# Rocking Media Over Ex Vivo Corneas Improves This Model and Allows the Study of the Effect of Proinflammatory Cytokines on Wound Healing

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**Purpose.** The aim of this work was to develop an in vitro cornea model to study the effect of proinflammatory cytokines on wound healing.

**Methods.** Initial studies investigated how to maintain the ex vivo models for up to 4 weeks without loss of epithelium. To study the effect of cytokines, corneas were cultured with the interleukins IL-17A, IL-22, or a combination of IL-17A and IL-22, or lipopolysaccharide (LPS). The effect of IL-17A on wound healing was then examined.

**Results.** With static culture conditions, organ cultures deteriorated within 2 weeks. With gentle rocking of media over the corneas and carbon dioxide perfusion, the ex vivo models survived for up to 4 weeks without loss of epithelium. The cytokine that caused the most damage to the cornea was IL-17A. Under static conditions, wound healing of the central corneal epithelium occurred within 9 days, but only a single-layered epithelium formed whether the cornea was exposed to IL-17A or not. With rocking of media gently over the corneas, a multilayered epithelium was achieved 9 days after wounding. In the presence of IL-17A, however, there was no wound healing evident. Characterization of the cells showed that wherever epithelium was present, both differentiated cells and highly proliferative cells were present.

**Conclusions.** We propose that introducing rocking to extend the effective working life of this model and the introduction of IL-17A to this model to induce aspects of inflammation extend its usefulness to study the effects of agents that influence corneal regeneration under normal and inflamed conditions.

Keywords: cornea organ culture, inflammation, wound healing

The cornea, through which light enters the eye, is a transparent, avascular tissue[1] that is responsible for keeping bacteria from entering into the eye[2] while at the same time allowing oxygen to enter.[3] This is achieved through tight junctions present at the superficial layer of the cornea, creating a barrier.[4] If violated, this barrier function makes the cornea and possibly the whole eye susceptible to infection.[5]

Physical damage to the corneal epithelium is the most common cause of wounding. Wound healing of the cornea involves the removal of necrotic cells, infiltration of neutrophils, and migration of cells from the wound edge covering the wound, along with simultaneous multilayer formation.[6]

Corneal injuries are usually associated with some level of inflammation that includes neutrophils,[7] macrophages, dendritic cells, and lymphocytes.[8] Neutrophils are recruited to the injury site from the limbal blood vessels[7] and tear fluid[6] just after stromal apoptosis takes place[9] due to the release of IL-1[10] and TNF-$\alpha$[11] by the injured epithelial cells. The neutrophils phagocytose cellular debris and pathogens,[12,13] clearing up the site of injury, and also release large amounts of cytotoxic agents that assist in the phagocytosis of microbes.[14]

Most cornea organ culture models employed to study the transfer of cells to the cornea or the effects of chemicals on the corneal epithelium and wound healing[15–19] have a life span of around 3 to 4 weeks at maximum, and most of these models are static cultures.[15,16,20]

The static models, while fairly simple to set up, do not mimic the situation in vivo in which the corneas are kept intermittently moist, through the blinking action of the eyelids, but are not submerged. In the current study the corneas were placed in a simple rocking system (based on a commercially available egg incubator modified for $CO_2$ perfusion). Using this technique, multiple corneas could be cultured simultaneously, and we demonstrate that the models survive for at least 4 weeks, which is twice as long as under static conditions.[15]

We then used these conditions to develop an in vitro cornea wound model and incorporated cytokines known to induce inflammation. We identified the cytokine IL-17A, which induced clear tissue disruption to the corneal epithelium, and studied wound healing in the model in the presence of this cytokine over 9 days under both static and rocking culture conditions. We show that rocking provides a model in which

