An ex vivo porcine skin model to evaluate pressure-reducing devices of different mechanical properties used for pressure ulcer prevention

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Pressure ulcers are complex wounds caused by pressure- and shear-induced trauma to skin and underlying tissues. Pressure-reducing devices, such as dressings, have been shown to successfully reduce pressure ulcer incidence, when used in adjunct to pressure ulcer preventative care. While pressure-reducing devices are available in a range of materials, with differing mechanical properties, understanding of how a material's mechanical properties will influence clinical efficacy remains limited. The aim of this study was to establish a standardized ex vivo model to allow comparison of the cell protection potential of two gel-like pressure-reducing devices with differing mechanical properties (elastic moduli of 77 vs. 35 kPa). The devices also displayed differing energy dissipation under compressive loading, and resisted strain differently under constant load in compressive creep tests. To evaluate biological efficacy we employed a new ex vivo porcine skin model, with a confirmed elastic moduli closely matching that of human skin (113 vs. 119 kPa, respectively). Static loads up to 20 kPa were applied to porcine skin ex vivo with subsequent evaluation of pressure-induced cell death and cytokine release. Pressure application alone increased the percentage of epidermal apoptotic cells from less than 2% to over 40%, and increased cellular secretion of the pro-inflammatory cytokine TNF-alpha. Co-application of a pressure-reducing device significantly reduced both cellular apoptosis and cytokine production, protecting against cellular damage. These data reveal new insight into the relationship between mechanical properties of pressure-reducing devices and their biological effects. After appropriate validation of these results in clinical pressure ulcer prevention with all tissue layers present between the bony prominence and external surface, this ex vivo porcine skin model could be widely employed to optimize design and evaluation of devices aimed at reducing pressure-induced skin damage.

INTRODUCTION

Over 2.5 million people in the USA develop pressure ulcers every year, and worryingly this figure includes 20% of patients hospitalized for non-related conditions. These complex wounds are caused by injury as a result of pressure- and shear-induced trauma to the skin and underlying tissues. Current treatments costs are in excess of $11 bn/year in the US.[1] Therefore, there remains a critical need to develop preventative methods to protect the skin from pressure-induced injury. One approach has been to develop pressure-relief dressings or padding, applied as an adjunct to pressure ulcer prevention. However, understanding of the specific mechanical properties required to protect the skin remains limited.

Consensus panel recommendations suggest that prophylactic dressings, which include gel pads, can reduce pressure, shear, and manage the microclimate of the skin.[2] Of the currently published studies, Call et al. evaluated commercially available dressings in a shear displacement skin analogue model, where the amplitude of shear stress and friction was reduced by varying degrees.[3] Moving to the clinic, Santamaria et al. report a 10% reduction in ulcer formation incidence in a large randomized controlled trial testing the application of Mepilex Border, a silicone bordered dressing, to the sacral

region and heel of intensive care patients.[4] An easy-to-apply, washable silicone pad (KerraPro[TM]) reduced the development of pressure ulcers in a series of case studies,[5] while another gel-like pad (Aderma[TM]), designed to mimic fatty tissue prevented re-ulceration in a nursing home pilot prevention program.[6] While these studies show promise, it remains unclear how the skin-protection efficacy of pressure-reducing devices is linked to their mechanical properties, or even what the ideal properties of a pressure relieving dressing would be.

An ongoing problem is the scarcity of appropriate biological experimental models to compare device mechanical properties to efficacy. Bronneberg et al. previously introduced a simple culture model, where a contact pressure of 13 kPa (100 mmHg) was applied to the commercial skin equivalent EpiDerm for 24 hours. They reported cell swelling, cell toxicity and release of the inflammatory cytokines, Interleukin-8 (IL-8), and tumor necrosis factor alpha (TNFα).[7,8] While EpiDerm resembles human epidermis, it is expensive and remains an artificially reconstructed epidermal substitute that lacks full skin structural properties. By contrast, ex vivo porcine skin is morphologically very similar to human skin, contains full skin architecture and stratification, and is readily available. Moreover, the secretion of inflammatory cytokines, e.g., IL-8, is more comparable than in murine skin, as there is a direct ortholog in pig skin but no homolog in murine skin.[9]

Here we report the results of a study designed to investigate the relationship between mechanical properties of gel-like pressure-reducing devices and their efficacy at protecting from pressure-induced cellular damage using an ex vivo porcine skin model. We show that dressing mechanical properties influence ex vivo efficacy, with a stiffer dressing providing protection across a range of compressive forces.

