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Authors
Kao, Bunsho
Kelly, Kristen M
Aguilar, Guillermo
et al.

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Evaluation of Cryogen Spray Cooling Exposure on In Vitro Model Human Skin

Bunsho Kao, MD, PhD,[1,2] Kristen M. Kelly, MD,[1,4]* Guillermo Aguilar, PhD,[1,3,5] Yoshiaki Hosaka, MD, PhD,[2] Ronald J. Barr, MD,[4] and J. Stuart Nelson, MD, PhD[1,3,4]

[1]*Beckman Laser Institute and Medical Clinic, Irvine, California*
[2]*Department of Plastic and Reconstructive Surgery, Showa University, Tokyo, Japan 142-8666*
[3]*Department of Biomedical Engineering, University of California, Irvine, California 92612*
[4]*Department of Dermatology, University of California, Irvine, California 92612*
[5]*Department of Mechanical Engineering, University of California, Riverside, California 92521*

Background and Objectives: Cryogen spray cooling (CSC) is commonly used during dermatologic laser surgery. The epidermal and dermal effects of CSC have not been adequately evaluated. To study the potential for epidermal and dermal injury after CSC using an in vitro model of human skin (RAFT).

Study Design/Materials and Methods: RAFT specimens were exposed to continuous CSC spurt durations of 10, 20, 40, 80, 100, 200, or 500 milliseconds. Biopsies were taken acutely, 3 and 7 days post-CSC exposure. Sections were stained with hematoxylin and eosin for evaluation of possible injury, Ki-67 to determine keratinocyte viability, and Melan-A, to identify and evaluate melanocytes.

Results: Minimal, transient epidermal changes were noted in specimens exposed to continuous CSC spurts of 80 milliseconds or less. Keratinocytes and melanocytes remained viable. Continuous CSC spurts of 100, 200, or 500 milliseconds (much longer than recommended for clinical use) resulted in significant epidermal injury acutely, with partial or full thickness epidermal necrosis at 7 days. Only the 500 millisecond specimen demonstrated dermal change, decreased fibroblast proliferation at 3 days.

Conclusions: Continuous CSC spurts of 80 milliseconds or less induce minimal, if any, epidermal or dermal damage and are unlikely to produce cryo-injury when used during dermatologic laser surgery. Lasers Surg. Med. 34:146–154, 2004. © 2004 Wiley-Liss, Inc.

Key words: RAFT; cryogen spray cooling; cryogen injury

INTRODUCTION

The risk of non-specific epidermal thermal injury must be addressed in order to safely and effectively perform laser treatment directed at subcutaneous targets. Epidermal melanin represents an "optical barrier" through which light must pass to reach targeted dermal chromophores. Melanin absorption of laser light causes localized heating of the epidermis, which may, if not controlled, produce permanent complications such as scarring or dyspigmentation. Selective cooling protects the epidermis, allowing the safe use of higher laser fluences, permitting treatment of darker skin types, and decreasing treatment pain, and has become an integral part of dermatologic laser surgery [1].

One effective and efficient method of selective epidermal cooling is cryogen spray cooling (CSC) or dynamic cooling which has been adapted for use in conjunction with laser therapy for a wide variety of clinical indications [2–4]. CSC has been incorporated into many commercially available laser devices currently used for treatment of vascular lesions, hair removal, and non-ablative skin rejuvenation [1]. A millisecond cryogen spurt is applied to the skin surface immediately before laser exposure. As liquid cryogen rapidly evaporates, the skin temperature is reduced as a result of supplying the latent heat of vaporization. Tetrafluoroethane ($C_2H_2F_4$), an environmentally compatible, non-toxic, non-flammable freon substitute [5], has been demonstrated in multiple studies to be a safe and effective cooling agent and is the only cryogenic compound currently approved for dermatologic use by the Food and Drug Administration.

Despite the excellent safety record of CSC, some investigators have recently expressed concern that CSC may, on occasion, induce cryo-injury or frostbite [6]. In order to address this concern, the effects on human skin of subzero temperature exposure after CSC must be clearly understood.

Previous studies of CSC have involved computational models [7–9], epoxy phantoms [10], animal models, and human subjects [3]. None of these methods allows easy and

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*Correspondence to: Kristen M. Kelly, MD, Beckman Laser Institute and Medical Clinic, 1002 Health Sciences Road East, Irvine, California 92612. E-mail: KMKelly@uci.edu

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accurate evaluation of the potential for acute CSC injury as well as observation of the recovery process. Computational models and epoxy phantoms obviously cannot show the response of human skin to CSC injury. The wound healing response in animal models differs from that of human skin after CSC. In human studies, it is difficult to compare the effects of multiple CSC parameters (e.g., spurt duration, cryogen mass flux, droplet size, velocity, and temperature) on a single subject.

In a previous study, we demonstrated the usefulness of an in vitro model of human skin (floating collagen gel: RAFT) to evaluate the wound healing response after irradiation with devices used for photorejuvenation [11]. The RAFT model mimics in vivo human skin in terms of structure, cellular activity, and function [12]. Cultured fibroblasts form dense collagen fibrils, which repress fibroblast growth, as seen in human dermis. The keratinocyte layers with melanocytes added, similarly mimic the epidermis of in vivo human skin. RAFT tissue culture overcomes many of the difficulties associated with other models as described above. Human tissue injury and healing can be approximated and a wide range of devices and parameters can be easily and simultaneously evaluated.

To study the potential for epidermal and dermal injury after CSC, we exposed RAFT specimens to cryogen spurt durations of 10, 20, 40, 80, 100, 200 or 500 milliseconds and then performed serial histologic evaluations over the subsequent 7 days.

MATERIALS AND METHODS

Cell Cultures

Human epidermal keratinocytes from neonatal skin (BioWhittaker, Walkersville, MD) were cultured in KGM-2 medium (BioWhittaker) at 37°C in 7.5% CO_2 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 ml, 20 IU of streptomycin per ml, and 0.4 mM L-glutamine.

