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Structural Changes in Skeletal Muscle Fibers after Icing or Heating on Downhill Running in Mice

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An experimental animal model that causes mild structural disorders of skeletal muscles is essential to understand general exercise-induced muscle damage. Thermal stimulations such as icing and heating are commonly used as treatments for muscle injuries in sports. We established a downhill running (DR) protocol that leads to structural muscle disorders without sarcolemmal disruption and directly compared the structural changes produced by icing and heating after DR. Male ddY mice were divided into the DR, DR plus icing (Ice), and DR plus heating (Heat) groups. All mice ran at 20 m/min, −20% grade on a treadmill for a total of 90 min (three rounds of 30 min). In the Ice and Heat groups, an ice pack and a hot pack were, respectively, applied to the exercised triceps brachii muscles for 20 min just after DR. The proportion of myofibers with structural disorders was higher in the Ice group than in the DR and Heat groups at days 1 and 7 after DR. Moreover, the structural disorder of myofibers was slightly improved in the Heat group at day 1 after DR compared with the DR group. These findings suggest that icing treatment might aggravate the structural changes after DR.

INTRODUCTION

Unaccustomed, very intense, and/or too frequent exercise often causes skeletal muscle injury, termed as exercise-induced muscle damage (EIMD) (17). EIMD includes a variety of symptoms such as delayed onset muscle soreness, loss of muscle strength, and structural disruption of muscle fibers (8, 18), and these symptoms occur in some sports activities (4, 9). Severe muscle damage such as sarcolemmal disruption and muscle fiber necrosis is rarely detected in human cases of EIMD after eccentrically biased exercise (17). By contrast, structural disorders, including disturbance of striation pattern and Z-line streaming in skeletal muscle fibers without sarcolemmal disruption, commonly occur (3, 21). Therefore, designing an experimental animal model that induces these mild structural disorders is essential to understand general EIMD in human sports activities. Downhill running (DR) has been well accepted as one of the EIMD models that involve eccentric contractions of exercised muscles. Previous studies have performed different DR protocols, causing various degrees of muscle damage in rodents (e.g., 1, 23). By establishing the proper DR protocol, some therapeutic interventions could be examined for use in a sports setting.

In the context of sports medicine, cryotherapy has traditionally been used as a therapeutic and restorative modality after excessive exercise and/or muscle injury under the premise that it alleviates pain, reduces tissue metabolism, and modifies vascular responses to decrease swelling (20). However, it has been demonstrated in animal studies that icing applied soon after severe muscle injury impairs muscle regeneration (11, 19, 22). In contrast to icing, heat stress after muscle injury is likely to facilitate the regenerative process of damaged myofibers (7, 15, 24). Although above-mentioned studies investigated the influence of icing or heating on muscle damage accompanied by sarcolemmal disruption and muscle fiber necrosis, little is known regarding the effects of these thermal treatments on muscle damage without sarcolemmal disruption.

The purpose of the present study was to examine the effects of icing and heating treatments on mild EIMD without sarcolemmal disruption of skeletal muscle fibers in mice. To accomplish this, we modified previously used DR protocols (1, 6, 23) to establish a new DR protocol that leads to relatively moderate structural muscle damage.

MATERIALS AND METHODS

Animals

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Eight-week-old male ddY mice (SLC Japan Inc., Tokyo) weighing 36–40 g were used. All mice were housed in a controlled environment at 22°C ± 2°C with a 12-h light/dark cycle. They were allowed access to food and water freely throughout the experiments. This study was approved by the Institutional Animal Care and Use Committee and performed according to Kobe University Animal Experimentation Regulations (approval No. A120904). Animals were randomly divided into three DR groups: DR (n = 30), DR plus icing (Ice: n = 24), and DR plus heating (Heat: n = 24). In addition to these groups, age-matched three mice were subjected to skeletal muscle crush injury (e.g., 10, 13) using the triceps brachii muscles as a positive control of sarcolemmal disruption.

