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Authors
Nelson, JS
Tuqan, AT
Kelly, KM
et al.

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ORIGINAL ARTICLE

Alia T. Tuqan · Kristen M. Kelly · Guillermo Aguilar
Julio C. Ramirez-San-Juan · Chung-Ho Sun
David Cassarino · Damian Derienzo
Ronald J. Barr · J. Stuart. Nelson

Evaluation of single versus multiple cryogen spray cooling spurts on in vitro model human skin

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Abstract Many commercially available dermatologic lasers utilize cryogen spray cooling for epidermal protection. A previous tissue culture study demonstrated that single cryogen spurts (SCS) of 80 ms or less were unlikely to cause cryo-injury in light-skinned individuals. More recently, multiple cryogen spurts (MCS) have been incorporated into commercial devices, but the effects of MCS have not been evaluated. The aim was to study an in vitro tissue culture model and the epidermal and dermal effects of SCS vs patterns of shorter MCS with the same preset total cryogen delivery time (Δt_c) and provide an explanation for noted differences. Four different spurt patterns were evaluated: SCS: one 40-ms cryogen spurt; MCS2: two 20-ms cryogen spurts; MCS4: four 10-ms cryogen spurts; MCS8: eight 5-ms cryogen spurts. Actual Δt_c and total cooling time (Δt_{Total}) were measured for each spurt pattern. RAFT tissue culture specimens were exposed to cryogen spurt patterns and biopsies were taken immediately and at days 3 and 7. Actual Δt_c was increased while Δt_{Total} remained relatively constant as the preset Δt_c of 40 ms was delivered as shorter MCS. Progressively more epidermal damage was noted with exposure to the MCS patterns. No dermal injury was noted with either SCS or MCS. For a constant preset Δt_c of 40 ms, delivering cryogen in patterns of shorter MCS increased the actual Δt_c and consequently the observed epidermal cryo-injury as compared to an SCS.

Keywords Cryogen spray cooling · RAFT tissue culture · Histology · Injury

A. T. Tuqan · K. M. Kelly (✉) · G. Aguilar
J. C. Ramirez-San-Juan · C.-H. Sun · J. S. Nelson
Beckman Laser Institute and Medical Clinic, 1002 Health Sciences Road East, Irvine, CA, 92612
E-mail: KMKelly@uci.edu
Tel.: +1-949-8247980
Fax: +1-949-8242726

K. M. Kelly · D. Cassarino · D. Derienzo · R. J. Barr
J. S. Nelson
Department of Dermatology, University of California, Irvine, CA

G. Aguilar
Department of Mechanical Engineering, University of California, Riverside, CA

J. S. Nelson
Department of Biomedical Engineering, University of California, Irvine, CA

J. C. Ramirez-San-Juan
Department of Optics, Instituto Nacional de Astrofisica, Optica y Electronica Puebla, Mexico

Introduction

Selective epidermal cooling has become an integral part of laser dermatologic surgery [1]. By protecting the epidermis, cooling allows the safe use of higher fluences, permitting treatment of darker skin types, decreasing treatment pain, and enhancing therapeutic outcome.

One effective and efficient method of selective epidermal cooling is cryogen spray cooling (CSC) (definitions of terms: Table 1), which delivers a millisecond domain cryogen spurt to the skin surface immediately before laser exposure. Cryogen spray cooling has been incorporated into a wide range of therapeutic devices including those used for laser hair removal, treatment of vascular lesions and acne, and non-ablative photorejuvenation [1, 2, 3, 4]. 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.

A previous study using an in vitro RAFT tissue culture model, demonstrated that single cryogen spurts

Table 1 Definitions of terms

Term	Definition
Cryogen spray cooling (CSC)	Technique used to cool skin in laser dermatologic procedures
Single cryogen spurt (SCS)	One cryogen spurt of a given Δt
Multiple cryogen spurts (MCS)	Pattern of multiple cryogen spurts separated by intervening delays
Spurt duration (Δt)	Time up to which the cryogen valve remains open
Total cryogen delivery time (Δt_c)	Sum of all Δt in a pattern
Delay time (Δt_d)	Time between consecutive cryogen spurts during which no cryogen is delivered to the skin surface
Total cooling time (Δt_{Total})	Δt_c + sum of all Δt_d (for MCS)
Sub-zero time (Δt_s)	Time up to which sprayed-surface remains below 0^o C

(SCS) of up to 80 ms induce minimal, if any, epidermal or dermal injury and were unlikely to produce cryo-injury in light-skinned patients when used in conjunction with laser dermatologic surgery [6].

