum-free, phenol red-free RPMI-1640 medium (Invitrogen). The cells were then exposed to light using the same BL filters described previously. To make sure that the differences were not merely due to the differences in cell numbers (as we have shown that BL affects proliferation), we shortened the protocol for only 1 day of exposure (3 hours). At the end of exposure, the media from six wells of each experimental condition was collected and combined to form one biological sample. Combining the media from the other six wells formed the second sample. The same was done for the control group. Then we centrifuged each sample at a rate of 2000 rpm at 4°C. The supernatant was then collected and stored at –80°C to be used later when performing the Quantibody® human angiogenesis array. The same experimental setup was used for the other experiment using the other filter.

3.1.6.2 Quantibody® human angiogenesis array:

We tested the effect of filtering BL on the secretion of ten different pro-angiogenic cytokines (VEGF, Angiogenin, ANG-2, epidermal growth factor; EGF, bFGF, heparin binding EGF-like growth factor; HB-EGF, hepatocyte growth factor; HGF, Leptin, platelet derived growth factor subtype BB; PDGF-BB and placental growth factor; PIGF) by both 92.1 and UW-1 cell lines using the Quantibody® Array (RayBiotech, Norcross, GA, USA). This array is based on the multiplex sandwich enzyme-linked immunosorbent assay (ELISA) technology to determine the concentration of multiple cytokines at the same time with high sensitivity and specificity. Briefly, a glass slide provided with the kit consists of 16 wells of identical cytokine antibody arrays for the aforementioned pro-angiogenic compounds. Each antibody is arrayed in

quadruplicates. All conditions and controls were tested in duplicates using the samples prepared previously. The array was conducted using the protocol and the material provided with the kit. First, the glass chip device was allowed to dry inside and then outside the sealed plastic bag for a total of 2 hours. The glass chip device was then blocked using the Sample Diluent provided with the kit for 30 minutes. Meanwhile, we created serial dilutions of cytokine standards by mixing a reconstituted cytokine mix with the same Sample Diluent as described in the protocol. After blocking, 100 µl of each of the seven cytokines standards prepared and 100 µl of a control composed of only the Sample Diluent were applied to the glass chip. This step generated standard curves of pre-determined standard concentrations in order to quantify the cytokines in our samples. In addition, 100 µl from each of the two biological samples of each filter and another 100 µl from the controls of both filters were also applied to the glass chip and then incubated at room temperature for 1 hour. After the incubation period, all the samples, including the standards and the controls, were decanted and the glass chip was washed with the wash buffer provided. Then the glass chip was incubated for 1 hour with 80 µl of a reconstituted detection antibody cocktail provided with the kit and then washed with the washing buffer. After washing, the glass chip was incubated with the Cy3 equivalent dye conjugated streptavidin provided in the kit for another 1 hour and then washed afterwards. The glass chip device was then disassembled and inserted into the glass dryer/washer; it was afterwards washed with wash buffer 1 and wash buffer 2 as described in the protocol.

3.1.6.3 Slide Scanning and data analysis:

After the last washing step, the glass was dried in a slide holder by centrifuging at 1000 rpm for a total of 3 minutes. It was then sent by priority mail in a cold container and wrapped with aluminum foil for scanning and analysis at RayBiotech. A laser scanner was used to detect the fluorescent dye and the data were quantified and analyzed using the Quantibody Q-Analyzer® software. The concentrations of the different angiogenic cytokines were determined by comparing the fluorescent dye signals from either the control or the condition group with the signals from the standard dilution curves.

3.1.7 Statistical analysis

For the 92.1 cell line, the proliferation rates (filtering 20% BL versus control and filtering 16% BL versus control) and the levels of the detected pro-angiogenic cytokines (filtering 20% BL versus control) were compared. For the UW-1 cell line, the proliferation rate (filtering 16% BL versus Control) and the levels of the detected pro-angiogenic cytokines (filtering 16% BL versus control) were compared. Statistical analysis was performed using Graphpad Prism (GraphPad Inc, San Diego, CA, USA). Normality was determined using the D'Agostino - Pearson omnibus test. The Student's *t*-test was performed to compare the values between each two groups and *P*-values <0.05 were considered significant.

