

# Proliferative Capacity of in Vitro Corneal Epithelium: Role of Acacia Honey in the Initial Step of Wound Healing

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**Abstract**—Proliferation of corneal epithelial cells (CEC) is vital in the initial stage of wound healing. This study aimed to investigate the proliferative capacity of Acacia Honey (AH) on rabbit CEC via assessment on morphology, proliferation, cell cycle, gene and protein expressions. The optimal dose of AH in basal medium (BM) and complete cornea medium (CCM) was identified via MTT assay. CEC cultured in both media supplemented with 0.025% AH showed optimal proliferative capacity compared to the control. There were no abnormal changes in morphology and cell cycle analysis. Gene and protein expression of CK3 was increased in the CEC cultured with 0.025% AH in both media. CEC cultured in media supplemented with 0.025% AH promotes proliferation while retaining its normal morphology, cell cycle, gene and protein expressions. These promising results serve as an impetus in realizing the proliferative potential of AH in promoting the initial step of corneal wound healing.

**Index Terms**—Acacia Honey, proliferation, corneal epithelial cells, corneal wound healing.

## I. INTRODUCTION

Corneal epithelium provides the initial physical barrier in preventing ocular infection and possesses a tear film-cornea interface which is important for the refractive power of the eye. Corneal epithelium possesses 4 to 6 layers of non-keratinized, stratified squamous epithelium. The most superficial layers of corneal epithelial cells (CEC) consist of 2-3 layers of squamous polygonal cells. Cells with lateral wing-like extensions were located overlying the superficial layers. The basal layer is the deepest layer of corneal epithelium which comprises a layer of columnar cells. Basal cells are capable of mitosis, besides the limbal stem cells and transient amplifying cells[1].

Trauma, chemical, keratectomy and microbial infections are common causes of eye injuries. This injury

may involve only the epithelium such as corneal abrasion [2]. The exposed corneal surface is susceptible to infection and diseases. During the corneal epithelium wound healing, the CEC migrate, proliferate and differentiate in an independent manner [3]. CEC proliferation is the key process in restoring the number of cells for wound closure. The conventional treatment for corneal injury is administration of topical antibiotic or antifungal eye drop to prevent secondary infection. However, long duration of treatment will lead to the development of resistance. In addition, preservatives such as Benzalkonium Ammonium Chloride (BAK) in the topical eye drop lead to side effects such as disruption of corneal epithelium, tear film instability, red eye, dryness, conjunctivitis and allergy [4].

Honey is a natural food which has a high content of sugars and small percentage of other active ingredients such as vitamins, organic acids, minerals, proteins, flavonoids, phenolic acids, enzymes and other phytochemicals, which vary according to its floral and geographical origin. Many studies have been reported on the role of various honey in promoting skin wound healing [5]. Since both the cornea and skin epithelia are derived from surface ectoderm embryologically, honey could be a potential agent in promoting proliferation of CEC in the corneal wound healing.

Acacia honey (AH) is produced by *Apis mellifera* honeybees, which harvest nectar from Acacia mangium trees. AH has been documented to have an anticancer effect on human and mouse melanoma cells *in vitro* [6]. However, its proliferative properties are yet to be studied. Hence, this study was designed to investigate the effect of AH on CEC proliferation *in vitro* which corresponds to the initial step of *in vivo* wound healing. The proliferative capacity of AH on CEC was performed via assessment on morphology, proliferation, cell cycle, gene and protein expressions.

## II. RESEARCH DESIGN AND SAMPLING

This research was approved by the Research and Ethical Committee of Faculty of Medicine, University Kebangsaan Malaysia (UKM project code: GGPM-2011-085) and Universiti Kebangsaan Malaysia Animal Ethics Committee (project code: UKMAEC Approval Number FP/ANAT/2012/ NORZANA/ 18-JANUARY/ 419-JANUARY-2011-DECEMBER-2013-AR-CAT2).

#### A. Acacia Honey

Acacia honey (AH) was obtained from Ministry of Agriculture, Malaysia. AH was gamma irradiated at 25 kGy at Ministry of Science, Technology and Innovation, Malaysia and stored at room temperature.

