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Effects of hydrogen peroxide in a keratinocyte-fibroblast co-culture model of wound healing

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ABSTRACT

Recently, there has been renewed interest in the role of reactive oxygen species (ROS), especially H_2O_2, in wound healing. We previously showed that H_2O_2 stimulates healing in a keratinocyte scratch wound model. In this paper, we used a more complex and physiologically relevant model that involves co-culturing primary keratinocytes and fibroblasts. We found that the two main cell types within the skin have different sensitivities to H_2O_2 and to the widely used "antioxidant" *N*-acetyl-L-cysteine (NAC).

Keratinocytes were very resistant to the toxicity of H_2O_2 (250 and 500 μM) or NAC (5 mM). However, the viability of fibroblasts was decreased by both compounds. Using the co-culture model, we also found that H_2O_2 increases re-epithelialization while NAC retards it. Our data further illustrate the possible role of ROS in wound healing and the co-culture model should be useful for screening agents that may influence the wound healing process.

1. Introduction

One of the most important repair processes after dermal wounding is the restoration of the epidermal barrier over the wound site so as to prevent dehydration and opportunistic infections. This is also known as re-epithelialization and is largely achieved by a combination of keratinocyte migration and proliferation [1,2].

Various models of different complexity have been developed to study the re-epithelialization process. The simplest of these models is the monolayer keratinocyte scratch wound in which a layer of confluent keratinocytes is mechanically injured with a pipette tip and the resurfacing of the denuded area is used as an indicator of re-epithelialization [3]. This model has already been used widely by us and others to examine the effect of soluble compounds on wound healing [4,5]. H_2O_2 has been shown to play an important role in the events following dermal wounding [6–8] and we have previously found that H_2O_2 can induce cell proliferation and migration in a keratinocyte scratch wound model by inducing a sustained activation of the ERK pathway [5]. Nevertheless, the scratch wound model is a considerable over-simplification of the re-epithelialization process, which involves multiple cell types [9].

Three-dimensional organotypic cultures of keratinocytes and fibroblasts as well as *ex vivo* culture of skin explants have emerged as additional models to study the re-epithelialization process. 3D organotypic cultures involve the culturing of keratinocytes on dermis equivalents, which are created by mixing fibroblasts with type I collagen and allowing the collagen to solidify [10]. Keratinocytes are seeded onto the dermis equivalent, allowed to attach and exposed to the air–liquid interface. The organotypic culture can then be mechanically wounded by complete bisection and the re-epithelialization process studied using histological techniques [11].

In the *ex vivo* explant model, skin biopsies of mice or human subjects are allowed to attach onto a tissue culture dish and over the following days, keratinocytes emerge from the edge of the explants, migrating steadily as a cell sheet [12]. The rate of re-epithelialization can be conveniently monitored using a normal phase contrast microscope. However the effect of any treatment on the fibroblasts in the explants would still need to be evaluated by histological techniques.

Two-chamber type co-culture models involve culturing fibroblasts and keratinocytes in two separate chambers separated by a semi-permeable membrane. Such models have long been used to study paracrine signaling between keratinocytes and fibroblasts [13]. In this model, keratinocytes are usually grown on a semi-permeable transwell membrane insert while fibroblasts are grown in a tissue culture dish. The keratinocytes are exposed to

Abbreviations: AUC, area under the curve; HKGS, human keratinocyte growth supplement; NAC, *N*-acetyl-L-cysteine; ROS, reactive oxygen species.

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the air–liquid interface and allowed to stratify and differentiate. Soluble factors secreted by the fibroblasts are able to pass through the membrane and affect the growth and differentiation of keratinocytes and vice versa. However such models have never been used for studying re-epithelialization.

In the present paper, we used a co-culture model to explore if our previous studies on the mitogenic effect of H_2O_2 in keratinocytes would still be relevant in a more complex and perhaps more physiologically relevant system. We developed a simple model based on the two-chamber type co-culture model which can be used to evaluate re-epithelialization rate and effects of added compounds much more easily compared to the 3D organotypic culture models and *ex vivo* explant models used by others previously.

