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Effect of Pulsed and Continuous Ultrasound Therapy on the Degree of Collateral Axonal Branching at the Lesion Site, Polyinnervation of Motor End Plates, and Recovery of Motor Function after Facial Nerve Reconstruction

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ABSTRACT

The aim of the present study is to test whether ultrasound therapy of muscles denervated by nerve injury would improve the quality of their reinnervation by reduction of the collateral axonal branching at the lesion site and poly-innervation degree at the neuromuscular junctions. After transection and suture of the buccal branch of the facial nerve, pulsed or continuous type of ultrasound therapy was applied to the paralyzed whisker pad muscles of rats in the course of 2 months. Instead of reduction, we found a significant increase in the collateral axonal branching after continuous ultrasound therapy when compared to the branching determined after pulsed or sham ultrasound therapy. Both types of ultrasound therapy also failed to reduce the proportion of polyinnervated end plates in the reinnervated facial muscles. Accordingly, continuous ultrasound therapy failed to restore any parameter of the motor performance of the vibrissal hairs. Application of pulsed ultrasound therapy promoted slight improvements of the functional parameters angular velocity and acceleration. The inhomogeneous structural and functional results achieved after both types of ultrasound therapy let us conclude that further studies are required to evaluate its effects on peripheral nerve regeneration. Anat Rec, 302:1314–1324, 2019. © 2019 Wiley Periodicals, Inc.

Key words: pulsed ultrasound; continuous ultrasound; polyinnervation; collateral axonal branching

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INTRODUCTION

Ultrasound therapy is one of the most commonly and frequently used electrophysical procedures for treatment of diseases of the peripheral nervous system such as neuralgia, neuritis, and inflammation (Bakhtiary and Rashidy-Pour, 2004; Huisstede et al., 2018; Moore et al., 2000). However, the underlying mechanism of ultrasound treatments is not completely understood (Mitragotri, 2005). Ultrasound waves have been shown to have a dual effect. First, the sonic energy produces internal heat, similar to diathermy. Second, the energy of the sound waves acts like a high-frequency massage to tissues (ter Haar, 1999).

Most studies that reveal beneficial effects of ultrasound therapy after injury on muscles and nerves acknowledge that the underlying mechanism is not clear. In our earlier work, we demonstrated that manual stimulation of denervated rat whiskerpad muscles reduced the proportion of polyinnervated neuromuscular junctions (NMJs) and improved the motor performance of the vibrissal hairs after facial-to-facial (Angelov et al., 2007) and hypoglossal-to-facial nerve suture (Guntinas-Lichius et al., 2007; Ozsoy et al., 2014). In this study, we likewise aim to test the effect of ultrasound therapy (with properties of a micromassage and thermal therapy) on polyinnervation of NMJ, degree of collateral axonal branching, and recovery of whisking function.

MATERIALS AND METHODS

Animals

Sixty-four young adult female Wistar rats were obtained from the Laboratory Animal Unit of the Akdeniz University, Antalya, Turkey (Protocol number: 2014.05.03). Before and after experiments, all rats were kept on standard laboratory food and tap water *ad libitum* with an artificial light–dark cycle of 12 hr light on, 12 hr off. All experiments were conducted in accordance with the Turkish Law on the Protection of Animals, and the procedures used were approved by the local animal welfare committee.

Experimental Animal Groups

Animals were divided into five groups. Groups 1 and 2 consisted of 8 and Groups 3–5 of 16 rats (Table 1).

Group 1 (Intact control) These animals were used to determine the number of neurons in lateral facial subnucleus, to establish the functional parameters and the innervation pattern of the vibrissal muscles.

Group 2 (Handling control) These animals were subjected to surgical operations: the marginalis mandibulae nerves were extirpated at the most proximal point and a buccal branch transection and suture repair (buccal-buccal anastomosis, BBA) were performed. Thereafter, they received no therapy and were only held by the experimentator.

Group 3 (Sham ultrasound control) After surgery (marginalis mandibulae extirpation and BBA), animals were subjected to daily ultrasound therapy for 2 months when the device was turned off (i.e., without ultrasound wave being applied).

Group 4 The rats received surgery (marginalis mandibulae extirpation and BBA). Twenty-four hours after the surgery rats were subjected to pulsed ultrasound therapy for 2 months daily.

Group 5 The animals from this group received surgery (marginalis mandibulae extirpation and BBA) and continuous ultrasound therapy for 2 months daily.

Surgery

Anesthesia of the animals was performed by intraperitoneal application of 100 mg/kg Ketamine (Ketasol; Richter Pharma AG, Wels, Austria) and 5 mg/kg Xylazine HCl (Xylazine; Alfasan, Woerden, Holland). Transection and end-to-end suture of the proximal and distal stump of buccal branch of the right facial nerve were performed under an operating microscope in 56 rats (Groups 2–5). Then, the marginalis mandibulae branch of the facial nerve (which sends a ramus to the muscles of the whiskerpad) was extirpated from the most proximal part of the facial nerve (Groups 2–5).

Ultrasound Therapy

Therapeutic ultrasound can produce both thermal and nonthermal effects (Rutjes et al., 2010) and the thermal effects are similar to those of general thermal therapy, including pain relief and acceleration of tissue repair (Xu et al., 1998). However, it is extremely difficult to consider the thermal and the nonthermal effects of continuous ultrasound separately. The continuous ultrasound produces heat in tissues, whereas the pulsed ultrasound does not; therefore, the pulsed ultrasound was used in order to evaluate only the nonthermal effect (only high-frequency massage effect) and the continuous ultrasound was used for both thermal and nonthermal effects.

