SOLUVILJEMIIN PERUSTUVAN SARVEISKALVOHAAVAMALLIN KEHITYS

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Tutkimuksen tavoitteena oli tutkia ihmisen sarveiskalvon soluista kehitetyn solumallin soveltuvuutta haavan paranemisen tutkimiseen sekä vertailla eri haavantekomenetelmien tehokkuutta. Tutkimuksessa käytettiin kuolematonta ihmisen sarveiskalvon epiteelistä eristettyä HCE-solulinjaa. HCE-solut kasvatettiin filtterien päällä, jotka oli päällystetty kollageeni I:lla. Solujen annettiin kasvaa tasaiseksi kerrokseksi minkä jälkeen solut siirrettiin ns. ilmakasvatukseen, jossa soluja kasvatettiin noin 3 viikkoa. Ilma-neste-rajapinta auttaa soluja kerrostumaan sarveiskalvon epiteelin kaltaisiksi kerroksiksi. Ilmakasvatukseen siirryttäessä solukerrokset jaettiin kolmeen ryhmään, joista jokaisella oli hieman erilainen kasvatusliuos. Tällä pyrittiin saamaan tietoa kyseisten aineiden vaikutuksesta solujen kasvuun ja kerrostumiseen.

Ilmakasvatukseen siirrettyjen solujen kerrostuneisuutta arvioitiin viikon välein valmistamalla parafiinileikkeitä ja tutkimalla niitä valomikroskoopilla. Kerrostuneiden solujen toiminnallisuutta tutkittiin solujen sähköneristyskykyä kuvaavan TER-mittauksen avulla. Haava tehtiin kerrostuneeseen soluviljelmään käyttämällä keltaista pipetin kärkeä tai pipetin suodatinta. Pipetin suodatin oli kastettu joko tislattuun veteen, alkoholiin tai natriumhydroksisidiliuokseen. Haavan teon jälkeen paranemista seurattiin valomikroskoopilla.

Tutkimuksessa huomattiin, että käyttämällämme menetelmällä solut kyllä kerrostuivat normaalisti, mutta TER-arvot jäivät selvästi odotuksia matalammiksi. Tämä tarkoittaa, että solukerroksen pinta ei ollut täysin tiivis ja erilaistuminen ei täysin onnistunut. Tutkimuksessa ei huomattu eroja eri kasvatusliuosten välillä. Solukerroksiin tehdyt haavat sulkeutuivat täysin viiden päivän kuluessa ja parhaaksi menetelmäksi osoittautui pyöreä haavamuoto, joka tehtiin etanoliin tai natriumhydroksidi liuokseen kastetulla pipetin suodattimella.

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1. INTRODUCTION

1.1 Structure of the cornea

The cornea is the front part of the eye that covers the iris (Figure 1A). It is a clear and avascular tissue in order to let light through and it also protects the anterior parts of the eye from injuries and inflammations. The surface is covered with a thin tear film on the other side and aqueous humor on the other. Cornea has the refractive power of up to 43 diopters and this is the highest power in eye, but the value is fixed. (Watsky et al 1995).