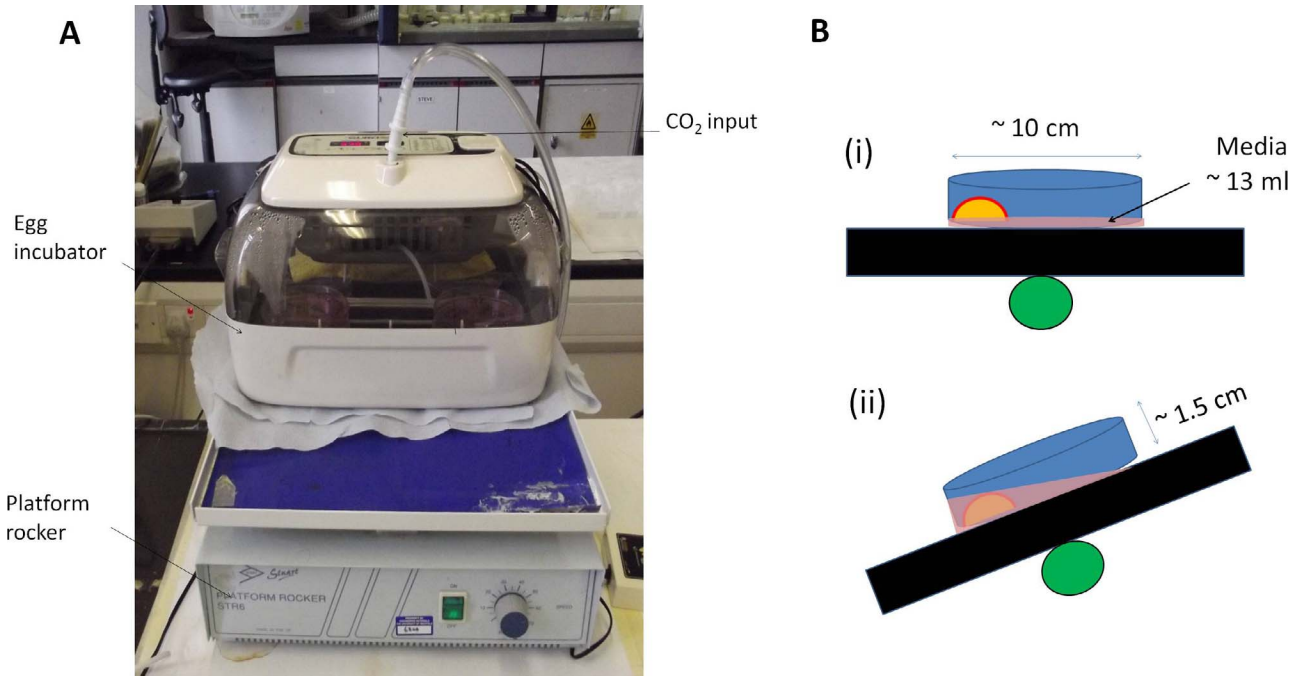


**Figure 1.** Photograph of a commercially available egg incubator on a commercially available platform rocker (**A**) and schematic of the cornea organ culture in a Petri dish at a (i) horizontal position and (ii) inclined position on a platform rocker (**B**).

wound healing occurs rapidly except when in the presence of IL-17A.

## Materials and Methods

### Cornea Organ Culture

New Zealand white rabbits were obtained from a rabbit farm (Hook Farm, Hampshire, UK). The rabbits weighed between 2.4 and 2.6 kg. Excess fat and tissue were excised from the eyes, which were then processed as described previously.[16] The corneoscleral buttons were cultured at an air–liquid interface in 1 mL medium consisting of Dulbecco's modified Eagle's medium (DMEM GlutaMAX; Gibco Life Technologies Ltd., Paisley, UK) and Ham's F12 medium (Biosera, Ringmer, UK) in a 1:1 ratio supplemented with 10% fetal calf serum (Biosera), 10 ng/mL epidermal growth factor (EGF), 5 μg/mL insulin, 2.5 μg/mL Amphotericin B, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich, Poole, UK). The corneas were cultured at 37°C in a humidified, carbon dioxide ($CO_2$) environment.

### Static Culture of Corneas

The medium was changed every other day, and the corneas were kept wet by dropping medium on the surface every day.

### Application of Cytokines to the Cornea

On day 7, the medium of the unwounded corneas was changed to contain 20 ng/mL recombinant human IL-17A (Peprotech, London, UK), 25 ng/mL IL-22 (Peprotech), a combination of IL-17A and IL-22 (20 ng/mL and 25 ng/mL, respectively), or 1 μg/mL lipopolysaccharide (LPS). All cytokines were added to media, and control organ cultures were cultured in media without cytokines. The medium was changed every other day with wetting of the corneas every day. On day 14, the samples were fixed using 3.7% buffered formaldehyde and processed for conventional histology.

### Rocking Culture of the Corneas

After preparation of the corneoscleral button, corneas were placed at the periphery of a 90-mm Petri dish and held in place by the end of a pipette tip glued onto the Petri dish using Araldite epoxy adhesive (Araldite; Huntsman Advanced Materials, Basel, Switzerland). The corneas were cultured in 13 mL culture medium and placed in our in-house rocking system consisting of an egg incubator (R-COM King Suro Egg Incubator; P&T Poultry, Powys, UK), which was subsequently placed on a platform rocker (Platform Rocker STR6; Stuart, Stone, UK) (Fig. 1) set at a speed of 10 rocks per minute and maximum tilt angle of 6°. The corneas were maintained at 37°C in the humidified chamber of the egg incubator (Fig. 1). Sixteen corneas could be handled in the incubator at one time.

### Assessment of the Effect of Including a $CO_2$ Supply in the In-House Rocking Incubator

Cornea organ cultures were cultured in the in-house rocking incubator in the absence and presence of 5% $CO_2$. $CO_2$ was added through a hole at the top of the egg incubator via tubing from a cylinder containing 5% $CO_2$ (see Fig. 1). The corneas were cultured for either 2 or 4 weeks and compared to corneas cultured under static conditions in a conventional cell incubator (in the presence of $CO_2$) with the media changed every week.

