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● *Original Contribution*

FOCUSED ULTRASOUND TREATMENT OF CERVICAL LYMPH NODES IN RATS WITH EAE: A PILOT STUDY

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Abstract—In this pilot study, focused ultrasound (FUS) was used to produce hyperthermia in cervical lymph nodes of rats having experimental autoimmune encephalomyelitis (EAE) to alleviate symptoms associated with EAE. EAE was induced in dark agouti rats, and EAE scores were recorded over 21 d. At the onset of EAE symptoms, rats were treated with FUS to induce temperatures of 43–44°C for 20 min in the superficial cervical lymph nodes. An EAE remittance score was tallied for all rats, defined as the maximum EAE score observed minus the minimum EAE score observed after the maximum EAE was reached. On average, the peak remittance score for FUS-treated rats was 1.14 ± 0.48 versus 0.33 ± 0.27 for sham-treated rats. These differences were statistically significant ($p = 0.037$). Therefore, FUS treatment of cervical lymph nodes in rats with EAE resulted in a significant reduction in EAE score. (E-mail: oelze@illinois.edu) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Focused ultrasound, Ultrasound therapy, Experimental autoimmune encephalomyelitis, Multiple sclerosis.**

INTRODUCTION

Multiple sclerosis (MS) is a prevalent neurologic disease among adults in the United States (≈500,000 cases) and worldwide (≈2.5 million cases) (Kobelt et al. 2006; Mayr et al. 2003). This disease initially involves immune-mediated demyelination and transection of axons within the central nervous system (CNS) and later transitions into a neurodegenerative condition associated with insufficient neurotrophic support in the CNS. The disease pathology causes white and grey matter deterioration and the progression of disability over time. Disease modifying therapies (*e.g.,* interferons) are only modestly effective for slowing long-term progression of pathologic and disability outcomes (Confavreux et al. 2003). This underscores the importance of considering other approaches for managing MS.

Immunomodulatory therapies represent the primary first line approach for managing MS (Galea et al. 2015; Thrower 2009). A review of the latest immunologic understanding of MS, roadblocks to treatment and therapeutic approaches to treat the disease can be found in Dendrou et al. (2015). Many immunomodulatory therapy agents have been developed to reduce the number and severity of relapses in people with relapsing forms of MS (Galea et al. 2015; Ingwersen et al. 2012; Steinman 2014). Many of these agents work by reducing the number of circulating white blood cells (lymphocytes) through a number of mechanisms (*e.g.,* inactivating the lymphocytes or sequestering the lymphocytes to the lymph nodes). For example, in a recent phase II clinical trial, two immunotherapeutic drugs (rituximab [MabThera; Roche, Basil, Switzerland] and ocrelizumab [Roche and Biogen, Cambridge, MA, USA]) showed promise in reducing relapse rates by depleting B cells in the CNS (Hauser et al. 2008; Kappos et al. 2011).

MS largely involves an inflammatory response that is hypothesized to occur from lymphocytes attacking the nerve fibers in the brain, spinal cord and optic nerve (Larocca 2011). By reducing circulating lymphocytes, fewer auto-reactive cells are available to enter into the CNS and attack nerve fibers, thereby reducing MS lesions. These immunomodulatory drug agents have

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demonstrated success in reducing relapses and disability progression in MS, but not without side effects. The side effects of greatest concern are rare, but include lethal infections and malignancies (Ingwersen et al. 2012). Other side effects include headache, flu, diarrhea, back pain, cough, macular edema, increased blood pressure and liver problems.

Recent studies have indicated that the cervical lymph nodes are responsible for the entrance and exit of lymphocytes into the CNS (Louveau et al. 2015). Related to this idea, one such experimental manipulation to reduce lymphocyte proliferation that has been successfully applied in a rat experimental autoimmune encephalomyelitis (EAE) model of MS is cervical lymphadenectomy (Phillips et al., 1997). In that study, cervical lymphadenectomy was used to test the hypothesis that lymphocytes originating from the cervical lymph nodes were responsible for the inflammatory response leading to MS lesions. By excising the cervical lymph nodes, cerebral EAE lesions were reduced by 40% compared to sham procedures. In a similar study, cervical lymph nodes and lumber lymph nodes were resected in EAE mice (van Zwam et al., 2009). In that study, a reduction in EAE incidence and delay in relapse was observed after resection of both cervical lymph nodes and lumbar nodes.

