In vitro models for evaluation of hyperbaric oxygen therapy in wound healing: a review

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Key words
Human skin equivalent, models, hyperbaric oxygen, chronic wounds, research, review article

Abstract

(Malda J, Dawson RA, Kairuz E, Topping G, Long R, Upton Z. *In vitro* models for evaluation of hyperbaric oxygen therapy in wound healing: a review. *Diving and Hyperbaric Medicine*. 2007; 37: 25-30.)
Chronic ulcers are a major problem affecting a significant number of people around the world. The condition is difficult to heal and often leads to amputation. Hyperbaric oxygen (HBO) has been used clinically for the treatment of chronic ulcers and positive outcomes have been reported. However, owing to the lack of large randomised controlled trials and some conflicting data, controversy regarding the effectiveness of HBO in chronic wound healing persists. Besides randomised controlled clinical trials, *in vitro* studies hold promise in providing further insight into the role of HBO in wound healing and in aiding the establishment of a scientific foundation upon which more rational and efficacious HBO therapeutic regimes may be developed. The present article provides an overview of the available *in vitro* data on HBO with regards to wound healing. In particular, it focuses on experimental design issues and future opportunities using human skin equivalent models to study HBO-mediated wound healing.

Introduction

Chronic leg and foot ulcers are a significant cause of pain and impaired quality of life. Even small lesions may become a long-term problem, resulting in partial lower-limb amputation, and creating a sustained demand on healthcare systems.[1] The associated loss of productivity and the requirement to provide support infrastructure places additional financial burden on the wider community.[1]

Hyperbaric oxygen treatment (HBO) has been suggested as a potential wound healing therapy due to the hypoxic nature of the chronic wound and the requirement for oxygen during the wound healing process.[2,3] Indeed, increased wound closure in response to HBO has been demonstrated using animal models and, importantly, clinical studies have demonstrated faster healing of chronic ulcers and a decreased risk of major amputation.[4–9]

These outcomes have been used as the justification of Medicare coverage for the treatment of these conditions with HBO (item numbers 13015 and 13020) in Australia.[10] However, due to the conflicting data and the lack of a large randomised controlled trial, controversy regarding the effectiveness and validation of treatment regimes of HBO for enhancing healing of chronic wounds still exists.[11] Thus, its clinical application is the subject of great debate. In view of this, elucidation of the pathophysiological mechanisms underlying the demonstrated success of HBO will aid the further validation and hence full exploitation of the therapeutic potential of HBO therapy in wound healing.

Evaluation of HBO *in vitro* using human cell monolayers

Two-dimensional (2D) *in vitro* monolayer cultures of keratinocytes, fibroblasts, melanocytes and endothelial cells have been used to evaluate the effects of HBO.[12–16] HBO has not been shown to affect keratinocyte proliferation, either positively or negatively, in two studies reported to date.[15,16] We have recently confirmed this in our laboratory using monolayer cultures of the human keratinocyte cell line, HaCaT (unpublished results). In addition, differentiation appeared not to be significantly affected by HBO, as evaluated by means of the expression of late differentiation markers cytokeratin 10 and involukrin.[16]

Data reported on the effects of HBO on human fibroblast proliferation is conflicting. For example, Hehenberger et al and Tompach et al observed a dose-dependent effect on proliferation after 24 hours following a single hyperbaric treatment.[12,13] In contrast, Piepmeier et al did not observe any effects with a single treatment and Dimitrijevich et al observed a mitogenic effect only after prolonged HBO exposure.[14,15] Interestingly, these authors also demonstrated that collagen production by fibroblasts is inhibited by HBO treatment, contrary to the widely accepted belief that HBO aids wound healing by up-regulating collagen synthesis.[15,17] Clearly, it is difficult to draw any hard conclusions based on the limited and contrasting data available, illustrating the need for more extensive *in vitro* studies.

