The effect of pH on cell viability, cell migration, cell proliferation, wound closure, and wound reepithelialization: In vitro and in vivo study

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ABSTRACT

Wound microenvironment plays a major role in the process of wound healing. It contains various external and internal factors that participate in wound pathophysiology. The pH is an important factor that influences wound healing by changing throughout the healing process. Several previous studies have investigated the role of pH in relation to pathogens but studies concentrating on the effects of pH on wound healing itself are inconclusive. The purpose of this study was to comprehensively and in a controlled fashion investigate the effect of pH on wound healing by studying its effect on human primary keratinocyte and fibroblast function in vitro and on wound healing in vivo. In vitro, primary human keratinocytes and fibroblasts were cultured in different levels of pH (5.5–12.5) and the effect on cell viability, proliferation, and migration was studied. A rat full-thickness wound model was used to investigate the effect of pH (5.5–9.5) on wound healing in vivo. The effect of pH on inflammation was monitored by measuring IL-1α concentrations from wounds and cell cultures exposed to different pH environments. Our results showed that both skin cell types tolerated wide range of pH very well. They further demonstrated that both acidic and alkaline environments decelerated cell migration in comparison to neutral environments and interestingly alkaline conditions significantly enhanced cell proliferation. Results from the in vivo experiments indicated that a prolonged, strongly acidic wound environment prevents both wound closure and reepithelialization while a prolonged alkaline environment did not have any negative impact on wound closure or reepithelialization. Separately, both in vitro and in vivo studies showed that prolonged acidic conditions significantly increased the expression of IL-1α in fibroblast cultures and in wound fluid, whereas prolonged alkaline conditions did not result in elevated amounts of IL-1α.

Wound microenvironment plays a major role in the process of wound healing. It is influenced by various external and internal factors that participate in wound pathophysiology.[1,2] The pH is an important factor that influences wound healing by changing throughout the healing process.[1] Intact skin is naturally acidic with a pH ranging from 4 to 6 due to organic acid secretion by keratinocytes in the epidermis. Upon injury, the barrier of the skin is destroyed and the pH of the wound surface increases due to leakage from the microvessels and will approximate a physiologic pH (7.4). Furthermore, there is a pH gradient in the wound bed, the value rising gradually with increasing depth.[3] During the healing process and depending on the pathophysiology of the wound, the wound environment may become alkaline, which has been associated with nonhealing wounds or acidic which has been associated with healing wounds.[4]

In acute wound healing, the pH decreases and the wound environment becomes acidic due to the energy required for cell proliferation, migration, and synthesis of extracellular matrix. A naturally occurring acidic environment is important for a wound, since it impairs the growth of microbes in the wound bed and enhances antimicrobial activity as a result of increasing solubility of free metal ions.[5] In addition, low pH is thought to support delivery of oxygen to the wound bed due to the Bohr-effect. As wound closure is achieved, pH peaks around 7.4 before decreasing to the pH level of an uninjured skin, once the skin barrier is reestablished.

The pH of a chronic nonhealing wound becomes alkaline upon failure to heal.[6] It has been suggested that ammonia, liberated from urea causes the wound pH to increase. Alkaline environment has been thought to be harmful for wound healing since it stimulates many

proteases to cleave proteins and their end products are toxic to wound tissue.[7] In addition, a slightly alkaline environment is less protective against bacteria than a slightly acidic environment, which causes higher risk of infection and subsequently biofilm formation, leading to increasing morbidity and mortality.[8]

Bacteria can be classified as acidophils, neutrophils, or basophils based on the pH for optimal growth. The optimal pH for growth of common bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* has been reported to be close to neutral (*S. aureus*: 7–7.5; *P. aeruginosa*: 6.5–7) with a minimum pH for growth being 4.2 and 5.6 and maximum 9.3 and 8, respectively.[9] Bacterial contamination alters the pH of the wound bed due to increased lactate deposition,[10,11] which in turn affects the expression of proinflammatory cytokines and matrix metalloproteinases.[12,13] Prolonged over expression of these factors keeps the wound stuck in a self-perpetuating inflammatory stage that may prohibit wound closure.[12] For this reason, alkaline dressings, with peroxide and bleach,[14] and acidic wound dressings, containing citric acid, boric acid or acetic acid,[15] have been used to treat infected wounds. These treatments aim at affecting pathogens as well as the function of the cells and enzymes in the wound.[1] Although both acidic and alkaline dressings have been used in the clinic, wound pH is not regularly monitored and treated in clinical practice and it is still not completely clear how an increase or decrease in the pH level affect the wound microenvironment and the healing process.

The purpose of this study was to comprehensively investigate the effect of pH on wound healing. Primary keratinocytes and fibroblasts were cultured in acidic and alkaline environments and their viability, proliferation, and migration were studied over time. In a full-thickness rat wound chamber model, the pH value of the wound environment was modified and maintained at the desired pH level during the whole experiment and the effect on wound closure and reepithelialization was studied. In addition, the effect of acidic, neutral, and alkaline pH levels on inflammation was investigated.

MATERIALS AND METHODS

Adjustment of the pH level

For experiments, 0.1% acetic acid was used to prepare acidic culture media and potassium hydroxide solution 1.0 N (Sigma-Aldrich, St. Louis, MO) for preparing an alkaline pH level. The different pH media were sterilized by collecting solutions in 50 mL syringes (BD, Franklin Lakes, NJ), which were then filtered through a 0.22 μM syringe filter (Fisher Scientific, Pittsburgh, PA). Before starting measurements for adjusting pH levels, by using the glass electrode pH meter Accumet Research AR15 (Fisher Scientific), buffer solutions at pH 4.0, 7.0, and 10.0 (Fisher Scientific) were used for calibration.