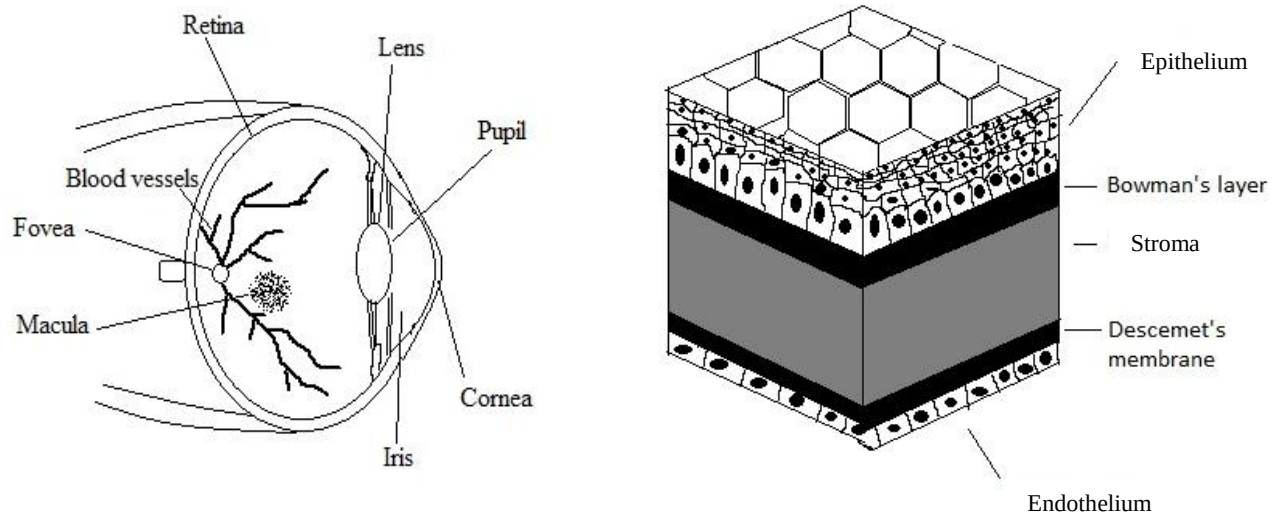

Figure 1. Structure of the human eye (A) and cornea (B).
(Revised from Hornof et al., 2005 and Sasaki et al., 1999)

The cornea is lined with a stratified epithelium which serves as a protective barrier for the cornea Figure 1b). It prevents pathogens from entering deeper and also limits the fluid flow into the transparent and dehydrated stroma. This epithelium usually has 5-7 cell layers on humans and consists of two layers of flattened superficial cells, multilayered polygonal-shaped wing cells and one layer of columnar basal cells. (Watsky et al 1995)

The corneal epithelium is catagorized to so called tight epithelium having a relatively high transepithelial electrical resistance (TER) of 1000 $\Omega x cm^2$ achieved through tight junctions of superficial cells (Rojanasakul et al. 1992). Wing cells are attached to other cells with desmosomes and most of the apical layer contains microvilli (Watsky et al 1995). In humans the cornea is 0,5–0,6 mm thick in the center and 0,6–0,8 mm at the periphery and has a diameted of 11,5mm at the periphery. Under the corneal epithelium lies a thin layer of extracellular matrix (ECM) which plays a role in epithelial adhesion to the underlaying stroma. This acellular Bowmans's layer consists

mainly of various types of collagen and this layer does not regenerate. Stroma consist mainly of hydrated type I collagen and differentiated keratocytes and represents 90% of corneal thickness (Watsky et al 1995). Basal lamina of the endothelium is called Descemet's membrane and corneal endothelium consist of a single layer of hexagonal cells covering the posterior surface. (Sasaki et al. 1999)

1.2 Renewal of the corneal epithelium

The turnover time of the corneal epithelium is estimated to be 7 days. Stem cells exist in the limbus which is located on the border of cornea and sclera and new basal cells are derived from these cells. As cells move towards the surface they undergo mitosis and differentiate into wing cells and finally into superficial cells (Boulton, Albon 2004).

Corneal epithelium can be injured by different mechanisms and disorders such as aging, tear deficiency, microorganism infiltration, chemicals, or mechanical damage. When the cornea is damaged the denuded area is rapidly covered by residual epithelial cells. Fast healing process is needed in order to re-establish barrier function to block pathogens from entering deeper into the eye. (Lyu, Joo 2005)

1.3 Cell culture models of cornea

For study purposes, different kinds of culture models have been developed. Cell culture models based on both primary and immortalized cells have been developed as potentially reliable models of the native human cornea. The main problem with primary cell lines is their owing to senescence after several passages. The use of corneal limbal cells has mainly focused on surgical studies and in addition the primary cells have been used in growing the epithelium on filters for permeability studies (Germain et al. 2000).

