Hyperbaric oxygen stimulates epidermal reconstruction in human skin equivalents

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Abstract

The crucial role of oxygen during the complex process of wound healing has been extensively described. In chronic or non-healing wounds much evidence has been reported indicating that a lack of oxygen is a major contributing factor. Although still controversial, the therapeutic application of hyperbaric oxygen (HBO) therapy can aid healing of chronic wounds. However, how HBO affects re-epithelisation, involving processes such as keratinocyte proliferation and differentiation, remains unclear.

We therefore, used a 3-dimensional human skin equivalent model to investigate the effects of daily 90-minute HBO treatments on the reconstruction of an epidermis. Epidermal markers of proliferation, differentiation and basement membrane components associated with a developing epidermis, including p63, collagen type IV and cytokeratins 6, 10 and 14, were evaluated.

Morphometric analysis of hematoxylin and eosin stained cross-sections revealed that HBO treatments significantly accelerated cornification of the stratum corneum compared to controls. Protein expression as determined by immunohistochemical analysis confirmed the accelerated epidermal maturation. In addition, early keratinocyte migration was enhanced by HBO.

Thus, HBO treatments stimulate epidermal reconstruction in a human skin equivalent. These results further support the importance of oxygen during the process of wound healing and the potential role of HBO therapy in cutaneous wound healing.

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Introduction

Re-epithelisation is a critical process during the cutaneous wound healing process, involving the migration, proliferation and differentiation of keratinocytes and the remodelling of the basement membrane to restore the integrity of the affected site.[1] In non-healing or chronic wounds this process is limited by a number of contributing factors, including local tissue hypoxia caused by limited supply of vascular oxygen.[2,3] Under hypoxia, as was demonstrated in animal studies, the healing of ulcers is delayed as a result of reduced granulation tissue production and delayed epithelialisation.[4]

Besides its role in epithelialization[4], oxygen is also involved in collagen synthesis[5], angiogenesis[6,7] and bacterial killing.[8] Hence, it is regarded as one of the critical nutrients during the reparative process.[9,10] Indeed, the requirement of oxygen during wound healing is the underlying rational for the use of hyperbaric oxygen (HBO) therapy in the treatment of chronic wounds. HBO, usually 100% oxygen inspired at 2.4 atmosphere absolute (ATA), results in higher oxygen concentrations within the capillaries hence increasing the perfusion distance of oxygen within the wounded tissue.[11] Despite findings that show that HBO elevates wound oxygen tension[12], the effectiveness of its clinical application for healing of chronic wounds is still the subject of great debate. While some studies have reported faster healing of chronic ulcers[13,14] and a decreased risk of major amputation,[15] there is still a need to verify possible benefits of HBO therapy in a large randomised controlled/matched trial[16] and relevant human *in vitro* models. Current *in vitro* data on the effect of HBO treatment on cells surrounding the wound, such as fibroblasts[17-20] and keratinocytes[17,21] is limited and inconclusive. For example, Hehenberger *et al.*[19] and Tompach *et al.*[18] observed a dose-dependent effect on fibroblast proliferation of a single hyperbaric treatment after 24 hours,

whereas Dimitrijevich *et al.*[17] only observed a mitogenic effect after prolonged exposure. In addition, the majority of the studies focussing on the effect of HBO on fibroblasts and keratinocytes have been performed, unlike the *in vivo* situation, in a 2-dimensional setting.

Three-dimensional human skin equivalent (HSE) models can provide an environment which is more similar to that found in normal *in vivo* skin, both morphologically and biochemically.[22-24] One such model utilises a human derived de-epidermised dermal scaffold with an intact basal lamina,[25] which contains essential extracellular matrix components for keratinocyte attachment. These models have been successfully used to study wound healing and evaluate treatments for a range of skin repair applications[22,25-31]. In view of this, we have applied this 3-dimensional *in vitro* HSE model to study the effects of daily 90-minute HBO treatments (for up to 5 days) on the reconstruction of an epidermis. We evaluated the influence of HBO on the thickness of the newly formed epidermis, as well as on expression of epidermal protein markers of proliferation, differentiation and the basement membrane that are associated with re-epithelisation and demonstrated that HBO stimulates the formation of an epidermis in a human *in vitro* model.