Primary keratinocyte and fibroblast cultures

Cells were isolated from discarded skin obtained from patients undergoing elective plastic surgery (Brigham & Women's Hospital IRB #2010P002947). To establish primary cultures of both fibroblasts and keratinocytes 0.5 cm^2 skin pieces were cut out and incubated in 6.5 U/mL dispase (Invitrogen, Carlsbad, CA) at 4°C overnight. The epidermis was separated from the dermis using sterile forceps and needles. To isolate keratinocytes, the epidermis was incubated with 0.25% trypsin/EDTA 1× (Invitrogen) at 37°C for 10 minutes. Keratinocytes were further isolated, by pipetting vigorously. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA) was added to inhibit trypsin. Subsequently, the cells were centrifuged at 300 × *g* for 5 minutes. The supernatant was discarded and the cell pellet resuspended in keratinocyte medium (Thermo Fisher Scientific). The keratinocytes were plated on to collagen-I coated flasks (Becton Dickinson, Bedford, MA) and cultured for the experiments. Media was changed every second day throughout culturing and everyday day throughout the experiment. Second passage of cells was used for experiments.

Primary human dermal fibroblasts were isolated from the remaining dermal pieces. Fibroblasts were grown from the dermal skin pieces in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific), with 1% penicillin-streptomycin (Thermo Fisher Scientific) and 10% FBS (Thermo Fisher Scientific). Media was changed every second day through culturing and everyday throughout the experiment. Passage numbers 2–5 were used for experiments. Cell supernatants from both cell types were collected for further analysis.

Cell viability assay

Cells were plated on 12 well plates (Becton Dickinson) at a density of 30,000 cells per well. The viability assay was initiated at 50% confluence. The cells were treated with media with a pH ranging from 5.5 to 12.5. Viability was measured at days 1, 3, and 5 using Tryban Blue cell viability assay (Sigma). Briefly, using 0.05% trypsin/EDTA (Thermo Fisher Scientific) the cells were detached and centrifuged for 5 minutes at 300 × *g*. Subsequently, the supernatant was discarded and the cells were resuspended in 50 μL of phosphate buffered saline solution. To stain the dead cells 5 μL of 0.4% Trypan blue solution (Sigma) were added to each tube. Cell viability was calculated under the microscope (Eclipse E400 light microscope, Digital Sight camera; Nikon, Kanagawa, Japan) using a hemocytometer (InCyto, Drive Covington, GA) after 5 minutes of incubation. In the viability assay N-number (number of wells) for each pH level and time point was 6–8.

Cell proliferation assay

Primary keratinocytes and fibroblasts were plated at a density of 10,000 cells per well in a 96-well plate (Becton Dickinson). After 48 hours, the cells were treated with media with pH ranging from 5.5 to 12.5. A CellTiter 96® Aqueous Proliferation Assay (Promega, Fitchburg, WI) was performed using manufacturer's protocol at days 1, 3, and 5. Briefly, proliferating cells produce formazan as a byproduct from tetrazolium, this conversion can be quantified by measuring the absorbance. Cells were incubated 60 minutes with tetrazolium and the absorbance was measured at 490 nm for each well in the 96-well plate using a SpectraMax M5 Microplate Reader and SoftMax Pro software (Molecular Devices, Sunnyvale, CA). In the proliferation

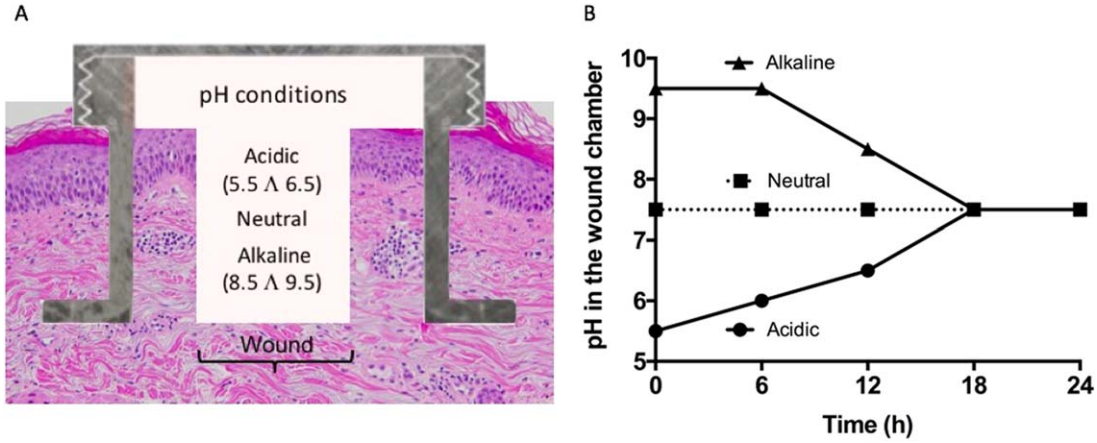


Figure 1. (A) Two circular concentric punch wounds, 4 mm and 10 mm in diameter, respectively, were created. The skin inside the 4-mm punch wound was removed creating a full-thickness wound in a skin island which was being studied. Following wound creation, customized round titanium wound chambers, with a squared rim and a titanium screw-top lid (Harvard School of Engineering; Cambridge, MA) were placed under the lateral skin edge of the 10-mm circular incision. The pH conditions of the wound were modified by adding acidic, neutral, or alkaline media into the wound. (B) The 24-hour development of the pH in the wound chamber was studied. Acidic (5.5) and alkaline (9.5) pH media were administered into the chambers and the pH value was measured every 6 hours. The results showed that both acidic and alkaline pH conditions returned to neutral approximately in 18 hours. Fresh media was added to the chambers daily to maintain the desired pH level in the wounds. [Color figure can be viewed at wileyonlinelibrary.com]

assay N-number (number of wells) for each pH level and time point was 6.

