Fatigue and damage to the masseter muscle by prolonged low-frequency stimulation in the rat

Konosuke Yamasaki a, *, Shuitsu Harada b, Itsuro Higuchi c, Mitsuhiro Osame d, Gakuji Ito a

a Department of Orthodontics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
b Department of Oral Physiology, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
c Division of Neurology, Neurology Disease Center, Kagoshima University Medical and Dental Hospital, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
d Department of Neurology and Geriatrics, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

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KEYWORDS
Muscle fatigue; Muscle damage; Low-frequency stimulation; Masticatory muscle; Sarcolemmal injury

Summary
This study aimed to examine peripheral fatigue and the resultant damage to the masseter muscle due to prolonged low-frequency stimulation. Thirty male rats were divided into S1, S2, S4, Dantr and Sham groups. The left masseters were used as experimental muscles. A pair of stimulation electrodes was placed on the left masseter. A stimulating session included rectangular electric pulses of 18 Hz (5 mA, 18 V, 0.7 ms) for 2 h with a 3 min rest period between sessions. One session was given to the S1 group, two sessions to the S2 group and four sessions to the S4 group. Four sessions were given to the Dantr group with administration of dantrolene to determine any artifacts of the electrical current. No electric stimulation was given to both side masseters in the Sham group or to the control (right) masseters in the other groups. In each session, jaw-closing force increased to a peak within 1 min and attenuated to the steady force. The peak force decreased as the session advanced in each group. Both side masseters were dissected after the stimulations and examined histologically. The experimental masseter was significantly heavier than that of the controls in the S1, S2 and S4 groups, and the muscle fibres showed irregularity of size and shape with enlargement of interstitial space and infiltration of mononuclear cells into the fibres. However, no such histological change was observed in the Dantr and Sham groups. It was confirmed that fatigue and damage to muscle fibres could be induced in masticatory muscles by prolonged low-frequency stimulation.

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Introduction

Prolonged bruxism, such as clenching and grinding of teeth, causes overactivity of the jaw-closing muscle, and is presumed to be a factor for initiating or perpetuating pain in TMD. Although mechanisms of muscle pain are not yet fully clarified, overactivity of the jaw-closing muscle is presumed to be one of the causes of pain, including myofascial pain. Therefore, prolonged low-level muscle activities could produce muscle fatigue, injury or damage, and may induce pain in masticatory muscles.

Low-frequency (1 Hz) electrical stimulation for 4 h has been shown to increase the uptake of Ca$^{2+}$ and the leakage of lactate dehydrogenase in rat skeletal muscle. Also, the sarcolemma of rat leg muscles was injured after 6–24 h of low-frequency (20 Hz) electrical stimulation. Lengthening contractions with high-frequency stimulation for 300 ms caused damage to masticatory muscles in rats and mice. However, the histological characteristics of masticatory muscles with physiological fatigue were not examined in detail. The aim of the present study is to examine the relationship between peripheral fatigue and resultant damage of the masseter muscle due to prolonged low-frequency stimulation.

Materials and methods

Materials

Thirty male Wistar rats aged 7–8 weeks were assigned into five groups: S1, S2, S4, Sham and Dantr. The rats were housed in a room maintained at 24 °C under a 12 h light–dark cycle. Food and water were provided ad libitum. The treatment and care of the rats used in this study followed the guidelines of the Animal Care and Ethics Committee of Kagoshima University Dental School.

Surgical procedure and electrode placement

The rats were anaesthetised with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body weight, Nembutal, Dainippon Pharmaceutical, Osaka, Japan). The trachea was secured with a polyethylene tube. The level of anaesthesia was maintained by supplemental injections. After shaving the surface of the left cheek, the animal was laid on a platform in a supine position with the head secured by a pillow of hard silicon rubber (Fig. 1). A resin pad was adhered to the anterior part of the maxilla including the upper incisors, and tied securely to the platform with stainless steel wire. To record the jaw-closing force, the lower incisors were connected with ligature wire to an isometric power transducer (Type UK-1K, Unique Medical, Tokyo, Japan). The position of an edge of the lower incisor was adjusted to the same level of the edges of the upper incisors, to keep the jaw-closing muscles in a relaxed position.

Two incisions of 3 mm length were made on the left cheek skin to place a pair of electrodes (Pt–Ir electrodes, KU201-013, Unique Medical, Tokyo, Japan), separated by 10 mm, on the left superficial masseter (Fig. 1). To prevent direct contact of the electrode with the muscle, the tips of the electrodes were covered with cotton balls held by a cotton ball holder. The cotton balls were kept in light contact with the fascia by wire springs from the platform. Sterile Ringer’s solution at 37 °C was administered through the holes of the resin holder every few minutes to keep the cotton balls wet. The right masseters were not treated and were used as controls.