#### B. Isolation And Culture of Rabbit Corneal Epithelial (CEC) Cells

CEC was isolated according to method by Norzana et al. 2007 [7]. Briefly, the corneas from six New Zealand white strain rabbits were excised followed by removal of connective tissue such as endothelium, ocular muscle, sclera and iris. The corneas were rinsed with phosphate buffered solution (Gibco Invitrogen, USA) and incubated in Dispase solution 2mg/ml (Sigma-Aldrich, USA) at 4 °C for 18 hours to separate the epithelium from the stroma. The epithelium was gently scrapped off using a fine surgical blade and then transferred into a centrifuge tube. Five ml of 0.05% trypsin-EDTA (Gibco Invitrogen, USA) was added for obtaining single epithelial cell suspension followed by addition of 5ml define trypsin inhibitor (Gibco Invitrogen, USA) to deactivate the activity of trypsin-EDTA. Then, the CEC was centrifuged at 500×g for 10 minutes with the supernatant discarded. Resultant pellet was suspended in Complete Corneal Medium (CCM) containing human corneal growth supplement (HCGS) and antibiotic antimetabolic (Gibco, Invitrogen, USA). The total number of cells was quantified using haemocytometer (Weber Scientific Int, Ltd. Middlx, England). Viable CEC was seeded with  $1 \times 10^5$  per well seeding density in six well-plates (BD Falcon, Franklin Lakes, NJ). CEC was cultured in 5% CO<sub>2</sub> incubator (Jouan, DuguayTrouin, SH) under 95% humidity at 37 °C. Media were changed every two days. Upon reaching 80% confluence, the primary culture (P0) was trypsinized using 1ml of versene (Gibco, Invitrogen, USA) and 0.05% trypsin-EDTA and then subculture with the same condition until passage 1(P1). Then, CEC were cultured in 4 different media namely; A) basal medium (BM), B) BM with supplementation of 0.025% AH, C) complete corneal medium (CCM) and D) CCM with supplementation of 0.025% AH. Morphological features of CEC in culture were assessed via inverted phase contrast microscope (Carl Zeiss, Germany).

#### C. MTT Assay

MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide; Sigma-Aldrich) assay was used for assessment of proliferative capacity of CEC in four different media where viable cells formed insoluble purple formazan. CEC at P1 were seeded in 96-well cell culture plate (Cellstar, Greiner Bio-one, Germany) at a density of  $5 \times 10^3$  cells per well. After 24 hours, the

medium was changed to both BM and CCM supplemented with different concentration of Acacia honey using two times dilution factor method ranging from 3.125% to 0%. The cells were incubated at 37 °C in a 5% CO<sub>2</sub> and 95% humidity. After 72 hours, MTT assay was performed by adding 10 µl MTT solution into each well and incubated for 4 hours in a dark condition. The purple formazan precipitate that formed by living cells were solubilized with 100 µl dimethylsulfoxide at each well, and the absorbance was measured at 570 nm by ELISA reader. Acacia honey concentration which produced the highest level of absorbance was chosen as the optimal dose of CEC proliferation. Inhibition CEC viability of 50% (IC<sub>50</sub>) values were obtained when 1.56nM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added in BM and CCM respectively during the pilot study. Hence, H<sub>2</sub>O<sub>2</sub> at a concentration of 1.56nM was chosen as positive control to ensure the optimal dose of AH does not reach the cytotoxic stage.