The RAFT model mimics in vivo human skin in terms of structure, cellular activity, and function [7]. Cultured fibroblasts form dense collagen fibrils, which repress fibroblast growth, as seen in human dermis. The keratinocyte layers with added melanocytes similarly mimic the epidermis of in vivo human skin. Human tissue injury and healing can be approximated using the RAFT tissue culture model while avoiding limitations of skin biopsies including risks to patients such as discomfort, scarring, and infection. As such, RAFT tissue cultures allow easy and simultaneous evaluation of a wide range of devices and treatment parameters.

In recent years, clinicians and engineers have sought to expand the boundaries of laser dermatologic surgery, using higher fluences to improve therapeutic outcomes in patients with darker skin types [8, 9, 10]. To accomplish these goals safely, epidermal cooling must be optimized [11, 12]. In an effort to enhance epidermal protection, several commercially available devices have been designed to deliver multiple intermittent cryogen spurts (MCS) which can be delivered before and after laser exposure or alternated with laser exposure in a variety of patterns. Cryogen evaporation initiates as soon as the cryogen is released from its pressurized container and is accelerated as there is deposition on the relatively hot skin surface. When multiple short spurts are utilized, evaporation of a cryogen spurt can be completed or nearly so before the next spurt arrives and this results in enhanced cooling efficiency [13].

Despite the fact that such devices are in clinical use, the effects of MCS have yet to be fully evaluated. We evaluated the actual total cryogen delivery time (Δt_c) and total cooling time (Δt_{Total}) of a 40 ms SCS (a spurt duration commonly utilized in clinical practice) and three different MCS patterns each with a preset Δt_c of 40 ms. Using in vitro RAFT model human skin, we evaluated the tissue effects of each of these CSC patterns. Serial histologic evaluations were performed immediately and at days 3 and 7 post-exposure to CSC.

Methods

Cell cultures and raft model construction

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), 100 IU of penicillin per ml, 100 IU of streptomycin per ml, and 2 mmol/l l-glutamine. The RAFT model was constructed as described in a previous publication [6] (Fig. 1).

Cryogen delivery and nozzle

Cryogen utilized was $C_2H_2F_4$ with boiling temperature $T_b = -26$°C at atmospheric pressure. Cryogen was kept in a container at saturation pressure (approximately 6.6 bar at 25°C), and delivered as either a SCS or as MCS through a standard high-pressure hose to a sole-