Materials and Methods

Isolation and culture of keratinocytes

Keratinocytes were obtained from 4 consenting adult patients undergoing routine cosmetic surgery, resulting in a surgical discard, with institutional ethics committee approval and in adherence to the Declaration of Helsinki Principles. Cells were isolated and cultured using a modification of the Rheinwald and Green method[32] as previously described[33]. In brief, pieces of skin were incubated in 0.125% trypsin (Invitrogen, Mulgrave, VIC, Australia) overnight at 4°C to promote separation of the epidermal and dermal layers. Cells were subsequently collected by gentle scraping of the newly exposed dermal and epidermal surfaces into a 10-fold volume of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.), then washed and resuspended in culture medium. The freshly isolated keratinocytes were expanded for one passage (P1) for 7 days on a feeder layer of lethally irradiated 3T3 fibroblasts (ATCC, Manassas, VA, U.S.A.; CCL-92) in keratinocyte culture medium consisting of a 3:1 mixture of DMEM and Ham's F12 medium (Invitrogen) supplemented with 10% FBS, 1 μg mL^{-1} insulin (Sigma-Aldrich, Castle Hill, NSW, Australia), 10 ng mL^{-1} human recombinant EGF (Invitrogen), 180 μM adenine (Sigma-Aldrich), 0.1 μg mL^{-1} cholera toxin (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), 0.4 μg mL^{-1} hydrocortisone (Sigma-Aldrich), 0.01% v/v non-essential amino acids solution (Invitrogen), and 1% v/v penicillin/streptomycin solution (Invitrogen). Culture medium was replaced every 2-3 days.

Preparation of dermal equivalent (de-epidermised dermis)

The dermal equivalent was prepared as previously described[34] with slight modifications. Briefly, 1-2 cm pieces of skin were incubated in 1 M sodium chloride (Sigma-Aldrich) at

37°C for 12 hours. The epidermis was removed using forceps, leaving behind the de-cellularised de-epidermised dermis (DED). This DED underwent several subsequent washes in culture medium before use.

Generation of skin equivalent and composite culture

The DED pieces were trimmed to approximately 1.4 cm x 1.4 cm and placed papillary side up in a 24-well culture plate. Sterile stainless steel rings (Aix Scientifics, Aachen, Germany) with a silicone washer base and a diameter of 7 mm were placed on top of the DED.[22,35] To each ring, 1.9 x 10^4 keratinocytes (P1), suspended in 200 μL of culture medium, were added and the DEDs were incubated at 37°C and 5% CO_2. After 1 day the rings were removed and the HSEs (dermis plus keratinocytes) were elevated to the air-liquid interface by transferring the composites to a stainless steel grid in a 6-well culture plate maintained in keratinocyte culture medium at 37°C and 5% CO_2 for up to 5 days. Each test was performed in triplicate and 3 independent replicates of the experiment were performed.

Hyperbaric oxygen treatments

HSEs were treated daily for up to 5 days in a temperature and humidity controlled custom-made 7-litre hyperbaric unit (Fink Engineering, Cheltenham, VIC, Australia). The metal chamber was sealed and flushed for 2 minutes with oxygen and subsequently the pressure was increased to 2.4 atmosphere absolute (ATA). The pressure was maintained for 90 minutes, after which the chamber was slowly de-pressurized over 5 minutes. Controls were treated for 90 minutes with air at 1 ATA under similar experimental conditions.

Migration measurements

Keratinocyte migration was examined by measuring the increase in surface area of the reconstructed epidermis. Samples were harvested after 0, 3 and 5 days of culture at the air-liquid interface (n = 3 per time point) and stained with 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to visualize the epidermis macroscopically. This involved submerging the samples in 6 mL of 0.5 mg mL^{-1} MTT dye (Sigma Aldrich) and incubating the samples at 37°C for 90 minutes. Subsequently, the stained reconstructed epidermis was photographed (Nikon Coolpix 4500; Maxwell Optical, Lidcombe, NSW, Australia) and the area (cm^2) of migration was quantified (3 independent measurements) using Scion Image software (Scion Corporation, Frederick, MD, U.S.A.).

Histology

Samples (n = 3 per time point) were taken after 0, 3 and 5 days of culture at the air-liquid interface, fixed in 10% formalin and dehydrated in a graded ethanol series. Samples were then embedded in paraffin and cut to yield 5 μm thick cross-sections. Sections were stained with hematoxylin and eosin (H&E; Sigma-Aldrich) for further histological analysis. Thickness of the cornified and viable epidermis was determined from 4 consecutive images acquired in the centre of the reconstructed dermis using a digital camera (Nikon DXM 1200; Maxwell Optical) mounted on a microscope (Leitz Laborlux S; Leica Microsystems, Gladesville, NSW, Australia). The average thickness (μm) of the layers was measured using Scion Image software (Scion Corporation) by averaging 10 measurements per image.