#### D. Reverse-Transcriptase Polymerase ChainReaction (RT-PCR) and PCR Amplification of CEC Cells

Total RNA from cultured CEC in BM and CCM media with or without supplementation of optimal dose of AH was extracted with TRI Reagent (Molecular Research Centre, Cincinnati, USA) according to the protocol recommended by the manufacturer. Briefly, chloroform was added into the TRI Reagent homogenate to separate the colourless aqueous containing total RNA. Isopropanol and Polyacryl carrier (Molecular Research Centre) was then added to precipitate the total RNA. The extracted RNA pellet was rinsed with 75% ethanol, and air dried. Then, Rnase and Dnase free distilled water (Invitrogen, Carlsbad, USA) was added to dissolve the RNA pellet. Complementary DNA (cDNA) was synthesised from total RNA using Superscript™ III First-Strand Synthesis Super Mix reverse transcriptase (Invitrogen, Carlsbad, USA) according to manufacturer's protocol. The protocol condition was: 10 minutes at 23 °C for primer annealing, 60 minutes at 50 °C for reverse transcription and 5 minutes at 85 °C for reaction termination. PCR was then performed using 1 µl of cDNA template, 1 µl of each forward and reverse primer, 12.5µl of iQ SYBR Supermix and deionised water. The expression of corneal epithelial cells specific marker, cytokeratin 3 (CK3) was analysed by qRT-PCR. The CK3 primers (sense and antisense) used for quantitative PCR reactions were designed based on the sequences published in GenBank using Primer-3 software as shown in Table I. Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) primers were used as internal control to ensure cDNA quality and loading accuracy. Using SYBR Green as the indicator, the two-step RT-PCR reaction was performed in a Bio-Rad iCycler (Bio-Rad, USA). The reaction condition was cycle 1: 95 °C for 3 minutes (1 ×), cycle 2: Step 1 95 °C for 10 sec and Step 2 61 °C for 30 sec (40 ×), followed by melting curve analysis. PCR products were visualised by gel electrophoresis on a 2% agarose gel containing 0.5µg/ml ethidium bromide.

#### E. Immunocytochemistry

CEC was cultured in chamber slides with 4 different media: A) BM, B) BM with supplementation of 0.025% AH, C) CCM and D) CCM with supplementation of 0.025% AH. The slides were fixed in 4% paraformaldehyde at 4 °C for 30 minutes and soaked in acetone for 5 minutes. Cells were stained using Animal Research Kit (Dako ARK, US) protocol with some modification. In brief, the slides were incubated with 0.03% hydrogen peroxidase blocking agent for 6 minutes and heated at 95 °C with Tris-EDTA for 20 minutes. The slides were incubated with a rabbit anti-CK3 antibody ( Abcam, UK) diluted 1:200 in blocking agent for 30 minutes. After washing with Tris-Buffered Saline for three times, the slides were incubated with HRP (Dako) secondary antibody for 30 minutes and DAB substrate for 7 minutes. Nuclei were stained blue with haematoxylin (Sigma). Positive stained cells exhibited brownish precipitate in the cytoplasm using confocal laser scanning microscopy (LSM-510, Zeiss).

F. Cell Cycle Analysis

CEC (5.0X 10<sup>5</sup> cells) was stained with Cycle Test Plus DNA Reagent Kit (Becton Dickinson, US) according to the manufacturer’s protocol. In brief, cells were centrifuged at 1000rpm and supernatant was discarded. Cells were resuspended with trypsin in a spermise tetra hydrochloride detergent buffer. After incubation, cells were suspended with trypsin inhibitor and ribonuclease A in citrate stabilizing buffer and transferred to a sterile flow cytometer glass tube. Then, 200 µl of propidium iodide (PI) was added, and incubation was done in the dark on ice. The percentages of CEC in G0-G1, S, and G2-M phases were determined by the Mod Fit software for cell cycle distribution. Cell cycle distribution was analysed using CellQuest™ software (Becton Dickinson) in the flow cytometry (Becton Dickinson, FACS Canto II). The DNA histograms for each sample were determined using BD FASCS Diva software.

TABLE I. PRIMERS SEQUENCE OF GENES FOR QUANTITATIVE PCR.

Gene	Gen Bank Accession Number	Primer Sequence 5'-3'	PCR product size (bp)
GADPH	NM_001082253	F:caacgaattggctacagca R:aaactgtgaagaggggcaga	186
CK3	XM_002711005	F:gactcggagctgagaagcat R:cagggtctcaggaagtga	198

III. DATA ANALYSIS AND STATISTICS

All data were analysed using Statistical package for Social Sciences (SPSS) version 20 for statistical significance. Values were expressed as mean ± standard error of mean (SEM) using Student’s t-test and One-way Analysis of Variance (ANOVA). A p-value of less than 0.05 was considered significant.