### Corneal Epithelial Wounding

After cleaning the eyes of excess tissue, the corneas were held in position and the central area of the cornea was wounded using 20% methanol within an 8-mm-diameter trephine. After 40 seconds, the area was rinsed with PBS to remove any excess methanol; and using a sclerotome knife, the epithelium was

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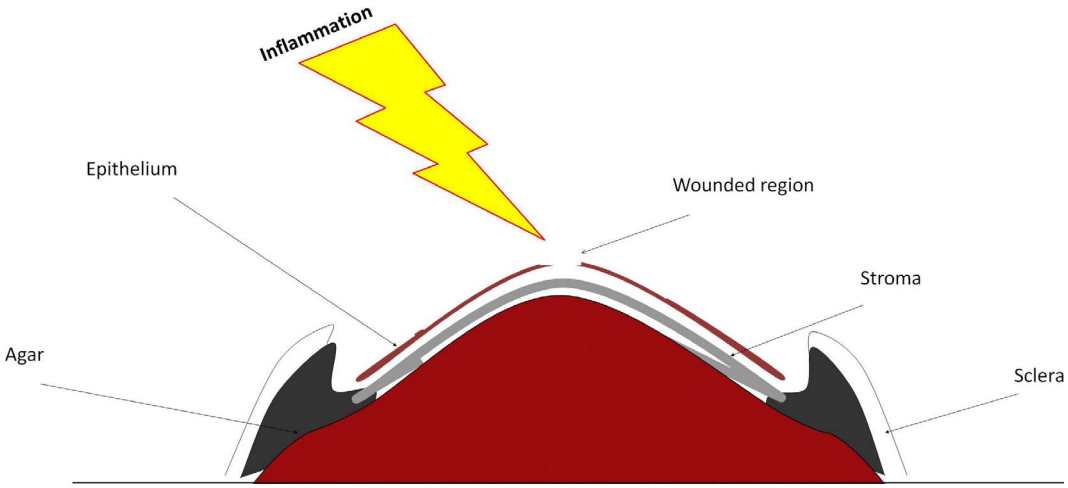


**Figure 2.** Schematic of an inflamed in vitro cornea wound model.

scraped from the treated area (Fig. 2). The corneoscleral buttons were excised and then processed to be placed in 90-mm Petri dishes as described previously in this paper. Organ cultures with unwounded epithelia were also set up as controls.

On day 2, the media of the wounded and unwounded corneas were changed to contain the cytokine that had the greatest effect on corneal morphology—IL-17A (Fig. 2). Controls were both wounded and unwounded corneas in organ culture medium without IL-17A. On day 7 the corneas in the rocker were transferred to 35-mm Petri dishes. On day 9 the corneas were fixed for 1 hour using 3.7% buffered formaldehyde and processed for conventional histology. This rocking protocol was compared to a previous protocol[16] in which corneas were cultured in a 35-mm Petri dish under static conditions in an incubator maintained at 37°C in a humidified, 5% $CO_2$ environment.

### Immunohistochemistry

Corneal sections of 4 μm were mounted onto Superfrost Plus microscopic slides (Thermo Fisher Scientific, Loughborough, UK) and stained for cytokeratin 3 (CK3) and P63 (stem cell marker) as described previously.[16] The samples were imaged using a Zeiss LSM 510 META confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) at the Kroto Research Institute Confocal imaging facility.

## Results

### Investigation of the Effect of Culturing Corneas Under Rocking Versus Static Conditions

We assessed the integrity of the corneal epithelium under static and rocking conditions. In order to mimic the blinking action of the eyelids and consequently keep the corneas wet, the corneas were placed on a rocker within an incubator maintained at 37°C with a small volume of media that flowed intermittently over the cornea as a result of the rocking action. These epithelia were compared to those of corneas cultured within static incubators at 37°C.

Figure 3 shows the effect of $CO_2$ on the corneas cultured under rocking conditions. It could be clearly seen that culturing the corneas under static conditions in the presence of $CO_2$ for 2 weeks (Fig. 3A) and 4 weeks (Fig. 3D) resulted in a epithelium one or two cell layers thick, while rocking conditions without (Fig. 3B) and with (Fig. 3C) $CO_2$ resulted in a epithelium five cell layers thick over the same period. On culturing the corneas under rocking conditions for 4 weeks, the epithelium was completely lost in the absence of $CO_2$ (Fig. 3E) but was retained in its presence (Fig. 3F). After 2 weeks it was observed that the organization of the epithelium was poor in the absence of $CO_2$ (Fig. 3B) compared to the corneas cultured in the presence of $CO_2$ (Fig. 3C); and after 4 weeks, even in the presence of $CO_2$ (Fig. 3F), the epithelium appeared to have lost the superficial desquamated cells compared to 2 weeks in culture (Fig. 3C).

### Effect of Inflammatory Cytokines on Corneal Epithelium

Previous studies[21] and unpublished studies in the group have shown that exposure to the cytokines IL-17A and IL-22 leads to inflammation of the epidermis, while a combination of IL-17A and IL-22 further enhanced inflammation of human colonic subepithelial myofibroblasts.[22] Similarly, LPS has been shown to induce inflammation of the epithelium.[23,24] Accordingly, these cytokines and LPS were selected as potential proinflammatory cytokines for this study.

