

Hypoxic conditioned culture medium from fibroblasts grown under embryonic-like conditions supports healing following post-laser resurfacing

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Summary

Objectives Treatment of facial skin perturbed by laser resurfacing with a novel, topical hypoxic conditioned culture medium (HCCM) product results in apparent, accelerated wound recovery time. The HCCM product is conditioned by neonatal fibroblasts under hypoxic conditions and used as the active ingredient in a formulated topical lotion. The HCCM contains significant quantities of growth factors such as vascular endothelial growth factor, keratinocyte growth factor, and interleukin-8. As these molecules are known to play an important role in normal wound healing *in vivo*, we conducted a pilot clinical evaluation “Proof of Concept” in which individuals, after receiving laser resurfacing, were instructed to use either active or placebo lotion on their abraded skin. **Methods** The end points used were clinical assessment of the time to complete healing, clinical and bioinstrumental mexameter measurements of erythema, and the number of days of rescue petrolatum use by patients, post-laser.

Results Day 7, post-laser treatment, resulted in a greater improvement in erythema, and re-epithelization of the peri-oral and peri-ocular regions in subjects using the active lotion vs. placebo control as determined by blinded, clinical evaluation of gross photographs and bioinstrumental mexameter measurements. A statistically significant reduction in rescue petrolatum use in active lotion-treated subjects was reported. Finally, no attendant cutaneous safety concerns (e.g., irritant/allergic dermatitis) were reported with either active or placebo lotion.

Conclusions This HCCM product may have broad applications within the field of skin wound repair.

Keywords: extracellular matrix, laser treatment, cosmetic procedure, erythema, tissue engineering, wound healing

Introduction

Laser resurfacing as a method to improve cosmesis was introduced in the 1980s.^{1,2} The first lasers used in this

way demonstrated significant rejuvenation of photodamaged skin; however, their side effects, which included prolonged recovery and patient discomfort, quickly led to the introduction of new laser systems that targeted deeper tissues and effectively stimulated collagen production with little patient discomfort.³ Currently, three types of lasers are used for treating photoaging—those that result in ablative, nonablative, or fractional resurfacing.

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The number of laser-resurfacing procedures is increasing in the United States on an annual basis and currently surpasses 500 000 procedures per year.⁴ Currently, a well-established, “gold-standard” product to treat the wounds caused by laser resurfacing does not exist. The topical treatments commonly recommended vary from petrolatum,⁵ to spa lotions to plant extracts that have little clinical evidence supporting their use. Furthermore, patients typically require upward of 2–4 weeks following laser therapy before they feel comfortable about returning to society without the use of some cover-up, such as heavy make-up, scarves, and/or sunglasses.

In the fields of tissue engineering and regenerative medicine, a number of novel ideas have been conceptualized and tested during the past several years. These include use of various scaffolds for growing cells *in vitro* and producing cell-based products. The therapeutic potential of cell-based treatments has been well described and evaluated in a number of clinical trials. These products, e.g., Apligraf^{®6} and Dermagraft^{™7,8} developed in the 1990s for treating leg ulcers, have helped vast numbers of patients and are true testaments to the clinical benefits of cell-based therapies. However, these products are not without challenges, including cell retention or cell engraftment, packaging and shelf life, the regulatory approval process, and managed care reimbursement. In contrast, products derived from human cells, such as naturally secreted extracellular matrix (ECM) or hypoxic conditioned culture medium (HCCM), represent tissue engineering and regenerative medicine solutions without the concern and challenges of the presence of living cells in the final product.

A tissue engineering technology using a platform to allow cell attachment has been developed recently. This process uses techniques for growing neonatal fibroblasts that induce the cells to produce a nonsoluble matrix material, most similar to early embryonic structural tissue(s). This material can provide the framework and signals necessary for later growth and development of blood, skin, muscle, and bone. During this manufacturing process the fibroblasts create a soluble product, the HCCM. In this study, we describe the use of a HCCM product, which is manufactured under unique conditions that mimic the characteristics of the embryonic environment.