IV. RESULTS

A. Cell Viability and Proliferation Assay

The CEC proliferation assay was performed using different composition of AH in BM and CCM is shown

in Fig. 1. CEC cultured in BM supplemented with AH at concentration of 0.012% AH to 0.39% AH showed significant higher proliferative capacity (p<0.05) compared to BM group [Fig. 1A]. CEC in CCM group exhibited a significant increasing proliferative potential (p<0.05) when supplementation with AH at the concentration of 0.025% to 0.1% [Fig. 1B]. AH at the concentration of 0.025% showed the highest proliferative capacity both in BM and CCM media. The optimal dose of AH obtained was greater than the inhibition value (IC50) of the H2O2 positive control indicating the excellent cytocompatibility property of AH with CEC. CEC cultured in CCM with or without supplementation of AH showed a higher cell proliferative capacity in all concentrations compared to BM group [Fig. 1C].

Supplementation of 0.025% AH promotes higher proliferative capacity in CEC in BM and CCM. Hence, this optimal dose is selected for the subsequent test.

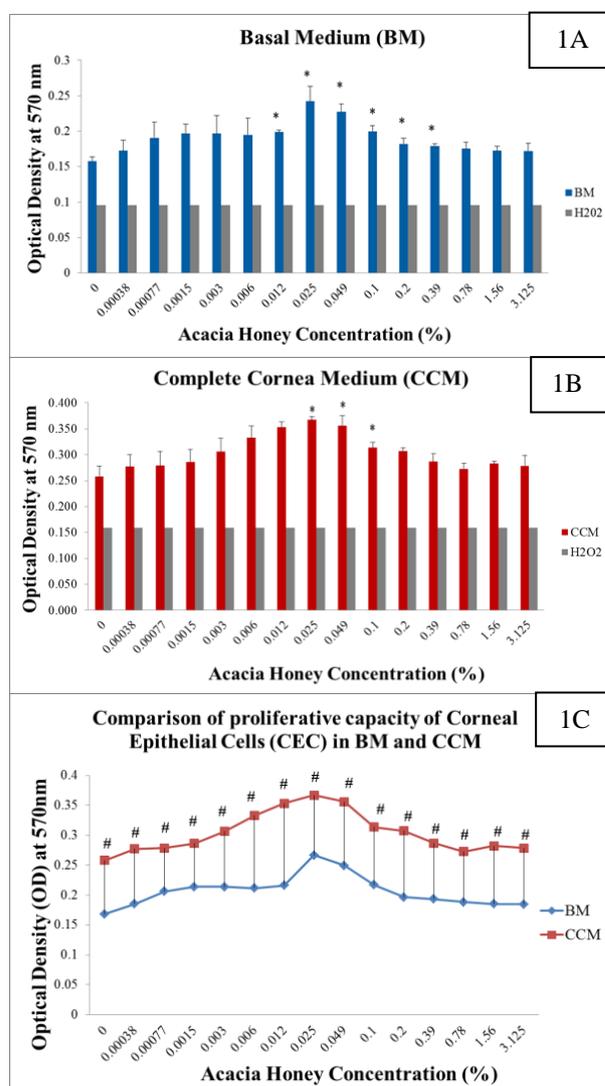


Figure 1. Viability of CEC cultured in basal medium (BM, 1A), complete corneal medium (CCM, 1B) supplemented with AH ranging from 0.00038% to 3.125%. Comparison of proliferative capacity of CEC between the two groups (1C). Significant difference (p < 0.05) between the same medium was marked with (\*) while (#) denotes significant difference (p < 0.05) between groups. Values were tested using Student t-test and expressed as mean ± SEM, n = 6.

**B. Phase contrast micrographs**

The morphological feature of CEC was observed to detect any abnormal changes in cell morphology. At day 3, CEC cultured in BM with or without 0.025% AH showed clusters of flattened, large and polygonal-shaped cells (Fig. 2A and Fig. 2B). CEC cultured in CCM with or without 0.025% AH showed smaller polygonal cells with cohesive appearance and distinct cell border (Fig. 2C and Fig. 2D). This indicates that CEC cultured in 0.025% AH in BM and CCM did not show any abnormal morphological changes. Higher cell density was observed in the cultured CEC supplemented with 0.025% AH in BM (Fig. 2B) and CCM (Fig. 2D) media when compared to control respectively (Fig. 2A and Fig. 2C). These results are in conformity with the MTT result.

