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Novel Model for Evaluation of Epidermal Preservation and Dermal Collagen Remodeling Following Photorejuvenation of Human Skin

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Background and Objectives: In order to optimize photorejuvenation of human skin, a method must be developed to reliably compare the potential for epidermal preservation and dermal fibroblast stimulation of different laser devices and irradiation parameters. We describe a novel human skin tissue culture model developed for this purpose.

Materials and Methods: An artificial skin model, consisting of human keratinocytes in the epidermis and human fibroblasts and rat-tail collagen in the dermis, was cultured using the floating collagen gel (RAFT) method. Repetitive low-fluence Er:YAG laser irradiation was applied to test the applicability of our RAFT model for characterization of epidermal preservation and dermal fibroblast stimulation post-laser treatment.

Results: Histopathologic evaluation revealed a thin layer of epidermal keratinocyte preservation immediately after low fluence sub-ablative Er:YAG laser irradiation. One-week post-laser irradiation, the average increase in number of dermal fibroblasts as compared to control was statistically significant ($P < 0.01$).

Conclusions: The RAFT model can be used to assess the potential for epidermal preservation and dermal fibroblast stimulation of different photorejuvenation devices and irradiation parameters and offers several advantages over traditional animal and human skin models. *Lasers Surg. Med.* 32:115–119, 2003. © 2003 Wiley-Liss, Inc.

Key words: floating collagen gel; RAFT model; artificial skin; laser skin resurfacing; photorejuvenation

INTRODUCTION

The efficacy of conventional laser skin resurfacing (LSR) for treatment of photodamage and rhytid reduction is well established [1]. However, this procedure ablates the epidermis, which subjects the patient to considerable discomfort and risks including infection, prolonged erythema, and dyspigmentation. An ideal method of photorejuvenation would achieve cosmetic improvement with minimal post-operative wound care and risk of adverse effects.

Histological assessment of actinically damaged dermis demonstrates increased glycosaminoglycans and abundant, thickened and tortuous elastic fibers. Because these

histologic changes associated with wrinkles are primarily present in the dermis, epidermal disruption may not be necessary for rhytid improvement [2].

In pursuit of a method to preserve the epidermis while stimulating dermal remodeling and collagen formation, investigators have proposed multiple techniques for photorejuvenation of human skin. Variable treatment outcomes have been achieved, but to date, an optimal technique has not been demonstrated [3]. Several irradiation parameters must be considered in development of photorejuvenation techniques, including wavelength, pulse duration, spot size, repetition rate, and light dose. However, a practical and reliable method to compare the potential for epidermal preservation and dermal fibroblast stimulation of different laser devices and irradiation parameters is not presently available.

Previous studies of photorejuvenation involved either animal models or human subjects, but neither method is completely satisfactory. Rat or porcine skin is commonly used in animal studies, but the wound healing response in these models certainly differs from that of human skin after laser treatment. As a result, conclusions from such studies may not be relevant for human skin [4]. In human studies, it is difficult to compare the effects of different devices or multiple irradiation parameters on a single subject. In addition, human studies are subject to concerns regarding post-irradiation medical care, cosmetic effects of biopsies, and regulatory obstacles.

We propose an alternative model for objective in vitro evaluation of photorejuvenation of human skin. The discussed model (floating collagen gel: RAFT) is a form of

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artificial skin, composed of human keratinocytes in the epidermal layer, and human fibroblasts and rat-tail collagen in the dermal layer. The term "RAFT" was coined by Michalopoulos and Pitot [5], and refers to the process whereby liver epithelial cells were exposed to air and prompted to differentiate after being "floated" on a collagen matrix. In 1984, Asselineau and Prunieras adapted this technique to skin research [6], and, with minor modifications, it has been used since for studies in dermatology, surgery, and pharmacology.

Our RAFT model mimics *in vivo* human skin in terms of structure, cellular activity, and function [7]. The keratinocyte layers on top of the dermal layer mimic the epidermis of *in vivo* human skin [8]. Cultured fibroblasts form dense collagen fibrils, which repress fibroblast growth, similar to that seen *in vivo* in the dermis.

This study introduces the RAFT model for evaluation of the potential for epidermal preservation and dermal fibroblast stimulation induced by a method of laser photorejuvenation. The laser irradiation parameters were selected to resemble those used successfully for laser-induced collagen stimulation and dermal remodeling in previous animal studies. In that work, repetitive low-fluence Er:YAG laser irradiation was proven to induce deep collagen coagulation and subsequent neo-collagen formation with epidermal preservation [9,10].

