CLINICAL AND ANTIBACTERIAL EFFECT OF TUALANG HONEY IN PSEUDOMONAS KERATITIS IN RABBIT EYES

By

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DISCLAIMER

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

Date: 23th May 2017 ……..

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ABSTRAK

PENGENALAN

Pseudomonas aeruginosa adalah salah satu penyebab keratitis bakteria yang boleh menjadi teruk sehingga menyebabkan kebutaan. Fluoroquinolones dan aminoglycosides mempunyai keberkesanan klinikal yang diiktiraf terhadap *Pseudomonas* keratitis. Memandangkan perintang antibiotik semakin meningkat pada masa kini, keberkesanan antibiotik ini telah berkurang dengan ketara. Madu tualang dihasilkan oleh lebah rock (Apis dorsata) yang membina sarang di dahan-dahan pokok tualang (Kompassia excelsa atau Mengaris). Ia digunakan secara umumnya sebagai produk perubatan dan makanan di Malaysia. Madu tualang mempunyai potensi untuk membunuh dan menyahaktifkan pelbagai jenis bakteria. Selain itu, ia juga mempunyai ciri-ciri anti-radang dan antioksidan yang boleh memudahkan proses penyembuhan luka. Ciri-ciri madu tualang ini menjelaskan potensinya untuk digunakan sebagai agen terapeutik alternatif untuk keadaan perubatan tertentu terutamanya jangkitan luka.

OBJEKTIF

Tujuan kajian ini adalah untuk menilai respon klinikal dan kesan antibakteria madu tualang sebagai agen terapeutik alternative di dalam rawatan *Pseudomonas* keratitis di mata arnab.

KAEDAH KAJIAN

Kajian kawalan rawak di dalam keratitis bakteria telah dijalankan menggunakan mata arnab dari September 2014 sehingga September 2016. 30 stroma kornea arnab telah disuntik dengan 1,000 unit '*colony forming units* (CFU)' *Pseudomonas aeruginosa* di

dalam setiap mata. Dua puluh empat jam selepas suntikan, arnab-arnab ini dibahagikan secara rambang kepada tiga kumpulan dengan 10 arnab di dalam setiap kumpulan. Kumpulan A telah dirawat dengan topikal gentamicin 0.3%, kumpulan B dengan topikal madu tualang 30%, dan Kumpulan C dengan gabungan topikal gentamicin 0.3% dan madu tualang 30%. Topikal antibiotik kemudiannya dititis setiap 2 jam untuk 24 jam pertama di dalam ketiga-tiga kumpulan. Selepas itu, antibiotik telah dititis setiap 4 jam selama 6 hari. Pemeriksaan klinikal dan min *'slit lamp examination* (SLE)' yang dibuat ke atas mata-mata arnab ini telah didokumenkan pada hari ke-1, 2, 3, 5 dan 7 selepas induksi *Pseudomonas aeruginosa.* Mata-mata ini telah diperiksa dan direkodkan secara klinikal menggunakan sistem pemarkahan ulser untuk menyusupan ke dalam kornea, ulser kornea, *hypopyon* dan penembusan kornea. Pada hari ke-7 selepas induksi *Pseudomonas aeruginosa*, arnab-arnab ini telah dieuthanasia dan kornea telah dituai untuk menentukan min CFU di dalam setiap kornea.

Data yang diperolehi dianalisis dengan menggunakan '*Statistical Package for Social Science* (SPSS)' versi 22. '*Repeated measure ANOVA'* dan '*multivariate analysis of variance'* telah digunakan untuk analisis statistik. Untuk data yang tidak sekata, ujian '*Kruskal Wallis'* telah digunakan. Oleh itu nilai-nilai ini dinyatakan dalam median (julat antara kuartil). Nilai $p < 0.05$ diambil sebagai tidak ketara secara statistik.