**C. Gene Expression Analysis**

CK3 is a specific marker for corneal epithelium. There was a significant increased mRNA expression of CK3 ( $p < 0.05$ ) in CEC cultured with 0.025% AH in both BM ( $p = 0.002$ ) and CCM ( $p = 0.021$ ) groups compare to control group respectively (Fig. 3). Gel electrophoresis showed specific size product of CK3 in all media (Fig. 4). These indicate AH retained the specificity and stability of CEC phenotype cultured in BM and CCM media.

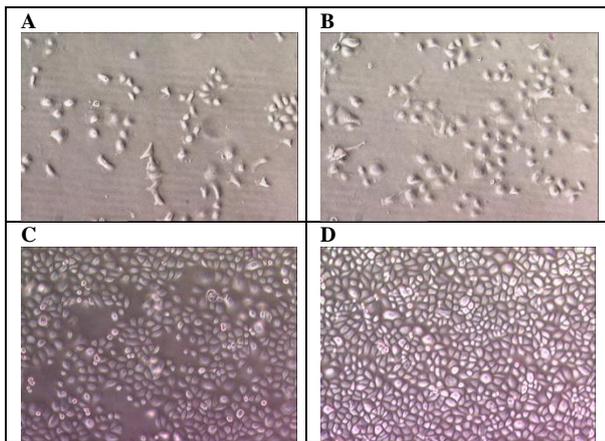


Figure 2. Phase contrast micrographs showing the morphology of CEC cultured in different media; A) Basal medium, BM, B) BM plus 0.025% AH C) Complete corneal medium, CCM, D) CCM plus 0.025% AH at day 3, passage 1. Supplementation of 0.025% AH was able to maintain CEC cell morphology and promote higher cells density.

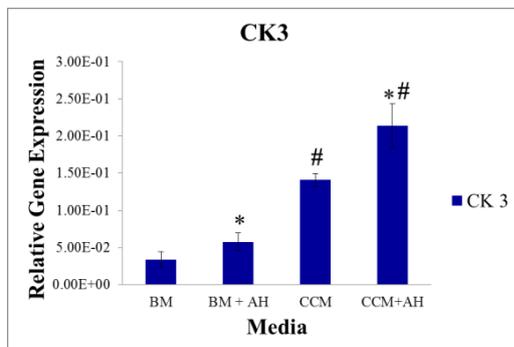


Figure 3. Gene expression of CK3 of CEC in four different media using qRT-PCR with GADPH gene as internal control. (\*) denotes significant difference ( $p < 0.05$ ) in the same group and (#) denotes significant difference ( $p < 0.05$ ) between groups. Value were tested using Student t-test and expressed as mean  $\pm$  SEM, n=6.

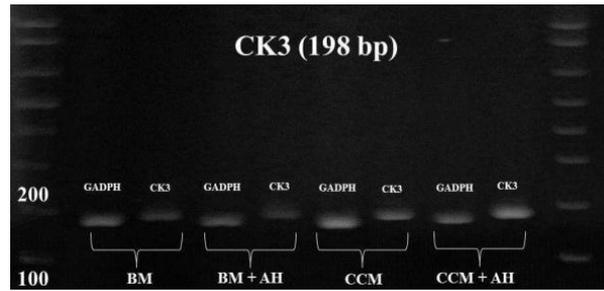


Figure 4. Gel electrophoresis of cultured CEC phenotype with GADPH gene as internal control.

**D. Protein Expression Analysis**

CEC showed positive stained cells for CK3 in all culture media. However, CEC cultured in media supplemented with 0.025% AH in BM and CCM showed a higher density of positive stained expression pattern for CK3 (Fig. 5). This result is in agreement with gene analysis by qRT-PCR.

**E. Cell Cycle Analysis**

Cell cycle analysis is performed to ascertain the DNA content of the cells. The ploidy state during cell culture is essential to detect any abnormal DNA property that leads to development of cancer cell.

There was no evidence of aneuploidy or tetraploidy detected in this study (Fig. 6). The CEC cultured in BM and CCM media with supplementation of 0.025% AH showed a lower percentage in G0-G1 phase compared to control. However, both supplemented groups showed significant increase in the percentage of S-phase (synthesis phase) and G2-M phase compared to control respectively (Table II). Higher percentage in S-phase indicates a greater proliferative capacity with higher DNA content and cell nuclei.