MATERIALS AND METHODS

Cell Cultures

Normal human epidermal keratinocytes from neonatal skin (BioWhittaker, Walkersville, MD) were cultured in

KGM-2 medium (BioWhittaker) at 37°C in 7.5% CO₂ atmosphere.

Normal human dermal fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 20 IU of penicillin per milliliter, 20 IU of streptomycin per milliliter, and 0.4 mM L-glutamine.

RAFT Model Preparation

Reconstitution buffer was made with 2.2 g NaHCO₃ and 4.77 g HEPES in 100 ml 0.05 M NaOH and sterilized with a 0.22 µm filter. Seven parts of rat-tail collagen, type 1 (BD Biosciences, Bedford, MA) were mixed with two parts of 5 × DMEM and one part buffer and neutralized with 1 M NaOH to pH 7.4 ± 0.2.

Fibroblasts were suspended in the collagen matrix at a density of 1 × 10⁵ cells/ml. For construction of the dermal layer, 1 ml of this suspension was allotted into each well of a 24-well plate (Corning, Corning, NY) and incubated for 24 hours at 37°C in a 7.5% CO₂ atmosphere. For construction of the epidermal layer, keratinocytes were suspended with KGM-2 medium at a density of 1 × 10⁵ cells/ml and seeded in 1 ml aliquots onto each dermal layer of the 24-well plate. All RAFT models were incubated under identical conditions throughout the study.

After 1 week, the models were lifted to the air-liquid interface using a stainless steel grid support (Fig. 1a). The keratinocytes were allowed to stratify and differentiate to form a 50 µm thick epithelium (i.e., 10–12 keratinocyte layers) (Fig. 1b). Two weeks after culture initiation, the tissue models were ready for laser irradiation.

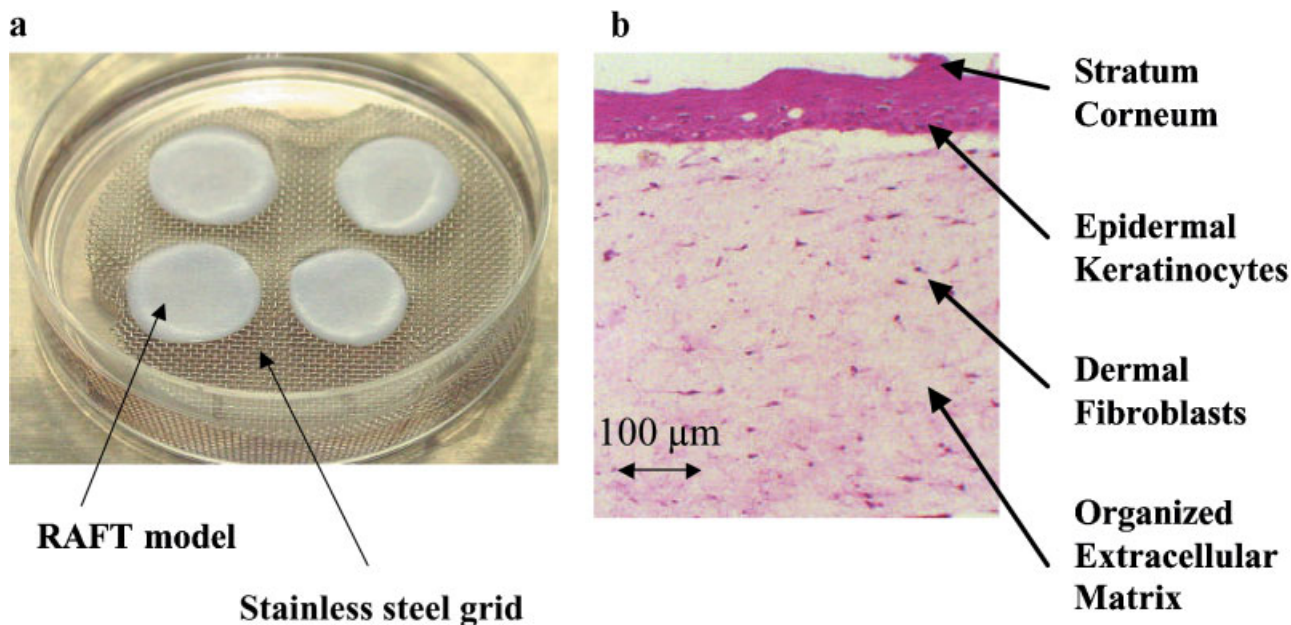


Fig. 1. RAFT model: (a) RAFT model on a stainless steel grid in a culture dish; (b) Histopathology of the RAFT model. [Hematoxylin and eosin (H&E) stain; original magnification 100×.]

