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Quantitative Histomorphometry and Quantitative Polymerase Chain Reaction (PCR) as Assessment Tools for Product Development

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Abstract

In the current chapter, 12 normal, healthy subjects were enrolled in a clinical study to assess the efficacy of a topically delivered therapeutic to improve the health and appearance of skin. Clinical and histological assessments along with immunohistochemistry and gene expression results were evaluated using quantitative methods for a comprehensive determination of the therapeutic effect. As described in the previous chapter, coupling of various analytic tools in this way can allow for a more complete assessment of a therapeutic activity, a biomedical device's success, or a combination therapy's clinical benefit where a drug coating may be delivered to a targeted area using a biomedical device as a delivery system (e.g., drug eluting stents).

The therapeutic evaluated in the current study was a topical dissolved oxygen dressing (OxygeneSys™ Continuous, AcryMed, Inc., Beaverton, OR). Clinical evaluations demonstrated that the dressing was well tolerated and several measures of skin health and integrity showed improvements compared to a control dressing site. Quantitative data from histology, immunohistochemistry, and gene expression studies demonstrated a general reduction in inflammatory response markers and transcription products (IL-6, IL-8, TNF-alpha, MMP-1, and MMP-12) while facilitating a general increase in structural skin proteins (collagen I, elastin, and filaggrin). Additionally, p53 signals from biopsy samples support the conclusion that the topical therapeutic presented no safety concerns. In summary, the data from this study demonstrated that the dressing had no deleterious effects and stimulated beneficial effects on intact, nonwounded skin. Additionally, quantitative histomorphometry and quantitative polymerase chain reaction (PCR) techniques provided unique tools to comprehensively assess clinical benefits.

Key words Quantitative histomorphometry, Histology, Immunohistochemistry, Quantitative polymerase chain reaction, qPCR, RT-PCR, Gene expression, Product development

1 Introduction

As an organ, skin serves numerous functions, including protection from external environmental insults such as pathogenic organisms, UV radiation, changes in water, humidity, and temperature, and also plays a significant role in the immune system (1). Skin health is dependent on a number of changing physiologic mechanisms that are often compromised with age. For example, wound healing is significantly compromised in the elderly (1) and these wounds can

become chronic in nature and present serious clinical issues if they are left untreated (2). Our elderly population is not the only subject group with issues or concerns about skin health. As the largest and most aesthetically important organ in the body, the skin is a growing area of focus for individuals from all age groups. Geriatric people are interested in curbing the effects of age while younger people are interested in maintaining a youthful, healthy skin condition.

It has become widely accepted in the field of skin care that the nutritional supply of oxygen to the skin is primarily supplied by internal circulation that is widely available in the deeper dermal layers. However, recent data have shown that significant amounts of oxygen may be available via diffusion from the external overlying surface (3). Bioavailability of oxygen in the skin is critically important for a number of reasons. There is a close dependency between tissue oxygenation and wound healing: wounds with a pO_2 less than 30 mmHg are considered to be hypoxic and have more clinically associated challenges such as being slow to heal, having little or no granulation tissue, and having accumulations of necrotic deposits (4). In contrast, those wounds with pO_2 levels greater than 30 mmHg usually have fewer longer term clinical issues and follow a normal course of wound healing (4). Furthermore, wounds deprived of oxygen deposit collagen poorly and are easily infected. Epithelialization represents a final resolution of the wound and its mechanisms are optimized at high oxygen levels (5).

Oxygen is essential for wound healing and normal skin organ function. Since there is limited diffusion across the stratum corneum into the epidermis, the goal of the current study was to evaluate if the topical delivery of a total dissolved oxygen in dressing form on intact human skin would improve clinical and histologic skin functioning. Biopsy samples were taken from subjects at active and control sites following 8 weeks of treatment. Biopsy samples were coronally sectioned, with one half processed for histopathology to assess impact on hydration, oxidative stress, and structural proteins and the second half processed for real-time RT-PCR analysis to assess impact on inflammatory markers. These data were correlated with clinically relevant markers such as desquamation, hydration, and roughness. Results from these evaluations suggest active mechanisms are in play with the use of topical oxygen therapy to intact, healthy skin. No safety issues were seen in the current study and structurally significant and biologically relevant differences were detected as a result of 8 weeks of active treatment.

