# THE ROLE OF RANIBIZUMAB AS AN ADJUNCTIVE AGENT IN MITOMYCIN-C-TREATED GLAUCOMA TUBE IMPLANTATION ON CONJUNCTIVAL FIBROBLAST TISSUE CULTURE

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**UNIVERSITI SAINS MALAYSIA**

**2018**

# THE ROLE OF RANIBIZUMAB AS AN ADJUNCTIVE AGENT IN MITOMYCIN-C-TREATED GLAUCOMA TUBE IMPLANTATION ON CONJUNCTIVAL FIBROBLAST TISSUE CULTURE

**by**

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**Thesis submitted in fulfillment of the requirements**

**for the Degree of**

**Master of Science**

**October 2018**

## ACKNOWLEDGEMENT

*(In the name of Allah, The Most Beneficient, The Most Merciful)*

My immense gratitude and deepest appreciation to my supervisor, Associate Prof Dr Azhany Yaakub and as well as my co-supervisors, Dr Azlina Ahmad, Professor Dr Hasnan Jaafar and Professor Dr Liza Sharmini Ahmad Tajudin for their constant support, precious guidance and advice throughout and until the completion of this study. This thesis would not have been possible without their guidance and help.

Special thanks to Dr Siti Azrin Abdul Hamid from Unit of Biostatistics and Research Methodology for her consultation regarding statistical analysis. I would like to sincerely thank to Mrs Asiah, Mrs Siti Fadilah, Mr Hairie, Mrs Khairiena and all the staff at the Craniofacial Laboratory, School of Dentistry, USM, for aiding me during my laboratory works. My deepest thanks to Mr Jamaruddin from Department of Immunology, School of Medical Sciences, USM and Mrs Hasikin from School of Health Sciences, USM.

I also wish to express my special thanks to my friends and colleagues, lecturers and staff from Department of Ophthalmology, School of Medical Sciences, USM, for their assistance, support and sharing throughout my Master's journey.

Finally, my heartiest gratitude goes to my beloved husband, my parents and family, for their invaluable help, encouragement and sacrifices that enable me to accomplish this postgraduate study. For their endless love and patience, no words can ever describe my deepest thanks, only God can repay you in kind.

Here, I would like to acknowledge Universiti Sains Malaysia for the research university (RUI) grant (1001/PPSP/813069) and USM Fellowship Scheme for the financial supports during the study.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Akt | Protein Kinase B |
| Caspases | Cysteine Proteases |
| $CO_2$ | Carbon Dioxide |
| DMSO | Dimethyl Sulphoxide |
| DPBS | Dulbecco's Phosphate Buffered Saline |
| DNA | Deoxyribonucleic Acid |
| ECM | Extracellular Matrix |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGF | Epidermal Growth Factor |
| *et al* | and others |
| FACS | Fluorescent Activated Cell Sorting |
| Fab | Fragments of antigen-binding |
| Fc | Fragment crystallizable |
| FBS | Fetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| FGS | Fibroblast Growth Supplement |
| FITC | Fluorescein Isothiocyanate |
| g | Gram |
| GDD | Glaucoma Drainage Device |
| HConF | Human Conjunctival Fibroblasts |
| HMDS | Hexamethyldisilazane |
| IOP | Intraocular Pressure |
| kD | kiloDalton |

| | |
|---|---|
| L | Liter |
| MMC | Mitomycin – C |
| min | minute |
| mg | milligram |
| ml | milliliter |
| µl | Microliter |
| nm | nanometer |
| $O_2$ | Oxygen |
| PDGF | Platelet-derived Growth Factor |
| PI | Propidium Iodide |
| PRF | Platelet-rich fibrin |
| PS | Phosphatidylserine |
| P/S | Penicillin/Streptomycin |
| p38 MAPK | p38 mitogen – activated protein kinase |
| p-ERK | Phosphorylation of extracellular signal – related kinase |
| p-FAK | Phosphorylation of focal adhesion kinase |
| RNA | Ribonucleic Acid |
| rpm | revolutions per minute |
| SEM | Scanning Electron Microscope |
| TGF-β | Transforming growth factor – beta |
| VEGF | Vascular Endothelial Growth Factor |
| VEGFR | Vascular Endothelial Growth Factor Receptor |
| ˚C | Degree Celcius |
| % | Percentage |

**PERANAN RANIBIZUMAB SEBAGAI AGEN ADJUNKTIF KEPADA RAWATAN-MITOMYCIN-C DALAM PENGIMPLANAN TIUB GLAUKOMA PADA KULTUR TISU FIBROBLAS KONJUNKTIVA MATA**

## ABSTRAK

Kadar rembesan *vascular endothelial growth factor* (VEGF) yang tinggi kebiasaanya berlaku selepas pembedahan glaukoma. Namun, hal ini tidak diingini oleh kerana ia menyebabkan berlakunya penyembuhan luka/parut yang berlebihan seterusnya menggagalkan tujuan sebenar pembedahan. Oleh yang demikian, kajian ini mencadangkan potensi fungsi ranibizumab, iaitu *anti – vascular endothelial growth factor* (anti – VEGF), sebagai agen adjunktif dalam mencegah/ menghalang pembentukan fibrosis yang berlebihan. Tujuan kajian ini dijalankan adalah untuk menentukan kesan rawatan ranibizumab bersama mitomycin – C ke atas proliferasi, toksisiti dan migrasi sel fibroblas konjunktiva mata manusia (HConFs). Dalam kajian proliferasi dan toksisiti, HConFs dikulturkan dalam medium fibroblas dan dirawat dengan mitomycin-C (MMC) 0.40 mg/ml. Kultur ini kemudian dirawat dengan ranibizumab yang berbeza kepekatan/dos (0 mg/ml, 0.30 mg/ml, 0.45 mg/ml dan 0.60mg/ml). Pembiakan kultur HConFs tersebut dinilai selepas 24, 48 dan 72 jam menggunakan ujian alamarBlue. Untuk toksisiti pula, kadar kematian sel secara apoptosis dibaca menggunakan mesin *flow cytometri* yang ditandakan dengan Annexin-V FITC dan propidium iodide, 72 jam selepas rawatan. Sementara itu, untuk kajian morfologi dan migrasi HConFs, model implantasi tiub glaukoma secara in vitro telah dibuat. Menggunakan model tersebut, HConFs telah dirawat mengikut 4 kumpulan iaitu; kawalan, MMC 0.40 mg/ml, ranibizumab 0.45 mg/ml and MMC