Models of entire cornea are constructed on cell culture inserts. This method is very time consuming since successive growth of corneal epithelial, stromal and endothelial cells are needed. These organotypic cornea constructs have been used in wound healing and surgery studies (Gipson et al. 2003).

There have also been other approaches to this subject. It has been shown earlier that ocular surface reconstruction with cultivated oral mucosal epithelial transplantation technique could be an option for severe ocular surface injuries and limbal cell deficiency. In this study the group was able to

produce stratified cell sheets without serum, conditioning of the medium or increased EGF concentration. It was also shown that these tissue-engineered cell sheets were transparent enough to allow recognition of text through the cell layer. (Ilmarinen et al. 2013)

Simian virus 40 (SV40) immortalized human corneal epithelial (iHCE) cell lines were independently developed by Kahn et al. (1993) and Araki-Sasaki et al. (1995) and these lines have been later proven useful in toxicology and absorption testing (Toropainen et al. 2001).This cell line has been proven to grow up to 400 generations through 100 passages and cells also survive being frozen. (Araki-Sasaki et al. 1995)

It has been proven that culturing the corneal epithelial cells on permeable filters and at an air-interface is a critical factor in the formation of tight cell layer and for normal differentiation. According to other studies, polyester filters coated with collagen or collagen-fibroblasts or collagen-laminin have been proven to be the most suitable for culturing HCE cell layers. (Toropainen et al. 2001).

Fetal bovine serum and steady concentration of epidermal growth factor is equally important. Cells that were exposed to this air-liquid -interface exhibited a significantly higher peak transepithelial electrical resistance. Desquamation of stratified epithelia has also been used as a marker of terminal differentiation. Other such markers include tight junction with its characteristic proteins and flattened topical cells. (Chang, Basu & Lee 2000; Robertson et al. 2005)

On the other hand it has also been shown that high serum consentrations disturb cell proliferation and differentiation (Kruse, Tseng 1992). Fortunately it has also been shown that it is possible to grow corneal cell lines in serum-free medium (Gipson et al. 2003; Mohan et al. 2012; Robertson et al. 2005). It has also been shown that EGF increment is not required for successful culture of cell sheets (Ilmarinen et al. 2013).

The most common method to study corneal wound healing is to harvest rabbit corneas for studies but it is very time consuming and can be considered unethical. It is estimated that the number of rabbits needed for a pharmacokinetic study is usually more than 20. There can also be differences between human and rabbit corneal cells (Makoid, Robinson 1979). Also immortalized HCE cells have been used for wound healing studies. However, if the study is performed without collagen coated filters and air-lifting HCE cells have remained undifferentiated (Obata, Tsuru 2007; Sharma, He & Bazan 2003).

The aim of this study was to develop an easy and repeatable method to mimic the corneal wound healing process. We selected immortalized HCE lines developed by Araki Sasaki et al 1995 for our studies. First goal of the study was to culture terminally differentiated HCE cells. The effects of air-liquid –interface on stratication process of the cells was examined by TER-values of the cell cheet and observing the cell morphology under lightmicroscope. Previosly it has been shown that EGF and FBS may intefere the normal HCE differentiation and wound healing –process. Therefore, we wanted to test the effect of different culture conditions for the differentiation process. Finally, we wanted to an optimal wound making technique for our wound healing study purposes.

2. MATERIALS AND METHOD

2.1 Cell Culture

The immortalization of the human corneal epithelial cells (HCE) has been described earlier(8 Araki-Sasaki,K. 1995). The cells were provided by Dr. K. Araki-Sasaki (Dept. of Opthalmology, Osaka University School of Medicine, Osaka, Japan). The HCE cells were cultured in DMEM/F12 1:1 medium (Gibco) supplemented with 15% heat-inactivated fetal bovine serum (FBS; Gibcp), 1% L-glutamine (Gibco), 5µg/ml insulin (Sigma), 10ng/ml EGF (Sigma) and 1% antibiotic/antimycotic solution (Gibco). Medium without EGF and medium without both EGF and FBS were also used. The cultures were maintained in 95% air and 5% CO_2 at 37°C, and subcultured twice a week. The cultures with the medium without both EGF and FBS were subcultured once a week.