KEPUTUSAN

Tiada perbezaan statistik yang ketara dalam min skor SLE (p = 0.209) di antara kumpulan gentamicin, madu tualang dan gabungan gentamicin-madu tualang di dalam rawatan *Pseudomonas* keratitis. Tiada juga perbezaan ketara dalam median CFU (p = 0,820) di antara ketiga-tiga kumpulan ni pada hari-7 selepas induksi *Pseudomonas aeruginosa.*

KESIMPULAN

Topikal gentamicin, topikal madu tualang dan topikal gabungan gentamicin- madu tualang menunjukkan tindak balas klinikal dan kesan anti-mikrob yang sama dalam rawatan *Pseudomonas* keratitis di model haiwan. Kajian lebih lanjut menggunakan saiz sampel yang lebih besar dan tambahan kumpulan kawalan adalah penting untuk membuktikan kesan antimikrobial madu tualang di dalam rawatan *Pseudomonas* keratitis.

ABSTRACT

BACKGROUND

Pseudomonas aeruginosa is a common cause of microbial keratitis that can be sufficiently severe to cause a significant loss in visual acuity. Fluoroquinolones and aminoglycosides have a well-recognized clinical effectiveness against *Pseudomonas*-induced keratitis. As resistant pathogens are becoming more potent nowadays, the effectiveness of these antibiotics are reduced conspicuously. Tualang honey is produced by the rock bee (*Apis dorsata*), which builds hives high up in the branches of the tualang tree *(Kompassia excelsa or Mengaris).* It is used commonly as medicinal product and food in Malaysia. Tualang honey has both bactericidal and bacteriostatic properties against a wide range of bacteria. Besides that, it has anti-inflammatory and antioxidant properties which can facilitate wound healing. The potency of tualang honey against microorganisms suggest its potential to be used as an alternative therapeutic agent for certain medical conditions especially wound infection.

OBJECTIVE

To evaluate the clinical response and antibacterial effect of tualang honey as an alternative therapeutic agent in *Pseudomonas*-induced keratitis in rabbit eyes.

METHOD

An experimental randomized control trial in bacterial keratitis was performed in rabbit eyes between September 2014 and September 2016. A total of 30 rabbits' corneas were

injected intrastromally with 1,000 colony forming units (CFU) of *Pseudomonas aeruginosa* in each eye. Twenty-four hours after the injection, the rabbits were randomised into three groups consisting of 10 rabbits in each group. Group A was treated with topical gentamicin 0.3%, Group B with topical tualang honey 30%, and Group C with combination of topical gentamicin 0.3% and tualang honey 30%. Topical antibiotics were then administered every 2 hours for the first 24 hours in all three groups. Subsequently, the medications were administered every 4 hours for 6 days. Clinical examination and mean slit lamp examination (SLE) of rabbits' eyes were documented at day 1, 2, 3, 5 and 7 post induction of *Pseudomonas aeruginosa*. The eyes of each group were examined and recorded clinically using ulcer scoring system for corneal infiltrate, corneal ulcer, hypopyon and corneal perforation. At day 7 post induction of *Pseudomonas aeruginosa*, rabbits were euthanized and corneas were harvested to determine the mean CFU per cornea.

The data collected were analyzed using Statistical Package for Social Science (SPSS) software version 22. Repeated measure ANOVA with multivariate analysis of variance were used for statistical analysis. For the data that was not normally distributed, Kruskal Wallis test was used. Thus the values were expressed as median (interquartile range). The p value of < 0.05 is considered statistically significant.

RESULT

There was no statistically significant difference in mean SLE score (p=0.209) between gentamicin, tualang honey and combination of topical gentamicin-tualang honey groups in *Pseudomonas*-induced keratitis. There was also no significant difference in the level of median CFU (p=0.820) among the three groups on day 7 post induction of *Pseudomonas aeruginosa.*

CONCLUSION

Topical gentamicin, topical tualang honey and combination of topical gentamicin-tualang honey shows similar clinical response and antimicrobial effect in treating *Pseudomonas*-induced keratitis in animal models. Further studies with bigger sample size and additional of a control group are essential to establish tualang honey's antimicrobial effect in *Pseudomonas*-induced keratitis.