In the literature, it is shown that using the 1.0 and 1.5 W/cm^2 intensity pulsed or continuous ultrasound therapy significantly enhanced proliferation of satellite cell in

TABLE 1. Experimental groups

Group No.	Surgery	Ultrasound type	Effect on tissue
1 (Intact control)	No	No	–
2 (Handling control)	• Transection and end-to-end suture of the buccal branch of the facial nerve (BBA) • Extirpation of the marginal mandibular branch	No	–
3 (Sham ultrasound control)	• Transection and end-to-end suture of buccal branch (BBA) • Extirpation of marginalis mandibulae	Sham	–
4	• Transection and end-to-end suture of buccal branch (BBA) • Extirpation of marginalis mandibulae	Pulsed	Mechanic massage
5	• Transection and end-to-end suture of buccal branch • Extirpation of marginalis mandibulae	Continuous	Mechanic massage and thermal

the injured muscle (Rantanen et al., 1999), increased immediate range of motion (Draper et al., 1998), significantly increased insulin-like growth factor-1Eb splice variant gene expression (Delgado-Diaz et al., 2011), diminished catalase and superoxide dismutase activities (Freitas et al., 2007), and inhibited the development of disuse muscle atrophy partially (Matsumoto et al., 2014). In this study, we aim to test the mechanic massage effect of 1.5 W/cm^2 intensity ultrasound by using pulsed ultrasound and the thermal effect with continuous one.

For the treatment, an ultrasound device (Palm size 103S Ito Physiotherapy and Rehabilitation Ltd., Tokyo, Japan) that can produce both pulsed and continuous ultrasound was used. According to FDA standards, the given values of the device by the manufacturer for beam nonuniformity ratio and the effective radiating area are 3.6 and 0.9 cm^2, respectively. Ultrasound therapy was initiated 1 day after surgery. Rats in Groups 4 and 5 were daily subjected to ultrasound by moving the sound head gently in circular pattern on the right vibrissae and whisker pad muscles for 5 days a week. The daily time period and dosage were determined by a pilot study (see below).

The depth of the effectiveness of ultrasound depends on the frequency. The lower the frequency, the deeper the ultrasound can reach. The ultrasound therapy with 1 MHz frequency can reach to the depth of up to 3–5 cm, while the 3 MHz ultrasound can only reach to a depth of 1–2 cm (Larsen et al., 2005). The rat vibrissal muscles are superficial; therefore, in our study, 3 MHz ultrasound was applied.

Specific temperature increases are required to achieve beneficial therapeutic effects in the tissue. Based on previous studies by Lehmann et al. (1967a, 1967b, 1974), where the baseline muscle temperature was 36–37°C, an increase of approximately 1°C (mild heating) accelerated metabolic rate in the tissue. An increase of up to 2–3°C (moderate heating) increased blood flow and reduced pain, muscle spasm, and chronic inflammation. Vigorous heat (>4°C) inhibited sympathetic activity and decreased viscoelastic properties of collagen.

Evaluation of Mechanical Effect by Using Pulsed Ultrasound

In this group (Group 4), the mechanical effect of ultrasound was examined. For this reason, the animals in this group were submitted to 10 min daily sessions of therapeutic ultrasound exposure in the pulse mode (1:1 duty cycle) and with a modulated frequency of 3 MHz, intensity of 1.5 W/cm^2.

Evaluation of Thermal and Mechanical Effects Together by Using Continuous Ultrasound

For this purpose, the animals in this group (Group 5) received 5 min daily sessions of therapeutic ultrasound exposure in the continuous mode and with a modulated frequency of 3 MHz, intensity of 1.5 W/cm^2.

Analysis of Vibrissal Motor Performance

All vibrissae were cut with the exception of two large vibrissae in row C (i.e., the third row from dorsal) on each side of the face. Active exploration movements of vibrissae were recorded for 3–5 min. Video records of each animal were examined and 1.5 sec sequences, in which the animal's head was stable, were selected for biometric analysis of whisking. The lines between the medial angles of both eyes and tip of the rat's nose were used as reference lines. The shaft of each vibrissea was represented by the line passing through the point on the base and a point on the shaft 0.5 cm away from the base.

The evaluated parameters were as follows:

1. Forward protraction of vibrissal movement was evaluated by measuring the rostral angle (in degrees) between the midsagittal plane and the whisker shaft; maximum protraction caused the lowest angle;
2. Whisking frequency was determined by the cycles of protraction and retraction (i.e., backward movement, per second);
3. Amplitude was the difference between maximal protraction and maximal retraction (in degrees);
4. Angular velocity was change of angular position relative to the origin by time (degrees per second);
5. Angular acceleration was the change in the angular velocity by time during protraction (degrees per second^2).

For further details an earlier study (Guntinas-Lichius et al., 2007) should be consulted.

Estimation of Axonal Branching by Fluorescence Labeling

Application of fluorescence tracers. Half of the animals in each group were used for this purpose. After sufficient anesthesia, the superior buccolabial and inferior buccolabial branches of the buccal branch of the facial nerve were instilled with crystals of Fast Blue (FB) and Fluoro Ruby (FR), respectively. Crystals were left *in situ* (60 min). Thereafter the application sites were rinsed, dried, and the wound was closed.

Animal sacrifice and tissue harvesting. Fifteen days after instillation of the fluorescent tracers, animals were transcardially perfused with 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. Brainstems were serially sectioned on a vibratome (frontal, 50 μm); sections were mounted on glass slides and air-dried at room temperature. For further details, please consult earlier work (Dohm et al., 2000).