To the authors' knowledge, no studies of cervical lymphadenectomy in humans with MS have been conducted; therefore, it is unknown whether cervical lymphadenectomy will produce comparable results in humans. Excising all of the cervical lymph nodes in humans is not without risk, and further complications will be associated with both the invasive procedure required to excise the lymph nodes and the long-term effects associated with nonfunctioning cervical lymph nodes. Nevertheless, this research further underscores the hypothesis that targeting lymphocytes in the CNS and draining lymph nodes can attenuate disease activity.

We hypothesize that the cervical lymph nodes can be spared from excision while the number and activity of lymphocytes can be reduced significantly through targeted hyperthermia of the cervical lymph nodes using focused ultrasound (FUS). Recently, FUS treatment (often termed high-intensity focused ultrasound) of cancer and other maladies has received much attention. FUS for medical therapy was first introduced more than 60 y ago by the Fry brothers (Fry et al. 1954; Kennedy et al. 2003) and has continued to expand in applications in recent years. Currently, in the United States, FUS has been approved to treat uterine fibroids and bone metastases. Further clinical trials are underway for treating different conditions, such as cancer and Parkinson's disease and essential tremor (Elias et al. 2013; Magara et al. 2014). Reviews of past and current applications of FUS can be found in Tyshlek et al. (2014) and Ebbini and ter Haar (2015). FUS ultrasound has been used to treat a variety of different diseases including neurologic disorders (Elias et al. 2013; Lipsman et al. 2013), bone metastases (Liberman et al. 2009; Turkevich et al. 2011), abdominal tumors (Aubry et al. 2013; Leslie et al. 2012) and prostate cancer (Chaussy and Thuroff 2010; Napoli et al. 2013; Royce and Sothilingam 2008). According to one source, over 80,000 patients have been treated globally with FUS (Tyshlek et al. 2014).

The fundamental principle of FUS therapy is that due to the nature of ultrasound wave propagation, ultrasound fields can be focused or targeted to a specific tissue location non-invasively. FUS can be used to produce therapeutic effects through two mechanisms: heating of tissues or mechanical action. For heating tissues, ultrasound energy is absorbed by the tissue at the ultrasound focal region resulting in localized heating of tissues. Therefore, FUS techniques can be used to treat different tissues, such as cancer (Zhou 2011), through ablation or provide targeted hyperthermia. The temperature elevation can be predicted from the bio-heat equation (Vanne and Hynynen 2003) and monitored using thermocouples or other methods of thermometry such as ultrasound (Bayat et al. 2015; Ghoshal et al. 2014; Seip and Ebbini 1995; Straube and Arthur, 1994) or MRI (Ries et al. 2010; Tempany et al. 2003). Mechanical action using FUS in combination with microbubbles has been used to initiate transient opening of the blood–brain barrier for the delivery of drugs or other molecules for therapy (Burgess et al. 2015; O'Reilly and Hynynen 2012; Wang et al. 2014).

In this pilot study, the feasibility of using FUS to treat rats with EAE by targeting hyperthermia to the cervical lymph nodes was assessed. Targeted hyperthermia through FUS is a minimally invasive therapy (*i.e.,* only ultrasound waves penetrate) that could specifically target the cervical lymph nodes and has the potential to retain long term viability of the cervical lymph nodes while killing lymphocytes or degrading the function of lymphocytes residing in the lymph nodes. Furthermore, in the case of the chronic remitting/relapsing forms of MS, FUS therapy has the potential to be applied repeatedly for each relapse episode.

MATERIALS AND METHODS

Animal handling

The experimental protocol was approved by the Institutional Animal Care and Use Committee, University of Illinois, Urbana-Champaign and satisfied all university

and National Institutes of Health rules for the humane use of laboratory animals. EAE was induced in 7–10-wk-old dark agouti rats (Harlan Laboratories, Indianapolis, IN, USA) through injection of myelin oligodendrocyte glycoprotein (MOG [1-125]) (AnaSpec, Inc., Fremont, CA, USA). The MOG (1-125) protein was used in the induction of EAE in the rats because it results in an EAE model that is relapsing remitting and has lesions in both the brain and spinal cord (von Budingen 2004). For each rat, 50 μg of MOG was mixed with Complete Freund's Adjuvant (Sigma-Aldrich, St. Louis, MO) and injected (total injection volume of 100 μL/animal) subcutaneously at the base of the tail.