2 Materials and Methods

2.1 Human Subjects

A total of 50 healthy subjects (men and women ages 50–69 years; mean age 58.4) completed a single-site, randomized, controlled,

8-week study. Of these 50 subjects, 12 were randomly selected for biopsy collection. Subjects had age-appropriate photoaging and stable concomitant medications. Informed consent was obtained from all subjects in the study, which was approved by the Concordia Clinical Research Institutional Review Board, New Jersey.

The semiocclusive, absorbent, oxygen-enriched dressing (Active Group, OxygeneSys™ Continuous, AcryMed, Inc., Beaverton, OR) was affixed to the skin covering the anterior tibia on one limb and the contralateral limb was covered with a Kling® bandage to function as the control. A computer-generated randomization scheme determined which limb (left or right) would receive the experimental dressing. The dressing was wet with an ampule of eye moisturizer and affixed to the shin with a Kling® dressing held together with paper tape. The dressing was applied daily by the subject following bathing and worn for 24 h continuously. The location of the dressing placement was noted by the investigator with black indelible ink. Subjects were permitted to continue using their own skin care, cleansing, and makeup products but were not allowed to begin any new products for the 8-week duration of the study. No skin care products of any kind were used on the shins where the dressing was applied.

2.2 Clinical Assessments

Study subjects evaluated in a blinded manner were assessed by the same investigator (clinician) throughout the study. Dressings were removed prior to clinical grading and all parameters were evaluated at 4 and 8 weeks on a 5-point ordinal scale, from 0 (no signs or symptoms) to 4 (very dramatic signs and symptoms resulting in discomfort, representing an adverse reaction). A compliance check visit was performed at 1 week. Clinical investigator assessed efficacy parameters were desquamation, roughness, erythema, and skin texture; and tolerability parameters were itching, stinging, and burning. Digital images of each shin were collected at baseline, 4 weeks and 8 weeks. Skin hydration and water loss were measured with the appropriate Dermalab instruments and probes (Cortex Technology, Denmark): corneometer, TEWL (transepidermal water loss), elasticity, and skin coloration (6). Sensory monofilament test was performed by drawing a cotton fiber over the skin.

2.3 Biopsy

One 3 mm full thickness skin biopsy was taken from each shin (randomized active and control) of 12 randomly selected subjects at week 8. Each biopsy was coronally sectioned in half (superficial to deep) with one half immediately placed in ice-cold fixative (2 % paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS and incubated at 4 °C for 48 h for histologic and immunohistologic analyses. The remaining half was placed in ice-cold RNAlater (Sigma Chemical Company, St. Louis, MO) and incubated overnight; then stored at −80 °C until processed for real-time RT-PCR analysis.

2.4 Histology

Histologic and histopathologic analyses were performed at the 8-week time point, comparing control to active site and assessed from 5 μm serially sectioned paraffin-embedded tissues, and either stained with hematoxylin and eosin for histopathology or primary antibodies for immunohistochemistry (IHC). Primary IHC antibodies were oxidative stress DNA adduct 8-hydroxy-2-deoxyguanosine (8-OHdG) (Abcam, Cambridge, MA), water-glycerol channel aquaporin-3 (AQP3) (Santa Cruz Biotechnology, Santa Cruz, CA), structural proteins filaggrin (Vector Laboratories, Burlingame, CA), collagen I (Abcam, Cambridge, MA), and elastin (Abcam, Cambridge, MA), and processed using standard immunohistochemistry methods.