Electrical stimulation and analysis of jaw-closing force

There are two types of muscle fatigue: (1) low-frequency fatigue (contraction fatigue) due to stimulation below approximately 20 Hz; and (2)
high-frequency fatigue (transmission fatigue) due to stimulation above approximately 80 Hz. In the present study, low-frequency (18 Hz) stimulation (5 mA, ~18 V, 0.7 ms) was applied by a stimulator (SEN-3201, Nihon Kohden, Tokyo, Japan).

A series of electrical stimulations for 2 h was defined as one session. One session was given to the S1 group, two sessions were given to the S2 group, and four sessions were given to the S4 and Dantr groups. Each session was separated by a 3 min rest period. Cotton balls were replaced during the break because they were gradually compressed such that the holders could contact the muscle. To confirm that muscle damage did not occur due to the application of the electrical current, a muscle relaxant, sodium dantrolene (Dantrium, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, 2 mg/ml of 5% mannitol sodium), was administered to the Dantr group through a cannula placed in the femoral vein 1 h before the start of the stimulation. Dantrolene was provided supplementarily several times up to a total dose of 5 mg. In the Sham group, two incisions were made in the left cheek and a pair of electrodes with cotton balls was set on the masseter for 12 h without stimulation. The cotton balls were soaked in sterile Ringer’s solution every few minutes.

The jaw-closing force was transmitted by an isometric power transducer, amplified by an AC amplifier (VC-10, Nihon Kohden, Tokyo, Japan), displayed on a digital oscilloscope (DS-8812, Iwatsu, Tokyo, Japan), and stored on a digital recorder (DRM3MK2, TEAC, Tokyo, Japan) at a sampling frequency of 1000 Hz. The data were analysed with a bio-information multitask analysing system (Kissei Comtec, Tokyo, Japan).

**Histological examination of muscle damage**

Twelve hours after the beginning of the first session, both experimental and control muscles were dissected and weighed. The rats were killed by over-injection of sodium pentobarbital. The muscles were mounted on a specimen holder with traganth gum (Wako, Tokyo, Japan) and OCT compound (Sakura Finetechnical, Tokyo, Japan), quickly frozen in isopentane cooled by liquid nitrogen, and stored at −80°C in a deep freezer. Cryostat serial transverse sections of 8 μm thickness were cut out from the middle part of the superficial masseter and picked up on the aminosilane-coated slides.

Sections were stained with haematoxylin-eosin, or immunohistochemically stained with monoclonal antibodies for dystrophin (dystrophin C terminus), dysferlin and α-sarcoglycan (Novocastra, Newcastle, UK, 1:50 dilution). Biotinylated antimouse IgG was used as a secondary antibody, and the ABC method was used for signal detection (ABC kit; Vector, CA, USA). Double staining was performed with haematoxylin for each specimen.

Muscle fibre injury was identified by the presence of histological damage to the muscle fibres, such as loss of sarcolemmal membrane, invasion of mononuclear cells, and/or loss or reduction of sarcolemmal protein. The number of muscle fibres with or without sarcolemmal injury was counted within five to nine microscopic fields (200×) in each specimen from both experimental and control muscles until the total count exceeded 1000 in each muscle. The ratio of the number of muscle fibres in the experimental muscle against that in the control muscle per field (Ex/C) was calculated in each rat. The percentage of muscle fibres with sarcolemmal damage was also calculated for each muscle.

**Data analysis**

To evaluate muscle fatigue, the force at the peak (peak force), the force at the steady level after the peak (steady force) at 120 min, and the time to reach peak force (peak time) in each session were measured. Differences in the effects of session on the forces in the S4 and Dantr groups were analysed by ANOVA; a multiple comparison post test (Bonferroni/Dunn) tested for statistical significance between each possible pair of mean forces. To evaluate muscle damage, weight ratios, Ex/C and percentage of the muscle fibres with sarcolemmal injury (damaged fibres) among the groups were analysed by ANOVA; Bonferroni/Dunn tested for statistical significance between each possible pair of variables. A P value of <0.05 was considered to be significant.