2. Materials and methods

2.1. Materials

Epilife medium with 60 μM calcium, human keratinocyte growth supplement (HKGS), primary human dermal fibroblasts and primary human keratinocytes (both derived from neonatal foreskin) were purchased from Invitrogen, Eugene, OR. Radioimmunoprecipitation assay (RIPA) buffer was purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-involucrin (Cat# SC 21748) and mouse monoclonal anti-GAPDH (Cat# SC 47724) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated goat anti mouse secondary antibody (Cat 0031430) was purchased from Pierce Chemicals (Rockford, IL, USA). 0.4 μm Pore size Transwell transparent polyester membrane inserts and their corresponding 6-well plates were purchased from Corning (USA). Thirty-five millimeter μ-dish inserts, purchased from Ibidi (München, Germany), were used as the wound assay inserts in the co-culture model. High glucose Dulbecco's Modified Eagle's Medium (DMEM) with added glutamine and fetal bovine serum was purchased from PAA (Linz, Austria). *N*-acetyl-L-cysteine (NAC), H_2O_2, phenylmethanesulfonylfluoride (PMSF), dimethyl sulfoxide and thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma–Aldrich (USA).

2.2. Cell culture

Primary fibroblasts were cultured in a humidified 95% air, 5% CO_2 incubator at 37 °C and maintained in DMEM supplemented with 10% FBS. Primary keratinocytes were cultured in EpiLife medium supplemented with HKGS at 100:1 ratio. Keratinocytes from passage 4–5 and fibroblasts from passage 6–8 were used for the experiments.

2.3. Co-culture wound model

An adhesive wound assay insert was stuck onto the centre of a 6-well transwell to occlude the centre of the transwell from the surroundings. Keratinocytes were trypsinized, re-suspended in Epilife medium supplemented with HKGS and seeded into the area outside the wound assay insert at 3×10^5 cells, 2 ml per transwell. Fibroblasts were trypsinized, re-suspended and seeded into 6-well plates at 3×10^5 cells, 2 ml per well. Keratinocytes and fibroblasts were grown separately for 48 h until confluent. The transwells containing the keratinocytes were then placed above the fibroblasts. Medium for both cell type was changed to the co-culture medium, which was basal Epilife medium:basal DMEM:FBS at a ratio of 49:49:2. The volume of medium was 2 ml per well and 1 ml per transwell insert (Refer to Fig. 1). After 2 days, the keratinocytes were exposed to the air–liquid interface by removing the medium in the transwells inserts while the medium at the bottom fibroblasts layer was replaced with fresh medium. Two days later, the wound assay insert was removed using a pair of sterile forceps. A sterile scalpel was also used to lightly dislodge the wound assay insert from the keratinocytes around it. With the wound assay insert removed, the keratinocytes could grow into the denuded area.

The co-cultures were treated with the cell permeable thiol compound, NAC or with H_2O_2 to evaluate their effects on re-epithelialization. As NAC is acidic, it was dissolved in the co-culture medium and the pH was adjusted to pH 7.4 with NaOH. Both NAC and H_2O_2 were filter-sterilized before use. 2 ml of co-culture medium containing H_2O_2 or NAC was added into the fibroblasts layer and 1 ml into the keratinocytes. After 30 min, the medium in the keratinocytes layer was aspirated leaving only the medium in the fibroblasts layer. Thirty minutes was chosen because we have previously shown that cells were able to decompose almost all the H_2O_2 present in the medium by 30 min [5].

The treatment was repeated every 24 h, over a period of 96 h. The same treatment was carried out for control experiments except that no test compounds were added. Images of the closure were monitored with a dissection microscope (Leica Microsystems, Wetzlar, Germany) for 4 days at 24 h intervals. The size of the denuded area was determined using the polygon tool in the image analysis software, ImageJ (NIH, USA) and expressed as percentage closure of the original wound.

