Temperature Changes During and after Eccentric Contractions and its Effect on Force and Desmin Loss in Rat

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Abstract- The typical features of eccentric exercise-induced muscle damage is prolonged loss of muscle strength and the most rapid structural change in the fibers is loss of immunostaining for the intermediate filament protein, desmin. In this study isolated perfused rat muscle was used to examine the direct effect of temperature changes on the eccentric contraction-induced force and desmin loss. The left medial gastrocnemius muscle was separated and the entire lower limb was transferred into a prewarmed (35^{o}C) organ bath. Temperature was adjusted to 31 or 39^{o}C during and after eccentric contractions. Maximal isometric force and desmin loss were measured after 15 isometric or eccentric contractions. According to our data, organ bath temperature changes during or after eccentric contractions had no significant effect on force loss. However, a strong correlation between desmin loss and temperature changes during ($r = 0.886$, $P< 0.05$) and a weak correlation between desmin loss and temperature changes after ($r= 0.699$, $P<0.05$) eccentric contractions was observed. Our results suggest that cooling during eccentric contractions may decrease desmin loss but temperature changes after eccentric contractions have no effect on desmin loss.

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Introduction

In many situations, contracted muscle may elongate. It is a kind of contraction called eccentric or lengthening contraction. These contractions occur during activities such as downhill running, walking downstairs or lowering a weight. For example, as we step down the slope, the contracting quadriceps muscle controls the rate of knee flexion against the force of gravity and in the process the muscle undergoes an eccentric contraction with each step (1). Unaccustomed eccentric muscle contractions result in muscle damage (2). Magnitude of the injury was closely related to the magnitude of the muscle strain on the fibers (3).

The typical feature of muscle damage are high plasma creatine kinase (CK) activity, force loss, range of motion loss, swelling, and delay onset muscle sorness (DOMS) (4). The timing of these changes suggests that the muscle weakness might be a primary consequence of the muscle damage (5). The histological feature of muscle damage is probably the extensive sarcomere disruption and Z-disk streaming. Using animal models, this ultrastructural evidence of muscle damage has been related to a loss of desmin (6, 7). Desmin is the main intermediate filament protein in skeletal and heart muscles. In mature skeletal muscle, desmin filaments encircle and interlink myofibrils at the level of the Z disks and connect them to the plasma membrane thus aligning the myofibrils (8). It is shown that eccentric contractions produce an early activation of the ubiquitous calpains 1 and 2 whose in vitro substrates include desmin and titin (9, 10).

Since *in vivo* changes in muscle temperature can increase sympathetic nerve activity via metaboreflexes

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and mechanoreflexes which result in systemic effects (11, 12), it is difficult to identify the direct effect of temperature. On the other hand, in isolated muscle, the eccentric contraction-induced force loss can be attributed to hypoxia (13). Moreover, although muscle's architecture is an important determinant of its force generating capacity (14), the orientation of muscle fibers in force transmission may be disturbed due to muscle dissection in isolated muscle preparation. In the present study to eliminate above factors we have used isolated perfused medial gastrocnemius muscle to evaluate the direct effect of temperature changes on the eccentric contraction-induced muscle damage.

Losses of force and desmin have been observed very soon (within minutes) (3, 7). For this reason and because studies on isolated muscle can analyzed force and desmin loss but not pain or tenderness, the focus of this article is on the early muscle weakness and desmin staining following eccentric muscle contractions.

The effect of temperature changes on the eccentric exercise-induced muscle damage has been investigated in different studies (4,13,15-17) but effect of temperature alternations on ultrastructural changes have not studied to day. Since desmin loss is dependent on enzyme activity, in this study we have investigated the effect of temperature changes during and after eccentric contractions on force and desmin loss.

Materials and Methods

Experiments were performed in accordance with the criteria outlined in the Guide for the Care and Use of Laboratory Animals (Copyright 1996 by the National Academy of Sciences; http://books.nap.edu/ readingroom/books/labrats/). The protocol of the present experiment was approved by the institutional ethics committee of Medical Sciences / University of Tehran. Male Sprague Dawley rats (n=49) weighting between 270 and 300 g (19 to 21 weeks) were used. All animals were maintained at a constant temperature (22 ± 0.5^{o}C) with 12/12 light/dark cycles and with free access to food and water.