Fluorescence microscopy. Sections were observed on a fluorescence microscope using a custom-made band pass filter set which eliminated the fluorescence cross talk between the tracers. Separate color images of retrogradely labeled facial motor neurons were generated and combined using image analysis software. The technique allowed identification and manual counting of all cells that were stained by FR only, FB only, and those double stained by FR + FB. For further details, an earlier study (Guntinas-Lichius et al., 2007) should be reviewed.

Counting. Every third section through the facial nucleus was counted by the optical dissector approach to determine the total number of motor neurons with axons projecting into the two labeled buccal nerve branches (Fig. 1). For further details, an earlier study (Angelov et al., 1999) should be reviewed.

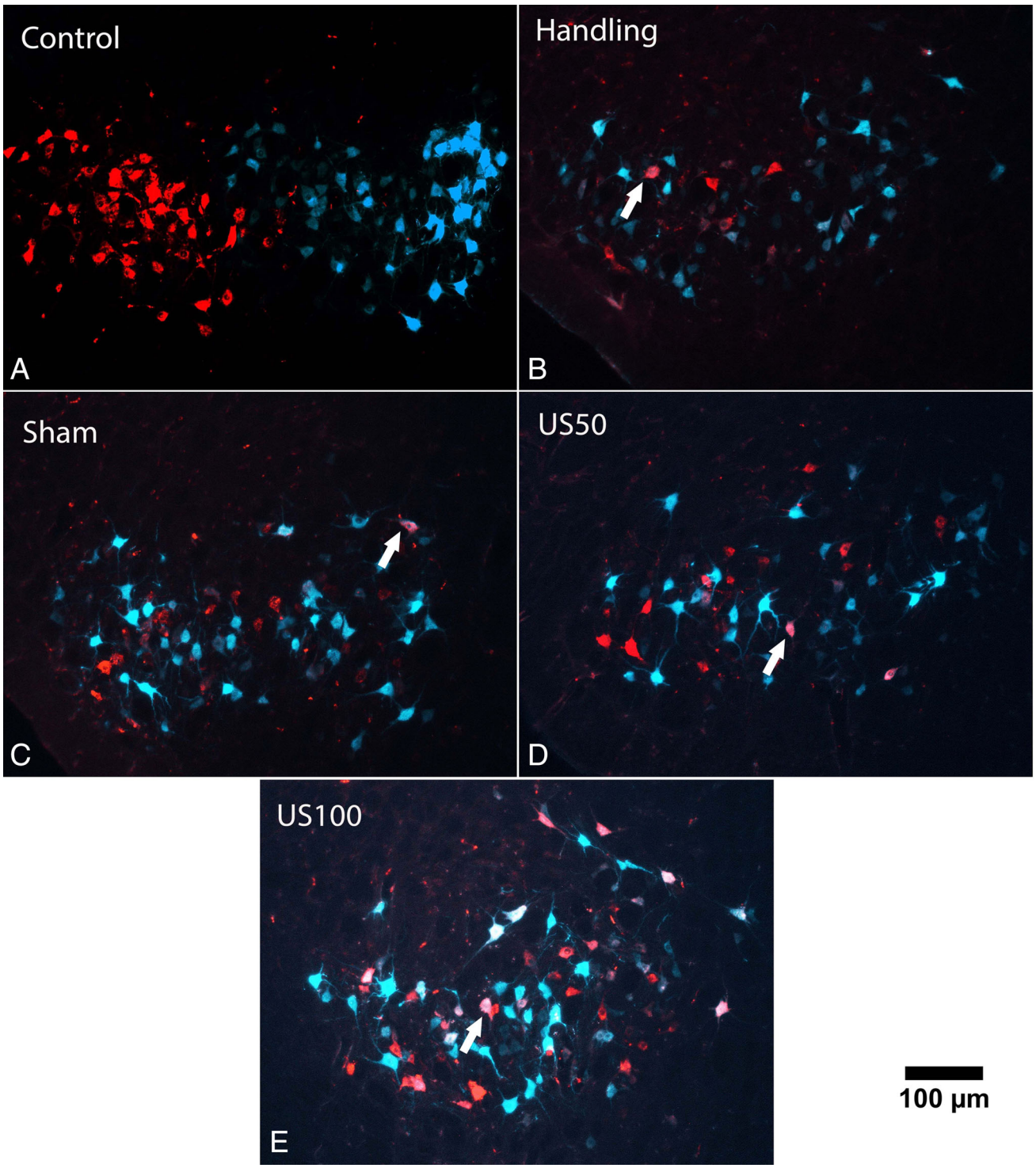


Fig. 1. Facial nucleus exhibiting a clear myotopic organization after application of Fluoro Ruby (FR) and Fast Blue (FB) for retrograde tracing to the r. buccolabialis superior and inferior branches of the facial nerve of unoperated rats, respectively. A myotopic organization is evident (**A**). In contrast, at 2 months after facial nerve transection followed by re-anastomosis and double retrograde labeling of the buccal rami, the myotopic organization disappears and many double-labeled neurons are observed after: handling (**B**), ultrasound therapy performed for 2 months when the device is off (**C**), pulsed ultrasound (1.5 W/cm^2 intensity, 50% duty cycle, 10 min, (**D**) continuous ultrasound (**E**), 1.5 W/cm^2 intensity, 100% duty cycle, 5 min).

