EXTRACORPOREAL SHOCK WAVE TREATMENT CAN SELECTIVELY DESTROY END PLATES IN NEUROMUSCULAR JUNCTIONS

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ABSTRACT: Introduction: This study assesses the effect of radial extracorporeal shock wave (rESW) exposure on neuromuscular transmission and neuromuscular junction (NMJ) morphology. Methods: We applied 2,000 rESWs at 0.18 mJ/mm² and a frequency of 15 Hz to the right calf of male rats, measured the compound muscle action potential (CMAP), and examined NMJ morphology using electron microscopy. Left calf muscles were used as controls. Results: rESW exposure significantly reduced CMAP amplitude without delayed latency in exposed muscles compared with controls. All rESW-exposed muscles exhibited NMJs with irregular end plates. Mean junctional fold interval was significantly increased compared with controls. However, axon terminals and muscle fibers surrounding NMJs with irregular end plates were unchanged. Discussion: This localized destruction of end plates may be caused by differences in acoustic impedance induced by the density of acetylcholine receptors. These results provide a possible mechanism for the effectiveness of rESW treatment for spasticity and dystonia.

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Extracorporeal shock wave therapy (ESWT) can improve function without inducing weakness in patients who suffer from spasticity and dystonia.1–4 However, the mechanism of ESWT in the treatment of impaired muscle coordination has not been clarified. In a previous study, we applied radial extracorporeal shock waves (rESWs) to the gastrocnemius muscle of Sprague-Dawley rats and discovered, using a rhodamine-a-bungarotoxin binding method, that rESW-exposed muscles exhibited degeneration of acetylcholine receptors (AChRs).5 Compared with controls, rESW-exposed muscles showed significantly reduced compound muscle action potential (CMAP) amplitudes immediately after treatment, which persisted for 8 weeks without delaying latency. These results suggest that ESWT had caused a transient dysfunction in nerve conduction at the neuromuscular junction (NMJ). However, we could not completely explain the mechanisms underlying the effects of rESWs on muscles. In particular, we could not confirm any structural changes in NMJs after exposure to rESWs. This study assesses changes to neuromuscular transmission and NMJ morphology after exposure to rESWs.

MATERIALS AND METHODS

A total of 20 male Sprague-Dawley rats (8 weeks old; Japan SLC, Shizuoka, Japan) were used in this study. The rats were housed in an animal resources facility under controlled light–dark conditions with free access to food and water. The experimental protocols for this study were approved by our institutional animal ethics committee (approval No.21-140).

Rats were anesthetized with an intramuscular injection of a mixture of 0.75 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol. A 15-mm applicator for the radial hand piece of the Swiss DolorClast (EMS, Nyon, Switzerland), a device that generates radial shock waves pneumatically, was used to apply 2,000 rESWs with an energy flux density of 0.18 mJ/mm² to the right calf of each rat. The frequency and working pressure of the rESWs were 15 Hz and 4 bar, respectively. The left calf of each rat was not exposed to rESWs and was used as a control. The rESWs were applied to the central area of the calf, corresponding to the primary location of end plates in the rat gastrocnemius muscle, which form an M-shaped band across the entire muscle.6

Measurement of Neuromuscular Transmission. Using the results of our previous report (difference in mean amplitude, 10.0 mV; SD, 7.0)5, we calculated the sample size required to show a significant reduction in CMAP amplitude with a statistically significant P value of 0.05 and power of 0.80. This analysis indicated that more than 9 limbs per group were required, so we measured the CMAP in 10 rats. Maximum CMAP amplitude and latency were used as a measure of neuromuscular transmission. CMAPs were measured with a Neuropack XI device (Nihon Kohden, Tokyo, Japan) immediately after application of rESWs.

Abbreviations: AChR, acetylcholine receptor; CMAP, compound muscle action potential; EM, electron microscopy; ESWT, extracorporeal shock wave therapy; NMJ, neuromuscular junction; rESW, radial extracorporeal shock wave

Key words: acetylcholine receptor; extracorporeal shock wave therapy; end plate; neuromuscular junction; synaptic cleft

Conflicts of Interest: We declare that all authors have no competing interests or other interests that might be perceived to influence the results and/or discussion reported in this article.

