Histological effects of focused ultrasound treatment on the sciatic nerves of rats: an experimental study

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ABSTRACT

In this study, the histological changes in the sciatic nerves of rats after exposure to different doses of focused ultrasound (FU) were investigated in order to provide an experimental basis for the application of FU to peripheral nerve disorders. Forty-eight healthy male SD rats were randomly divided into six groups. Each group was subjected to different FU exposure times, including 10 s, 30 s, 1 min, 2 min, 3 min and 4 min of exposure. The irradiated target area was centered 0.5 cm behind the greater trochanter of the left femur with a 1-cm radius. A 1-cm-long segment of the sciatic nerve within the irradiated target area of each rat was selected for observation. The corresponding region of the right sciatic nerve of each rat was used as the control group. The results indicated that the degree of nerve injury is positively related to the dosage of FU exposure, i.e. a higher dosage leads to a greater degree of injury. Thus, FU is expected to become a new method for treating chronic neuropathic pain. Furthermore, different dosages of FU could be selected to irradiate nerves so as to cause different degrees of blockage in nerve conduction function, thus meeting the requirements of different patients suffering from pain.

KEYWORDS
Focused ultrasound; rat; sciatic nerve; histological changes; ultrastructure

Introduction

In recent years, there have been numerous breakthroughs regarding the development of less invasive surgical methods, such as laparoscopic surgery, radiofrequency ablation and cryoablation. Although these methods have replaced many open operations, they still have invasive effects.

In the current clinical practice, ultrasounds are one of the most useful diagnostic tools with which most clinicians are familiar. Nonetheless, not many physicians are familiar with focused ultrasound (FU), which is a new non-invasive clinical treatment technique that utilizes the beam radiation-convergence properties of ultrasound together with good tissue penetration capability to focus the FU exposure within the target area [1,2]. The great advantage of FU is that it can focus energy to a small region (approximately 1 mm x 10 mm ellipsoid) inside the body without damaging intervening tissue [3]. Recent symposia and workshops in addition to an increasing number of FU surgery publications demonstrate that the technology is viable and is likely to see significant development.

At present, FU is primarily used to treat gynecological diseases [4,5], brain tumours [6–8], painful bone metastases [9] and urological disorders [10]. However, research regarding the use of FU therapy to treat neurological disorders by selectively targeting the brain, spinal cord and nerves has been promising.

In this study, FU was used to irradiate the sciatic nerves of rats in order to observe the histological changes in response to different dosages of FU exposure and, thereby, establishing an experimental basis for the application of FU to peripheral nerves disorders.

Subjects and methods

Apparatus

A focused ultrasound therapeutic system (Yuande Biomedical Co., Ltd. Beijing, FEP—BY02), a transmission electron microscope provided by the Institute of Biomedical Engineering at Nanchang University (HITACHI H-600) and a light microscopy system (Olympus, BX-40) were used for the purposes of this study.

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Animals and categorization

The subjects consisted of 48 healthy male SD (Sprague–Dawley) rats weighing 450 ± 20 g. The rats, which were provided by the Animal Center of the Nanchang University College of Medicine, were raised conventionally after procurement. The experiment started after one week of non-abnormality. The rats were randomly divided into six groups, which were exposed to 10 s, 30 s, 1 min, 2 min, 3 min or 4 min of FU therapy (see details below).

Preparation

The rats were anaesthetized using a hypodemic injection of 10% chloral hydrate (0.3–0.4 mL/100 g). After administering the anesthetic, all hair was removed from the left rear side of the irradiated site and the left front side opposite to the irradiated site of each rat, and the locations were cleaned with 8% sodium sulphide. The left rear side of each rat was exposed to FU therapy, while the left front side of each rat was used for observation purposes. The rats were then fastened in the supine position on the metal framework. The skin within the target areas was submerged in deaerated treatment water.

FU irradiation

After the rats were properly positioned, the target area of each rat was identified using diagnostic ultrasound technology and manual positioning. The area selected for irradiation was centered 0.5 cm behind the greater trochanter of the left femur with a 1-cm radius. The target area was only exposed to FU once throughout the entire experimental procedure.