Digital, whole-slide scans (Aperio ScanScope CS) were used for all evaluations to quantify changes in levels, using established digital pathology algorithms (7). All artifacts were manually excluded prior to digital algorithm. H-score, a widely used pathology method for quantitatively evaluating staining features, was used to determine changes in staining intensity. The H-score is directly related to staining intensity, scored as 0, 1+, 2+, or 3+ of the area, cell, or object and calculated by the formula: $(3 \times \text{percentage of } 3+) + (2 \times \text{percentage of } 2+) + (\text{percentage of } 1+)$. Results range from 0 to 300 (8).

2.5 Real-Time PCR

Nucleic acids were extracted from tissues frozen in RNAlater by homogenizing in RLT lysis buffer on ice (Qiagen, Valencia, CA) with an Omni THq rotor stator (Omni International, Kennesaw, GA) for 30 s per sample. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). First strand cDNA was reverse transcribed with the Superscript III synthesis system (Invitrogen, Carlsbad, CA) and was diluted 2:1 prior to addition to 10 μl PCR reactions containing 2× Taqman Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) and amplified on a 7900 Real-Time PCR System (Applied Biosystems, Carlsbad, CA) (Fig. 1). Real-time analysis was performed with the following Taqman probes: IL-6, IL-8, MMP-1 (collagenase), MMP-12 (elastase), TNF-α, TP53, VEGF and GAPDH. Samples were analyzed using the $2^{-\Delta\Delta Ct}$ method, using GAPDH as the housekeeping gene to normalize for sample to sample variations in RNA/cDNA. Data is presented as "fold change" in active vs. control.

2.6 Statistical Analysis

A paired student's *t* test was used to determine differences between the active group vs. the control group. A *p* value less than 0.05 was considered to be statistically significant.

Fig. 1 Applied Biosystems, Carlsbad, CA. 7900 Real-Time PCR System

3 Results

3.1 Human Subjects

A total of 50 subjects completed the study, 12 of which participated in the biopsy collection for this paper, without any major adverse events or deviations from the study design.

3.2 Clinical Measurements

There was a statistically significant increase in stratum corneum hydration from baseline to 4 weeks, 4–8 weeks, and baseline to 8 weeks ($p < 0.05$ at each time point) with an average increase in hydration of 41 μS (micro Siemens) in active vs. control from baseline to 8 weeks (Table 1) (6). There was a statistically significant decrease in the treatment efficacy parameters of desquamation, roughness and skin texture. There were no statistically significant changes in transepidermal water loss (TEWL), skin coloration monofilament sensorial measurement, or elasticity measures across all time points (data not shown).

3.3 Histology

There were no noticeable histopathologic differences between active vs. control sites at the 8-week time point with respect to acanthosis, spongiosis, chronic inflammation, hyperkeratosis, epidermal mononuclear infiltration, focal acantholysis, or dermal edema. Subtle differences were evident in epidermal thickness, vascular prominence, and occasional perivascular mononuclear cells. However, these features were concluded to be consistent with normal human skin and no trend change was observable for any of these characteristics between active and control samples. Representative histopathology is shown in Fig. 2. While there was a slightly lower level of rete pegs in the active vs. control sites, it was not statistically significant (6).

Table 1
Clinical measurements between control and treatment sites (6)

		Control	Treatment	*p* Value
Skin hydration (μS)	0–4 Weeks	−8.18 ± 44.01	15.88 ± 46.17	0.010
	4–8 Weeks	−2.04 ± 32.34	15.22 ± 41.96	0.025
	0–8 Weeks	−10.22 ± 33.91	31.10 ± 44.71	<0.001
Desquamation	0–4 Weeks	−0.27 ± 1.09	−0.49 ± 1.29	0.356
	0–8 Weeks	−0.12 ± 0.99	−1.08 ± 1.19	<0.001
Roughness	0–4 Weeks	−0.33 ± 1.12	−0.53 ± 1.24	0.396
	0–8 Weeks	−0.18 ± 0.99	−1.14 ± 1.15	<0.001
Skin texture	0–4 Weeks	−0.33 ± 1.12	−0.53 ± 1.24	0.396
	0–8 Weeks	−0.18 ± 0.99	−1.14 ± 1.15	<0.001