2.4. Cell viability assay

Cells were grown in the co-culture system but without the wound assay inserts. Samples were treated with H_2O_2 and NAC every 24 h over a period of 96 h, using the same method as described for the co-culture wound model. At 96 h, the keratinocyte layer and the fibroblast layer were separated. Cell viability was determined using the (Thiazolyl Blue Tetrazolium Bromide) MTT reduction assay. At 96 h, the transwell inserts (keratinocytes) were separated from the tissue culture dish (fibroblasts). 5 mg/ml MTT dissolved in the co-culture medium was added to the keratinocytes (1 ml) and fibroblasts (2 ml) respectively. The cells were incubated at 37 °C for 15 min before the medium was aspirated and DMSO was added to each well to dissolve the purple formazan product. 1 ml of DMSO was added to the keratinocyte layer and 2 ml of DMSO was added to the fibroblast layer. The extracted formazan dye from each well was then further diluted 5 times in a 96-well plate and the absorbance was read at 570 nm against a reference wavelength of 690 nm on a microplate reader (Tecan, Switzerland). Cell viability was computed by comparing absorbance of different treatments against that of untreated cells and expressed as a fraction.

2.5. Western blot analysis

Cells were washed twice with ice-cold PBS and 150 μL RIPA buffer supplemented with 1 mM PMSF was added per transwell membrane insert. Cell lysates were briefly ultrasonicated in a sonicator bath before centrifuging for 10 min at 10,000*g*, 4 °C. The remaining cell pellet was further extracted with 6 M urea to dissolve insoluble proteins that might be present in the suprabasal layers of keratinocytes. Depending on the size of the insoluble cell pellet, different volumes of 6 M urea were added. Five micro liters of 6 M urea was added to day 0 samples and 50 μl was added to day 4 and day 10 samples. The extracted insoluble proteins were then pooled together with the RIPA buffer extract.

Samples were separated on a 10% SDS–polyacrylamide gel and wet-transferred onto nitrocellulose membranes. Membranes were blocked with 5% skim milk in tris-buffered saline (TBS) with 0.1% Tween-20 for 1 h at room temperature and washed with TBS containing 0.1% Tween-20 prior to incubation with the antibodies