Experimental protocol

To create mild structural damage of the skeletal muscle, a total of 90 min, semi-continuous interval DR (three rounds of 30 min separated by 10 min rest intervals) on a 20°-declined treadmill (MK-680, Muromachi Co., Kyoto, Japan) at a constant speed of 20 m/min were performed in the present study. In the previous studies, various DR settings (i.e., duration, repetition, decline, and speed) have been used. For example, Armstrong et al. (1983) conducted a continuous DR for 90 min on a 16°-declined treadmill at a constant speed (16m/min), Takekura et al. (2001) performed an interval DR for a total of 90 min (5 min×18, 2 min-rest between sets) on a 16°-declined treadmill at a constant speed (18m/min), and Haramizu et al. (2013) used a speed-incremental DR (16m/min for 5 min, 18m/min for 5 min, 20m/min for 10 min, 22m/min for 130 min) on a 14°-declined treadmill. Some of the studies reported that DR causes muscle fiber necrosis in exercised triceps brachii muscles (1, 23). With reference to these studies, here we set a new DR protocol that generates mild structural damage of the muscle fibers (Fig. 2a) without sarcolemmal disruption (Fig. 1a). Before DR, all mice warmed up for 10 min on a horizontal treadmill at a speed of 10 m/min. None of the mice ran on the treadmill before DR. When the animals did not run properly, they received a mild touch on their hips with a brush to make them continuously run, and previously reported electrical stimulation (1) was not employed to alleviate potential pain.

Just after DR, the animals in the three experimental groups were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body weight) for each treatment. The methods of icing and heating treatments were identical to earlier reports (7, 13, 22, 24). In the Ice and Heat groups, the ice pack or hot pack was transcutaneously applied to the posterior surface of the bilateral forelimbs for 20 min with minimum compression controlled just to touch the skin at a room temperature maintained at 25°C. The ice pack was prepared by enclosing crushed ice in polyethylene bags, and the surface temperature of the ice pack was 0.3°C–1.3°C. Polyethylene bags filled with 42°C warm water were used as hot packs, and the bags were exchanged every 2 min during the heating treatment to keep a constant temperature. During icing and heating treatments for 20 min, the skin surface temperature of the triceps brachii muscles was measured every 5 min with a rod-shaped digital thermometer (CT250, Custom) without touching to the ice or hot pack. During the 20 min of treatment period, animals in the DR group were simply anesthetized with no interventions. At 20 min, days 1, 3, and 7 after DR, animals were sacrificed, and right triceps brachii muscles were harvested and used for analyzes. The time point of the “20 min after DR” indicates the end-point of the treatment period after DR in this study.

Evans blue staining

Evans blue staining is widely used to examine sarcolemmal disruption (5). Evans blue staining was performed for three mice in the DR group and three crush-injured mice to verify sarcolemmal disruption. Before DR and the crush injury, 1% evans blue (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) dissolved in phosphate-buffered saline (PBS) was injected into the peritoneum of animals at a volume of 1% body mass. One day after DR and the crush injury, mice were sacrificed by an overdose of pentobarbital sodium with intraperitoneal injection. Soon after the sacrifice, the triceps brachii muscles were removed, frozen in dry ice-cooled acetone without fixation, and stored at −80°C until analysis. Cross-sections were made with a cryostat (CM-1510S; Leica Microsystems, Wetzlar, Germany), mounted on glass slides, and viewed under a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

Toluidine blue staining

At 20 min, days 1, 3, and 7 after DR (n = 6 per group per time point), animals were anesthetized and sacrificed by intracardiac perfusion initially with Millonig’s phosphate buffer, followed by fixation with 4% paraformaldehyde and 2.5% glutaraldehyde dissolved in Millonig’s phosphate buffer. The muscles were removed and immersed in the same fixative for 24 h at 4°C. Both ends of the muscle tissue (tendinous parts) were pinned on a corkboard during immersion to prevent muscle tissue shortening by fixation. After immersion, these samples were cut into small pieces, post-fixed in 1% osmium tetroxide, dissolved in the Millonig’s phosphate buffer, dehydrated with a series of graded alcohols, cleared with propylene oxide, and then embedded in an epoxy resin mixture (Quetol 812; Nisshin EM, Tokyo, Japan). Approximately 1-μm-thick longitudinal

sections were made with glass knives, stained with 1% toluidine blue, and viewed with light microscopy (BX50; Olympus, Tokyo, Japan).