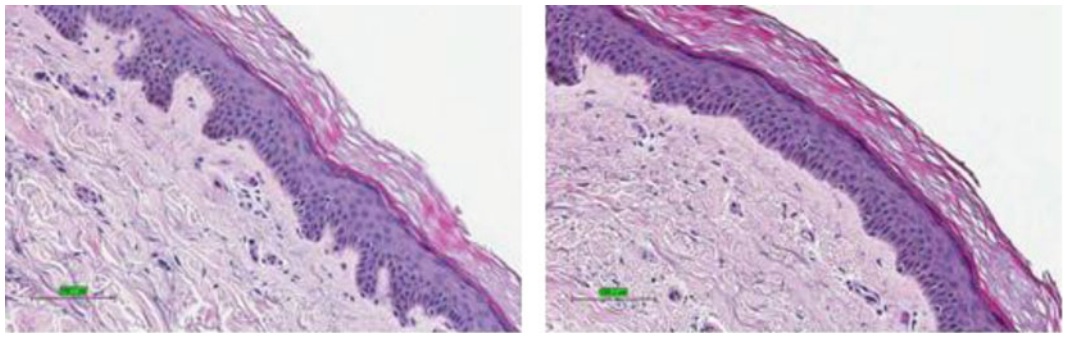

Fig. 2 Representative histopathology (hematoxylin and eosin stain). *Left*, subject #10, control site at the 8-week time point. *Right*, subject #10 active site at the 8-week time point. Scale bars = 100.5 μm (6)

3.4 Immuno histochemistry

Immunohistochemical (IHC) analysis of coronal, serially sectioned biopsies from the 12 subjects revealed a modest increase in 8-OHdG levels in active vs. control sites suggesting increased oxygen was penetrating the epidermis through the dressing, resulting in a measurable increase in DNA adduct formation.

The aquaglyceroporin channel, AQP3, showed a slight decrease in active vs. control sites; however, active sites exhibited a more "circumferential" or membrane-localized staining pattern, suggesting recruitment of AQP3 from the cytoplasm to the membrane to facilitate water and glycerol transport (Fig. 3) (6).

IHC analysis revealed an increase in H-score of filaggrin, collagen I, and elastin in active vs. control sites. While not statistically significant, these key structural protein data collectively show a trend increase, suggesting an influence on the structural organization in the skin (Fig. 4) (6).