Raft Model Construction

Reconstitution buffer was made with 2.2 g $NaHCO_3$ and 4.77 g HEPES in 100 ml of 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 concentrated 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 4×10^5 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 at 37°C in a 7.5% CO_2 atmosphere on day 0 (Fig. 1a). Six hours after construction of the dermal layer, melanocytes (Cell Culture Core Facility, Department of Dermatology, Yale University, New Haven, CT) were suspended with KGM-2 medium at a density of 5×10^4 cells/ml and seeded in 1 ml aliquots into each dermal layer of the 24 well plate.

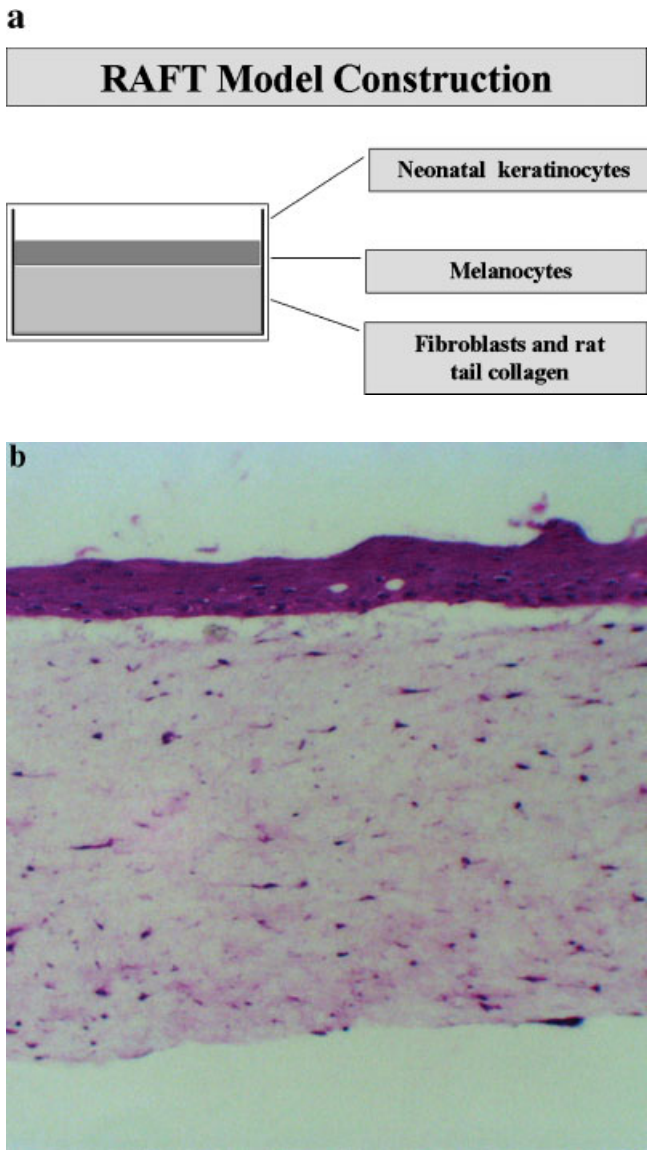

Fig. 1. RAFT model: (**a**) RAFT model construction. **b**: Histopathology of the RAFT model (H&E stain; original magnification = 100×). [Figure can be viewed in color online via www.interscience.wiley.com.]

Keratinocytes were suspended with KGM-2 medium at a density of 3×10^5 cells/ml and seeded in 1 ml aliquots into each well of the 24 well plate on day 1. All RAFT models were incubated under identical conditions throughout the study.

Models were lifted using a stainless steel grid support to form an air-liquid interface on day 2. A 3 μm pore size polycarbonate membrane filter (Osmonics, Minnetonka, MN) was placed between the specimen and stainless steel grid to avoid adhesion to the grid. Thereafter, cells were nourished only by diffusion from under the matrix. The keratinocytes were allowed to stratify and differentiate into a 50 μm full-thickness epithelium consisting of 10–12 layers (Fig. 1b). On day 11, the models were exposed to cryogen spurts of varying durations.

Cryogen delivery and nozzle. The cryogen utilized was $C_2H_2F_4$, also known as R134a, with boiling temperature $T = -26^{\circ}C$ at atmospheric pressure. Cryogen was kept in a container at saturation pressure (approximately 6.6 bar at 25°C), and delivered as a continuous spurt through a standard high-pressure hose to an identical solenoid valve and nozzle used in several commercial CSC devices (GentleLASE®, Smoothbeam™, and Vbeam®, Candela Corporation, Wayland, MA). The distance of the nozzle tip to the sprayed RAFT specimens was identical to that of the Vbeam laser (35 mm). Spurt durations, defined as the time the valve remained open, were electronically controlled by a digital delay generator (DG535, Stanford Research Systems, Sunnyvale, CA). Continuous cryogen spurts of 10, 20, 40, 80, 100, 200, or 500 milliseconds were evaluated.

Histopathology

RAFT specimens were biopsied acutely, 3 and 7 days post-CSC exposure. Control specimens (not exposed to CSC) were harvested at identical time points. All specimens were fixed for 24 hours in buffered 10% formalin and then transferred to phosphate buffered saline. Specimens were embedded in paraffin, cut into 6 μm thick sections, and mounted onto albumin-coated slides for hematoxylin and eosin (H&E) staining. Ki-67 staining was performed on selected sections to evaluate cell viability and proliferation. Melan-A staining was performed on selected sections to identify melanocytes in order to evaluate the effects of CSC on those cells. For Ki-67 and Melan-A stains, specimens were cut into 4 μm thick sections and mounted onto Chem Mate™ capillary gap plus slides (BioTek, Winooski, VT).

Three digital images were taken from each slide using an optical microscope (Olympus, model BH-2) and digital camera (Olympus, DP10), and then standardized using computer software (Adobe Photoshop).

RESULTS

Figure 1b shows a histological section of a control specimen not exposed to CSC showing the normal epidermal thickness and dermal structure of the RAFT model.