MATERIALS AND METHODS

Ethical considerations

Human skin was obtained with patient consent and all work performed on human skin was ethically approved (13/SC/0499). Porcine skin was collected from animals slaughtered in accordance to European EEC export standards. No experimental procedures were performed on live animals.

Mechanical testing

Porcine abdominal skin was obtained from female large white pigs of 6–8 months age within 15 minutes after sacrifice. The skin was then shaved with a surgical prep razor, cleaned with alcohol wipes, and transported in Hank's buffered salt solution, on ice. Human abdominal skin was obtained from a female subject undergoing breast reconstruction surgery at the University Hospital of South Manchester (UK). Skin was cut into roughly 5 cm × 5 cm pieces and the subcutaneous fat was cut away. Two self-adherent, gel-like pressure-relieving devices were tested and compared in this study; they were silicone-based KerraPro Pressure Reducing Pads Strip, 0.3 cm thickness (Crawford Healthcare Ltd, Cheshire, United Kingdom), and Aderma Dermal Pad Sheet, 0.3 cm thickness (Smith & Nephew Medical Ltd, Hull, United Kingdom).

Skin (2 mm thick) and device samples (KerraPro 3 mm thick, Aderma 4 mm actual thickness) of 6 mm diameter were made using a disposable biopsy punch. Mechanical testing was performed using an Instron 5943 load frame with a 10 N load cell, fitted with Bluehill 2 software (Instron Inc, Norwood, MA). Samples were subjected to cyclic compression of five cycles up to 0.2 N at displacement rates of 0.5, 1, 2, 5, and 10 mm/min and compressive creep up to 0.2 N at a displacement rate of 5 mm/min, and then maintaining a 0.2 N compressive load for 10 minutes. Samples of the pressure-reducing devices [50 mm in length (20 mm test region) and 3 mm in width] were subjected to cyclic tensile strain at five cycles up to 0.2 N at each displacement rate of 5, 10, 20, 40, and 100 mm/min and tensile stress-relaxation with the samples strained up to 0.2 N at a displacement rate of 1 mm/min and the maintaining the set strain for 10 minutes. Analyses of mechanical testing data were performed using OriginPro 8.5 (OriginLab Corporation, Wellesley Hills, MA) (see Supporting Information Figure S1 for full details). The elastic modulus was calculated from the slope of the stress–strain curves on the increasing part of the strain cycle. Energy dissipation measurements were obtained from the stress–strain hysteresis loops. The compressive creep curve was fitted to a sum of two exponential growth curves to yield the estimated limiting strain and two characteristic compression creep time constants. The tensile stress–relaxation curve was fitted to a sum of two exponential decay curves to yield the baseline stress and two characteristic relaxation time constants.

Autoclave of pressure-reducing devices

Pieces (5 × 5 cm) of KerraPro Pressure Reducing Pads Strip, 0.3 cm thickness were subjected to repeated cycles (5, 10, or 20 times) of sterilization using a benchtop autoclave (Prestige Medical).

Ex vivo culture model

Porcine skin pieces were incubated three times in ice-cold PBS containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Sigma, St. Louis, MO) for 15 minutes on ice. After, 18 mm full-thickness biopsies were made using a sterilized cork borer. The skin was cultured on a stack of three absorbent pads (Merk Millipore) topped with a 0.45 μm nylon membrane (Merk Millipore, Bedford, MA) in a well in a 6-well plate (Corning) containing 3 mL DMEM supplemented with 10% FCS and 100 U/mL penicillin, 100 μg/mL streptomycin and 250 ng/mL amphotericin B (all from Sigma). For ex vivo culture of porcine skin under pressure, a system adapted from a previous study[8] was used where a controlled weight was applied perpendicular to the epidermis via a flat surface indenter of 6 mm diameter (see Figure 3C and D). Weights of 0.19, 0.30, and 0.66 N were used to apply pressures of 6.6, 11, and 20 kPa, respectively, for 24 h, at 37°C, in 5% CO_2. Pressure-reducing devices of 6 mm diameter, made using a biopsy puncher, were placed between the indenter and the skin. Control skin was cultured under pressure without any device between the indenter and the skin.