The corneas were cultured for 7 days prior to the application of the cytokines. Figure 4 shows that IL-17A (Fig. 4B) or a mixture of IL-17A and IL-22 (Fig. 4D) resulted in the formation of a poor epithelium with a very loosely attached multilayer of cells. Addition of IL-17A alone led to a split in the epithelial multilayer. The basal cells remained well adhered to the corneal surface with a suprabasal split of the cells above this. The control cornea that had not been exposed to the cytokines showed a normal multilayer of cells on the corneal surface (Fig. 4A), while corneas exposed to IL-22 (Fig. 4C) and LPS (Fig. 4E) formed an epithelium that was similar to that seen in the control.

### Effect of Cytokines on Wound Healing

Corneas were initially cultured for 2 days prior to the application of IL-17A. Figure 5 shows the effect of IL-17A on wound healing of the cornea. Figure 5A shows a normal cornea under static conditions. Here there was no multilayer formation, and after 9 days in culture there was just a single layer of cells on the corneal surface. Interleukin-17A (Fig. 5B)

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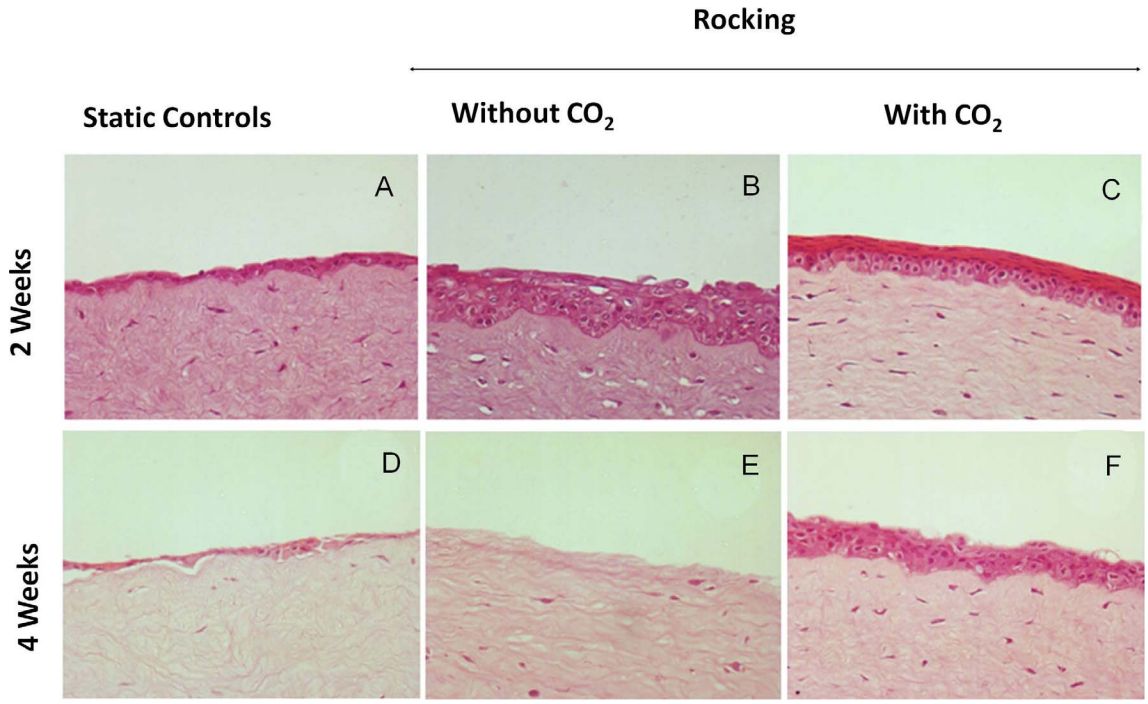


**Figure 3.** Hematoxylin and eosin of cornea organ cultures at 2 weeks under static conditions (**A**) and rocking conditions without (**B**) and with (**C**) $CO_2$ and at 4 weeks under static conditions (**D**) and rocking conditions without (**E**) and with (**F**) $CO_2$.

on the unwounded cornea again resulted in a single layer of cells on the surface, with the cells appearing abnormal with some level of perinuclear vacuolation. The wounded cornea (Fig. 5C) was approximately one layer thick, and a similar epithelium was seen when the wounded cornea was exposed to IL-17A (Fig. 5D). Although the images show the central area of the cornea where wounding was carried out, the epithelium shown in the images is representative of the whole corneal epithelium as seen by visual examination.

Under rocking conditions, however, the control unwounded cornea had a much improved epithelium compared to the cornea cultured under static conditions, with approximately seven layers in thickness (Fig. 5E). Interleukin-17A had an adverse effect on the unwounded cornea resulting in the loss of most of the epithelium, and only a very few cells remained present on the surface (Fig. 5F). Nine days after wounding the control cornea, a multilayered epithelium had formed of approximately five cell layers thick under rocking conditions