Analysis of target muscle reinnervation. End plate analysis is an effective indicator of muscle reinnervation. It is a commonly used technique for evaluating the effectiveness of different methods of muscle reinnervation (Thanos and Terzis, 1995; Thanos et al., 2001). The ratio of monoinnervated to polyinnervated

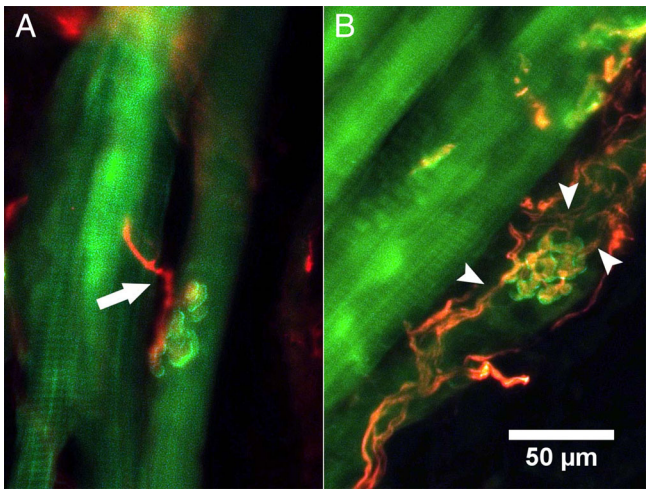


Fig. 2. Panels (**A**) and (**B**) show examples of monoinnervated and polyinnervated end plate, respectively. The monoinnervated end plate is reached by a single axon (arrow in A). In contrast, three branched axons (arrowheads in B) reach the border of polyinnervated motor end plate stained by α-bungarotoxin.

motor end plates (neuromuscular junctions, NMJ) was approximated as previously defined (Guntinas-Lichius et al., 2005, 2007).

The target muscle reinnervation analysis was performed by using the remaining eight animals per group 2 months after surgery. The quality of end plate reinnervation was determined by the ratio of monoinnervated (innervated by one axon) to polyinnervated end plates (innervated by two or more axons) (Guntinas-Lichius et al., 2005). Levator labii superioris muscle was selected as a representative of the external vibrissal muscles. Following transcardial perfusion the levator labii superioris muscle on the operated and unoperated sides of the face was dissected free under a surgical microscope and cryoprotected by immersion in 30% sucrose. Longitudinal sections (30 μm thick) were cut on a cryostat and mounted on slides. To visualize intramuscular axons and motor end plates, every third section through the muscle was double stained with antineuronal class III β-tubulin and α-bungarotoxin (Fig. 2). Sections were observed with a fluorescence microscope. For further details, an earlier work (Bischoff et al., 2009; Guntinas-Lichius et al., 2005, 2007) should be reviewed.

RESULTS

Determination of the Dose Required to Increase the Intramuscular Temperature in a Pilot Study

Continuous ultrasound. In this study, 2–3°C moderate increase of temperature was aimed. In a pilot study, we determined the ultrasound dosage in order to achieve targeted thermal increment in the muscle during the continuous ultrasound exposure. We tested different intensities of insonation. For this purpose, we inserted thermocouple 26-gauge thermistor needle (Phystek MT-23/5; Physitemp Instruments, Clifton, NJ) into the muscle, which was connected to a monitor (Bailey Instruments BAT-10; Physitemp Instruments) that displayed the temperature in degrees Celsius (Fig. 3). In the pilot study, six sides of three animals were used for measurements prior to the ultrasound application: the mean (SD) baseline muscle temperature was 36.5 ± 1°C. Temperature was monitored continuously and recorded every 10 sec during