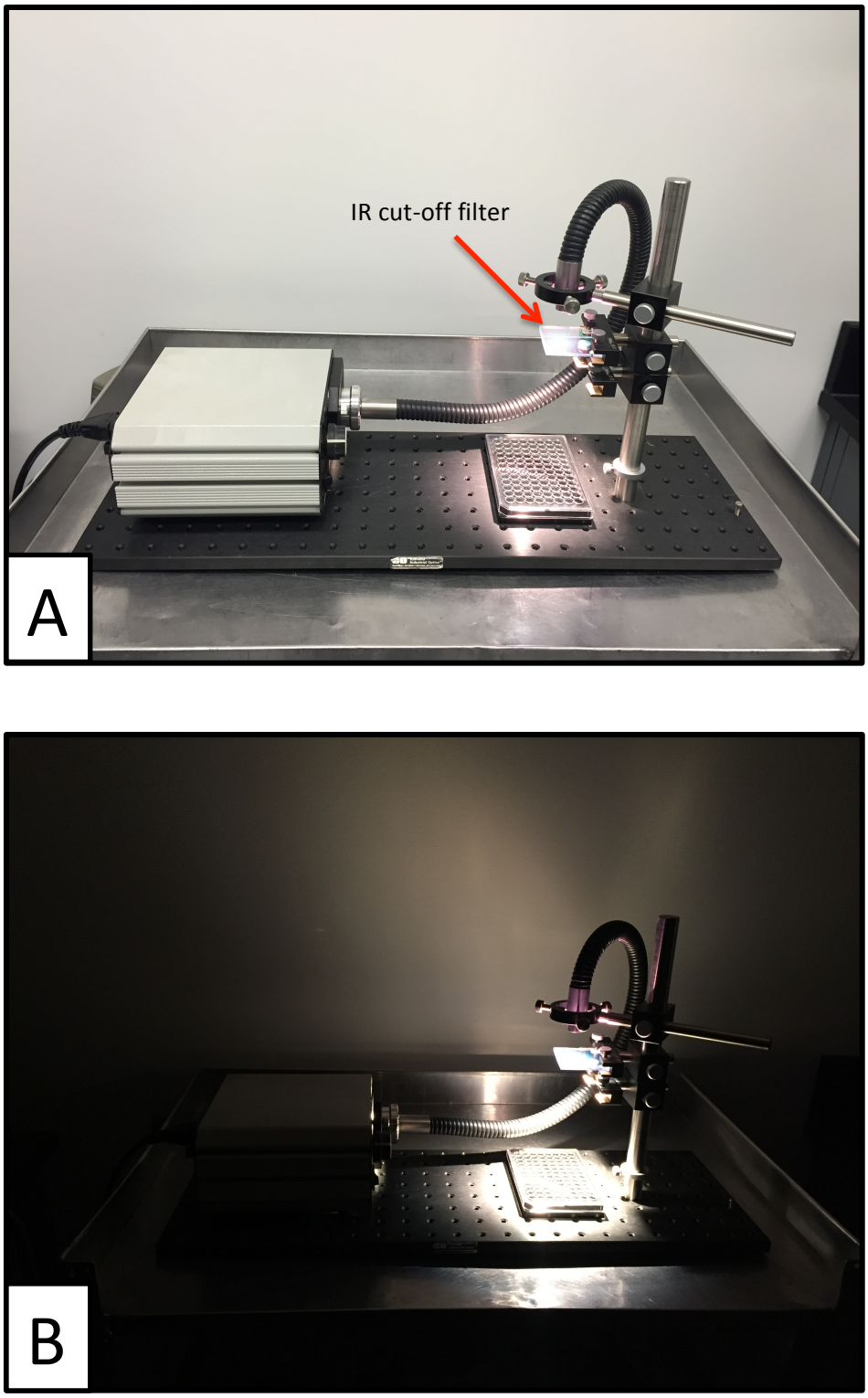

Figure 5. The experimental setup showing the fiber-optic illuminator and the fiber-optic guide during both open light (A) and dark room (B) conditions. Note the IR cut-off filter preventing IR rays from reaching the cells.