Surgery

The animals were anesthetized with sodium pentobarbital (50 mg/Kg, intra-peritoneally) and placed supine on the operating table. The skin was totally removed from the entire left lower limb. Femoral artery was exposed and cannulated with heparinized (200 U/cc) PE–10 catheter. To restrict perfusion to the lower portion of the limb, the catheter was advanced as far as possible toward the popliteal space. The muscle was perfused at 6 ml/min with modified Krebs Henseleit solution (containing in mM: NaCl; 118.5, KCl; 4.7, $CaCl_2$; 2.5, $MgSO_4$; 1.2, KH_2PO_4; 1.2, $NaHCO_3$; 25, glucose; 11) by a peristaltic pump (Meredos GmbH, Germany). The perfusate was gassed with 95% O_2 and 5% CO_2 (final pH 7.4) and its temperature was maintained at 35^{o}C and continuously recorded by a digital thermometer at the point before entering the catheter into the artery.

After cannulation, the thigh muscles and femur bone were cut. The bone was clamped in horizontal position. Medial gastrocnemius muscle was exposed by removing the semitendinosus and biceps femoris muscles as previously described by Rijkelijkhuizen *et al.* (18). This muscle has its own separate nerve and vessels and can be isolated without injury to its structure (18). It can also be perfused via femoral artery cannula.

Briefly, the calcaneal bone and Achilles tendon were identified, the calcaneal bone was cut whereas Achilles tendon left intact. The soleus muscle was removed and the plantaris muscle identified and its tendon cut from musculotendon junction to keep its attachment into Achilles tendon intact. The medial and lateral gastrocnemius muscles were separated by cutting fibers in such a way that the medial gastrocnemius remained undamaged. The femur was clamped in a vertical position¸ whereas the medial gastrocnemius was fixed horizontally.

Left lower limb and medial gastrocnemius muscle were transferred into water-jacket perfusion chamber. At the end of surgery, animals were killed with high doses of sodium pentobarbital.

Temperature selection

Since the perfusion fluid temperature was kept constant during the study and the eccentric contraction heat production was low (2, 19), the organ bath temperature may be the main factor determining muscle temperature. The temperatures selected for use were based on the following reasons. First, superficial and deep muscle temperatures of rats at rest are normally 35.5-37.2^{o}C (20). Similar values have been observed for human, but temperatures in the 30-35^{o}C range are not uncommon (21, 22). Second, different studies have shown 1 to 4^{o}C temperature rise during active and passive warm-up (22, 23, 24, 25,26) and icing decrease muscle temperature at least 5^{o}C (15). Third, although muscle temperature during exercise exceed 40^{o}C, experiments were not performed at temperature above 39^{o}C because of concerns about muscle viability in our set-up. Therefore, organ bath temperature was adjusted to 35 ± 4^{o}C.

Experimental protocol

An important issue in all studies of eccentric muscle damage is to distinguish between the reduction in force caused by fatigue and that caused by the eccentric contractions. For this reason, it is important to compare the eccentric exercise against an isometric (or concentric) control and to design the study so that the reduction in force after the isometric series is minimal (2). Thus this study was conducted in isometric and eccentric sections. In each section the experimental protocol was the same.

Groups

After transportation of each muscle into chamber, tissue was allowed adapting for 45 min (Organ bath temperature was adjusted to 35^{o}C) and then the experimental protocol continued in 5 different ways:

1. G35 (control): the temperature was set at 35^{o}C during the entire experiment.
2. G31D, the temperature was decreased to 31^{o}C during contractions for 30 min.
3. G31A: The temperature was decreased to 31^{o}C for 30 min after contractions.
4. G39D, the temperature was increased to 39^{o}C during contractions.
5. G39A, The temperature was increased to 39^{o}C for 30 min after contractions.

Contractions duration was about 30 minutes**.**

Muscle perfusion

To ascertain complete perfusion, we have conducted a pilot study in which medial gastrocnemius muscle samples were frozen after, a. about 160 min perfusion (with or without isometric contractions) and b. non-perfusion period. Then transverse slices of 2 mm thickness were made and incubated in 1% triphenyltetrazoliumchloride (TTC) in phosphate buffer solution (pH 7.4) for 30 min at 37^{o}C. To enhance the contrast between stained and unstained tissues, slices were immersed in 10% formalin. Stained slices were viewed under a light stereoscope. Brick red stained tissues were taken as viable, whereas pale or white ones were considered as necrotic.