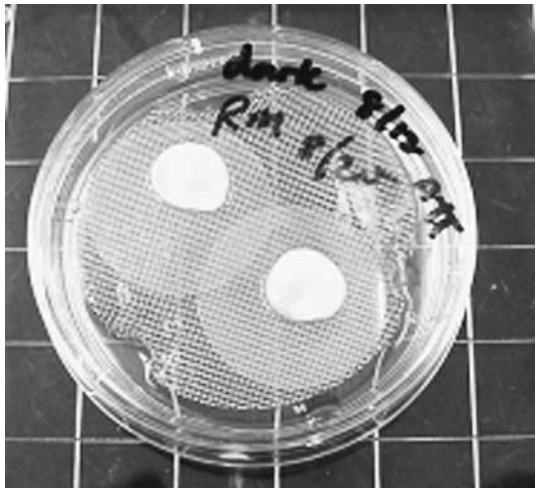

Fig. 1 RAFT cultures lifted on a stainless steel grid

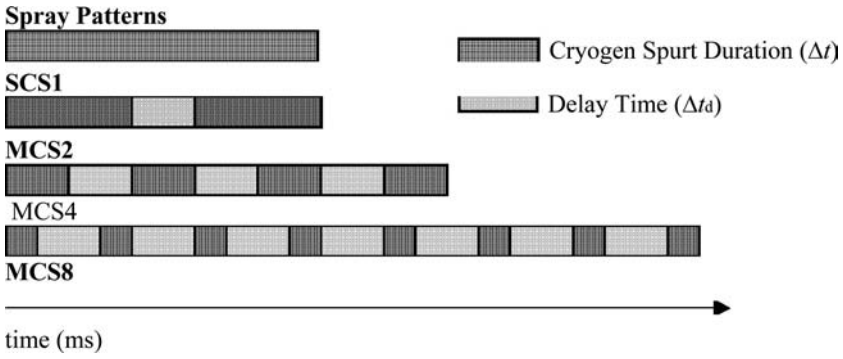

Fig. 2 A timing diagram of spurt patterns showing alternating cryogen spurts and delay time (when no cryogen is sprayed)

noid valve and nozzle identical to that used in a commercial CSC device (GentleLASE, Candela Corporation, Wayland, MA). Spurt durations (Δt), defined as the time the valve remained open and summed to yield Δt_c, were electronically controlled by a digital delay generator (DG535, Stanford Research Systems, Sunnyvale, CA). Four different spurt pattens were evaluted (Fig. 2). SCS: one-40 ms cryogen spurt; MCS 2: two 20-ms cryogen spurts; MCS 4: four 10-ms cryogen spurts; MCS 8: eight 5-ms cryogen spurts. MCS patterns all had an identical preset Δt_c of 40 ms, but with a constant time interval between consecutive spurts, delay time (Δt_d) of 10 ms, resulting in a variation of Δ_{Total} from 50 to 110 ms (Table 2). A commonly utilized spurt duration in clinical practice is 40 ms.The nozzle-to-sprayed surface distance, z, was 31 mm for the SCS and MCS2-8 patterns diagrammed in Fig. 2. A distance of 31 mm is used in many commercially available devices including the GentleLASE, Smoothbeam, and V-beam (Candela Corporation, Wayland, MA).

In order to evaluate preset vs actual Δt_c and Δt_{Total} for SCS of 40, 50, 60, 70, 80 ms and MCS 2–8, we employed the experimental setup diagrammed in Fig. 3. A horizontal (0.63 mm diameter) He–Ne laser beam was placed 31 mm away and perpendicular to the nozzle. When cryogen droplets crossed the laser beam, light was attenuated and light intensity as a function of time was measured by a high speed photodetector (DET110; Thorlabs. Inc; Newton, NJ) placed across the spray cone and aimed at the laser beam. The electric pulses that control the solenoid valve and the signal from the photodetector were, generated and acquired by an input/output data acquisition board (100B Instrument, Omega Engineering, Stamford, CT) at 10 kHz sample rate. Measuring the full-width at half maximum (FWHM) of the photodetector signal and comparing that to the input signal to the valve, allowed comparison of the preset and actual Δt_c and Δt_{Total}.

Histopathology

Two RAFT specimens were biopsied at each of the following time points: immediately, three, and seven -days post-exposure to CSC. All specimens were fixed for 24 h 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.

Results

Cryogen delivery

Measurements revealed that for SCS, the actual Δt_c is very similar to the preset Δt_c (Fig. 4, Table 2). However, for MCS, as the Δt_c is divided into multiple shorter spurts (the longer the preset Δt_{Total}), the bigger the difference between the preset and actual Δt_c. For both SCS and MCS, the preset Δt_{Total} approximates the actual Δt_{Total} (Fig. 5, Table 2).

