Effect of local cooling on excitation-contraction coupling in myasthenic muscle: Another mechanism of ice-pack test in myasthenia gravis

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Abstract

Objective: The ice-pack test is a convenient diagnostic testing procedure for myasthenia gravis (MG). We investigated the underlying mechanism of the ice-pack test performed on bilateral masseters.

Methods: We performed trigeminal repetitive nerve stimulation (RNS), excitation-contraction (E-C) coupling assessment (Imai’s method) and bite force measurement before and after cooling of the masseters in MG patients and normal controls. After placing the ice-pack on the masseters for 3 min, serial recordings of the three tests were performed at various time intervals during 10 min after cooling.

Results: The bite force increased significantly after cooling in ice-pack-positive MG patients. The acceleration and acceleration ratio (acceleration at a given time to baseline acceleration) of jaw movement increased significantly after cooling of the masseters in ice-pack-positive MG patients compared to ice-pack-negative patients and normal controls. The prolonged effect of cooling continued until the end of recording even though decremental response to RNS had returned to baseline value.

Conclusions: Cooling of myasthenic muscle may induce two effects. One is relatively short effect on electrical synaptic transmission at the endplate, and another is prolonged effect on E-C coupling in the muscle.

Significance: The ice-pack test induces a prolonged effect of ameliorating impaired E-C coupling in MG.

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1. Introduction

Ptosis, the most common symptom in myasthenia gravis (MG) (Murai et al., 2011), can be improved by local cooling of the eyelid in some patients with MG. This phenomenon has been used in the “ice-pack test” to diagnose MG (Saavedra et al., 1979; Sethi et al., 1987; Ertas et al., 1994). Possible physiological mechanisms of how cooling improves neuromuscular transmission include: (1) increased postsynaptic receptor sensitivity to acetylcholine (ACh) (Harris and Leach, 1968); (2) facilitated transmitter replacement in the presynaptic terminal (Hubbard et al., 1971); (3) efficient utilization of ACh (Borenstein and Desmedt, 1974; Ricker et al., 1977); (4) decreased hydrolysis of ACh by acetylcholine esterase (AChE), allowing sustained action of the transmitter already released from the axon terminal (Foldes et al., 1978); and (5) reduced rate of removal of calcium ions from the nerve terminal following stimulation (Maddison et al., 1998).
Although previous studies focused on the amelioration of neuromuscular transmission at the endplate by local cooling as described above, cooling of the skeletal muscle is known also to induce contractile responses, probably caused by Ca²⁺ release from the sarcoplasmic reticulum (Talon et al., 2000; Protasi et al., 2004). The release of Ca²⁺ plays a significant role in the events that link the electrical stimulus to the mechanical response, which is excitation-contraction (E-C) coupling. Therefore, the ice-pack test may involve another mechanism that increases contractile response by enhancing E-C coupling in the myasthenic muscle.

Our group has developed a method for in vivo assessment of E-C coupling, so-called Imai’s method. Using this method, we have found that bite force correlates significantly with masseteric E-C coupling time (iECCT) [difference in onset latencies between masseteric compound muscle action potentials (CMAPs) and mandibular movement-related potentials (MRPs)] (Tsuda et al., 2010). We have also revealed that anti-ryanodine receptor (RyR) antibody contributes to E-C coupling impairment in MG (Imai et al., 2011, 2012a), and that tacrolimus is a RyR enhancer in MG (Imai et al., 2012b).

In the present study, we attempted to elucidate another mechanism of the ice-pack test in MG by performing serial recordings of trigeminal repetitive nerve stimulation (RNS), mandibular MRP, and bite force before and after local cooling of bilateral masseters. We demonstrated the enhancement of E-C coupling by cooling.