CHAPTER 1:

INTRODUCTION

Bacterial keratitis is a sight-threatening infectious disease of the cornea. *Pseudomonas aeruginosa* is an important opportunistic human pathogen and the most common causative organism in cases of contact lens related bacterial keratitis (Bourcier et al., 2003; Green et al., 2008; Goh et al., 2010). Other risk factor for bacterial keratitis are ocular surface diseases, ocular trauma, use of immunosuppressive medications and ocular surgery (Bourcier et al., 2003). Corneal ulceration, stromal abscess formation, corneal oedema, and anterior segment inflammation are characteristic of infective keratitis. *Pseudomonas* keratitis can cause rapid progression and corneal perforation within 24 to 48 hours due to its virulence factors (Tang et al., 2013).

In cases of severe inflammation, formation of a deep ulcer results in thinning of the cornea. The most feared complications of *Pseudomonas* keratitis are corneal perforation and secondary endophthalmitis, which can lead to loss of the eye. Morbidity occurs with a severe corneal scar and vascularization, which eventually threaten the vision. Furthermore, irregular astigmatism is a major issue in healed keratitis patients.

Goh and colleagues in Malaysia reviewed 202 contact-lens related corneal ulcer patients between 2007 and 2008. The mean age was 26 years old and 71.3% patients were females. The commonest organism cultured was *Pseudomonas aeruginosa* (79.7%).

A retrospective analysis of clinical and microbiological characteristics of bacterial keratitis survey done in Paris, France by Bouncier and co-workers (2003) revealed that the mean patient age was 39 years. 152 patients were female while 139 patients were male, giving gender distribution close to 1:1 ratio. Among the predisposing factors, contact lens wear was the most common and 30% of isolated bacteria were Gram negative, mostly *Pseudomonas aeruginosa*. Ocular surface diseases were present in 21% of cases and acute corneal trauma in 15% of cases. Gram negative bacteria were associated with severe anterior chamber inflammation and greater infiltrates.

Topical antibiotics constitute the mainstay of treatment in cases of *Pseudomonas* keratitis, with systemic antibiotics used only in cases of corneal perforation or endophthalmitis. The most commonly used therapies to treat *Pseudomonas* keratitis are fluoroquinolones or fortified aminoglycosides (Willcox, 2012). Other antimicrobials can be used, depending on the clinical progress and laboratory findings. If the corneal ulcer is small, peripheral and no impending perforation is present, intensive monotherapy is adequate. Combination therapy is indicated in severe cases. Ceftazidime, gentamicin, ciprofloxacin and their combinations are commercially prepared antimicrobial agents in Malaysian market. They are proven safe and effective clinically (Jones, 1979; O'Brien, 2003).

Bacterial keratitis remains one of the most important potential complications of contact lens use and refractive corneal surgery. Keeping this in mind, early diagnosis and treatment are key to minimizing any vision-threatening sequela. In addition, close follow-up, attention to laboratory data, and changing antimicrobials if no clinical improvement is evident are important elements for successful outcome.

Generally *Pseudomonas aeruginosa* is sensitive to fluoroquinolones and aminoglycoside, but there have been multi-resistant *Pseudomonas aeruginosa* strains reported in Australia. The strains were resistant to ciprofloxacin, gentamicin, tobramycin, and amikacin but were sensitive to ceftazidime, imipenem, meropenem, and timentin (Ku et al., 2010). A retrospective study of antibiotic resistance in microbial keratitis in the United Kingdom by Shalchi and co-workers (2011) revealed that resistance to Gram positive fluoroquinolones was higher than previously reported in the United Kingdom. The same study reported a higher rate of Gram negative bacterial keratitis due to increased usage of contact lenses.

Honey has been widely accepted as food and medicine by all generations, traditions, and civilizations, both ancient and modern. It is produced by honey bees. Honey has been reported to contain about 600 compounds. These include carbohydrates such as fructose (38.5%) and glucose (31.0%), proteins, lipids, mineral, vitamins and antioxidants (Bogdanov et al., 2007; Israili, 2014).