HCE cells, passages 59 to 78, were seeded onto the rat tail collagen type 1 coated polyester cell culture inserts (filter surface area, 0,9 cm^2; pore size, 0,45µm; Biocoat, BD) using 200 000 cells per insert. The cells were then grown at 37°C in humidified air with 5% CO2 with medium both in apical and basolateral chambers for 7 days. The cells were then exposed to an air-liquid interface for at least 3 weeks. The culture medium was replaced three times a week. After the culture (P60-68) was confluent 400µl medium was administered in the apical chamber and medium from the basolateral chamber was removed. The cells were grown in three different kinds of medium: one containing EGF and FBS (+/+), one only FBS (-/+) and one without both (-/-) for 0-30 days before fixation and paraffin sectioning. Finally paraffin slices were evaluated for their consistency under the microscope. Using only 300µl of medium with EGF and FBS was also tested. This time cells were grown 25-38 days before fixation, paraffin slicing.

2.2 Light Microscopy

Properties of the stratified HCE cultures were evaluated on basis of paraffin sections which were made after every week of air-liquid interface culture until the cells had been cultured for 5 weeks. These samples were also used to determine the effects of EGF and FBS on stratification process. Stratified corneal epithelial cells were fixed in 4% paraformaldehyde, dehydrated in a graded ethanol series (80%, 94%, 99%) and embedded in paraffin. Paraffin sections were cut, stained with eosin and hematoxylin solution and photographed with Nikon Eclipse TS100 microscope equipped with. Nikon DS-5M camera with DS-L1c control unit.

2.3 Transepithelial electrical resistance

We used an Millicell electrical resistance system (Millipore, Bedford) to measure TER-values at different phases of stratification process and after using different culture mediums. Cells were washed with PBS from both sides, and after equilibration time, TER measurement was taken. TER-values were measured with 0,5ml culture medium without EGF and FBS in apical and 1,5ml in basolateral chamber. TER-values for filters without cells were also measured. All TER values were calculated by subtracting the resistance measured from the blank insert without any cells from the resistance measured from the inserts with HCE cells, taken in count the effective surface area of 0,9cm^2. After measuring TER-value for one cell sheet, the system was cleaned with 70% alcohol solution.

2.4 Wound

The wound was made in a stratified HCE cell culture after exposure to air-liquid interface. Methods we tested were scrape wounds with Pasteur pipette tip, yellow plastic pipette tip or with a surgeon's knife, or round shaped wounds made with Pasteur pipette and suction or with pipette filter (Safe-Cone filter, standard ø 1.83 mm**)** bathed in purified water, 70% ethanol or 1M NaOH solutions.

3. RESULTS

3.1 HCE cells differentiate in air-liquid interface

First we examined the effects of air-liquid –interface on stratification process. Culture medium contained both EGF and FBS. After the culture was confluent 400µl of medium was administered in the apical chamber and medium from the basolateral chamber was removed. The cells were

grown 0-30 days in air-liquid interface before fixation and paraffin slicing. 8 days air-liquid time was enough to produce a 6 cells thick layer. However, the surface structure was still irregular (Figure 2). At day 21 stratified epithelium contained 12 cells thick layer with a regular surface structure and flattened superficial cells. At day 28 the structure was looking the same with no significant change. By the day 30 the epithelium had grown to 15 cells thick and the surface structure was regular and the most superficial cells were flattened. It is clearly seen that longer air-lifting time produce more cell layers. (Figure 2).

Figure 2. Morphology of representative samples of the immortalised human corneal epithelium cell sheets visualized with haematoxylin and eosin (HE) staining. The cells were cultured on porous collagen IV–coated culture inserts and differentiated under airlifting with all the additional supplements described earlier. Days(d) that each cell sheet spent under air-lifting are represented above corresponding pictures.