The HCCM material contains a variety of valuable growth factors and cytokines, i.e., vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF), and interleukin-8 (IL-8) that have been previously reported to play key roles in the wound healing process. We hypothesized that the growth of human neonatal

fibroblasts under hypoxic and reduced gravitational forces culture conditions, simulating the early embryonic environment prior to angiogenesis, would generate a HCCM material with embryo-like properties, and that this product would be capable of supporting wound healing in humans following post-laser therapy.

In this study, a pilot clinical evaluation was performed to determine whether a topical skin care gel supplemented with HCCM would mitigate common post-laser symptoms, e.g., erythema, edema, and flaking of the skin.

Materials and methods

Active material production and characterization

Scalable 1 L bioreactors were used to grow neonatal fibroblasts on dextran microcarrier beads under hypoxic conditions using standard tissue culture procedures and media. Within 4–8 weeks, two unique products were produced. The first product consists of an embryonic-like ECM and the second product is the HCCM (ReGenica[™] Facial Rejuvenation Complex; Histogen Aesthetics, San Diego, CA, USA) that contains various growth factors known to be critical in wound healing. These culture conditions have been optimized without the need for a fetal bovine serum additive in the final product. These cultures are monitored and regulated for their oxygen concentration (1–5%) and controlled during the entire culture period. The cultures are then harvested after 8 weeks of culture. The raw HCCM is then concentrated using a 10 kDa filter and tested for endotoxin, sterility, VEGF, and KGF concentration levels.

Clinical evaluation

After obtaining human subject approval and informed consent from the volunteers, 49 subjects were enrolled. For the clinical evaluation of the HCCM product, healthy subjects between 18 and 70 years of age were enlisted. Inclusion criteria included a Fitzpatrick score of I–III and no history of facial laser resurfacing within the last 12 months. Patients were treated with an ablative Fractional Erbium Laser in the peri-oral and peri-ocular regions (Starlux 500 2940 Laser; Palomar Medical Technologies, Burlington, MA, USA), as well as a nonablative Fractional Erbium Laser on the remainder of the face (Starlux 500 1540 Laser; Palomar Medical Technologies). ReGenica[™] was applied immediately after the resurfacing was performed. Photographs were taken immediately before treatment (day 1, baseline)

and on days 3, 5, 7, and 14 after treatment using a Canfield system and Nikon d80 camera (Canfield, Fairfield, NJ, USA).

Clinical grading

To grade the amount of erythema, two measurements were used: clinical evaluation (blind-controlled) and bioinstrumental assessment using the mexameter MPA device (see below).

For the clinical evaluation of erythema, two independent dermatologists were provided with blind-coded photographs and were asked to score the erythema levels (between 1 and 5) using the following criteria.

- 5 Severe erythema (deep color including any breaks in skin surface)
- 4 Moderate erythema (less pronounced color in comparison with #5 above)
- 3 Mild erythema (less pronounced color in comparison with either #4 or #5)
- 2 Resolving erythema (minor residual areas of mild erythema)
- 1 No evidence of erythema

Mexameter MPA 5 device

As clinical grading of erythema is subjective and variable from investigator to investigator, instrumentation also was used to quantitate the erythema. For the instrument reading, erythema values were measured using a Mexameter MPA 5 device (Courage-Khazaka, Koeln, Germany).

Study subjects participated in a 3-day washout of all facial products prior to their laser procedure, as well as throughout the 14-day follow-up period. Subjects were instructed to use the HCCM (active lotion) product or the placebo control twice a day for 8 days and were monitored for the entire 14-day period of the study.

Results

mRNA microarrays

The HCCM raw material was produced by culturing primary, neonatal foreskin fibroblasts under hypoxic conditions. This HCCM material was compared with a cell-conditioned medium created by an identical fibroblast cell line grown in monolayer under normoxic tissue culture conditions. Samples of total RNA from both products were compared using AgilentTM (Agilent Technologies, Santa Clara, CA, USA) whole human genome microarrays for global gene expression

(>40,000 genes) (Table 1). Results indicate that the hypoxic culture conditions result in a 14.78-fold increase in mRNA expression for hypoxia-inducible factor (HIF1A) and a 4.9 decrease in its respective inhibitor. This suggests that this HCCM material is experiencing a low oxygen tension environment (hypoxia) because the messenger RNA for HIF1A that codes for the translation of the protein is upregulated and its inhibitor is downregulated. Further, VEGFB (4.33-fold increase), KGF (11.51-fold increase), and IL-8 (5.81-fold increase) levels were also upregulated under these culture conditions. Both VEGF and KGF have been reported to be upregulated in hypoxic environments and facilitate events in the wound healing process.