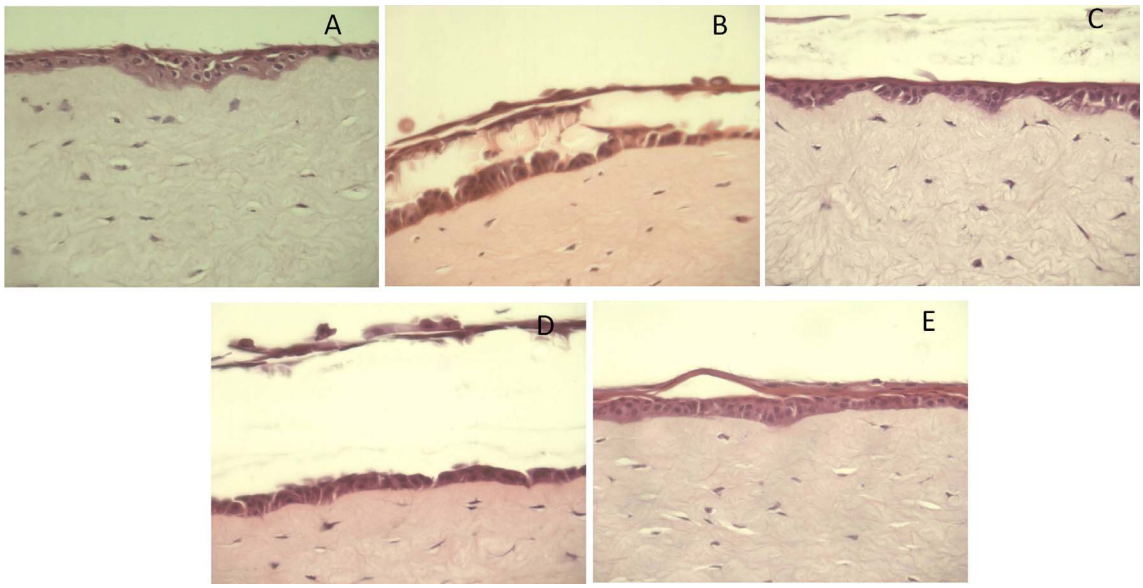


**Figure 4.** Hematoxylin and eosin of cornea organ culture exposed to cytokines. (**A**) The control cornea without cytokine exposure; (**B**) exposed to IL-17A (20 ng/mL); (**C**) exposed to IL-22 (25 ng/mL); (**D**) exposed to both IL-17A and -22 (20 ng/mL and 25 ng/mL, respectively); and (**E**) exposed to lipopolysaccharide (1 μg/mL).

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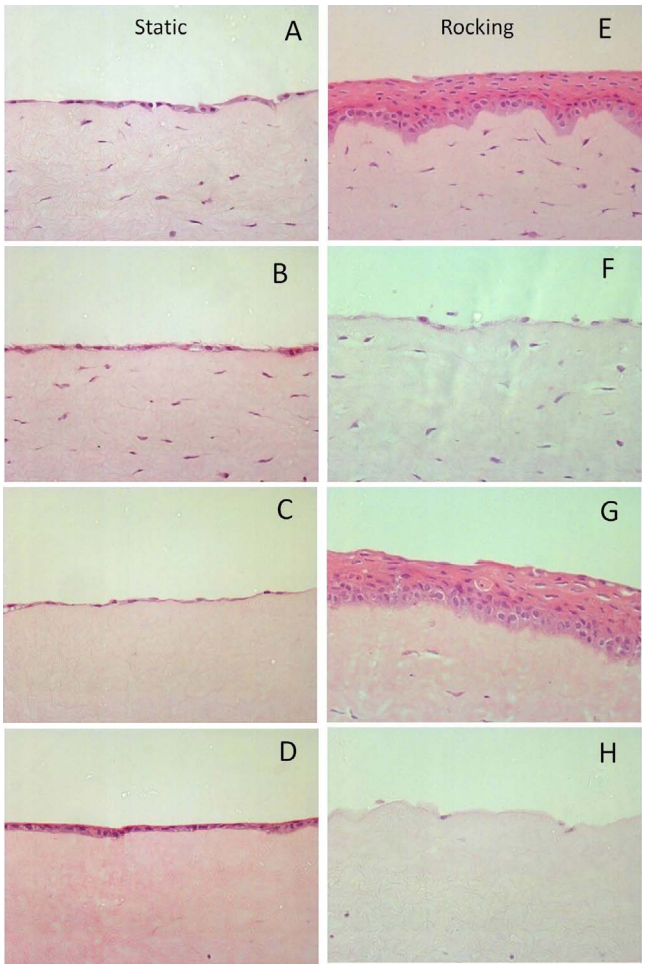


**Figure 5.** Hematoxylin and eosin of in vitro cornea organ culture after 9 days under static (**A**–**D**) and rocking (**E**–**H**) conditions. (**A**, **E**) Unwounded corneas; (**B**, **F**) unwounded corneas exposed to IL-17A; (**C**, **G**) a wounded cornea; and (**D**, **H**) a wounded cornea exposed to IL-17A.

(Fig. 5G); however, on exposing the wounded cornea to IL-17A, the cornea lost all the epithelial cells and there was no regeneration of epithelium visible even under rocking conditions (Fig. 5H). Also in the wounded corneas it was noted that the stromal cells were not present in the area where wounding had taken place (Figs. 5C–H).