EAE scores were recorded from day 5 post MOG injection to day 21. Rat weights were recorded before injection of MOG and once weekly after injection of MOG until an EAE score of 2 was reached at which point the rat weights were recorded daily. Animals were euthanized at day 21 or earlier due to severity of the EAE response or weight loss of 20% or more. The day 21 was chosen as the endpoint for euthanasia because this allowed approximately 2 wk of observation of the progression of EAE after the first visible signs. FUS-treated animals were necropsied after euthanasia to determine if damage consistent with thermal coagulation was present in and around the cervical lymph nodes. EAE disability was scored on a scale from 0 to 5: 0, no clinical signs; 1, loss of tip-of-tail reflex, or complete loss of tail tonus and unsteady gait; 2, paresis of the hind legs; 3, complete paralysis of the hind legs; 4, complete paralysis of lower part of body; 5, death due to EAE (Huitinga et al., 1995). To quantify the remittance of EAE, a peak remittance score (PRS) was calculated and defined as the maximum EAE score observed minus the minimum EAE score observed after the maximum EAE score was reached. In addition, a relapse rate was scored for each animal group. A relapse is defined here as a sustained increase (>2 d) of at least one full grade in the EAE score after the animal had remitted at least one full EAE score grade for at least 2 d. The relapse rate is the total number of relapses in a group of rats during the observation period divided by the total number of rats in the group (Theien et al. 2003). Finally, an average disability index (ADI) was calculated by taking an average of the EAE scores per group divided by the number of animals in the group starting on the last day of therapy (day 12). This was used instead of a cumulative disability index score because of the small number of animals in the pilot study and the early euthanasia of several animals due to severe ataxia or weight loss. Statistically significant differences for the PRS, average rat weight at euthanasia and relapse rate were assessed using a single-variable analysis of the variance between the FUS-treated and sham-treated rats.

Before the study, the timing and course of EAE in rats after injection of 50 μg of MOG and without treatment was recorded in order to plan the FUS therapy. In these animals (n = 6) it was observed that the first symptoms of EAE (*i.e.,* an EAE score of 1) were present at 7–8 d post injection in the six rats. No remittances were observed in the groups up to day 14. Therefore, day 9 post-MOG injection was chosen as the start day for the FUS therapy.

FUS methods

The rats first showed EAE symptoms (*e.g.,* limp tail, hind limb weakness, hind limb paralysis and weight loss) at approximately 7–8 d post injection. Six rats were given a sham treatment, that is, the rats were anesthetized and the exact same experimental setup was used as for FUS-treated animals but with no ultrasound turned on. Seven animals underwent FUS treatments. At days 9 and 12 post-injection, presumably after the first symptoms were observed, FUS therapy was applied. The rationale for using two treatments separated by 3 d is that while some lymphocytes are in the cervical lymph nodes, other lymphocytes would be in circulation in the CNS. By supplying two treatments spaced a few days apart, it was hypothesized that additional lymphocytes associated with the cervical lymph nodes and CNS could be killed or damaged. In addition, hyperthermia is known to provide thermotolerance to tissues such as lymphocytes for a period of at least 24 h (Boreham et al. 1997; Dieing et al. 2007). Therefore, the treatments were spaced more than 24 h apart to reduce the likelihood of thermotolerance reducing the ability of FUS to kill or degrade the function of lymphocytes residing in the targeted cervical lymph nodes.

In the exposures, a 500 kHz, f/3 transducer with a 1-in diameter (IL0508 HP; Valpey-Fisher, Hopkinton, MA, USA) was used to expose the superficial cervical lymph nodes of the rats to ultrasound. The nominal −3-dB transmit beamwidth of the source at the focus was estimated to be 9 mm. The experimental configuration is shown in Figure 1. Animals were exposed to ultrasound

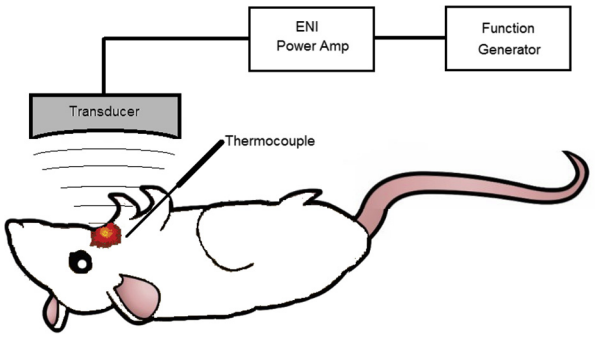

Fig. 1. Experimental configuration.