Laser Irradiation

Two sets of irradiation parameters were tested: a sequence of 5 or 10, 0.8 J/cm^2 sub-ablative pulses from an Er:YAG laser (UltraFine™ by Coherent, Santa Clara, CA), delivered at a repetition rate of 5 Hz. Laser irradiation procedures resembled those used in previous animal studies in our laboratory [9,10]. The 4 mm laser hand-piece was mounted 25 cm above the target surface to increase the spot size, resulting in a single-pulse energy density of $1.4 \pm 0.2 \text{ J/cm}^2$ in the central region of the irradiated spot. Two external attenuators were inserted into the beam to reduce the fluence to 0.8 J/cm^2 . Three test spots were created with each set of laser parameters.

Histopathology

RAFT specimens from test sites and controls were biopsied at baseline (immediately post-irradiation for the test sites and a comparable time point for controls) and 1 week post-irradiation. All samples were fixed for 24 hours in buffered 10% formalin and then transferred to phosphate buffered saline. Samples were embedded in paraffin, cut into $6 \mu\text{m}$ sections and mounted onto albumin-coated slides for hematoxylin and eosin (H&E) staining.

Three digital images were taken from each slide using an optical microscope (Olympus, model BH-2, Melville, NY) and digital camera (Olympus, DP10), and standardized using computer software (Adobe Photoshop). The number of fibroblasts in a $900 \times 600 \mu\text{m}$ area was counted from each of the printed images by three examiners. The numbers in Table 1 represent averaged values of these counts.

A paired *t*-test was performed to determine the statistical significance of the differences in dermal fibroblast counts at baseline and 1 week post-laser irradiation.

RESULTS

Representative histologic sections of control samples are presented in Figures 2a,b. The majority of the epidermis is missing in these specimens as it was in the irradiated samples. We believe this to be a processing artifact as discussed in more detail later.

Figures 2c,d demonstrate the histology obtained using $0.8 \text{ J/cm}^2 \times 5$ pulses and are representative of results obtained after laser irradiation. The sections obtained at baseline (immediately post-irradiation) demonstrate a few

preserved keratinocytes (Fig. 2c). There was essentially no fibroblast change and only mild shortening of upper dermal collagen fibers. After 1 week (Fig. 2d), the fibroblast density was increased in the irradiated samples as compared to controls ($P < 0.01$) (Table 1).

DISCUSSION

In our study, we were able to preserve a thin layer of RAFT epidermis after sub-threshold Er:YAG laser irradiation. In addition, the average dermal fibroblast number was significantly increased at 1 week post-laser irradiation, relative to non-irradiated control samples harvested at the same time. These results are comparable to those achieved in our previous animal study and approach the goals of non-ablative photorejuvenation: epidermal preservation and dermal fibroblast stimulation.

The RAFT model offers a variety of benefits over previously utilized test media. This model mimics human skin better than animal models because it contains human keratinocytes and fibroblasts and also has human skin-like structure. Many identical specimens can be tested simultaneously without the difficulties and limitations inherent to animal and human studies. The model can be engineered as desired by varying the size, dermal thickness, and cell density. Further, there is the potential with this model, for manipulation of other factors relevant to the healing process including inflammatory cells, cytokines, and perhaps even blood flow.

In both the irradiated and control samples, only a few epidermal cells were visualized after the histologic processing. Prior to processing, we did visualize a thicker epidermal layer with a microscope set up adjacent to the RAFT irradiation equipment (a system which did not permit photography). We believe that due to the immature nature of our RAFT samples (2–5 weeks old), the adhesion between the cell layers was not sufficiently strong to endure the histologic processing. This can be corrected in future work by reducing the peak temperature during histologic processing to 55°C , below the collagen melting point, preventing dermal–epidermal junction separation.

In conclusion, with our RAFT model, we were able to demonstrate a thin layer of epidermal preservation and simultaneous stimulation of dermal fibroblasts after multiple-pulse, low fluence Er:YAG laser irradiation, results comparable to previous animal studies. This article

TABLE 1. Average Fibroblast Count (Number Per Viewing Field) for Control and Irradiated Samples at Baseline and 1 Week Post-Irradiation

Pulses \times energy density	Baseline	One week	
Control	43.5	48.5	+11%
$5 \times 0.8 \text{ J/cm}^2$	58.8	92.2	+57%**
$10 \times 0.8 \text{ J/cm}^2$	41.7	52.5	+26%*
Average of all irradiated samples	50.3	72.3	+43%**

The percent change is calculated relative to baseline.

* $P < 0.05$.