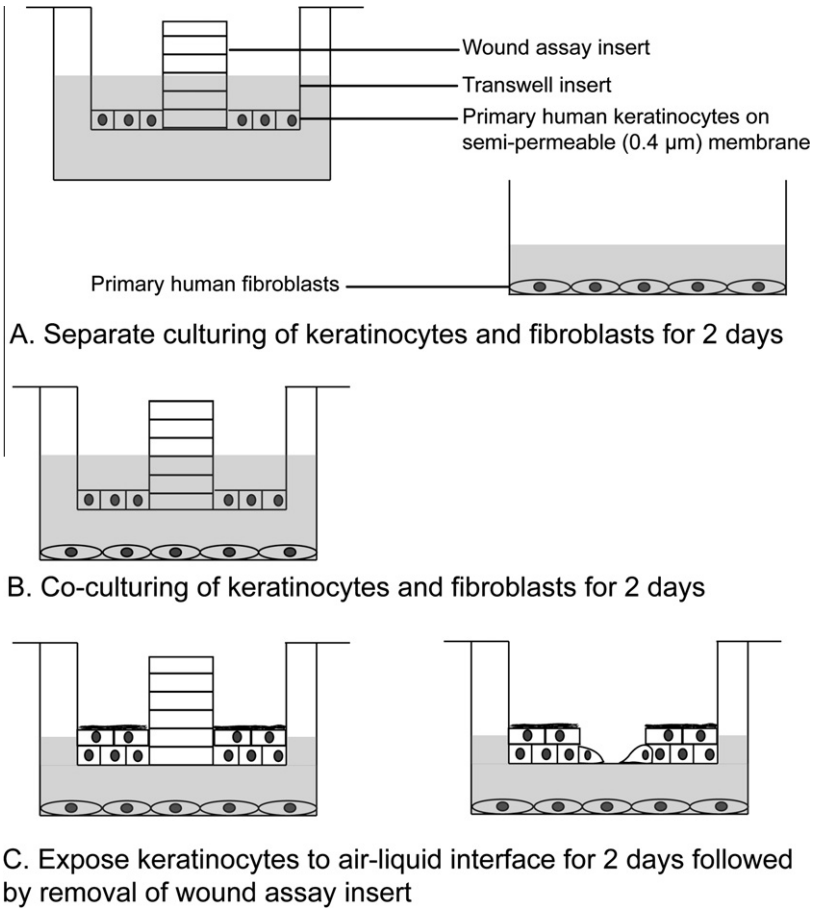

Fig. 1. Schematic diagram depicting creation of the co-culture wound model. (A) Keratinocytes and fibroblasts were initially cultured separately. (B) Keratinocytes and fibroblasts were put into co-culture for 2 days. (C) Keratinocytes were exposed to the air–liquid interface followed by removal of the wound assay insert.

overnight at 4 °C. Antibodies against β-actin and involucrin were diluted 5% BSA at 1:2000 and 1:1000, respectively. Proteins were detected with horseradish peroxidase-conjugated goat anti-mouse and goat anti-mouse secondary antibody, and imaged using enhanced chemiluminescence substrate on a Carestream IS4000MM Imaging System (Rochester, NY, USA).

2.6. Statistics

All results are expressed as mean ± SEM. The data were analyzed and compared using 1-way ANOVA with Graphpad Prism 5.0 where appropriate. Differences between means at $p < 0.05$ were considered significant.

The wound closure curves were integrated using the trapezoid rule and the area under the curves (AUC) analyzed by 1-way ANOVA. The wound healing curves could have been analyzed by a 2-factor ANOVA with one factor being time and the other the different treatments. However, it is clear that wound closure must increase with time hence there is no need to test if time affects wound closure. By performing statistical testing on the integral, we were able to use the more powerful 1-way ANOVA instead of 2-way ANOVA.

3. Results

3.1. Involucrin expression in keratinocytes after co-culturing

Keratinocytes will differentiate and stratify when cultured at the air–liquid interface. Involucrin is a precursor of the cornified envelope and its expression in the keratinocytes has been suggested to be a marker of stratification in such co-culture systems [14]. Thus, involucrin levels were measured on days 0, 4 and 10 after co-culture, which correspond to the keratinocytes just before co-culturing, the day of "wounding" and 6 days after "wounding". It was found that there was a significant increase in involucrin expression on the day the keratinocytes were wounded and the increased expression was sustained for at least 10 days of co-culture (Fig. 2).

3.2. Keratinocytes and fibroblasts display different sensitivities to H_2O_2 and NAC

In view of our previous observations that H_2O_2 stimulated proliferation in keratinocytes, we examined its effects in the co-culture model. It was found that keratinocytes and fibroblasts have very different susceptibilities to H_2O_2 (Fig. 3). The co-cultures were treated with H_2O_2 at 250 and 500 μM and cell viability was evaluated using the MTT reduction assay. Results were then expressed as fraction viable compared to untreated cells.

Keratinocyte viability was analyzed by 1-way ANOVA and it was found that there was a significant difference between the different treatments ($p = 0.0098$). Post-hoc analysis was carried out using Dunnett's multiple comparison test and it was found, consistent with our previous results, that 250 μM H_2O_2 increased keratinocyte proliferation by 20% ($p < 0.05$) while 500 μM induced a slight but statistically insignificant increase in keratinocyte proliferation.