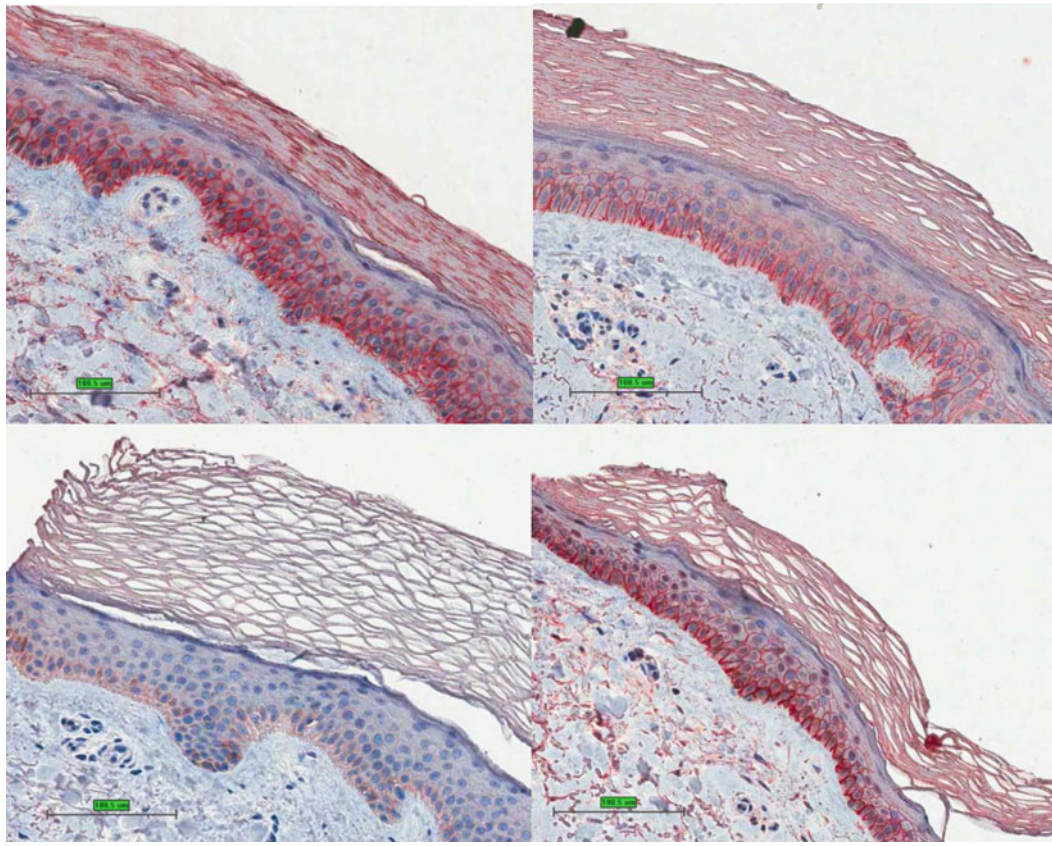

Fig. 3 Representative aquaporin-3 levels at 8 weeks. *Upper left*: subject #10, Control site. *Upper right*: subject #10, Active site. *Lower left*: subject #13, Control site. *Lower right*: subject #13, Active site. Scale bars = 100.5 μm (6)

3.5 Real-Time RT-PCR

GAPDH was used as the housekeeping gene to normalize for sample to sample variations in mRNA. Analysis was performed on a range of inflammatory, structural, angiogenic, and cellular stress genes. Pro-inflammatory cytokines IL-6, IL-8, and TNF-α showed a modest decrease in active vs. control sites. MMP-1 (collagenase) and MMP-12 (elastase) showed a more robust decrease. During inflammation or damage, both MMP-1 and -12 are upregulated to degrade and remodel extracellular matrix (ECM). Trend analysis of these markers suggests consistency in the data and a mechanism of downregulating the expression of inflammatory markers (Fig. 5).

VEGF expression levels were unchanged, suggesting the oxygen dressing neither decreased tissue pO_2 levels to hypoxic levels nor increased pO_2 to hyperoxic levels, both of which would lead to an increase in VEGF expression. TP53 (p53) levels were unchanged. As a central monitor of cellular stress and its environment, including sensing reactive oxygen species (ROS)