Parameters

Different FU parameters can cause different effects on a tissue (Figure 1). FU application to the sciatic nerve within the body can be described using the standard treatment parameter of ‘dose (J/cm²).’ An FU dose is quantified in a similar way to other forms of medical treatment where energy is applied to tissue, which is the average acoustic intensity I (W/cm²) multiplied by the duration t (s) of the exposure (Dose = I · t) in units of J/cm². The experimental parameters were as follows: 8 mm diameter of the FU treating head; 300 W/cm² average acoustic intensity; voltage of 140 V; step distance of 1 mm; row pitch of 1 mm; stratigraphical gap of 2 mm; element launch time of 0.13 s; duty time of 0.15 s; 30 single-point emissions; six different total treatment launch times (10 s, 30 s, 1 min, 2 min, 3 min and 4 min) with total irradiation dosage of 3000, 9000, 18,000, 36,000, 54,000 and 72,000 J/cm², respectively. The treatment head frequency was 1.356 MHz.

Collection and preparation of the samples

All of the rats were alive after the FU irradiation. The samples were collected and prepared within 24 h of FU irradiation. First, the rats were euthanized with a hypodermic injection of 10% chloral hydrate (1.0 mL/100 g). One-centimeter-long segments of the sciatic nerve of each rat were extracted at the FU treatment site.

Light microscopy

The tissue samples of four rats in each group were fixed in 4°C Bouin fluid for light microscopy and were embedded in paraffin and cut into either cross-sections or longitudinal sections with respect to the axons and were stained with either a combination of hematoxylin and eosin (H&E) or Masson’s trichrome. Then observation was done using a light microscope. The corresponding region of the right sciatic nerve of each rat was used as the control group.

Electron microscopy

The tissue samples of the other four rats in each group were double fixed in 3% glutaraldehyde for transmission electron microscopy (TEM) and were cut into 1-mm cross-sections, postfixed in 1% osmium tetroxide, dehydrated using a series of increasing concentrations of ethanol and embedded in plastic (3:2 ratio of Spurr’s epoxy and propylene oxide). Then, the samples were cut into 1-µm-thick semi-thin sections, post-stained with toluidine blue stain and observed with a light microscope.
Next, the samples were cut into approximately 100-nm-thick thin sections, post-stained with uranyl acetate and lead citrate, and observed with a TEM. The corresponding region of the right sciatic nerve of each rat was used as the control group.

Results and discussion

Light microscopy observations

Control group
As shown in Figures 2(A,B) and 3(A), the nerve fibers of each sample in the control group were arranged closely and in parallel to one another. In addition, the nerve fibers were corrugated in shape. The axons were continuous and surrounded by thick medullary sheaths. Furthermore, the nuclei of the Schwann cells were regular in shape, and the endoneural membranes were blue.

Ten-second-exposure-time group
The nerves in this group exhibited normal morphology, as shown in Figures 2(C,D) and 3(B).

Thirty-second- and one-minute-exposure time groups
As shown in Figures 2(E,F) and 3(C), the nerves in these groups exhibited minor lesions with some nerve fiber vacuoles, mild axon curling and axonotmesis, and mild demyelination.

Two-minute-exposure-time group
As shown in Figures 2(G,H) and 3(D), the nerves in this group exhibited considerable lesions with abundant axonal degeneration, numerous vacuoles, myelin lysis and amytalination, and Schwann cell nuclei lysis.

Three- and four-minute-exposure time groups
As shown in Figures 2(I,J) and 3(E), the nerves in these groups exhibited significant lesions with disordered nerve fiber structures, abundant demyelination and myelin degeneration, significant demyelination and Schwann cell lysis, and blood cells with significant hyperaemia.

TEM observations

Control group
As shown in Figures 4(A,B) and 8(A), the nerve fiber cross-sections were approximately circular. In addition, the axons were surrounded by thick medullary sheaths, and the myelin lamellar structures had a homogeneous texture and were closely arranged without cracks. Furthermore, the Schwann cell nuclei were regular in shape with intact karyothecas and clearly-defined, unexpanded nucleopores.

Ten-second-exposure-time group
The nerves in this group did not exhibit any noticeable changes, as shown in Figures 4(C,D) and 8(A).

Thirty-second- and one-minute-exposure time groups
As shown in Figures 5 and 8(B), the nerves in these groups exhibited minor lesions with vague myelin lamellar structures, cracks and vacuoles. The Schwann cell axonal complexes did not exhibit any significant changes.