0.40 mg/ml+ranibizumab 0.45 mg/ml. Kadar migrasi HConFs ke dalam tiub diperhatikan pada hari ke – 7, 14 dan 21 menggunakan ujian alamarBlue dan *scanning electron microscope* (SEM). Aplikasi ranibizumab bersama MMC telah menghalang proliferasi HConFs secara signifikan pada 48 dan 72 jam rawatan. Kadar proliferasi HConF bergantung kepada dos, iaitu proliferasi telah dikurangkan secara signifikan oleh ranibizumab pada dos 0.45 mg/ml, ($p$<0.05). Menariknya, jumlah HConFs didapati tinggi pada dos ranibizumab 0.60 mg/ml berbanding kumpulan rawatan lain. Selain itu, rawatan ranibizumab juga didapati merangsang apoptosis dan mengurangkan nekrosis HConFs. Kadar apoptosis yang tertinggi serta kadar nekrosis yang terendah diperhatikan dalam kumpulan rawatan ranibizumab 0.45 mg/ml. Sementara itu, kajian migrasi fibroblas ke dalam tiub selepas 7 hari menunjukkan kadar migrasi yang rendah dalam kumpulan MMC dan MMC+ranibizumab berbanding kawalan (p<0.05). Rawatan MMC menunjukkan peratusan migrasi yang sedikit rendah berbanding MMC+ranibizumab, (p=0.792). Berbeza pula dengan kumpulan yang dirawat dengan ranibizumab, peratusan migrasi fibroblas yang lebih tinggi berbanding kawalan, (p=0.985). Manakala, pada hari ke – 14 dan 21, peratusan migrasi fibroblas didapati hampir sama dalam semua kumpulan (p<0.05) dan (p=0.248). Kesimpulannya, aplikasi ranibizumab telah mengurangkan lagi proliferasi HConFs yang dirawat oleh MMC dengan menambahkan lagi apoptosis serta mengurangkan nekrosis. Namun, ranibizumab sahaja tidak berupaya untuk mengurangkan kadar migrasi HConFs ke dalam tiub glaukoma. Sebaliknya, rawatan adjunktif MMC bersama ranibizumab boleh mempengaruhinya.

**THE ROLE OF RANIBIZUMAB AS AN ADJUNCTIVE AGENT IN MITOMYCIN-C-TREATED GLAUCOMA TUBE IMPLANTATION ON CONJUNCTIVAL FIBROBLAST TISSUE CULTURE**

## ABSTRACT

Elevated expression of vascular endothelial growth factor (VEGF) usually occurs after the glaucoma surgery. The elevated expression of VEGF is unnecessary as it contributes to excessive scarring which will fail the surgery. Therefore, this study investigated the potential role of ranibizumab, an anti – vascular endothelial growth factor (anti – VEGF), as anti – adjunctive agent in preventing the excessive fibrosis. The aim of this study is to determine the effect of adjunctive use of ranibizumab with mitomycin – C on proliferation, cytotoxicity and migration of human conjunctival fibroblasts (HConFs) cell line. In proliferation and cytotoxicity study, HConFs were cultured in fibroblast medium and treated with 0.40 mg/ml mitomycin – C (MMC). The cultures were then administered with ranibizumab at different concentration (0 mg/ml, 0.30 mg/ml, 0.45 mg/ml and 0.60mg/ml). Viability and proliferation of HConFs were assessed at 24, 48 and 72 hours by alamarBlue assay. For cytotoxicity, the apoptosis rate of treated HConFs was evaluated via flow cytometry using Annexin-V FITC and propidium iodide staining after 72 hours treatment. Meanwhile, for morphology and migration study, in vitro model of glaucoma tube implantation was used. HConFs were treated according to 4 groups; control, MMC 0.40 mg/ml, ranibizumab 0.45 mg/ml and MMC 0.40 mg/ml +ranibizumab 0.45 mg/ml. Migration of HConFs onto the tube was observed at day 7,14 and 21 through alamarBlue assay and scanning electron microscope (SEM).

Application of ranibizumab following MMC caused a dose – dependent inhibition of HConF's viability and proliferation which significant after 48 and 72 hours. It was shown that HConF's proliferation was significantly reduced by ranibizumab at concentration 0.45 mg/ml, ($p$<0.05). In fact, surprising higher number of HConF was noted in 0.60 mg/ml ranibizumab group in contrast to other treatment groups. Concurrently, treatment of ranibizumab also found to induce apoptosis and reduced necrosis among HConFs. The highest level of apoptotic HConFs and the least necrotic HConFs were observed at concentration 0.45 mg/ml. While in tube migration study, 7 days treatment resulted in lower percentages of HConFs migrated on to the tube observed in MMC and MMC+ranibizumab treated group compared to control, (p<0.05). Treatment of MMC showed lower percentage of migrated fibroblasts than MMC+ranibizumab group, (p=0.792). In contrast, higher percentage of migrated fibroblasts was observed in ranibizumab group with respect to control, (p=0.985). Whereas, at day 14 and 21, percentage of migrated fibroblasts is almost the same in all groups, (p<0.05) and (p=0.248), respectively. In conclusion, application of ranibizumab could further inhibit the proliferation of MMC treated HConFs by increasing the apoptosis and reducing the necrosis. It was also discovered that ranibizumab alone was not effective in suppressing the migration of HConFs onto the glaucoma tube. Instead, the adjunctive treatment of MMC and ranibizumab could significantly affect the migration of fibroblasts on to the glaucoma tube.

# CHAPTER 1

# INTRODUCTION

## 1.1 Overview on Glaucoma

Glaucoma is defined as optic neuropathy characterized with specific visual field defect and specific optic disc cup ratio. It is a pathologic condition in which the optic nerves called ganglion cell axons undergo a progressive loss causing visual field damage (**Figure 1.1**). This condition usually caused by abnormal high pressure inside the eye known as intraocular pressure (IOP). If left untreated, glaucoma may lead to irreversible blindness. Several treatments are available to prevent the further loss of vision caused by glaucoma. One of them is glaucoma filtration surgery for implantation of glaucoma drainage devices (Rhee, 2012).

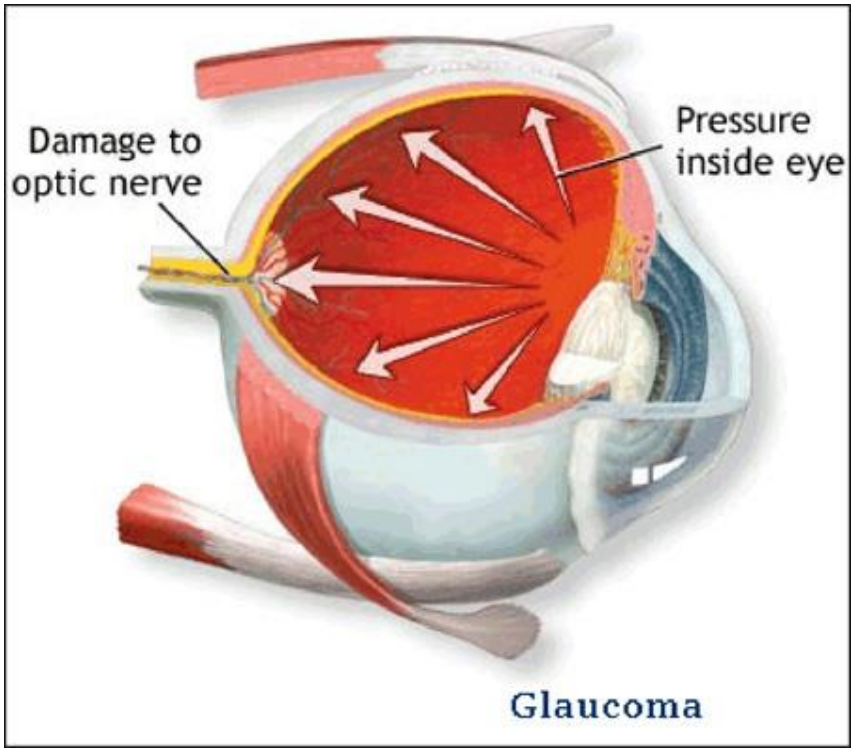


**Figure 1.1** Pathologic condition of glaucoma due to abnormal high intraocular pressure (A.D.A.M, 2018)

## 1.2 Glaucoma Filtration Surgery

Glaucoma filtration surgery including trabeculectomy, non – penetrating surgery technique and aqueous shunts functions to drain out aqueous humor to the sub – tenon space. All these types of filtration surgery depend on external resistance created by wound healing. The success of these surgical procedure is hindered by the variability of wound healing response. Inadequate healing response will results in hypotony while vigorous healing response causes high IOP to re – occur (Mathew and Barton, 2011).