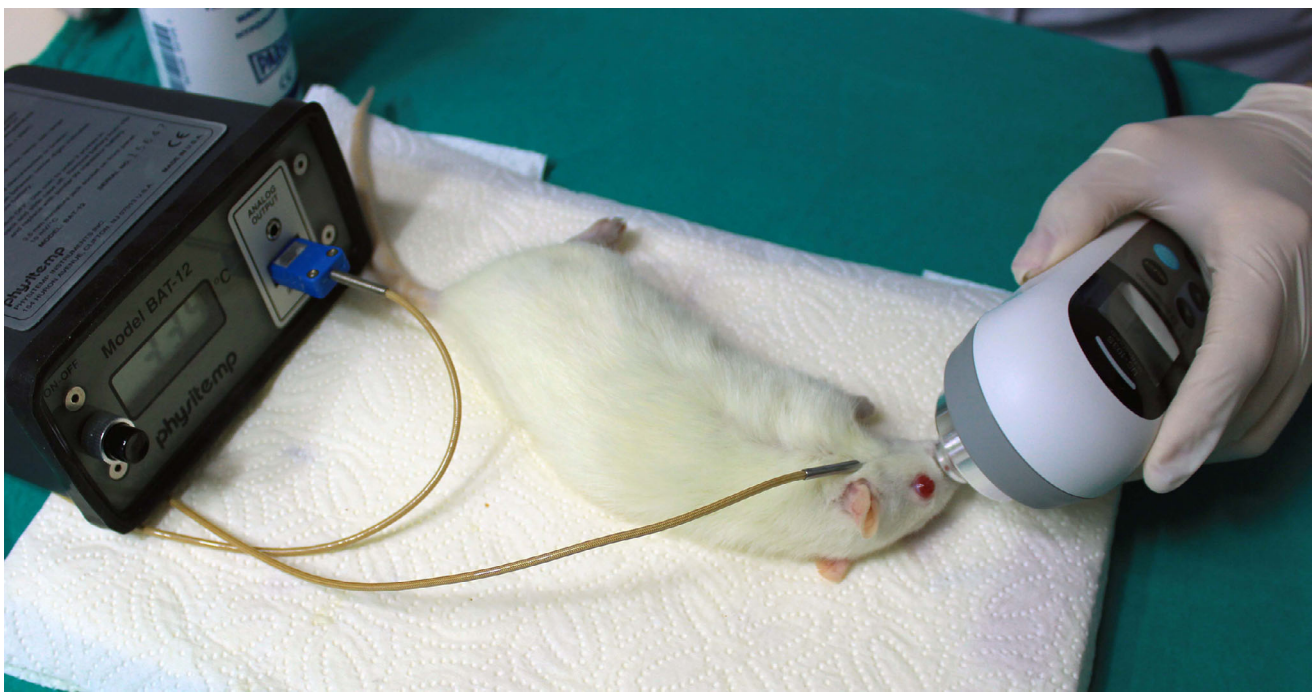


Fig. 3. Determination of ultrasound dosages to achieve targeted thermal changes with a pilot study. The picture shows application of ultrasound, and measurement of intramuscular temperature changes with a needle type thermocouple inserted into the whisker muscles and connected monitor.

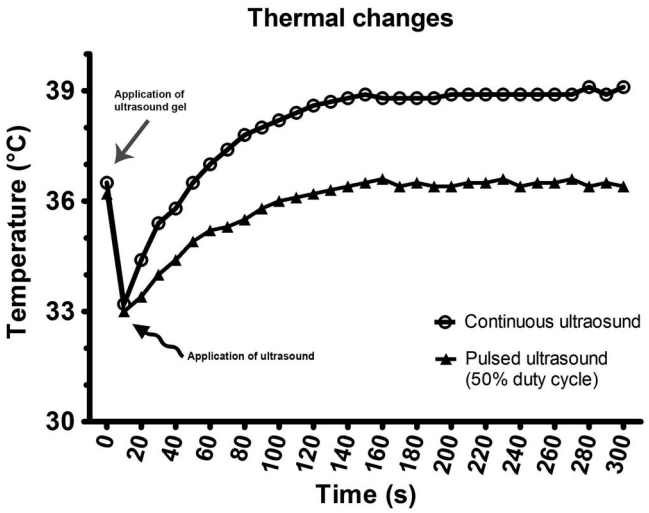


Fig. 4. Diagram illustrating the thermal changes after application of continuous and pulsed type of ultrasound. Application of ultrasound gel (arrow) causes thermal decrease in the muscle. While the application of pulsed ultrasound (curly arrow) increased intramuscular temperature to the normal body temperature, the continuous type caused 2–3°C increase. Data obtained from our pilot study.

5 min of ultrasound application. Application of ultrasound gel caused 3 ± 0.5°C decrease. After ultrasound insonation, we showed that at 1.5 W/cm^2 intensity, 2–3°C thermal increase from normal body temperature could be achieved in around 60 sec, which peaked at 39 ± 0.5°C. This temperature could be maintained for 5 min during the application (Fig. 4).

Pulsed ultrasound. For the second purpose of our study, we tested the required dose of insonation without any thermal changing in the muscle. In order to provide the same value of energy just like in the thermal application but without causing any thermal alteration we tested the same intensity with lower duty cycle (50%) in two times longer time period. We demonstrated that the application of ultrasound with 1.5 W/cm^2 intensity, 50% duty cycle increased the intramuscular temperature to the normal body temperature after ultrasound gel application. However, it did not cause any thermal increment over normal body temperature in 10 min.

Biometric Analysis of Whisking Behavior

The video-based motion technique provides rapid and robust functional analyses of vibrissal movements (Guntinas-Lichius et al., 2002). The healthy animals get information about their immediate vicinity by moving each of their vibrissae ("whiskers") in a coordinated fashion and by sweeping motion ("whisking") at a frequency of 6 Hz (Bermejo et al., 1996; Carvell and Simons, 1990; Semba et al., 1980). A typical whisking consists of protraction and retraction phase of the vibrissae movements. Both phases are under control of active muscular contraction. The protraction and retraction are performed in rostral and in caudal direction, respectively (Berg and Kleinfeld, 2003). In our study, the amplitude of whisking was about 62-degree angle (fourth column in Table 2).

Examination of motor functions showed that, when compared to intact animals, the mean amplitude was poor in handled rats (Handling, Group 2), in rats receiving sham ultrasound (Sham ultrasound Group 3) or postoperative ultrasound (Group 4 and Group 5) of the vibrissal muscles. The mean amplitude was reduced to 50%. The reductions in angular velocity according to intact rat were 66%, 66%, 40%, and 53% in Groups 2, 3, 4, and 5, respectively. Similar reduction was observed in angular acceleration data (Table 2).

Degree of Collateral Axonal Branching

We assessed the degree of axonal branching and the projection patterns of motor axons originating from lateral facial subnucleus by means of double retrograde labeling (Dohm et al., 2000). In intact animals, no double-labeled motor neurons were observed because intact motor neurons send only one unbranched axon to one of the facial rami (Fig. 1A). Thus, the index of axonal branching in the facial nerve trunk of intact animals calculated from the buccal motor neurons was 0% (Table 3).

Two months after buccal nerve cut/anastomosis and handling or sham ultrasound (Groups 2 and 3) myotopic organization into subnuclei was not observed anymore. All retrogradely labeled motor neurons were diversified throughout the facial nucleus (Fig. 1B, C).