Histological analyses

Samples were fixed in 10% buffered formalin and embedded in paraffin. 6 μm wax sections were subjected to staining with H&E, immunofluorescence for Ki67 or TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining. For detection of Ki67, de-waxed and rehydrated sections were treated with citrate antigen retrieval buffer, blocked with 10% cold water fish gelatin (Sigma), 1% BSA (Sigma), 0.2% Triton X-100 in PBS, incubated with Ki67 antibody (clone MM1, Vector Labs, Burlingame, CA) diluted 1:100 in blocking buffer at 4 °C, overnight, incubated with Alexa 594-conjugated secondary antibodies (A21203, Life Technologies, Grand Island, NY) diluted 1:100 in blocking buffer at RT for 1 hour. TUNEL staining was performed using an In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) according to manufacturer's instructions. Fluorescent images were taken using a digital camera attached to an Olympus BX51 microscope and captured using MetaVue imaging software (Molecular Devices, Sunnyvale, CA). H&E Images were acquired using a [20×/0.80 Plan Apo] objective using the 3D Histech Pannoramic 250 Flash II slide scanner. Images were analyzed using ImageJ software.

ELISA

Culture medium collected after 24 hours was subjected to ELISA for TNFα and IL-8 (Abcam, Cambridge, United Kingdom; ab100756 and ab113352), according to manufacturer's protocol.

Statistical analyses

Mean and standard error were calculated for each data set from replicates. All experiments were performed with an n of at least three biological replicates in the porcine skin ex vivo model or three different biopsies in mechanical tests. Data were evaluated using nonparametric Mann–Whitney (t-tests). Data from the TUNEL experiment was evaluated using One-way ANOVA with Dunnett's post hoc test. The p values are expressed in the figure legend where applicable.

RESULTS

Mechanical characterization of pressure-reducing devices

Two comparable gel-like pressure-reducing devices (KerraPro Pressure Reducing Pad and Aderma Dermal Pads) were selected for this study. Both KerraPro and Aderma were highly pliable, resisting tensile and compressive forces without breaking. As the devices would be used to redistribute pressure on the skin, their compressive properties were first analyzed. Discs (of 6 mm in diameter) of each dressing were prepared. The 6 mm discs were subjected to cyclic compression tests between two flat surfaces (Figure 1A and B), which revealed that KerraPro was significantly stiffer, by two-fold, than Aderma at all the displacement rates tested (Figure 1C). Cyclic compression loading-unloading tests revealed that KerraPro experienced significantly less energy loss (calculated from the hysteresis of loading and unloading stress–strain curves) than

Aderma (Figure 1D), which suggests that there is higher internal friction within the Aderma material and thus higher energy loss. Compression creep tests used 0.2 N force, equivalent to the 6.6 kPa experienced by areas of the body on a hard surface when in supine position. Creep tests revealed that Aderma experienced almost double the strain (28.9%) of KerraPro (14.5%; Figure 1E), reaching statistical significance. Indeed, a constant 0.2 N force applied for 10 minutes led to an additional 1% reduction (0.02 mm) in KerraPro thickness vs. an additional 2% reduction in Aderma thickness (0.08 mm; Figure 1F), with KerraPro reaching the maximum strain more quickly than Aderma (Figure 1G and H).

Tensile strength evaluated by cyclic tensile loading-unloading at increasing displacement rates revealed a stark contrast in tensile elastic modulus between the devices; KerraPro was significantly stiffer (1.4-times the elastic modulus) than Aderma at all the displacement rates tested (Supporting Information Figure S2A). The energy dissipation was quite different for both samples with KerraPro showing an energy loss of around 2% at all displacement rates but this value reduced rapidly with increasing displacement rate reaching very low values at the highest rate (Supporting Information Figure S2B). Tensile stress–relaxation tests revealed that KerraPro maintained significantly higher force (from 0.2 N) and relaxed significantly faster than Aderma, as indicated by the baseline stress and time constant values, respectively (Supporting Information Figure S2C–E).

Mechanical characterization of autoclaved KerraPro

KerraPro, but not Aderma, was uniquely able to withstand sterilization by autoclaving. To assess potential detrimental effects of autoclaving on mechanical properties, compressive mechanical properties of samples from KerraPro autoclaved 5, 10, or 20 times were compared with unautoclaved KerraPro. The only visual change with autoclaving was a change in coloration with increasing number of autoclave cycles (Supporting Information Figure S3A). No significant difference in elastic modulus (calculated from cyclic compression tests) was observed between untreated and autoclaved KerraPro (Supporting Information Figure S3B). However, a clear positive correlation was observed between energy dissipation and number of autoclave cycles (Supporting Information Figure S3C). Compression creep tests revealed no significant differences in the estimated limiting strain or time constants between untreated and autoclaved KerraPro (Supporting Information Figure S3D–F).

