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
\text{c}) and provide an explanation for noted differences. Four different spur 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
\text{c} and total cooling time (Δt
\text{Total}) were measured for each spur pattern. RAFT tissue culture specimens were exposed to cryogen spurt patterns and biopsies were taken immediately and at days 3 and 7. Actual Δt
\text{c} was increased while Δt
\text{Total} remained relatively constant as the preset Δt
\text{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
\text{c} of 40 ms, delivering cryogen in patterns of shorter MCS increased the actual Δt
\text{c} and consequently the observed epidermal cryo-injury as compared to an SCS.

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

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 photo-rejuvenation [1, 2, 3, 4]. Tetrafluoroethane (C₂HF₄), 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
(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 ($\Delta t_d$) and total cooling time ($\Delta t_{Total}$) of a 40 ms SCS (a spurt duration commonly utilized in clinical practice) and three different MCS patterns each with a preset $\Delta t_c$ 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% 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), 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 C₂H₅F₄ 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](image-url)
noid valve and nozzle identical to that used in a commercial CSC device (GentleLASE, Candela Corporation, Wayland, MA). Spurt durations ($\Delta t$), defined as the time the valve remained open and summed to yield $\Delta t_c$, were electronically controlled by a digital delay generator (DG535, Stanford Research Systems, Sunnyvale, CA). Four different spurt patterns were evaluated (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 $\Delta t_c$ of 40 ms, but with a constant time interval between consecutive spurts, delay time ($\Delta t_d$) of 10 ms, resulting in a variation of $\Delta t_{Total}$ 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 $\Delta t_c$ and $\Delta t_{Total}$ 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 $\Delta t_c$ and $\Delta t_{Total}$.

**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 $\Delta t_c$ is very similar to the preset $\Delta t_c$ (Fig. 4, Table 2). However, for MCS, as the $\Delta t_c$ is divided into multiple shorter spurts (the longer the preset $\Delta t_{Total}$), the bigger the difference between the preset and actual $\Delta t_c$. For both SCS and MCS, the preset $\Delta t_{Total}$ approximates the actual $\Delta t_{Total}$ (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

| Pattern   | Preset $\Delta t_c$ (ms) | Actual $\Delta t_c$ (ms) | Preset $\Delta t_{Total}$ (ms) | Actual $\Delta t_{Total}$ (ms) | $\Delta t$(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            |
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.
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.
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 $\Delta t_c$ is divided into multiple shorter spurts, the preset and actual $\Delta t_c$ diverge while preset and actual $\Delta t_{\text{Total}}$ 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 $\Delta t_{\text{Total}}$ as the spurt is divided. This observation indicates that $\Delta t_{\text{Total}}$ rather than $\Delta t_c$, either preset or actual, should be used to select CSC duration.

This effect on $\Delta t_c$ 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 ($\Delta t_s$). This skin phantom model approximates human skin and has proven to represent reliably surface temperature variations during CSC [15]. Table 2 shows $\Delta t_s$ for the patterns evaluated in this study. The values for $\Delta t_s$ 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 $\Delta t_c$, preset and actual $\Delta t_{\text{Total}}$, and $\Delta t_s$ 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 $\Delta t_c$ 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 $\Delta t_{\text{Total}}$ 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 $\Delta t_{\text{Total}}$ rather than $\Delta t_c$. However, it should be noted that in this study all evaluated MCS patterns had a $\Delta t_{\text{Total}}$ of 110 ms or less. It is not clear whether for CSC patterns with longer $\Delta t_{\text{Total}}$, 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, $\Delta t_c$ 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 $\Delta t_c$ of 40 ms, delivering cryogen in patterns of shorter MCS increased the actual $\Delta t_c$ and consequently the observed epidermal cryo-injury as compared to an SCS.

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