Cell migration assay

Fibroblasts and keratinocytes were plated on 12 well plates (Becton Dickinson) at a density of 30,000 cells per well. After reaching 90% confluence, a scratch wound was made across the well, using a pipette tip. Cells were then treated with media with a pH ranging from 5.5 to 12.5. At days 1, 3, and 5 after treatment, the wound area was measured and cell migration was quantified as the remaining scratch wound area relative to the initial wound area using ImageJ software (NIH, Bethesda, MD). In the migration assay N-number (number of wells) for each pH level and time point was 6–8.

Animals

All the rat procedures were approved by the Harvard Medical Area Standing Committee on Animals. Female Wistar rats (Charles River, Wilmington, MA) weighing about 200 g were used for this study. Rats were allowed to acclimatize for 72 hours before the experiments. Anesthesia was induced with 4% isoflurane and was maintained with 1–3% isoflurane (Novaplus, Hospira) and oxygen via snout mask. After the procedure, rats were transferred back to the cage and monitored during recovery from anesthesia. Buprenorphine 0.005 mg/kg was administered subcutaneously for pain control during the first 24 hours after the procedure.

Wound creation

The dorsum of the rat was shaved to remove all hair and disinfected twice using 70% isopropanol (Contec, Spartanburg, SC). Two circular concentric punch wounds, 4 mm and 10 mm in diameter, respectively, were created. The skin inside the 4-mm punch wound was removed creating a full-thickness wound in a skin island which was being studied. Following wound creation, customized round titanium wound chambers, with a squared rim and a titanium screw-top lid (Harvard School of Engineering; Cambridge, MA) were placed under the lateral skin edge of the 10 mm circular incision (Figure 1A). The chamber was secured to the muscle fascia with two 3-0 prolene sutures (Ethicon Inc, Somerville, NJ) in diametrically opposite holes. To obtain a water tight seal around the wound chamber a purse string suture was placed in the skin around the circular rim of the chamber using 4-0 Vicryl (Ethicon Inc). A total of four wounds with titanium chambers were made on the dorsum of each rat. The model has been extensively described in a previous communication.[16]

Modification of the wound pH environment

Five hundred microliter of medium with different pH (5.5, 6.5, 7.5, 8.5, and 9.5) was administered to each chamber. To ensure that the pH would stay at the desired level, the pH of the media in the chamber was measured and changed every day throughout the experiment. Wound fluid was also collected daily from the chambers and used for further analysis (Figure 1).

Wound healing

Wound healing was measured on day 4 and 8. Digital photographs of the wounds were taken at the time of surgery and on day 4 and day 8 (Nikon Corporation). Wound surface area was measured within the wound margins from

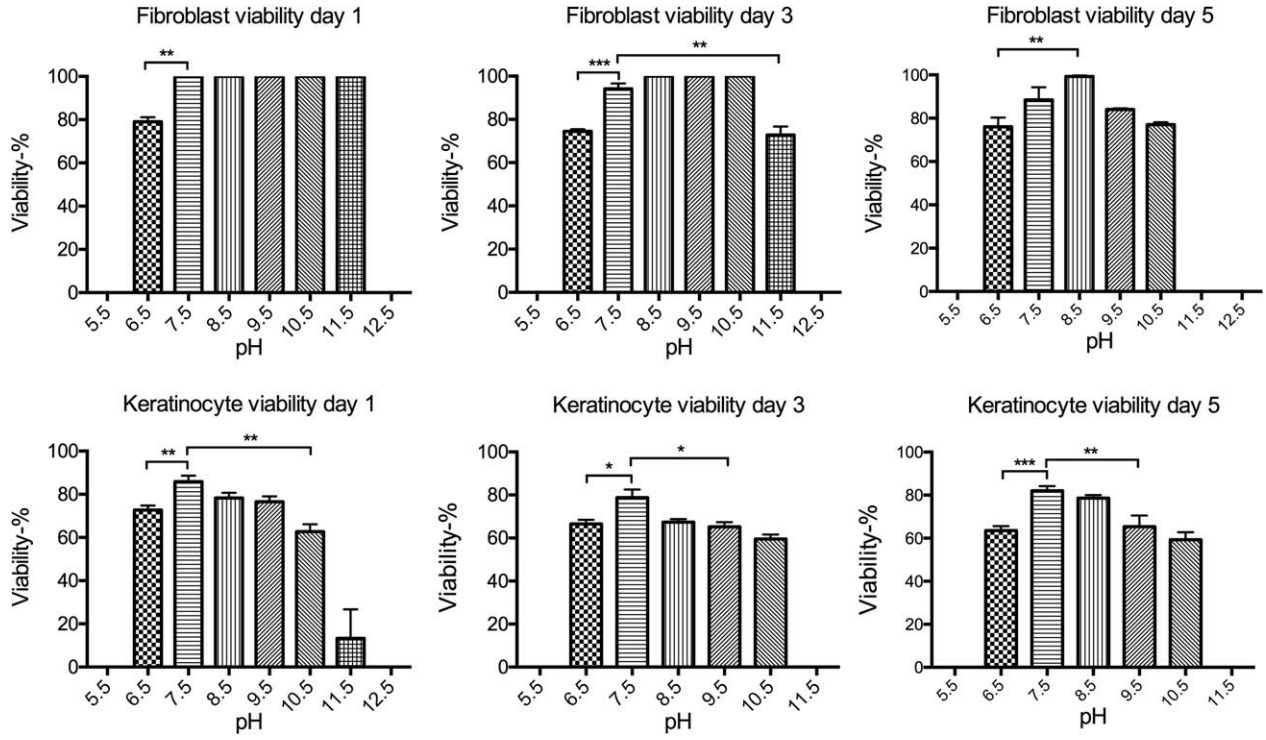


Figure 2. Cell viability: Primary keratinocytes and fibroblasts tolerate a wide range of pH. No significant differences were seen in viability when the cells were cultured within a pH range of 6.5–10.5 for 5 days. Both keratinocytes and fibroblasts died by day 1 when cultured in pH <6.5 or >11.5. The optimal viability percentage (100%) in fibroblasts was found at pH levels of 7.5–11.5 on day 1, 8.5, to 10.5 on day 3 and at 8.5 on day 5. The best viability percentages for keratinocytes were measured at pH 7.5 (Day 1: $85 \pm 2.7\%$; Day 3: $82 \pm 2.2\%$; Day 5: $79 \pm 1.3\%$). *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