3.2 EGF and FBS are not necessary for the stratification process

During the air-lifting the HCE cells were grown using either 300µl or 400µl of medium in the apical chamber of the cell culture insert. Cells grown in 400µl had more cell layers and the surface was more flat than with the cells grown with 300µl (Figure 3). We decided to use 400µl in our further studies. Then we examined the possibility to grow cells without EGF and FBS. Cells were grown in three different kinds of medium: one containing EGF and FBS (+/+), one only FBS (-/+) and one without

both (-/-). We found it was possible to grow stratified cells in a medium without EGF and FBS, and even the surface appeared flat and tight in these samples. The fixation process caused damage to the stratified epithelium. (Figure 3.)

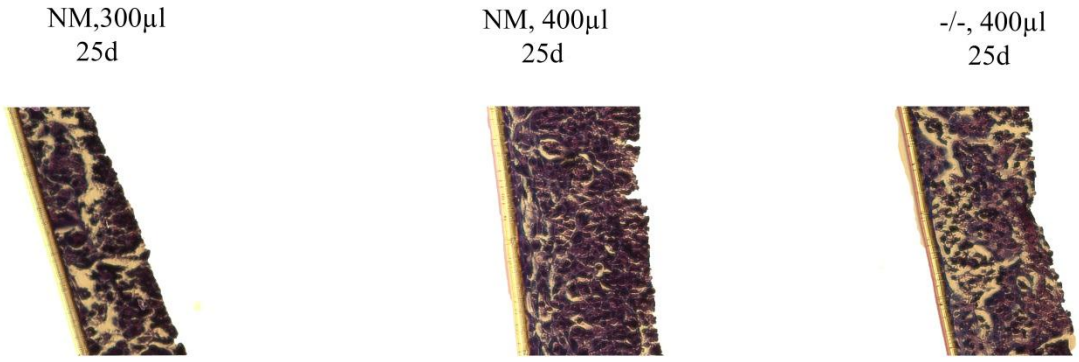

Figure 3. Morphology of representative samples of the immortalised human corneal epithelium cell sheets visualized with haematoxylin and eosin (HE) staining. The cells were cultured on porous collagen IV–coated culture inserts and differentiated under airlifting. All three were cultured with different mediums. First one from left with 300µl of normal medium(NM) in the filter chamber, second with 400µl of normal medium and third 400µl of medium missing both EGF and FBS. Days(d) that each cell sheet spent under air-lifting are also represented above corresponding pictures.

3.3 Stratified epithelium does not show functional properties

The integrity of the stratified epithelium grown under different culture conditions was analyzed using transepithelial electrical resistance (TER) measurements. TER-values of the cultivated cell sheets were followed for 37 days. Results showed a significant drop after the first week in all three culture conditions. After the initial drop, the values got significantly higher towards the end-point in the medium without EGF and FBS supplements. With other mediums the values remained low throughout the study. (Table 1.)

Paraffin slices were made after the testing was complete. Morphology analysis of the cell sheets showed approximately 10 cell layers thick sheet but the structure was not confluent. (Data not shown.)

Table 1. The mean TER-values (n=3) with different mediums and different air-lifting times. -/- represents a medium without EGF and FBS, -/+ a medium without EGF but with FBS and +/+ means a medium with both EGF and FBS. The value of a similarly treated blank insert without the cells has been subtracted from the measurements with the cells.

Days	-/- Ωxcm^2	-/+ Ωxcm^2	+/+ Ωxcm^2
0	305	290	109
3	325	375	255
10	62	23	18
17	90	29	17
23	117	23	18
30	180	15	37
37	188	23	25

We speculated that the measuring process could disturb the differentiation process so we also wanted to perform measurements with end-point method as described earlier. The highest TER-values were again achieved with a medium without EGF and FBS supplements (Table 2). After the measurements, the morphology of these cell sheets were analyzed and light microscopy revealed confluent and tight cell sheets after 16 days. The cell layer was 6 cells thick in medium without supplements and 13 cells thick in medium with both EGF and FBS (Data not shown).