** $P < 0.01$.

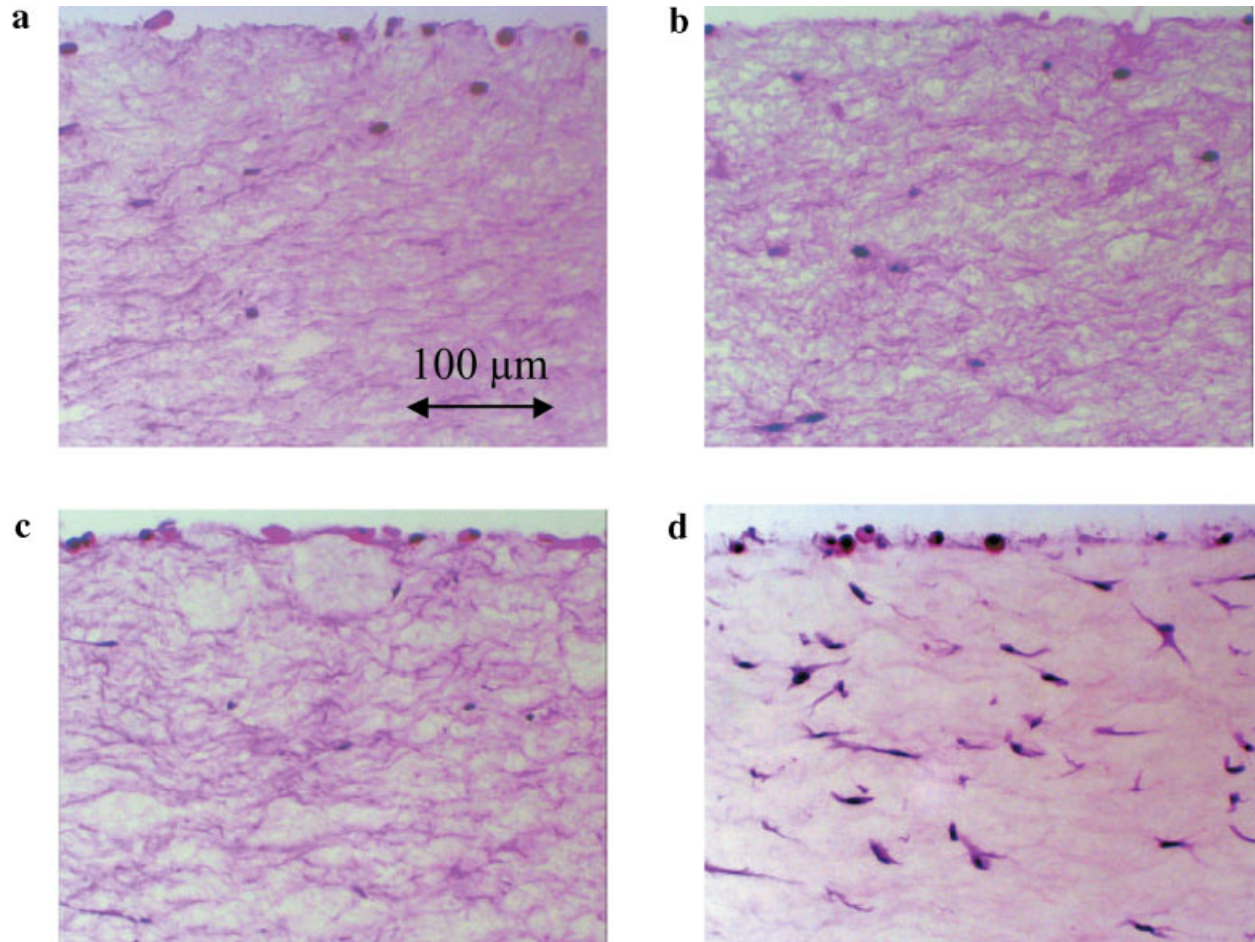


Fig. 2. RAFT control samples harvested at: (a) baseline; (b) 1 week. RAFT samples irradiated with $0.8 \text{ J/cm}^2 \times 5$ pulses, harvested at: (c) baseline; (d) 1 week. [Bar in Fig. 2a is $100 \mu\text{m}$; H&E stain; original magnification $200\times$.]

provides our preliminary results with the use of this model, but we believe that with minor adjustments, the RAFT model can be used to compare the potential for epidermal preservation and dermal fibroblast stimulation of different photorejuvenation devices and irradiation parameters. We have recently amended our RAFT construction process by increasing the number of fibroblasts added during dermal layer construction. This should allow more reliable comparison of subtle fibroblast number changes in our future studies. Overall, the RAFT model offers several advantages over human and animal skin models and further study is recommended to elucidate its potential.

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