4 Chapter Four

4.1 Results

4.1.1 Proliferation assay

As expected based on previous studies, exposing UM cells to white light for 3 hours daily for a total of 4 days caused an increase in the proliferation rate. Moreover, filtering BL decreased the proliferation rate of both cell lines. Interestingly, both cell lines showed different sensitivities for BL filtration. For instance, filtering 16% of BL reaching the 92.1 cell line did not change the proliferation rate significantly when compared to the control with full light exposure (1.22 ± 0.12 versus 1.29 ± 0.10 OD; P = 0.175). However, filtering 20% of BL caused a significant reduction in the proliferation rate compared to the control (1.16 ± 0.12 versus 1.33 ± 0.08 OD; $P < 0.01$) (Figure. 6). Conversely, the UW-1 cell line showed a significant reduction in proliferation when filtering 16% of BL compared to full exposure to light (1.46 ± 0.07 versus 1.72 ± 0.05 OD; $P < 0.01$; Figure. 7). Table 2 shows the effect of BL filtering on the proliferation rates of both cell lines. These results indicate that filtering 16%–20% of BL is enough to offer a protective effect.

Table 2. The effect of BL filtering on the absorbance levels (proliferation rates) of the tested cell lines.

Cell line	BL filtering level at 450 nm (%)	Average absorbance of the filter group (OD) ± SD	Average absorbance of the control group (OD) ± SD	*P* value
92.1				
	16	1.22 ± 0.12	1.29 ± 0.10	0.175
	20	1.16 ± 0.12	1.33 ± 0.08	< 0.01
UW-1				
	16	1.47 ± 0.07	1.72 ± 0.05	<0.01