Electrical stimulation

Medial gastrocnemius muscle has its own nerve and vessel branches and can be easily stimulated (18). To isolate the effect of temperature on nerve conduction, the muscle was stimulated directly. Stimulating electrodes (0.2 mm diameter platinum wire) were implanted into each end of the muscle (out of tissue sampling area) and connected to an electrical stimulator (Harvard 6002, Harvard Apparatus, Holliston, Massachusetts USA). Supramaximal stimulation (15-20 V) consisted of 100-Hz stimulus (square pulse, 0.5 ms width) with a train duration of 0.7 s.

Eccentric contractions

The Achilles tendon together with a piece of calcaneal bone was connected to a force transducer (MLT50 ADInstruments, Australia) mounted on a servomotor. Acceleration, velocity, start length, onset of movement, stimulation algorithm, stimulation frequency and duration of the muscle contractions were controlled by a computer. Force and length outputs from the servomotor were sampled at 1 kHz.

The optimum length (L_o) of the medial gastrocnemius was defined as the muscle length at which active force was maximal. Eccentric contractions were performed for a range of 8 mm below to 4 mm over L_o. For each eccentric contraction, the muscle was first placed at L_o - 8 and isometrically activated until tension stabilized (300 ms) and then lengthening change was imposed at a rate of 17 mm/s for 0.7 s. Thus total duration of each contraction was approximately 1 second. After each contraction, the muscle was allowed to recover at L_o – 8 mm for 2 minutes to minimize the effect of fatigue. Fifteen eccentric contractions were carried out. Recording was performed by Power Lab System (4SP, ADInstruments, Australia). Force data were also saved on a compact disk for later analysis.

Isometric force loss

Isometric force measuring has been the most widely used method of determining muscle function after eccentric exercise (27). To determine force loss due to eccentric contractions, maximal isometric force was measured before and 10 min after isometric or eccentric contractions. Supramaximal isometric stimulation (15-20 V) was applied with a frequency of 100 Hz and train duration of 300 ms. Percentage change in maximal isometric force from pre- to post-eccentric contractions was used to indicate the magnitude of the eccentric contraction-induced force loss. In all groups, maximal isometric force was recorded at 35^{o}C.

Desmin staining

Muscle sections were immunostained with antidesmin (monoclonal mouse anti-human clone D33, from Dako, Glostrum, Denmark) to evaluate the structural integrity of the cytoskeletal network (6). All muscle tissue samples were obtained from predetermined location of medial gastrocnemius muscle. Since, perfused muscles with isometric contractions

gave typical cross-sectional patterns at different temperatures and did not show desmin loss only eccentric contractions groups were studied in this stage.

For semiquantitative analysis (28), three independent observers blindly examined cross sections from muscles exposed to eccentric contractions at different temperatures. For each section, each observer counted the number of fibers that demonstrated overtly altered staining patterns after eccentric contractions (reduced staining of desmin protein, Figure 1), normalized this number to the total number of fibers in the section, and normalized values were averaged between the three observers. Desmin staining was calculated from the following formula:

Desmin staining = 100 - Desmin loss

Statistical analysis

Data were expressed as mean ± SEM. Comparison between isometric force before and after eccentric and isometric contractions was done by Student's paired *t* test. Groups were compared by one-way ANOVA and Tukey's post hoc test. Pearson's correlation coefficient (r) was used for desmin loss in different temperatures. All statistical analyses were performed using SPSS software (SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant in all groups.

Results

TTC staining

TTC staining was used to ensure muscle perfusion. It didn't reveal any necrotic area in perfused muscles (with and without isometric contractions) after 160 min, but in non-perfused samples the necrotic areas and ischemic slices were distinguishable.