Buccal-buccal anastomosis without and with ultrasound therapy for 2 months caused similar phenomena (Groups 4 and 5): transection and suture of buccal branch resulted in the loss of myotopic organization in the lateral facial subnucleus. This is probably due to enormous branching of regenerating axons and poor axonal pathfinding.

TABLE 2. Biometrics of vibrissal motor performance in intact group and after buccal-buccal anastomosis (BBA); in handling group (only held by the experimenter), in sham group (ultrasound therapy was performed for 2 months when the device is off), in US50 group (pulsed ultrasound therapy with 1.5 W/cm^2 intensity and 50% duty cycle for 10 min) and in US100 group (continuous ultrasound therapy with 1.5 W/cm^2 intensity and 100% duty cycle for 5 min)

	n	Frequency (in Hz)	Angle at maximal protraction (in degrees)	Amplitude (in degrees)	Angular velocity during protraction (in degrees/sec)	Angular acceleration during protraction (in degrees/sec^2)
1. Intact	8	8.1 ± 1.5	79 ± 12	62 ± 9	$1{,}501 \pm 430$	$75{,}056 \pm 21{,}500$
2. Handling	8	6.9 ± 1.6	104 ± 12*	32 ± 9*	587 ± 185*	$29{,}325 \pm 9{,}258$*
3. Sham US	16	7.1 ± 1.4	106 ± 14*	29 ± 10*	553 ± 156*	$27{,}625 \pm 7{,}815$*
4. US50	16	7.6 ± 1.4	99 ± 12*	31 ± 5*	$895 \pm 265^{\#}$	$44{,}767 \pm 13{,}246^{\#}$
5. US100	16	8.9 ± 2.4	99 ± 14*	32 ± 8*	696 ± 262*	$34{,}786 \pm 13{,}121$*

Group mean values ± SD are given. *Statistical significance versus intact, $^{\#}$versus sham and handling animals (Kruskal-Wallis, Dunn's test $P < 0.05$).

TABLE 3. Number of buccal motoneurons with axons projecting only in the superior (FR-only) or inferior (FB-only) buccal branch of the facial nerve in intact group and after buccal-buccal anastomosis (BBA); in handling group (only held by the experimenter), in sham group (ultrasound therapy was performed for 2 months when the device is off), in US50 group (pulsed ultrasound therapy with 1.5 W/cm^2 intensity and 50% duty cycle for 10 min) and in US100 group (continuous ultrasound therapy with 1.5 W/cm^2 intensity and 100% duty cycle for 5 min)

	n	Neurons projecting only into the superior branch of buccal nerve (FR-only)	Neurons projecting into the inferior branch of buccal nerve (FB-only)	Neurons projecting into the superior and inferior branches of buccal nerve (FR + FB)	Degree of collateral axonal branching Percentage of FR + FB
1. Intact	8	477 ± 67	1,025 ± 137	0	0
2. Handling	8	422 ± 141	914 ± 361	98 ± 55	7 ± 3
3. Sham US	8	409 ± 181	838 ± 557	92 ± 76	6 ± 3
4. US50	8	572 ± 261	955 ± 478	94 ± 56	6 ± 2
5. US100	8	601 ± 245	765 ± 182	167 ± 48	12 ± 1*

Group mean values ± SD are given. *Statistical significance versus all groups (analysis of variance, Kruskal–Wallis, Dunn's test $P < 0.05$.

TABLE 4. Reinnervation pattern of the vibrissal muscle motor end plates in intact group and after buccal-buccal anastomosis (BBA); in handling group (only held by the experimenter), in sham group (ultrasound therapy was performed for 2 months when the device is off), in US50 group (pulsed ultrasound therapy with 1.5 W/cm^2 intensity and 50% duty cycle for 10 min) and in US100 group (continuous ultrasound therapy with 1.5 W/cm^2 intensity and 100% duty cycle for 5 min)

	Monoinnervated NMJ (%)	Polyinnervated NMJ (%)	Non- innervated NMJ (%)
1. Intact	100	0	0
2. Handling	75 ± 4	23 ± 3	2 ± 2
3. Sham US	77 ± 3	21 ± 3	2 ± 2
4. US50	81 ± 4	18 ± 3	1 ± 2
5. US100	76 ± 2	23 ± 2	1 ± 2

Group mean values ± SD are given.

Double labeling was regularly observed (Fig. 1B–E). This can be explained by the retrograde axonal transport of two different fluorescent dyes (taken up by two axonal branches projecting either into the superior or into the inferior buccolabial branches of the buccal branch of the facial nerve) to one facial motoneuronal perikaryon.

In the present study, the index of axonal branching following BBA and either of handling, sham or the ultrasound treatment was 6%–7% (sum of the percentages of FR + FB retrogradely labeled perikarya in Table 3). However, the index of axonal branching in Group 5 (12%) was significantly higher than the other groups. In conclusion, the total loss of myotopic organization and increased degree of collateral axonal branching indicated that ultrasound therapy in low intensity did not influence the patterns of axonal projection. In contrast, ultrasound therapy in higher intensity increased the degree of axonal branching (6B–E).

Reinnervation Pattern of Vibrissal Muscles

Although it is claimed that the poly-innervation of the end plates is transient after the lesion (Hennig and Dietrichs, 1994), recent studies have shown that this phenomenon may be permanent after the establishment of nerve-muscle contact (Choi and Raisman, 2005; Esslen, 1960; Jergovic et al., 2001; Mackinnon et al., 1991; Madison et al., 1999; Reynolds and Woolf, 1992) and our previous study shows that it has a negative effect on functional recovery (Guntinas-Lichius et al., 2005).