BL: blue light; OD: optical density; SD: standard deviation; nm: nanometer

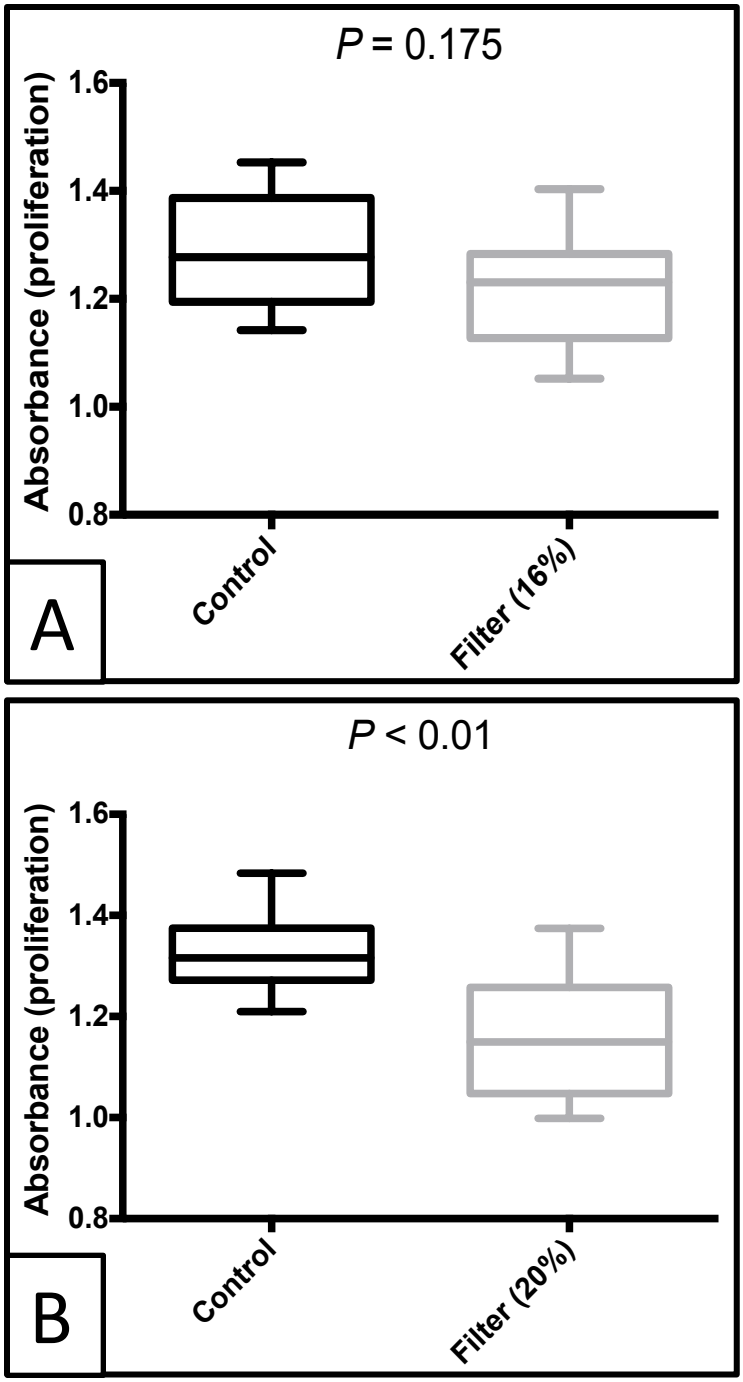

Figure 6. A box-and-whisker plot showing the average absorbance for the 92.1 cell line after light exposure using cell counting kit-8 (CCK-8) proliferation assay. (A) Filtering 16% of BL did not decrease the rate of proliferation significantly (P = 0.175). (B) Filtering 20% of BL resulted in a significant reduction in the proliferation rate (as shown by the lower absorbance) when compared to the full spectrum exposure ($P < 0.01$).

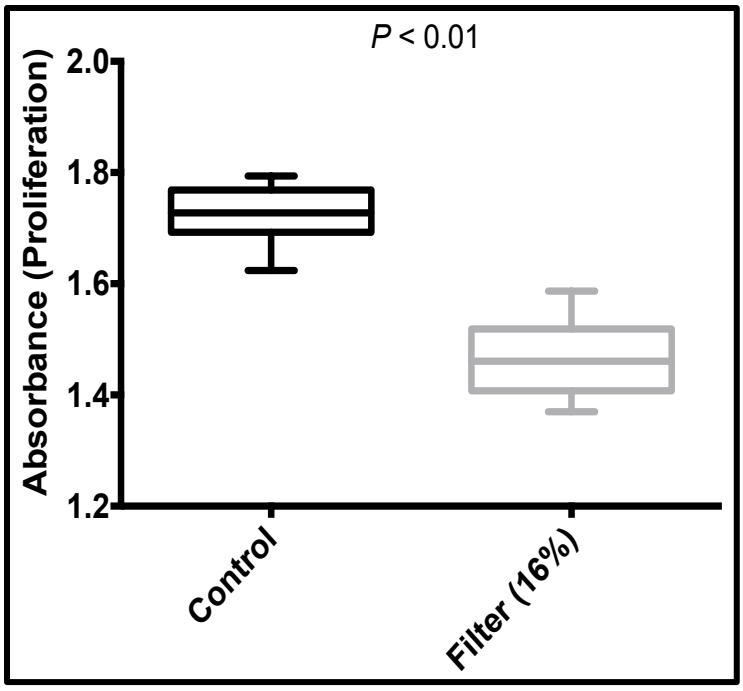

Figure 7. A box-and-whisker plot showing the average absorbance for the UW-1 cell line after light exposure using cell counting kit-8 (CCK-8) proliferation assay. Filtering 16% of BL was sufficient to decrease the rate of proliferation significantly ($P < 0.01$).