Histopathology

40 ms SCS

Acutely, the stratum corneum exhibited mild parakeratosis. In the epidermis, rare vacuolated keratinocytes and scattered apoptotic cells were noted, although the

Table 2 Total cryogen delivery time (Δt_c), total cooling time (Δt_{Total}) and duration of sub-zero time (Δt_s) for study patterns (Δt_s extrapolated from Ramirez-San-Juan et al. [14])

Pattern	Preset Δt_c (ms)	Actual Δt_c (ms)	Preset Δt_{Total} (ms)	Actual Δt_{Total} (ms)	Δt_s(ms)
40 ms SCS	40	41.9	40	41.9	1.6
40 ms MCS2	40	47.5	50	53.2	1.9
40 ms MCS4	40	53.4	70	73.6	2.3
40 ms MCS8	40	63.8	110	112.3	3.2

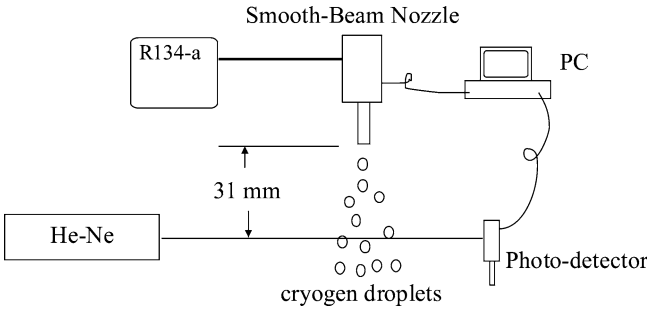

Fig. 3 Experimental set-up employed to measure the actual vs preset Δt_c and Δt_{Total} for SCS and MCS patterns

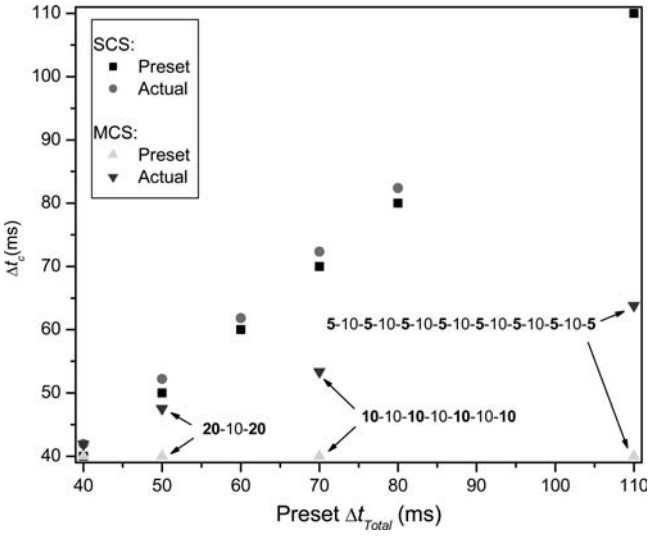

Fig. 4 Preset and actual Δt_c vs preset Δt_{Total} for both SCS and MCS sequences. For SCS, the actual Δt_c is very similar to the preset Δt_c; however, for MCS, the longer the preset Δt_{Total}, the bigger the difference between the preset and actual Δt_c

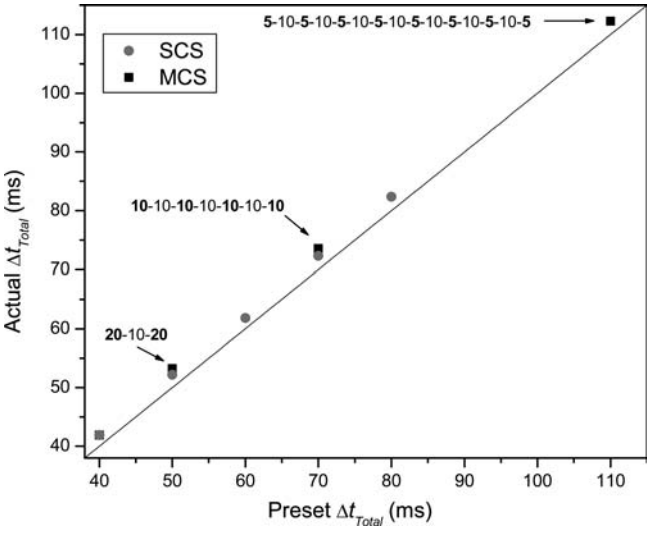

Fig. 5 Actual Δt_{Total} vs preset Δt_{Total} for SCS and MCS sequences. For both SCS and MCS the actual Δt_{Total} is very similar to the preset Δt_{Total}