**Results**

**Attenuation of jaw-closing force**

Upon stimulation, the jaw-closing force increased rapidly and reached its maximum within approximately 1 min, decreased rapidly within several minutes, and reached a steady level that was maintained until the end of the session (Fig. 2). Stimulation with 18 Hz produced a series of twitches, but did not result in the summation of contractions (Fig. 2). This pattern of jaw-closing force was similar among the S1, S2 and S4 groups. Detailed data of the jaw-closing force in the S4 and Dantr groups are shown in Table 1. The peak force at the start of the session and the steady force at 120 min were used from this point onwards.
The mean peak force in the S4 group was significantly larger than the steady force in the S4 group (ANOVA, d.f. = 3, \( F = 11.718 \), \( n = 24 \), Bonferroni/Dunn, \( P < 0.05 \)), the peak force in the Dantr group (ANOVA, d.f. = 3, \( F = 11.7 \), \( P < 0.05 \)), and the steady force in the Dantr group (ANOVA, \( n = 24 \), d.f. = 3, \( F = 11.7 \), Bonferroni/Dunn, \( P < 0.05 \)) (Fig. 3).

The peak time in the S4 group increased as the sessions advanced (9.3 ± 1.0 s in session 1, 15.3 ± 6.3 s in session 3, and 20.6 ± 7.2 s in session 4) (Fig. 4). In the Dantr group, the peak time in session 1 (33.6 ± 4.5 s) was significantly less than that in sessions 3 (61.1 ± 6.3 s) and 4 (58.5 ± 3.0 s) (ANOVA, d.f. = 3, \( F = 9.483 \), Bonferroni/Dunn, \( P < 0.05 \)). Although the peak time increased until session 4 in the S4 group, the increasing rate was less than that in the Dantr group. The peak time in the Dantr group was significantly greater than that in the S4 group (ANOVA, d.f. = 1, \( F = 95.193 \), Bonferroni/Dunn, \( P < 0.05 \)) (Fig. 4).

### Muscle damage

### Muscle weight

There was no significant difference among the body weights in the five groups (Table 2). There was,

![Figure 2](image-url)  
**Figure 2** A recording of the jaw-closing force in a rat, showing the first 2 h session (a part between the two oblique strokes is abbreviated), the successive break time for 3 min, and the beginning part of the second session. A part of the steady force for 1500 ms in the first session is expanded showing individual twitches.

![Figure 3](image-url)  
**Figure 3** Changing of peak and steady forces at 120 min from the start of each session in the S4 and Dantr groups.

<table>
<thead>
<tr>
<th>Session</th>
<th>Time (min)</th>
<th>S4 group</th>
<th>Dantr group</th>
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<tr>
<td>0</td>
<td>0</td>
<td>70.0 ± 7.7</td>
<td>51.1 ± 11.2</td>
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<tr>
<td></td>
<td>5</td>
<td>40.7 ± 4.2</td>
<td>33.7 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>40.0 ± 4.1</td>
<td>32.7 ± 3.9</td>
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<tr>
<td></td>
<td>30</td>
<td>42.1 ± 3.9</td>
<td>31.0 ± 3.4</td>
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<td></td>
<td>60</td>
<td>42.2 ± 4.0</td>
<td>27.3 ± 3.0</td>
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<td></td>
<td>90</td>
<td>41.7 ± 3.7</td>
<td>25.7 ± 2.6</td>
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<tr>
<td></td>
<td>120</td>
<td>41.4 ± 2.8</td>
<td>26.9 ± 3.3</td>
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<td>50.7 ± 3.1</td>
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<tr>
<td></td>
<td>5</td>
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<td>28.7 ± 3.6</td>
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<td>10</td>
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<td>28.8 ± 3.6</td>
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<td>30</td>
<td>38.8 ± 1.6</td>
<td>27.5 ± 3.8</td>
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<td>38.6 ± 1.8</td>
<td>25.9 ± 2.9</td>
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<td>90</td>
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<td>24.0 ± 2.8</td>
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<td></td>
<td>120</td>
<td>39.4 ± 3.0</td>
<td>21.0 ± 3.0</td>
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Table 1 Jaw-closing force (cN) in the S4 and Dantr groups.