Documentation of the rate of wound healing using photography

In this pilot clinical evaluation active lotion (HCCM containing) was evaluated as a post-laser resurfacing treatment in 49 healthy volunteers. Twenty-four of the subjects received the active material; the remaining 25 subjects were treated with a placebo consisting of the vehicle gel formulation without the active HCCM agent. Photographs taken immediately before treatment and on four visits after treatment suggest that patients receiving the active formulation had a more rapid recovery, post-laser treatment, compared with the placebo group (Fig. 1a). As can be seen, the slightly older (69 years) subject, who was treated with active formulation, had virtually healed by day 7, whereas the individual (64 years old) who was treated with placebo had not totally healed on that day.

Table 1 Samples of total RNA from HCCM of primary neonatal foreskin fibroblast cultures (under hypoxic conditions) compared with the conditioned culture medium created by an identical fibroblast cell line grown under normoxic conditions. Both products were compared using AgilentTM whole human genome microarrays for global gene expression (>40 000 genes).

Gene	Fold increase	Fold decrease
HIF1A	14.18	
HIF1AN		4.9
VEGFB	4.33	
VEGFC	3.84	
KGF	11.51	
IL-8	5.81	

HIF1A, hypoxia-inducible factor; HIF1AN, hypoxia-inducible factor inhibitor; VEGF, vascular endothelial growth factor; KGF, keratinocyte growth factor; IL, interleukin.



Figure 1 (a, b) Representative photographs taken on 1, 3, 5, 7, and 14 days post-laser therapy of patients treated with either active formulation or placebo. The active formulation product was evaluated as a post-laser resurfacing treatment in 49 patients (active vs. placebo). The products were applied after a micro-fractional ablative erbium laser (Palomar Lux 2940 nm) and nonablative (Palomar Lux 1540 nm) treatment to the face. The results suggest that patients receiving the active formulation product had a more rapid recovery time post-laser treatment compared with the placebo group.

In a comparison of two younger patients (Fig. 1b) the clinical benefit of active formulation is less pronounced. In the subject treated with active formulation (44 years), a benefit can be seen by day 5. In the placebo subject (39 years), the healing results at day 7 were the most dramatic. One limitation in the

comparison of these two subjects is the variation in severity of laser treatment. This limitation exists throughout the study. In Figure 1b, the placebo subject received slightly more aggressive laser treatment than did the active formulation-treated, which makes direct comparisons difficult.

Days of use of an ointment to treat residual crusting and flakiness

After laser treatment, all subjects were provided a jar of petrolatum to take home and use “as needed” on their healing skin, in addition to the active formulation or placebo, as long as the subjects felt it necessary to emolliate their treated skin. Most individuals using active formulation stopped using the petrolatum at 4.13 days (± 0.56) after treatment (Fig. 2). By contrast, the individuals treated with placebo used petrolatum for 6.69 days (± 0.40). The difference in the days of petrolatum use is statistically significant ($P = 0.0004$), suggesting that the active formulation enhances reconstitution of a skin barrier.

Grading of erythema on treated skin

Clinical grading

The amount of erythema in the facial skin after laser treatment was graded clinically (using gross photographs at each time points) by two independent dermatologists, blinded to the type of treatment the subject received. The difference in the erythema score between the active formulation and placebo was not statistically significant on the first few days after laser therapy. However, starting on day 5 after treatment, the amount of erythema was less for the active formulation-treated skin compared with the placebo (Fig. 3).

Measurement of erythema by instrumentation

The erythema remaining in the skin after laser therapy also was judged using a Mexameter MPA 5 device. Using this instrument the amount of erythema recorded at each scheduled office visit over the 14-day study period was captured (Fig. 4). As can be seen, the values paralleled the clinical, *in vivo* findings exactly, although the differences were not statistically significant.