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ESWT Can Destroy End plates in NMJs
Measurements were performed at room temperature (controlled between 27°C and 30°C), and skin temperature was maintained between 35°C and 37°C during the measurements by the same investigators. After application of rESWs, rats were fixed in the prone position. The skin and muscle were incised, and the sciatic nerve was exposed. Two small monopolar needle electrodes were used for stimulation; the stimulating electrodes were placed on the sciatic nerve, a recording needle electrode was inserted subcutaneously over the gastrocnemius muscle, and the ground electrode was positioned under the abdomen of the rat. To detect maximal CMAP amplitude, electric stimulation was applied from 1 mA to a maximal stimulus that was at least 20% greater than the maximum amplitude of the CMAP. Stimuli were at 1 HZ and 0.2 ms in duration. All rats were humanely killed after measurements.

Morphological Assessment of NMJs With Electron Microscopy. Transmission Electron Microscopy. Five 8-week-old male Sprague-Dawley rats were used in transmission electron microscopy (EM) studies. Rats were transcardially perfused with 0.9% saline, followed by 500 mL of 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) 2 hours after exposure to rESWs. The gastrocnemius muscles were resected bilaterally, and whole muscle specimens were prefixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1.5–3 days. After fixation, tissues were washed with wash buffer at 4°C, postfixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 h, and washed with 0.1 M cacodylate buffer (pH 7.4). Specimens were then dehydrated in a graded series of ethanol solutions in water (60%, 70%, 80%, 90%, 95%, 98%, 100%, 100%, and 100%; 10 min each), followed by propylene oxide for 20 min. After dehydration, specimens were infiltrated with combinations of TAAB Epon 812 resin mixture (TAAB, Aldermaston, UK) and propylene oxide, respectively, at 33% and 67% for 1 h, and 50% and 50% for 1 h, and 67% and 33% for 1 h, before 100% resin infiltration overnight at room temperature. The infiltrated specimens were placed in 12-mL vessels with pure TAAB Epon 812 resin mixture and polymerized at 37°C, 45°C, 60°C, and 65°C. Sections 80-nm thick were cut with an Ultracut S Ultramicrotome (Leica, Wetzlar, Germany) and stained with 3% uranyl acetate in deionized water and lead citrate (Reynold’s method) for 5 min before examination with an H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV.

To assess the morphological changes in NMJs resulting from rESW exposure, we measured and compared the thickness of end plates, the width of synaptic clefts, and the interval between junctional folds in transmission EM images of normal NMJs in control and irregular NMJs in rESW-exposed muscle (Figs. 1, 2). End plate thickness was used to assess the vertical change, synaptic cleft width was used to assess changes in the relationship between presynapses and postsynapses, and the interval between junctional folds was used to assess changes around junctional folds induced by rESWs. End plate thickness was assessed only in NMJs that were cut in the direction of the muscle fiber by measuring 2 thicknesses: thickness A, defined as the distance from the muscle fiber to the top of the end plate and thickness B, defined as the distance from the muscle fiber to the bottom of the synaptic cleft (Figs. 1, 2). The width of a synaptic cleft was defined as the distance between the narrowest point on the presynaptic membrane and each junctional fold (Fig. 2). We also counted the number of end plates in the slice using a monohole grid for transmission EM. To enhance the reliability of this quantitative morphometric assessment, we counted only end plates with nucleated cells that included chromatin.