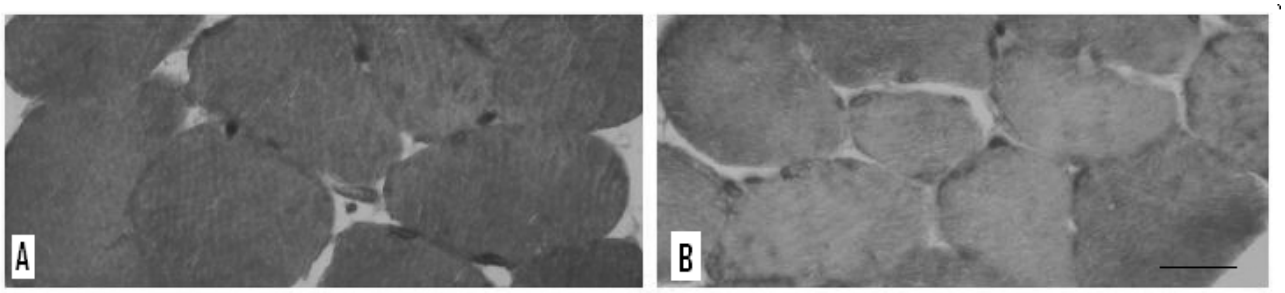

Figure 1. Eccentric contraction-induced desmin loss. A, desmin staining of muscle sample after isometric contractions, that doesn't show any desmin loss. B, muscle sample after eccentric contractions in which desmin staining is lost. Bar 100 µm.

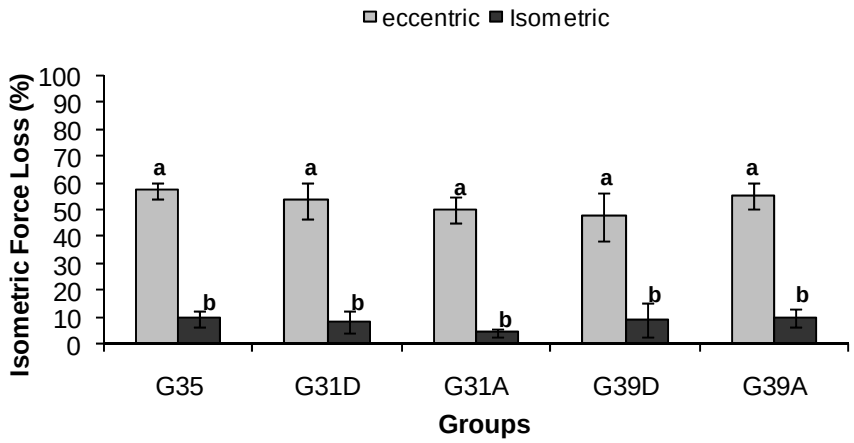

Figure 2. Effect of organ bath temperature changes on contraction-induced isometric force loss. There were no significant differences among isometric (n=3-4) or eccentric (n=6-7) groups, P > 0.05. G35, the temperature was set at 35°C during the entire experiment. G31D, during contractions, the temperature was decreased to 31°C for 30 min. G31A, after contractions, the temperature was decreased to 31°C for 30 min. G39D, during contractions, the temperature was increased to 39°C for 30 min. G39A, after contractions, the temperature was increased to 39°C for 30 min. Values with the same letter are not significantly different. Values are mean ± SEM.