with peak pressure levels between 366–540 kPa at a duty cycle of 60% (I_{SPTA} = 4.5 to 9.7 W/cm^2). Pressure levels were estimated by using a calibrated hydrophone (GEC Marconi Ltd, Great Baddow, UK). A tone burst with a 60% duty cycle was generated by an arbitrary waveform generator (W1281 A; Tabor Electronics, Tel Hanan, Israel), based on settings for FUS exposure from a previous study (Ghoshal et al. 2014), and then amplified by an 2100 L RF power amplifier (ENI, Rochester, NY, USA). Ultrasound was coupled to the skin surface by placing the transducer in a specially designed holder filled with degassed water heated to 37°C. The holder consisted of a plastic bowl with a hole cut out in the bottom, which was covered with plastic wrap to act as an acoustic scanning window and the bowl filled with degassed water. The bottom of the plastic wrap was in contact with the rat skin and coupled with mineral oil.

Based on several studies, it was estimated that exposing lymphocytes to temperatures of 43–44°C for 20 min or more would result in significant lymphocyte death or dysfunction (Boreham et al. 1997; Dewhirst et al. 2003; Dieing et al. 2007). To achieve these temperature elevations in the rats at the location of the cervical lymph nodes, different exposure conditions were used in a group of rats (n = 7) not included in the EAE study in order to provide a basis for ultrasound exposure conditions in the EAE study. The transducer was positioned over the jaw of the rat, and the focus of the transducer was placed above the skin surface to heat the superficial cervical lymph nodes just under the skin surface. By placing the focus above the skin surface the diverging part of the ultrasound beam was just under the skin surface. This allowed the beam to widen and cover a slightly larger surface area just under the skin (>9 mm spot size). The superficial cervical lymph nodes exist just under skin surface as a chain of small bead-like structures (each lymph node or bead around a millimeter in diameter). The chain of the superficial cervical lymph nodes spans a distance of less than a 9 mm, allowing the whole chain on one side to be covered by the ultrasound beam. In a therapy session, animals were exposed once on the left side of the jaw for 20 min and then once on the right side for 20 min. A thermocouple was used to record the temperature near the lymph nodes during exposures, and a rectal thermocouple was used to record the body temperature during exposures. Temperature values of the thermocouple were monitored in order to keep the temperature between 43 and 44°C during the exposures. When temperatures were observed less than or greater than the desired temperature range, the ultrasound pressure levels were adjusted in an attempt to bring the temperature back into the desired range. After each therapy session, rats were allowed to recover and monitored up to day 21 post-MOG injection.

RESULTS

On average the PRS for FUS-treated animals was 1.14 ± 0.48 versus a PRS of 0.33 ± 0.27 for sham-treated animals. These differences were statistically significant (p = 0.037). The relapse rate for the FUS-treated rats was 0.57; the relapse rate for the sham-treated rats was 0.17. These differences were statistically significant. However, the relapse rate for the sham-treated animals was low because only two animals underwent a remittance during the 21-d observation period. The average rat masses at the d of MOG injection were 127 ± 15 g and 127 ± 16 g for the FUS-treated and sham-treated groups, respectively. The average rat masses at the d of euthanasia were 118 ± 20 g and 116 ± 13 g for the FUS-treated and sham-treated groups, respectively. Figure 2 shows the average EAE score for FUS-treated versus sham-treated animals. The sharp remittance in EAE symptoms after the second treatment (day 12) can be observed from Figure 2 for the FUS-treated animals. Temperatures, recorded from the rectal thermometer, were on average 33.7 ± 1.3°C and 33.8 ± 1.3°C before and after treatment, respectively, for sham-treated animals. For FUS-treated rats the average temperatures were 33.2 ± 1.8°C and 36.1 ± 1.7°C before and after treatment, respectively. This indicates that the core temperature was slightly elevated using the FUS therapy.