macroscopic wound photos using ImageJ software. The area of each wound closure was expressed as a percentage of its original size on day 0. The wounds were excised, using an 11-blade scalpel and fixed in formalin for histologic analysis. The biopsies were embedded in paraffin, cut in sections, and stained with Hematoxylin and Eosin (H&E). The sections were examined using an Eclipse E400 light microscope, and images captured using a DS-Fi1 camera (Nikon Corporation). Quantitative measurements were performed using NIS-Elements D3.0 digital image analysis software (Nikon Corporation). Reepithelialization was defined as the sum of the new epithelium divided by the original wound area. Total of 12 rats (48 wounds) were used in the study and the N-number for each treatment and time point in the in vivo experiments was at least 4.

ELISA

Amounts of interleukin 1-alpha (IL-1α) in both wound fluid and cell culture supernatant were measured using a quantitative IL-1α ELISA assay per manufacturer's protocol (Thermo Fisher Scientific). Plates were read using a SpectraMax M5 Microplate Reader (Molecular Devices) and IL-1α concentrations were quantified against a standard curve. The N-number for each pH and time point was 6.

Statistical analysis

All statistical analyses were performed in GraphPad Prism (GraphPad, La Jolla, CA). Data is presented as mean ± SEM. Comparison of groups was performed using t test and p-values < 0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p > 0.001$).

RESULTS

In vitro

Cell viability

Both primary keratinocytes and fibroblasts tolerated pH from 6.5 to 10.5 very well and no major differences were seen in viability when the cells were cultured within this pH range for 5 days. The results indicated that slightly alkaline conditions were comparable to neutral conditions (no statistically significant differences) as slightly acidic conditions showed a small decrease in viability. Fibroblasts viability was 100% at pH levels 7.5–11.5 on day 1, at pH levels 8.5–10.5 on day 3 and at pH level 8.5 on day 5. The best viability percentages for keratinocytes were measured at pH 7.5 (Day 1: $85 \pm 2.7\%$; Day 3: $82 \pm 2.2\%$; Day 5: $79 \pm 1.3\%$). Both keratinocytes and fibroblasts died already by day 1 when cultured in pH < 6.5 or >11.5 (Figure 2).

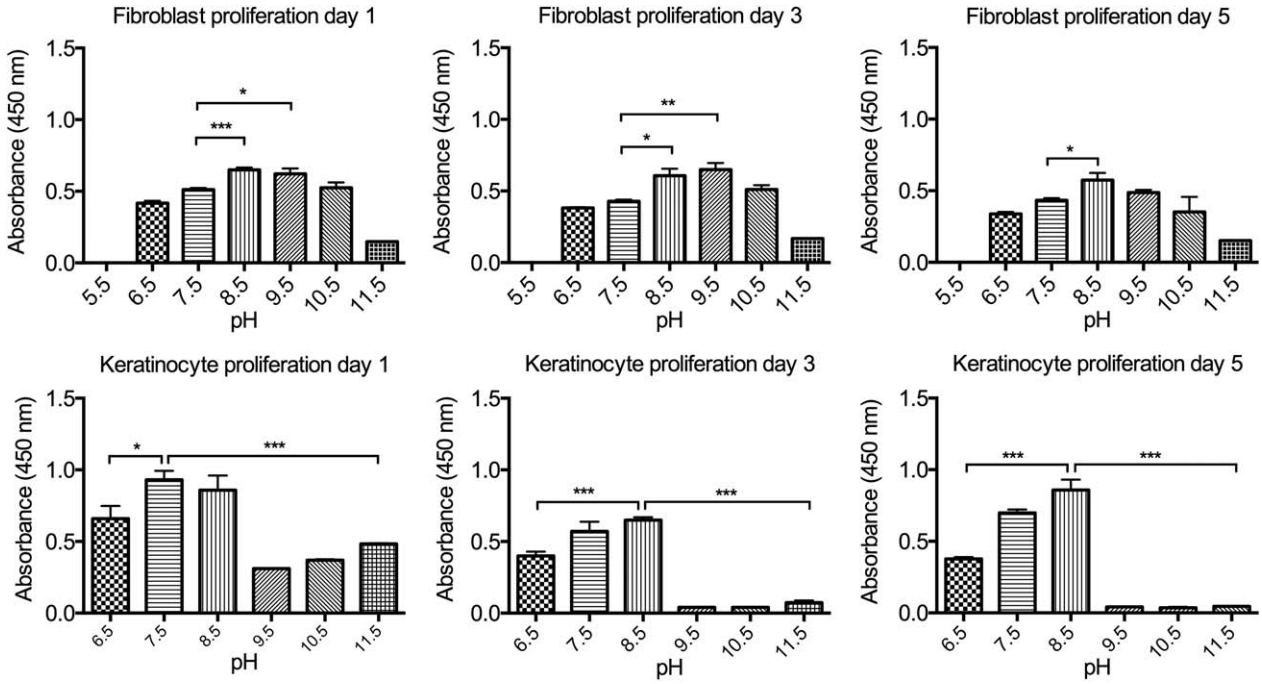


Figure 3. Cell proliferation: Fibroblasts proliferated within a wide range of pH (6.5–10.5). Keratinocytes proliferated within a pH range of 6.5–8.5. The proliferation was decreased outside these ranges. The optimal pH for fibroblast proliferation was seen at a pH of 8.5 at day 1, 8.5, and 9.5 at day 3, and 8.5 at day 5. The optimal pH for keratinocyte proliferation was found at a pH of 7.5 at day 1, 8.5 at day 3, and 8.5 at day 5. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