Tualang honey is produced by the rock bee (*Apis dorsata*), which builds hives high up in the branches of the tualang tree (*Kompassia excelsa* or *Mengaris*). In Malaysia, these trees can reach up to 250 feet in height and are plentiful in the north-eastern region in the state of Kedah (Mohamad et al., 2010; Erejuwa et al., 2011). More than 100 nests may be found on a tualang tree. Such tree can yield about 450 kg (about 1000 pounds) of honey. Tualang honey is brown in colour with pH of 3.55-4.00 and specific gravity of 1.335 (Ghazali et al., 2009).

Physicians have been using honey for therapeutic purposes for a long time. Honey has antimicrobial properties and is used widely for wound dressings. Its antimicrobial effect is mainly due to the osmotic effect of the substance's sugars, its acidity, and the peroxidase activity (Terrab et al., 2003; Tan et al., 2009; Ghazali et al., 2009; Nasir et al., 2010). Tualang honey has both bactericidal and bacteriostatic properties against a wide range of bacteria (Tan et al., 2009; Ghazali et al., 2009). This has been reported in a study where superior healing was achieved with honey dressings compared to conventional silver and aquacel dressings (Halim et al., 2010).

In ophthalmic use, it has been reported to have almost equal efficacy as conventional treatment in the treatment of alkaline injuries to the cornea (Bashkaranet et al., 2011). A double blinded clinical trial by Salehi et al (2014) showed that topical honey can improve vernal keratoconjunctivitis symptom such as redness, limbal papillae, and allergic reactions. It can reduce the usage of topical steroid to prevent steroid-related complications.

Antimicrobial agents are important in preventing progression of infectious diseases worldwide. However, as resistant pathogens are becoming more potent nowadays, the effectiveness of these antibiotics is reduced to a great extent. This bacterial resistance to the antimicrobial agents brings severe adverse effect to public health (Levy and Marshall, 2004). Therefore, as an alternative, antimicrobial strategies include revising and re-evaluating the therapeutic use of ancient remedies, such as honey (Basualdo et al., 2007; Mandal et al., 2010a; Mandal et al., 2010b) which is known to have antimicrobial and wound healing properties (Khoo et al., 2010; Sukur et al., 2011; Zainol et al., 2013).

CHAPTER 2:

STUDY PROTOCOL

CLINICAL AND ANTIBACTERIAL EFFECT OF TUALANG HONEY ON PSEUDOMONAS KERATITIS IN RABBIT EYES

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2.1 INTRODUCTION

Pseudomonas aeruginosa is one of the most common causes of microbial keratitis that cause sight threatening condition (Jeng and McLeod, 2003). Widespread use of contact lenses has greatly increased the incidence of *Pseudomonas aeruginosa* keratitis which can progress rapidly to corneal perforation in less than 24 hours (Dart et al., 1991; Ostler, 1993; Bourcier et al., 2003; Fong et al., 2004). Therefore, timely antimicrobial treatment must be initiated for rapid eradication of this infecting organism.

Ceftazidime, gentamicin, ciprofloxacin or their combinations have been the first line of treatment in the current practice. They are proven safe and effective clinically. (Jones, 1979; O'Brien, 2003). However, there are recent reports regarding resistance to gentamicin and ciprofloxacin in treating microbial keratitis in Australia, Europe and North America (Ly et al., 2006; Green et al., 2008; Wilcox, 2011; Lichtinger et al., 2012). At present, there is no incidence reported in South East Asian countries.

Honey is a natural sweetener produced by honey bees. It is a concentrated aqueous solution of inverted sugar with a complex mixture of fructose (38%), glucose (31%), and other sugars. Honey contains more than 180 substances, including amino acids, vitamins, minerals and enzymes like oxidase, amylase and catalase (Perez et al., 2002; Terrab et al., 2003). Tualang honey is taken from beehive on tualang tree (*Kompassia excelsa or Mengaris*) which is produced by the rock bee (*Apis dorsata*). In Malaysia, it is commonly found in Kedah rainforest (Mohamad et al., 2010; Erejuwa et al., 2011).