Comparison of mechanical properties of human and porcine skin

Macroscopically, human and porcine skin appeared very similar. Excised skin was first trimmed to a thickness of 2 mm, and 6 mm biopsies were made (Figure 2A). The skin was subjected to compression testing placed between two flat surfaces, and hydrated with the addition of PBS (Figure 2B). No significant difference was observed between the compressive elastic modulus of human and porcine skin samples, however, energy loss was significantly lower in human skin (Figure 2C and D).
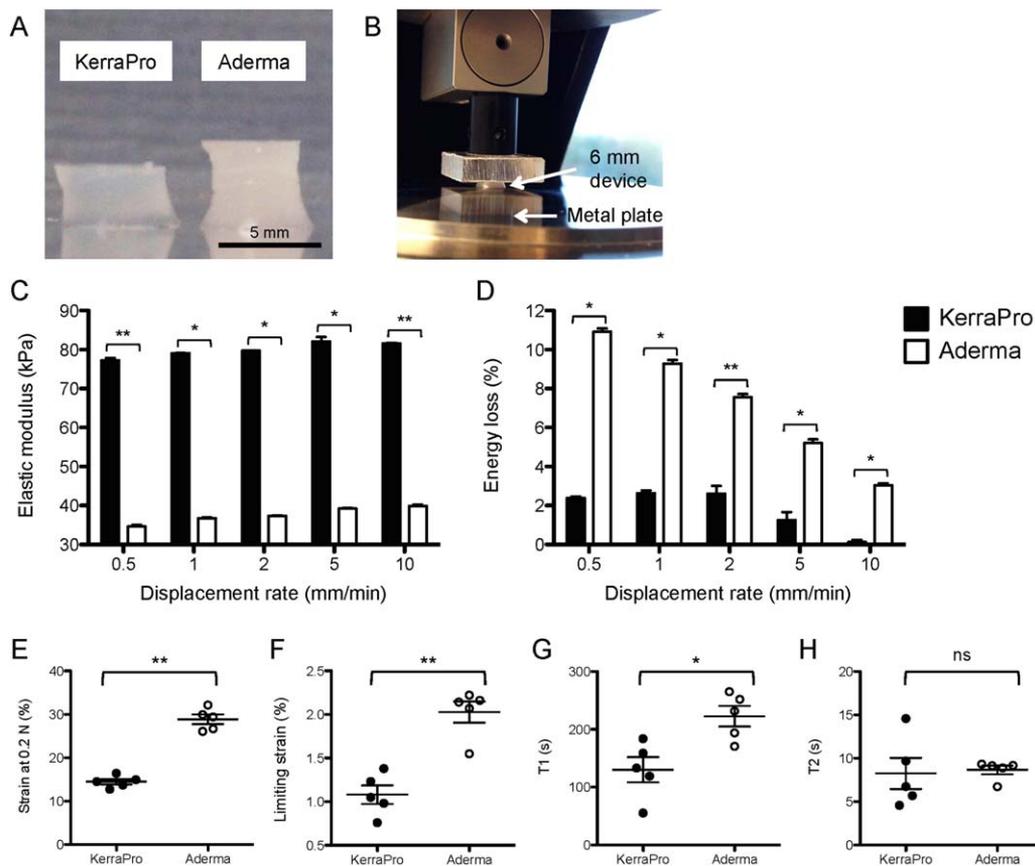
Figure 1. Comparison of compressive properties of pressure-relieving devices. (A) Height (thickness) of 6 mm pressure-relieving devices tested. (B) Device undergoing compression test using an Instron. (C) Elastic modulus calculated from compressive stress-strain curved at increasing displacement rates, up to 0.2 N load shows significant differences between KerraPro and Aderma ($*p = 0.0119$, $**p = 0.0079$). (D) Energy loss was calculated from cyclic compressive loading-unloading tests at different displacement rates, up to 0.2 N load shows significant differences between KerraPro and Aderma ($*p < 0.0286$, $**p = 0.0097$). (E) The strain at the start, (F) maximum change in strain, and time constants, (G) T1 and (H) T2, were calculated from compressive creep tests, where 0.2 N load was applied to the material at 5 mm/min, and then the force was maintained for 10 minutes ($*p = 0.0159$, $**p = 0.0079$).

Compression creep tests also revealed no significant difference in estimated limiting strain or time constants between human and porcine skin (Figure 2E–H). The skin is an important tissue layer for pressure ulcer development. Collectively, these data validate porcine skin as mechanically comparable to human skin.