10 and 20 Millisecond Cryogen Spurts

Acute specimens exposed to 10 or 20 millisecond cryogen spurts did not demonstrate any significant epidermal or dermal injury. At 3 days, no injury was noted in the specimens exposed to 10 millisecond cryogen spurts. In the 20 millisecond specimens, there was focal mild parakeratosis and scattered apoptotic cells. At 7 days, mild hyperkeratosis and parakeratosis with rare apoptotic cells were noted in the 10 and 20 millisecond specimens. In as much as similar changes were noted in control specimens, we believe the latter can be attributed to the normal RAFT aging process. No dermal injury was noted in any of the specimens exposed to 10 or 20 millisecond cryogen spurts.

40 and 80 Millisecond Cryogen Spurts

Figures 2 and 3 show histological sections of the RAFT exposed to spurt durations of 40 and 80 millisecond,

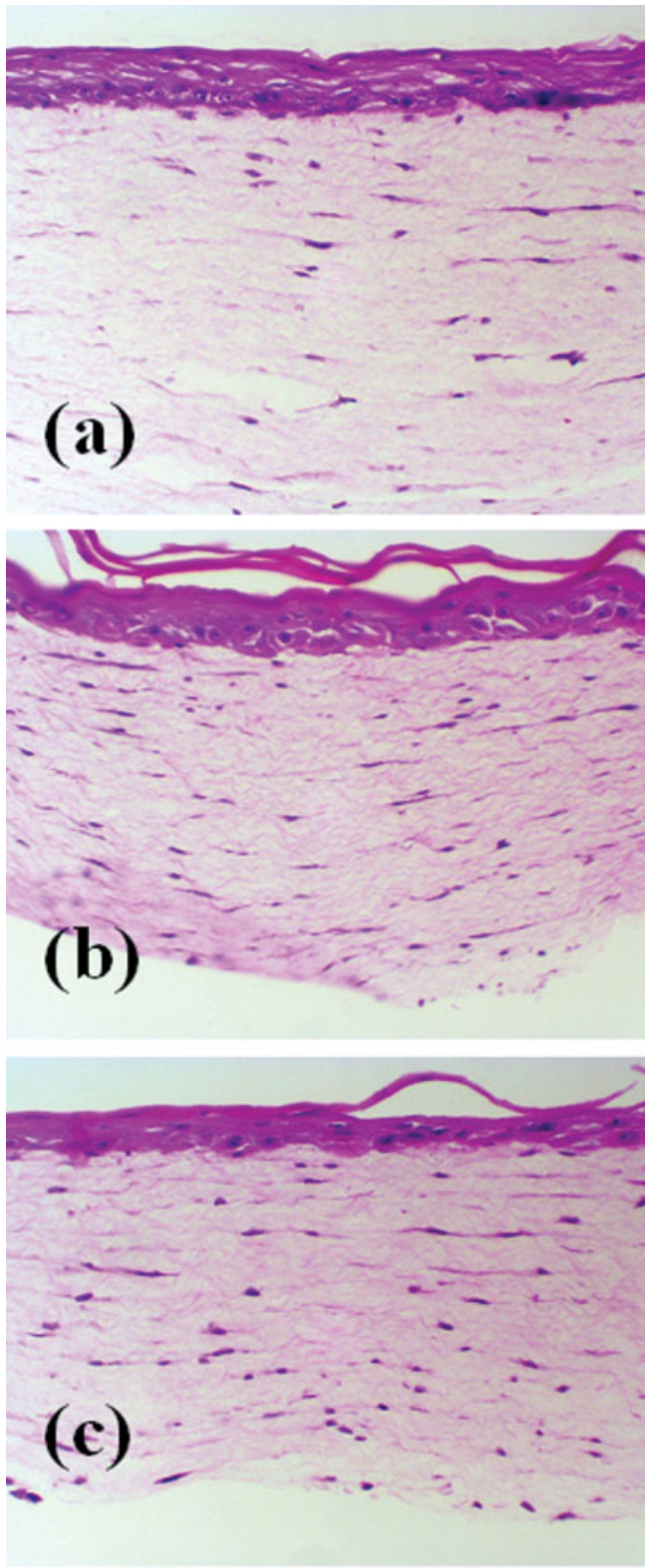

Fig. 2. RAFT specimens exposed to 40 millisecond spurt of CSC harvested (**a**) acutely; (**b**) 3 days; and (**c**) 7 days after cryogen exposure (H&E stain; original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