4.1.2 Human angiogenesis array

Using the Quantibody® human angiogenesis array, four different pro-angiogenic factors (VEGF, angiogenin, leptin and PIGF) were detected in the media of both the control group and the BL filter group (filtering 20% of BL) for the 92.1 cell line. The other cytokines were not produced in detectable quantities. When comparing both groups, the BL filtered group showed decreased levels of the excreted VEGF (1029 ± 350 pg/ml versus 1314.6 ± 259 pg/ml), angiogenin (2586.6 ± 77.6 pg/ml versus 2764.8 ± 327.4 pg/ml), leptin (35.7 ± 8.5 pg/ml versus 36.1 ± 11.4 pg/ml) and PIGF (79.3 ± 2 pg/ml versus 100 ± 28 pg/ml); however, the changes were non-significant with *P* values of 0.45, 0.53, 0.97 and 0.41, respectively (Figure. 8). For the UW-1 cell line, only VEGF and angiogenin were detected in the media of both the control and the filter group (filtering 16% of BL), while the other cytokines were not produced in detectable quantities. Moreover, filtering 16% of BL decreased the production of both VEGF (357.3 ± 32.8 pg/ml versus 438.9 ± 10.1 pg/ml) and angiogenin (1777.1 ± 33.5 pg/ml versus 1919.3 ± 2.6 pg/ml). This decrease was not significant for VEGF ($P = 0.14$; Figure 9); however, a trend towards a significant decrease was seen with angiogenin ($P = 0.06$; Figure 9). These results indicate that filtering BL may have an effect on angiogenic properties of the tested cells, which is an important step for metastasis in patients diagnosed with UM.

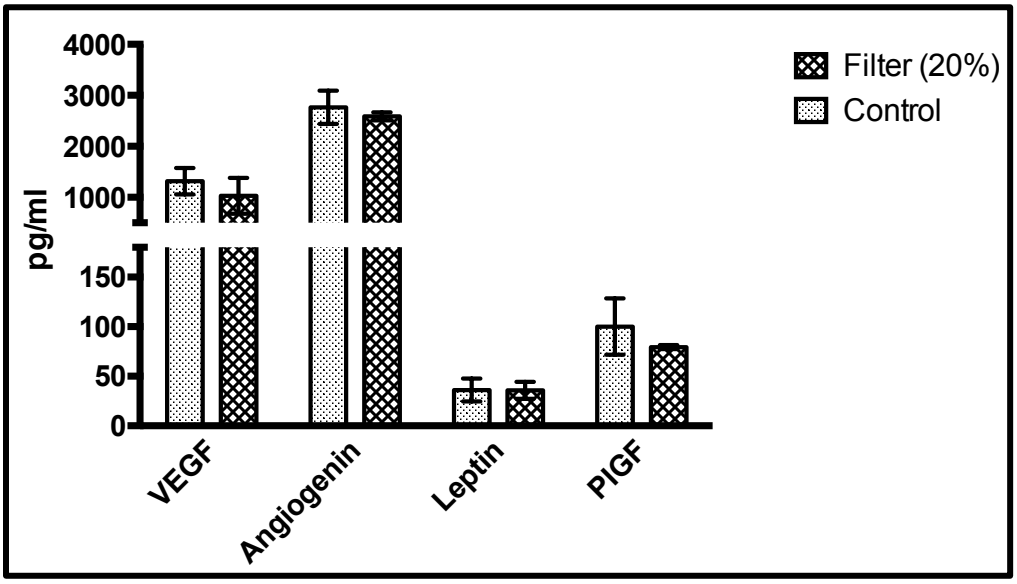

Figure 8. Levels of the different pro-angiogenic factors detected in the media of the 92.1 cell line after exposure to light or filtering 20% of BL. The differences between the two conditions for VEGF, angiogenin, Leptin and PIGF were not significant with *P* values of 0.45, 0.53, 0.97 and 0.41, respectively.