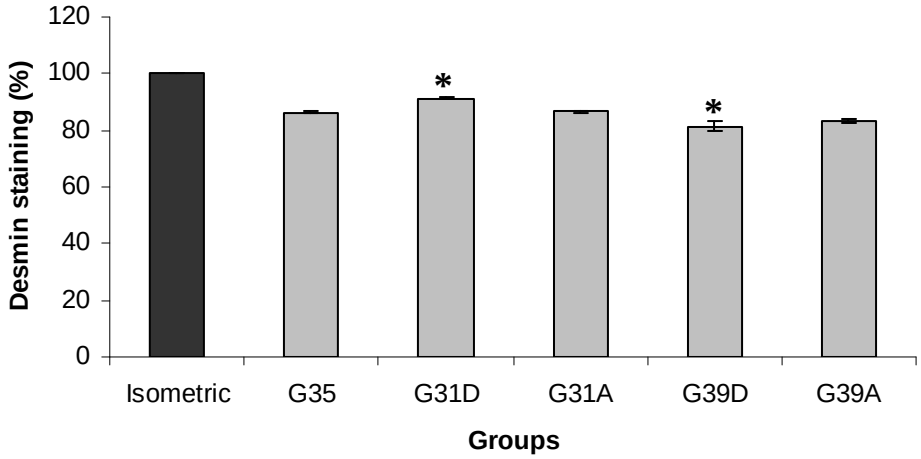

Figure 3. Effect of organ bath temperature on desmin loss. G35, the temperature was set at 35°C during the entire experiment. G31D, during eccentric contractions, the temperature was decreased to 31°C for 30 min. G31A, after eccentric contractions, the temperature was decreased to 31°C for 30 min. G39D, during eccentric contractions, the temperature was increased to 39°C for 30 min. G39A, after eccentric contractions, the temperature was increased to 39°C for 30 min.* Significant difference from G35. Values are mean ± SEM. $P<0.05$ as determined by ANOVA.

Isometric force loss

As mentioned before, percentage change in maximal isometric force from pre - to post- contractions was used to indicate the magnitude of the eccentric contraction-induced force loss (27). By using Student's paired *t* test, isometric contractions showed no significant force loss ($P> 0.05$) but, exposure to eccentric contractions was associated with a significant reduction in maximum isometric force ($P<0.05$). However, organ bath temperature changes during or after isometric or eccentric contractions had no statistically significant effect on force loss (Figure 2, $P > 0.05$).

Desmin loss

The observations were blindly done (inter reliability, ICC= 0.801). Isometric contractions had no effect on desmin pattern but in eccentric groups, there was a significant difference ($P<0.05$) in desmin loss among different groups (Figure 3).

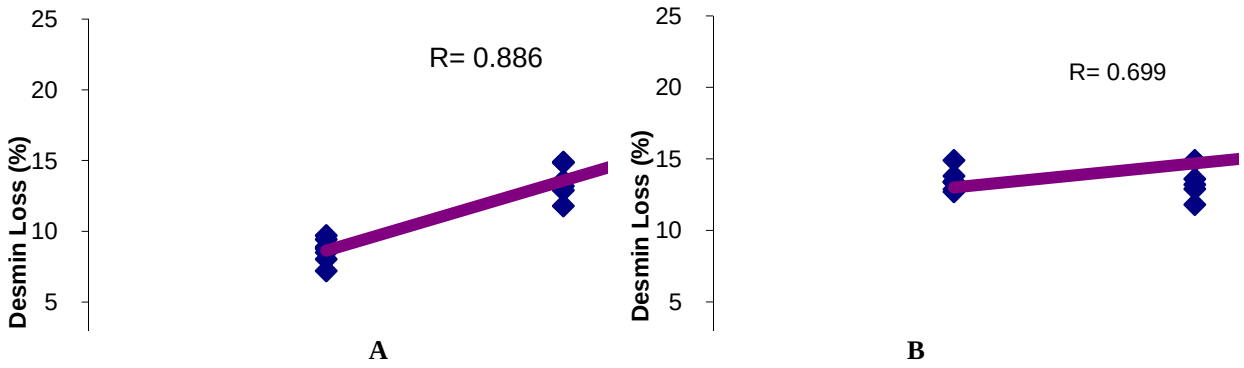

Figure 4. Scatter plots of desmin loss vs organ bath temperature. There is a strong correlation between desmin loss and organ bath temperature during (A) and weak correlation between desmin loss and organ bath temperature after (B) eccentric contractions.

As it shown in Figure 3, when temperature was changed during eccentric contractions they had significant difference comparing with control group (G35) but if temperature was change after eccentric contractions they did not showed any significant difference comparing with G35. The most desmin loss was seen in G39D. However, there is no significant difference in desmin loss when organ bath temperature decreased or increased to 31 or 39^{o}C after eccentric contractions.

Pearson's correlation was performed to see if any relationship existed between desmin loss and temperature changes (Figure 4). According to our data, there was a strong correlation between desmin loss and temperature changes during (r= 0.886, $P< 0.05$) eccentric contractions (Figure 4A) but correlation between desmin loss and temperature changes after eccentric contractions was weak (r= 0.699, $P< 0.05$).

Discussion

We have investigated the effect of temperature changes after and during eccentric contractions on force and desmin loss resulting from eccentric contractions of isolated perfused medial gastrocnemius muscle. In this study, there was a strong effect of temperature on the desmin loss when organ bath temperature was changed during eccentric contractions. In contrast, there was no effect of temperature changes on eccentric contraction-induced force loss.