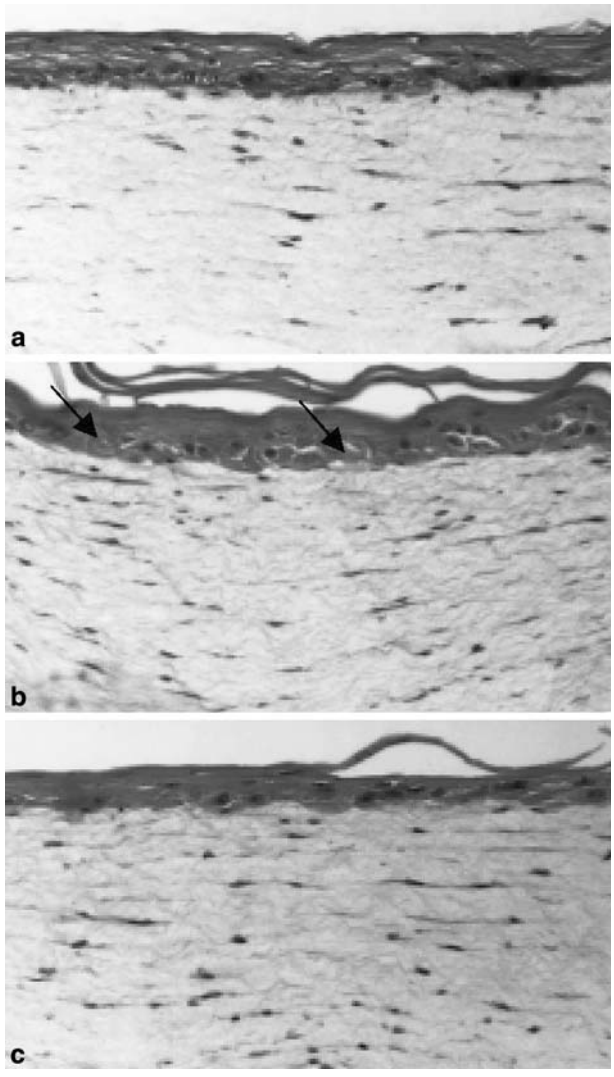

Fig. 6 RAFT specimens exposed to 40 ms SCS harvested: **a** acutely; **b** three days and **c** seven days after cryogen exposure (H&E stain; original magnification = 200×). Apoptotic cells are indicated by the *arrows* and identified by appearance: an eosinophilic, round, homogeneous colloid body

vast majority of epidermal cells were unaffected (Fig. 6a). At three days, parakeratosis and more scattered apoptotic cells were identified, but once again, the majority of the epidermis appeared healthy and intact (Fig. 6b). At seven days, mild hyperkeratosis and parakeratosis with rare apoptotic cells were seen (Fig. 6c). These changes were also seen in control specimens and are believed to be the result of normal RAFT aging. No dermal injury was noted in any of the specimens.

MCS2

Acutely, the only epidermal change was scattered apoptotic cells, but their numbers were increased compared to those observed in the acute SCS biopsies