Scanning Electron Microscopy. Five 8-week-old male Sprague-Dawley rats were used in scanning EM studies. After

FIGURE 1. Morphometric analysis of neuromuscular junctions (NMJs) in gastrocnemius muscles of rats imaged with transmission electron microscopy. End plate thickness was assessed in NMJs that were cut in the direction of the myofibrils by measuring thickness A, defined as the distance from the myofibrils to the top of the end plate and thickness B, defined as the distance from the myofibrils to the bottom of synaptic cleft. Solid arrows, thickness A; dashed arrows, thickness B.
rESW exposure, muscle tissue specimens in saline were fixed by gradually replacing the saline solution with a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1.5–3 days. After washing with 0.1 M cacodylate buffer (pH 7.4) at 4°C, we used the KOH-collagenase digestion method as follows. The fixed muscle was immersed in 5 N KOH for 10 min at 60°C, rinsed in 0.1 M phosphate buffer for 3 h, and incubated in a collagenase solution (Sigma type II, 0.5–0.6 mg/ml in 0.1 M phosphate buffer, pH 6.8; Sigma-Aldrich, St Louis, MO) for 3 h at room temperature. Muscle specimens were rinsed with 0.1 M cacodylate buffer (pH 7.4) 5 or 6 times and conductively stained by treatment with 2% OsO₄ for 1 h at room temperature. Specimens were dehydrated in a graded series of ethanol, transferred to t-butyl alcohol, and freeze dried (Eiko ID-2, Eiko, Japan). The dried specimens were coated with gold–palladium in an ion coater and observed by using a scanning electron microscope (S-4500 FE type; Hitachi, Chiyoda, Japan) at an accelerating voltage of 5 kV.

We assessed the horizontal change induced by rESWs by outlining the perimeter of end plates and measuring their area. Only end plates that were captured from directly above were measured (Fig. 3). Transmission EM and scanning EM images were assessed in Win ROOF version 7.2.0 (Mitani, Tokyo, Japan).

**RESULTS**

**Amplitude and Latency of CMAP.** There was a significant reduction in CMAP amplitude between control and rESW-exposed muscles on the day of shock wave application (34.2 ± 6.2 vs. 24.0 ± 4.6, \(P = 0.0006, \text{power} = 0.98\)). However, there was no significant difference in CMAP latency between control and rESW-exposed muscles on the day of rESW application (1.47 ± 0.18 vs. 1.52 ± 0.14, \(P = 0.55, \text{power} = 0.09\)).

**Morphological Assessment of NMJs.** Transmission Electron Microscopy. Transmission EM showed that both normal NMJs and end plates with irregular synaptic clefts were present in all rESW-exposed muscles (Fig. 4a–d). We measured 11 NMJs in the control group, with a mean slice per NMJ of 3.3 (range 2–9 slices), and 10 NMJs in the ESW group, with a mean slice per NMJ of 3.0 (range 1–5 slices). We measured thickness A of end plates at 52 points in the control group and 28 points in the ESW group and thickness B at 43 points in the control group and 22 points in the ESW group. Mean thickness A and thickness B of NMJs exposed to rESWs were significantly reduced compared with controls (Table 1).

Synaptic cleft width was measured at an average of 118.7 points per NMJ in the control group (range 14–178 points) and 84.6 points per NMJ in the ESW group (range 10–123 points). Mean synaptic cleft width of irregular NMJs was significantly narrower than that of normal NMJs (Table 1). Some vacuoles were observed around NMJs and muscle fibers (Fig. 4b). Furthermore, all end plates remained in contact with axon terminals. There was no apparent damage to the terminal endings of presynaptic fibers, and there was no change in the morphology of vesicles and presynaptic membranes of NMJs with irregular postsynaptic membranes (Fig. 4c,d). We measured the interval between junctional folds at 164 clefts in controls and 143 clefts in the ESW group and found no
significant increase after rESW exposure compared with controls (Table 1). In contrast, there was no apparent damage to muscle fibers surrounding NMJs with irregular postsynaptic membranes.

We counted 8 NMJs per 16.0 \( \mu \text{m}^2 \) in slices from rESW-exposed specimens and 10 NMJs per 4.2 \( \mu \text{m}^2 \) in slices from controls. Therefore, the number of NMJs in rESW-exposed muscle was reduced to one-fifth of that in control specimens.