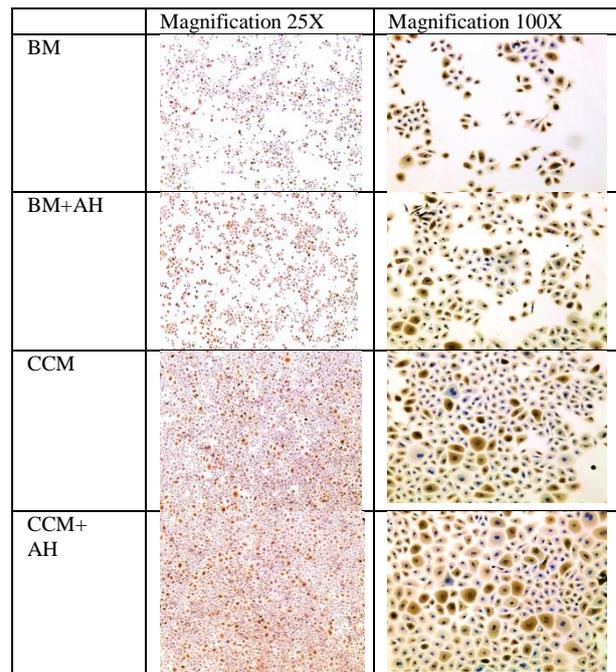


Figure 5. Immunocytochemistry of monoclonal antibody against CK3 of CEC cultured in 4 different media.

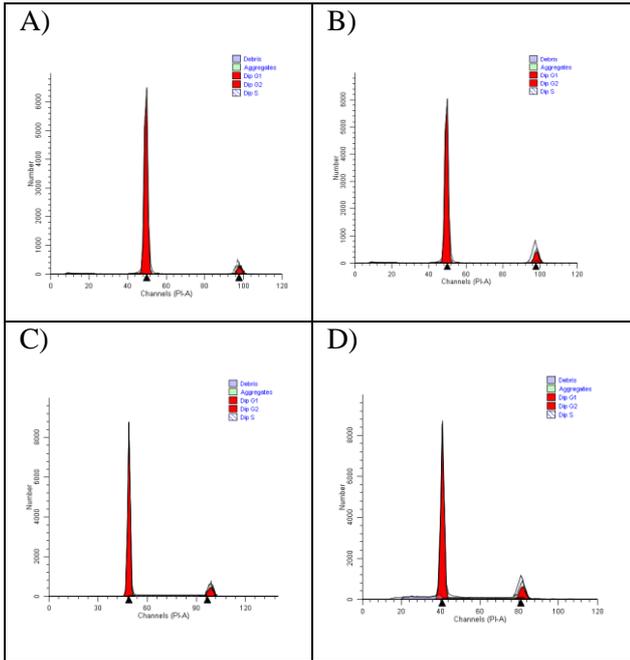


Figure 6. DNA histogram generated from CEC cells cultured in BM and CCM media with or without supplementation of 0.025% AH showing the percentage of G0-G1, S and G2- M phases. X-axis represents the relative fluorescence intensity proportional to DNA content. DNA histogram showed CEC cells cultured in (A) BM medium, (B) BM supplemented with 0.025% AH, (C) CCM medium, (D) CCM supplemented with 0.025% AH. The data analysis showed absence of aneuploidy or tetraploidy was detected in both histograms, indicating CEC were all in diploid state.

TABLE II. AVERAGE PERCENTAGES OF DNA CONTENT OF CEC CELLS IN DIFFERENT STAGES OF CELL CYCLE IN DIFFERENT MEDIUM. SIGNIFICANT DIFFERENCES WHEN BM WITH SUPPLEMENTATION OF 0.025% AH WERE COMPARED WITH THE CONTROL, BM (\*P<0.05) AND CCM WITH SUPPLEMENTATION OF 0.025% AH WERE COMPARED WITH THE CONTROL CCM (\*\*P< 0.05) AT DIFFERENT CELL CYCLE STAGES. VALUE WERE EXPRESSED AS MEAN ± S.E.M.