2. Materials and methods

2.1. Subjects and protocol

Twenty-five patients (9 males and 16 females) aged 29–72 years (mean, 53.7 years) diagnosed with MG at Sapporo Medical University School of Medicine, Sapporo, Japan were studied. MG was diagnosed when a patient manifested typical clinical findings and defect in neuromuscular transmission revealed by electrophysiological tests. Such defect was indicated by either an abnormal decrement in repetitive nerve stimulation tests conducted on the nasalis, trapezius, abductor pollicis brevis and abductor digitii minimi; or increased jitter observed on concentric needle single fiber electromyography conducted on the extensor digitorum communis and orbicularis oculi (Kouyoumdjian and Stålberg, 2008; Kokubun et al., 2012). Disease severity was graded according to the Myasthenia Gravis Foundation of America (MGFA) clinical classification (Jaretzki et al., 2000). Acetylcholine receptor (AChR) binding antibodies, anti-muscle specific receptor tyrosine kinase (MuSK) antibodies (Shiraishi et al., 2005) and anti-RyR antibodies (Mygland et al., 1992; Takamori et al., 2004) were measured. Thymic lesions were diagnosed histopathologically after extended thymectomy.

At the time of this study, 11 of 25 patients were naive to specific MG therapy, while the remaining 14 patients had MG exacerbation during treatment with corticosteroids alone or corticosteroids with tacrolimus (4 and 10 patients, respectively). For patients who were taking pyridostigmine bromide, they stopped taking the drug more than 12 h before the study. Eight patients had undergone extended thymectomy.

We classified MG patients into two groups according to the ice-pack test for chewing. Ice-packs were placed on bilateral masseters (see: Section 2.2) for 3 min, and whether chewing ability improved after cooling was subjectively assessed by the MG activities of daily living profile (MG-ADL) (Wolfe et al., 1999; Tsuda et al., 2010). Twenty-five MG patients were divided into ice-pack-positive group (13 patients; 4 males and 9 females; aged 30–72 years, mean 52.2 years) or ice-pack-negative group (12 patients; 5 males and 7 females; aged 29–72 years, mean 55.3 years).

Each assessment was performed in the supine position. After cooling of bilateral masseter muscles by ice packs for 3 min, serial recordings of CMAP and MRP were performed at 15, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600 s, and RNS at 30, 210, 390, 570 s after cooling. At the same time of the electrophysiological assessments, bite force was also measured before and within 2 min after the 3-min cooling, allowing adequate rest between recordings. Bite force was measured only once after cooling to avoid chewing fatigue. Normal ranges for the methods used in this study were established using the data of 9 healthy control subjects (5 males and 4 females) aged 35–66 years (mean, 49.3 years).

The study was approved by the ethics committee of Sapporo Medical University School of Medicine, Sapporo, Japan (number: 16–81, 23–86). All healthy control subjects and patients gave informed consent for participation in this study.

2.2. Cooling of masseter and temperature monitoring

We used commercially available cold packs (3 M Reusable Cold/Hot Pack; 3M Consumer Health Care, St Paul, MN, USA) to cool bilateral masseters. After the cold packs were stored in freezer for at least 2 h, two packs were wrapped in cloth and applied to both cheeks simultaneously for 3 min. The skin temperature above the masseters was measured by non-contact thermometer (Visiofocus 06400; Tecnimed Sri, Italy) just before each electrophysiological recording.

2.3. Bite force measurement

Details of the procedures to measure bite force have been described previously (Tsuda et al., 2010; Imai et al., 2012a). Briefly, a commercially available pressure-sensitive sheet for dentistry (Dental Prescale 50H-R; Fuji Photo Film, Japan) was used. With the pressure-sensitive sheet positioned on the upper dental arch, the patient was instructed to clenched the teeth with maximum force for 5 s. The sheet was then analyzed with a computer analyzing system (FPD-707; Fuji Photo Film, Japan) and the bite force was calculated.