In the intact group, all motor end plates were determined to be monoinnervated (Fig. 2A). Following the repair of facial nerve damage, polyinnervated motor end plates were observed. The percentages, which we determined were 23 ± 3% in handling group, 21 ± 3% in sham stimulation group, 18 ± 3 in pulsed ultrasound, and 23 ± 2% in continuous ultrasound group (Fig. 2B; Table 4). Thus, ultrasound failed to improve the quality of muscle reinnervation.

DISCUSSION

In the present study, we showed that ultrasound therapy five times in a week for 2 months initiating 1 day after transection and repair of the buccal nerve by end-to-end suture could not provide noteworthy improvement in motor recovery. Accordingly, ultrasound therapy could not achieve a decrease in the number of pathologically polyinnervated end plates. It was observed that, regardless of whether ultrasound therapy or sham stimulation was applied, collateral axonal branching increased. Furthermore, continuous-type ultrasound was observed to cause more collateral axonal branching.

The continuous and pulsed mode of therapeutic ultrasound is one of the physical therapy modalities suggested for the management of loss of function and can be used as a part of rehabilitation. The ultrasound has two effects over the tissue. The first one is thermal increment, which has the same effect as thermal therapy. The second one is the micro-massage effect of ultrasound sonication. It is difficult to distinguish both effects in continuous

ultrasound therapy (Mitragotri, 2005; ter Haar, 1999). Therefore, we used pulsed mode of ultrasound to determine the micro-massage effect and the continuous mode to determine thermal effect.

Our pilot study showed that application of ultrasound transmission gel caused a cooling effect at the beginning. Similar degrees in the temperature of the muscle were also reported in the previous studies (Matsumoto et al., 2014). The degrees of body temperature because of anesthesia could be a misleading result of pilot study. In order to eliminate this factor, we used hot plate to keep core body temperature and we monitored it with a rectal thermometer. Additionally, the needle thermometer inserted to the muscle tissue assured that pulsed ultrasound did not cause increment over baseline of core body temperature and for the continuous ultrasound showed the 3°C increment. The aimed increment was achieved in around 2 min and sustained stable throughout the insonation. When these all findings are considered together, we can conclude that our method is suitable for comparing the thermal and nonthermal effect of ultrasound therapy.

Effect of Therapeutic Ultrasound on Injured Skeletal Muscle

The therapeutic effect of ultrasound on injured skeletal muscle has been shown in several *in vivo* and *in vitro* reports. The reports have shown that the application of ultrasound promoted protein synthesis, proliferation of cell, and production of cytokines (Fu et al., 2008). The contused rat gastrocnemius muscle was treated with a pulsed ultrasound (3 MHz, 1.5 W/cm^2) which promoted satellite cell proliferation in the early stages of the regeneration (Rantanen et al., 1999). Nevertheless, the study could not prove whether ultrasound would also promote formation of myotube and other morphological differentiations. Treatment of lacerated rat gastrocnemius muscle with pulsed ultrasound confirmed that this therapy protected the damaged muscle by reducing catalase and superoxide dismutase activities, that is, from oxidative damage (Freitas et al., 2007). Pulsed ultrasound therapy (0.57 W/ cm^2, 1 MHz), which was applied on the first day after injury, has been shown to support repair of lacerated rat gastrocnemius muscle by promoting myoblast differentiation (Piedade et al., 2008). The therapeutic effect of continuous ultrasound on contraction injury was confirmed. The ultrasound (0.5 W/cm^2, 1 MHz) significantly improved muscle contraction (Karnes and Burton, 2002). Muscle atrophy caused by immobilization of the joints for 4 weeks has been shown to reduce by the application of pulsed-type ultrasound therapy. The nonthermal mechanical effect of the pulsed ultrasound might have effectively protected the muscle fiber volume by activating the satellite cells (Matsumoto et al., 2014).

Molecular Changes in Muscles after Ultrasound Stimulation

Several previous reports showed that the irradiation of pulsed ultrasound increased vascular endothelial growth factor and fibroblast growth factor in fibroblasts and angiogenic cells in culture (Reher et al., 1999; Toyama et al., 2012). Furthermore, low-intensity pulsed ultrasound promoted the differentiation of osteoblasts and the proliferation of Schwann cells in culture (Tsuang et al., 2011; Ying et al., 2012), and pulsed ultrasound induced an increase in insulin-like growth factor-1 gene expression in undamaged skeletal muscle in humans (Delgado-Diaz et al., 2011).

Effects of Therapeutic Ultrasound on Nerve Regeneration

Most of the work employing ultrasound to promote regeneration following injury of peripheral nerve has been performed in the sciatic nerve of the rat. Low intensity ultrasound (16 mW/cm^2) therapy was performed for 20 min a day for 12 days following transection and repair of the rat sciatic nerve. Comparison of ultrasound-treated animals with sham-treated group showed that the ultrasound-treated animals had an increased number of regenerating nerve fibers, higher myelination, larger size in fiber dimension, and activated Schwann cell (Crisci and Ferreira, 2002). The authors attributed this enhancement to an increased activity of the Schwann cells, which caused a rapid recovery of myelin sheaths. In another study, following a crush injury, it was shown that the ultrasound treatment (0.25 W/cm^2, 1 min per day, three times a week for 30 days) promoted significantly earlier recovery of function of the foot in comparison to nontreated rats (Mourad et al., 2001). It was speculated that accelerated wound healing and a "regenerative effect" of ultrasound therapy promoted functional recovery. After nerve repair by Schwann cell seeded conduit, pulsed ultrasonic stimulation (0.3 W/cm^2, 5 min/day) was found to have significant enhancing effect on number and area of regenerated axons at the midconduit of implanted grafts (Chang et al., 2005). Both, improved seeded cell survival and proliferation were attributed to an enhancing effect of ultrasound on cell wall flexibility and cell membrane fluidity.