Figure 2. Light microscopy images of the longitudinal segments of rat sciatic nerves stained with H&E (A, C, E, G, I) and Masson’s trichrome (B, D, F, H, J). Normal, untreated nerve sections (A, B); normal, 10-s exposure-time nerve sections (C, D); nerve sections with minor lesions (E, F), considerable lesions (G, H) and significant lesions (I, J); × 400. Scale bars = 50 μm.
Two-minute-exposure-time group
As shown in Figures 6 and 8(C), the nerves in this group exhibited considerable lesions, debonded myelin lamellar structures with inhomogeneous thickness, abundant cracks and numerous vacuoles. The axons were pressurized, and fewer Schwann cells were observed. The nuclei of the Schwann cells were irregular in shape with impaired karyothecas and distended nucleopores.

Three- and four-minute exposure time groups
As shown in Figures 7 and 8(D), the nerves in these groups exhibited significant lesions. The nerve fiber structures were disordered, and only the outlines of the nerve fibers were discernable. In addition, the nerve fiber structures appeared diminished with a honeycomb-like appearance, and rarefaction of the nerve fibers was observed. Furthermore, the nuclei of the Schwann cells were severely deformed. Finally, vacuoles were observed around the nucleus, and the karyotheca was nearly dissolved and broken.

Feasibility for treatment of peripheral nerves disorders
FU is a non-invasive treatment method that offers several key advantages, including satisfactory therapeutic effects, low associated risks and few side effects. In addition, FU can be used repetitively until the desired effects have been achieved. Notably, recent studies [11–14] have shown that high doses of FU exposure can result in neural injury, such as axonal demyelination and axonotmesis, thereby hindering nerve conduction. Thus, FU therapy could be used to treat intractable neuropathic and cancer-related pain [15,16]. The thermal cavitation effects of ultrasonic sound result in coagulative necrosis [17,18]. In addition, the thermal effects associated with high doses
of FU exposure can suppress nerve function and potentially lead to the permanent or semi-permanent degeneration of axons and the necrosis of supporting Schwann cells. Furthermore, FU therapy at high temperatures (≥70°C) could possibly be used to completely block nerve conduction [18]. It is likely that cavitation is involved in the demyelination of axons, and stable cavitation could cause demyelination without resulting in the total disruption of axons. Thus, stable cavitation could possibly be used to partially block nerve conduction, thereby decreasing the intensity of nerve signals without resulting in the total degeneration of nerve cells.

Although the pathological changes caused by FU-induced peripheral nerve damage have been studied, few studies have been conducted on the ultra-microstructural changes in nerve tissue resulting from different dosages of FU irradiation. In this study, the effects of different FU irradiation dosages on the general histology and ultra-microstructure of nerves were investigated. At an FU power of 300 W/cm², the following results were obtained.

- No pathological changes in the nerves were observed after an exposure time of 10 s (total dosage of less than 3000 J/cm²).
- Minor pathological changes in the nerves were observed after exposure times of 30 s and 1 min (total dosage ranging from 9000 to 18,000 J/cm²).
- Considerable lesions in the nerves were observed after an exposure time of 2 min (total dosage of 36,000 J/cm²).

**Figure 6.** Transmission electron micrograph images of the myelin of rat sciatic nerve cross-sections with considerable lesions (exposure time of 2 min). The myelin lamella of each nerve fiber is debonded and inhomogeneous in thickness, with numerous cracks and vacuoles. Note: × 5000 (A, B), × 6000 (C, D).

**Figure 7.** Transmission electron micrograph images of the myelin of rat sciatic nerve cross-sections with significant lesions (exposure times of 3 and 4 min). The nerve fiber structure of each nerve is disordered, and only the outline of each nerve is discernable. Each nerve appears diminished and rarefaction with a honeycomb appearance. Note: × 5000 (A, B); × 8000 (C, D).

**Figure 8.** Transmission electron micrograph images of the Schwann cell nuclei of rat sciatic nerve cross-sections. (A) Normal, untreated nerve section with Schwann cell nuclei regular in shape, an intact karyotheca and clearly defined, unexpanded nucleopores; (B) nerve section with minor lesions and no significant changes in the Schwann cell axonal complexes; (C) nerve section with considerable lesions, Schwann cell nuclei irregular in shape, an impaired karyotheca and a distended nucleopore; (D) nerve section with significant lesions, severely deformed Schwann cell nuclei and a lysed karyotheca. Note: × 12,000 (A–D).
— Significant lesions in the nerves were observed after exposure times of 3 and 4 min (total dosage greater than 54,000 J/cm²).