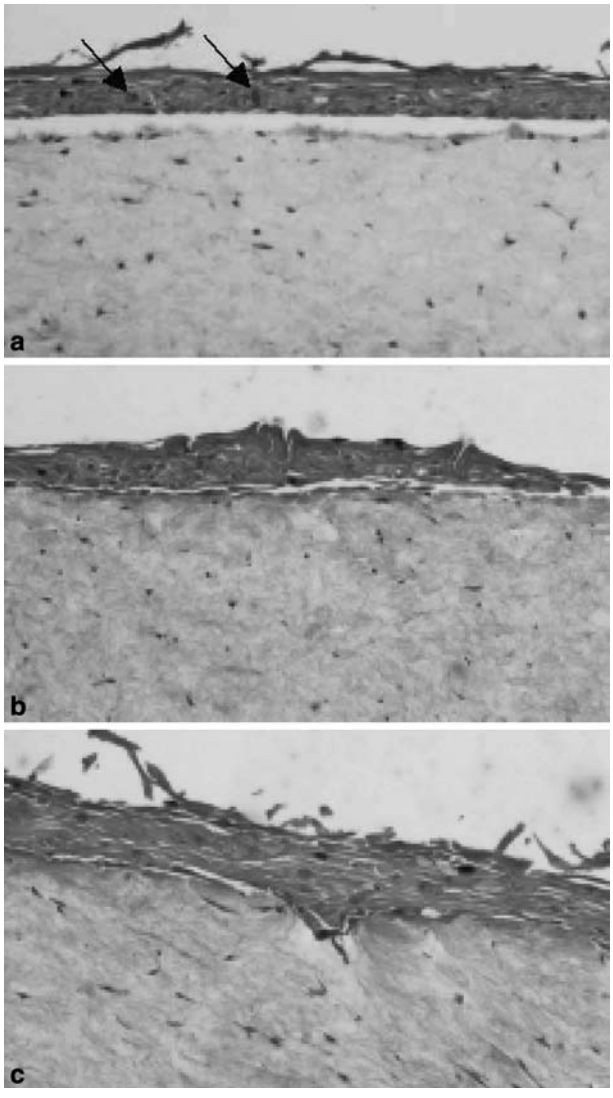

Fig. 7 RAFT specimens exposed to the MCS2 pattern harvested: **a** acutely; **b** three days and **c** seven days after cryogen exposure (H&E stain; original magnification = 200×; Note: Basement membrane separation is a processing artifact). More apoptotic cells are present in these sections. Two of the apoptotic cells are indicated by *arrows* in **a**

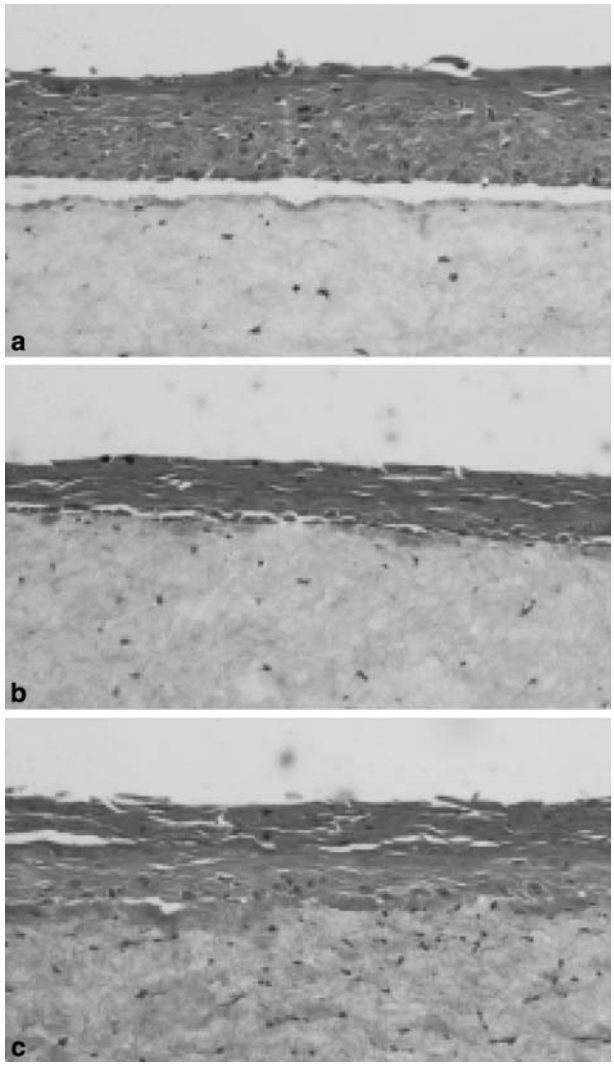

Fig. 8 RAFT specimens exposed to the MCS4 pattern harvested: **a** acutely; **b** three days and **c** seven days after cryogen exposure (H&E stain; original magnification = 200×; Note: Basement membrane separation is a processing artifact)

(Fig. 7a). At three days, parakeratosis was now evident in the stratum corneum and epidermal apoptosis was again observed (Fig. 7b). The same changes were seen in the seven-day biopsy with some increase in the degree of parakeratosis (Fig. 7c). No dermal injury was observed in any of the specimens.