Cell proliferation

Overall fibroblasts proliferated within a pH range of 6.5–10.5 and keratinocytes within a pH range of 6.5–8.5 and outside these ranges proliferation was poor or nonexistence for both cell types. The results indicated that alkaline pH conditions (pH: 8.5–9.5) increased fibroblasts proliferation statistically significantly and similar trend was observed in keratinocytes (pH: 8.5). The best conditions for fibroblast proliferation were at pH 8.5 on day 1, at pH 9.5 on day 3, and at pH 8.5 on day 5. Keratinocytes proliferated the best at pH 7.5, 8.5, and 8.5 on days 1, 3, and 5, respectively (Figure 3).

Cell migration

Fibroblasts migrated well when cultured in pH levels from 6.5 to 9.5. The fibroblast migration was greatest at pH 7.5. Both acidic and alkaline pH conditions decreased the migration of fibroblasts. The best pH for keratinocyte migration was also 7.5 and migration was likewise decelerated in pH conditions below 7.5 and above 8.5 (Figure 4).

The effect of pH on wound healing

The 24-hour development of the pH level in the wound chamber was observed. The measurements showed that both acidic and alkaline pH conditions in the chamber returned to neutral approximately in 18 hours (Figure 1B). Therefore, fresh pH media was added to the wound chambers daily during the experiment to maintain the desired pH of the environment (Preferably, the fresh pH media would have been administered every 18 hours but the animal protocol regulations allowed only once a day administration).

Wound closure

Macroscopic wound closure was evaluated as the size of the wound area on days 4 and 8 compared to the original wound area at day 0. The best pH condition for wound healing was 7.5. Our results showed that on day 4 both acidic and alkaline conditions slowed down wound closure but by day 8 only the strongly acidic wound environment ($pH < 6.5$) decelerated healing ($p < 0.05$), whereas an alkaline wound environment ($pH > 7.5$) did not seem to negatively affect wound closure (Figure 5A).

Reepithelialization

On day 4, the wounds in neutral pH conditions showed the best reepithelialization percentage ($35 \pm 21\%$) and by day 8 wounds in strongly alkaline (pH 9.5) conditions had reepithelialized the best ($84 \pm 6\%$). Similarly, to the wound closure data, the results suggested that an acidic environment ($pH < 7.5$) decelerated reepithelialization although no statistically significant differences were seen between the groups at day 8. Interestingly, alkaline environment did not have a negative effect on wound reepithelialization. Moreover, a trend was seen showing that alkaline conditions could even increase reepithelialization (Figure 5B).

The effect of pH on IL-1α expression

The IL-1α concentration was measured from wound fluid harvested from full-thickness rat wounds treated with a pH