Immunohistochemistry showed that the cells on the corneal surface were not affected by the addition of IL-17A. Cytokeratin 3 was expressed despite being exposed to IL-17A under both static and rocking conditions (Figs. 6A–G). P63 continued to be expressed in all the corneas whether exposed to IL-17A or not (Figs. 6I–O). However, this was more prominent at the peripheral area of the cornea than the central area. P63 could also be visualized in the basal layer of cells in the central corneal region, where a multilayer of cells formed in the unwounded (Figs. 6M, 7A–C) and wounded (Figs. 6O, 7D–F) corneas under rocking conditions, but could not be seen where only a monolayer of cells had formed (Figs. 6I–L, 6N). In the wounded corneas exposed to IL-17A under rocking conditions (Figs. 6H, 6P), no cells were present on the corneal surface, as seen using hematoxylin and eosin (H&E) staining; hence it was not possible to examine CK3 or P63 expression (Fig. 5).

## Discussion

From the current study we suggest that the epithelia of three-dimensional (3D) cornea models can be improved by simply rocking the corneas from side to side to mimic the blinking action of the eye. Blinking provides the cornea with fresh tears and nutrients to the corneal cells.[25] Richard et al.[19] in 1991 placed corneas on a standard laboratory rocking platform where the intermittent movement of fluid over the cornea was used to mimic blinking. They compared the rocking technique to corneas cultured under static submerged conditions. Results showed that culturing the corneas intermittently at an air–liquid interface reduced epithelial, stromal, and endothelial intercellular edema. However, placing the rocker in an incubator does not seem to have been taken up by others—possibly because this can make the incubator system susceptible to overheating. In 2006, a more complex perfusion model[18] was published in which media were delivered drop-wise onto the cornea to mimic the blinking action instead.

In the current study the corneas were placed in a simple rocking system (based on a commercially available egg incubator modified for $CO_2$ perfusion). Using this technique, it was readily demonstrated that the 3D models could be maintained in culture for twice as long as under static conditions.

When culturing corneal limbal stem cells for use in transplantation, some groups have cultured cells on an amniotic membrane at an air–liquid interface[26] to form a multilayer of cells prior to transplantation, while others have cultured the cells under submerged conditions[27] forming only a monolayer of cells, and both report clinical success. With the current technique it would be possible to place an amniotic membrane or a synthetic membrane on the cornea and look at the formation of an epithelium from cultured cells, or indeed from explants of tissue under rocking conditions, to see which provides best epithelial regeneration in vitro.

In order to establish the model, the effect of including a $CO_2$ supply in the rocking incubator was first studied. The presence of $CO_2$ was crucial for pH maintenance around 6.9 to 7.8, which is important for cells to grow.[28] In cells cultured in bicarbonate-buffered media, the bicarbonate leaves the medium in the form of carbon dioxide, making the medium very alkaline. The presence of 5% $CO_2$ in the incubator maintains an equilibrium in the tissue culture dish, preventing the increase in the pH to over 8.5,[29] which is toxic to the cells.[30] Our results, as expected, confirmed the necessity of a $CO_2$ supply to our in-house incubator, especially while culturing these corneas for long periods of time (4 weeks) compared to shorter periods (2 weeks).

In the immune system, T helper cell functions[31] can be divided into Th1, Th2, and Th17.[32] Th-17 is a T helper cell subset that on differentiation releases IL-17, IL-21, and IL-22 with IL-1, IL-6, and TGFβ1 playing a major role in driving the differentiation process,[33] while IL-22 is also sourced from Th2 cells, which are involved in immunity and remodeling.[34] Interleukin-17A, a member of the IL-17 cytokine family, is known to contribute to the host defense against pathogens in both mucosal and epithelial tissue; and IL-22, a member of the IL-10 cytokine family, plays a pivotal role in epithelial wound healing.[35,36]

Interleukin-17 and -22 are known to stimulate neutrophil infiltration and metalloproteinases (MMPs).[37] Studies have shown that IL-17 in particular contributes to disrupting the corneal barrier function due to the MMPs produced. This can have a large effect on the epithelial cell multilayer, causing erosion and surface irregularity.[38] Studies have also shown that IL-17RA (the receptor of IL-17A) is highly expressed in the