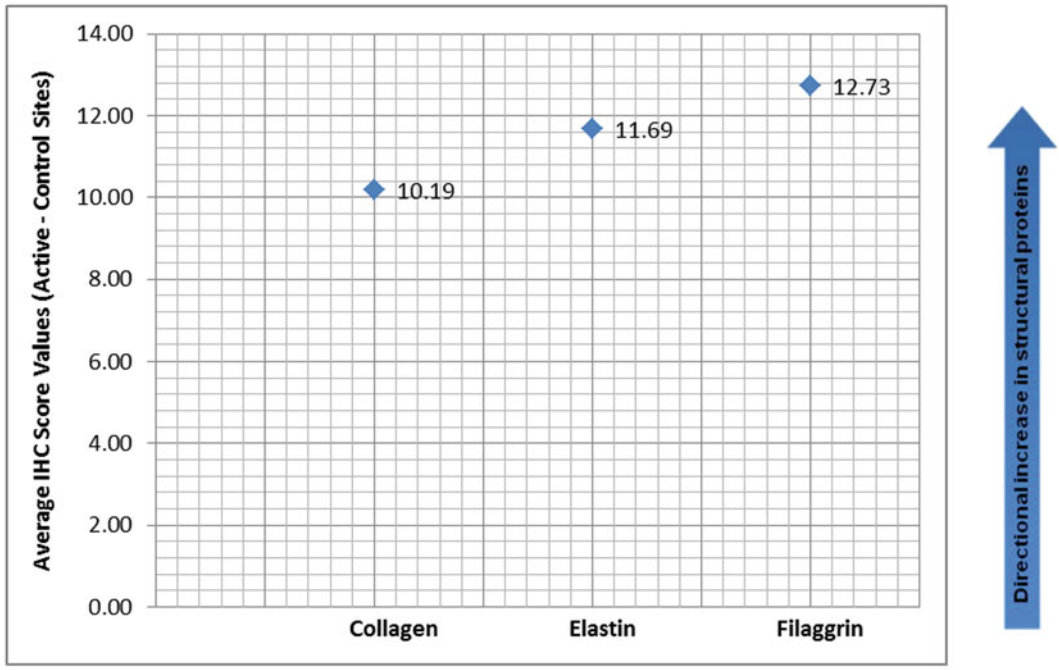

Fig. 4 Immunohistochemistry trend plot of structural proteins collagen I, elastin, and filaggrin at 8 weeks (6)

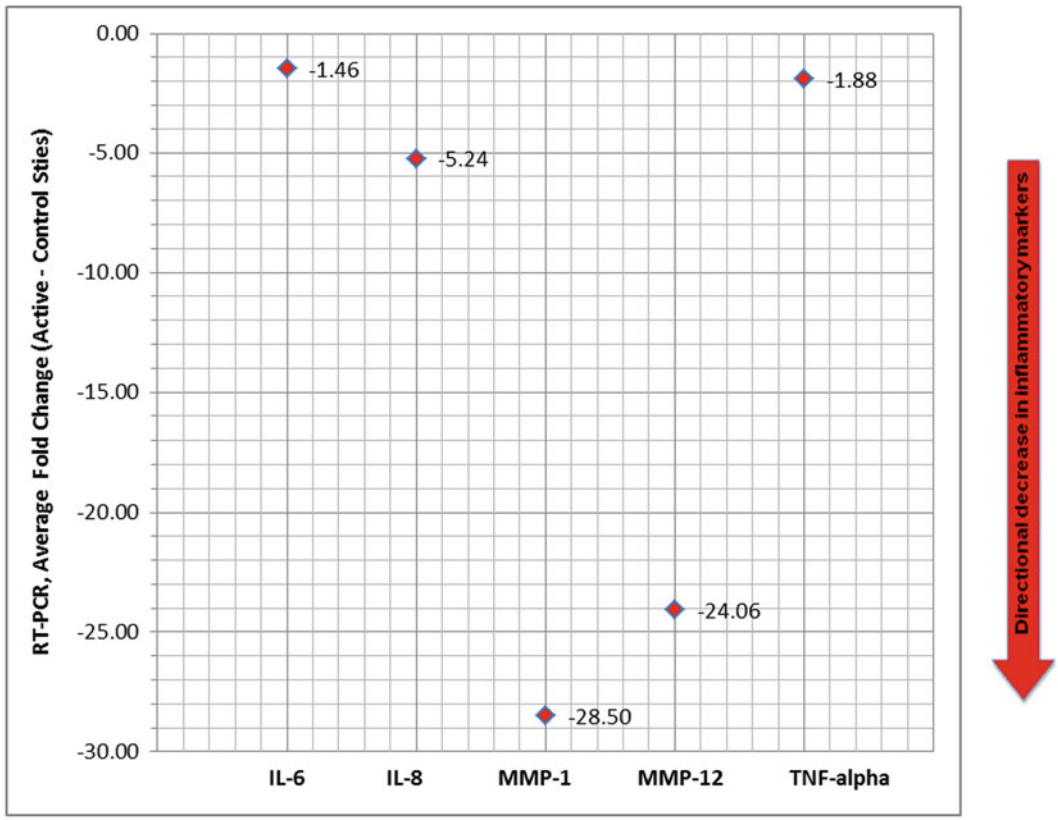

Fig. 5 Real-time RT-PCR trend plot of Inflammatory Markers at 8 weeks (6)

levels, the data suggests the skin covered by the topical oxygen dressing remained healthy with respect to p53 levels, similar to the control.