Filtration surgery remains as the most effective therapy in lowering intraocular pressure (IOP) in glaucoma patients. However, excessive post – operative scarring of the conjunctiva and tenon at the surgical site leads to the failure of the surgery. Excess scar formation because of increased wound healing response after the surgery, obstructs the aqueous humor outflow causing poor IOP control, hence reduced the surgical outcome (Bochmann *et al.*, 2011; How *et al.*, 2010; Li *et al.*, 2009).

## 1.3 Glaucoma Drainage Devices

Glaucoma drainage devices (GDDs) are devices that was designed to lower intraocular pressure (IOP) (**Figure 1.2**). They are also known as aqueous shunts, aqueous shunting devices or tube shunts. GDD is traditionally used in cases of failed filtration surgery, recalcitrant and complex glaucomas such as inflammatory, neovascular and traumatic glaucoma. Nowadays, increasing number of publications on GDDs allow the use of GDDs as primary glaucoma surgery (Rhee, 2012).

Made of medical grade silicone, there are two types of GDDs which are *restrictive* (Ahmed and Krupin) and *nonrestrictive* (Molteno and Baerveldt) models (**Figure 1.3**). *Non-restrictive* or *non-valved* devices permit the free flow of aqueous from inside the eye to episcleral platewhile the *restrictive* or *valved* devices have a flow – controlling element within the posterior part of the tube to limit the aqueous flow (Rhee, 2012).


**Figure 1.2** Parts of glaucoma drainage device (GDD).

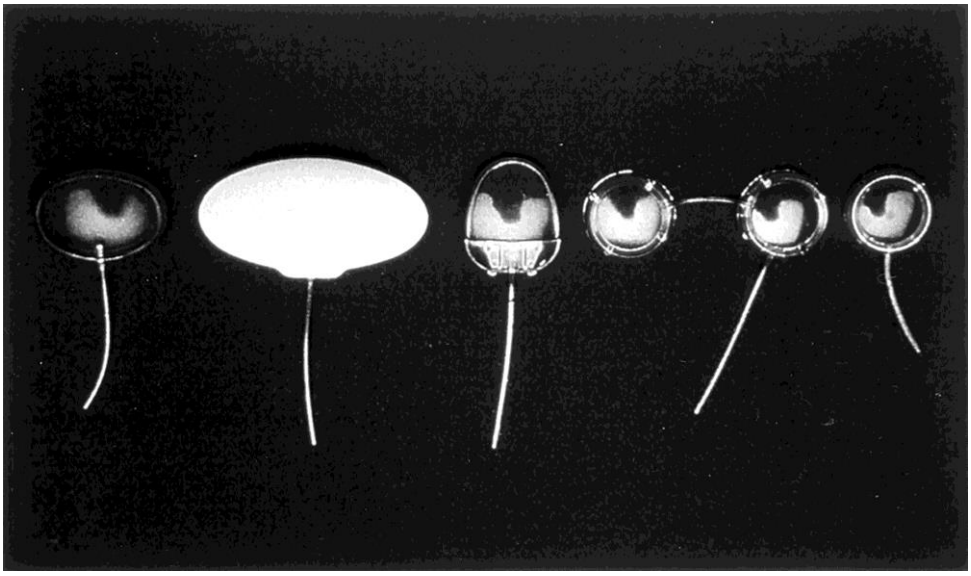

**Figure 1.3** Different types of GDD. From left to right showing Krupin, Baerveldt, Ahmed valve, two – plate Molteno and Molteno implants. Picture adapted from (Ceballos and Parrish, 2002)

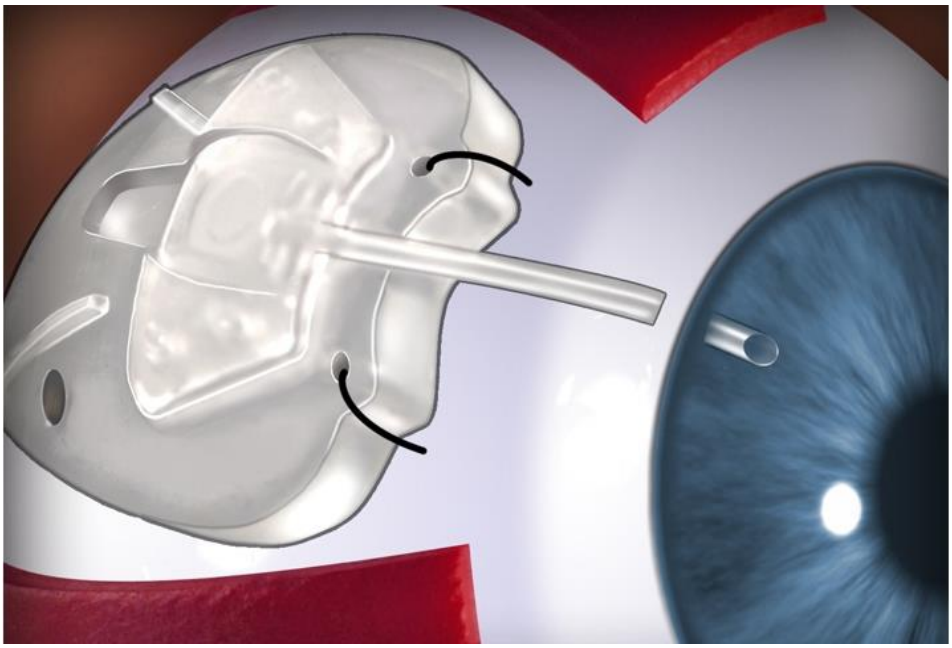

**Figure 1.4** Glaucoma tube shunt implantation in patient's eye. End of the tube is inserted into the anterior chamber while the plate is placed on the surface of eyeball, underneath the conjunctiva (MohawkValleyRetina, 2017).

## 1.4 Ocular Wound Healing

Fibroblasts in the tenon's capsule are the key player in ocular wound healing (Li *et al.*, 2009). They are the cells that function for generating a contractile force, which is essential in the post – operative scarring process. The scarring can develop through the proliferation, migration and contraction of the fibroblasts. Fibroblasts are stimulated by growth factors to differentiate into myofibroblasts. Myofibroblasts are responsible for fibrosis through the increased of extracellular matrix (ECM) synthesis, for granulation tissue formation, and wound contraction (Honjo *et al.*, 2007; Horsley and Kahook, 2010).