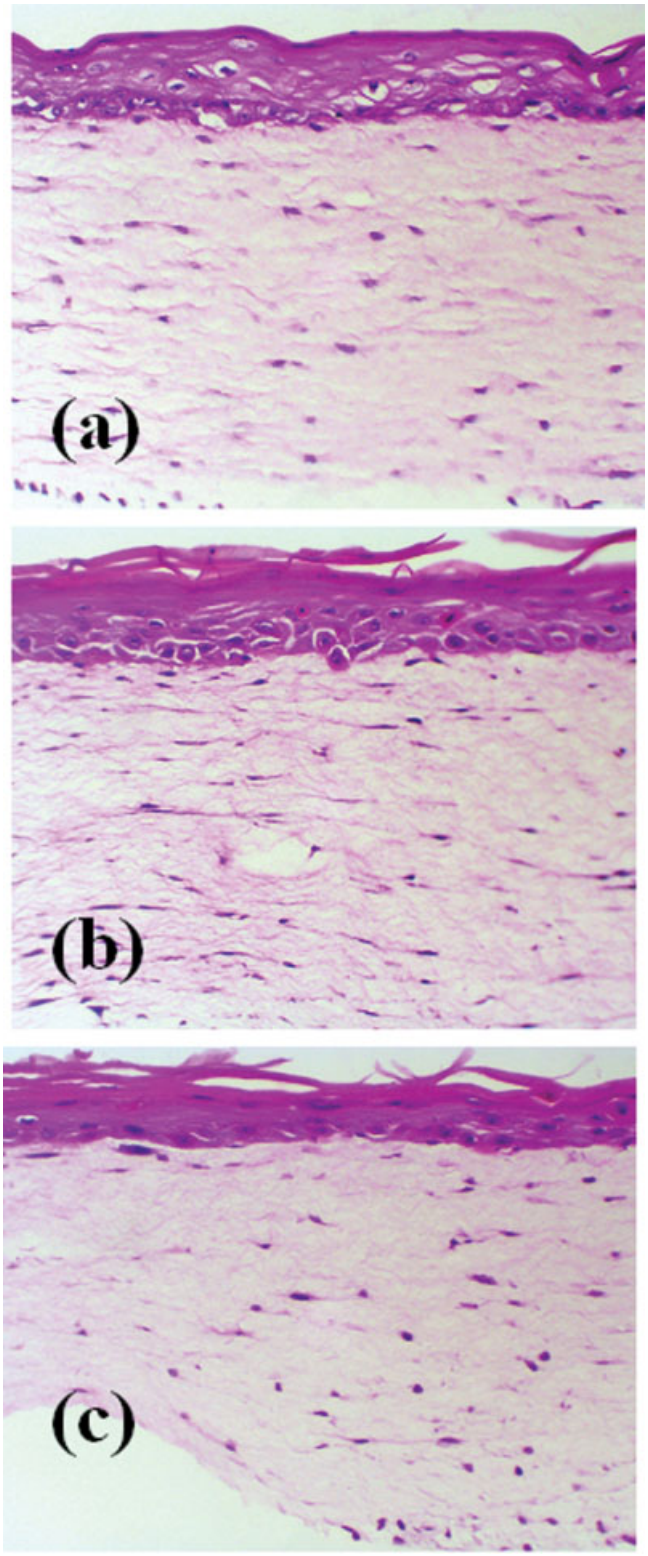

Fig. 3. RAFT specimens exposed to 80 millisecond spurt of CSC harvested (**a**) acutely; (**b**) 3 days; and (**c**) 7 days after cryogen exposure (H&E stain; original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

respectively. Acutely, minimal epidermal injury was noted in specimens exposed to 40 or 80 millisecond cryogen spurts. In the 40 millisecond specimens, the stratum corneum exhibited mild parakeratosis and rare vacuolated keratinocytes and scattered apoptotic cells were identified. In the 80 millisecond specimen, parakeratosis was increased and more vacuolated keratinocytes and apoptotic cells were noted. However, the vast majority of epidermal cells appeared unaffected by cryogen exposure. At 3 days, parakeratosis and more scattered apoptotic cells were noted, but once again, the majority of the epidermis appeared healthy and intact. At 7 days, mild hyperkeratosis and parakeratosis with rare apoptotic cells were noted, changes that were found similarly in control specimens and, as previously noted, are believed to be the result of normal RAFT aging. No dermal injury was noted in any of the specimens exposed to 40 or 80 millisecond cryogen spurts.

100 and 200 Millisecond Cryogen Spurts

Figure 4 shows histological sections of the RAFT exposed to a spurt duration of 100 millisecond. Acutely, the epidermis was compacted and a considerable number of apoptotic cells were noted in the 100 millisecond specimen. Similar changes were evident, as well as concomitant hydropic degeneration, in the 200 millisecond specimen. At day 3, in both the 100 and 200 millisecond specimens, parakeratosis was noted with superficial epidermal necrosis and apoptotic cells. At 7 days, the upper half of the epidermis was necrotic after exposure to 100 or 200 millisecond cryogen spurts. Once again, no dermal injury was noted in any of the specimens exposed to 100 or 200 millisecond cryogen spurts.

500 Millisecond Cryogen Spurt

Figure 5 shows histological sections of the RAFT exposed to a spurt duration of 500 millisecond. Acutely, many apoptotic cells and hydropic degeneration were noted after exposure to 500 millisecond cryogen spurts. At 3 days, the upper half of the epidermis was necrotic and at 7 days, the entire epidermis was necrotic. Dermal injury was not evident on H&E specimens but decreased fibroblast activity was noted with Ki-67 staining (see below).

Ki-67 Staining

Ki-67 staining identifies proliferating, viable cells of any origin [13] and was performed on day 3 specimens after exposure to 40, 80, 100, 200, or 500 millisecond cryogen spurts (Fig. 6). Ki-67 staining was similar to control in those specimens exposed to 40 or 80 millisecond cryogen spurts, confirming that both keratinocytes and fibroblasts were viable and normally proliferating. Ki-67 staining of 100 or 200 millisecond specimens on day 3 demonstrated a decreased number of proliferating keratinocytes but continued proliferation of dermal fibroblasts. In the 500 millisecond specimen, Ki-67 staining on day 3 revealed only rare proliferating cells in the epidermis. At this very long spurt duration, decreased fibroblast staining was also observed.