Figure 1
See caption on next page for explanation

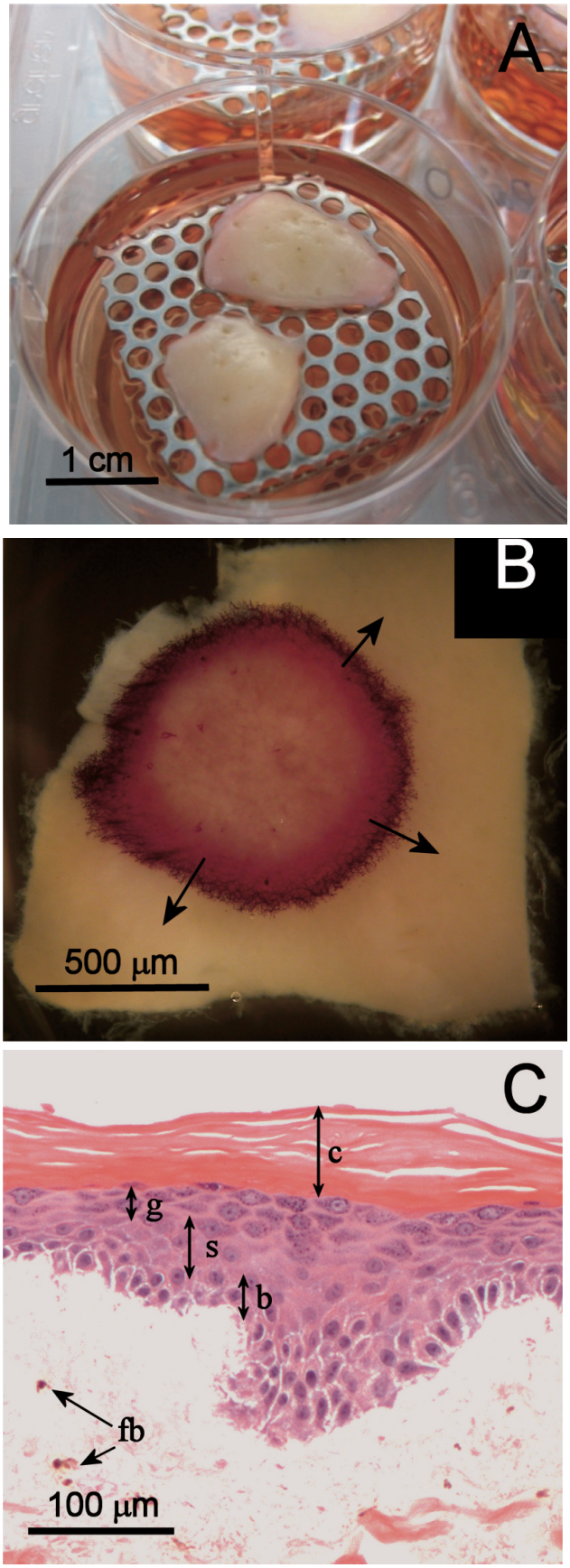

Figure 4
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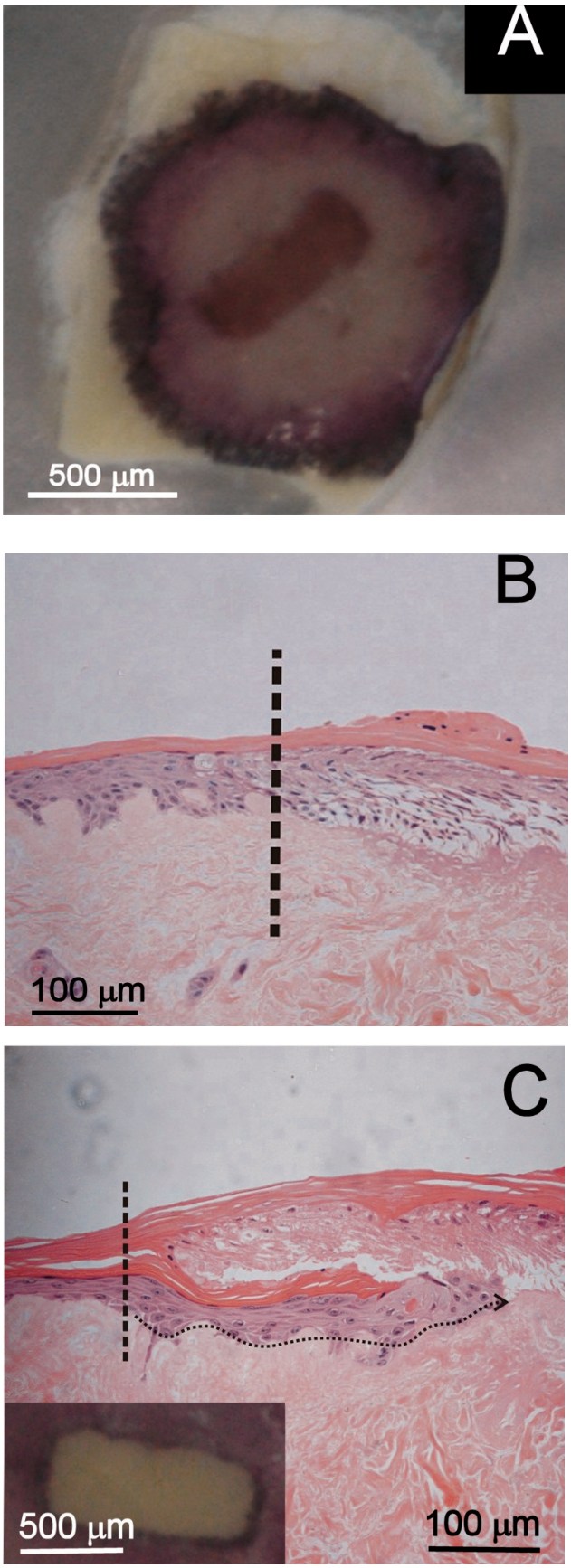


Figure 1
(A) The human skin equivalent (HSE) model; appearance at Day 10 of culture at the air-liquid interface
(B) A MTT-stained HSE after 5 days of culture; arrows indicate lateral migration of the newly-formed epidermis
(C) Histological cross section of the HSE at Day 10 demonstrating the stratum basale (b), stratum spinosum (s), stratum granulosum (g) and stratum corneum (c) and fibroblasts (fb) in the dermal component

Figure 4
(A) MTT and (B) histological analyses of the wounded HSE model immediately after burning. The model was burnt using a heated metal rod after 8 days' culture. The dashed line shows the margin of the burn
(C) Histology 6 days after wounding; the dotted arrow running beneath the basement membrane shows the migration of keratinocytes from the wound margin. The inset shows MTT analysis of the burn area (reproduced from Topping et al[22] with permission)

Two-dimensional cell culture has proven to be a valuable research tool, but its limitations have become increasingly recognised. Because of the highly unnatural geometric and mechanical constraints imposed on cells, these cultures only approximate properties of normal tissues.[18] Moreover, this approximation is almost always limited to single cell types and does not take into account the impact of the other cells and the supporting environment that surrounds tissues.[18] Thus, the results obtained on the effects of HBO using 2D cell culture may be misleading and non-predictive for *in vivo* responses.

Evaluation of HBO using three-dimensional *in vitro* models

To study the complex process of wound healing *in vitro*, more physiological three-dimensional (3D) models have been developed.[19] These have become known as human skin equivalent (HSE) models, reflecting the intent to more closely mimic the *in vivo* situation. HSEs are reproducible *in vitro* models of skin that allow the culture to occur at the air/liquid interface and provide a valuable tool when investigating factors and treatments, including HBO therapy, that can improve or impair wound healing.