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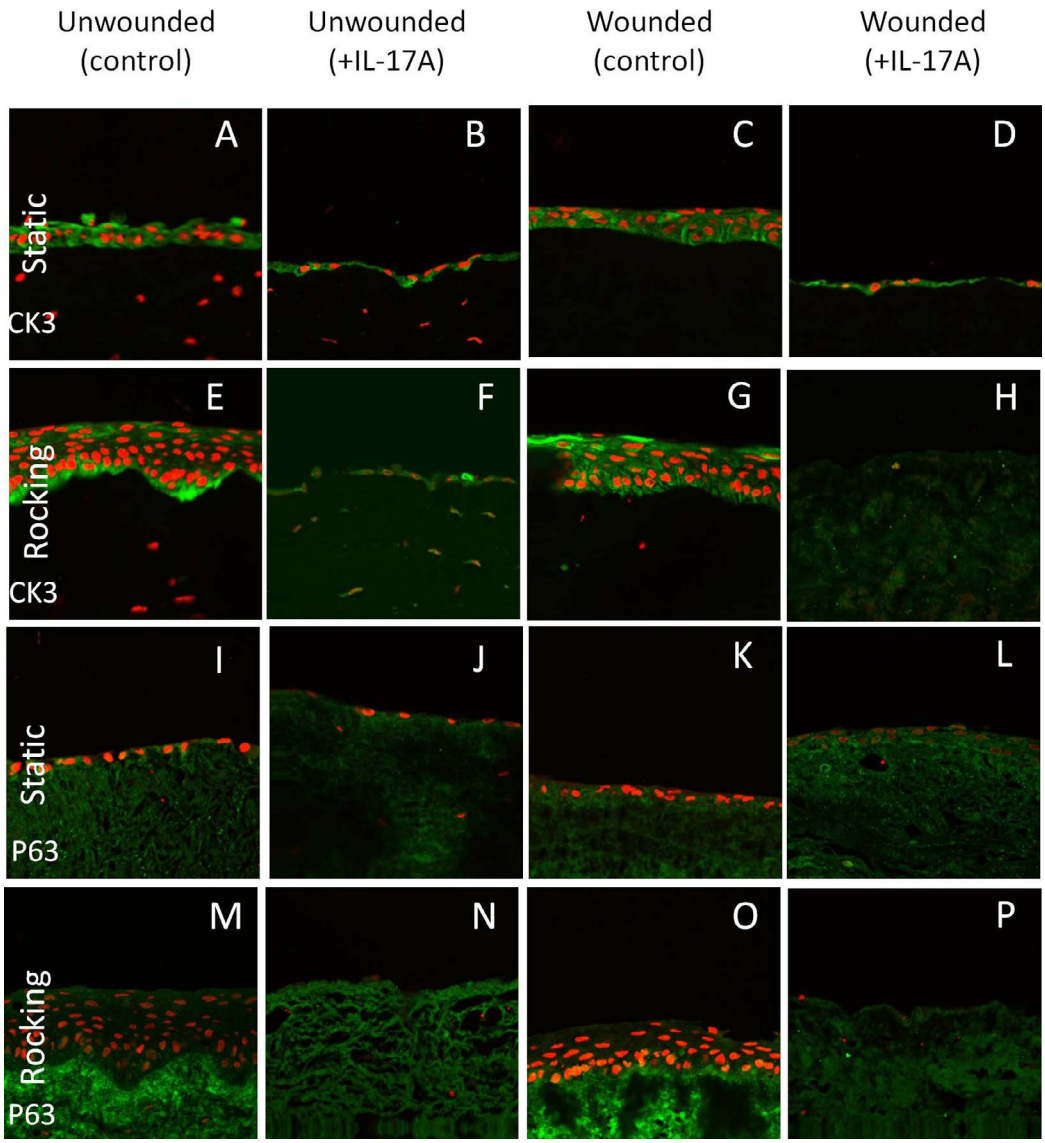


**Figure 6.** Expression of CK3 (**A–H**) and P63 (**I–P**) (shown in *green*) in cells on an in vitro corneal organ culture model under static (**A–D**, **I–L**) and rocking (**E–H**, **M–P**) conditions. (**A**, **E**, **I**, **M**) An unwounded cornea (control); (**B**, **F**, **J**, **N**) an unwounded cornea exposed to IL-17A; (**C**, **G**, **K**, **O**) a wounded cornea; (**D**, **H**, **L**, **P**) a wounded cornea exposed to IL-17A. Nuclei were counterstained with propidium iodide (*red*).

basal limbal region, suggesting that the region is susceptible to the inflammatory action of IL-17.[39] Donetti et al.[40] reported that IL-17 reduces proliferation, is destructive to tissue, and is described as proinflammatory.[34] Interleukin-22 has been reported to be responsible for the inhibition of differential gene expression while inducing proliferation and migration of keratinocytes.[41] Interleukin-17 alone has been implicated in dry eye,[38] scleritis, experimental autoimmune uveoretinitis,[42] herpetic stromal keratitis,[43] and multiple sclerosis,[44] while IL-17 and IL-22 together have been implicated in diseases related to skin inflammation, rheumatoid arthritis,[44–46] and uveitis.[42] This may explain the results in this study (Fig. 4) showing that IL-17 or the combination of IL-17 and -22 affected the epithelial morphology on the corneal surface.

In this particular study, LPS, which is found on the cell wall of Gram negative bacteria and is an endotoxin known to cause inflammatory responses to the host tissue,[47,48] had little or no effect on the appearance of the corneal model. Studies have shown that LPS may cause an inflammatory response only if there is an entry site into the cornea, but certainly in these studies we failed to find any convincing response to LPS.

