PRELIMINARY STUDY ON THE EFFECT OF COMMERCIALLY AVAILABLE EYE TONERS ON RABBIT CORNEAL KERATOCYTES PROLIFERATION AND MIGRATION

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ABSTRACT

Devoid of scientific proofs, the use of commercially available natural product-based eye toners for therapeutic purposes incites controversies surrounding it. This study aims to evaluate the effect of selected commercially available eye toners on in vitro cellular model using rabbits’ corneal keratocytes. The primary cells were cultivated in culture media supplemented with or without eye toners as control. Growth kinetic profile, cells proliferation assay, scratch assay and immunocytochemistry analysis were performed in comparing both culture media. There were no significant differences between control and the commercially available eye toners in the growth kinetics and cell proliferation. Microscopic observation of the scratch assay suggested the eye toners did not promote cells migration as compare to control. However, in the immunohistochemistry study the eye toners are able to sustain the expression of Connexin-43 and Lumican. This preliminary study indicates that the selected eye toners do not promote the proliferation and migration of corneal keratocytes. They are able to maintain the expression of Connexin-43 and Lumican which might have beneficial effect in corneal transparency. Studies with individual element of the eye toners need to be done.

1.0 Introduction

Back in 2009, the Ministry of Health (MOH) Malaysia had recalled 27 local cosmetic products from the market [1]. It was reported that the products did not comply with the definition of cosmetics. Cosmetics are preparations to be used on external parts of the body that include the skin, hair, nails, lips, teeth or the oral cavity for the purpose of cleaning, perfuming, changing or improving appearance, protecting or keeping in good condition and correcting body odours [2].

Among the products recalled were eye toners manufactured by several local cosmetic companies [3]. The eye toners were originally notified as cosmetic products. Various local eye toners that include in these categories are commercially available in the market such as Permata Hijrah, As-Syifa Herb, Ainun Theraphy, Qurrotaaini, Al-Jabbar, B-Trang as well as Dr. Eyes. Most of the eye toners gained an overwhelming acceptance by many Malaysian users. They were meant for external applications claim for moisturize, reduce the dark circle and wrinkles around the eye area. Improvement of vision and short- or long-sightedness are categorized as medicinal claims and thus, not allowed for cosmetics. Other claims prohibited to be used for cosmetic products include to refresh eye nerves, to remove foreign materials from the eyes, to treat conjunctivitis as well as to reduce eye itchiness or redness.
Despite notified as cosmetics, the eye toners were misused for treating eye conditions through direct instillation into the eyes as eye drops. Eye drops related products do not fall under the definition of cosmetics. They should be controlled as pharmaceutical products. The products must be manufactured in licensed manufacturing premises that comply to Good Manufacturing Practices (GMP) [4].

The cornea is vulnerable to a host of pathologies, which can be classified into hereditary, age-related, traumatic or infectious causes. Any of the complex processes and structure governing the successful function of the cornea can be affected and impair normal vision. To date, the most viable treatment option for permanently opacified or severely malformed corneas is by corneal transplantation [5]. Yet, the commonest drawback, lack of donor supply, has led to development of corneal tissue equivalents in vitro via tissue engineering approach [6,7,8]. This approach using corneal cells is expected to have higher successful degree of host integration after transplantation and hoped to circumvent the need for donor corneas. Beside for transplantation purposes, tissue engineering techniques can be used in other biomedical applications, e.g. drug permeation studies, and in toxicology as an alternative for animal experimentation [9,10].

Corneal models, constructed by cell culturing methods, vary from simple monolayer cultures to stratified cell cultures, to epithelium-stroma co-cultures, and to more complex tissue-engineered three-dimensional corneal tissue equivalents. Toxicity has been assessed by various methods such as cell count, cell detachment, colony forming efficiency, morphological changes, nutrient transport changes, measurement of cellular protein, energy metabolism disturbances, membrane changes, and transparency, histology and cytokine evaluations of the corneal model [9,10].

The false impression on the use of eye toners due to the dearth of scientific evidences is understandable. Therefore, this study aims to fill the gap by evaluating the potential impact of selected commercially-available eye toners from the local market namely Permata Hijrah Eye Toner, Dr. Eyes Eye Toner and Qurrataaini Eye Toner, using an in vitro cellular model. This approach employed rabbit’s corneal keratocytes as a model.