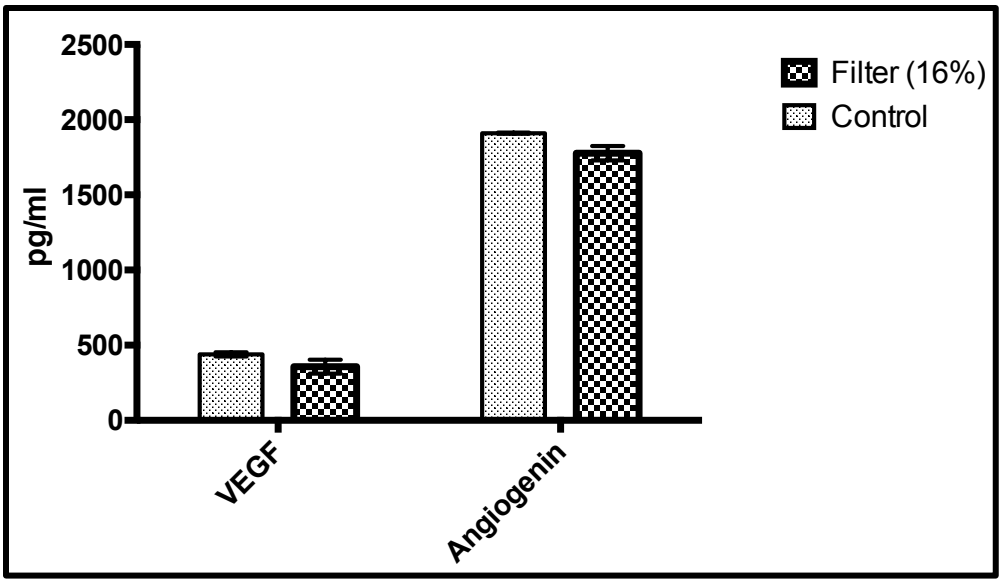

Figure 9. The levels of the different pro-angiogenic factors detected in the media of the UW-1 cell line after both exposure to light and filtering 16% of BL. The differences between the two conditions for both VEGF and angiogenin were not significant with *P* values of 0.14 and 0.06, respectively.

5 *Chapter Five*

5.1 Discussion

UM is the most common primary intra-ocular malignancy in adulthood and is a potentially life-threatening disease. It originates from the melanocytes of any of the uveal structures, namely the choroid, ciliary body and the iris. The age-adjusted incidence of UM is 5.1 cases per million in the United States (15). Despite the recent advances in the diagnosis and treatment of this type of cancer, up to 40% will die from liver metastasis within 10-year-period (15, 126).

There are several risk factors that increase the chances of developing UM. Fair skinned people, people with light irises, and people who find difficulty tanning, are all at risk for developing UM (19, 20). Moreover, pre-existing nevi in the choroid, especially if they are large, are capable of malignant transformation making an individual at risk for developing UM (139).

Interestingly, the blue component of white light has been linked to the development and progression of UM in both *in vivo* and *in vitro* studies (79, 80). In addition, Marshall et al (79) found that filtering BL using the SN60AT IOL (Alcon, Inc.), which filters 50% of BL at 450 nm, significantly decreased the proliferation rate of four different human UM cell lines. Therefore, implanting BL filtering IOLs during cataract surgery might confer protection against the development and progression of UM.

Based on the aforementioned risk factors, it is evident that different people have different risk profiles for developing UM and that a “one-size-fits-all” approach to blue-

light filtering (50% at 450 nm) might not be ideal. For instance, someone with blond hair and blue eyes might require the traditional 50% filtering, but someone with dark hair and dark irises might achieve protection against UM development with less filtering, thereby subjecting themselves to fewer side effects of filtering BL. Therefore, in the present study, we hypothesized that a protective effect against BL-induced changes in UM cells can be achieved by filtering less than 50% of BL. To test this hypothesis, we decided to test the effect of filtering less than 50% of BL (450 nm) on the proliferation rates of one human UM cell line, 92.1, and one transformed uveal melanocyte cell line, UW-1. These cell lines represent both ends of the spectrum with the 92.1 cell line being characterized by high proliferative, migration and invasion potential, while the UW-1 cell line is known to have benign morphologic features and lower proliferation rates (140, 141). We noticed that filtering 20% of the BL spectrum in the 92.1 cell line significantly decreased the proliferation rate compared to full light exposure. On the other hand, filtering 16% of BL (450 nm) was enough to decrease the proliferation rate significantly of the UW-1 cell line compared to a control with full light exposure. This difference may be explained by the fact that the 92.1 is a highly malignant cell line, which makes it more vulnerable to BL-induced mutations leading to increased aggressiveness; therefore, filtering more BL (20%) was necessary to prevent these changes. However, for the UW-1, which is a transformed uveal melanocyte cell line, we needed to filter less BL (16%) to have the protective effect.