Table 2. The mean TER-values when the values were taken as an end-point –measurement. -/- represents a medium without EGF and FBS, -/+ a medium without EGF but with FBS and +/+ means a medium with both EGF and FBS. The value of a similarly treated blank insert without the cells has been subtracted from the measurements with the cells.

Days	-/- Ωxcm^2 (n)	-/+ Ωxcm^2 (n)	+/+ Ωxcm^2 (n)
0	119 (2)	15 (2)	40 (2)
2	278 (1)	271 (1)	56 (1)
9	23 (2)	32 (2)	33 (2)
16	32 (2)	26 (2)	48 (2)
23	104 (1)	70 (1)	15 (1)
30	144 (1)	70 (1)	18 (1)

3.4 The small pipet filters created optimal round wounds to the epithelium

Different wound making techniques were tested in order to find an easy and repeatable method for future wound healing study purposes. In our testing, scraping the cell sheets with pipet tip damaged theunderlying insert filter. The wound was also irregular, as is seen in the figure 4A. We managed to do almost perfectly round wounds using suction pipet filters soaked in different solvents (Figures 4C-E). It was also possible to make wounds without using NaOH or ethanol solutions (Figure 4B). Results varied from almost perfectly round to wounds with debris in the middle. In every scenario there where at least some cells in the middle.