**Scanning Electron Microscopy.** Morphological assessment of NMJs by scanning EM showed that exposure to rESWs destroyed end plates in the

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Shock wave</th>
<th>( P ) value (power)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness A, ( \mu \text{m} )</td>
<td>3.9 ± 0.9</td>
<td>2.8 ± 1.0</td>
<td>&lt;0.0001 (0.99)</td>
</tr>
<tr>
<td>Thickness B, ( \mu \text{m} )</td>
<td>2.1 ± 0.6</td>
<td>1.2 ± 0.6</td>
<td>&lt;0.0001 (0.99)</td>
</tr>
<tr>
<td>Synaptic cleft width, nm</td>
<td>76.5 ± 18.1</td>
<td>68.2 ± 16.5</td>
<td>&lt;0.0001 (1.00)</td>
</tr>
<tr>
<td>Interval between junctional folds, nm</td>
<td>91.6 ± 22.7</td>
<td>115.7 ± 33.4</td>
<td>&lt;0.0001 (1.00)</td>
</tr>
<tr>
<td>Area, ( \mu \text{m}^2 )</td>
<td>300.7 ± 47.1</td>
<td>320.9 ± 133.7</td>
<td>0.83 (0.09)</td>
</tr>
</tbody>
</table>
right calf muscles of rats (Fig. 5). The extent of damage to end plates after rESW exposure differed among individuals (Fig. 6). We measured the area of 9 and 10 end plates in control and rESW-exposed calves, respectively, and found no significant difference between the 2 groups (Table 1). Moreover, there was no apparent damage to the muscle fibers surrounding irregular end plates, regardless of the degree of damage to end plates.

FIGURE 5. Morphological appearance by scanning electron microscopy of neuromuscular junctions (NMJs) in gastrocnemius muscles of rats exposed to radial extracorporeal shock waves (rESWs). Compared with normal NMJs in control muscle (a,c), NMJs in the calf muscles of rats that were exposed to rESWs (b,d) were destroyed. There was no apparent damage to the muscle fibers surrounding NMJs with irregular postsynaptic membranes.

FIGURE 6. Differences in the extent of damage to end plates after radial extracorporeal shock wave (rESW) exposure. (a) Compared with Figure 5d, part of the sarcolemma (arrows) is visible. (b) There was more damage to the left side of the end plate compared with the right side, suggesting that the rESWs may have passed through the left side. However, damage could not be confirmed on the surface of muscle fibers around the left edge of the end plate.
DISCUSSION

Using electron microscopy, we found that damage to the gastrocnemius muscle of rats due to rESW exposure was confined to end plates, whereas axon terminals and muscle fibers surrounding the destroyed end plate were unaffected. Shock waves can pass through fluid and soft tissue but are reflected at the borders of tissues with different acoustic impedances. Kinetic energy that can induce tissue degeneration is released at the junction of structures with different acoustic impedances. This principle may explain why axon terminals and muscle fibers were unaffected by rESWs, given that the acoustic impedance of muscle and peripheral nerve are similar to that of fat tissue. Furthermore, our findings suggest that there is a difference in acoustic impedance at the postsynaptic membrane of NMJs.

The difference between the postsynaptic membrane of NMJs and the extrajunctional membranes is the density of AChRs. AChRs are highly concentrated in the postsynaptic membrane but diffusely distributed in muscle membrane (2.2 × 10^7 per end plate vs. <10 per μm² in the muscle membrane of rat diaphragm). This increased density of AChRs thickens the postsynaptic membrane, which may change the acoustic impedance between the presynaptic and postsynaptic membranes. In addition, we previously confirmed the degeneration of AChRs after rESW exposure. Therefore, the high density of AChRs at the end plate may explain why the effect of rESW exposure is confined to end plates.

Morphometric assessment by transmission EM revealed that muscles exposed to rESW had thinner irregular end plates, narrower synaptic clefts, and wider interjunctional fold intervals compared with controls. However, scanning EM showed no significant difference in end plate area between damaged and normal end plates. These results suggest that, rather than flattening, rESW exposure may cause local collapse of the postsynaptic membrane.