According to the manufacturer, Permata Hijrah Eye Toner formulation is based on 40% of aqua (zam-zam water), 30 % of margarita or pearl, 20% Ostrea shell extract and 10% Corallina officinalis extract. This combination is chosen maybe due to the ability of Ostrea shell to heal infected sores or wound [11], pearl that contains approximately 20 amino acids that can heal and maintain the cells [12], as well as the antimicrobial properties of Corallina officinalis [13]. While, zam-zam water has been proven to have high amount of mineral composition when compared to normal tap water [14]. Dr. Eyes Eye Toner is the combination of euphrasia, cineraria and pearl water formulated using nanotechnology. Both euphrasia and cineraria are herbaceous flowering plants. Euphrasia has been used in Scotland in the 14th and 15th centuries for various eye problems e.g. conjunctivitis [15]. While, cineraria have been used homeopathically for over one hundred years ago in Europe, India and South America to treat cataract [16]. Another formula added in the eye toner is pearl water. In Chinese traditional medicines, pearl powder is a natural source of calcium which is taken internally and topicaly to treat visual weakness, inflammation of the eyes or growth over the eyes [17]. The manufacturer claimed that the eye toner has various advantages since it is formulated through homeopathy, far infrared (FIR) and nanotechnology which are very effective in treating eye problems.

The main ingredients of Qurrataaini Eye Toner are Epiphyllum oxypetalum flower extract, fruit of Melaleuca leucadendra, piper betel, Orthosiphon spicatus extract, pandan leaves, honey and zam-zam water. Epiphyllum oxypetalum (Bunga Bakawali) through the dew collected from its blooming flower was known for years to treat eye problem and improve user’s vision [18]. While, the oil of Melaleuca leucadendra (kayu putih) had traditionally applied on skin to relieve respiratory discomfort and sickness. It has the ability to treat hypertension [19] and has hypoglycaemic properties [20]. However, nothing was found about the effect of M. leucadendra on the eyes. In contrast, piper betel (sirih) was reported to cure inflammation of the mucous membranes of the eyes and help in treating trachoma [21]. It also had antifungal, aflatoxin suppressive and antioxidant characteristics [22]. Since ancient era, honey has been used as a traditional medicine to treat throat, flu and fever. It has anti-angiogenic and anti-inflammatory properties that are useful in corneal inflammatory and infectious conditions [23].

From the information above, it is evident that the effects of the individual eye toners constituents were studied extensively. However, to the best of authors knowledge, there is no study on the potential impact of the ingredients that are readily available for human use in such combination. Other than medical or therapeutic claims, one common ground shared by these three selected eye toners is the method of administering; that is to spray directly into the eyes which contradicted the cosmetic product definition [2]. Hence, it is felt that the scientific community has the responsibility to reach out the local society to educate and promote the awareness of evidence-based healthcare practices.

2.0 Material and Method

The study was approved by the Kulliyyah Ethics Committee Meeting No. 3/2011, on 5th December 2011 at the Kulliyyah of
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Medicine, International Islamic University Malaysia, Kuantan Campus.

2.1 Preparation of Eye Toner Solutions.

Commercially available eye toners namely Permata Hijrah Eye Toner, Dr. Eyes Eye Toner and Qurrateaunini Eye Toner were bought from the local shop. Throughout this study, the eye toners were coded or labelled as ET01, ET02 and ET03 respectively. They were aliquot into sterile containers using 0.22 µm sterile syringe filter. The eye toners were kept at room temperature until used. The dosage of each eye toner was determined in accordance with the manufacturer’s recommendation. Based on simple calculation, one spray of eye toner solution is equal to 160 µl. Corneal absorptions were reduced to approximately 5% after administration [24]. Therefore, based on the assumption, 5% from 160 µl eye toner was supplemented into each sample.

2.2 In vitro Conical Keratocytes Model

Six rabbits’ cornea were obtained from locally available slaughterhouse. All corneal tissues were washed with phosphate buffered saline (PBS) (Gibco, Invitrogen, USA) prior to enzymatic digestion using 0.3% Collagenase A (Roche Applied Science, Germany) for 6 hours in 5% CO2 incubator (Thermo Scientific, USA) at 37° C. The cells suspension was centrifuged and the pellet was suspended in PBS for total cell count with haemacytometer (Rohem, Rohem Instruments. Pvt. Ltd, India).

Cell viability was determined using trypan blue dye exclusion test (Gibco). The cells were co-cultured with the initial seeding of 10,000 cells/cm2 in 6-well plate (Nunc, Denmark) prior to isolation of keratocytes for further subcultivations. Each sample was assigned into four groups - corneal keratocytes were cultured in culture media supplemented with eye toner solutions namely ET01, ET02 and ET03. Corneal keratocytes cultured without any eye toner solutions was used as control. The culture media were also supplemented with 10% Foetal Bovine Serum (FBS) (Gibco), antibiotic and antimycotic (Gibco), 200 mM L-glutamine (Gibco), 50 µg/ml of ascorbic acid (Gibco) and HEPES buffer (Gibco). All cultures were maintained in 5% CO2 incubator at 37°C with the medium changed every other day. Once confluence, the primary culture, P0 was trypsinized using Trypsin-EDTA (Gibco) and counted for total cell and viability by using trypan blue exclusion method. The cultures were expanded for three more passages (P1, P2 and P3) and grown in the same condition as the primary culture.