Immunohistochemistry

For desmin immunohistochemistry, three mice in the DR group at day 1 after DR were sacrificed by intracardiac perfusion initially with PBS, followed by the fixative containing 2% paraformaldehyde dissolved in PBS. Soon after the perfusion, muscles were removed and immersed in the same fixative for 24 h at 4°C. After that, the samples were treated with 10% sucrose for 4 h, 15% sucrose for 4 h, and 20% sucrose overnight, frozen in dry ice-cooled acetone and stored at −80°C until analysis. Longitudinal sections (5 μm) were cut with a cryostat and mounted on glass slides, air-dried for 15 min at room temperature, and rinsed in PBS for 5 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide diluted with methanol for 30 min at room temperature. After washing in PBS, these sections were incubated with an anti-desmin antibody (sc-14026, lot# B0310; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing in PBS, they were incubated with the secondary antibody, MAX-PO (R) (Nichirei Biosciences, Tokyo, Japan), for 30 min at room temperature and reacted with 3, 3′-diaminobenzidine reaction solution.

Quantitative analyzes

After eccentric exercise, the disturbance of the striation pattern in skeletal muscle fibers is detectable on toluidine blue-stained longitudinal sections (3). To quantify the myofiber with structural disorders (disturbance of the striation pattern and/or mild disorganization) in DR mice, 10 fields were captured at 10× magnification of objective lens, and the ratio of the muscle fibers exhibiting structural disorders to total muscle fibers was calculated per animal per time point. At least 150 muscle fibers per animal were included for the analysis. In addition, lengths from the center of the I-band to the adjacent center of the I-band were measured at 20 min and day 1 after DR by analyzing the muscle fibers that did not exhibit any structural disorders. At 40× magnification of objective lens, lengths of 50 units per animal (300 units per time point in each group) were measured. All quantitative analyzes were performed using Image J software (http://rsbweb.nih.gov/ij).

Statistical analyzes

Data are expressed as the mean ± standard error of the mean (SEM). All statistical analyzes were conducted using statistical software (R version 4.0.2; R Foundation for Statistical Computing Platform, Vienna, Austria). Statistical significance was assessed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. P value <0.05 was considered statistically significant.

RESULTS

Verification of sarcolemmal conditions after DR

After DR, positive signals of evans blue were observed in the interstitial space of skeletal muscle fibers but not in their sarcoplasm (Fig. 1a). By contrast, after the crush injury, the sarcoplasm of the myofibers was mostly stained with evans blue (Fig. 1b). These results indicate that the sarcolemma was not disrupted after the DR setting used in this study.

The skin surface temperature of the muscle during icing or heating

Before icing or heating applications, the skin surface temperature of muscles was 30.5°C ± 0.1°C (n = 3). Owing to icing, the temperature decreased over time, and the lowest temperature was observed at the end of the icing application (8.3°C ± 0.3°C, n = 3). Owing to heating, the temperature gradually increased, and the highest temperature was noted at the end of the heat application (39.1°C ± 0.1°C, n = 3). These changing patterns of temperature values measured at skin surface were similar to the patterns measured at muscle surface in the

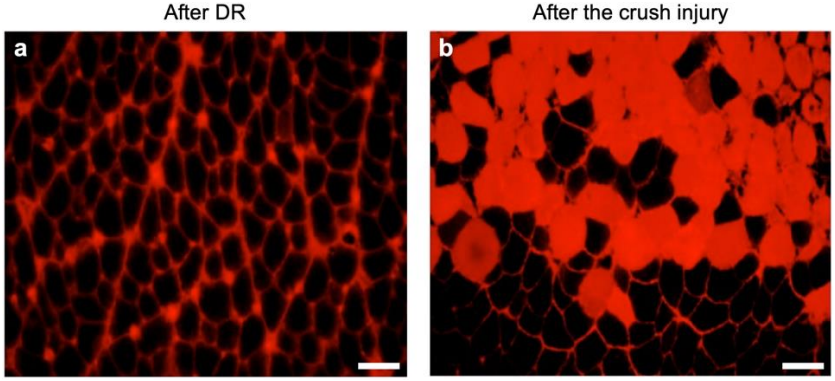

Figure 1. Evans blue stained-cross sections of the exercised triceps brachii muscle at day 1 after DR (a) and crush-injured triceps brachii muscle at day 1 after the crush injury (b). Positive signals of evans blue (red) in the sarcoplasm are detectable in the crush-injured muscle but not in the DR-exercised muscle. Scale bars, 100 μm.

previous studies (22, 24) that performed icing or heating treatment after skeletal muscle crush injury.