The clinical implications of our results is that we can achieve the protective effect of filtering BL using less than 50% BL filtration (between 16%–20%). This will allow patients at low risk of developing UM undergoing cataract surgery to avoid the potential

unwanted side effects reported when implanting the currently available BL filtering IOLs (50% of BL at 450 nm), such as decreased blue color perception, abnormal circadian rhythm and poor dark adaptation (109, 113, 115, 117-121). In other words, the decision of implanting an IOL with specific filtration level for BL will be based on the individualized risk of each patient for developing UM. This approach is fundamental in the practice of personalized medicine where each treatment is tailored based on the individual patient need. One way of implementing this practice clinically can be achieved by conducting a risk-profile scoring system that can be filled before the day of surgery by the attending ophthalmologist. This risk profile scoring system can predict the likelihood of a person to develop UM based on the total score given for his/her risk factors. For example, people with fair skin and light irises are at higher risk for developing UM than people with dark skin and therefore will be given a higher score. Moreover, people with fair skin and light irises who have choroidal nevi are at higher risk for developing UM than people with fair skin and light irises but without choroidal nevi. Therefore, based on the final score, a decision can be made as to which BL filtration level is needed to protect against UM development. It is worth mentioning that when considering the potential side effects of BL filtering, avoiding these side effects with less than 50% filtration is only theoretical and how much less side effects we can get with only 16% or 20% BL filtration is unknown. In order to know so, retrospective clinical studies with IOLs that filter less than 50% BL are needed.

When discussing BL as a risk factor for developing UM, the concept of BL filtering should be extended beyond just patients undergoing cataract surgery and the implantation of BL filtering IOLs. While it is known that UM develops in old people and

that as we age, our lens yellows and thereby naturally filters BL (40, 42), we must consider that there are millions of people that have already implanted with IOLs that do not filter BL. For those individuals, sporting sunglasses with built-in BL-filtering properties should be a serious consideration. Moreover, for certain occupations with high emission for UV and BL such as welding, wearing protective shields is highly recommended (142).

In the present study, we did not use a control protected from light, as was done by Marshal et al. in their previous work (79). This decision was made because both groups, the control and the condition groups, should be tested under a similar environment and shielding the control group from light will completely alter this environment by increasing humidity and reducing temperature. Moreover, our eyes are constantly exposed to light (except during sleep), thus we believe that having controls with full-spectrum light exposure represent a more realistic scenario.

In addition to their high proliferation rate, UM cells depend on angiogenesis as an important step for metastasis. In fact, UM cells only metastasize hematogenously (124, 125). Therefore, the idea of targeting angiogenesis is appealing for decreasing metastasis rates. In 2010, an *in vivo* model showed that targeting VEGF using bevacizumab suppressed primary tumor growth and decreased the number of liver micrometastases in a dose dependent manner (143). Accordingly, in addition to measuring proliferation rates, we also tested the effect of BL filtering on the secretion of different pro-angiogenic molecules by the tested cell lines, which, to the best of our knowledge, is the first study to do so. Both cell lines are known to synthesize and excrete various pro-angiogenic cytokines including VEGF, angiogenin, EGF and HB-EGF (144). Of the ten pro-