Immunohistochemistry

For immunohistochemical analysis, paraffin sections were cut and deparaffinised in ethanol and xylene. After incubation with primary anti-bodies for either p63 (1:100), cytokeratin 6 (1:1), cytokeratin 14 (1:20), cytokeratins 1/10/11 (1:400 RDI Research Diagnostics, MA,

U.S.A.), or collagen type IV (1:10, Developmental Studies Hydroma Bank, Iowa City, IA, U.S.A.), sections were stained using a diaminobenzidin (DAB) DAKO Envision kit (Dakocytomation, Botany, NSW, Australia) according to manufacturers' instructions. All sections were counterstained with Mayer's hematoxylin and examined by light microscopy. Immunostainings leaving out the primary antibody were used as negative control.

Statistics

All measurements are expressed as mean ± standard deviation. Statistical significance was determined using a paired Student *t*-test. A value of $p < 0.05$ is considered significant.

Results

In determining the effect of HBO treatment on the formation of re-epithelialisation, human skin equivalents (HSEs) were HBO-treated in a laboratory scale HBO unit according to regimes similar to that routinely used for clinical treatment of non-healing wounds.[11]

Accelerated epidermis formation in human skin equivalents treated with HBO

To evaluate the effect of HBO on the reconstruction of an epidermis, dermal substrates were seeded with expanded keratinocytes and kept submerged for 24 hours to allow initial cell attachment and expansion. At transfer to the air-liquid interface (day 0) a thin epidermal layer, 2-3 cell layers thick, had developed (Figure 1a). The thickness of the layer gradually increased and acquired an epidermal morphology during the course of culture (Figure 1b, 1c). The morphological differences expected between the basal and uppermost layers were observed for cultures established under all conditions. However, HBO-treated constructs displayed trends towards greater thickness and degree of maturation of the upper layer (better developed stratum corneum (SC)) when compared to control cultures. Thus, a distinct SC layer was not observed until after 5 days in samples cultured under control conditions, whilst a SC was already noted within 3 days in the HBO-treated samples (Figure 1d). In addition, with the HBO-treated constructs the number of SC layers had further increased and a basket weave-like appearance was observed by day 5 (Figure 1e).

Image analysis of cross-sectioned composites stained with hematoxylin and eosin (H&E) revealed that the reconstructed epidermal layer in HBO-treated samples was significantly thicker ($p<0.05$, paired two tailed Student *t*-test) at both day 3 (mean ± SD; 33.7 ± 0.7 μm and 81.4 ± 18.0 for control and HBO, respectively) and day 5 (mean ± SD; 65.0 ± 14.2 μm and

113.2 ± 17.9 for control and HBO, respectively) (Figure 1e). In addition, the SC layer was also found to be significantly greater after both 3 (mean ± SD; 1.7 ± 0.8 μm and 10.2 ± 3.0 for control and HBO, respectively) and 5 days (mean ± SD; 8.3 ± 1.2 μm and 19.9 ± 6.9 for control and HBO, respectively), whereas additional samples treated daily for 90 min, either with 100% oxygen at 1 ATA or air at 2.4 ATA, were not found to be significantly different from the controls (air at 2.4 ATA) (results not shown).

Enhanced proliferation and differentiation in human skin equivalents treated with HBO

Immunoperoxidase staining with a monoclonal antibody raised against the nuclear transcription factor p63, a marker of undifferentiated proliferating cells,[36] revealed the presence of clonogenic cells shortly after the establishment of the model (Figure 2). At day 0, sporadic clusters of p63 positive cells were observed within the basal epithelial layer and p63-specific staining further intensified after 3 and 5 days. The majority of cells, located within the suprabasal and basal layers of the HBO-treated epidermal constructs, stained positive for p63 at day 3, but at day 5 expression was decreased and was limited to the deeper basal layers.

Immunoperoxidase staining with a monoclonal antibody raised against cytokeratins 1, 10 and 11 (K1/10/11) revealed enhanced epidermal differentiation[37] within the HBO-treated constructs (Figure 2). Differentiated squamous cells were found to be present within in the uppermost layer of HBO-treated constructs after 3 days, while they could not be detected until after 5 days within the control constructs.

Deposition of the basal cell marker cytokeratin 14 (K14)[37] could not be detected after 3 days (Figure 2). After 5 days, only faint staining for K14 was observed in the basal layers of the control samples (arrows; Figure 2). In contrast, when HSE constructs were exposed to daily

HBO treatments, staining was observed throughout the basal layer of the regenerated tissue at day 5, indicative of the more advanced maturity of the HBO-treated tissue.