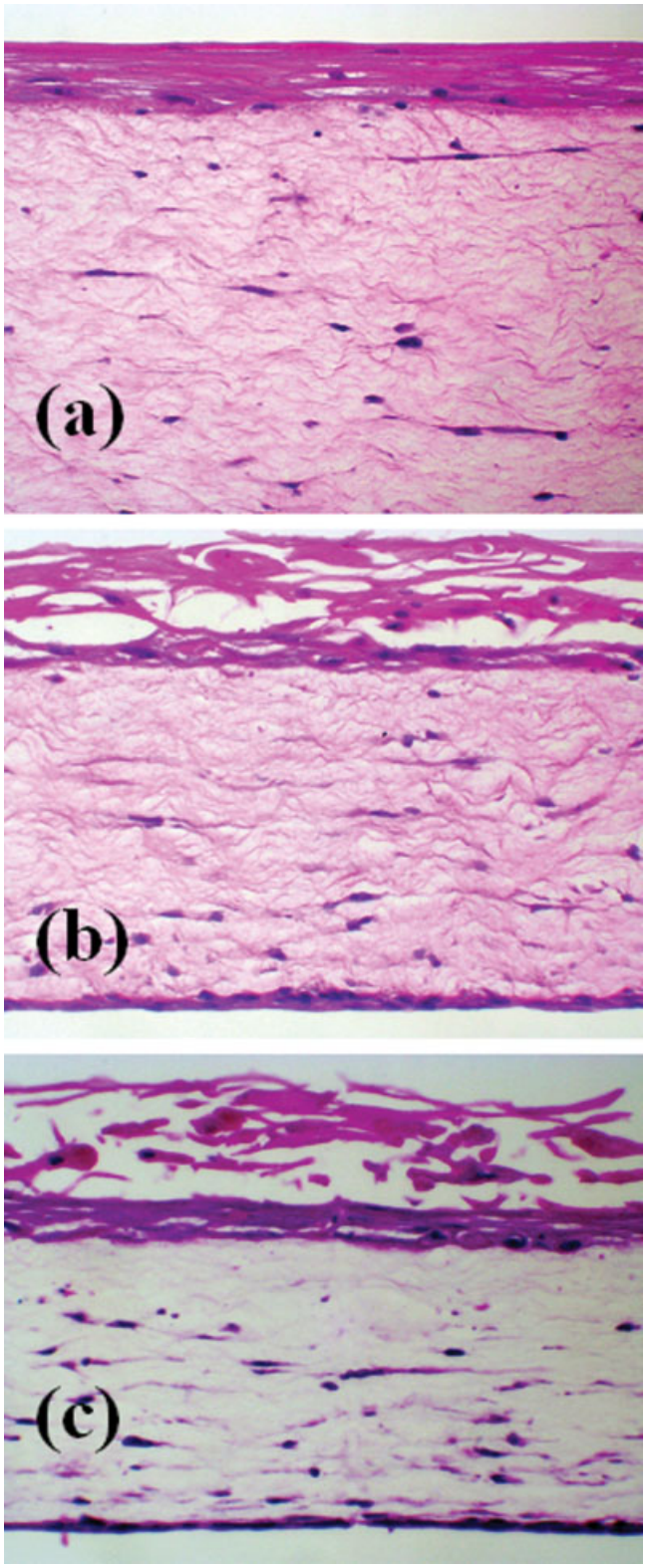

Fig. 4. RAFT specimens exposed to 100 millisecond spurt of CSC harvested (**a**) acutely; (**b**) 3 days; and (**c**) 7 days after cryogen exposure (H&E stain; original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

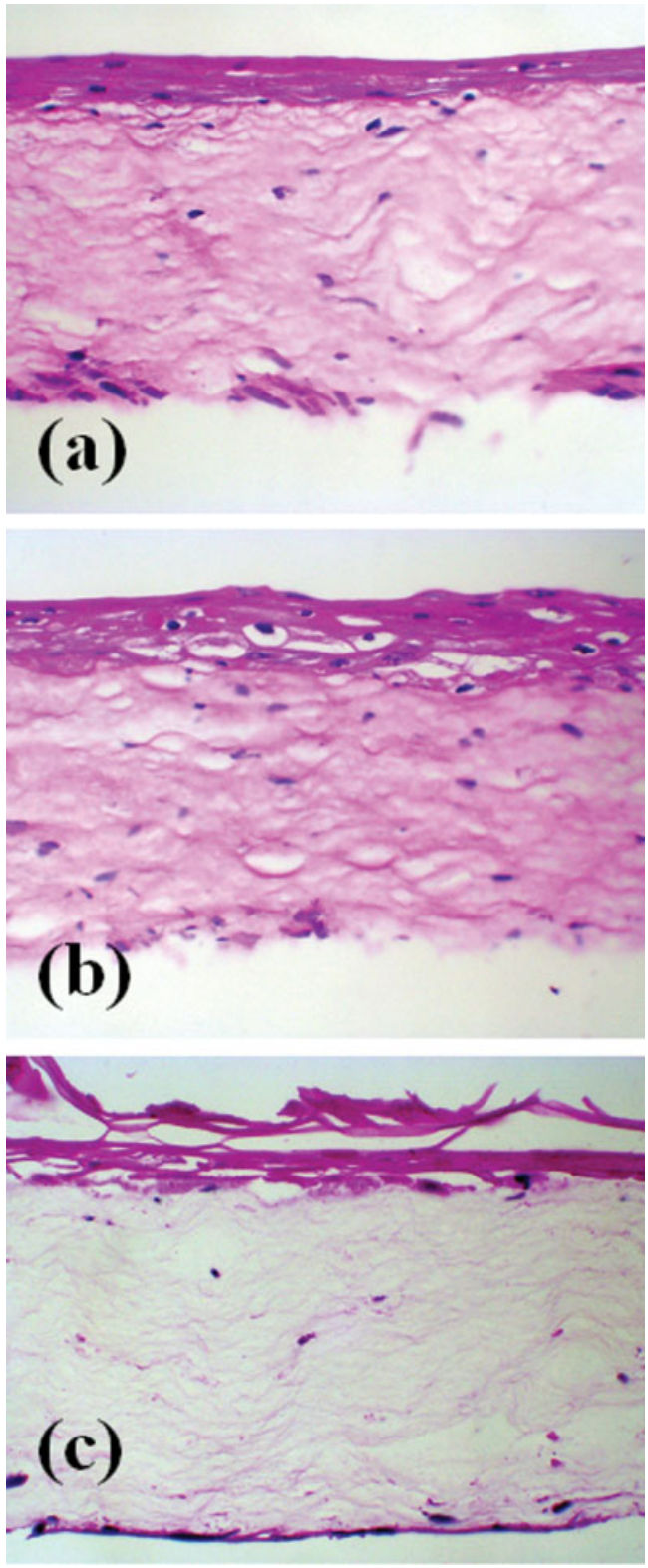

Fig. 5. RAFT specimens exposed to 500 millisecond spurt of CSC harvested (**a**) acutely; (**b**) 3 days; and (**c**) 7 days after cryogen exposure (H&E stain; original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