Structural changes of muscle fibers on icing or heating after DR

After DR, structural disorders of the skeletal muscle fibers such as the disturbance of the striation pattern and mild disorganization (arrows in Fig. 2a) were observed at all-time points in all DR groups and quantified (Fig. 2b). At 20 min after DR, the proportions of muscle fibers exhibiting structural disorders in the three DR groups were still low, and no significant difference was observed between the groups. At day 1 after DR, the proportion of fibers with structural disorders in the Ice group (16.7% ± 3.0%) was significantly higher than the proportions in the DR (8.8% ± 0.1%, $P < 0.05$) and Heat (2.9% ± 0.03%, $P < 0.01$) groups, and there was a tendency toward a difference between the DR and Heat groups ($P = 0.084$). At day 3 after DR, the difference was detected between the Ice (11.9% ± 2.3%) and Heat (5.6% ± 0.04%) groups ($P < 0.05$). At day 7 after DR, the proportion of muscle fibers displaying structural disorders in all groups recovered to initial levels (20 min after DR) but the Ice group had a higher proportion (4.8% ± 0.1%) than the DR (2.0% ± 0.1%, $P < 0.05$) and Heat (1.6% ± 0.1%, $P < 0.01$) groups.

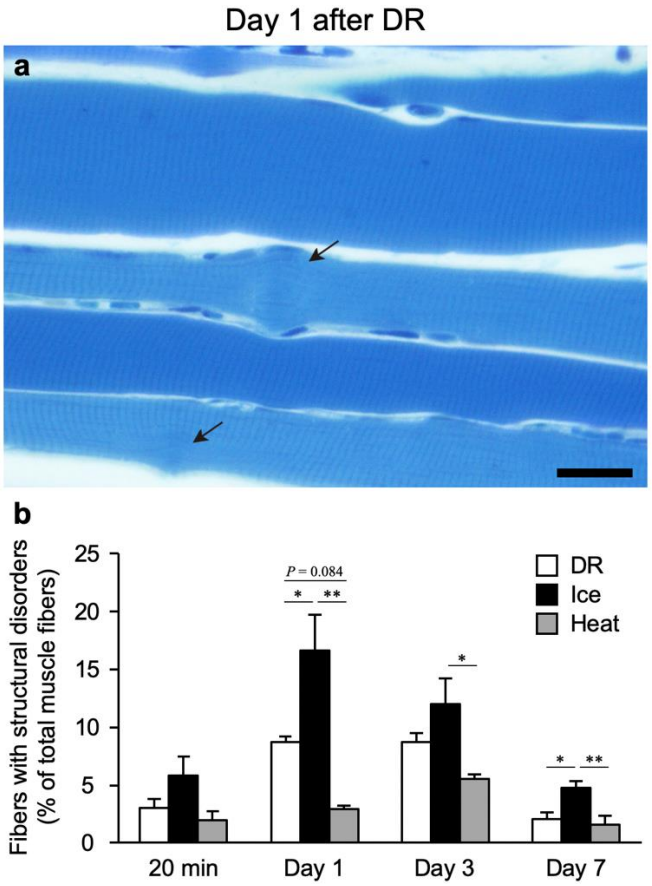

Figure 2. Structural disorders of muscle fibers after DR. (a) A representative image of longitudinal muscle sections stained with toluidine blue at day 1 in the DR group. Arrows indicate the structural disorders of muscle fibers. Scale bars, 20 μm. (b) Histogram showing the proportion of muscle fibers with structural disorders to total muscle fibers at 20 min, days 1, 3, and 7 after DR in the DR, Ice, and Heat groups. Values are expressed as the mean ± SEM (n = 6 per group per time point). Statistical significance was assessed with one-way ANOVA followed by Tukey's post hoc test. *$P < 0.05$. **$P < 0.01$.