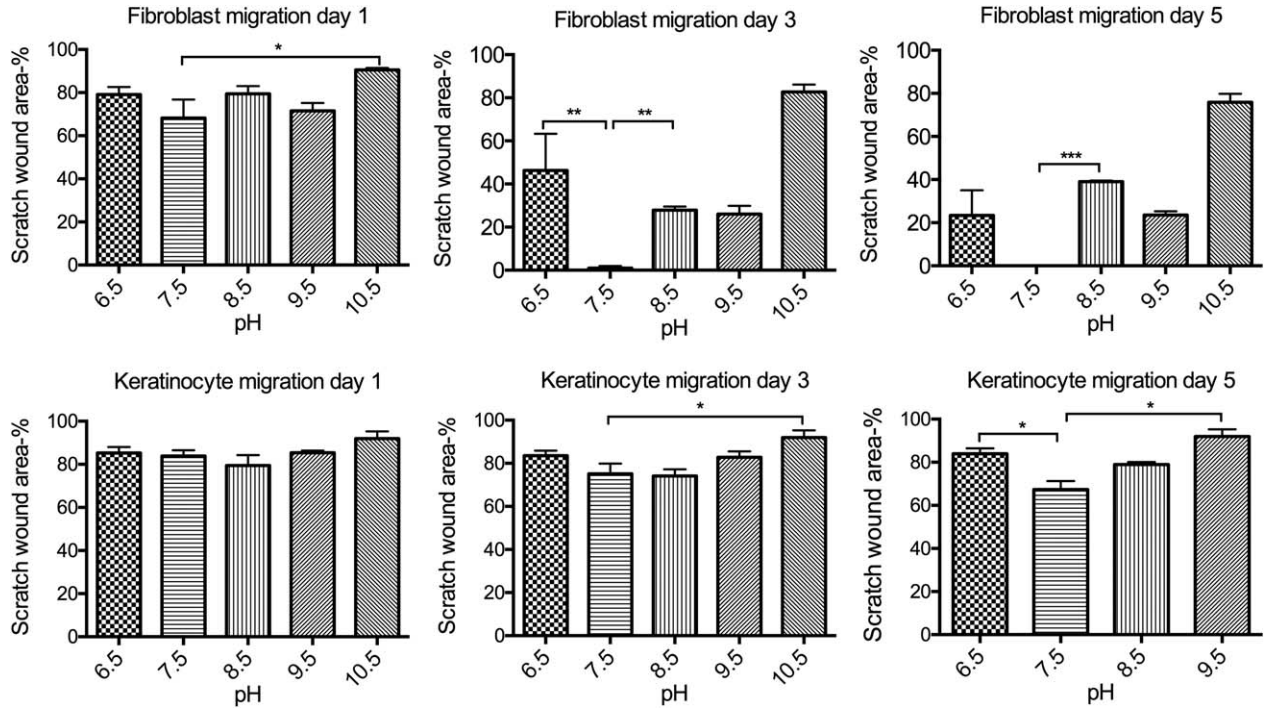


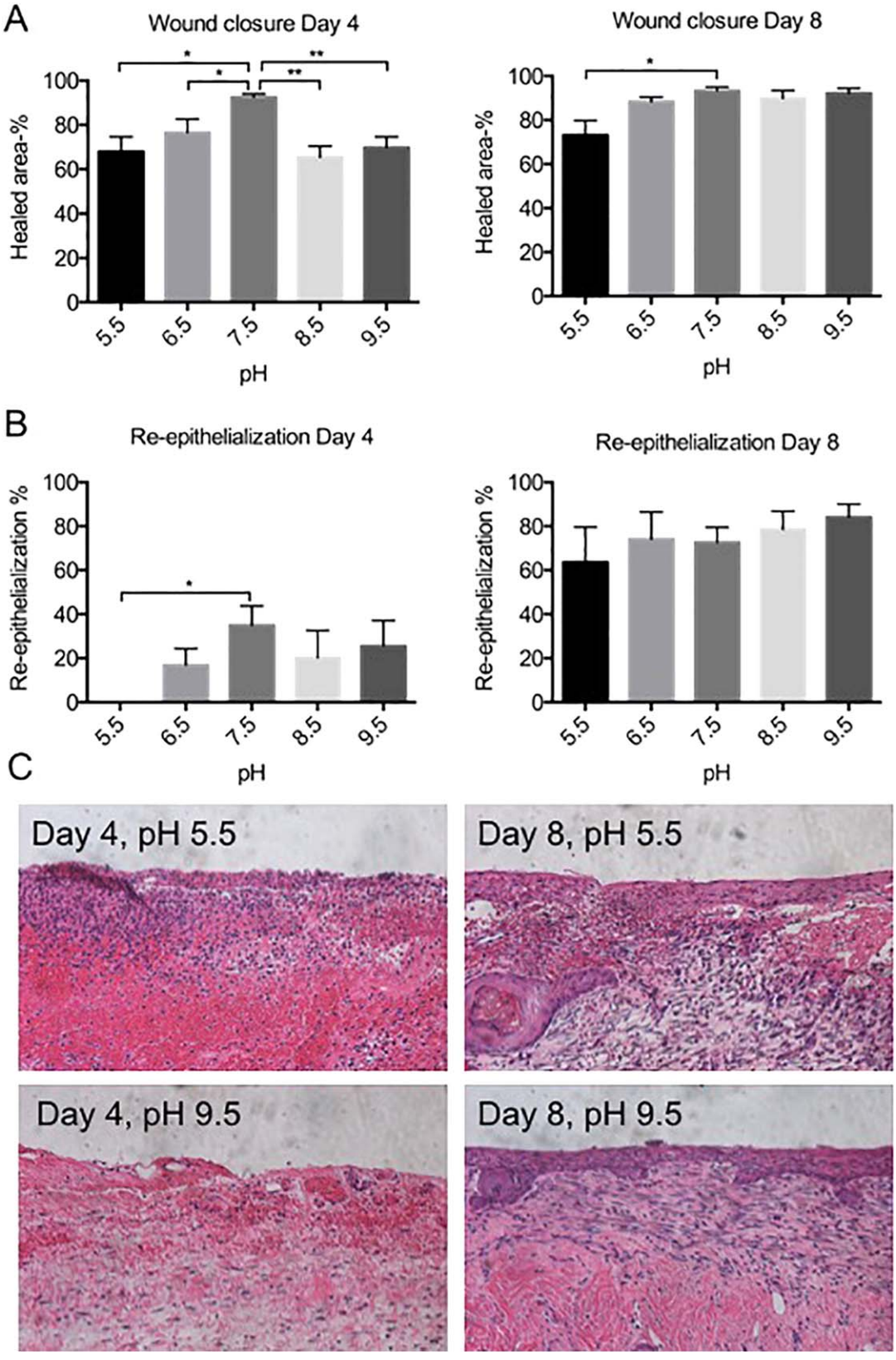
Figure 4. Cell migration: Fibroblasts migrated well when cultured in pH levels from 6.5 to 9.5. Optimal migration for fibroblasts was seen at a pH of 7.5 at day 3 and 5. The best pH for keratinocyte migration was found at a pH of 7.5. Both slightly acidic and alkaline pH conditions decreased migration in fibroblasts and migration was completely inhibited in a pH of 10.5. $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$.

of 5.5, 7.5, or 9.5 on days 1, 4, and 8 (Figure 6A). In addition, the amount of IL-1α was measured from fibroblast culture supernatants (pH: 6.5, 7.5, and 8.5) on days 1, 3, and 5 (Figure 6B). On days 1 and 4 no significant differences in amounts of IL-1 α in the wound fluid were observed among acidic, neutral, and alkaline environments. On day 8, the results indicated significantly elevated ($p < 0.01$) amounts of IL-1α in the acidic wounds in comparison to both neutral and alkaline wounds. Similar findings were observed in the in vitro study. On day 1, the fibroblasts cultured in pH of 8.5 showed elevated ($p < 0.05$) IL-1α amounts compared cells cultured in neutral or acidic (6.5) pH conditions. After 3 days in culture no differences in the IL-1α concentrations were seen but prolonged acidic conditions significantly ($p < 0.05$) increased the expression of IL-1α in fibroblast cultures by day 5 similarly to the wound fluid (Figure 6).