Correlation between proliferation and lateral migration of keratinocytes

After removal of the silicone washer rings at day 0 the cells migrated from the centre seeding region to the edge of the dermal substrates regardless of experimental conditions (Figure 3). The migrating edges of the reconstructed epidermis demonstrated an intense staining compared to the centre after incubation in MTT solution, indicative of a high metabolic activity within the outer regions. At day 5 the expression of the wound healing-associated marker, cytokeratin 6 (K6),[38,39] was more pronounced at the peripheral boundaries in comparison to the centre of both HBO-treated and control cultures (Figure 3a-3d). Interestingly, in line with the quantification of the overall epidermal thickness (Figure 1B), the layer of K6 expressing cells appeared thicker within HBO-treated samples (Figure 3b and 3d). Additionally, after 3 days of culture at the air-liquid interface, the populated surface area of the dermal scaffolds was significantly greater ($p<0.05$, paired two tailed Student *t*-test) for the HBO-treated samples compared to the controls (Figure 3e; mean ± SD, 0.46 ± 0.03 cm^2 and 0.58 ± 0.06 cm^2 for control and HBO, respectively). Although a similar situation was observed after 5 days, this was not significant.

Basement membrane remodelling is stimulated in human skin equivalents treated with HBO

Immunoperoxidase staining with a type IV collagen antibody revealed the presence of an intact basement membrane in control samples without seeded keratinocytes (Figure 4a) and in non-epithelialised areas of the dermal scaffold at all time points (not shown). In contrast, in regions of control samples where a new epidermis was formed, there was minimal collagen type IV staining within the basal membrane region after days 3 (Figure 4b, 4d). After 5 days,

minimal staining was observed in the control samples (Figure 4c), whilst collagen type IV had been re-deposited in the regions of re-epithelialisation of HBO-treated HSEs (arrows; Figure 4e).

Discussion

The effect of hyperbaric oxygen therapy on the reconstruction of an epidermal layer on dermal substrates was evaluated to further understand the critical role of oxygen during the process of wound repair. In this study we utilised a 3-dimensional human skin equivalent (HSE) model to evaluate the effects of daily HBO treatments and employed a protocol similar to that used to clinically treat chronic wounds. In particular, we focussed our investigations on the reconstruction of the epidermis. We have demonstrated that HBO treatment markedly modulates epidermal formation. In addition, we also report, for the first time, that these changes in epidermal formation are supported by differences in the markers of proliferation, differentiation and basement membrane components.

The benefits of HBO in wound healing are not without controversy.[16] Previously, toxic effects of HBO treatments on 2-dimensional (2D) primary human keratinocytes cultures[17,21] and cell lines (HaCaT)[40] have been observed. This phenomenon is most likely to be due to the increased oxidative stress generated by hyperoxia.[40] However, this response may be misleading and non-predictive for the *in vivo* response, since the resistance of 2D cultured skin cells to oxidative stress is markedly decreased as compared to 3D cultured cells.[41] Benefits of HBO therapy have, on the other hand, been demonstrated using various animal models, including an ischemic rabbit ear ulcer model[4] and mice models.[42] Nevertheless, these models are generally not considered optimal for studying human wound healing.[43]

HSE models can provide an *in vitro* 3-dimensional environment, possessing many of the properties of *in situ* skin, for studying wound healing. Preliminary data using a collagen gel-based HSE model suggested that daily HBO treatment (90 minutes at 2.0 ATA) over 10

consecutive days stimulates cornification of the epidermis.[17] For the current studies we have utilised a HSE based on a dermal scaffold; this model possesses significant advantages over other scaffolds [44]. Specifically, it is composed of an acellular dermal matrix and has an intact basement membrane, elements that have been shown to be important for the adherence of the epidermis to the dermis and for the differentiation of keratinocytes.[45,46] Hence, the epidermis formed on these scaffolds has a high degree of similarity to the epidermis *in vivo*, with the three main regions clearly visible; a rapidly proliferating basal layer, a differentiating suprabasal layer and an uppermost stratified cornified layer.[22,25] Indeed, on the air control dermal scaffolds there was a visible cellular layer after 3 days with what appeared to be a developing cornified layer. In the absence of HBO treatments, this layer further matured after 5 days and a SC could clearly be distinguished. However, after daily 90-minute HBO treatments, the formation of the epidermis was significantly greater after both 3 and 5 days, which was illustrated by a thicker epidermis and earlier onset of stratification. These findings support previous reports that oxygen controls the rate of epithelialization.[2]