Peak force was measured at 0—2, 4—5, 9—10, 29—30, 59—60, 89—90 and 119—120 min, shown as 0, 5, 10, 30, 60, 90 and 120 min, respectively. Mean ± S.E. (\( n = 6 \)).
however, a significant difference \((t\text{-}test, \, P < 0.05, \, n = 6)\) between the mean weight for the experimental muscle and that for the control muscle in the S4, S2 and S1 groups, while no significant difference was observed in the Dantr and Sham groups (Table 2). The mean weight ratio of the experimental/control muscle in the S4 group was the highest and significantly greater (ANOVA, \(d.f. = 4, \, F = 16.138, \, \text{Bonferroni/Dunn}, \, P < 0.05\)) than that in the S1, Dantr and Sham groups. The ratio in the S2 group followed that in the S1 group, and was significantly larger than that in the Dantr group (ANOVA, \(d.f. = 4, \, F = 16.138, \, \text{Bonferroni/Dunn}, \, P < 0.05\)) and the Sham group (ANOVA, \(d.f. = 4, \, F = 16.138, \, \text{Bonferroni/Dunn}, \, P < 0.05\)) (Table 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>(n)</th>
<th>Body weight (g)</th>
<th>Muscle weight (g)</th>
<th>Experiment/control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6</td>
<td>210.7 ± 5.8</td>
<td>0.75 ± 0.05</td>
<td>0.76 ± 0.05</td>
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<tr>
<td>Dantr</td>
<td>6</td>
<td>226.7 ± 19.3</td>
<td>0.87 ± 0.04</td>
<td>0.86 ± 0.04</td>
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<tr>
<td>S1</td>
<td>6</td>
<td>215.7 ± 16.9</td>
<td>0.79 ± 0.03</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>S2</td>
<td>6</td>
<td>230.5 ± 20.3</td>
<td>0.93 ± 0.17</td>
<td>0.85 ± 0.18</td>
</tr>
<tr>
<td>S4</td>
<td>6</td>
<td>210.5 ± 6.3</td>
<td>0.92 ± 0.06</td>
<td>0.77 ± 0.03</td>
</tr>
</tbody>
</table>

Mean ± S.E.

**Table 2**  Body weight, muscle weight and ratio of experiment/control.

**Figure 4**  Changing of the peak time defined as the time from the start of the stimulation to reach the peak force in each session.

**Figure 5**  Microscopic images of transverse sections of the masseter muscles. In the Sham and Dantr groups, no pathological finding is observed in the experimental (left side) muscles. In the S1 group, enlargements of interstitial space, slight infiltration of mononuclear cells and rounded, swollen muscle fibres are observed and some fibres are stained lightly (arrows). In the S2 and S4 groups, enlargements of interstitial space, infiltration of mononuclear cells and swollen muscle fibres are observed. Some fibres are densely stained (filled stars) or necrotic with invasions of mononuclear cells (open stars). Haematoxylin and eosin staining. Horizontal bar: 50 \(\mu\)m.
Histological and immunohistological findings

The experimental muscles in the Sham and Dantr groups showed no pathological characteristics similar to the control muscles in all groups (Fig. 5). The experimental muscles in the S1 group showed enlargements of interstitial space, slight infiltration of mononuclear cells, and rounded swollen muscle fibres with irregular size and shape (Fig. 5). In the S2 group, enlargements of interstitial space, infiltrations of mononuclear cells and swelling of muscle fibres were more advanced than in the S1 group. Some fibres were hypercontracted or necrotic with invasion of mononuclear cells. In the S4 group, enlargements of interstitial space, infiltrations of mononuclear cells and the swelling of muscle fibres were more advanced than in the S2 group. More fibres were hypercontracted or necrotic with invasion of mononuclear cells (Fig. 5).

The mean Ex/C was $1.00 \pm 0.02$ ($n = 6$) in the Sham group and $0.93 \pm 0.04$ ($n = 6$) in the Dantr group, indicating that the numbers of muscle fibres in the experimental and control muscles per microscopic field were equal in these two groups (Fig. 6). However, the mean Ex/C in the Sham group ($1.00 \pm 0.02$, $n = 6$) was significantly greater than that in the S4 group ($0.63 \pm 0.04$), the S2 group ($0.74 \pm 0.05$) and the S1 group ($0.81 \pm 0.04$) (ANOVA, d.f. = 4, $F = 14.953$, Bonferroni/Dunn, $P < 0.05$). Also, the mean Ex/C in the Dantr group was significantly greater than that in the S4 and S2 groups (ANOVA, d.f. = 4, $F = 14.953$, Bonferroni/Dunn, $P < 0.05$).

Immunohistochemical experiments revealed the prominent reduction of dystrophin among fibres of the experimental muscles in the S1, S2 and S4 groups; however, the reduction of sarcoglycan and dysferlin was smaller than that of dystrophin in the same fibres (Fig. 7). Such a reduction was not observed in the Sham and Dantr groups.