2.4. Repetitive nerve stimulation and movement-related potentials recording

Detailed procedures of repetitive stimulation of the masseteric branch of the trigeminal nerve and recording of potentials have been described previously (Tsuda et al., 2010; Imai et al., 2012a). Briefly, trigeminal RNS was delivered via a cathode (bare-tipped monopolar needle) placed approximately 15 mm into the mandibular notch between the coronoid process and condyle, and an anode (surface electrode) placed on the ipsilateral zygomatic process (Fig. 1). CMAP was recorded from surface disc electrodes placed on the antero-inferior region of the muscle and over the mandibular angle, with a ground electrode placed on the neck. A 0.2-ms rectangular pulse was delivered with gradual increasing intensity to reach a supramaximal response. Once a supramaximal CMAP was obtained, a train of ten stimuli was given at a frequency of 3 Hz. We define abnormal decrement as over 10% reduction in amplitude on the fifth CMAP compared to the first, which indicates impairment of synaptic transmission at the end-plate (Ozdemir and Young, 1971; Mayer and Williams, 1974).

The masseteric ECCT and acceleration of jaw movement were measured by Imai’s methods, with the mouth opened gently with an inter-lip distance of 2.5 cm. The stimulating and CMAP recording techniques were the same as described above for trigeminal RNS. The mandibular MRP was recorded after a single supramaximal stimulation using an acceleration converter (SV1101; NEC, Tokyo, Japan) taped at the chin (Tsuda et al., 2010). The ECCT was defined as the difference in onset latencies between the mas-
We also assessed the maximal acceleration of jaw movement as a significant parameter of the twitch force, by measuring the maximal amplitude of MRP (Yamamoto et al., 2016). An electromyograph (Nicolet Viking IV; Nicolet Biomedical, Madison, WI) was used in all the stimulating and recording procedures.

2.5. Statistical analysis

The Mann-Whitney U-test was used to analyze differences of baseline parameters between normal and patient groups. Wilcoxon matched-pairs signed-ranks test was used to compare the bite force before and immediately after cooling. Multivariate analysis of variance (MANOVA) was used to analyze differences in serial data of skin temperature, CMAP amplitude, CMAP area, % decrement, ECCT, MRP amplitude (=maximal acceleration of jaw movement) and the ratio of maximal acceleration (acceleration at a given time to baseline acceleration) at various times after cooling between 2 groups (ice-pack-positive/negative MG, ice-pack-positive MG versus normal, ice-pack-negative MG versus normal). The effect of group (GROUP: ice-pack-positive/negative MG, ice-pack-positive MG versus normal, ice-pack-negative MG versus normal), the time after cooling (TIME) and the interaction between group and time (GROUP*TIME) were analyzed using MANOVA. When GROUP*TIME interaction was found to be statistically significant, the intergroup differences for each TIME were further analyzed using Mann-Whitney U-test. A p value less than 0.05 was considered to indicate statistical significance. Statistical analyses were conducted using the JMP statistical program (SAS Institute Inc., Cary, NC).

3. Results

3.1. Clinical features

Twenty-five MG patients comprised 4 patients with a purely ocular form (MGFA class 1) and 21 patients with generalized form (MGFA class 2–3). Eighteen of 21 patients with generalized MG had chewing disability of various degrees. Serum antibodies against AChR were detected in 20 MG patients, and MuSK in one of the patients. One anti-AChR positive patient also had anti-RyR antibodies. At the time of this study, 8 MG patients had undergone extended thymectomy. Histopathological study on surgical samples revealed thymoma in 6 patients and remnant thymus in 2 patients. Extended thymectomy performed after this study revealed thymoma in 3 other patients.

There were no significant differences in sex, age, MGFA class, MG-ADL score including chewing disability, antibody status, thymectomy and treatment between ice-pack-positive and -negative MG patients (Table 1).