Characterisation of newly-formed tissue using antibodies generated against epidermal markers, including p63,[36] cytokeratin 1/10/11 (K1/10/11),[37] cytokeratin 6 (K6)[38,39] and cytokeratin 14 (K14),[37] revealed the accelerated maturation of the epidermis on HBO-treated HSEs. p63 expression was mainly localised to the basal layers and the number of positive cells was particularly abundant after 3 days of HBO treatments, illustrating the rapid proliferation of the cells. The number of positive cells decreased after 5 days while the tissue further matured, as was indicated by the increased expression of K1/10/11 and K14, as well as the increase in cornification. These differentiation-promoting effects of HBO on keratinocytes are consistent with earlier reports.[17,21]

Migration of keratinocytes is crucial to obtain coverage of a wound bed. Quantification of the lateral migration on the dermal scaffolds demonstrated that HBO stimulated the early migration of the keratinocytes, whilst still allowing a more rapid differentiation. K6 expression was largely localised to the suprabasal region of the migrating front of keratinocytes, where the cells are undergoing rapid turnover. The inducible expression in keratinocytes in response to disturbances of epidermal homeostasis,[38] as well as the down regulation after maturation of cultured skin,[39] is a likely explanation for the topographical differences observed and illustrates the value of these models in wound healing research.

Substantial evidence reveals the important role that extracellular matrix components play in mediating epithelial cell morphogenesis.[47] At the wound site the keratinocytes on the migration edge remodel the basement membrane to promote migration over the denuded area. In view of this, we investigated changes in collagen type IV, a major component of the basement membrane. Prior to seeding the keratinocytes on the DED, type IV collagen expression was continuous throughout the basal membrane as is found for normal skin. After 3 days collagen type IV could not be detected in the basal membrane region regardless of treatment regime, indicating the remodelling of the basement membrane.[30] In line with previous investigations[48-50], collagen type IV was not re-deposited in the fibroblast-free control samples by day 5. In contrast, when samples were exposed to daily HBO treatments collagen type IV was re-deposited after 5 days, further supporting the above findings of accelerated epithelial maturation by HBO.

HSEs have been developed as a testing and research model for the cosmetic, pharmaceutical and chemical industries[51] and also to study skin wound healing.[31,51] On the other hand, they also have potential application in the treatment of burns and other acute or chronic wounds.[52-

[54] Hence, insight in the stimulatory effects of HBO on epidermal reconstruction will also impact on the *in vitro* production of such composite grafts of cadaver dermis and epidermal sheets.

There are a number of important differences between the HSE model and the clinical *in vivo* situation that should be noted. For example, the mechanism through which the oxygen is supplied to the tissue is somewhat different due to the lack of vascularisation. During HBO therapy *in vivo* it is the high arterial oxygen concentration which plays a critical role in driving angiogenesis into hypoxic spaces,[7] which in turn is believed to support wound healing. However, since oxygen will also be supplied through the culture medium, we believe that the adopted strategy has significant *in vivo* relevance.[17] In addition, this study focussed on the response of keratinocytes alone in a 3-dimensional model to HBO treatments over 5 days, hence the model did not contain fibroblasts in the dermal component. The responses of the HSE models containing fibroblasts will, however, be the focus of future research, because the presence of fibroblasts has been shown to be an important factor in the regulation of epidermal formation.[28,55]

In conclusion, the present study demonstrates that HBO stimulates the reconstruction of an epidermis in a human skin equivalent model by enhancing different stages of the wound healing cascade (proliferation, early migration and differentiation of the keratinocytes, as well as remodelling of the basement membrane). These results further illustrate the critical role of oxygen during the process of wound repair and may aid further understanding of the therapeutic benefit of hyperbaric oxygen in dermal wound healing, and may provide critical information to improve current treatment protocols.