After the glaucoma surgery, conjunctival and episcleral fibrosis (which is fibroblast mediated) occurs due to progressive fibroblast migration, proliferation, collagen deposition and angiogenesis at the site of the filtration bleb. From histologic

studies, the maximum proliferation of subconjunctival fibroblasts occurs in the third to fifth postoperative day (Mathew and Barton, 2011; Memarzadeh *et al.*, 2009).

There are four sequential processes in wound healing which are angiogenesis, migration and proliferation of fibroblasts, deposition of ECM (scar formation) and maturation and reorganization of the fibrous tissue (remodelling) (Kumar, 2007). These healing phases do not happen in series but relatively overlap in time (**Figure 1.5**).

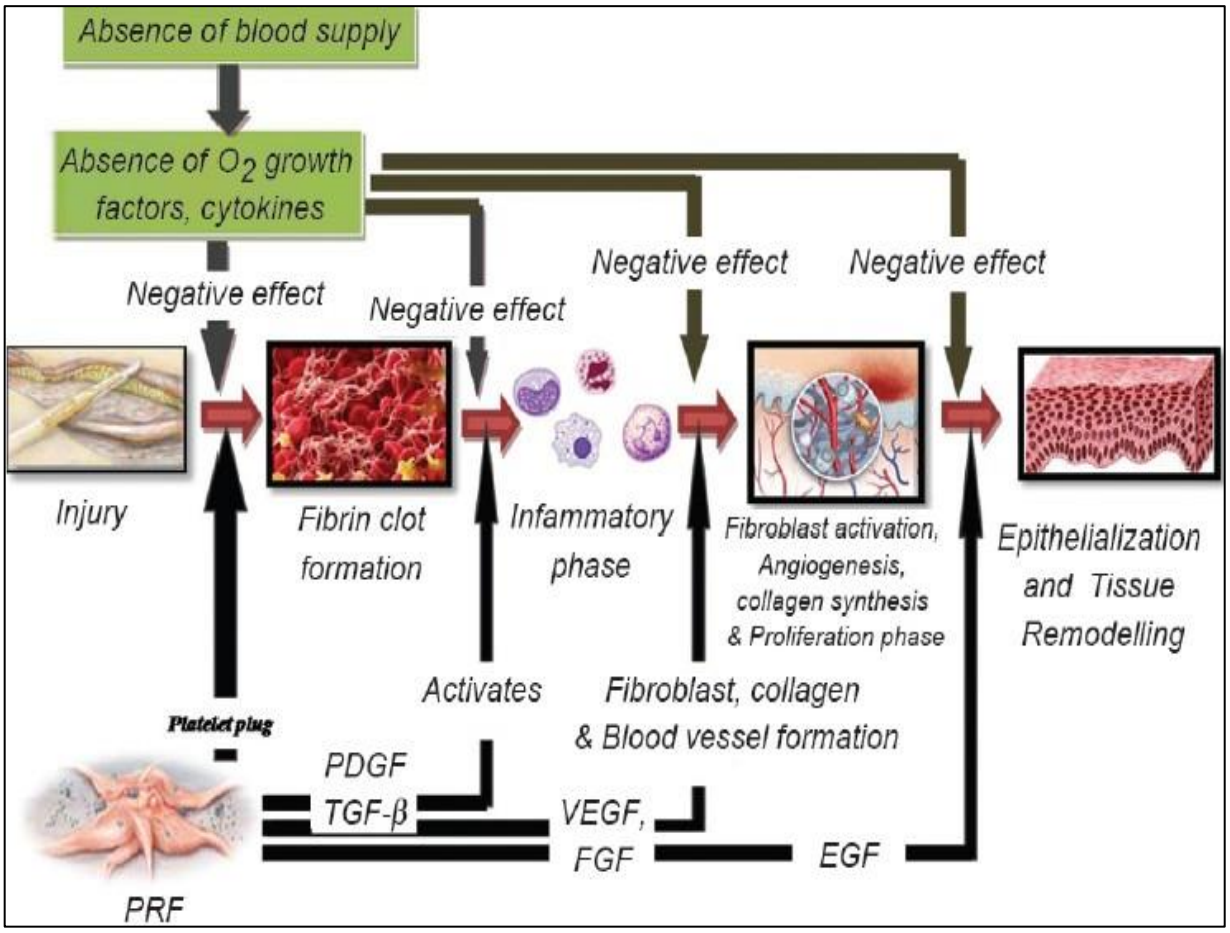


**Figure 1.5** Pathway of wound healing and scar formation (Jain *et al.*, 2012).

### 1.4.1 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is the protein growth factor that plays an important role in building up the circulatory system under the physiological conditions (Barrett *et al.*, 2010; Takahashi, 2011). It was first described as a molecule that increases the permeability of blood vessels and stimulates the production of new blood vessels (Bochmann *et al.*, 2011). VEGF induced the formation of vascular (vasculogenesis) during embryonic development and growth of blood – vessels (angiogenesis) in adults (Ferrara, 2004; Olsson *et al.*, 2006).

Cell-mediators and growth factors such as VEGF are essential in wound healing mechanism. Once the balance of these growth factors is disturbed, altered wound-healing processes with extensive scar formation may occur. In cutaneous wounds, high level of VEGF induces scar formation through increasing vascularity and collagen deposition, whereas neutralization of the VEGF reduces angiogenesis and cutaneous fibrosis (Li *et al.*, 2009).

### 1.4.2 Angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vasculature (Olsson *et al.*, 2006). It is a critical process in healing that provides the blood supply for the tissues. Abnormally, it is important for tumor growth in which if tumors do not develop a blood supply, they do not grow. VEGF is a key compound in physiological angiogenesis and serves as a major stimulator in pathological angiogenesis (Barrett *et al.*, 2010; Stewart, 2012; Takahashi, 2011). In condition of neovascularisation and in malignant tumor, VEGF is over expressed enable them to growth and metastasize (Bochmann *et al.*, 2011).

Angiogenesis allows the early migration of inflammatory cells and fibroblasts into wound and release the key mitogenic cytokines such as fibroblast growth factor (FGF). Blocking the angiogenic cascade will reduce the migration and proliferation of the fibroblast and consequently slow down the healing response (Memarzadeh *et al.*, 2009). This inhibition can be occur through the use of selective inhibitor of growth factor such as anti – VEGF. Anti – VEGF monoclonal antibodies can be used as an approach in preventing or treating extensive wound healing with over expression of VEGF (Bochmann *et al.*, 2011).

### 1.4.3 Fibroblast Migration and Proliferation

Few days after the wound occurs, fibroblast begin to migrate from adjacent uninjured tissue to the wound site through fibronectin fibrils (Hsieh and Chen, 1983). It then proliferates and accumulates in the wound to reconstitute a variety of connective tissue components. This proliferation phase occured simultaneously with angiogenesis.

Fibroblasts migration and proliferation are vital in wound granulation. Initially, cellular blood constituents such as platelets and monocytes release various peptide growth factors [for instance platelet – derived growth factor (PDGF) and transforming growth factor-β (TGF-β)] to stimulate the migration of fibroblasts. More mediators are produced which draw the fibroblasts, neutrophils, mast cells, endothelial cells and basal cells to migrate to wound. Finally, the tissue granulation and remodeling take place (Schreier *et al.*, 1993).