3.2. Comparison of baseline values

At baseline, the mean skin temperature (SD, range) was 32.7 °C (1.3, 30.5–34.8) in normal controls, 33.1 °C (1.6, 30.4–35.4) in ice-pack-positive patients and 33.3 °C (1.5, 30.2–35.0) in ice-pack-negative patients (Table 2). The mean amplitude and area of CMAP were 2.9 (0.7, 1.9–4.2) mV and 7.1 (0.9, 4.0–10.9) msV, respectively, in normal controls, 1.9 (1.1, 0.3–3.8) mV and 5.2 (2.9, 1.0–9.6) msV in ice-pack-positive patients and 2.9 (1.3, 1.3–6.1) mV and 8.3 (4.3, 2.7–19.3) msV in ice-pack-negative patients. The mean ECCT was 3.5 (0.5, 2.8–4.5) ms in normal controls, 3.8 (0.7, 2.6–4.7) ms in ice-pack-positive patients and 3.4 (0.6, 2.4–4.3) ms in ice-pack-negative patients. The mean MRP amplitude was 2.8 (0.9, 1.3–4.3) m/s² in normal controls, 1.8 (1.0, 0.9–4.6) m/s² in ice-pack-positive patients and 3.4 (2.1, 0.8–8.9) m/s² in ice-pack-negative patients. There were no significant differences in the above baseline parameters between ice-pack-positive patients, ice-pack negative patients and normal controls, except a significant difference between baseline CMAP amplitude between ice-pack-positive patients and normal controls.

On the other hand, the mean baseline bite force (SD, range) was 852.8 (367.6, 183.4–1234.0) N in normal controls, 321.9 (218.1, 12.0–719.3) N in ice-pack-positive patients and 404.7 (170.7,
Table 1

Comparison of disease severity, antibody status and treatment in 25 patients with myasthenia gravis (MG) classified by ice-pack test.

<table>
<thead>
<tr>
<th>Subject group</th>
<th>Number</th>
<th>Age (male, female)</th>
<th>MGFA class (Thymoma)</th>
<th>Antibody</th>
<th>Thymectomy (Thymoma)</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ice-pack-positive MG patient</td>
<td>13 (4, 9)</td>
<td>52.2 (12.9)</td>
<td>2.0 (2.2, 1–3)</td>
<td>7 (6.7, 3–11)</td>
<td>321.9 (218.1) **</td>
<td>371.9 (252.0) **</td>
</tr>
<tr>
<td>Ice-pack-negative MG patient</td>
<td>12 (5, 7)</td>
<td>55.3 (14.6)</td>
<td>2.0 (2.1, 1–3)</td>
<td>5 (5.5, 2–8)</td>
<td>404.7 (170.7) *</td>
<td>403.9 (244.9) *</td>
</tr>
<tr>
<td>Normal controls</td>
<td>9 (5, 4)</td>
<td>49.3 (11.7)</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>

Data are shown as mean (SD) for age and bite force; median (mean, range) for MGFA class and MG-ADL score; and number of patients for antibody, thymectomy (thymoma) and treatment. Eleven patients were untreated. Single p asterisk and double asterisks denote p < 0.05 and p < 0.01, respectively, compared to normal values, as analyzed by Mann-Whitney U-test. MGFA, clinical classification of myasthenia gravis foundation of America; MG-ADL, myasthenia gravis activities of daily living profile; AChR, acetylcholine receptor; RyR, ryanodine receptor; MuSK, muscle-specific tyrosine kinase.

3.3. Chronological changes after cooling

3.3.1. Bite force

The mean bite force (SD, range) immediately after cooling was 818.0 (310.5, 213.7–1138.0) N in healthy control subjects, 371.9 (252.0, 24.4–891.9) N in ice-pack-positive patients and 403.9 (244.9, 257.0–779.8) N in ice-pack-negative patients (Table 1). Wilcoxon matched-pairs signed-ranks test showed a significant increase in bite force after cooling compared to baseline in ice-pack-positive patients (p = 0.02).

3.3.2. Skin temperature

After 3-min cooling, the mean skin temperature (SD, range) decreased to 15.0 °C (4.1, 10.1–22.8) in normal controls, 14.9 °C (3.9, 7.4–19.8) in ice-pack-positive patients and 14.6 °C (2.4, 11.5–21.0) in ice-pack-negative patients at 15 s. The mean skin temperature gradually recovered, and finally returned to 30.8 °C (1.7, 28.8–34.5) in normal controls, 31.4 °C (2.3, 26.3–35.7) in ice-pack-positive patients and 32.2 °C (1.9, 28.2–35.2) in ice-pack-negative patients at 600 s after cooling (Table 2). MANOVA showed no significant change in skin temperature between the three groups.