The morphological features of cultured corneal keratocytes were examined daily using inverted light microscope (Inverted CK30, Olympus, Shinjuku, Tokyo) and recorded using a portable camera (AM4023X, Dino-lite, Europe). The viability and total number of cell were recorded in every passage for growth kinetic evaluation by cell growth rate (cells/day/cm2), and population doubling time (days). The keratocytes were sub-cultured for several passages (P1, P2 and P3) for cells proliferation assay, in vitro scratch assay study and immunocytochemistry analysis.

Results were analysed using Student’s t-test and the difference was considered significance when p≤ 0.05. Data were expressed as mean ± standard error of the mean (SEM) of six samples (n=6).

2.3 Growth Kinetic Assessment

The total number of cell was calculated by the “total live cell” / 9 X 104 X suspension volume (ml). The viability of cell was recorded as percentage of the “total live cell” / “total live and dead cells”.

Growth rate is the subtraction of total cell number with the initial cell seeding / h where “h” is the time (day) required for cell to grow into confluence. Population doubling times were calculated assuming that keratocytes developed from single cell. Thus, average population doubling times are given by h / D; where “D” is equal to the number of cell doublings. “D” is defined by the expression of log N log No/2 given by h / D; where “D” is equal to the number of cell doublings. “D” is defined by the expression of log N log No/2 log 2 where “N” is the cell number after “h” days and No is the original cell number. The values obtained provide a minimum estimate for the maximum rate of cell division and show that keratocytes are dividing at a similar rate.

2.4 Cells Proliferation Assay

Cell proliferation, viability and toxicity were assessed by a modified XTT proliferation assay (XTT Proliferation Assay Kit, ATCC, USA). In this assay, 96-well plate was used to seed and sub-culture the cells for several passages (P1, P2 and P3) with and without eye toners. The test was conducted after the cultured keratocytes achieved 80% to 90% confluent.

2.5 Scratch Assay

An in vitro wound healing model was established by using monolayer cultured cells. When the monolayer cultured keratocytes reached confluence, cells in the middle of culture plate was scraped out using 5 mm sterile cell scraper, producing emptied 5 mm straight line. The cultures were then supplemented with the respective amount of ET01, ET02 and ET03. Cultured cells without eye toners served as control. They were observed every 4 hours until both edges of the straight line were merged completely.

2.6 Immunocytochemistry Analysis

Lumican and Connexin-43, positive markers of corneal keratocytes were used for immunocytochemistry analysis. In
this study, two antibodies used for immunocytochemistry (ICC) were monoclonal antibody to lumican (Mdbioproducts, Mdbiosciences, North America) and anti-connexin 43/ GJA1 antibody (CXN-6) (Abcam, Abcam plc, UK). After incubation and reaching about 50% to 60% confluency, the cells were fixed with 4% paraformaldehyde for 24 hours at 4°C. Then ICC analysis was performed in accordance with the manufacturer protocol. The respective antibodies were applied to the cells for 60 minutes. After washing with PBS, UltraTek Anti-polyvalent biotinylated antibody (secondary antibody) was applied to the slides and incubated for 20 minutes. Slides were then incubated with streptavidin-peroxidase for 20 minutes. Freshly prepared chromogen substrate AEC was applied to visualize brownish precipitate in the slides. Slides were counterstained with Mayer’s Haematoxylin and mounted using permanent aqueous medium.

3.0 Result and Discussion

3.1 Morphological observation

In this study, cultured cells demonstrated the typical morphology of corneal keratocytes (flat, dendritic-like with long interconnecting cytoplasmic processes extended to adjacent cells). However, the cells became more flattened and larger with a reduction in cytoplasmic processes and many cells were polarized with filopodial extensions as passage number increased. These results were consistent with the data from earlier studies that keratocytes subjected to serial passages in serum-containing media would lose the typical morphology of keratocytes [25]. Though the control cells appeared more stellate-shape and have longer cytoplasmic processes compared with eye toner groups at passage 1 but the eye toner groups were better in retaining the expression until passage 3 which elucidated the efficiency of eye toner in maintaining the intercellular communication in keratocytes culture. However, the length of interconnecting processes of keratocytes in both groups at passage 2 looked rather equivalent. During the characterization of corneal cells, Norzana et al. (2015) reported that most of keratocytes became elongated with scanty cytoplasm and exhibited spindle-shaped morphology throughout culturing process. While at the later stage of the culture, the keratocytes showed fibroblastic appearance with mitotic figures [6,26].