Honey has used widely for wound dressing due to its bactericidal and bacteriostatic properties against a wide range of bacteria (Tan et al., 2009; Ghazali et al., 2009). It is reported in a study where superior healing achieved with honey dressing compared to conventional silver and aquacel dressings (Khoo et al., 2010). The antibacterial effect of tualang honey was concentration dependent and bactericidal effect was observed at concentration of 25% or more for Pseudomonas aeruginosa isolates tested (Tan et al., 2009). Besides its antimicrobial properties, honey also anti-inflammatory and an antioxidant properties which can be used to treat infection (Tan et al., 2009; Zainol et al., 2013). Tualang honey also was proven to facilitate wound healing such as moist burns and several other types of wounds (Ahmed et al., 2013).

As antibiotic resistance of bacteria in on the rise, the discovery of alternating therapeutic agents is essential (Tan et al., 2009). Since Malaysian tualang honey is known to have antimicrobial and wound healing properties, it can be used as an alternative for therapeutic purpose (Khoo et al., 2010; Sukur et al., 2011; Zainol et al., 2013).

Our literature review revealed that there is no published report on tualang honey use to treat infective condition in the eyes. Thus, it is our sincere hope that this study will be able to provide new knowledge regarding possibly of its role in the treatment of ocular infection.

2.2 OBJECTIVES

2.2.1 General Objective

To evaluate the clinical response and antibacterial effect of tualang honey in keratitis induced by *Pseudomonas aeruginosa* in rabbit eyes.

2.2.2 Specific Objectives

i. To compare mean Slit Lamp Examination (SLE) score of *Pseudomonas*-induced keratitis between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% in rabbit eyes on day 1, 2, 3, 5, and 7 post induction of *Pseudomonas aeruginosa* .

ii. To compare mean bacterial colony forming units (CFU) of *Pseudomonas*-induced keratitis between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% in rabbit eyes on day 7 post induction of *Pseudomonas aeruginosa*.

2.3 RESEARCH HYPOTHESIS

i. There is difference of mean SLE score of *Pseudomonas*-induced keratitis in eyes of rabbits between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% on day 1, 2, 3, 5, and 7 post induction of *Pseudomonas aeruginosa*.

ii. There is difference of mean bacterial CFU in *Pseudomonas*-induced keratitis in eyes of rabbits between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% on day 7 post induction of *Pseudomonas aeruginosa*.

2.4 DEFINITION OF TERMS

i. Tualang honey: 100% pure honey supplied by FAMA, Malaysia and was taken from the beehive on tualang tree in Kedah rainforest.

ii. *Pseudomonas*-induced keratitis: a disease of the cornea that result from direct infection of *Pseudomonas aeruginosa* and complicated with massive destruction of the cornea (Wilson, 1970).

iii. SLE score: grades of ulcer scoring system assessed using slit lamp examination based on corneal infiltrates, corneal ulcer, hypopyon and corneal perforation (Dong et al., 2012).

iv. Bacterial CFU: a quantitative bacterial colony analysis on the diseased corneal button (Barequet et al., 2004; McCormick et al., 2008).

2.5 METHODOLOGY

2.5.1 Study Design

Randomized control trial in animal study.

2.5.2 Population and Sample

Study population: New Zealand white adult rabbits will be obtained from Animal Research and Service Centre, Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan, Malaysia.

Place of study: Animal Research and Service Centre, Microbiology Laboratory and Pharmacological Laboratory, Universiti Sains Malaysia, Health Campus, Kubang Kerian, Kelantan, Malaysia.

Duration of study: 2 years (September 2014 – September 2016).

2.5.3 Selection Criteria

i. Inclusion Criteria:

- Healthy adult rabbits with clear cornea.
- Successfully induces *Pseudomonas aeruginosa* keratitis.

ii. Exclusion Criteria:

- Severe systemic infection with *Pseudomonas aeruginosa.*

2.5.4 Sampling and Sample Size

Sample size was calculated based on the first and second objectives. Sample size for the first and second objective is calculated by using G power and sample size calculation version 3.1.9.

i. First Objective: To compare mean SLE score of *Pseudomonas*-induced keratitis between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% in rabbit eyes on day 1, 2, 3, 5, and 7 post induction of *Pseudomonas aeruginosa*.