3.3.3. CMAP and % decrement in RNS

Serial recordings showed the effects of cooling on CMAP amplitude and % decrement in RNS after cooling in MG patients and normal controls (Table 2). Although analysis of GROUP×TIME interaction showed no significant differences in CMAP parameters (amplitude and area) and % decrement in RNS between ice-pack-negative group and normal controls, MANOVA showed that the time courses of these parameters were significantly different between ice-pack-positive group and the other two groups. After cooling, CMAP amplitude was significantly lower in ice-pack-positive group than in ice-pack-negative group and normal controls at all recordings up to 300 s. On the contrary, CMAP amplitude was not significantly different between ice-pack-negative group and normal controls at any of the recordings. CMAP area in ice-pack-positive group was significantly smaller than in ice-pack-negative group at 150, 300, 450, 600 and 800 s after cooling, and also significantly smaller than in normal controls at 15, 60 and 240 s after cooling. The rates of abnormal decrement (over 10% reduction in amplitude on the fifth CMAP compared to the first) in RNS were not different between ice-pack-positive and negative groups at baseline. In 4 ice-pack-positive and 4 ice-pack-negative patients with abnormal decrement at baseline, 2 ice-pack-positive patients showed no decrease in % decrement in the early stage after cooling, while the remaining 6 patients showed decreases in % decrement after cooling followed by recovery to baseline values in the late stage (Fig. 2). However,
Table 2
Changes in skin temperature, compound muscle action potential and movement-related potential (MRP) parameters after cooling in ice-pack-positive and -negative myasthenia gravis (MG) and normal controls.