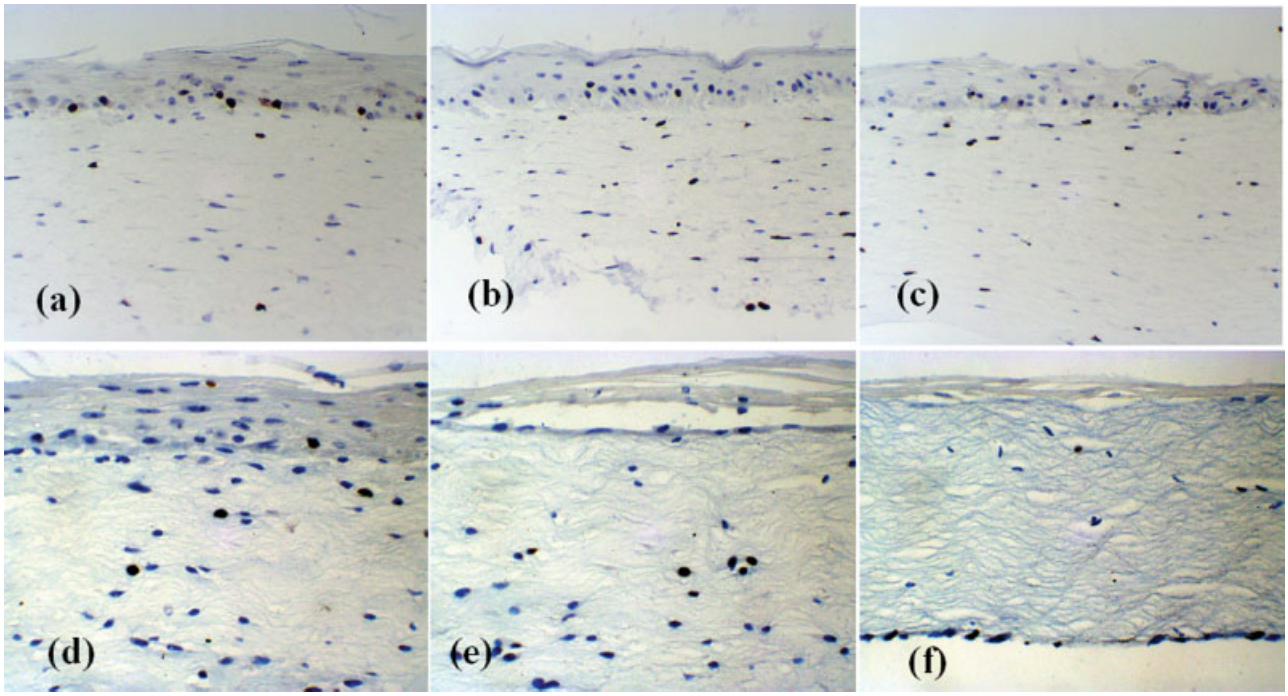

Fig. 6. Ki-67 stain of specimens harvested 3 days after cryogen exposure. Proliferating and thus, viable cells, are stained brown. **a**: Control (no cryogen exposure), (**b**) 40 millisecond, (**c**) 80 millisecond, (**d**) 100 millisecond, (**e**) 200 millisecond, (**f**) 500 millisecond (original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

Melan-A Staining

Melan-A staining identifies only melanocytes, but will stain those cells whether viable or not. Melan-A staining (Fig. 7) of day 3 and 7 specimens allowed clear identification of melanocytes, which interestingly were present not only in the basal layer, but also scattered throughout the epidermis. In the 100, 200, or 500 millisecond specimens at day 3, the number of viable appearing melanocytes was decreased as compared to control and 10, 20, 40, or 80 millisecond specimens. This observed reduction in the number of viable appearing melanocytes, which was apparent somewhere between spurt durations of 80 and 100 millisecond, was similarly observed in the 7 day specimens.

DISCUSSION

Dermatologists are familiar with the destructive effects induced on skin by several seconds of cryogen application, as liquid nitrogen is used successfully for treatment of a wide range of benign and malignant skin conditions. Generally, the cryosurgeon aims to achieve tissue necrosis by applying liquid nitrogen to the skin for several seconds, rapidly freezing the target to a temperature of −50 to −60°C. Cryogen-induced skin response depends on both the degree and time of skin temperature alteration.

CSC differs significantly from standard dermatologic cryosurgery utilizing millisecond spurts of tetrafluoroethane, applied to the skin as a fine mist. A thin layer of liquid is deposited on the skin surface, which evaporates rapidly. In laser surgery, continuous cryogen spurt durations of 10–80 millisecond are commonly utilized. These very abbreviated (millisecond) applications expose the skin surface to sub-zero temperatures for less than 2 seconds (Fig. 8a,b). Longer continuous spurt durations of 100, 200, or 500 millisecond induce prolonged sub-zero skin surface temperature reductions (Fig. 8b). So far, the tissue effects of millisecond sub-zero temperature reductions induced by CSC have not been evaluated despite its successful clinical use in conjunction with dermatologic laser surgery for nearly a decade.

Further, a model that predicts reliably the extent of epidermal damage due to freezing is still unavailable. In contrast, epidermal damage due to excessive heating (e.g., laser irradiation) can be calculated using a first order kinetic model, the Arrhenius equation:

$$\Omega(t) = \int_0^t A \exp(-E_a/RT)\mathrm{d}t$$

where A is a frequency factor [1/second], E_a an activation energy barrier [J/mole], R the universal gas constant (8.32 J/mole K), and T the absolute tissue temperature [K]. The empirical values for A and E_a must be found from systematic experimentation and, have been reported for the epidermis [14]. It would be useful to attempt to determine freezing damage constants, A and E_a that would allow prediction with reasonable accuracy of the extent of