## 1.5 Cell Death

Cell death is necessary for development, growth and survival of any living thing. For instance, cell death is required during embryogenesis in tissue sculpting, development of the immune system and destruction of damaged cells (Tait *et al.*, 2014). There are two broad, mutually exclusive classification of cell death; necrosis and apoptosis (Galluzzi *et al.*, 2015). In senescent human fibroblasts in vitro, it was suggested that both apoptosis and necrosis are involved in the cell death (Ohshima, 2006).

### 1.5.1 Necrosis

Severe insults/injury due to physical (e.g.,heat or radiation), chemical (e.g.,extreme changes in pH or potent detergent) and mechanical (e.g., shearing) stimuli caused the cell to die through necrosis (Galluzzi *et al.*, 2015). These acute injuries typically results in cells swelling and burst. Their contents are spilled all over the adjacent uninjured cells causing potentially destructing inflammatory response (Alberts *et al.*, 2002). Necrosis is commonly associated with pathological process (Adigun R, 2018).

### 1.5.2 Apoptosis

Meanwhile, another distinct cell death mode is a programmed cell death called apoptosis. The term of apoptosis was introduced by Kerr in 1971 (derived from a Greek word meaning “falling off” like leaves from a tree (Alberts *et al.*, 2002; Kerr, 1971). Apoptosis normally occurs in multicellular organism, as homeostasis mechanism to sustain the cell population in tissue by control of cell division and death. When the cells are no longer needed, they commit suicide by apoptosis (Alberts *et al.*, 2002; Elmore, 2007). Apoptosis also occurs as defense mechanism to

the various of noxious stimuli. Both physiological and pathological may trigger the apoptosis (Adigun R, 2018; Elmore, 2007; Norbury and Hickson, 2001).

In apoptosis, the cell dies neatly with few morphological changes including cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies (Alberts *et al.*, 2002; Elmore, 2007; Pollack and Leeuwenburgh, 2001). The dying cell is later phagocytosed either by a specialized phagocytic cell, macrophage, or neighbouring cell. This way permits the macrophage/neighbouring cell to recycle the organic components of the dead cell (**Figure 1.6**) (Zhang *et al.*, 1997).

Apoptosis is irreversible and energy – dependent process, which mediated by proteolytic enzymes called caspases. Caspases initiate the cell death by cleavage of specific proteins in the cytoplasm and nucleus. They are found in the cells in inactive form called procaspases. Activation of caspases through cleavage from other caspases produced a proteolytic caspase cascade (Alberts *et al.*, 2002; Elmore, 2007). This mechanism triggered by either extrinsic or intrinsic apoptotic pathway (Wu *et al.*, 2008).

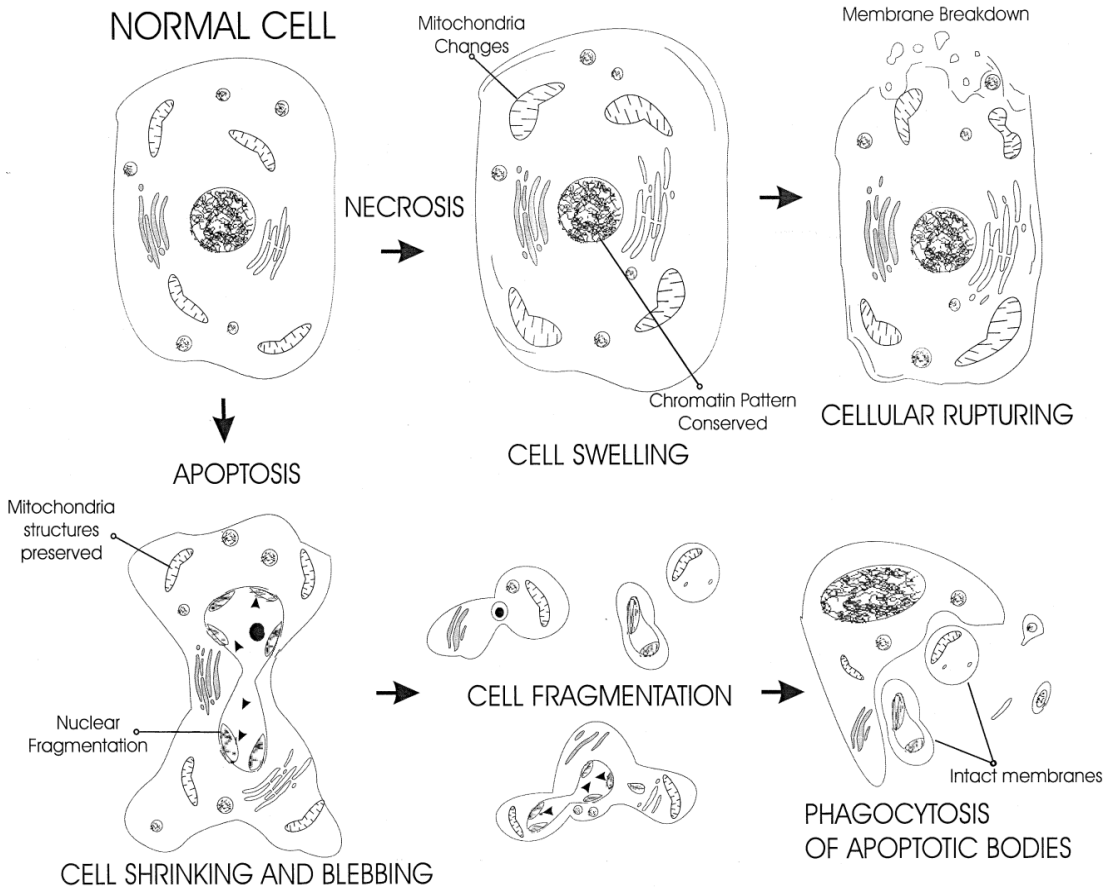


**Figure 1.6** Comparison of the cells undergo necrosis and apoptosis. Cellular rupturing in necrosis caused inflammation while in apoptosis, no inflammation occured because the cell fragmented and phagocytosed by macrophage (Pollack and Leeuwenburgh, 2001)

## 1.6 Wound Healing Modifying Agent

As the development of fibrosis became the post – operative complication of the filtration surgery, wound healing modifying agent such as mitomycin – C (MMC) and 5 – fluorouracil (5-FU) has been used widely to enhance the surgical outcome. Adjunctive use of these chemotherapy drugs help to prevent the post surgical scarring and significantly improved the success rate of filtration surgery.

However, these agents have the non – specific mechanism of action that can cause the widespread cell death and apoptosis, associated with potentially sight – threatening complications such as hypotony, blebitis and endophthalmitis (Vijaya *et al.*, 2011; Yoon and Singh, 2004). These agents are not recommended to be applied repeatedly due to mentioned complications. Thus, alternative agents are needed for specific and effective anti – scarring treatment which can work synergistically with MMC or 5-FU  without serious side effects.