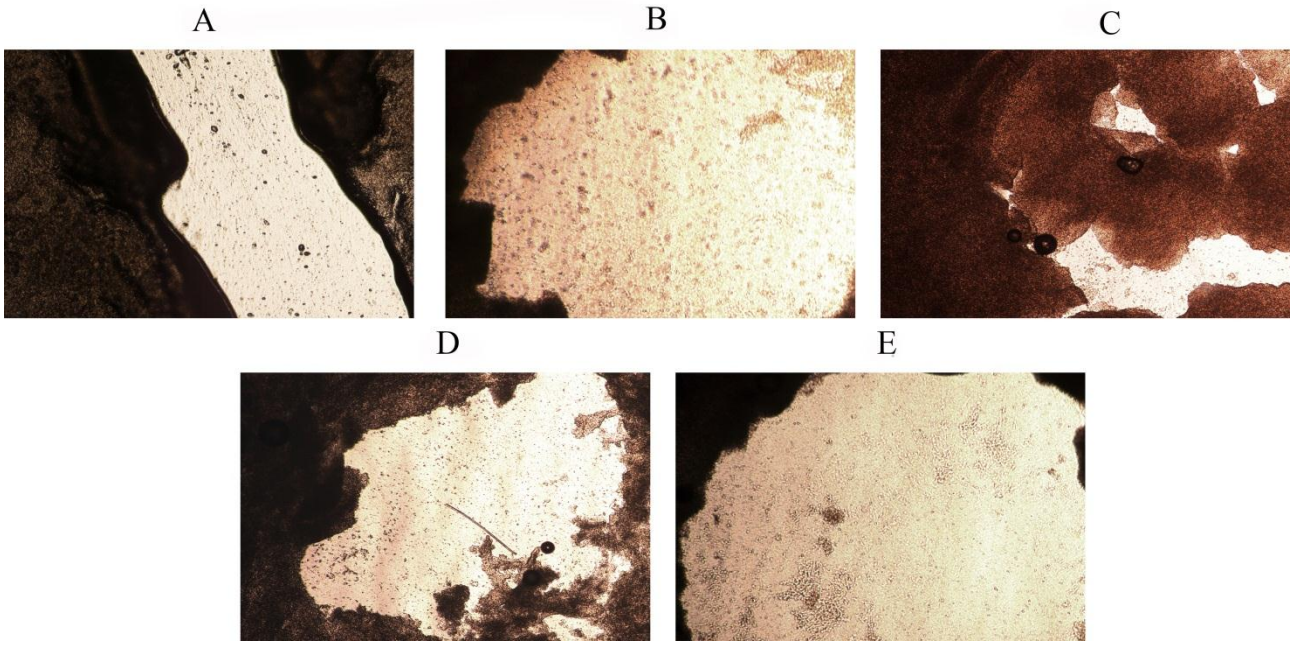

Figure 4. Cells sheets and different wound making techniques. The wound was made with A). pipet tip and a scraping motion, B). a suction pipet filter dipped into water and pressed on to the cell sheet, C). twisting the filter, D). a pipet filter that has been soaked into 70% ethanol solution and then pressed on to the cell sheet briefly once, E. pipet filter soaked into 1mol/l NaOH solution and then pressed on the cell sheet for one minute.

3.5 The speed of the wound healing process is not dependent on the wound making technique

The pipet filter –technique were the filter was soaked into water, ethanol or NaOH solutions was chosen for the further studies. Results showed that the wound process was not dependent on the solvent used in wound making. Wounds were closed even when made using NaOH or ethanol (Figure 5). Cell proliferation was evident on the second day after the wounding and on the fifth day there was at least one cell layer thick cell sheet covering the wound in every scenario. This was clearly seen when using a light microscope. No difference in healing process between different wound making methods was noticed. The speed of the healing seemed to be the same with these three different agents. (Figure 5.)