The major finding of this study was the extent to which subjecting corneas to gentle rocking improved the maintenance of the epithelium. Not only did they survive longer than under static conditions (4 weeks compared to 2 weeks), but there was a clear benefit when a wound was made in the model and it was allowed to recover. Recovery from wounding was very effective when corneas were kept under rocking conditions, much less so under static conditions. For the maturation of most 3D epithelial tissues in vitro, exposure to an air–liquid interface is essential, as is the case for skin, buccal mucosa, and esophageal mucosa.[49–51] However, in the case of the cornea, it has not been clear whether for in vitro experimentation it is best to keep the epithelia submerged or at an air–liquid interface or, as in this study, subject to intermittent bathing with media. This study clearly shows the benefit of subjecting these epithelia to the intermittent movement of media.

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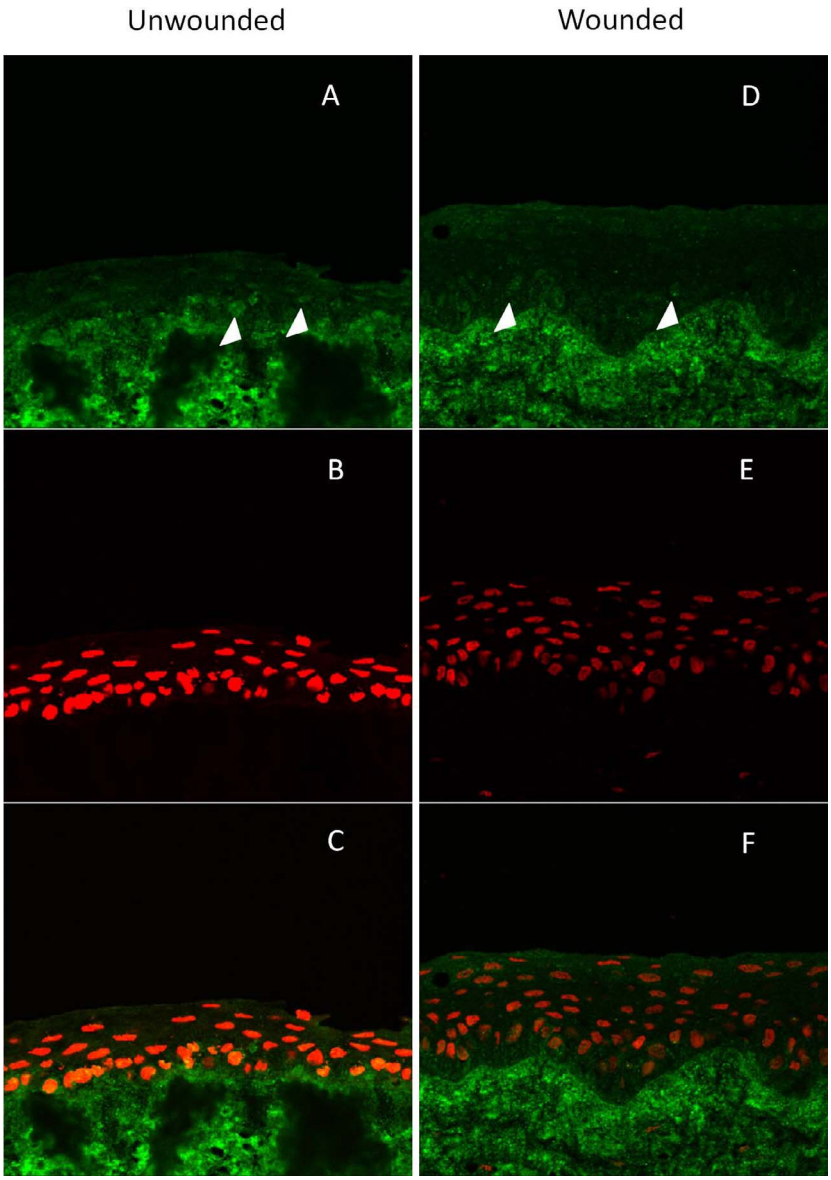


FIGURE 7. Expression of P63 in unwounded (**A**–**C**) and wounded (**D**–**F**) corneas cultured under rocking conditions. Images show P63 (*green*) expressed in nuclei (indicated by *white arrows*) in (**A**, **D**); nuclei counterstained with propidium iodide (*red*) in (**B**, **E**); and the merged images in (**C**, **F**).

When epithelia are injured, one of the first responses of the stromal cells is apoptosis of keratocytes, which can continue for 1 week after the injury has taken place.[52] It was noted in this study that most of the stromal cells close to the epithelial surface had died, giving a clear indication of the area where the wounding had taken place on the cornea during these experiments as shown in Figure 5. However, our attempts to confirm whether the stromal cells had undergone apoptosis were unsuccessful (results not shown), so we cannot make any conclusions regarding how these cells died.

With respect to expression of CK3 and P63, CK3 is known to be associated with the differentiated cells of the central corneal region,[53] while Pellegrini et al.[54] suggested that P63 is a stem cell marker expressed only in the basal layers of the limbal region. In characterization studies it was seen that wherever cells were present on the corneal epithelium, CK3 was expressed toward the central region of the cornea while P63 was expressed at the periphery, which contains the limbus as well as the basal region of the corneal epithelium (Fig. 7).

We suggest that this relatively simple ex vivo rabbit cornea model can now more usefully be used to look at some of the influences on corneal repair and regeneration that are currently tested in live rabbit eyes.

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