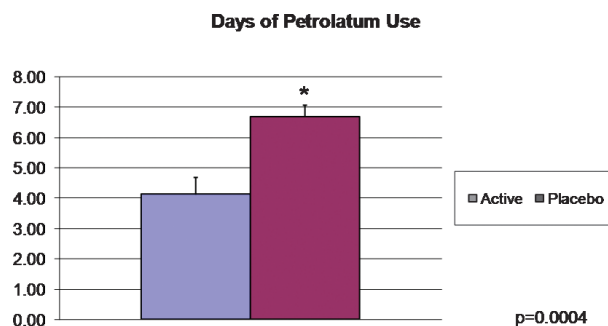


Figure 2 Documentation of the use of petrolatum in the active formulation and placebo treatment groups throughout the study. * $P = 0.0004$.

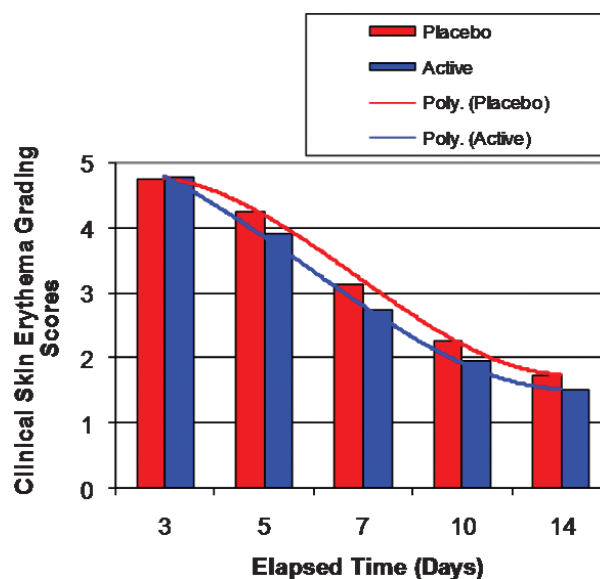


Figure 3 Clinical erythema grading scores. Two independent dermatologists were provided with blind-coded photographs and asked to score the erythema levels.

In summary, at day 7, more improvement in erythema, and re-epithelization of the peri-oral, and peri-ocular regions in subjects using the active formulation vs. placebo had occurred. Additionally, instrument evaluation of hemoglobin capillary extravasation supported the findings from the blinded, clinical evaluation of the gross photographs that demonstrated a trend in the reduction in erythema in the active formulation-treated subjects vs. placebo control. Self-administered subject questionnaires revealed a perceived, positive experience with the active formulation (no itching, burning, stinging) and a statistically significant reduction in reported rescue petrolatum use in subjects treated with active formulation vs. placebo. Finally, no attendant cutaneous safety concerns (e.g., irritant/allergic dermatitis) with either product use occurred over the course of the study.

Discussion

In the current study, the HCCM product was used as the active ingredient the active formulation. This HCCM product is the result of a well-established tissue engineering process in the field that has provided a number of unique products for clinical use. Specifically, using neonatal fibroblasts to develop naturally occurring products was first introduced as a product known as TransCyte^{TM9} (Advanced Tissue Sciences, Inc., La Jolla, CA, USA). TransCyteTM is a tissue-engineered human