4 Discussion

In the current study, normal, healthy subjects were enrolled in a pilot clinical evaluation to assess the efficacy and tolerability of a dissolved oxygen dressing for improving skin health and appearance. The dressing provided an oxygen-enriched environment that may promote a favorable milieu for the promotion of healing in patients. Clinical analysis was performed on 50 subjects; histological and gene expression analyses were performed from biopsies on 12 randomly selected subjects. Clinical data indicates that the dressing is well tolerated and several measures of skin health and integrity showed improvement compared to the dressing-only control site. No safety issues were seen during the 8-week study period. This data was supported by the immunohistochemistry and gene expression studies, which showed a general reduction in inflammatory response markers and transcription products concomitant with a general increase in structural proteins. Additionally, there was a significant decrease in investigator-measured desquamation, roughness, and skin texture in active vs. control sites.

The increase in 8-OHdG levels in active vs. control sites suggests the dissolved oxygen dressing increased oxygen levels in the skin. More specifically, there was an increase in 8-OHdG in active vs. control epidermis, suggesting additional oxygen, delivered by the dressing, penetrated the skin and caused an increase in *OH-intermediates. The DNA analogues appear to be managed by normal physiological processes without deleterious consequences. If the DNA damage were beyond the capacity of the cell to manage, we would anticipate seeing an upregulation of p53 mRNA expression.

The levels of both VEGF and p53 were unchanged, suggesting the increased oxygen is within acceptable levels within the cells. Since there was an increase in production of 8-OHdG yet no change in p53 expression, we can infer that the topical oxygen therapy is penetrating the skin and resulting in positive changes that are not causing excessive stress to the integument. This strongly suggests an active therapy mechanism is in effect without negatively impacting cell and tissue health. Furthermore, VEGF levels were unchanged in active vs. control sites, suggesting the dissolved oxygen dressing created no hypoxic or hyperoxic states (9). VEGF expression is extremely sensitive to deleterious physiologic changes in oxygen levels. Therefore, since VEGF expression in the current study was unchanged and p53 levels were effectively

unchanged, we can conclude that the therapeutic conditions were safe at the cell and tissue level.

Decreased expression of IL-6, IL-8, TNF-α, MMP-1, and MMP-12 also suggests insignificant stress within the cells and skin. Inflammatory markers IL-6, IL-8, TNF-α, and the matrix metalloproteinases MMP-1 (collagenase) and MMP-12 (elastase) are known to be upregulated when inflammatory processes or mechanisms are in play. They are secreted by leukocytes and regulate a wide range of cellular and tissue responses, recruiting macrophages, neutrophils, inducing angiogenesis, and inducing remodeling of damaged tissue (10). In this study, expression of these markers was downregulated in active vs. control sites, suggesting a decrease in inflammatory pathways or mechanisms and a decrease in breakdown or remodeling of the extracellular matrix within the integument following active treatment.

The increased structural protein levels of collagen I and elastin may be due to reduced protein turnover by a decrease in MMP-1 and MMP-12 mRNA levels. These structural proteins are critically important to the architecture of the skin as an organ. Greater collagen I presence in the dermis may provide skin with greater structural integrity. Further consistency in the data within the current study lies in the fact that three major structural skin proteins (filaggrin, elastin, and collagen I) all increase in their expression in active sites when compared to control sites in the same subject. Furthermore, elastin is a structural protein found in the dermis as well as other critical tissues such as blood vessels, heart, bladder, and ligaments where it provides physiologically relevant elasticity. Elastin levels appear to be higher in active vs. control sites. Greater elastin presence in the dermis may provide skin with greater structural integrity and elasticity. Filaggrin facilitates the organization and condensation of keratinocytes at maturation, contributing to the functional stratum corneum. The increased levels of filaggrin in active vs. control sites, suggests an increased production of filaggrin, likely contributing to more structurally sound barrier and water retention functions. Increased filaggrin levels correlate with the increase in investigator-measured skin hydration and decreases in skin texture, roughness, and desquamation. A trend increase of these three structural proteins suggests consistency in the data and a mechanism of positively influencing the expression of key structural proteins (Fig. 4). Because these proteins trend together, we can conclude with a higher level of confidence that the active treatment is stimulating beneficial structural changes within the underlying skin. A summary of the overall findings in the current study is given in Table 2.