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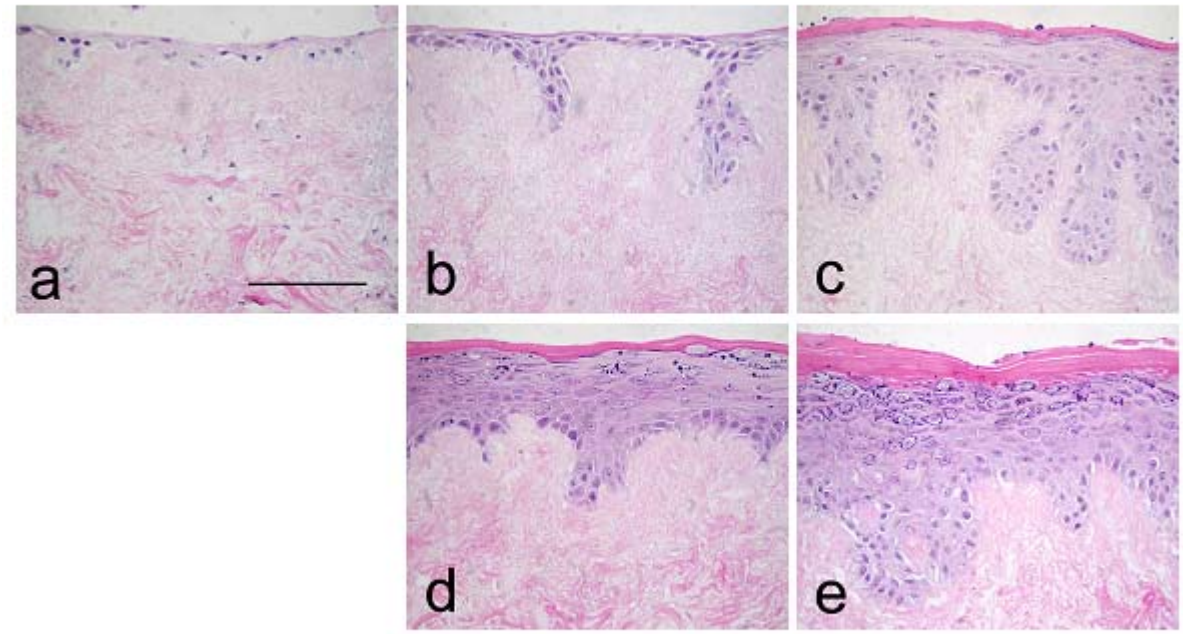
Figure legends



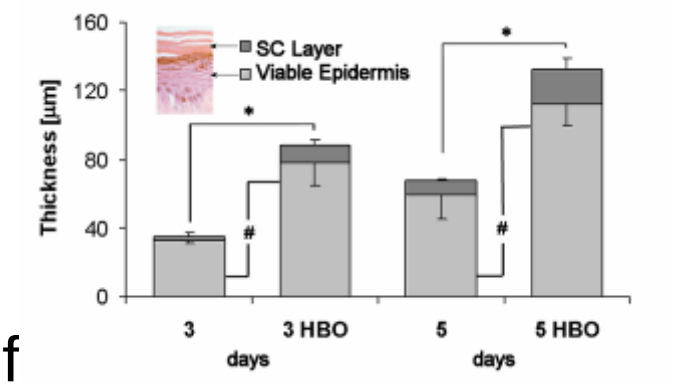


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Figure 1. Hematoxylin and eosin staining of cross-sections of reconstructed epidermis after transfer to the air-liquid interface after (a) 0, (b, d) 3 and (c, e) 5 days of daily 90-minute treatments with (b, c) air at 1 ATA or (d, e) 100% oxygen at 2.4 ATA: upper epidermis stains purple, lower dermis stains a lighter violet. Scale bar represents 100 µm. (f) Effect of hyperbaric oxygen treatment on the thickness of the cellular and cornified layer of the

*reconstructed epidermis on dermal substrates after 3 and 5 days of culture at the air-liquid interface (# and * indicate significant differences, $p<0.05$).*