Porcine skin survival ex vivo

Here we describe an ex vivo porcine skin culture model. To first test viability without mechanical loading, skin samples were collected at 0, 1, 2, and 3 days post culture. Tissue viability was assessed by histological quantification of proliferative cells and apoptotic cells indices. The number of Ki67-positive proliferative cells in the epidermis declined with increasing culture time, from 50 cells/mm at day 0 to 20 cells/mm after 3 days in culture (Figure 3A). By contrast, dermal Ki67 positive cells remained relatively constant across all time points examined (Figure 3A). Quantification of apoptosis was performed by terminal deoxynucleotidyl

transferase dUTP nick end labeling (TUNEL) assay, which is an established method for detecting DNA fragments characteristic of cell death or apoptosis.[10] The staining revealed low levels of epidermal TUNEL-positive cells at all time points but increasing dermal apoptosis over time (Figure 3B). Collectively, these data suggest that the ex vivo model porcine skin model was viable for the full 3 days in culture. For subsequent analysis in this manuscript we have used culture time of no longer than 24 hours.

Ex vivo model of pressure application to porcine skin

Porcine skin was cultured and subjected to surface pressures between 0 and 20 kPa across a 6 mm diameter area with no device (control), with KerraPro or Aderma (Figure 3C). After 24 hours the skin exposed to pressure exhibited visible surface indentations both macroscopically (Figure 3D), and histologically (Figure 3E). Quantification of apoptotic keratinocytes revealed a marked increase in epidermal cell death, even at the lowest pressure application (6.6
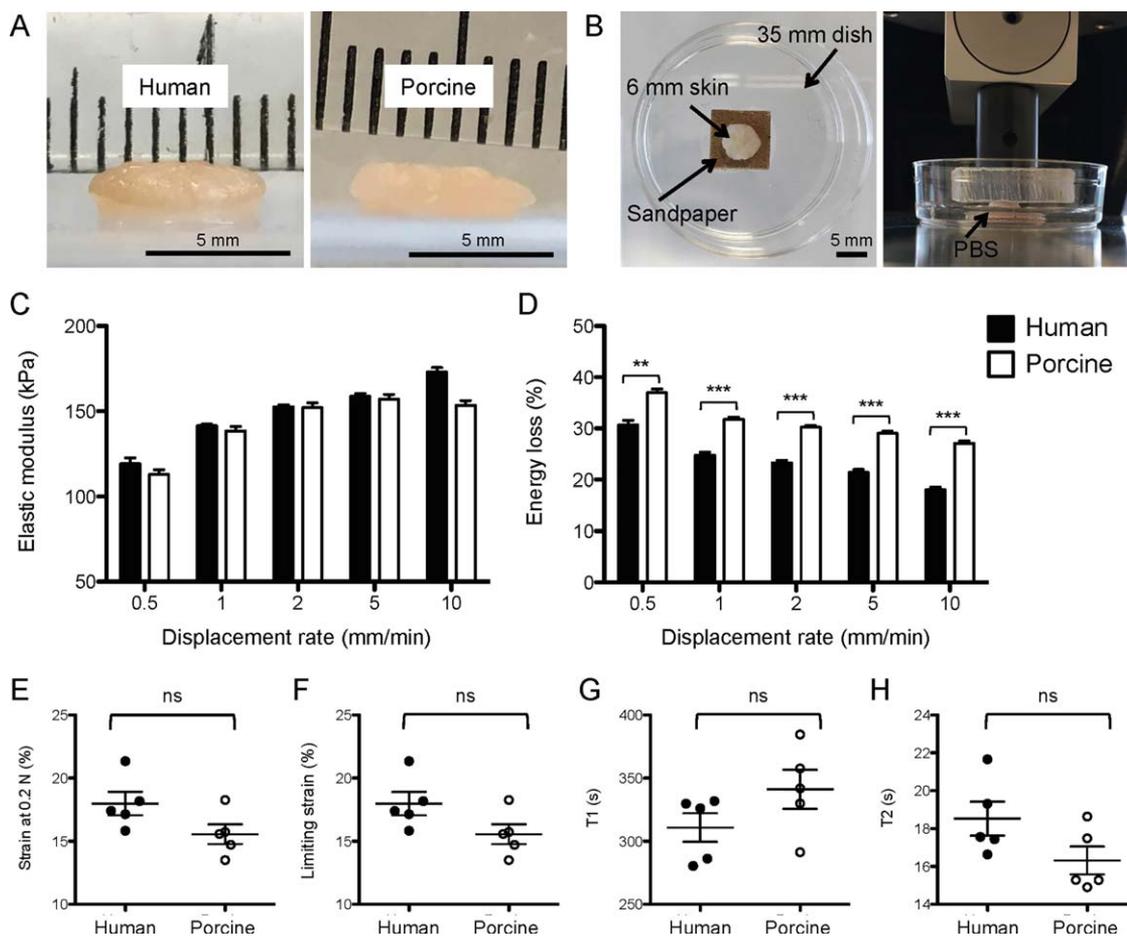
Figure 2. Comparison of compressive properties of human and porcine skin. (A) Height (thickness) of 6 mm human and porcine skin. (B) Set up for skin biopsy in PBS undergoing compression test using an Instron. (C) Elastic modulus calculated from compressive stress–strain curved at increasing displacement rates, up to 0.2 N load shows no significant differences between human and porcine skin. (D) Energy loss was calculated from cyclic compressive loading-unloading tests at different displacement rates, up to 0.2 N load shows significant differences between human and porcine skin ($**p < 0.0005$, $***p < 0.0001$). (E) The strain at the start, (F) maximum change in strain, and time constants, (G) T1 and (H) T2, were calculated from compressive creep tests, where 0.2 N load was applied to the material at 5 mm/min, and then the force was maintained for 10 minutes.