HSEs, consisting of a fibroblast-containing collagen gel with a layer of seeded keratinocytes on top, were first employed by Dimitrijevich et al for the study of HBO-mediated epithelialisation.[15] Their preliminary study provided histological indications that HBO enhances epidermal differentiation after 10 successive 90-minute treatments at 202.6 kPa.[15] For our studies, we adopted an HSE based on a de-epidermised dermal scaffold.[20] After seeding a layer of human keratinocytes (4.9×10^4 cells.cm^{-2}) on the top of this scaffold, the models were submerged in standard keratinocyte culture medium for one to three days to allow cell expansion prior to subsequent culture at the air/liquid interface (Figures 1a and b).[21,22]

This model possesses significant advantages over other skin equivalents with scaffolds. Specifically, it is composed of a dermal matrix (with or without incorporated fibroblasts) 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.[23] Hence, the epidermis formed on these scaffolds has a high degree of similarity to the epidermis *in vivo*, with the main regions clearly visible: a rapidly proliferating basal layer, a differentiating supra-basal layer and an uppermost, stratified cornified layer (Figure 1c).[20,22]

HSE models have been used as a testing and research platform for the cosmetic, pharmaceutical and chemical industries, as well as for the study of skin wound healing.[19,24] For example, the HSE model based on the de-epidermised dermis has been used as a model for contraction, cell invasion and angiogenesis.[25–27] In addition, it was used clinically as a skin replacement following release of contractures in previously burnt patients.[27]

Using this particular HSE model, we have recently demonstrated that daily hyperbaric treatments (90 min, 100% oxygen at 243 kPa) accelerate the reconstruction of an epidermis compared with air treatments at 101.3 kPa (1 ATA) (Figure 2).[21] Immunohistological characterization of the HSEs using various epidermal markers, including cytokeratins 1/10/11 (primary proteins of skin), revealed the earlier onset of epidermal differentiation within the HBO-treated constructs compared with air (Figures 2c and d). Moreover, the reconstructed epidermal layers in HBO-treated samples were significantly thicker at both Day 3 and Day 5 compared with the non-treated controls (Figure 3).

Additionally, after three days of culture at the air/liquid interface, the populated surface area of the dermal scaffolds, as visualized using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; stains living cells purple) staining (Figure 1b), was significantly greater ($p < 0.05$) for the HBO-treated samples than for the controls (mean ± SD, 0.46 ± 0.03 cm^2 and 0.58 ± 0.06 cm^2 for control and HBO, respectively) due to an increase in cell migration and proliferation.[21] Although a difference was observed after five days, this was not significant. Thus, using the HSE model and employing a protocol similar to that used to treat chronic wounds clinically, we demonstrated that HBO stimulates the reconstruction of the epidermis. Moreover, we showed, for the first time, that these changes in epidermal formation are supported by differences in markers of proliferation, differentiation and basement membrane components.

Experimental design issues for *in vitro* HBO treatment

Although the HSE model offers numerous opportunities to

further dissect the role of HBO in the healing of chronic wounds, there are differences between the model and the *in vivo* situation that should be considered and will impact on the experimental design. The HSE is a simplified model and lacks both innervation and vascularisation. However, oxygen will directly diffuse into the culture medium and has been shown to result in a significant rise in the partial pressure of oxygen (pO_2) over a 90-minute treatment; these values correlate with the values observed in normal tissue at 100% O_2 inspired within the range of 101.3–253 kPa.[15] The fall in tissue pO_2, observed after clinical HBO exposure, will be faster *in vitro* than *in vivo* and therefore the HSE model is a somewhat conservative indicator of the potential *in vivo* benefits of HBO therapy.

Rapid gas exchange can also influence the pH in the culture medium. Optimal growth of cells *in vitro* is dependent on maintaining a physiologic pH. Although the changes in the pH of the medium during a 90-minute HBO treatment were shown to be less than 0.10 pH units, depending on the conditions, pH changes can be significant and should thus be considered.[15] The most commonly used culture systems employ incubation in a high carbon dioxide (CO_2) environment, typically 5%. Hence, bicarbonate has to be present in the medium at a concentration of about 25 mM to reach the physiologic pH of about 7.4. When handling cells for extended periods of time in the absence of high CO_2 concentrations, e.g., in hyperbaric oxygen conditions, bicarbonate is not an adequate buffer and the pH of the media can rise to non-physiological levels. Therefore, other buffers with appropriate pKa levels, such as HEPES (N-[2-Hydroxyethyl] piperazine-N'-[2- ethanesulfonic acid]) for example, could be used.