### 1.6.1 Mitomycin-C

Mitomycin – C (MMC) is used widely in glaucoma filtering surgery as a standard treatment due to its modulatory effects on wound healing (Khouri, 2014). Isolated from soil bacterium of the *Streptomyces caespitosus,* MMC is an anti – neoplastic or alkylating agent that inhibit the DNA, RNA and protein synthesis of the cell. It inhibits the DNA by forming linkage at the N position of Adenine and at 06 and N position of Guanine. The most affected cell cycle is during late G-I and early S-phase. Chemical formula of MMC is $C_{15}H_{18}N_4O_5$ (Salmon SE, 1987; Singh and Singh, 2013)

In glaucoma surgery, MMC is use by various techniques such as sponge application or injection at dosage between 0.20 mg/ml and 0.40 mg/ml (Khouri, 2014). It has increased the success rate of surgery by inhibiting the fibroblasts proliferation and consequent scarring of filtration bleb (Al Habash *et al.*, 2015; Jampel, 1992). In spite of this, use of MMC also leads to several side effects for instance development of thin walled avascular/cystic blebs which might cause the bleb to leak and expose eyes to infection (Al Habash *et al.*, 2015; Singh and Singh, 2013). Previous study also reported the complication of hypotonous maculopathy, significant cataract and scleral necrosis exposing the ciliary body (Casson *et al.*, 2001; Singh *et al.*, 1995).

### 1.7 Anti- Vascular Endothelial Growth Factor (VEGF): Ranibizumab

There are three common therapeutic anti – VEGF antibodies available – bevacizumab, ranibizumab and pegaptanib. Ranibizumab will be used for the current study. Ranibizumab (Lucentis TM, Genentech Inc, San Francisco, United States of America) is a small and fully humanized monoclonal antibody – fragment. It is produced in *Escherichia coli* cells using recombinant DNA technology. It has a low molecular weight of 48 kDa with a good tissue penetration characteristic (Bastion *et al.*, 2011; Bochmann *et al.*, 2011; Carneiro *et al.*, 2012).

Ranibizumab was designed to bind and neutralize all isoforms and active degradation products of vascular endothelial growth factor A (VEGF-A). It differs from bevacizumab by its size and affinity for VEGF. Bevacizumab is humanized, murine full – length protein with two VEGF binding sites, whereas, ranibizumab is murine antigen – binding fragment (Fab) with only single affinity – matured VEGF binding site (Kim, 2007).

Ranibizumab is injected intravitreally which passes to all the retinal layers reaching the retinal pigment epithelium – choroid complex. The maximum tolerated single intravitreal dose of ranibizumab is 0.50 mg. Each vial of ranibizumab contains 2.30 mg in 0.23 ml, in which overfilling is considered necessary to achieve an injectable dose of 0.50 mg. Ranibizumab has a short systemic half – life (12 hours) and a long intravitreal half – life (6 days) (Bastion *et al.*, 2011; Bochmann *et al.*, 2011; Michels and Rosenfeld, 2005).

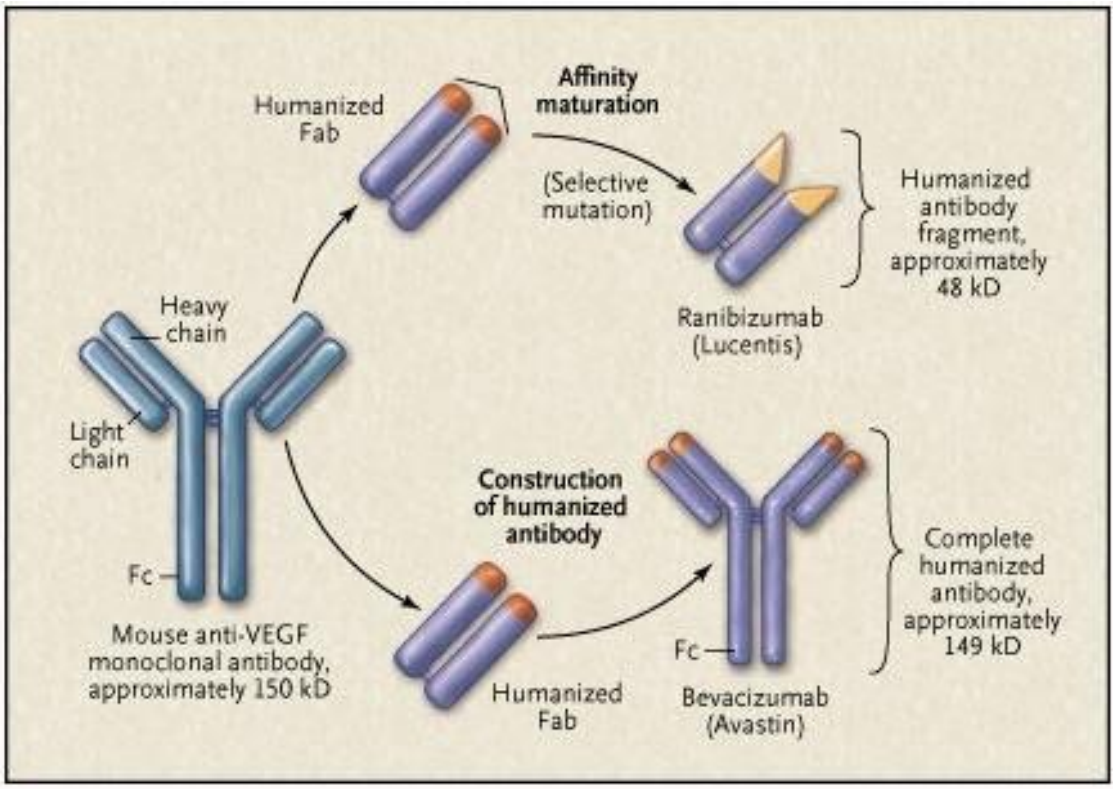


**Figure 1.7** Molecular structure of ranibizumab in comparison with bevacizumab (Steinbrook 2006).

## 1.8 Problem Statement and Rationale of Study

Previous experimental animal models and clinical trials have proved that ranibizumab is safe and effective to be used for the treatment of diabetic macular oedema, subretinal choroidal neovascularisation and neovascular age – related macular degeneration. Lately, it has been proposed that ranibizumab can be used in augmentation of glaucoma filtration surgery by preventing the epi – scleral fibrosis after the surgery. Adjunctive use of this anti – VEGF with MMC may also overcome the complications that caused by MMC (Bandello *et al.*, 2011; Elmekawey and Khafagy, 2014; Michels and Rosenfeld, 2005).

However, there are a limited number of studies regarding on the role of ranibizumab as anti – scarring agent in glaucoma filtration surgery. Available evidence is insufficient to evaluate the effectiveness of this anti – VEGF treatment in glaucoma. Therefore, current study will be conducted to further evaluate the effectiveness of ranibizumab treatment in glaucoma drainage device surgery.

## 1.9 Objectives of Study

### 1.9.1 General Objective

To determine the effects of anti – VEGF, ranibizumab, as anti – scarring agent on conjunctival fibroblast tissue culture following MMC.

### 1.9.2 Specific Objectives

1. To compare the level of proliferation of conjunctival fibroblasts treated at different concentration of ranibizumab following MMC treatment.