Changes in lengths between I-bands of muscle fibers on icing or heating after DR

To examine the impact of icing or heating after DR on the architecture of the sarcomere, lengths from the center of the I-band to the adjacent center of the I-band at 20 min and day 1 after DR were measured and quantified (Fig. 3). At 20 min after DR, lengths between I-bands in the Ice group (1.76 ± 0.02 μm) were

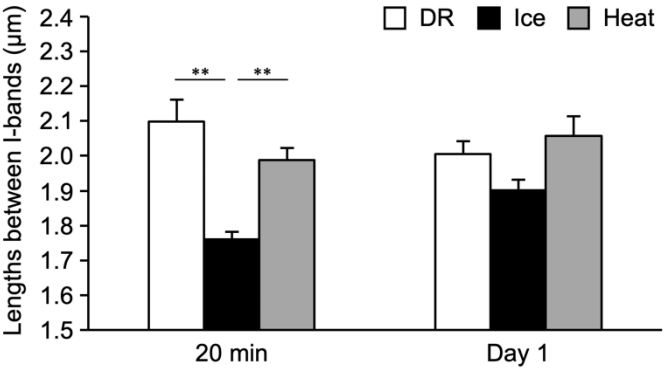

Figure 3. Lengths from the center of the I-band to the adjacent center of the I-band at 20 min and day 1 after DR in the DR, Ice, and Heat groups. Values are expressed as the mean ± SEM (n = 6 per group per time point). Statistical significance was assessed with one-way ANOVA followed by Tukey's post hoc test. **$P < 0.01$.

significantly shorter than those in the DR (2.10 ± 0.06 μm, $P < 0.01$) and Heat (1.99 ± 0.03 μm, $P < 0.01$) groups. At day 1 after DR, no difference between the three groups was observed.

Disturbance of desmin arrangement after DR

Desmin is one of the anchoring molecules located at the periphery of Z-lines and just beneath the sarcolemma in muscle fibers (16, 25). After DR, the immunoreaction of desmin in the sarcoplasm became partially irregular, in agreement with structural disorders on toluidine blue-stained sections (Fig. 4a, b).

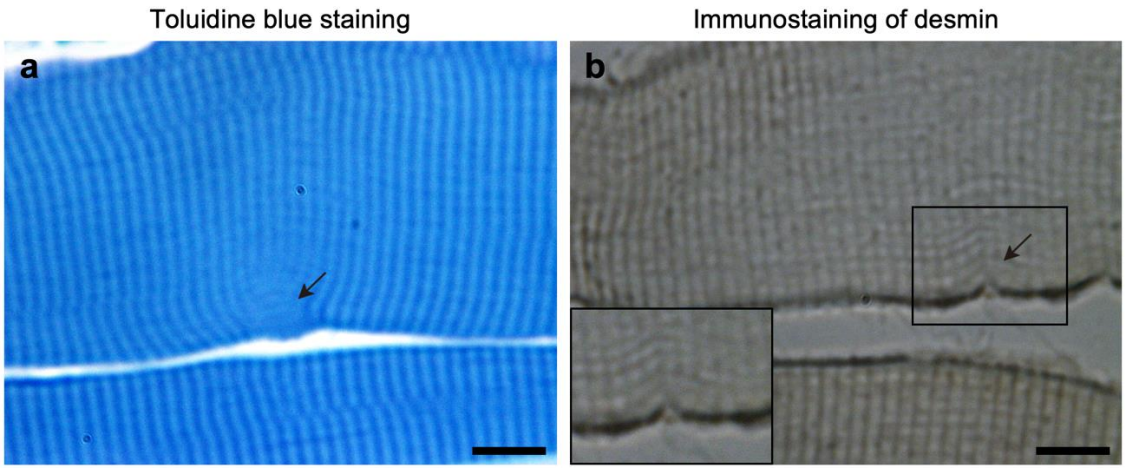

Figure 4. Representative images of toluidine blue staining (a) and immunostaining of desmin (b) on longitudinal muscle sections at day 1 after DR. Immunostaining of desmin was performed only in the DR group. Arrows in (a) and (b) indicate the structural disorders of muscle fibers and disturbance of desmin arrangement, respectively. Inset in (b) shows a higher magnification image of the desmin-disturbed area indicated by the black rectangle. Scale bars, 10 μm.