Effect size = 0.4

Power = 80%

Alpha level = 5% (0.05)

Number of group = 3

Number of evaluation = 5

Calculated sample size is 12 rabbits per group

ii. **Second Objective:** To compare mean bacterial CFU of *Pseudomonas*-induced keratitis between topical gentamicin 0.3%, topical tualang honey 30% and combination of topical gentamicin 0.3% and tualang honey 30% in rabbit eyes on day 7 post induction of *Pseudomonas aeruginosa.*

Effect size = 0.4

Power =80%

Alpha Level = 5% (0.05)

Number of group = 3

Calculated sample size is 22 rabbits per group

In view of the largest sample size (Second objective = 22 rabbits) is too big, sample size is chosen as 10 rabbits per group as suggested by the Animal Research Committee.

Number of rabbits needed for the preliminary study = 3

Number of rabbits per group in actual study = 10

Total number of rabbits needed = (10 x 3) + 3 = 33

2.5.5 Randomization Method

New Zealand white adult rabbits (aged 8 – 10 months) weighting between 2.5 and 3.0 kg will be used in this study. The rabbits will be randomly divided into 3 groups (A, B and C) consisting of 10 rabbits in each group.

a) The Group A will be treated with topical gentamicin 0.3%.
b) The Group B will be treated with topical tualang honey 30%.
c) The Group C will be treated with combination of topical gentamicin 0.3% and topical tualang honey 30%.

2.5.6 Study Material

i. Animals handling

New Zealand white adult rabbits

Rabbit cage and bedding

Elizabeth collar

ii. Drugs and Solution

Ketamine hydrochloride (100mg/ml)

Xylazine hydrochloride (20mg/ml)

Pentobarbitol (60mg/ml)

Povidone iodine (10mg/ml)

Propacaine hydrochloride 0.5% eye drop

Gentamicin 0.3% eye drop

Tualang honey 30% eye drop

Fluorescein ophthalmic strip (1mg/strip)

Pseudomonas aeruginosa (strain 27853)

Trypticase soy agar plates

Trypticase soy broth

iii. Surgical Instruments

Needles (22G, 27G and 28G)

Syringes (1ml, 5 ml)

Tuberculin syringes

Caliper

Paediatric Barraquer wire speculum

Conjunctival forcep

Surgical glove

Surgical drape

Shaver blade

Corneal scissors

Heine HR Binocular loupe with LED light

iv. Laboratory Instruments and Disposables

Topcon SL-7E biomicroscope

Weighing scale machine

Centrifuge tube (2ml, 5ml)

Beaker (150ml)

Goggles

Repeat pipette

Disposable biohazard bag

Gauze

2.5.7 Study Procedures

a) Phase 1 (Preliminary Study)

A preliminary study will be carried out prior to the actual study to establish the *Pseudomonas*-induces keratitis injury in rabbits. Three rabbits will be used in the preliminary study.

i. Maintenance of rabbits

The rabbits will be maintained and handled according to the recommendations of the animal ethics guidelines. Animals will be housed individually in stainless steel cages under controlled temperature, humidity and 12 hour light; dark cycle. Food and water will be provided ad libitum. The eyes of all rabbits will be examined with a microscope before the induction of *Pseudomonas aeruginosa* to make sure that their corners are normal.

ii. Preparation of *Pseudomonas aeroginosa* colony

Pseudomonas aeroginosa, strain 27853, will be used in this study. It is well documented strain for the testing of treatment response and courses well characterized keratitis in rabbits (McCormick et al., 2008). Single colony of *Pseudomonas aeroginosa* will be cultured in tryptic soy agar (TSA) overnight at 37°C. The overnight culture was inoculated into fresh tryptic soy broth (TSB) (1:100) and grown at 37°C. To infect rabbit corneas, the bacteria were serially diluted

in TSB to 10,000 CFU per ml. Diluted bacteria then plated and verified by a quantitative bacterial count on TSA.

iii. Preparation of topical tualang honey

Tualang honey will be obtained from Federal Agriculture Marketing Authority (FAMA), Kedah. The honey will be stored in the dark at room temperature and then will be subjected to sterilization by irradiation followed by sterility test prior to treat rabbits' eyes. Tualang honey with 30% concentration of will be freshly prepared everyday by mixing 1.5 ml of honey with 3.5 ml of distilled water in the morning just before the first instillation.