The control of temperature is an additional challenge, since changes in the temperature will affect the cellular responses, including proliferation.[28] Thus, it is important to carefully control the temperature in order to obtain reliable outcomes. Hence, a monoplace or multiplace chamber is less suitable than a research or custom-designed hyperbaric chamber that allows the control of temperature via a water jacket.[13,21]

Figure 2
(A, B) Hematoxylin and eosin staining and (C, D) expression of the differentiation marker cytokeratin 1/10/11 (K1/10/11) of cross-sections of reconstructed epidermis after 5 days of daily 90-minute treatments with air at 101.3 kPa (A, C) or 100% oxygen at 243 kPa (B, D)

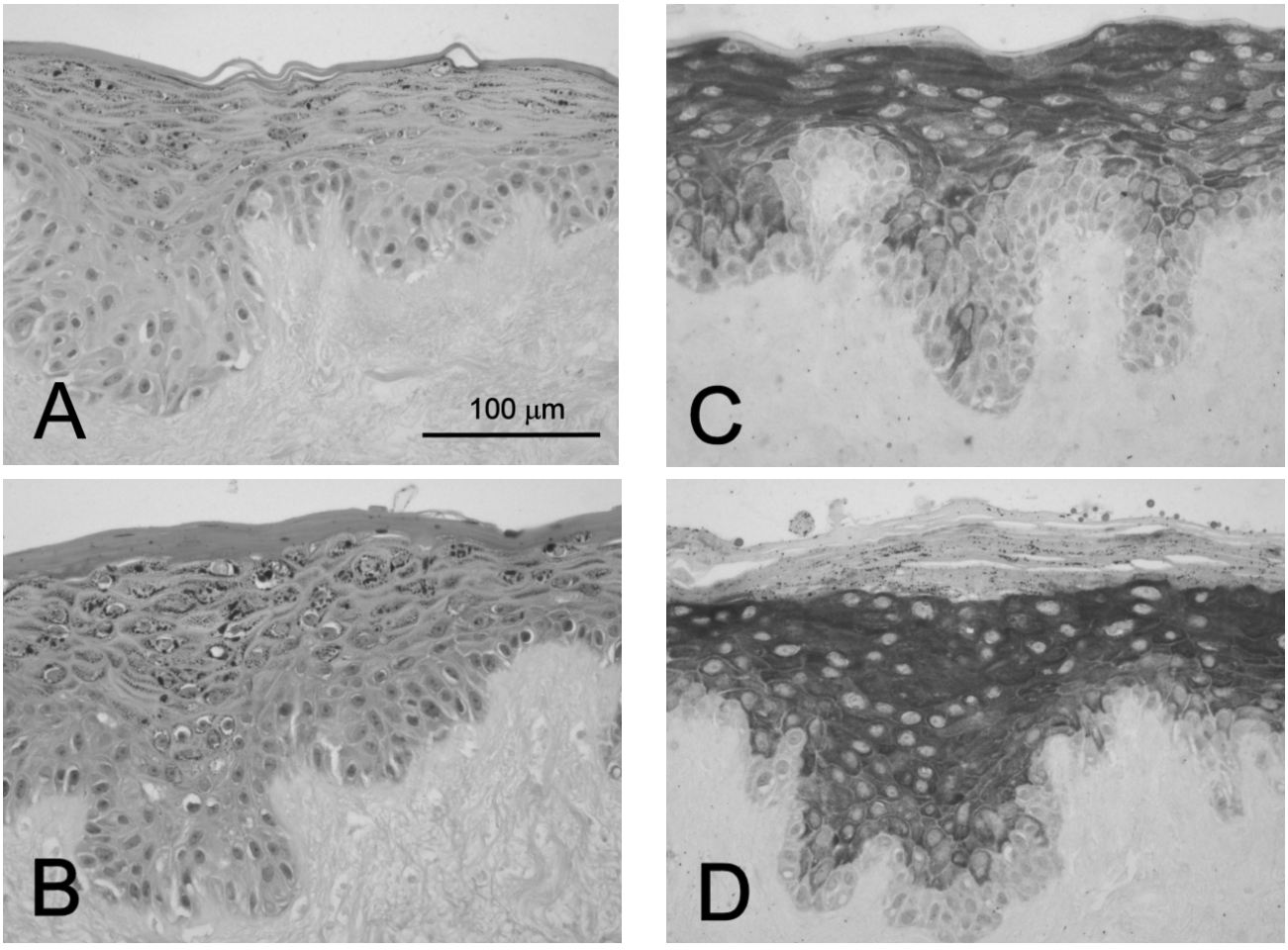
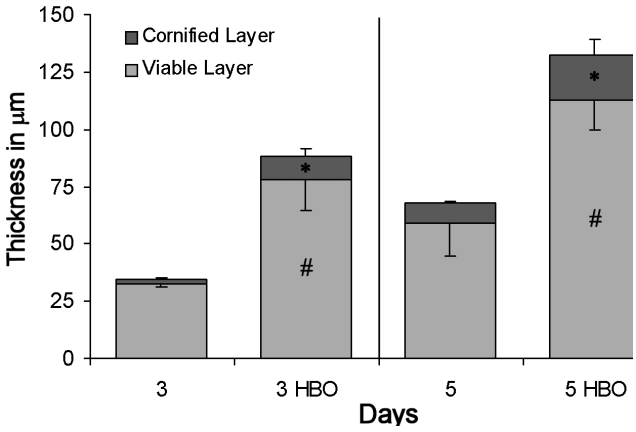