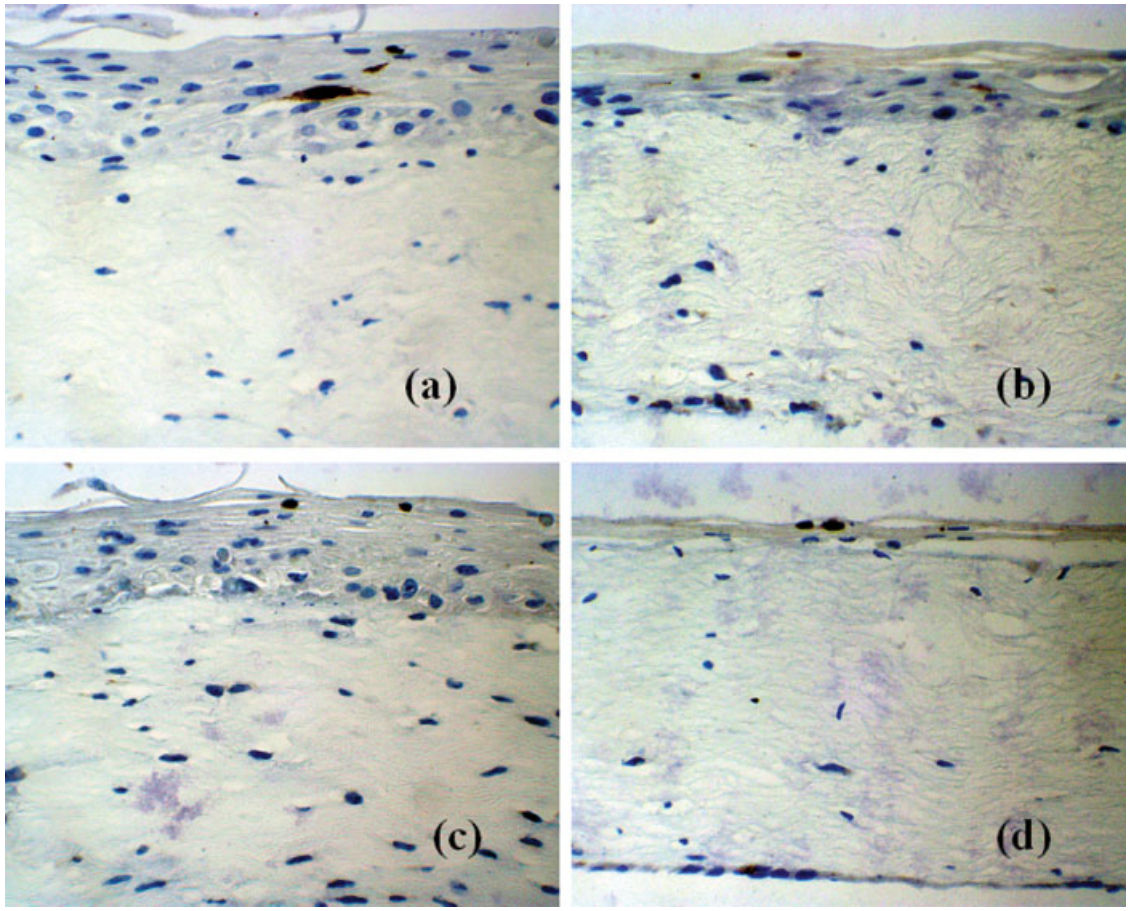

Fig. 7. Melan-A stain of specimens harvested 3 days after cryogen exposure. Melanocytes are stained brown. **a**: Control (no cryogen exposure), (**b**) 80 millisecond, (**c**) 100 millisecond, (**d**) 500 millisecond (original magnification = 200×). [Figure can be viewed in color online via www.interscience.wiley.com.]

epidermal damage due to extended freezing. In this study, we take the first step towards such a comprehensive analysis and evaluate the effects of CSC alone on human keratinocytes, melanocytes, and fibroblasts in an in vitro model of human skin.

The RAFT model allowed evaluation of epidermal and dermal effects induced after exposure to continuous cryogen spurts of varied duration. No significant epidermal or dermal injury was noted after spurt durations of 10 or 20 millisecond. Minimal epidermal injury was noted after exposure to 40 or 80 millisecond spurt durations, and complete recovery to normal histological architecture was evident in specimens harvested at 7 days. Ki-67 staining identifies proliferating, viable cells of any origin [13] and was similar to control in those specimens exposed to 40 or 80 millisecond cryogen spurts, confirming that keratinocytes remained viable after CSC exposure. Melan-A staining identifies only melanocytes, but will stain these cells whether viable or not. Evaluation of cell morphology of identified melanocytes suggests that these cells were healthy after spurt durations up to 80 millisecond. No dermal injury was noted in the specimens exposed to 40 or 80 millisecond.

This study demonstrates that at clinically relevant spurt durations (10–80 millisecond), CSC does not induce significant epidermal or dermal damage and is unlikely to cause cryo-injury. A different picture emerges with very long continuous spurts, duration of 100 millisecond or more. RAFT specimens exposed to these long spurt durations demonstrated significant epidermal cryo-injury with standard H&E histology and a decrease in Ki-67 staining, indicating a reduction in the number of proliferating keratinocytes after CSC exposure. After exposure to a 500 millisecond continuous spurt, dermal injury was also noted as demonstrated by decreased Ki-67 staining of fibroblasts.

Table 1 shows the duration of sub-zero temperature tissue exposure, $t_{sub0,}$ determined using a Plexiglas skin phantom model, for spurt durations evaluated in this study. This skin phantom has proven to represent reliably surface temperature variations during CSC [15]. As we noted significant epidermal damage for continuous spurts longer than 80 millisecond, 1.85 seconds appears to be the maximum permissible time that the skin surface can be exposed to sub-zero temperatures without inducing cryo-injury.