Fibroblast viability was analyzed similarly. Whereas keratinocytes can tolerate 500 μM H_2O_2 without any viability loss, H_2O_2

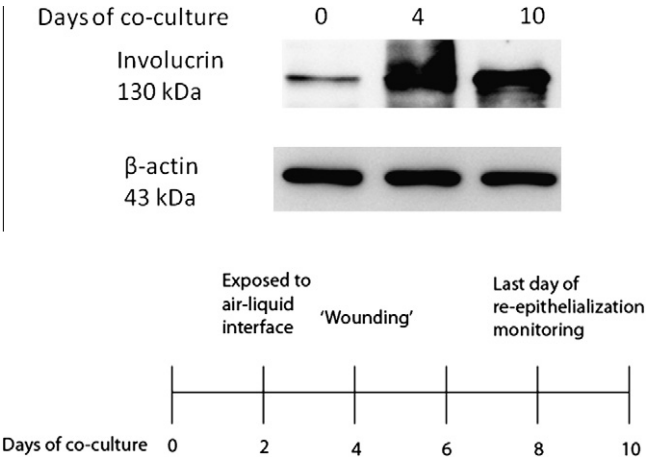

Fig. 2. Involucrin expression in keratinocytes when co-cultured with fibroblasts. A western blot of involucrin is shown with β-actin as the loading control. The blots shown are representative of 3 independent experiments. Accompanying is the timeline indicating the main steps of the co-culture model. Day 0 represents keratinocytes just before co-culture, day 4 corresponds to the day of 'wounding' and day 10 is 6 days after 'wounding'.

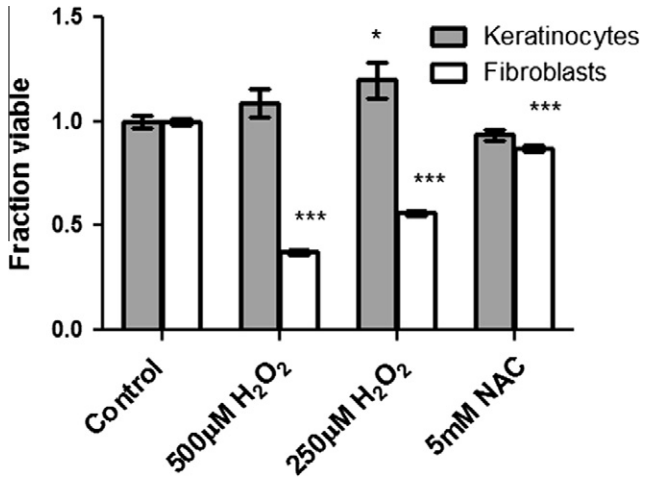

Fig. 3. Cell viability was measured using MTT assay and expressed as fraction viable compared to untreated keratinocytes or fibroblasts. Cells were exposed to the stated agent every 24 h for 96 h. 5 mg/ml of MTT dissolved in co-culture medium was added to keratinocytes (1 ml) and fibroblasts separately (2 ml). After 15 min, the medium was aspirated and the purple formazan dye was extracted with 1 and 2 ml of DMSO for the keratinocyte and fibroblast layer respectively. Results are average of 9 replicates ± SEM. p values were calculated using one-way ANOVA with Dunnett post hoc test where each column was compared to its respective untreated cell type. $^{*}p < 0.05$, $^{***}p < 0.001$.

was found to be more cytotoxic to fibroblasts, reducing cell viability by 63% at 500 μM (Fig. 3).

3.3. H_2O_2 promotes re-epithelialization but NAC retards it

The co-culture model was then used to evaluate the effects of H_2O_2 and NAC on re-epithelialization. As shown in Figs. 1 and 4A, a 'wound' of consistent size was created by seeding keratinocytes onto an area occluded by a wound assay insert. The rate of keratinocyte in-growth after removal of the insert was used as an indicator of re-epithelialization (Fig. 4B). We integrated the curves in Fig. 4B by the trapezoid rule approximation to obtain the area under the curve. A lower AUC indicates slower closure whereas a higher AUC indicates faster closure. Results are shown in Fig. 4C and analyzed by 1-factor ANOVA. There was a significant difference in migration rate between the different treatments ($p < 0.0001$). Post-hoc analysis was carried out using Dunnett's multiple comparison test to compare the effect of each treatment against that of untreated cells.