angiogenic cytokines tested, only four were detected in the media of the cultured 92.1 cell line: VEGF, angiogenin, leptin and PIGF. Conversely, only two pro-angiogenic factors were detected in the media of the UW-cell line: VEGF and angiogenin. Moreover, although we seeded the same number of cells (7,500 cells/well) for both cell lines, 92.1 cell line showed increased baseline levels of both VEGF and angiogenin compared to the UW-1 cell line. These differences could be related to the high migratory and metastatic potential of the former cell line (140, 141). Interestingly, filtering 20% and 16% of BL for the 92.1 and the UW-1 cell lines, respectively, decreased levels of excreted pro-angiogenic cytokines. Although these changes were not significant, angiogenin excreted by the UW-1 cell line was very close to meeting the statistical threshold. These results, for the first time, indicate the effect of BL and BL filtering on the levels of the different pro-angiogenic factors excreted by UM cell lines in culture. Combined with the protective effect of decreasing the proliferation rate, BL filtering may also protect against metastasis by decreasing the angiogenic potential of UM cells. Therefore, BL filtering IOLs for patients with high-risk profiles for developing UM, such as patients with large choroidal nevi, might have a tangible impact on survival. However, to validate these claims, further *in vivo* studies using animal models are warranted.

Throughout our study, we attempted to create a light exposure environment that simulates real life BL exposure. By comparison, in Marshal et al. experiments (79), cells were exposed to pure blue light; however, outside of very specific work-related conditions (such as welding), this scenario is unrealistic. In addition, using a thermometer during each experiment ensured that the exposure environment was monitored for possible temperature alterations. Another strength for our model is our proliferation assay

choice. Prior to using CCK-8, we used a sulforhodamine B assay to estimate the number of cells present in a given well. However, this assay is time consuming and requires multiple steps, all of which are subject to error, which was evident during our test experiments. Using CCK-8, on the other hand, involves only adding the chemical reagent to the well of interest and then incubating the experimental plate from 1–4 hours before reading the OD. Therefore, our model improved on previously studied models and we believe that, to date, it is the gold standard *in vitro* model for determining the effect of BL on UM cells.

Despite all the strengths of our model, it still suffers from some limitations. For example, the fiber-optic light source used in our experiment was not compatible for use inside the incubator. As a result, cells were stressed during exposure time. Moreover, exposing cells to light inside the incubator would have allowed us to increase exposure time, representing a chronic, instead of acute, situation, which is more representative of real life exposure.

It is clear from our results that filtering 16%-20% of BL can provide a protective effect against the development and progression of UM. This will allow customizing each BL filtering IOL based on the risk profile of each patient for developing UM. Moreover, the changes we made to the previously reported *in vitro* model testing the effect of BL on UM cells better simulate a real life exposure scenario. In addition, this study is the first to demonstrate the effect of BL and BL filtering on the angiogenic properties of UM cells.

5.2 Conclusion

By developing and implementing an *in vitro* BL exposure model, we showed that filtering BL decreases the proliferation rates of two different human UM cell lines, 92.1 and UW-1. However, it was apparent that both cell lines had different sensitivities when it comes to the percentage of BL filtered. In addition, BL filtering also resulted in a non-significant decrease in the secretion of various pro-angiogenic factors by both cell lines.

Based on the results of the current study and in line with the concept of personalized medicine, we recommend that individuals can be offered BL filtering IOLs with different filtering properties based on their individualized risk for developing UM. This will decrease the unnecessary associated side effects that, while rare, some people may experience with BL filtering, while maintaining the same protective effect against UM pathogenesis and progression.

Future perspectives involve manufacturing IOLs with specific BL filtering properties. Based on our results, the protective effect against aggressive UM changes was achieved by filtering 16% and 20% of BL, indicating that we may not need yellow IOLs that filter more than 20% of BL. In fact, these numbers can be used as reference points during the manufacturing process of these IOLs. After manufacturing these customized IOLs, the next step will be testing their protective effect against the development and progression of UM using an *in vitro* model. Moreover, we believe that an animal model using the same lenses is important to validate any result. Another future perspective, as discussed previously, is the development of risk-profile scoring system. This will make the clinical application of personalized medicine in regards to BL filtering IOLs more

feasible. With this scoring system, patients at high risk of developing UM can be offered more BL filtration than people with lesser risk.

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