To eliminate the effects of different body control systems on the procedures, isolated perfused muscle has been used. Since working muscle is affected by its perfusion, to ensure muscle full perfusion and oxygen supply, TTC staining, a fast and cheap method for detecting tissue injury by measuring dehydrogenase activity, was used (29, 30). TTC staining in present study showed complete medial gastrocnemius muscle perfusion.

Force loss is a marker of muscle injury. The eccentric contractions-induced force loss after unaccustomed eccentric contractions is a long-lasting phenomenon and complete recovery can take place in more than 1 month (31). The exact cause of the eccentric contraction-induced force loss is unknown (2, 7, 4, 32). According to our results, there were no significant differences in eccentric contraction-induced isometric force loss and the time of temperature change among the isolated perfused skeletal muscles tested at three temperature points. In human studies temperature changes were applied before eccentric contractions (4, 15,16, 33). For example, Nosaka et al (15). found that passive warm-up (short wave diathermy) and cool-down (ice pack) before eccentric contractions had no effect on eccentric contraction-induced isometric force loss. Our pervious study showed that organ bath temperature changes before eccentric contractions had no significant effect on force loss (34). Present study data showed that temperature changes during or after eccentric contractions had no effect on isometric force loss in isolated perfused skeletal muscle too. Warren et al (13) reported the effect of temperature changes on isometric force loss in isolated muscle. They changed the temperature during eccentric contractions and found strong positive relationship between temperature changes and eccentric contraction-induced isometric force loss in isolated muscle. The contrast between the two studies may arise from more simulation of normal condition in present study such as the preservation of tendon-bone complex or full perfusion of muscle in our set-up.

After eccentric contraction, the most rapid structural change in the fibers is loss of immunostaining for the intermediate filament protein, desmin (35). Desmin loss after eccentric contraction is extremely rapid and occurs within 5 min. This dramatic and rapid desmin loss, which does not occur after either isometric or concentric contraction, points to some type of enzymatic hydrolysis or protein phosphorylation as a likely mechanism rather than gene regulation, which requires much more time. (3, 7). It was shown that exercise-induced muscle damage produces an early activation of the ubiquitous calpains 1 and 2 whose in vitro substrates include desmin and titin (9). Ultrastructural evidence of muscle damage has been related to a loss of desmin (6, 7).

According to our results, as the organ bath temperature was increased during eccentric contractions, desmin loss was increased too, and a stronger correlation was seen (r= 0.886, $P<0.05$) between them. On the other hand, isometric contractions at different temperatures had no effect on desmin staining. In our pervious study, there was a strong correlation between desmin loss and temperature changes before (r= 0.93, $P<0.05$) eccentric contractions and the most desmin loss was seen in group which before contractions the temperature was set at 39^{o}C (34). According to present study, it seems that enzymatic activity is affected by temperature changes during eccentric contractions and temperature alternations which were applied after the eccentric contractions had no effect on desmin loss. Unfortunately, we did not examine the calpain enzyme activity in our studies Although the specific mechanisms

involved in this response remain to be determined, our results suggest that the mechanism(s) that underlying the desmin loss act better at higher temperatures especially when the organ bath temperature changes were set at 39^{o}C before or during the eccentric contractions. Accordingly, it is suggested to examine the effect of temperature changes on calpains enzyme activity in eccentric contractions in the separate study in the future. During the past 20 years the structural changes that occur in response to eccentric exercise have been interpreted as muscle injury but Yu *et al.* (36) findings have suggested an active remodeling process in response to eccentric exercise in humans. It seems that after a focal initial loss of some myofibrillar proteins, an addition of new sarcomeres into pre-existing sarcomeres can be observed. Although Yu *et al.* (36) did not see desmin loss 1 h, 2-3 and 7-8 days after eccentric exercise in human samples, in several other studies on both human and animals, desmin loss were seen (6, 34, 37, 38). In our study desmin loss was occurred and it was temperature sensitive.

In conclusion, the present study may provide some insight into the factors that affect the desmin loss in eccentric contractions. Our findings suggest that desmin loss in spite of the force loss is temperature sensitive and additional research on the use of thermal modalities is encouraged to provide better rationale for application of them.

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