kPa) in control skin, an effect that was abrogated by both KerraPro or Aderma treatments (Figure 4A). At all three pressures tested (6.6, 11, and 20 kPa) both as KerraPro and Aderma significantly reduced epidermal apoptosis vs. control (Figure 4B). At the highest pressure tested, KerraPro was significantly more effective at preventing epidermal cell death than Aderma.

KerraPro was more effective than Aderma in reducing pressure-induced cell death. To test if inclusion of KerraPro could also reduce the release of inflammatory cytokines, secreted TNFα and IL-8 were quantified after 24 hours of culture. Little TNFα (<4 pg/mL) was detected in porcine skin samples that were not subjected to pressure, however, this increased to greater than 10 pg/mL when high pressure was applied to control samples (Figure 4C). In the presence of KerraPro skin was protected from TNFα induction following application of pressure compared to control, with significantly reduced TNFα secretion (Figure

4C). By contrast, IL-8 was readily detectable in the medium of porcine skin cultures, however, the levels were not significantly raised when pressure was applied and no significant differences were observed between control and KerraPro-treated skin (Figure 4D).

DISCUSSION

Here we report a side-by-side comparison of the biological efficacy of two pressure-reducing devices comprising materials with different mechanical properties. Using a new porcine skin ex vivo model we report quantitative evaluation of direct effects on epidermal keratinocytes, where dressings significantly reduced pressure-induced cell death and pro-inflammatory cytokine release. Moreover, we report differences in the protective capacity of dressings linked to differing mechanical properties. This study
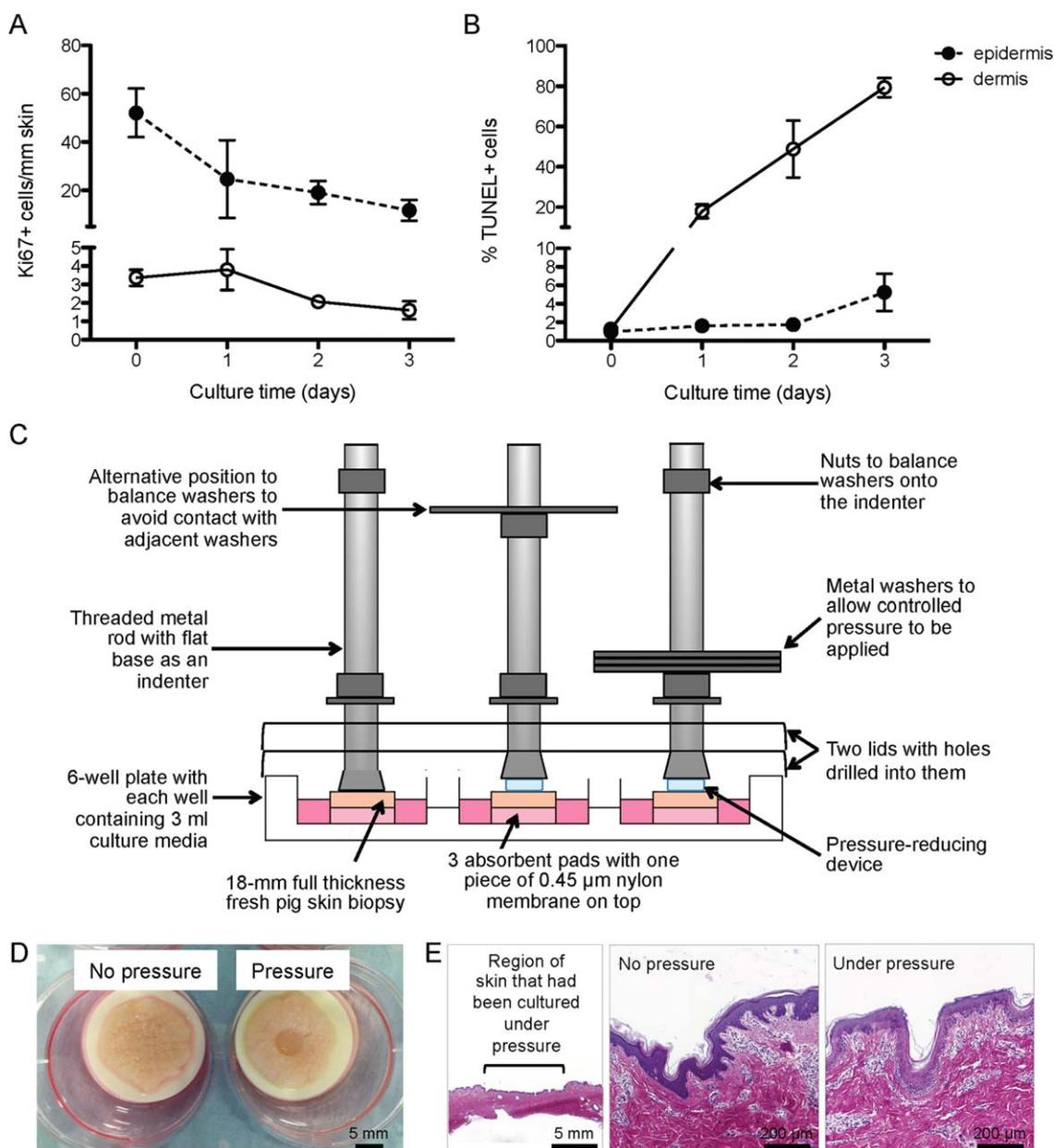