Figure 3
Thicknesses of the cellular and cornified layers after 3 and 5 days as determined by image analysis (n = 3 in 3 independent experiments, # and * indicate significant differences from control, p < 0.05). Adapted from Kairuz et al[21] with permission.



Future opportunities using skin equivalent models in wound healing research

HSE models provide an exciting means to improve and extend our knowledge regarding the effects of HBO on biological processes during the healing of chronic ulcers. Clearly, to further resemble native skin, various additional types of cells can be incorporated into the HSE, including melanocytes and Langerhans cells in the epidermal compartment, and fibroblasts and endothelial cells in the dermal compartment.[19] In addition, wounds can be created in the model and the healing response can be monitored.[22,29] Figures 4a and b show the wounded HSE model immediately after burning with a heated metal rod after eight days' culture, and Figure 4c the subsequent migration of keratinocytes facilitating wound re-epithelialisation.[22]

The model could be further improved to specifically mimic the *in vivo* chronic wound environment. Generally, this environment is of a hypoxic and highly proteolytic nature and diabetes is often an underlying cause of the condition.[2] The hypoxic nature of chronic wounds *in vivo* can be reconstructed by culturing the HSEs in a low-oxygen environment using a low-oxygen cell culture incubator or chamber. Similarly, the proteolytic environment of the chronic wound could be simulated by bathing the wounds created in the HSE model in chronic wound exudate obtained from consenting patients suffering from non-healing wounds. In addition, hyperglycaemia, as seen in diabetes, can be reproduced in the *in vitro* models by the application of abnormally high levels of glucose (up to 100 mM). Subsequently, intermittent HBO treatments can be administered and changes in the healing response can be evaluated histologically, as well as genetically. In this respect, the current advances in proteomics and genomics are of particular interest and can be incorporated into the research approach.

Conclusions

HSE models have advanced our understanding of wound healing, as well as of basic skin biology. Such models provide a more physiological 3D *in vitro* model of human skin that allows the incorporation of various cell types and circumvents the disadvantages associated with 2D *in vitro* models. Moreover, HSEs can be wounded and the healing can be studied. In addition, cultures can be maintained in the presence of chronic wound fluid, high glucose or hypoxia, thus simulating to some extent the inhibiting chronic wound environment. HSEs are therefore a valuable tool in furthering our understanding of the effects of HBO and can aid the further establishment of a scientific foundation upon which more rational and efficacious HBO therapeutic regimes may be developed.

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