As can be observed from Figure 2 there was a difference in the progression of the disease after day 8 between sham-treated and FUS-treated (and before any treatments were applied). On average the progression of EAE symptoms was delayed in the shams by about a day. Two of the six sham-treated animals did not show signs of EAE until day 10 post-injection. However, to be consistent with the therapy time applications between all animals, the two sham-treated animals that had delayed EAE symptoms still underwent the sham treatment on days 9 and 12 post-MOG injection.

Table 1 provides information about the maximum EAE score reached for each animal in the study and the day post-injection when remittance of EAE score was observed, if the animal did remit. All but one of the FUS-treated animals underwent remittance during the 21-d post-MOG injection. The single FUS-treated rat (rat 7 of the FUS treatment group) that did not remit during the 21 d only reached an EAE score of one and never progressed beyond an EAE score of one. In the sham treatment group only two animals ever remitted their symptoms over the 21 d.

Figure 3 shows the time progression of the ADI for the sham-treated and FUS-treated rats. The FUS-treated rats had a decrease in the ADI following the last day of therapy (day 12), which recovered at about day 18. For

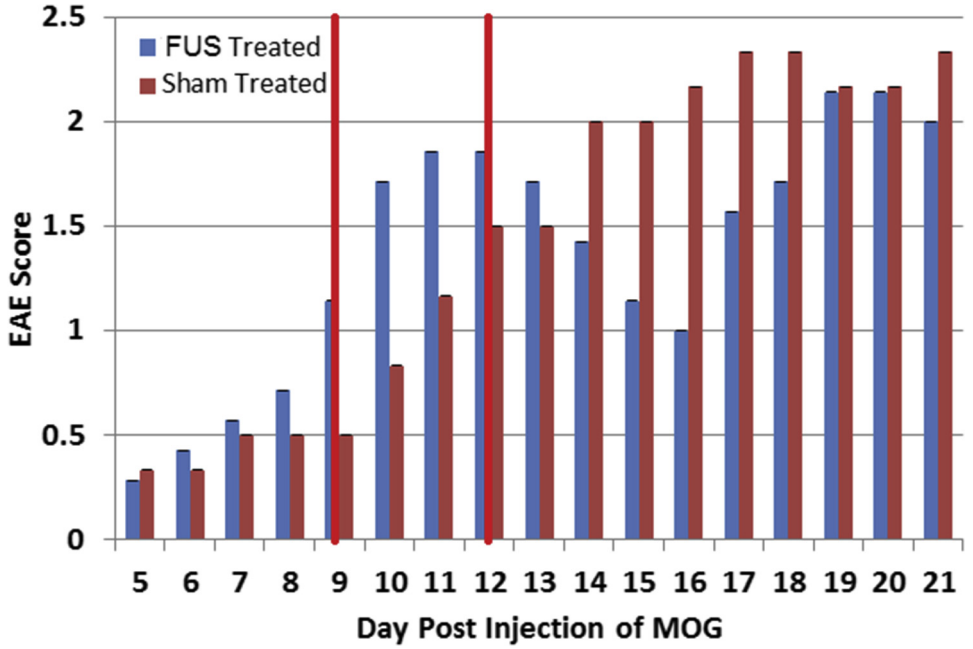

Fig. 2. Plot of the average experimental autoimmune encephalomyelitis (EAE) score versus day post-injection of MOG. Therapy was administered on days 9 and 12 and is denoted by the red bars. The graph shows the focused ultrasound (FUS)-treated rats increasing in average EAE score until the second day of treatment (day 12) where EAE score remits followed at day 17 by an increase in EAE score. The average EAE score increased for the sham-treated rats until a remittance at day 19.

the sham-treated rats, the ADI continued to increase with a short drop at day 19 and recovery at day 20. The ADI was always larger for the sham-treated rats compared to the FUS-treated rats after the day of treatment, indicating a higher disability among the sham-treated animals.

In addition, it was also noted that two of the six animals in the sham treatment group had signs of severe ataxia, a characteristic of atypical EAE brought on by cerebellar inflammation and demyelination, resulting in early euthanasia of the animals before day 21 (specifically, on day 17 and day 19). In stark contrast, no rats in the FUS treatment group developed signs of ataxia. Necropsy of euthanized animals did not provide any visible evidence that thermal coagulation of cervical lymph nodes or surrounding areas occurred due to the FUS treatments.