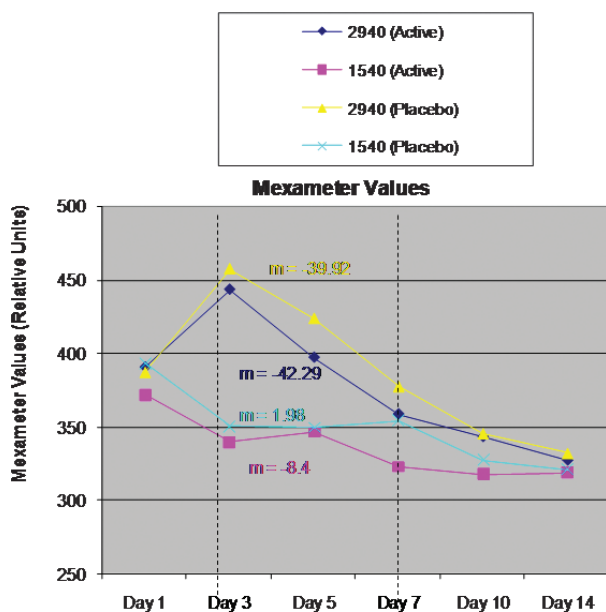


Figure 4 Mexameter values obtained from both placebo and active treatment groups that had received the 2940 or 1540 nm laser treatment(s). While not significant, the group receiving active formulation after the Palomar Lux 2940 resurfacing had a more rapid decrease over time in Mexameter values, as indicated by the slope values between days 3 and 7 ($m = -42.29$ vs. $m = -39.92$). Thus, these patients demonstrated a more rapid decrease over time in extravasation of hemoglobin from the capillary plexus in the dermis (i.e., less erythema) leading to a more rapid recovery).

ECM product with premarket approval for partial and full thickness burns. The ECM is created by seeding neonatal fibroblasts onto a Biobrane[®] (Smith & Nephew Wound Management, UK) scaffold placed within a closed bioreactor system that supports the uniform deposition of human collagens and glycosaminoglycans during a 3-week manufacturing process. At the end of the process cells are lysed and the product is thoroughly rinsed before being frozen. The human tissue-engineered ECM of Trancyte[™] induces rapid epithelialization and has demonstrated statistically significant faster healing, pain reduction as well as reduction in patient care time in mid-dermal facial burns.⁹

The next generation product using this fibroblast technology was Dermagraft[™] (Advanced BioHealing, La Jolla, CA, USA). Dermagraft[™], a living human dermal implant, was invented to provide living cells, human matrix components, and growth factors into wounds not responding to standard wound care management techniques.^{7,8} The growth factors produced by the fibroblasts within the Dermagraft[™] product include VEGF, KGF, and numerous others that have been well described to influence and augment the natural wound healing

process.^{7,10} These early tissue engineering technologies provided clinically significant benefits to patients by augmenting or enhancing the wound healing process leading to recovery.

Additionally, a paramount goal within the field of skin wound healing is the ability to reduce scar formation. Specifically, the concept of augmenting normal adult wound healing to be more representative of wound healing or tissue generation as it first occurs *in utero* would have vast clinical utility. Others have reported on the unique hypoxic environment during fetal development.¹¹ The influence of this hypoxic environment, in combination with many other aspects of developmental biology, contributes to the phenomenon of scarless wound repair. We hypothesize that culturing neonatal fibroblasts in suspension under low oxygen (hypoxic) conditions provides key signals, representative of developmental conditions, which encourage the conditioning of the culture medium. This HCCM product may have broad applications within the field of skin wound repair.

The characteristics of the HCCM material, as detected with microarray (Table 1), indicate that mRNA for key growth factors and cytokines is influenced by the culturing process. Specifically, VEGF and KGF are upregulated in their expression. Additionally, mRNA expression for IL-8 is increased. These molecules have been well described in the literature to facilitate normal wound healing events and may be partially responsible for the clinical benefits described in the current study.¹⁰ In the field of post-laser resurfacing care, numerous products exist in the marketplace. While some have reported their findings in the literature,¹² few report components that are well described to facilitate the normal wound healing process and be relatively abundant *in utero* during development.

In the clinical trial reported here, 49 healthy volunteers were enrolled in a clinical evaluation to test the efficacy of an active formulation (containing HCCM) in accelerating wound healing after laser resurfacing of the face. Use of a controlled-production culture medium that contains products produced by neonatal fibroblasts grown under embryo-like conditions appears to accelerate wound healing. Some of the results of this proof of principle study are statistically significant and have encouraged us to continue to pursue the concept that this type of product may be useful in the treatment of superficial injuries, iatrogenic or otherwise, of human skin. Additional studies using this novel HCCM functional ingredient in controlled application following split-face laser treatment as well as long-term maintenance post-laser cosmetic applications will need to be evaluated.

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