Medium	Cell cycle phases		
	G0-G1	G2-M	S
BM	89.73 ± 0.8734	3.97 ± 1.0208	4.72 ± 0.2340
BM+ 0.025% AH	85.34 ± 0.7646 *	5.93 ± 0.9879*	6.95 ± 0.3734*
CCM	81.85 ± 0.9783	6.82 ± 0.8686	9.86 ± 0.5678
CCM+ 0.025% AH	79.56 ± 1.6821 **	7.67 ± 1.1723**	10.97 ± 0.9764* *

V. DISCUSSION

Cornea wound healing is a dynamic physiological process. Repairing and restoring the cell layer is essential for retaining corneal transparency and normal visual acuity. Generally, the mechanism of the cornea wound healing following injury is characterized into three phases, which are epithelial cell migration, proliferation, and differentiation, which occur in a continuous and independent manner [3]. After cornea injury, CEC begins to flatten and migrate until the wounded area is covered. Migration of epithelial cells is associated with the cell proliferation to restore cell number during re-

epithelialization. Corneal wound healing process is completed when epithelial cells differentiated, closure of wound and restoring the well-layered structure of the corneal epithelium [2]. Hence, proliferation of epithelial cells is the key process in corneal wound healing.

Cell proliferation in corneal wound healing is an active and energy consuming process [2]. Corneal epithelial cell migration and proliferation depends on metabolic support provided by glucose in the aqueous humor and epithelial glycogen stores in vivo [3]. In this study, we have demonstrated that supplementation of AH at concentration of 0.025% in BM and CCM significantly promote the proliferation of CEC. Acacia honey is a type of honeydew honey with its major constituents of monosaccharide, which consists of mainly fructose and glucose. The sugars in honey have physicochemical properties such as viscosity, hygroscopicity, granulation and providing source of energy [8]. In comparison to other honey in Malaysia, Acacia honey possesses the highest total sugar content [9] which could explain the energy provider for the proliferation of CEC.

Hydrogen peroxide which is produced through oxidation of glucose in honey by enzyme glucose oxidase from honeybees has been documented as the main contributor for antibacterial property even when the honey is diluted [10]. This antibacterial property provides an optimal environment for the proliferation of CEC in a medium with additional AH. High acidity of AH also contributed to stability against the growth of bacteria [10].

Acacia honey contains the highest amount of trace element content compared to other nectar honey such as Tualang honey and Gelam honey in Malaysia [11]. Trace element such as zink, cuprum and magnesium are also known to stimulate keratinocyte proliferation through modulating integrins expression during re-epithelialization [12].

Human Corneal Growth Supplement (HCGS) in CCM consists of two main components, which are epidermal growth factor (EGF) and bovine pituitary extract (BPE). The critical processes in corneal wound healing are cell migration and proliferation, which are influenced by growth factors. EGF has been proven to possess an essential role in cell proliferation, migration, differentiation as well as metabolic regulation in different cell lines, including epithelial cells [13]. BPE is a mitogenic supplement which is often used in a serum-free growth medium which contains various growth factors and hormones, and possessed antioxidant activity [14]. It has been proven to induce proliferation for other epithelial cells such as human keratinocytes [15] and human mammary epithelial cells [16]. In our study, CEC showed a higher proliferation capacity in CCM medium compared to BM medium in all honey concentrations. This indicates that there is synergistic effect of AH with the growth factor in CCM.

Cytokeratin 3 (CK3) is a basic keratin pair of cytokeratin 12 in corneal epithelial cells. CK3 is a corneal specific marker which was strongly expressed by

all corneal epithelial cells and superficial limbal cells during advanced stage of cell differentiation [17]. CK3 is a specific corneal marker which plays an important function in maintaining corneal epithelial integrity [3]. Our study showed up-regulating of mRNA expression of CK3 in medium supplemented with 0.025% AH. This is in accordance to the findings of proliferation assay.

Cell cycle analysis is used to determine the cell proportion during proliferation at various phases; G0, G1, S-phase (synthesis) and G2-M (mitosis) during cell division. It is essential that the cultured CEC do not possess aneuploidy state that leads to malignant phenotype. Additional AH to the culture media increases the proliferation of CEC (increase in S phase and G2-M phase), whilst maintaining the normal diploid state.

## VI. CONCLUSION

In conclusion, Acacia honey at concentration of 0.025% promotes proliferation of cultured corneal epithelial cells whilst maintaining its phenotype and retaining normal cell cycle. Further studies need to be done to ascertain the specific component of AH which promote proliferation of corneal epithelial cells in a wound healing model.

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