2. To determine the cytotoxic effect of different concentration ranibizumab following MMC treatment on conjunctival fibroblasts.
3. To compare the level of migration and morphology of the conjunctival fibroblasts on the silicone tube under the influence of ranibizumab and/or MMC.

# CHAPTER 2

# METHODOLOGY

## 2.1 Study Design

### 2.1.1 Background of Study

The present study is an experimental study comprises of two phases of in vitro experiments. Primary human conjunctival fibroblasts (HConFs) were used in both phases of the study.

In the first phase, viability, proliferation and cytotoxicity assays were carried out in which HConFs were treated with different concentration of ranibizumab following single application of MMC. From these assays, the effective dose of ranibizumab was selected for the next phase.

Second phase of the study involved in implantation of the glaucoma tube on the culture plate to observe the morphology and migration of HConFs under the influence of ranibizumab and MMC, both as single agent and combination. A summary of the whole study is represented in **Figure 2.1** and **Figure 2.2**.

### 2.1.2 Flowcharts of Study

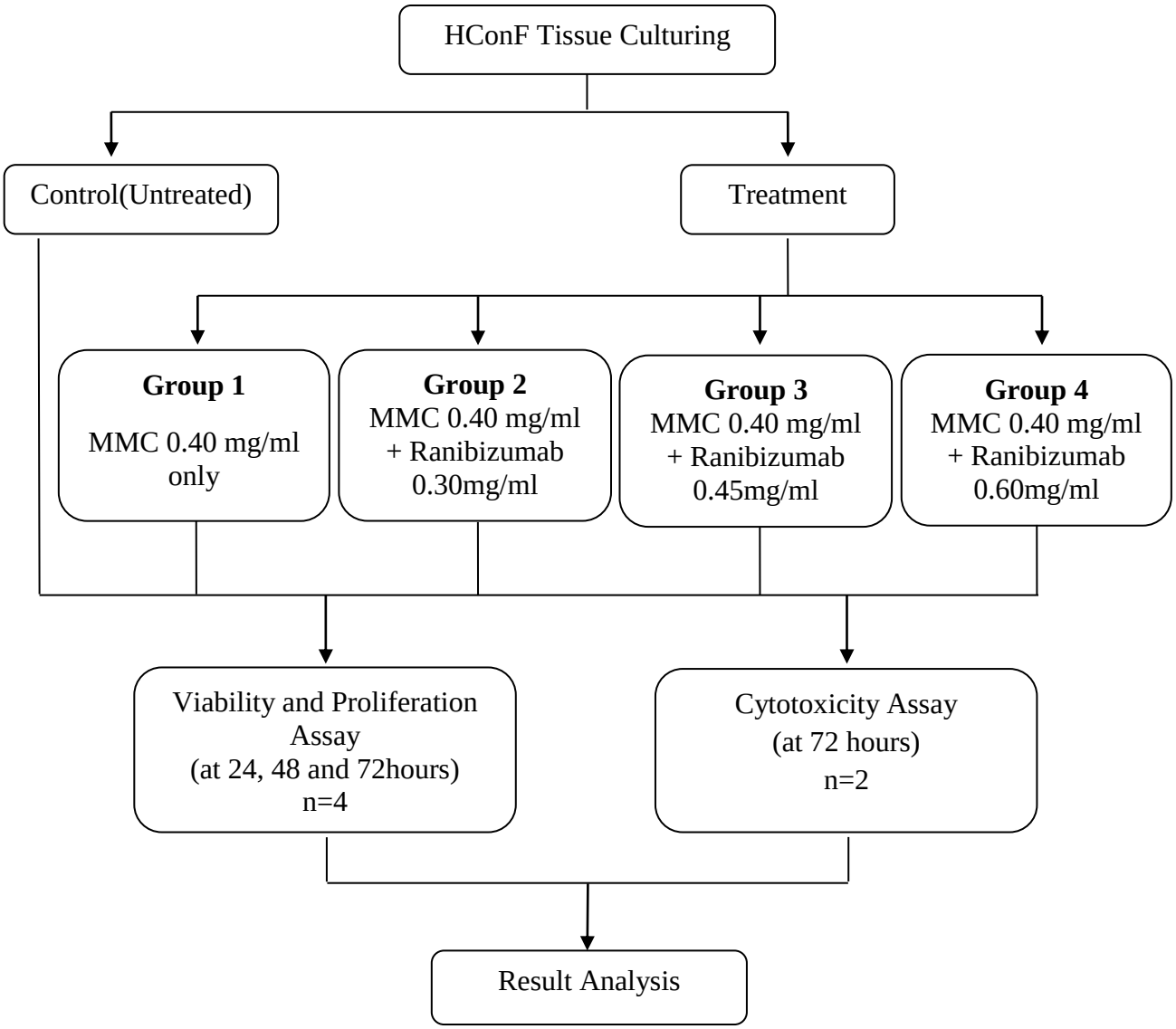


**Figure 2.1** Flow chart of study – Phase I

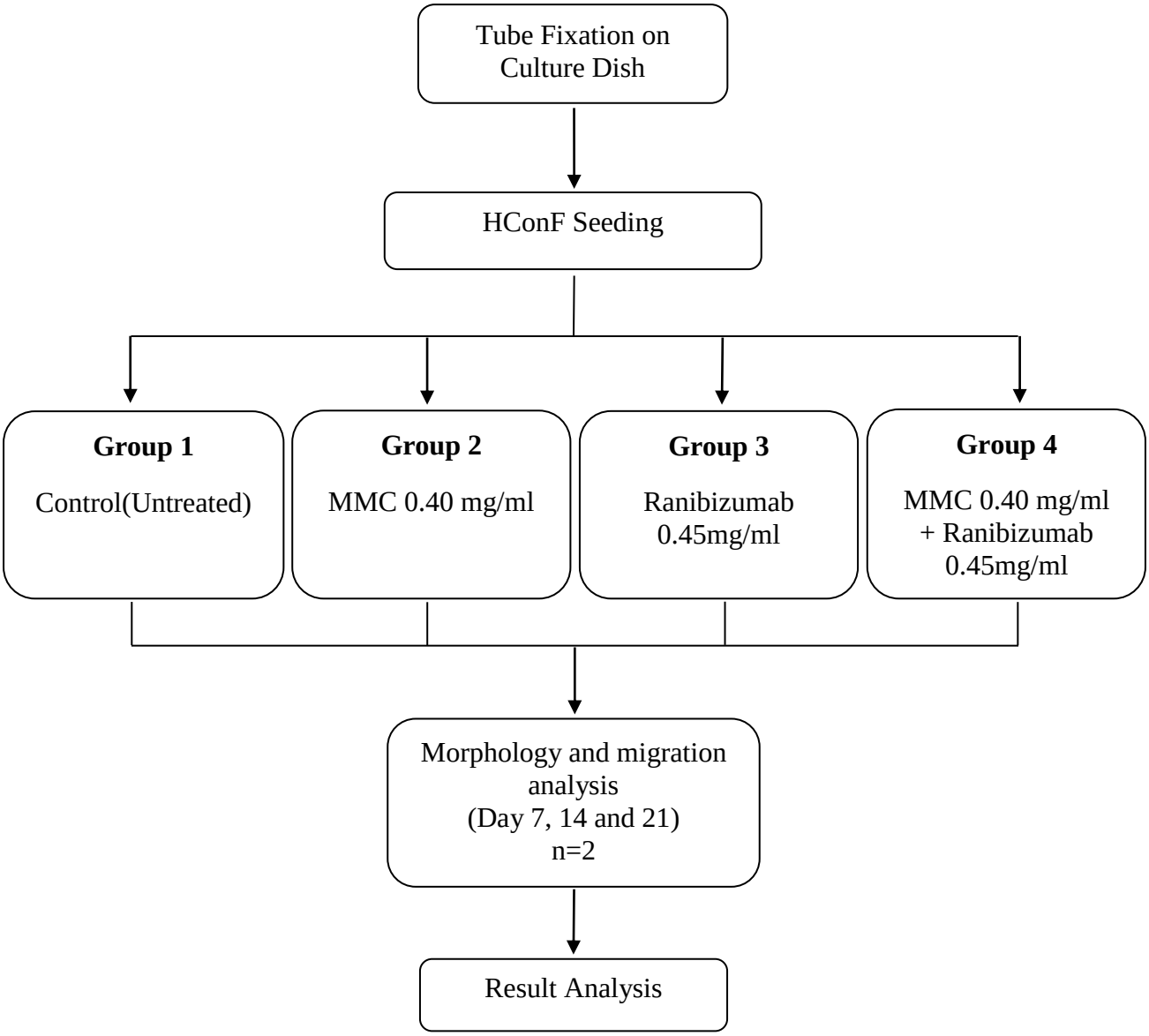


**Figure 2.2** Flow chart of study – Phase II

## 2.2 Sampling and Sample size

Primary culture of Human Conjunctival Fibroblast (HconF) were obtained commercially from Sciencell Research Laboratories (California, United States).

Sample size calculation was based on previous study (Md Noh *et al.*, 2014).

1. Level of proliferation of conjunctival fibroblasts treated at different concentration of ranibizumab following MMC treatment.

   6 study groups

   Consists of 1 negative control (medium only), 1 positive control (untreated cell), 1 standard treatment (0.40 mg/ml MMC treatment), 3 different concentration of treatment group (Ranibizumab 0.30 mg/ml, 0.45 mg/ml, 0.60 mg/ml in addition to 0.40 mg/ml MMC treatment). Each group was conducted in 4 replicates (Md Noh *et al.*, 2014).

2. Cytotoxic effect of different concentration ranibizumab following MMC treatment on conjunctival fibroblasts.

   5 study groups

   Consists of 1 positive control (untreated cell), 1 standard treatment (0.40 mg/ml MMC treatment), 3 different concentration of treatment group (Ranibizumab 0.30 mg/ml, 0.45 mg/ml, 0.60 mg/ml in addition to 0.40 mg/ml MMC treatment). Each group was conducted in 2 replicates.

3. Level of migration and morphology of the conjunctival fibroblasts on the silicone tube under the influence of ranibizumab and/or MMC.

   4 study groups

Consists of 1 positive control (untreated cell), 1 standard treatment (0.40 mg/ml MMC treatment), 2 treatment groups (Ranibizumab 0.45 mg/ml and MMC 0.40 mg/ml+Ranibizumab 0.45 mg/ml). Each group was conducted in 2 replicates.

## 2.3 Ethical Approval

This study was a part of big study entitled "Potential Anti Scarring Role of Anti – TGF and Anti – VEGF as Tube Drainage Device Coated Agent and Trabeculectomy Augmentation Agent in Glaucoma Surgery" (USM Animal Ethics Approval/2012/(81)(428)). It was approved on 1st September 2012 – 30th September 2015 and extended until 31st January 2019.