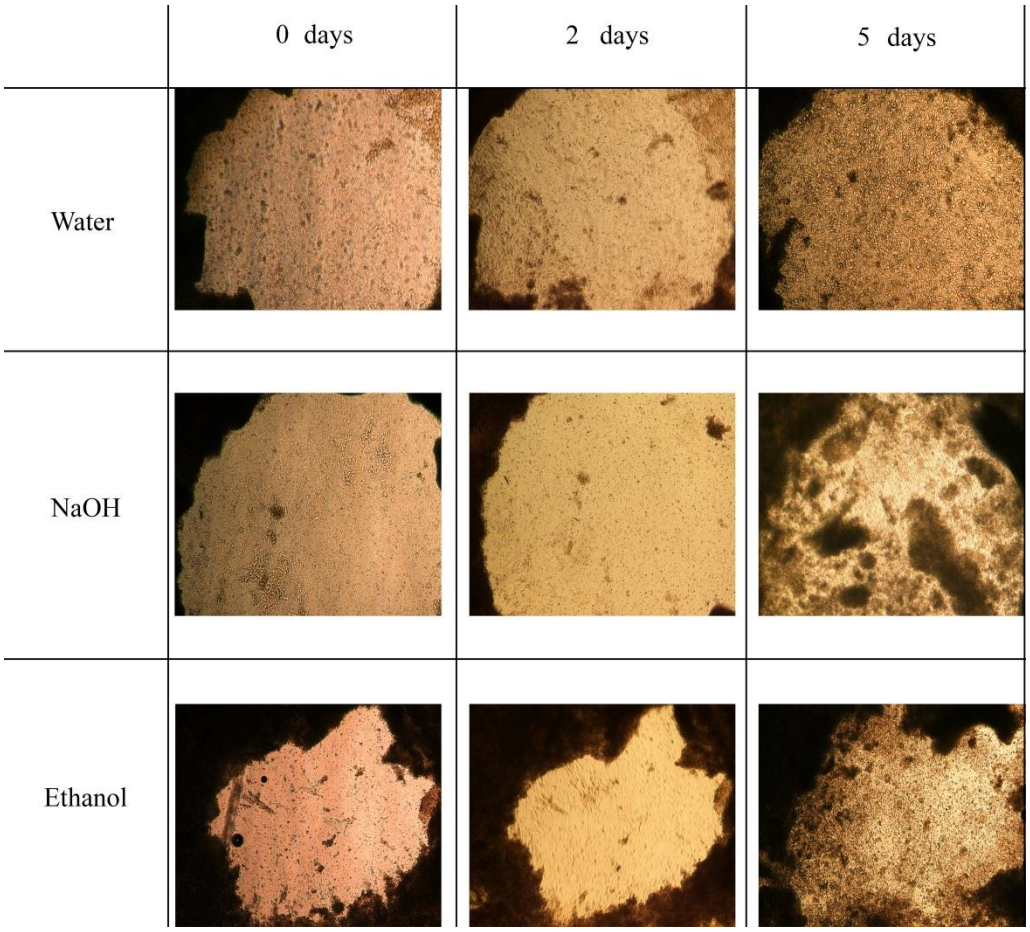

Figure 5. Wounds made with suction pipet filters that were soaked in different agents and then pressed against the cell sheets. Healing process was filmed with Nikon Eclipse TS100 microscope equipped with Nikon DS-5M camera using 40x magnification. Corresponding agent is featured in the left column and days after the wound was made on the top row. We used distilled water, 1M NaOH solution and 70% ethanol.

4. DISCUSSION

To our knowledge, all the previously reported studies of corneal wound healing have used either undifferentiated HCE cells or corneas harvested from animals. As mentioned earlier, corneal wound healing consists of three phases: Cell proliferation in the peripheral epithelium, cell migration in the central epithelium and differentiation (Boulton, Albon 2004). When studying this without air-lifting, it is only possible to measure the proliferation rate. Our study aimed to also assess differentiation and stratification back to normal corneal epithelium. This can be considered as a better end-point for the wound healing as it is the most time consuming process and is essential for normal barrier function (Lyu, Joo 2005).

We were also able to produce stratified iHCE cell sheets with our three different conditions and found no difference between the groups in either EGF or FBS increments. We also found out that there was a great variance between the degree of stratification among the samples. A similar phenomenon has previously been reported with HCE cells and one possible reason for this can be the location of the cells in the culture plate which can affect the microenvironment (Toropainen et al. 2001).

In our study the measured TER values for the cell sheets were also lower than in a normal human cornea or in previous studies. Human corneal epithelium models typically have TER between 400 and 800 $\Omega x cm^2$ (Hornof et al. 2005). In our study the maximum TER in every scenario was achieved on the second or the third day under air-lift. Our measurements were only taken once a week so by the time the next values were taken the cells had been under air-lift for 9-10 days. This could be important since the turnover time for human corneal epithelium is estimated to be around 7 days. This could mean that the most superficial cells were in senescence and would be shed in the human cornea. It is also known that the surface of the corneal epithelium produces about 70% of the total electrical resistance (Klyce 1972). In vitro this could mean that the barrier function was already lost since constant expression of the tight junction genes is needed. On the other hand, there are reports of rising TER values for up to 25 days under lifting (Toropainen et al. 2001). In light microscopy we discovered that the HCE cells stratified well under our culture environment. There can also be other explanations why our TER values differ from others, but our other findings implicate that the problem was with the cell sheets' surface structure.

Because our TER results were lower than usually, we chose not to use this marker with the wound healing studies before the reason was fully clear to us. With light microscopy we were able to show that it is possible to make clean-cut round wounds using the pipet filter. This was only a preliminary test, but it also showed us that the wounds made using water, ethanol or NaOH healed roughly in the same manner. We speculated that it could be better to use either ethanol or NaOH since these substances were more likely to also harm the cells left intact in the middle of the wound. Wounds made with pipet tips have previosly been used in corneal wound healing studies but in these cases there have been no coated filters. We found out that scraping the coated filters with these tips also damaged the coated surface which in our in vitro study couldn't regenerate like it would in vivo. We speculated that this could prove out to be a harmful effect in our further studies and chose not to use this method.

In summary, it was possible to produce stratified epithelium from HCE cells without growt factor increments. We also found a way to make wounds on stratified epithelium for further studies. We suggest immunostaining as a method to pinpoint the reason for lower than expected TER values in further studies.

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