It was found that both concentrations of H_2O_2 increase re-epithelialization rate but 250 μM increases wound closure more than 500 μM. We also examined the effects of the widely used "antioxidant" NAC. It had no effect on the viability of keratinocytes but decreased the viability of fibroblasts slightly (~13%) and the results were statistically significant ($p < 0.001$). Strikingly however, NAC was a powerful inhibitor of the wound closure process (Fig. 4).

4. Discussion

There have been reports of increased production of H_2O_2 in wounds by non-phagocytic cells [6]. Low concentrations of H_2O_2 have long been identified to have growth factor-like properties. H_2O_2 at low levels can increase DNA synthesis in quiescent cells and induce the expression of genes such as c-*fos* and c-*myc*, which are associated with proliferation [15,16]. Therefore there has been increased interest on whether low concentrations of H_2O_2 might be beneficial in wound healing [17]. On the other hand, it is well known that even low concentrations of H_2O_2 can induce cell senescence or death.

We observed that H_2O_2 at concentrations between 250–500 μM can stimulate keratinocyte re-epithelialization in our co-culture model of wound healing without inducing cell death. This concentration is much lower than the 1–3% solution (324–972 mM) typically used for wound disinfection purposes. However, 250 μM H_2O_2 had a stronger effect on re-epithelialization than 500 μM H_2O_2 which might be due to the cytotoxic effect higher H_2O_2 levels on the fibroblasts. Fibroblasts are known to secrete various soluble factors such as keratinocyte growth factor (aka FGF7) that can stimulate keratinocyte proliferation [18]. Decreased fibroblast viability could adversely affect keratinocyte re-epithelialization through reduced paracrine signaling. Previous studies have also shown differential sensitivity of keratinocytes and fibroblasts to H_2O_2. Keratinocytes are resistant to H_2O_2 up to at least 700 μM [19] but fibroblasts shows signs of toxicity at concentrations as low as 10 μM [20]. Using the co-culture model, we were able to show that the difference in susceptibility still occurs within a co-culture system.

An interesting aspect of our results was that NAC was found to inhibit wound closure in our model very strongly. Whether this is due to its effects as an antioxidant or due to dysregulation of redox signaling processes remains to be investigated since NAC has been shown to induce a wide variety of cellular effects by perturbing the thiol redox status [21]. Other studies have also shown that low concentrations of H_2O_2 can stimulate healing while NAC retards healing in organ culture as well as *in vivo* models of corneal injury [22].

NAC was shown to inhibit the wound closure process but did not affect the viability of the keratinocytes. It is likely that NAC inhibited cell proliferation in the keratinocytes but did not kill them. Hence, they were unable to re-epithelialize but remained viable. Our observation is consistent with previous observations that a more reduced redox status is associated with decreased cell proliferation [23] and NAC can induce cell cycle arrest [21]. NAC has been shown to induce cell cycle arrest at the G1 phase, without causing cell death in hepatic stellate cells [24] and mouse embryonic fibroblasts [25]. It is not entirely clear if NAC has a cytotoxic or a cell cycle arresting effect on the fibroblasts. The decrease in MTT reduction by NAC treated fibroblasts could be explained be due to either cell death or cell cycle arrest, where untreated fibroblasts proliferated but NAC treated fibroblasts did not.