Table 1. List of the maximum experimental autoimmune encephalomyelitis (EAE) scores recorded for each animal in the study and associated day post-injection when a remittance in EAE symptoms was observed

Rat identification	Max EAE score recorded	Day remittance observed
FUS-1	2	14
FUS-2	1	Not observed
FUS-3	3	16
FUS-4	1	12
FUS-5	3	11
FUS-6	3	14
FUS-7	3	13
Sham-1	3	19
Sham-2	3	Not observed
Sham-3	2	Not observed
Sham-4	2	Not observed
Sham-5	2	16
Sham-6	3	Not observed

DISCUSSION

Targeted hyperthermia using FUS of the superficial cervical lymph nodes in rats with EAE resulted in a statistically significant improvement in the remittance of EAE. Two treatments using FUS were spaced 3 d apart (days 9 and 12 post-MOG injection) to reduce the load of lymphocytes residing in the cervical lymph nodes. Based on the occurrence of the remittance of the EAE score in FUS-treated rats (Fig. 2), the remittance was closely associated with the timing of the treatments. In the sham-treated animals, remittance, if it occurred, was observed at least 4 d after the last sham treatment. Therefore, the timing of the remittances of EAE also suggests that the EAE remittance was associated with the FUS therapy. Furthermore, the timing of the ADI also suggests a relationship between (or an association between) the remittance to the actual therapy. The ADI was observed to decrease from day 13 and only recovering again at day 18 post-injection in the FUS-treated rats. Whereas

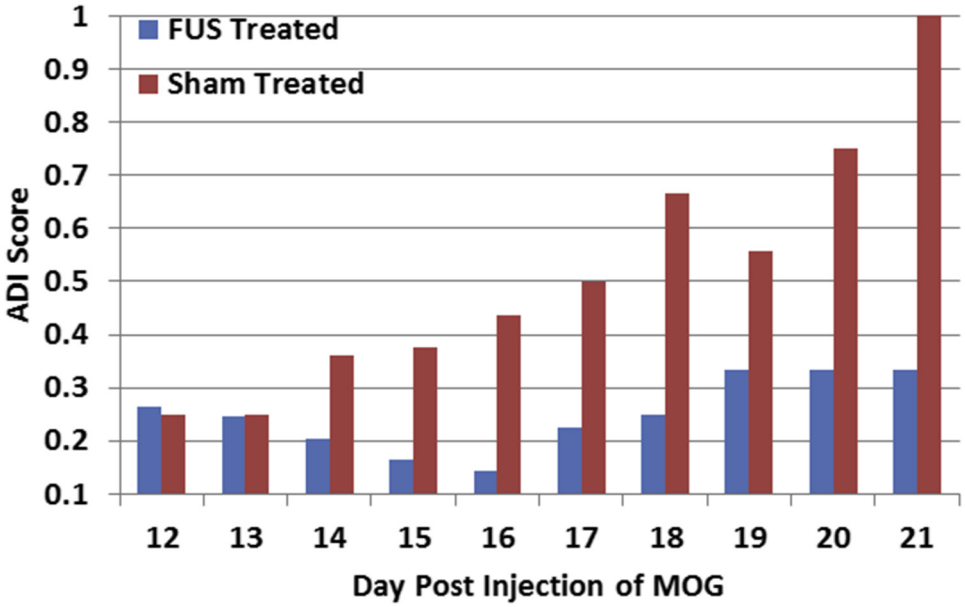

Fig. 3. Average disability index (ADI) score for focused ultrasound (FUS)-treated and sham-treated animals, which show that the ADI for the FUS-treated rats decreasing at day 13 (just after therapy) and not increasing again until day 17. The ADI for the sham-treated rats increased up to day 19.

in the sham-treated rats, the ADI increased up until day 19 and then recovered by day 20 post-injection.

The relapse rate for the FUS-treated animals (0.57) was higher than for the sham-treated animals (0.17). Only one sham-treated animal had a relapse of EAE symptoms while only two sham-treated animals of the six had remittance in their EAE score. The remittance of the sham-treated rat that did not relapse occurred at day 19 of the study, and it is possible that after day 21 the rat would have undergone a relapse. Therefore, for this study design, the relapse rate was not a good indicator of disease modification. Extending the period of observation in future studies would provide a better understanding of relapse rates for shams versus FUS-treated groups.