<table>
<thead>
<tr>
<th>Time after cooling (s)</th>
<th>Baseline</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>210</th>
<th>240</th>
<th>300</th>
<th>360</th>
<th>390</th>
<th>420</th>
<th>480</th>
<th>540</th>
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<th>600</th>
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<tbody>
<tr>
<td><strong>Ice-pack-</strong></td>
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<tr>
<td>Skin temperature (°C)</td>
<td>33.1 ± 1.6</td>
<td>14.9 ± 3.9</td>
<td>20.7 ± 4.1</td>
<td>24.2 ± 3.7</td>
<td>26.5 ± 3.4</td>
<td>27.7 ± 3.2</td>
<td>28.7 ± 3.1</td>
<td>29.5 ± 2.9</td>
<td>30.3 ± 2.7</td>
<td>30.6 ± 2.6</td>
<td>31.4 ± 2.6</td>
<td>31.4 ± 2.3</td>
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<tr>
<td>CMAP amplitude (mV)</td>
<td>1.9 ± 1.1#</td>
<td>2.3 ± 0.9#</td>
<td>2.2 ± 0.5#</td>
<td>2.1 ± 1.0#</td>
<td>2.0 ± 0.9#</td>
<td>2.0 ± 1.0#</td>
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<tr>
<td>CMAP area (msmV)</td>
<td>5.2 ± 2.9</td>
<td>13.8 ± 19.8#</td>
<td>3.8 ± 0.7</td>
<td>3.6 ± 0.8</td>
<td>3.6 ± 0.6</td>
<td>3.7 ± 0.6</td>
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<tr>
<td>Acceleration (%)</td>
<td>13.8 ± 19.8#</td>
<td>3.8 ± 0.7</td>
<td>3.6 ± 0.8</td>
<td>3.6 ± 0.6</td>
<td>3.7 ± 0.6</td>
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<tr>
<td>Acceleration ratio</td>
<td>3.8 ± 0.7</td>
<td>3.6 ± 0.8</td>
<td>3.6 ± 0.6</td>
<td>3.7 ± 0.6</td>
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<tr>
<td>Skin temperature (°C)</td>
<td>33.3 ± 1.5</td>
<td>14.6 ± 2.4</td>
<td>22.4 ± 2.7</td>
<td>25.5 ± 2.7</td>
<td>27.2 ± 2.3</td>
<td>30.3 ± 2.2</td>
<td>30.8 ± 2.1</td>
<td>31.4 ± 2.1</td>
<td>31.9 ± 2.0</td>
<td>32.2 ± 1.9</td>
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<tr>
<td>CMAP amplitude (mV)</td>
<td>2.9 ± 1.3</td>
<td>3.6 ± 1.9</td>
<td>3.5 ± 1.5</td>
<td>3.3 ± 1.5</td>
<td>3.4 ± 1.5</td>
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<tr>
<td>CMAP area (msmV)</td>
<td>8.3 ± 4.3</td>
<td>9.9 ± 6.0</td>
<td>9.5 ± 5.1</td>
<td>9.3 ± 4.7</td>
<td>9.6 ± 5.0</td>
<td>9.3 ± 4.9</td>
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<td>Acceleration (%)</td>
<td>5.9 ± 11.1</td>
<td>3.4 ± 0.6</td>
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<td>29.3 ± 1.8</td>
<td>29.9 ± 1.8</td>
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<tr>
<td>CMAP amplitude (mV)</td>
<td>2.9 ± 0.7</td>
<td>3.3 ± 0.6</td>
<td>3.3 ± 0.7</td>
<td>3.2 ± 0.7</td>
<td>3.1 ± 0.7</td>
<td>3.2 ± 0.7</td>
<td>3.0 ± 0.7</td>
<td>3.0 ± 0.8</td>
<td>2.9 ± 0.8</td>
<td>3.0 ± 0.7</td>
<td>2.9 ± 0.7</td>
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<tr>
<td>CMAP area (msmV)</td>
<td>7.1 ± 1.9</td>
<td>8.5 ± 1.6</td>
<td>8.2 ± 1.8</td>
<td>8.1 ± 1.9</td>
<td>7.9 ± 2.0</td>
<td>7.8 ± 1.8</td>
<td>7.6 ± 1.8</td>
<td>7.5 ± 1.9</td>
<td>7.2 ± 2.0</td>
<td>7.5 ± 1.9</td>
<td>7.3 ± 1.8</td>
<td>7.3 ± 1.5</td>
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<tr>
<td>Acceleration (%)</td>
<td>-10 ± 2.3</td>
<td>-1.8 ± 2.3</td>
<td>0.4 ± 1.7</td>
<td>-0.4 ± 1.7</td>
<td>0.5 ± 3.9</td>
<td>0.5 ± 2.3</td>
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<tr>
<td>Acceleration ratio</td>
<td>3.5 ± 0.5</td>
<td>3.7 ± 0.5</td>
<td>3.7 ± 0.5</td>
<td>3.6 ± 0.4</td>
<td>3.6 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>3.6 ± 0.4</td>
<td>3.5 ± 0.5</td>
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Data are expressed as mean ± SD. CMAP: compound muscle action potential; ECCT: excitation-contraction coupling time; Acceleration: maximum acceleration of jaw movement (=MRP amplitude); Acceleration ratio: acceleration at each time point/acceleration at baseline;

- # p < 0.05 compared to the ice-pack negative group.
- * p < 0.05 compared to normal controls. Statistical difference was determined by Mann-Whitney U-test.
mean % decrement in ice-pack-positive group was significantly greater than that in ice-pack negative group at baseline and at 30 s after cooling, and was significantly greater than that in normal controls at all recordings. In contrast, % decrement was not significantly different between ice-pack negative group and normal controls at any of the recordings.