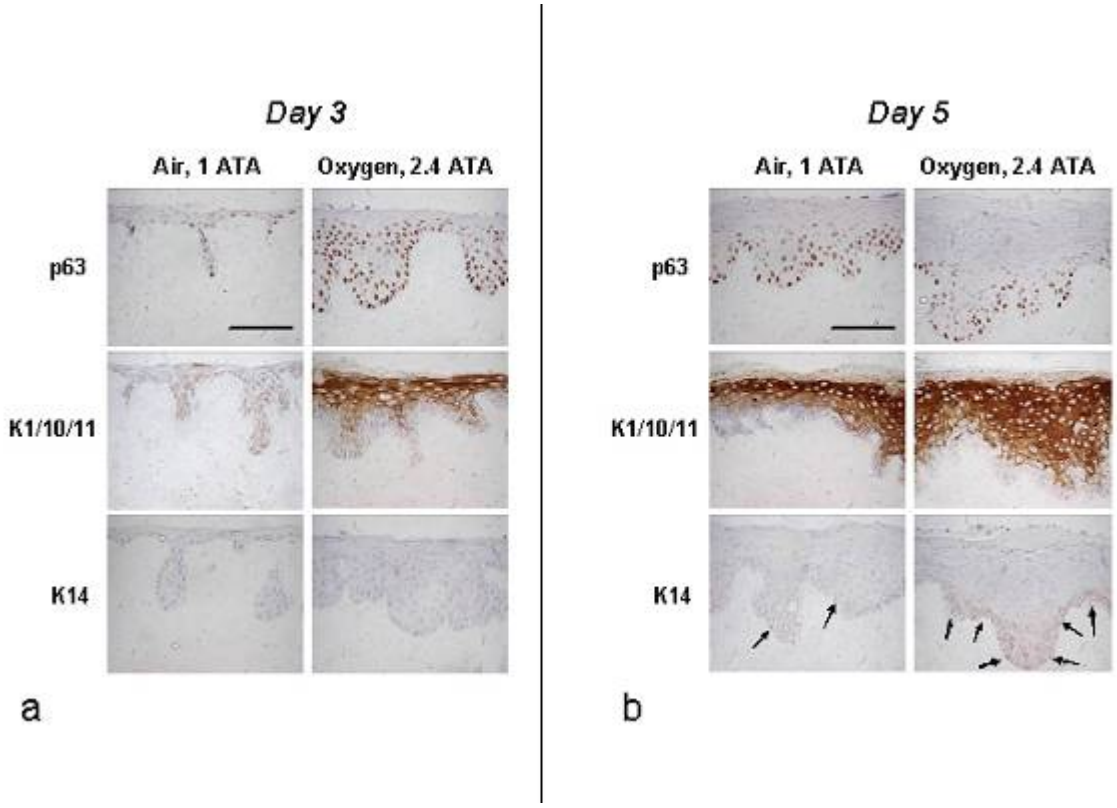

Figure 2. Expression of the early proliferation marker p63, the differentiation marker cytokeratin 1/10/11 (K1/10/11) and the basal cell marker cytokeratin 14 (K14) in reconstructed epidermis after transfer to the air-liquid interface after 3 and 5 days of daily 90-minute treatments with air at 1 ATA (Control) or 100% oxygen at 2.4 ATA (HBO). Scale bar represents 100 μm.