FUS could have been applied earlier than day 9 after the injection of MOG to test if FUS could protect against initial onset of EAE symptoms; such an application would not be consistent with human translation wherein therapy is initiated after disease onset. The decision to provide treatment after the initial occurrence of EAE symptoms was intended to mimic what might happen clinically with MS in humans. While certain factors are associated with the relapse of MS in humans, it is not always possible to predict when an MS relapse will occur or when the initial onset of MS might happen. Therefore, treatments of MS will always take place after a diagnosis of MS based on clinical symptoms.

The ultrasound therapy system was built with a single-element ultrasound source without image guidance to place the source fields (*i.e.,* target the superficial cervical lymph nodes). Targeting was accomplished by simply lining up the transducer above the skin visually where the cervical lymph nodes were known to reside in the rat. Monitoring was conducted though a small thermocouple placed only at one location. Therefore, the ultrasound therapy system was a basic system and the potential for inconsistent treatment was high. Yet, even with the limitations of the ultrasound therapy system and the limited number of animals in the pilot study, statistically significant differences in remittances of EAE (*i.e.,* PRS) were observed between FUS-treated and sham-treated animals. The FUS system can be improved through more rigorous monitoring of the transducer placement through image guidance and improved mapping of the temperature elevations during exposures.

The pilot study explored FUS exposure conditions and provided strong evidence that targeted hyperthermia of superficial cervical lymph nodes in rats with EAE using FUS ($n = 7$) improved the remittance of EAE symptoms compared to sham-treated rats ($n = 6$) with EAE. This indicated that heating of the cervical lymph nodes of rats with EAE using FUS provided preclinical evidence of a benefit. Therefore, further development, improvement and verification of this technique has the potential to affect clinical care of MS and offer a novel and safe approach to treating this debilitating disease.

This pilot study is the first ever FUS treatment of EAE in a rat model. Furthermore, the FUS technique represents a first for a non-pharmaceutical disease-modifying therapy for EAE and potentially in the future for

treating MS. In the FUS technique, only the superficial cervical lymph nodes were treated, which primarily service the CNS, thereby retaining the immune capacity provided by other lymph nodes throughout the body. The goal of our pilot study was not to ablate tissues but to elevate temperature for a defined duration to cause cell death or dysfunction of lymphocytes residing in the cervical lymph nodes while allowing for the lymph nodes themselves to survive. While not demonstrated *in vivo,* evidence suggests that tissues are more tolerant to heat than individual cells, that is, it takes a lower thermal dose to kill individual cells than tissue in the body (Dewhirst et al. 2003). Based on several studies, it was estimated that exposing lymphocytes to temperatures of 43–44°C for 20 min or more would result in significant lymphocyte death or dysfunction (Boreham et al. 1997; Dieing et al. 2007). The hypothesis was that by elevating the temperature in the cervical lymph nodes for a defined duration, the overall lymphocyte action in the CNS would be markedly reduced, resulting in mitigation or remittance of EAE severity. Further tests are needed to verify if lymphocytes are being killed or if their function is degraded due to the FUS therapy. Such tests would include immunohistological staining to quantify the presence of lymphocytes in the lymph nodes and to quantify demyelination in the brain and CNS.

Ultrasound devices to raise the temperature of tissues, such as muscle, are already used in physical therapy in the United States. We envision a future therapy applicator that positions small ultrasonic transducers around the neck of a human to produce elevated temperatures in the superficial cervical lymph nodes for a defined thermal dose to treat periods of disease activity (*i.e.,* relapses) and perhaps ongoing disease activity in MS. More immediate future directions for this work include improving the ultrasound exposure system and FUS monitoring, optimizing the exposure conditions of FUS for EAE remittance (which could include different pressure levels and number and placement of treatments) and conducting assays to quantify the functionality of lymphocytes in the cervical lymph nodes that have been exposed to therapy.

CONCLUSIONS

A novel FUS therapy hypothesized to reduce the number and function of lymphocytes that service the CNS, resulting in a remittance of the EAE symptoms, was applied to rats with EAE. Statistically significant differences in the remittance scores between FUS-treated and sham-treated animals were observed. In addition, no FUS-treated animals developed severe ataxia over the course of the disease. While the FUS system was crude (*i.e.,* without image guidance and temperature monitored only at a single location), a significant therapeutic response for EAE was observed. This technique represents the first reported FUS and non-pharmaceutical therapy approach to treating EAE and could potentially be translated to treating MS in humans.

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