Figure 3. Survival of porcine skin in ex vivo model. (A) The number of Ki67-positive cells and (B) the percentage of TUNEL-positive cells in the epidermis and dermis of porcine skin in culture for up to 3 days. (C) Schematic showing side view of 6-well plate set up to culture 18 mm porcine skin biopsies under controlled pressures using indenters and weights. (D) Photograph showing 18 mm porcine skin that had been in culture for 24 hours under 0 and 20 kPa pressure. (E) Corresponding H&E stained on a transverse section indicating the region of porcine skin that had been cultured under pressure. Representative higher magnification images illustrate porcine skin cultured with 0 or 20 kPa in the absence of a pressure-reducing device.

is the first to compare pressure-reducing devices in a skin culture model.

As the mechanical properties and structural features of porcine skin closely mirror human skin, these results represent an important step toward understanding the response of isolated human skin to pressure. Clearly this model is limited to the epidermal and dermal layers of the skin and therefore further research into the subcutaneous fat and other tissue layers and their role in pressure redistribution is required. Additionally, this model does not address the contribution of the systemic inflammatory response, although local inflammatory cells will be present. An important next set will be to correlate the results reported here with an appropriately designed clinical comparison on common
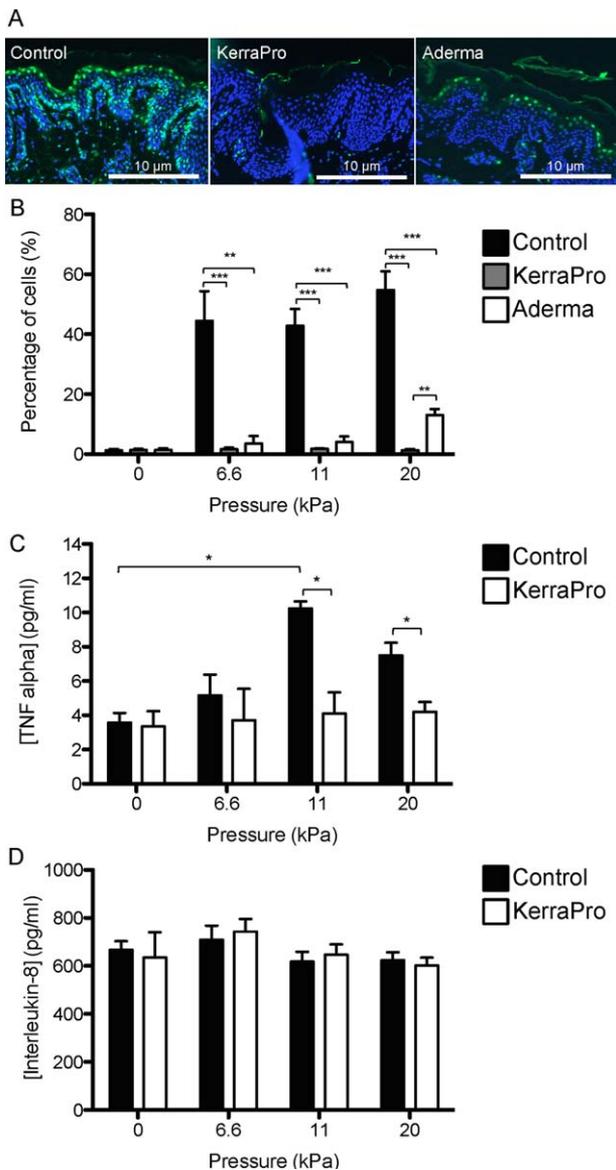
Figure 4. Evaluation of efficacy of pressure-relieving devices. (A) Images showing TUNEL-positive cells in porcine skin that had been cultured under 20 kPa pressure for 24 hours without (control) and with KerraPro or Aderma. (B) Percentage TUNEL-positive epidermal cells 24 h after culture following application of pressure alone, or pressure combined with KerraPro or Aderma (compared with control **$p = 0.0004$, ***$p < 0.0001$; KerraPro vs. Aderma **$p = 0.0049$). (C) TNF alpha and (D) interleukin-8 concentrations detected in the media of porcine skin cultures 24 h after application of pressure alone pressure plus KerraPro (*$p < 0.0212$).