The percentage of the number of injured fibres was only $1.4 \pm 1.0\%$ in the S1 group, and increased with the number of sessions (S2, $5.9 \pm 4.1\%$; S4, $15.5 \pm 6.1\%$) (Fig. 8). The percentage in the S4 group was significantly greater than that in the Sham, Dantr, S1 and S2 groups (ANOVA, d.f. = 4, $F = 20.967$, Bonferroni/Dunn, $P < 0.05$).

Discussion

Prolonged stimulation and masseter fatigue

Muscle fatigue was defined as ‘a reduced capacity for force development’ or ‘a response that is less...
Histological muscle damage

The current study may be the first attempt to examine damage, such as inflammatory response and sarcolemmal injury, induced by prolonged low-frequency isometric contractions in masticatory muscles. Various markers have been used to detect sarcolemmal injury, e.g., influx of fluorescent marker into muscle fibres, leakage of intramuscular proteins, such as plasma creatine kinase or myoglobin, and reduction of sarcolemmal protein. In the soleus and extensor digitorum longus in the rat, the reduction of dystrophin after low-frequency stimulation was more evident than the other sarcolemmal proteins, i.e., dystroglycan and sarcoglycan. In the current study, dystrophin was greatly reduced by prolonged stimulation, while sarcoglycan and dysferlin were relatively preserved in spite of the prolonged stimulation. The structure of the dystrophin molecule is long and flexible, and highly exposed to the action of proteolytic enzymes, whereas α-sarcoglycan and dysferlin are transmembrane proteins. Therefore, dystrophin might be more vulnerable to proteolytic enzymes produced by prolonged contractions than sarcoglycan and dysferlin.

In the current study, the increased weight of the muscle and the decrement of Ex/C were dependent upon the total duration of the experimental sessions. Even one session of stimulation induced enlargement of the interstitial space with slight infiltration of mononuclear cells and swollen fibres with irregular sizes and shapes. Also, abundant necrotic and hypercontracted fibres observed in the S2 and S4 groups indicate that the severity of the inflammatory responses was dependent upon the total duration of the experimental sessions. These results are in good agreement with the invasion of mononuclear cells into interstitial spaces in the rat soleus observed after treadmill running for 2 h. Histological masseter muscle damage with stimulation time dependency in the present experiment might be produced by intracellular Ca²⁺ accumulation and successive activation of Ca²⁺-dependent enzymes, such as calpain I or phospholipase A₂, similar to that in rat limb muscle by low-frequency contractions in the isometric position. This process may cause degradation of the sarcolemma, which might result in leakage of intracellular enzymes such as lactate dehydrogenase, and successive destruction of the sarcolemmal membrane. Therefore, the necrosis observed in the current study is the last stage in the process of sarcolemmal injury.

Figure 8  Percentages of the number of injured fibres against the total number of fibres in the experimental muscles for each group. Mean ± S.E., n = 6.
Dantrolene treatment

Dantrolene sodium, a peripheral muscle relaxant, attenuated limb muscle contraction and prevented skeletal muscle damage by reducing the release of Ca²⁺ from the sarcoplasmic reticulum of the skeletal muscles. In the present study, dantrolene was used to reduce muscle contractions to prevent fatigue in order to examine the effects of electrical current through the muscle during stimulation.

The peak time in the Dantr group was much longer than that in the 54 group. The peak time in the rat gastrocnemius muscle was prolonged after 10 Hz stimulation for 5 min and also subsequent to dantrolene administration, which might be caused by a reduced release of Ca²⁺. These results suggest that the physiological characteristics of fatigue in the rat masseter muscle are fundamentally similar to those occurring within the limb muscles.

Inflammatory responses and sarcolemmal damage to masticatory muscles subjected to sustained low-level activities were reduced. These results are consistent with a previous report that dantrolene treatment prevented damage of limb muscle in response to 2 h sessions of treadmill running in the rat.

Gissel hypothesised that muscle injury during continuous activity was caused by intracellular Ca²⁺ accumulation. On the other hand, Armstrong et al. hypothesised that the damage was caused by physical stress on muscle fibres in eccentric contractions. The effect of dantrolene may be caused by both the depression of Ca²⁺ from sarcoplasmic reticulum, which may cause intracellular Ca²⁺ accumulation, and by attenuation of continuous/repetitive mechanical stress on the vulnerable contractile elements, such as the dystrophin complex. Dantrolene sodium has already been used successfully in the treatment of malignant hyperthermia and was shown to reduce elevated serum creatinine kinase in patients with Duchenne muscular dystrophy. The current results suggest the possibility that dantrolene might be effective in the treatment of myogenous pain with prolonged muscle activity.

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References