DISCUSSION

In this study, we have investigated the effect of pH on wound healing by studying its effect on human primary keratinocyte and fibroblast migration, proliferation, and viability in vitro and on wound healing in vivo in a rat full-thickness wound model. We have also studied the effect of pH on inflammation during wound healing by measuring IL-1α concentrations from wounds and cell cultures exposed to different pH environments. Table 1 summarizes the effects of different pH conditions on skin cell behavior and wound healing.

Lönnqvist et al. studied the effect of acidic pH on keratinocyte viability and similar to our study, they showed that strongly acidic conditions are detrimental to cells even with limited duration of exposure. Our study showed that both cell types had most optimal migration when cultured in neutral conditions and both increase or decrease in pH decelerated their movement.[17] Lönnqvist et al. also studied keratinocyte migration by culturing them in pH levels of 5, 6, and 7 and their results also indicated that lower pH values prevented cell migration. Similarly, Schreml et al.[18] showed in 2D and 3D in vitro wound healing models that keratinocyte migration and wound closure was impaired in low pH levels. In addition, supporting our results, Lengheden and Jansson[19] showed almost a linear decrease in fibroblasts migration with an increase in pH. The study showed that when culturing the cells in pH ranging from 7.2 to 8.4 cell migration as well as DNA synthesis decreased. Interestingly, our results showed that an alkaline environment seemed to increase cell proliferation. This was especially evident in fibroblasts, which even showed increased proliferation at pH level of 9.5 in comparison to neutral or acidic conditions. Previous studies have also reported that alterations in pH affect cell proliferation but the results are somewhat contradictory.[20] Supporting our findings Schreml et al.[18] and Sharpe et al.[21] have both shown that keratinocyte proliferate better in slightly alkaline conditions. Liu et al.[22] investigated the role of pH on fibroblast proliferation and showed on the contrary to our findings that fibroblasts cultured in an acidic platelet-rich plasma lysate proliferated more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Wound closure Day 4
Healed area-%
100
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20
0
5.5
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Day 8, pH 5.5
Day 4, pH 9.5
Day 8, pH 9.5

Figure 5. (A) Wound closure: The optimal pH condition for wound closure was 7.5. Further, an acidic wound environment (pH < 6.5) decelerated wound closure, whereas an alkaline wound environment (pH > 7.5) did not seem to affect macroscopic wound closure significantly. (B) Reepithelialization: At day 4 wounds treated with a pH of 7.5 were more reepithelialized (35 ± 21%) than wounds treated with a pH above or below 7.5. Further, wounds treated with a pH of 5.5 did not show any reepithelialization at day 4. At day 8 wounds treated with a pH of 8.5 and 9.5 showed a trend of increased reepithelialization compared to wounds treated with a pH of 5.5. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. (C) Histology (H&E) of the wounds treated with a pH of 5.5 and 9.5 over time. [Color figure can be viewed at wileyonlinelibrary.com]

compared to cells cultured in alkaline conditions. Borsi et al.[20] in turn reported that fibroblasts when exposed to an acidic culture media lose their ability to proliferate. Bumke et al. performed an analysis on gene expression in human dermal fibroblasts upon altering the pH and the serum concentration. They found that expression of genes associated with cell proliferation was decreased when cells were cultured in an acidic environment in comparison to neutral.[23]

Traditionally it has been thought that a slightly acidic environment is beneficial for wound healing and alkaline conditions have been associated with chronic wound healing. Many studies have shown the efficacy of an acidic environment against pathogens but only few studies have investigated the effect of pH on wound healing in itself.[24–26] Lima et al., treated rat wounds with an ascorbic acid cream (10%) and followed wound healing for 14 days. Their results showed that in comparison to control wounds (neutral environment) the ascorbic acid had anti-inflammatory effects by reducing the number of macrophages in the wound. Additionally, they showed increased proliferation of fibroblasts and new vessels when compared to control wounds.[27] In a rat burn model Andrews et al., used 5% acetic acid to topically neutralize alkaline burns. Their results showed that burns treated with acetic acid demonstrated a more rapid return to physiologic pH, increased depth of dermal retention, decreased leukocyte infiltrate, and improved epithelial regeneration when compared with wounds treated with water irrigation.[28]

Our results from the full-thickness rat model indicated that a strongly acidic (pH 5.5) environment clearly prevents wound closure as strongly alkaline conditions do not have an effect on wound closure since even wounds maintained in a pH 9.5 environment healed similar to wounds in a neutral environment. Paralleling the effects on wound closure, our study suggested that an acidic environment