Scanning EM and transmission EM confirmed normal muscle fiber structure around irregular end plates, indicating that there was no muscle injury. Furthermore, transmission EM assessment confirmed that end plates remained in contact with axon terminals, even for NMJs with irregular end plates, and that the presynaptic membrane and vesicles in axon terminals were not affected by rESWs. This suggests that neuromuscular transmission in irregular NMJs may not be affected, despite the reduced number of NMJs, as long as AChRs are functional. We previously have shown that rESW causes degeneration of AChRs and reduces the number of normal AChRs, suggesting that some of these remaining AChRs may not be functional. However, normal NMJs can still fire if AChRs in irregular NMJs are not functional. Therefore, the reduction in CMAP amplitude could be due to the reduced number of NMJs and/or AChRs. Additional studies are required to determine the exact mechanisms involved.

CMAPs are used for the diagnosis of various nervous system disorders and to assess the pharmacological effects of muscle relaxants such as botulinum toxin, a neuromuscular transmission inhibitor that reduces CMAP amplitude in a dose-dependent manner. In this study, CMAP amplitude of rESW-exposed muscle was reduced to about 70% compared with control muscle. However, the number of NMJs with nucleated cells was reduced to approximately 20% of control. This was evident even though we used controls from the same rat and observed an approximate M-shaped band in both rESW-exposed and control calf muscles. It is possible that the number of end plates that degenerated as a result of ESW exposure was greater than that estimated by the reduction in CMAP amplitude. Given that not all end plates degenerated after ESW exposure, the decrease in CMAP amplitude might not be proportional to the stimulation. However, we could not confirm a correlation between irregular end plates and CMAP amplitude because we could not perform separate CMAP measurements in normal versus irregular NMJs. In addition, the NMJ is a complex three-dimensional structure; hence, our two-dimensional analysis may not be sufficient to measure the extent of the damaged area in end plates. Thus, we could not estimate the percentage of irregular NMJs resulting from rESW exposure.

Another explanation for the discrepancy between the magnitude of reduction in number of the NMJs and CMAP amplitude may be that NMJs located outside the M-shaped band in the central area of the calf where rESWs were applied might contribute to neuromuscular transmission. The energy level of shock waves is a major factor influencing the effectiveness of ESWT. Shock waves are transmitted radially and decrease in energy with distance from the applicator. Thus, our results suggest that application of rESWs to whole muscle is effective for reducing CMAP amplitude.

Several types of tissue damage have been observed by EM after exposure to ESW lithotripsy, including intercellular connection of bladder tissues, swelling of mitochondria, cytoplasmic vacuolization, and cell membrane micropores. Therefore, ESW exposure can destroy the contents of cells. However, lithotripsy uses approximately 3–6 times higher energy shock waves than that used for ESWT.
in the present study. Recommended treatment of tendinitis or plantar fascioplasty is an ESWT protocol of 3 treatment sessions at 1-week intervals, with 2,000 impulses per session at the highest energy flux density that can be tolerated by the patient. However, the use of high-energy ESWs in a rabbit Achilles tendon dose–effect study resulted in tendon necrosis.16 Thus, higher energy ESWs may not be a desirable way to increase the efficacy of ESW treatment. Additional research is required to determine the optimal dosage of rESWs for disrupting neuromuscular junction to reduce CMAP amplitude.

A major limitation of this study is that we did not conduct a prespecified primary investigation to determine the sample size required for NMJ morphological analysis by EM. Compared with the total number of NMJs in the muscle, the number of NMJs that met our criteria and were used in the assessment of end plate area was very small. Second, we did not conduct an in-depth study on the impact of rESWs on motor axons or motor nerve terminals. Hausdorf et al.22 reported that ESW exposure caused substantial selective loss of unmyelinated nerve fibers within the nerves of treated limbs without neurogenic disorders. The reduced CMAP amplitude might be an indication of motor axon disorder; however, additional studies are required to confirm this.

In conclusion, morphological assessment of NMJs by EM showed that ESWT destroyed end plates in NMJs. Although all end plates remained in contact with axon terminals, end plates of ESW-exposed muscles were significantly thinner, and the interval between junctional folds was increased compared with controls. In contrast, axon terminals and muscle fibers were unchanged. Therefore, ESW-induced damage was confined to the postsynaptic membrane. This morphological change may be one explanation for the significant reduction in CMAP amplitude without delayed latency after rESW exposure.

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