DISCUSSION

The present study established a new DR protocol causing the structural disorders of skeletal muscle fibers, such as disturbance of the striation pattern and mild disorganization, but without sarcolemmal disruption (Fig. 1). The damage level of muscle fibers observed in our data could mimic that of the EIMD by voluntary muscle actions in humans (2). With this new DR protocol, the impacts of icing and heating treatments on EIMD were compared. Our results first demonstrated that the structural disorder of muscle fibers after DR was aggravated by icing and slightly ameliorated by heating.

In this study, the new DR setting caused structural disorders of muscle fibers (Fig. 2). It has been reported that structural changes of exercised muscle fibers under light microscopic analysis clearly coincide with ultrastructural damage (i.e., Z-line streaming and smearing) under electron microscopic analysis (2, 21). Therefore, Z-line streaming at the ultrastructural level is more likely to be reflected in the structural disorders of muscle fibers in our results.

Previous studies revealed that icing applied after skeletal muscle injury with sarcolemmal disruption and necrosis impaired muscle regeneration (11, 19, 22). The present study using mild EIMD showed that icing treatment exacerbated the structural disorder of muscle fibers after DR (Fig. 2). Additionally, owing to icing after DR, lengths between I-bands became shorter than those in the DR group at the end-point of the 20-min treatment period (Fig. 3). This phenomenon preceded the detrimental effect of icing on structural changes at day 1 (Fig. 2). Thus, the disorganized architecture of the sarcomere, transiently induced by icing, might be involved in the mechanism underlying the aggravation of subsequent structural disorders after DR in the Ice group.

Desmin, a muscle-specific intermediate filament protein, is known for forming longitudinal connections between the peripheries of successive Z-lines and along the plasma membranes of striated muscle fibers and for maintaining structural integrity (16). At day 1 after DR, in this study, the desmin arrangement became irregular, congruent with structural disorders on toluidine blue-stained sections (Fig. 4). A previous investigation reported that after eccentric exercise, attenuation in immunostaining of cytoskeletal proteins such as desmin and dystrophin is mediated by the increased activity of calpain, a calcium-activated protease (26). Additionally, it has been demonstrated that calpain activity and degradation are affected by temperature changes (12, 14). In this study, the skin surface temperature of exercised muscles decreased to 8.3°C ± 0.3°C by icing and increased to 39.1°C ± 0.1°C by heating. Therefore, we speculate that the different thermal stimulations may alter calpain activity after DR, affecting the structural disorder of muscle fibers. Future research will elucidate the mechanism

governing the effects of icing and heating treatments on EIMD, including the link between temperature changes and enzyme activities.

One of limitations in the present study was that structural changes of muscle fibers after DR were not observed at the ultrastructural level. The results of the present study demonstrated that icing exacerbated structural disorder of myofibers and lengths between I-bands after DR under light microscopic analysis. Although previous studies showed that structural damage of exercised muscle fibers detected with light microscope is consistent with ultrastructural damage observed with electron microscope (2, 21), we cannot conclude whether icing provided a greater damage in the ultrastructure of sarcomere after DR. Examining ultrastructural changes under electron microscopic analysis in future studies may provide more detail regarding the effects of thermal treatments. Additionally, the present study used a single treatment for 20 min in agreement with previous investigations (7, 13, 22, 24), but other researchers selected different duration and repetition of icing and heating treatments (11, 15). Furthermore, as the data in the present study did not include biochemical analysis, we could not disclose the mechanism underlying our histological observation. Additional research performing various settings of thermal stimulations and biochemical analysis after muscle-damaging exercise will foster better understanding of thermal therapies to sports injuries.

CONCLUSION

We newly established the DR protocol to investigate the effects of icing and heating treatments on structural damage after eccentrically biased exercise and found that the structural disorder of muscle fibers after DR was exacerbated by icing and improved slightly by heating. Our results provide additional information to understand the effects of icing and heating treatments after EIMD in sports medicine.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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