MCS4

Acutely, there was epidermal apoptosis that had increased as compared to the SCS and MCS2 specimens (Fig. 8a). At three days, parakeratosis was observed and notably, superficial epidermal necrosis was present for the first time (Fig. 8b). In the seventh day specimens, parakeratosis was increased, acanthosis was now present and superficial epidermal necrosis was again observed (Fig. 8c). No dermal effects were evident in any of the specimens.

MCS8

Acutely, the changes in these biopsies were similar to those observed in the MCS4 specimens with fairly marked apoptosis (Fig. 9a). The third day specimens revealed parakeratosis, acanthosis, and superficial epidermal necrosis (Fig. 9b). Epidermal necrosis was increased in the seventh day specimens and was much more prominent as compared to the SCS and MCS2–4 specimens (Fig. 9c). Once again, no dermal changes were evident.

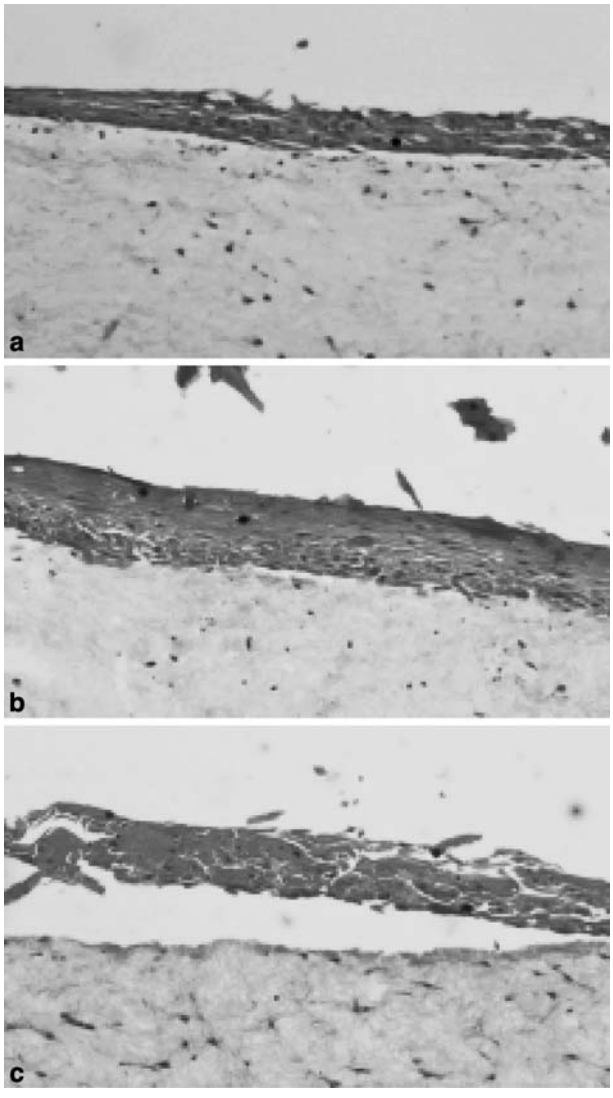

Fig. 9 RAFT specimens exposed to the MCS8 pattern harvested: **a** acutely; **b** three days and **c** seven days after cryogen exposure (H&E stain; original magnification = 200×; Note: Basement membrane separation is a processing artifact)

Discussion

It should be noted that clinically, CSC is not used alone but rather is combined with laser heating. However, it is useful to evaluate the effects of CSC alone for several reasons. First, CSC in combination with laser heating involves complex temperature interactions, which are difficult to understand if the effects of the separate components are unknown. Further, the skin coverage area of the cryogen spray is frequently larger than the laser spot in order to incorporate a margin of safety, and as such, some skin regions are exposed only to CSC.