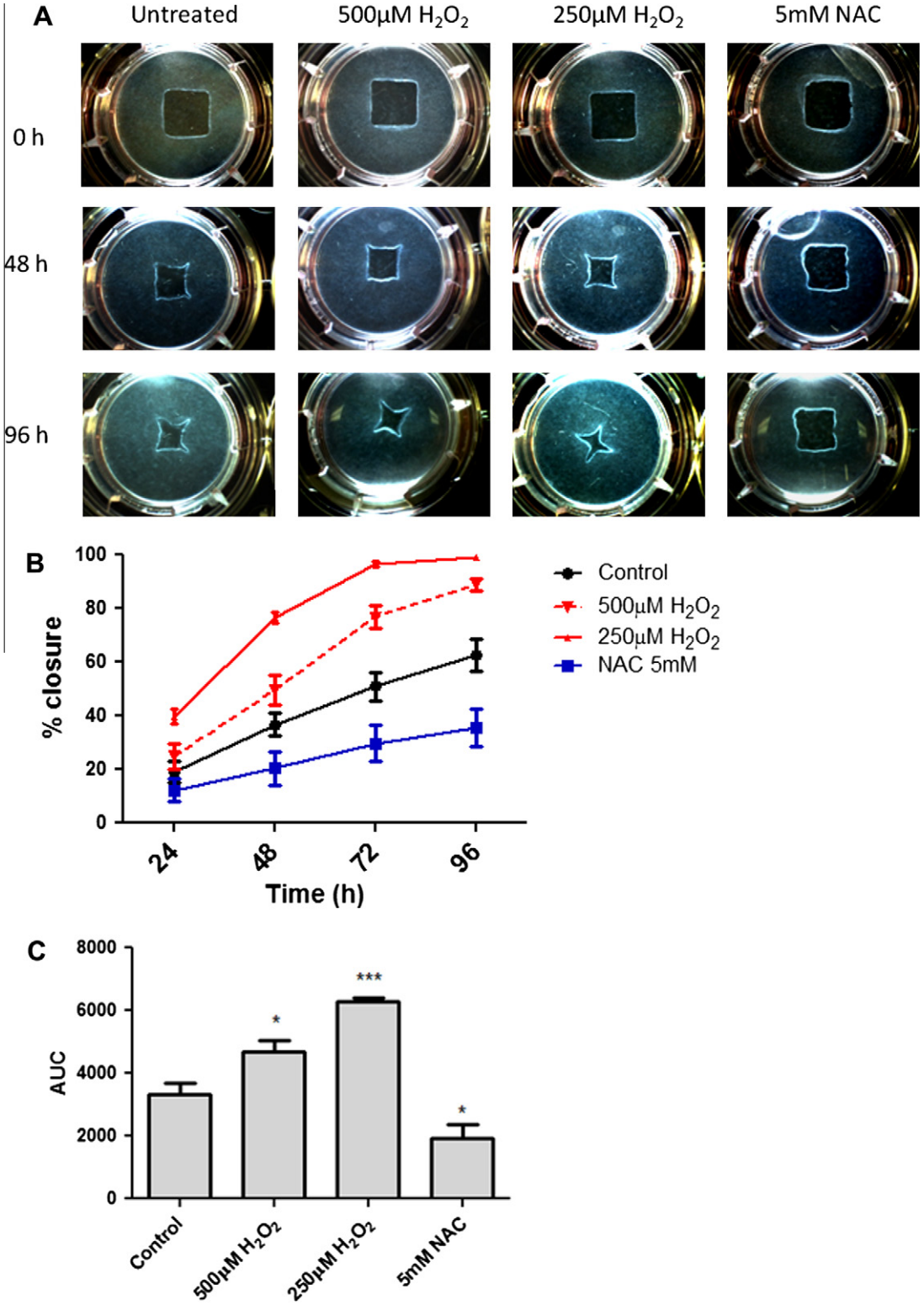

Fig. 4. H_2O_2 promotes keratinocyte migration in a co-culture model of re-epithelialization and NAC retards it. (A) Representative micrographs of the re-epithelialization process over time. (C) The rate of re-epithelialization for cells under different treatment. Medium was changed every 24 h over a period of 96 h and the treatment was re-applied. Results are average ± SEM of 8 replicates. (D) Results for the line graphs in (C) are integrated by the trapezoid rule approximation. The resulting area under the curve (AUC) is plotted as a bar graph and analyzed using 1-way ANOVA. *p*-Values were calculated Dunnett post hoc test where each column was compared to the control column.$^{*}p < 0.05$, $^{***}p < 0.001$.

Future investigations on the potential use of H_2O_2 or antioxidants in wound healing should take note of the differential susceptibility of the different cell types to these chemical agents. The co-culture wound model described is a simple and easy to perform experiment that can be used for the screening of potential lead compounds to be used in wound healing.

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