iv. Induction of *Pseudomonas aeruginosa* keratitis

The first rabbit will be anaesthetised with an intramuscular dose of ketamine hydrochloride (100mg/ml; 35mg/kg) and xylazine hydrochloride (20mg/ml; 2.4mg/kg) approximately 45 minutes prior to induction of *Pseudomonas aeruginosa* keratitis. The experiment will be carried out only on the right eye of each rabbit. A drop of propacaine hydrochloride 0.5% will be instilled to the right eye followed by insertion of barraquer wire speculum. Corneal stromal of anaesthetised rabbits will be injected with 1000 CFU of bacteria in 0.1ml of TSB (Thibodeaux et al, 2004; McCormick et al, 2008; Nejabat et al, 2009).

v. Clinical eye examination

The right eye of the rabbits will be examined to evaluate the clinical features with a microscope at day 1, 2, 3, 5, and 7 post induction of *Pseudomonas aeruginosa*. SLE of pathological changes in rabbit eyes will be performed by a single blinded examiner. The scoring system for SLE of the cornea will be done as per in Table 1.

Table 1: SLE score

Grade	Focus of infection		
0	No focus of infection		
1	Corneal infiltrate	1.25	Corneal infiltrate limited in the inoculated area
		1.50	Corneal infiltrate ≤ 1/2 corneal thickness
		1.75	Corneal infiltrate > 1/2 corneal thickness
2	Corneal ulcer	2.25	Diameter ≤ 3 mm
		2.50	> 3 mm diameter <5 mm
		2.75	Diameter ≥ 5 mm
3	Hypopyon	3.25	Altitude ≤ 1/3 corneal thickness
		3.50	> 1/3 corneal thickness altitude <1/2 corneal thickness
		3.75	Altitude > 1/2 corneal thickness
4	Hypopyon and corneal perforation		

Note: Adapted from Dong et al, 2012

vi. Preparation of corneal button

The rabbit will be euthanized after anaesthetized and administered a lethal dose of injection of intravenous pentobabitol (60 mg/kg) on day 7 post induction of *Pseudomonas aeruginosa*. The cornea will be excised and will be send for microbiological examination.

vii. Calculation of bacterial CFU

Cornea will be placed into sterile phosphate-buffered saline (0.1M, pH 7.2; PBS) and homogenized. Homogenized cornea will be serially diluted (1:10) in fresh TBS and plated, in triplicate, on TSA. Plates will be incubated at 37°C overnight. The bacterial colonies were identified using the colony morphology and Gram staining, then counted to determine the mean CFU per cornea. Sterility was maintained throughout the procedures to avoid cross-contamination.

b) Phase II (Actual Study)

Actual study will be conducted with 10 rabbits in each group.

i. Maintenance of rabbit

Rabbits will be maintained as per mentioned in 5.7.1.1.

ii. Preparation of *Pseudomonas aeruginosa* colony

Pseudomonas aeruginosa will be prepared as per in 5.7.1.2.

iii. Preparation of topical tualang honey

Topical tualang honey will be prepared as per in 5.7.1.3.

iv. Induction of *Pseudomonas aeruginosa* keratitis

Pseudomonas aeruginosa will be induced as mentioned in 5.7.1.4.

v. Treatment regime

24 hours after the injection, the rabbits will be randomized into the following groups;

i) Group A – gutt gentamicin 0.3%.

ii) Group B – gutt tualang honey 30%.

iii) Group C – gutt gentamicin 0.3% and gutt tualang honey 30%.

The eye drops will be administered every 2 hours for first 24 hours in all 3 groups. Subsequently, the medications will be administered every 4 hours until day 7 post induction of *Pseudomonas aeruginosa*.

vi. Clinical eye examination

Clinical features will be evaluated at day 1, 2, 3, 5, and 7 as per mentioned in 5.7.1.5.

vii. Preparation of the corneal button

The cornea will be excised as per mentioned in 5.7.1.6.

viii. Calculation of CFU count

The procedure will be performed as described in 5.7.1.7.