3.3.4. ECCT and MRP

MANOVA detected significant differences in the time courses of some MRP parameters between ice-pack-positive group and the other two groups (Table 2). The effects of cooling on ECCT and MRP were evident in some ice-pack-positive patients (Fig. 3). However, although the mean ECCT apparently decreased in ice-pack-positive group and was unchanged or increased in the other two groups after cooling, MANOVA detected no significant differences between 3 groups (Table 2). On the other hand, MANOVA showed significant differences in MRP amplitude (acceleration, m/s²) and ratio of acceleration (relative to baseline) between ice-pack-positive group and the other two groups. Acceleration was significantly lower in ice-pack-positive group than in the other two

Fig. 2. Effect of local cooling (3 min) of masseter muscles on % decrement in repetitive nerve stimulation (RNS) in 4 ice-pack-positive patients (open symbols) and 4 ice-pack-negative patients (closed symbols) with abnormal decrement at baseline. Six patients (*) showed slightly improved decrement, but returned to baseline after several minutes.

Fig. 3. Changes in maximal amplitude of MRP after cooling in an ice-pack positive patient. Four traces are superimposed. The mean amplitude of MRP increases from 5.1 m/s² to 7.8 m/s² at 2 min after cooling. The calibration scales indicate 5 ms and 10 mV for CMAP, and 5 ms and 5 m/s² for MRP.

Fig. 4. Changes in maximum acceleration (MRP amplitude) after cooling. The open and closed circles indicate data from the ice-pack-positive and -negative patients, respectively. MANOVA detected significant difference in the time course of maximum acceleration between 2 groups. At baseline (B), the acceleration in the ice-pack-positive patients was significantly lower than that in the ice-pack-negative patients (p < 0.05, Mann-Whitney U-test).
groups at baseline, but was not significant different between 3 groups after cooling (Table 2, Fig. 4). On the other hand, the acceleration ratio was significantly higher in ice-pack-positive group than in the other two groups after cooling consistently until 600 s (Table 2, Fig. 5).

4. Discussion

The effect of temperature has been studied extensively in patients with MG. Exacerbation of myasthenic symptoms by heat is a well known phenomenon (Simpson, 1974; Gutmann, 1980). Applying an ice pack to the eye improves ptosis (Sethi et al., 1998). In addition, the importance of keeping the extremity warm while performing repetitive stimulation has been noted (Borenstein and Desmedt, 1975). Rutkove et al. (1998) have also demonstrated that high temperature greatly enhances decrement on repetitive nerve stimulation. MG predominantly affects proximal muscles, probably because disease severity correlates with the temperature of the affected muscles, and proximal muscles are generally warmer (Jablecki and Benton, 1982).

The most striking finding of the present study is the prolonged effect of cooling on maximal acceleration of jaw movement in the myasthenic muscle in ice-pack-positive MG. The prolonged effect was quite specific to the myasthenic muscle, and the time course in ice-pack-positive MG was significantly different from those in ice-pack-negative MG and normal controls. The acceleration was significantly lower in ice-pack-positive group than in the other two groups at baseline. The greater % decrement in ice-pack-positive group than in the other two groups after cooling consistently until 600 s (Table 2, Fig. 5).

Muscle contraction occurs when Ca\(^{2+}\) is released from the sarcoplasmic reticulum following depolarization of the muscle membrane, resulting in binding of actin and myosin filaments and increased tension within the muscle. Then, Ca\(^{2+}\) is again taken up into the sarcoplasmic reticulum and muscle contraction ends.

The effect of temperature on E-C coupling is known to be complex even under normal conditions. Although several investigators in 1950's to 70's reported that the potentiation of twitch tension during post-tetanic potentiation was diminished by cooling the muscle (Walker, 1951; Close and Hoh, 1968; Hanson, 1974; Hoh, 1974), Krapur (1981) clearly demonstrated that some kind of potentiation remained at low temperature and during recovery from low temperature in a rat model. Recently, laboratory data showed that hypothermia increased the gain of E-C coupling in skeletal and cardiac muscles of mammals (Talon et al., 2000; Protasii et al., 2004; Shutt and Howlett, 2008). The cooling effect may reflect an increase in cytoplasmic Ca\(^{2+}\) concentration probably due to Ca\(^{2+}\) release from the sarcoplasmic reticulum (Protasii et al., 2004). It is likely that amelioration of impaired E-C coupling in MG may be another mechanism for the prolonged effect of cooling. We found smaller CMAPs in ice-pack-positive group than in ice-pack-negative group. Smaller CAMPs may accompany muscle atrophy, which may result in decreased twitch force. Although study of aged skeletal muscle (Delbano, 2011) and molecular biological study of skeletal muscle (Manring et al., 2014) suggest a relationship between muscle atrophy and impairment of E-C coupling, the relationship remains to be validated.