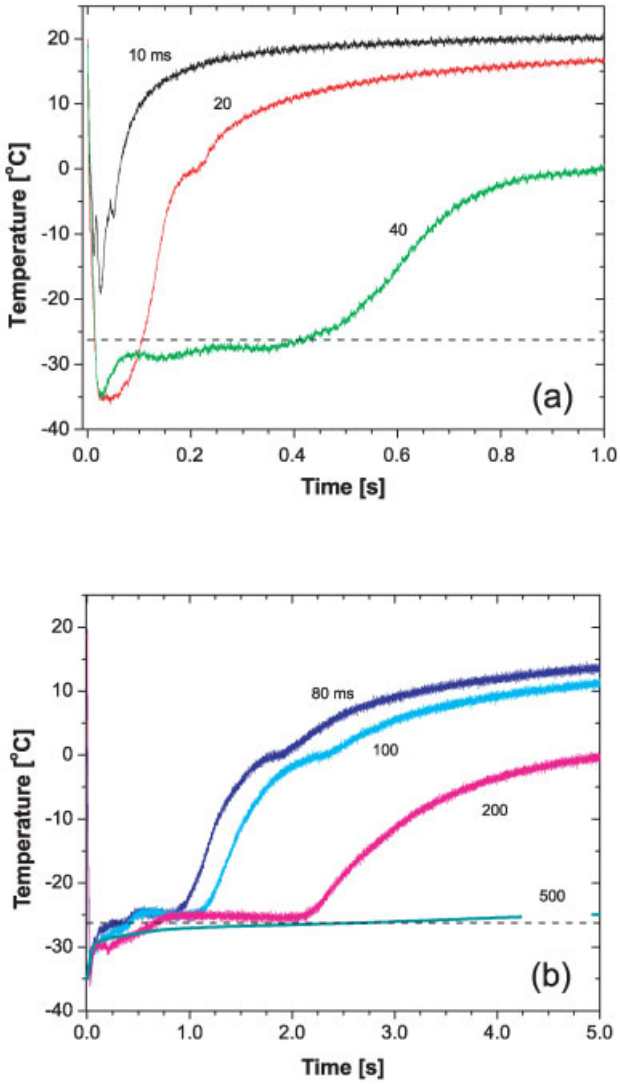

Fig. 8. Surface temperature versus time by spurt duration (**a**) $\Delta t = 10-40$ millisecond and (**b**) 80–500 millisecond with constant spray distance (D) = 35 mm. Dotted line = T_b = Boiling point of tetrafluoroethane (−26°C). Note the different time scales for figures a and b. [Figure can be viewed in color online via www.interscience.wiley.com.]

TABLE 1. Duration of Sub-Zero Temperature Tissue Exposure, t_{sub0}, as a Function of CSC Spurt Duration

Spurt duration Δt [milliseconds]	Time below sub-zero °C, t_{sub0} [seconds]
10	0.058
20	0.210
40	0.965
80	1.850
100	2.300
200	5.200
500	10.500

As described in the Introduction, we have previously demonstrated the usefulness of the RAFT model for evaluating wound healing response after irradiation with photorejuvenation devices [11]. This paper identifies an additional indication for this tissue culture model, evaluation of cryogen-induced effects. The RAFT model mimics in vivo human skin in terms of structure, cellular activity, and function. However, there are important differences between the in vitro RAFT model and in situ human skin. Most relevant to the current study is the lack of blood vessels, which would serve as a heat source for the epidermis and presumably mitigate any cryogen-induced injury. Further, the in vitro cells of the RAFT specimens are likely to be less robust than their in situ normal skin counterparts. Consequently, while we have demonstrated the safety of cryogen spurts up to 80 millisecond, it is possible that in situ experiments on human skin may demonstrate little, if any, damage after much longer cryogen spurt durations.

In conclusion, we were able to demonstrate with our RAFT model, that continuous CSC spurts of up to 80 millisecond induce minimal, if any, epidermal or dermal damage and are unlikely to produce cryo-injury when used in conjunction with dermatologic laser surgery.

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REFERENCES

1. Nelson JS, Majaron B, Kelly KM. Active skin cooling in conjunction with laser dermatologic surgery: Methodology and clinical results. Sem Cut Med Surg 2000;19:253–266.
2. Nelson JS, Milner TE, Anvari B, Tanenbaum BS, Kimel S, Svaasand LO. Dynamic cooling of the epidermis during laser port wine stain therapy. Lasers Surg Med 1994;6S:48.
3. Nelson JS, Milner TE, Anvari B, Tanenbaum BS, Kimel S, Svaasand LO. Dynamic epidermal cooling during pulsed laser treatment of port-wine stain. A new methodology with preliminary clinical evaluation. Arch Dermatol 1995;131: 695–700.
4. Nelson JS, Milner TE, Anvari B, Tanenbaum BS, Svaasand LO, Kimel S. Dynamic epidermal cooling in conjunction with laser-induced photothermolysis of port wine stain blood vessels. Lasers Surg Med 1996;19:224–229.
5. Nelson JS, Kimel S. Safety of cryogen spray cooling during pulsed laser treatment of selected dermatoses. Lasers Surg Med 2000;26:2–3.
6. Hirsch RJ, Farinelli WA, Anderson RR. A closer look at dynamic cooling. Lasers Surg Med 2002;Suppl 14:36.
7. Aguilar G, Diaz SH, et al. Cryogen spray cooling efficiency: Improvement of port wine stain laser therapy through

multiple-intermittent cryogen spurts and laser pulses. Lasers Surg Med 2002;31:27–35.
8. Aguilar G, Wang GX, Nelson JS. Dynamic behavior of cryogen spray cooling: Effects of spurt duration and spray distance. Lasers Surg Med 2003;32:152–159.
9. Tunnell JW, Torres JH, et al. Methodology for estimation of time-dependent surface heat flux due to cryogen spray cooling. Ann Biomed Eng 2002;30:19–33.
10. Torres JH, Nelson JS, et al. Estimation of internal skin temperatures in response to cryogen spray cooling: Implications for laser therapy of port wine stains. IEEE J Sel Topics Quant Elect 1999;5:1058–1066.
11. Kao B, Kelly KM, Majaron B, Nelson JS. Novel model for evaluation of epidermal preservation and dermal collagen remodeling following photorejuvenation of human skin. Lasers Surg Med 2003;32:115–119.
12. Asselineau D, Bernard BA, Bailly C, Darmon M, Prunieras M. Human epidermis reconstructed by culture: Is it "normal"? Soc Invest Dermatol 1986;86:181–186.
13. Endl E, Kauschl I, Baack M, Knippers R, Gerdes J, Scholzen T. The expression of Ki-67, MCM3, and p27 defines distinct subsets of proliferating, resting, and differentiated cells. J Pathol 2001;195:457–462.
14. Weaver JA, Stoll AM. Mathematical model of skin exposed to thermal radiation. Aerosp Med 1969;40:24.
15. Aguilar G, Wang GX, Nelson JS. Effect of spurt duration on the heat transfer dynamics during cryogen spray cooling. Physics in Medicine and Biology 2003;48:2169–2181.