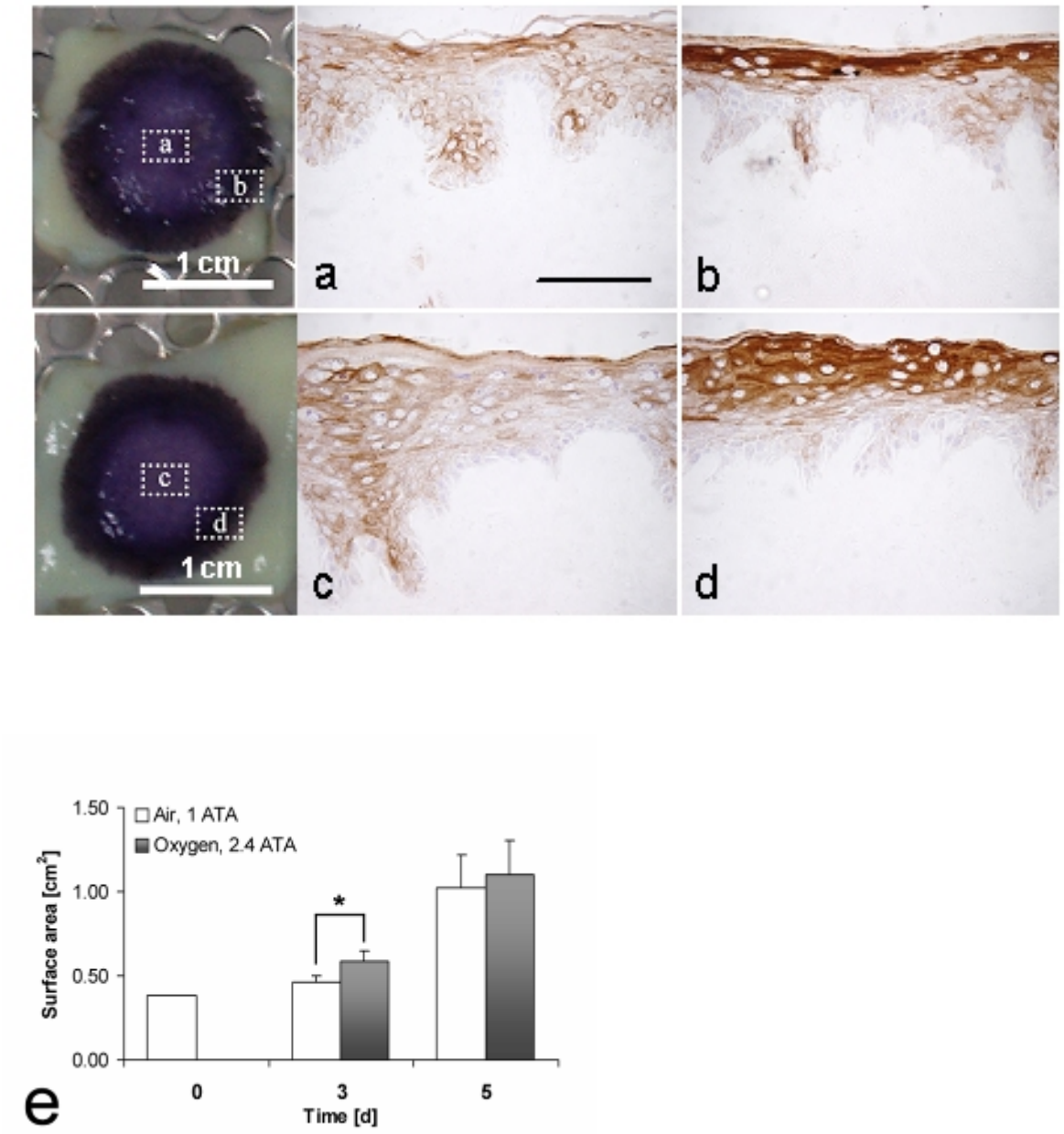

Figure 3. Lateral migration of the keratinocytes over the de-epithelialized dermal substrate. Local differences in cytokeratin 6 expression in the reconstructed epidermis after transfer to the air-liquid interface after 5 days of daily 90-minute treatments with (a, b) air at 1 ATA or (c, d) 100% oxygen at 2.4 ATA. Scale bar represents 100 μm. (a, c): Central area and (b, d): outgrowing area of the epidermis. (e) The effect of hyperbaric oxygen (HBO) treatment on the

lateral migration on dermal substrates after 3 and 5 days of culture at the air-liquid interface. (= statistically significant from the control, $p<0.05$).*

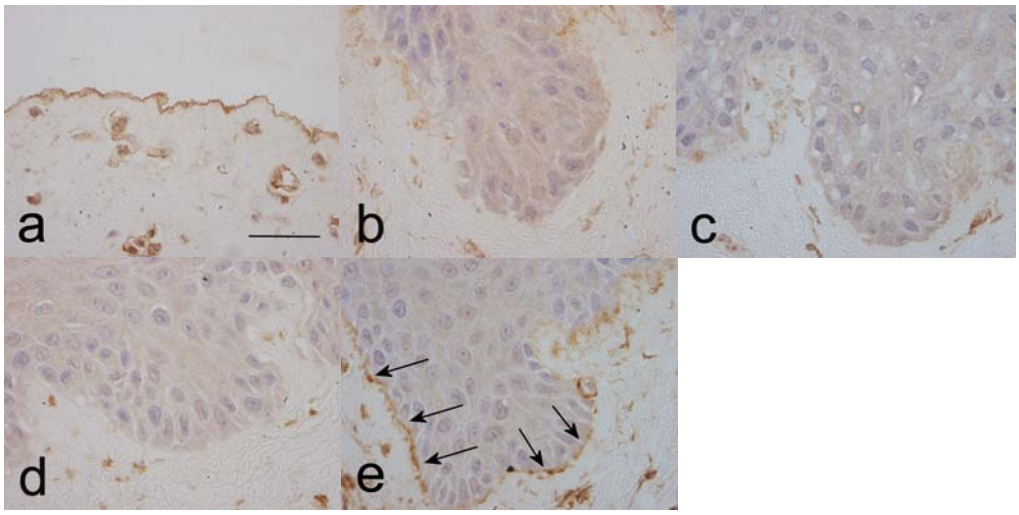

Figure 4. Expression of the basement membrane marker collagen type IV in the dermal scaffold (a) and the reconstructed epidermis after transfer to the air-liquid interface after 3 days (b, d) and 5 days (c, e) of daily 90-minute treatments with air at 1 ATA (b, c) or 100% oxygen at 2.4 ATA (d, e). Scale bar represents 15 μm.