Our studies indicated that as the Δt_c is divided into multiple shorter spurts, the preset and actual Δt_c diverge while preset and actual Δt_{Total} remain similar, although with a slight shift in time. Variability in cryogen delivery may occur due to the intrinsic response time of the solenoid valve, the time required for flow of cryogen along the valve and nozzle, and the time involved in spraying of the droplets from the nozzle to the skin surface. These sources of imprecision accumulate in the MCS sequences, resulting in an increased Δt_c as the spurt is divided. This observation indicates that Δt_{Total} rather than Δt_c, either preset or actual, should be used to select CSC duration.

This effect on Δt_c may help explain the histological effects observed in this study. Consistent with the results of our previous work, we saw no significant epidermal or dermal injury with the 40 ms SCS. Progressively more epidermal injury (but no dermal change) was noted as the same amount of cryogen was delivered over a longer time period using shorter multiple intermittent spurts. For example, epidermal necrosis was noted for the first time in the MCS4 pattern and was most prominent in the MCS8 pattern.

Recent work by Ramirez-San-Juan [14] provides a further explanation of the histological results obtained in this study. They used a Plexiglas skin phantom model to measure for SCS vs MCS, the time the skin surface temperature remains below 0°C, the so-called sub-zero time (Δt_s). This skin phantom model approximates human skin and has proven to represent reliably surface temperature variations during CSC [15]. Table 2 shows Δt_s for the patterns evaluated in this study. The values for Δt_s are extrapolated from the measurements obtained by Ramirez-San-Juan et al. [14]. Note that as cryogen delivery is divided among shorter individual spurts, actual Δt_c, preset and actual Δt_{Total}, and Δt_s are all progressively prolonged. The degree of cryogen-induced injury would be expected to increase as skin is exposed to more cryogen resulting in sub-zero temperatures for longer periods of time and this was confirmed by our histological observations.

Our results have important implications for laser dermatologic surgery. Commercially available devices, which utilize MCS patterns, may increase the risk of cryo-injury, which may be manifested as acute erythema and post-inflammatory hyperpigmentation. This does not imply that MCS patterns should not be used. However, the increased risk of injury with MCS must be taken into account by the clinician who should be aware that an MCS sequence with a preset Δt_c of 40 ms carries an increased risk of skin injury as compared to an SCS of 40 ms. Laser devices utilizing MCS patterns may rely on a delicate balance between heating and cooling and the operator must choose CSC parameters carefully, especially in those with increased risk of injury (including patients with sensitive skin and Fitzpatrick skin types IV–VI).

Although the RAFT is very useful, there are important differences between this in vitro model and in situ human skin. Most relevant to evaluation of tissue injury is the lack of blood vessels. However, for the maximum Δt_{Total} considered in this study, the cooling effects are limited to the epidermis and superficial papillary dermis, where minimal blood perfusion exists. As such, the

majority of heat transfer back to the epidermis and papillary dermis during and shortly after cryogen delivery comes from the surrounding air rather than the tissue itself and lack of blood vessels in the RAFT is a minor limitation. Further, it is likely that the in vitro cells of the RAFT specimens may be less robust than their in situ normal skin counterparts. As such, the MCS patterns evaluated in this study may not induce the same degree of damage in in vitro human skin. Future studies will evaluate clinically, the cutaneous effects of MCS patterns alone and in combination with lasers on human skin.

Our results suggest that SCS and MCS comparisons should be discussed in terms of Δt_{Total} rather than Δt_c. However, it should be noted that in this study all evaluated MCS patterns had a Δt_{Total} of 110 ms or less. It is not clear whether for CSC patterns with longer Δt_{Total}, dividing cryogen delivery into short MCS would result in increased injury. In fact, there is evidence that for cryogen spray patterns with $\Delta t_{\text{Total}} > 110$ ms, Δt_s increases more gradually as the cryogen is divided among multiple smaller spurts, as compared to an SCS [14]. Further studies will be required to evaluate the histological effects of MCS with a $\Delta t_{\text{Total}} > 110$ ms.

In conclusion, in this study we used RAFT in vitro model human skin to demonstrate that for a constant preset Δt_c of 40 ms, delivering cryogen in patterns of shorter MCS increased the actual Δt_c and consequently the observed epidermal cryo-injury as compared to an SCS.

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