In skeletal muscle, the major mechanism of Ca\(^{2+}\) release from the sarcoplasmic reticulum is related to the ryanodine receptor (RyR), which is coupled to voltage sensors on the sarcosomal membrane. Some congenital myopathies are known to be caused by RYR1 mutation (Wu et al., 2006; Ferreiro et al., 2002; Clarke et al., 2010; Wilmshurst et al., 2010; Kondo et al., 2012). RyR1 deficiency alters the expression pattern of several proteins involved in intracellular calcium homeostasis, and this may...
influence the manifestation of these diseases (Treves et al., 2005; Zhou et al., 2013). However, the underlying immunological mechanism involving RyR function in MG (Iwasa et al., 1998) is not fully understood. In a previous study, we demonstrated the contribution of anti-RyR antibody to impairment of E-C coupling in MG (Imai et al., 2012a). In this study, we were not able to examine the correlation between anti-RyR antibody and amelioration of E-C coupling by cooling in MG because of the small number of anti-RyR positive patients.

The ameliorating effect of the ice-pack test is due to multiple factors as described in the introduction section, such as reduced acetylcholinesterase activity (Shukuya, 1953; Foldes et al., 1978) and increased amplitude of ACh-induced depolarization due to increased Na’influx (Harris and Leach, 1968; Lass and Fischbach, 1976). While cooling may reduce mobilization of secondary storage of ACh, it also increases facilitation secondary to prolonged half-life of Ca2+ in the nerve terminal, allowing more ACh to be released with subsequent stimuli (Delaney and Tank, 1994). Moreover, the present study may add another mechanism; amelioration of impaired E-C coupling. Given the multiple reasons for improvement of the postsynaptic function by cooling, it is not surprising that wide ranges of sensitivity (80–100%) and specificity (25–100%) of the ice-pack test for the diagnosis of MG have been reported in the literature (Sethi et al., 1987; Ertas et al., 1999; Golnic et al., 1999; Chatzistefanou et al., 2009; Mittal et al., 2011). In fact, the present study showed relatively low sensitivity (13/25, 52%) of the masseteric ice-pack test for diagnosing MG.

There seems to be an optimal temperature to obtain a pronounced relief of weakness in MG. Previous investigators reported that reducing the intramuscular temperature to or below 27 °C caused a decrease in muscle twitch as a result of the affected contractile mechanisms (Borenstein and Desmedt, 1975). Decreased muscle twitch was observed in our MG patients and also normal controls immediately after 3-min cooling. Although we cannot directly compare the local temperature of our study with that in the previous report because we used surface measurement instead of a needle thermistor inserted into the muscle for temperature monitoring, excessive lowering of surface temperature may affect contractile mechanisms even in myasthenic muscle. The present study suggests not to cool surface temperature excessively to below 20 °C when performing ice-pack test in MG patients.

In summary, the present study strongly suggests that the effect of cooling on ice-pack-positive myasthenic muscles may involve two mechanisms of action. One is electrical synaptic transmission at the endplate, and another is E-C coupling linking electrical stimulus to mechanical response in the muscle. The ice-pack test may induce amelioration of twitch force for a short duration when the muscle temperature is relatively low, and for a longer duration when the muscle temperature is relatively high. On the other hand, twitch force did not increase after cooling in ice-pack-negative muscles, probably because the normal twitch force was not impaired at baseline.

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Competing interests

None declared.

References


Competing interests

None declared.