pressure ulcer sites, either intra-operatively or in high-risk patients.

The ex vivo model we used is an adaptation of that originally described by Bronneberg et al.[7] Our model has

been developed for increased simplicity, reproducibility and increased flexibility in the low pressure range. We use flat-based stainless steel threaded metal rods (6 mm diameter) as the indenter and interchangeable stainless steel washers to apply load via a standard (slightly modified) 6-well culture plate (Figure 3C). Moreover, to support reproducible, high throughput studies we have adapted the model and analysis to porcine skin, a readily available and ethically neutral tissue source. We employed pressures of between 6.6 and 20 kPa in this study. This is in line with pressures of 4–9 kPa, measured in the skin of healthy volunteers in supine position on operating mattresses[11] and 13–17 kPa in elderly patients undergoing hip surgery.[12] Literature suggests pressure induced cell death to be the result of tissue ischemia, with pressures as low as 4 kPa reducing vascular blood flow.[13,14] However, it is now understood that pressure in fact physically distorts tissue creating tensile, shear and compression stresses.[15] These lead to virtually immediate cell death, while hypoxia-induced cell death takes many hours. Surgery times as short as 2.5 hours can also result in direct changes to the skin.[16] Therefore it is likely that cell death in this porcine skin ex vivo model is a direct consequence of pressure exerted via the indenters and not by nutrient restriction.

In a previous study, pressure application to EpiDerm caused cell expansion,[8] however in this current porcine study no cell expansion was observed. Human skin ex vivo subjected to up to 300 kPa pressure also did not display cell expansion.[17] Thus, these studies highlight potential differences between bona fide skin models and those using skin equivalents, which may not replicate the in vivo situation. In this porcine skin model, the TNFα secretion but not IL-8 secretion by epidermal keratinocytes observed in EpiDerm in response to pressure was recapitulated.[8,18] It remains unclear whether IL-8 secretion is also elevated in pressure ulcers in vivo.

KerraPro is durable and washable[5] and in this study it maintained its integrity after repeated autoclaving especially in compressive creep tests. The compressive creep test is clinically relevant as pressure forces are sustained between repositioning of patients, typically every 3 hours.[19] The creep tests also showed significant differences between the materials of KerraPro and Aderma, in particular, it demonstrated that KerraPro experiences very little strain under constant load. This is likely the key mechanical property responsible for the rescue effect observed in pressure-induced cell death and TNFα secretion.

In conclusion, this study is the first to describe a simple and reliable ex vivo model to evaluate the effect of pressure-reducing devices. Using this model we show that mechanical properties of seemingly similar gel-like materials can respond very differently to pressure, while compression creep tests reveal the clinical relevance. With further validation the mechanical testing techniques described here should provide a new modality to evaluate future pressure-reducing devices, while correlative biochemical analyses will allow better prediction of clinical efficacy.

ACKNOWLEDGEMENTS

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Supporting Information

Additional Supporting Information may be found in the online version of this article.