The blinded clinical investigator's measurements of decreased desquamation, roughness, and skin texture in treated vs. control sites correlate with the significant increase in skin hydration values. This data is supported histologically by an increase in filaggrin,

Table 2
Data summary (6)

	Marker	Analysis	Result
Stress monitor	8-OHdG	IHC	Increase
	p53	PCR	No change
	VEGF	PCR	No change
Stratum corneum barrier	AQP-3	IHC	Slight decrease redistributed to membrane
	Hydration	Clinical	Increase
	Desquamation	Clinical	Decrease
	Roughness	Clinical	Decrease
	Skin texture	Clinical	Decrease
Inflammation markers	IL-6	PCR	Decrease
	IL-8	PCR	Decrease
	TNF-α	PCR	Decrease
	MMP-1	PCR	Decrease
	MMP-12	PCR	Decrease
Epidermis structure	Filaggrin	IHC	Increase
	Elastin	IHC	Increase
	Collagen I	IHC	Increase

resulting in increased production of natural moisturizing factors and a redistribution of aquaglyceroporin, AQP3, from the cytoplasm to the membrane.

In summary, the quantitative histomorphometry and quantitative PCR data from this study demonstrate that the dressing has no deleterious effects and appears to stimulate beneficial effects on intact, nonwounded skin. Additionally, clinical assessments, histomorphometry, and RT-PCR data correlate with one another which helps to strengthen study conclusions from the 12 patient subset that was evaluated via skin biopsies. In this way, these methods can be used to help corroborate findings in studies where sample sizes for available biopsies may be limited due to study or clinic restraints.

References

1. Gosain A, DiPietro LA (2004) Aging and wound healing. World J Surg 28:321–326
2. Lazarus GS, Cooper DM, Knighton DR et al (1994) Definitions and guidelines for assessment of wounds and evaluation of healing. Arch Dermatol 130:489–493
3. Roe DF, Gibbons BL, Ladizinsky DA (2010) Topical dissolved oxygen penetrates skin: model and method. J Surg Res 159:e29–e36
4. Heng MC, Harker J, Bardakjian VB, Ayvazian H (2000) Enhanced healing and cost-effectiveness of low-pressure oxygen therapy in healing necrotic wounds: a feasibility study of technology transfer. Ostomy Wound Manage 46:52–60
5. Cianci P, Hunt TK (1993) Adjunctive hyperbaric oxygen therapy in the treatment of diabetic wounds of the foot. In: Levin ME, O'Neal LW, Bowker JH (eds) The diabetic

foot, 5th edn. Mosby Year Book, St. Louis, MO, pp 305–319

6. Kellar RS, Audet RG, Roe DF, Rheins LA, Draelos ZD (2013) Topically delivered dissolved oxygen reduces inflammation and positively influences structural proteins in healthy intact human skin. J Cosmet Dermatol 12:86–95
7. Potts SJ, Young GD, Voelker FA (2010) The role and impact of quantitative discovery pathology. Drug Discov Today 15:943–950
8. McCarty KS Jr, Miller LS, Cox EB et al (1985) Estrogen receptor analysis: correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. Arch Pathol Lab Med 109:716–721
9. Valko M, Rhodes CJ, Moncol J et al (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160:1–40
10. Lee Y, Je YJ, Lee SS et al (2012) Changes in transepidermal water loss and skin hydration according to expression of aquaporin-3 in psoriasis. Ann Dermatol 24:168–174