3.2 Growth Kinetic Study

The profile includes cell viability, total cell yield, growth rate, population doubling time and total number of cell doubling. Cell viability is used to determine the number of viable cells present in a cell suspension based on the principle that live cells possess intact cell membranes that exclude certain dyes, e.g. trypan blue, whereas dead cells do not [27]. The viable (unstained) and non-viable (stained) cells can be visualized under light microscope and counted separately using the haemacytometer. In this study, cultured cell viability range from 72.6% to 91.6%. Corneal keratocytes cultured with or without eye toners showed no significant difference at all passages. However, ET01 showed a significant higher cell viability than ET02 (p=0.007) at passage 2 but no significant different compared to control.

Total cell yield or count is equivalent to the number of cells (x 10^5) per ml after cells harvesting (trypsinisation). In this study, number of cultured cells in all groups increased after every passages. No significant difference was observed in all groups. Sinha and Kumar (2008) indicated that population of cells cultured in vitro increase in number as the individual cell divides mitotically [28]. Multiplication starts only after period of adjustment and stops when a number is reached that is saturating for the system.

Growth rate is the increase of cell mass per unit time (day). Each time a cells is subcultured, it will grow back to the cell density that existed before sub-culture [29]. Figure 1(a) showed no significant difference at all passages except between control and ET02 group at passage 3 (p<0.05).

Number of cell doubling is to determine the total number of times the cells in the population have doubled post-isolation and sub-culturing. According to Freshney (2006), most normal cell will undergo a limited number of subcultures or passages [29]. This is referred to as finite cell lines. The limit is determined by the number of doublings that the cell population can go through before it stops growing because of senescence that reduces the cell’s ability to differentiate. In this study, number of cell doubling showed no significant difference at passage 1 and 2 for both groups. However, at passage 3, the number was lower in the eye toner groups compared to control, with significant difference between control and ET02 (p<0.05 and ET03 (p<0.05). Among eye toner groups, ET01 showed significantly higher number of cell doubling compared with ET03 group (p<0.05) at passage 2. This suggested that ET01 somehow help the cell population to speed up their number at this passage. The results were summarised in Figure 1(d).

Eslaminejad et al. (2008) defined population doubling time as the time by which the given cell population double their numbers by undergoing proliferation [30] which is similar to this present study. According to the result shown in Figure 1(e), both control and eye toner groups showed no significant difference at passage 1 and 2. However, at passage 3, keratocytes cultured with ET02 took a significant longer population doubling time than control (p<0.05). Meanwhile, at P2 eye toner groups noted a significant difference between ET01 and ET03 (p<0.05). ET03 group required significantly longer population doubling time than ET01. This may serve as an indicator that ET01 helped the cells to double their
3.3 Cells Proliferation Assay

The assay was used to evaluate potential toxicity effect of eye toner on corneal keratocytes. It is believed that in one way or another this cell proliferation assay shall support the growth kinetics profile. As shown in Figure 2, no significant difference was noted between keratocytes cultured with or without eye toners at passage 1 and 3. However, at passage 2, the eye toner groups showed a significant lower cells number than control, with p<0.05. This can be an indication that the eye toners have some inhibitory effects on corneal keratocytes proliferation.

population within short period of time better than ET02 and ET03.
Fig. 2: Comparison on cell viability between keratocytes cultured with and without eye toners showed no significant differences at passage 1 and 3. However, significant differences were noted at passage 2 (marked with *), with p=0.009, p=0.005 and p=0.05 for ET01, ET02 and ET03 respectively.

Although the actual ingredients were not specified in details, the indicated substances among those are the *zam-zam* water, the selected herbs, pearl extract and sea-based materials. The integration of prophetic medicine and the use of natural products for alternative treatment are becoming more common as people are becoming more conscious about health. In many aspects of treatment or medication, the trend is now deviating from synthetics drugs to going back to nature. On the same account, previous studies on the other prophetic medicine and natural products showed that honey [31] and edible bird’s nest [32], in its optimum concentration, enhanced the proliferative capacity of corneal keratocytes.