## 2.4 Financial Support

This study received USM Research University Individual grant (RUI 1001/PPSP/813069) for the purchased of primary cell culture, consumables items and study drugs.

## 2.5 Materials

### 2.5.1 Chemicals, Reagent and Solutions

All chemicals, reagent and solutions used in this study are listed in **Table 2.1**

### 2.5.2 Commercial Kits and Consumables

All commercial kits and consumables used in this study are listed in **Table 2.2**

### 2.5.3 Computer Application Programs and Software

All computer application programs and software used in this study are listed in **Table 2.3**

### 2.5.4 Drugs

All drugs used in this study are listed in **Table 2.4**

### 2.5.5 Laboratory Apparatus and Equipments

All laboratory apparatus and equipments used in this study are listed in **Table 2.5**

**Table 2.1** List of chemicals, reagent and solutions

| No. | Chemicals/ Reagent/ Solutions | Supplier |
|---|---|---|
| 1 | Dimethyl sulphoxide (DMSO) | Sigma-Aldrich, UK |
| 2 | alamarBlue Cell viability assay reagent | Thermo Scientific, USA |
| 3 | Fetal bovine serum (FBS) | Invitrogen, USA |
| 4 | Basal Medium | Sciencell, USA |
| 5 | Fibroblast growth supplement | Sciencell, USA |
| 6 | Penicilin/Streptomycin | Sciencell, USA |
| 7 | Dulbecco's Phosphate-Buffered Solution (DPBS) | Sciencell, USA |
| 8 | Poly-L-Lysine | Sciencell, USA |
| 9 | Trypsin/EDTA solution | Sciencell, USA |
| 10 | Trypsin Neutralization Solution | Sciencell, USA |
| 11 | Trypan blue solution (0.4%) | Sigma-Aldrich, UK |
| 12 | 70 % alcohol (Denatured) | HmbG Chemicals. Germany |

**Table 2.2** List of commercial kits and consumables

| No. | Consumables / Commercial Kits | Supplier |
|---|---|---|
| 1 | Cell culture flasks ($25cm^2$ and $75cm^2$) | SPL Life Sciences, Korea |
| 2 | Cell culture dish 35mm | SPL Life Sciences, Korea |
| 3 | Cell culture plate (6, 24 and 96-well plate) | SPL Life Sciences, Korea |
| 4 | Serological pipette (5ml and 10ml) | SPL Life Sciences, Korea |
| 5 | Cryovial | SPL Life Sciences, Korea |
| 6 | 5ml centrifuge/conical tube | SPL Life Sciences, Korea |
| 7 | 10ml centrifuge/conical tube | Biologix,USA |
| 8 | Micropipette tips | Axygen, USA |
| 9 | Annexin V-FITC Apoptosis Detection Kit | Invitrogen, USA |
| 10 | Clear RTV Silicone glue | X'traseal, Malaysia |

**Table 2.3** List of computer application programs and software

| No. | Software Packages | Supplier |
|---|---|---|
| 1 | Image – Pro Express Software | Media Cybernetics, USA |
| 2 | CellQuest Software | BD Biosciences, USA |
| 3 | MagellanData Analysis Software 4.0 | Tecan, Austria |
| 4 | Quanta FEG 450 | Fei, Netherlands |
| 5 | Excel Software | Microsoft Corp, USA |
| 6 | IBM SPSS Statistics 22 | IBM Corp |

**Table 2.4** List of drugs

| No. | Drugs | Supplier |
|---|---|---|
| 1 | Mitomycin – C | Kyowa Hakko Kogyo Co, Japan |
| 2 | Ranibizumab/Lucentis® | Novartis, Switzerland |