Therefore, the extent of nerve tissue damage was significantly related to the length of FU exposure. In addition, there were lesions of varying severities in the tissues surrounding the nerves, which is possibly due to the low accuracy of the ultrasound positioning. Magnetic resonance (MR), computed tomography (CT) and ultrasound imaging could all be used to improve the positioning accuracy of FU irradiation. In fact, these methods have all been successfully incorporated into current FU systems. However, ultrasound imaging would likely be the most appropriate in this setting because it could provide real-time guidance, and the progression of FU treatments could be monitored by detecting the formation of hyperechoic regions within the site of focus. Thus, an ultrasound positioning system should be applied to FU systems in order to minimize the side effects.

Nerve fibers are classified as either sensory or motor branches. Sensory fibers can be further categorized into Aδ fibers, C fibers, Aα fibers and Aβ fibers. Aδ and C fibers transmit algaesthesia and thalposis, while Aα and Aβ fibers transmit haptics. Studies have shown that nerve fibers possess varying degrees of temperature resistance. Nerve conduction is slightly affected at temperatures ranging from approximately 41 to 45 °C. Aδ and C fiber conduction is completely blocked at temperatures ranging from approximately 70 to 75 °C. In contrast, Aα, Aβ and motor fibers can endure higher temperatures [19,20].

Foley et al. [11,12] indicated that FU irradiation could be used as a new method for treating chronic neuropathic pain because it can disrupt nerve conduction. The FU-induced histological changes reported in this study could be used as a theoretical basis for the treatment of chronic neuropathic pain with FU irradiation. The results obtained in this study indicated that minor lesions were observed in the nerves after 30 s and 1 min of FU exposure, and it was further observed that considerable lesions appeared in the nerves after 2 min of FU exposure. According to these histological results, the temperature of the target area after 30 s and 1 min of exposure could have been less than 70–75 °C, and the temperature of the target area after 2 min of exposure could have been higher than 70–75 °C. Thus, accurate temperature gauges should be used in FU systems in order to maintain the temperature of the target area within 70–75 °C. This temperature range would selectively block Aδ and C fibers, which conduct algaesthesia, but would not affect Aα, Aβ and motor fibers, which conduct haptics. With these changes, FU irradiation therapy could effectively reduce pain without affecting other nerve functions.

FU could result in a variety of biological effects when applied to the structures of the nervous system. As a result, further research should be conducted on the optimization of FU parameters and elucidation of the acoustic mechanisms responsible for various effects on neural structures. Before safe and effective clinical treatments can be developed and applied in clinical practice, extensive in vivo studies regarding the various biological effects associated with the parameters of FU irradiation must be performed. In addition, a precise temperature measurement system should be implemented in order to control the temperature and, thereby, accurately position ultrasonic energy within the target area. FU transducers with more precise focusing capabilities could be used to treat the small branches of peripheral nerves, spinal nerves or sensitive regions of the brain and spinal cord. Thus, further studies are needed in order to improve the efficacy and minimize the side effects of FU therapy.

Conclusions

In this study, the effects of FU on nerves were investigated. The extent of nerve damage was positively related to the dosage of FU exposure. When the exposure dosage was less than 3000 J/cm², nerve function was unaffected. When the exposure dosage ranged from 9000 to 18,000 J/cm², the nerve conduction was partially disrupted. When the exposure dosage was greater than 36,000 J/cm², the nerve conduction was completely disrupted. However, these high dosages could cause some side effects. When the exposure dosage was greater than 54,000 J/cm², all of the nerve cells died. Therefore, for patients with spasmodism and pain that require retaining autonomic functions, the treatment aim is partial prevention of nerve conduction, and the FU dosage should be set in the 9000 to 18,000 J/cm² range. In contrast, if the treatment does not require retaining autonomic functions or the patients do not have autonomic functions, the aim is to completely prevent nerve conduction, so the FU dosage should be set in the 18,000 to 36,000 J/cm² range. It is important to note that future work expanding upon this research will need to include optimization of FU parameters and elucidation of the acoustic mechanisms responsible for the different effects on neural structures. Therefore, before safe and effective clinical treatments can be developed and accepted, extensive in vivo studies are necessary for determining the full range of biological effects associated with the various parameters of FU.
Disclosure statement
The authors declare no conflict of interest.

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