Fig. 3: (a) Photomicrographs of wound healing model in passage 2 under 4x original magnification. A, F, K and P represented cultured rabbit corneal keratocytes plate viewed immediately (0 hours) after wound induction using 5 mm cell scraper. At 24 hours, the cells cultured in ET01, ET02 and ET03 began to appear within the magnification field indicating faster proliferation rate compared to control (B). The average hour for wound to close completely was 55 hours for control and within 48-54 hours for cell cultured in eye toners. (b) Wound closure time showed no significant differences between keratocytes cultured with and without eye toners at all passages. (c) Wound closure rate showed no significant differences between keratocytes cultured with and without eye toners at all passages.
3.4 Scratch Assay

A straight-line scratch was performed using a sterile cells scraper. The technique may be considered as a simple wound healing study. The results were analysed according to wound closure time and rate. Based on sound logic, the results of this assay shall mirror the growth kinetics profile or the proliferative capacity of corneal keratocytes. The wound closure time refers to how long did the wound take to completely close or heal after induction using 5 mm cell scraper at 0 hour whereas the wound closure rate refers to the wound closure size (5 mm) taken to completely closed for a certain time (day) for every passage. The photomicrographs of wound healing model was shown in Figure 3(a). The measurement of the size of wound closure at every four-hour interval was taken immediately after wound induction using the photomicrograph ruler provided by Dino-eye software. From the figures, generally at 24 hours, the cells cultured in ET01, ET02 and ET03 began to appear within the magnification field indicating faster proliferation rate compared with control. In addition, the average hour for wound to close completely was 55 hours for control and within 48 to 54 hours for cell cultured in eye toners. However, no significant differences were observed in all groups at all passages.

3.5 Immunocytochemistry Analysis

Fig. 4: (a) Photomicrograph of immunocytochemistry staining of rabbit corneal keratocytes cultured with and without eye toners at different passage (P1, P2 and P3). Connexin 43 antibody dilution is 1:500. (b) Photomicrograph of immunocytochemistry staining of rabbit corneal keratocytes cultured with and without eye toners at different passage (P1, P2 and P3). Lumican antibody dilution is 1:50.
Immunohistochemistry or immunocytochemistry is a microscopic method used to identify a tissue/cell constituent such as antigens in situ by the use of a specific antibody through antigen-antibody interactions which can be visualized and examined under a microscope [33]. The interactions can be visualized by a marker such as fluorescent dye, colloidal gold or more commonly, an enzyme for light microscopy that makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context [34]. It provides the most direct method for identifying both the cellular and subcellular distribution of protein. The distribution of protein is being confirmed with the use of antibodies in situ. Specific antibodies allow examining protein modifications and expression [35].

Figure 4(a) demonstrated the outcomes of immunocytochemistry staining on cultured rabbit corneal keratocytes against Connexin-43. The brownish precipitation of control groups looked gradually decreased from passage 1 until passage 3. However, all eye toner groups were able to sustain the expression of Connexin-43 throughout passages. Among eye toner groups, ET01 group showed a more pronounced Connexin-43 expression throughout all passages. Similar pattern can be observed in the immunocytochemistry staining against Lumican (Figure 4(b)). ET01 indicated the strongest Lumican expression in the culture at all passages.

Lumican plays an important role in modulating the matrix for corneal transparency. Lack of Lumican can lead to deregulation of collagen fibril assembly and corneal opacification [35]. Other notable feature of corneal stroma is the unique gap-junction mediated intercellular network presents between keratocytes. In normal corneal stroma, the flattened, quiescent, and dendritic (stellate)-like cells linked to each other by their multiple extended processes formed by a pair of Connexin-43. The role of gap junctions is to facilitate the diffusion of ions and molecules smaller than 1 kDa and thus contributing to the regulation of cornea differentiation and homeostasis [36]. Hence, the reduction of the intercellular processes presented by keratocytes may decrease the number of gap junctions and expression of Connexin-43 subsequently. Overall results suggested, the eye toners may sustain the expression of Connexin-43 and Lumican that perhaps promote the gap-junction mediated intercellular communication and regulate collagen fibril organization that is conducive to corneal transparency.

4.0 Conclusion

This present study indicates that the commercially available eye toners do not have significant effect on corneal keratocytes proliferation and in vitro wound healing. The cell assay proliferation also indicates that all the commercially available eye toners have inhibitory effect on cell growth as compare to control. The eye toners express Connexin-43 and Lumican however the above finding needs further evaluation. The use of eye toners sparks controversies due to scientific inadequacy, it is hoped that this finding will benefit the local society through educating and promoting awareness of evidence-based healthcare practices. In fact, based on Islamic teaching, the importance of proof is stressed upon in the Quran as stated in Surah Al-Baqarah, Chapter 1, Verse 111: “Produce your proof if you are truthful”.

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