Our present results show that, despite daily ultrasound therapy, recovery of whisking after BBA was not complete. However, slight improvement was achieved in rats of Group 4 treated with pulsed ultrasound for angular velocity and angular acceleration. The therapeutic dosage of ultrasound is matter of concern and it is critical to determine the proper type for tissue healing and function (McDiarmid et al., 1996; Stratton et al., 1984). Therefore, the reason for inadequate recovery could be the improper intensity or duration of sonication. Such variable role of intensity of therapeutic ultrasound on nerve regeneration was shown in a study demonstrating that while lower intensity (0.5 W/cm^2) promoted an earlier regeneration, the higher intensity caused a later regeneration (1 W/cm^2) when compared to nontreated nerves (Lowdon et al., 1988).

The results of our study examine the essential importance of collateral axonal branching as an important part of the phenomenon of misdirected reinnervation: increased rate of branched motor neurons to 12% in rats of Group 5 treated with continuous ultrasound versus 6% in rats of Group 4 treated with pulsed ultrasound had no effect on function. The result obtained from the present experimental evidence is that there is not any correlation between functional restoration and reduced collateral axonal branching. These findings are in parallel with our previous studies, in which we aimed to repair hypoglossal (Ozsoy et al., 2011) and facial nerve (Hizay et al., 2012)

injuries over a Y-tube conduit to diminish collateral axonal branching.

Possible Cellular and Molecular Mechanism Underlying Therapeutic Effect of Ultrasound

Although the underlying mechanisms that are responsible for the therapeutic effects of ultrasound are unclear, it has been confirmed that ultrasound induces protein synthesis, release of angiogenic factors and cellular proliferation (Doan et al., 1999). Ultrasound is known to facilitate molecule passage across membranes by altering membrane properties (Doan et al., 1999; Fischell et al., 1991; Hogg and Landis, 1993; Maxwell et al., 1994; Steffen et al., 1994; Unger et al., 1997; Young and Dyson, 1990; Zur et al., 1993). Possibly, activation of signal transduction pathways causes this permeability changing. Disruption of normal functions of membrane permits Ca^{2+} influx into the cell (Mortimer and Dyson, 1988). In the cell, calcium is employed as a cofactor in regulation of enzymes activity, which is mostly related with signal-transduction pathways. The activation of calcium-sensitive signal-transduction pathways generally causes gene activation for a certain protein synthesis. Consequently, the intracellular functions and activities of the surrounding cells could be modulated by the resultant proteins (Johns, 2002; Karin and Smeal, 1992; Shirakawa and Mizel, 1989). Another hypothesis concerning mechanical effect of ultrasound is the frequency resonance hypothesis, which suggests that ultrasonic energy alters enzymatic activity by changing conformation of the enzymatic protein (Chetverikova et al., 1985; Olsson et al., 1994; Tachibana and Tachibana, 1997). Alternatively, energy provided to the enzyme by the ultrasound wave may spoil the integrity of functional enzymatic protein complex (Chetverikova et al., 1985) or dislodge an inhibitor molecule from the complex (Johns, 2002).

Lessons from Earlier Therapeutical Approaches

It has been shown that small amplitude movements and soft tissue massage promote muscle blood flow and maintain the optimum condition for the skin and muscles until the reinnervation (Beurskens, 2005; Coulson, 2005; Frach et al., 1992; Hovind and Nielsen, 1974). Furthermore, it is also thought that such therapeutic interventions limit the enlargement of motor units (Diels, 2005). This means preventing overflow or hyperinnervation of muscle targets caused by extensive axonal sprouting occurs intramuscularly (Gordon et al., 2004; Son et al., 1996). Additionally, the quantitative increase in the innervation also leads to a decrease in the quality that occurs as a result of the stimulation in an asynchronous manner by two or more motor neurons.

Current studies and our previous results indicate that soft tissue massage improves clinical outcomes whereby limiting the degree of motor end plate polyinnervation within target muscles (Angelov et al., 2007; Evgenieva et al., 2008; Pavlov et al., 2008). We suggest that during the phase of synaptic formation artificial passive movement of the muscle by manual stimulation decreases intramuscular sprouting and thus the degree of polyinnervation. However, although we expected that the poly-innervation of motor end plates would be decreased by the micro massage effect of therapeutic ultrasound, the poly-innervation patterns were unchanged in both ultrasound groups. Examination of target muscle showed abnormally dense meshwork of intramuscular axonal branches and approximately 20% polyinnervated end plates.

CONCLUSION

The results of the present study show different impact of pulsed and continuous ultrasound therapy on the functional and structural recovery after nerve injury. Although our results did not reveal any noteworthy recovery, we think that these variable effects are still unclear and are worth further investigating. In line with the results from previous studies, which emphasized the critical importance of type and concentration of the ultrasound sonication (McDiarmid et al., 1996; Stratton et al., 1984), we believe that no final conclusions should be made until more studies are conducted on ultrasound therapy.

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