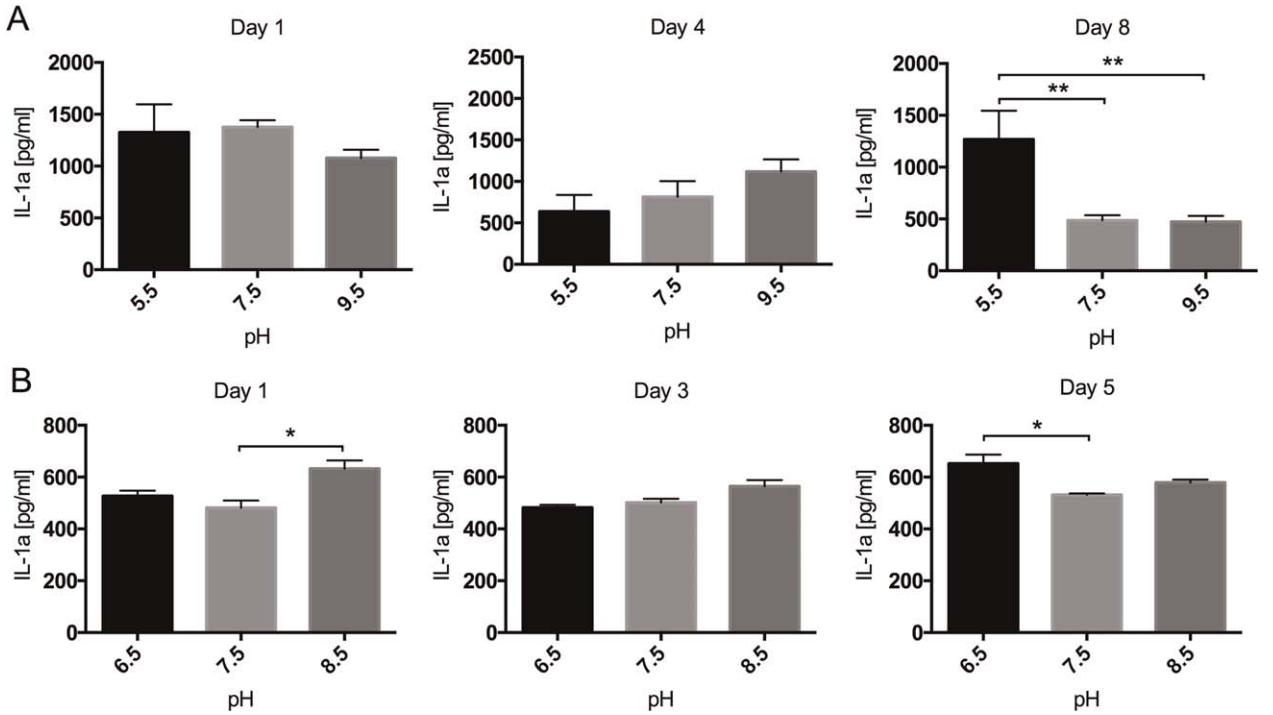

Figure 6. (A) IL-1α level in wound fluid: At day 1 and 4 no statistically significant differences were seen between the level of IL-1α in wound fluid from wounds treated with a pH of 5.5, 7.5, or 9.5. At day 8, the level of IL-1α was significantly higher in wounds treated with a pH of 5.5 compared to wounds treated with a pH of 7.5 or 9.5. (B) IL-1α level in fibroblast supernatant: At day 1 and 3 no statistically significant differences were seen between the level of IL-1α in the supernatant of fibroblasts treated with a pH of 6.5, 7.5, or 8.5. At day 8, the level of IL-1α in the supernatant from fibroblasts treated with a pH of 6.5 was significantly higher than in the supernatant from cells treated with a pH of 7.5. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

Table 1. Summary of the effects of pH on primary cell function and wound healing.

Function	Effect	Figure
In vitro		
Cell viability	Both cell types tolerated wide range of pH (6.5–10.5). Outside of this range the cells die. The best pH for fibroblast viability was 8.5 and for keratinocytes	See Figure 2
Cell proliferation	Alkaline environment (pH: 8.5–9.5) increased fibroblast proliferation statistically significantly ($p < 0.05$) in comparison to neutral pH. Similar trend was observed in keratinocytes.	See Figure 3
Cell migration	Fibroblasts and keratinocytes migrated best at neutral pH and both acidic and alkaline pH conditions decreased migration of the cells.	See Figure 4
In vivo		
Wound closure	The best pH condition for wound closure was 7.5. Strongly acidic wound environment ($pH < 6.5$) decelerated wound closure, whereas an alkaline wound environment ($pH > 7.5$) did not negatively affect wound closure.	See Figure 5A
Wound reepithelialization	No statistically significant differences were seen between the different pH conditions at the end of the experiment (day 8). A trend was seen showing that an acidic environment could decelerate and an alkaline environment could increase reepithelialization.	See Figure 5B

($pH < 7.5$) decelerated reepithelialization and alkaline environments did not have a negative effect on wound reepithelialization. Moreover, a trend was seen showing that alkaline conditions could even increase reepithelialization. IL-1α measurements from the wound fluid and cell culture supernatant showed that over time prolonged acidic environment increased the amount of IL-1α and interestingly a prolonged alkaline environment did not. These findings suggest that a prolonged acidic environment could increase the inflammation in the wound that could further explain the decreased wound closure and reepithelialization observed in the wounds treated with a pH below 6.5. However, these results are preliminary and more research is needed to confirm the connection between an acidic environment and inflammation. In acute wounds IL-1α is highly expressed immediately after wounding and during the inflammatory phase,[29,30] but the level drops dramatically as the wound begins to heal. A persisting high level of IL-1α has previously been found in chronic wounds, suggesting that a continuous high expression of IL-1 α prevents the wound from healing.[31]

Based on our literature review, this is the first study that has investigated the role of pH on wound healing in a controlled fashion both in vitro and in vivo. In conclusion, our result showed that both skin cell types tolerated wide range of pH very well. They also showed that both acidic and alkaline environments decelerated cell migration in comparison to neutral environments and interestingly alkaline conditions significantly enhanced cell proliferation. Results from the in vivo experiments indicated that a prolonged strongly acidic wound environment prevents both wound closure and reepithelialization as a prolonged alkaline environment did not have any negative impact on wound closure or reepithelialization (Table 1). Besides, both in vitro and in vivo studies showed that prolonged acidic conditions significantly increased the expression of IL-1α in fibroblast cultures and in wound fluid. Prolonged alkaline conditions did not result in elevated amounts of IL-1α.

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Conflict of Interest: All authors declare no conflict of interest.

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