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ABSTRACT :

The blood spinal cord barrier (BSCB) considerably limits the delivery and efficacy of treatments for spinal cord diseases. The blood brain barrier can be safely opened with low intensity pulsed ultrasound when microbubbles are simultaneously administered intravenously. This technique was tested on the BSCB in a rabbit model in this work. Twenty-three segments of spinal cord were sonicated with a 1MHz unfocused pulsed ultrasound device and compared to non-sonicated segments. BSCB disruption was assessed using Evan's blue dye (EBD) extravasation. Tolerance was assessed by histological analysis. An increased EBD concentration indicating BSCB disruption was clearly observed in sonicated segments compared to controls (p=0.004). On one animal, which received 10 sonications, repetitive BSCB disruptions showed no evidence of cumulative toxicity. BSCB can be disrupted using an unfocused pulsed ultrasound device in combination with microbubbles without neurotoxicity even in case of repeated sonications.

KEYWORDS:
Blood spinal cord barrier, ultrasound, blood brain barrier, spinal cord, drug delivery.

INTRODUCTION

Similarly to the blood–brain barrier (BBB), the blood–spinal cord barrier (BSCB) plays a protective and regulatory role for the spinal cord parenchyma. The BBB and BSCB are specialized passive and active structures between capillaries and parenchyma that protect the central nervous system from chronic exposition of potentially harmful substances. Only small (<400 Da), lipophilic compounds (2% of small molecules) can cross the BBB. The barrier function of spinal cord capillaries is based on tight junctions between nonfenestrated endothelial cells, together with the basement membrane, pericytes, and astrocytes. In addition, there are limited transport vesicles across endothelial cells of the BBB, thereby limiting transcellular transport(Abbott et al. 2010; Bartanusz et al. 2011; Pardridge 2005). For only a few substances, the BSCB has been shown to be slightly more permeable than the BBB (i.e cytokines) due to a lower cell junction protein expression (ZO-1 and occludin)(Bartanusz et al. 2011). Nevertheless, the BSCB impedes drug delivery for the treatment of various spinal cord disease including tumors, inflammatory disease (e.g. multiple sclerosis), neurodegenerative conditions (e.g. amyotrophic lateral sclerosis). Drugs, such as trophic factors have shown promising efficacy in preclinical animal models of spinal cord diseases but failed to translate in clinical trials (Kaspar et al. 2003). An important explanation is that, due to the BSCB, high doses that are not tolerated in humans would be required for the drug to effectively reach its target in the spinal cord.(Pardridge 2005)

Since 2001, it is known that the BBB can be safely and transiently opened using ultrasound (US) combined with intravascular systemic injection of microbubbles in pre-clinical studies(Beccaria et al. 2013; Beccaria et al. 2016; Hynynen et al. 2001). When the microbubbles pass through the ultrasound beam, they oscillate, exerting mechanical stress on the endothelium, leading to a transient increase in permeability to circulating drugs.(Burgess and Hynynen 2013; Downs et al. 2015; McDannold et al. 2005; Sheikov

et al. 2004) In a first-in-man safety and feasibility trial, it was shown that ultrasound induced BBB opening prior to carboplatin administration is well-tolerated in patients with recurrent glioblastoma.(Carpentier et al. 2016)

Our study was designed to evaluate if the BSCB could be safely disrupted using a 1 MHz unfocused pulsed ultrasound device in rabbits. BSCB disruption was quantified using Evans blue dye (EBD) extravasation. In addition, we examined if optimal acoustic parameters could be defined for sonications and evaluated the safety of multiple sonications.

MATERIAL AND METHODS

Animal protocol and surgical spinal cord exposition

Fifteen healthy New Zealand white rabbits, weighing 3.0–4.3 kg, were obtained from a research animal provider (CEGAV, SSC, France). Animals were housed with free access to food and water. All experimental procedures were approved by the Paris Descartes University Animal Ethics Committee (n° 2016061715547663) and were in accordance with the standards of the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research

Since ultrasound is absorbed and distorted by the spinal sagittal median posterior process, surgical removal of the bone was performed to insure reliable acoustic exposures. Animals were anesthetized by intramuscular injection of Xylazine (5 mg/kg-2% solution) and Ketamine (50 mg/kg-1000mg/10mL solution). If needed, additional injections were performed during the experiments. Animals were either ventilated with a mixture of isoflurane and oxygen using a laryngeal mask airway or allowed to spontaneously breathe ambient air. A catheter was inserted into the marginal ear vein

for perfusion of US-contrast agent and Evan's blue dye. The rabbit spinal cord extends from C0 to S2 (Greenaway et al. 2001). Spinal cord surgical exposition for ultrasound exposures and controls could be performed at multiple levels (T4, T7, T11 and L3 vertebras). On each level, the skin was infiltrated with a 1% lidocaine solution and laminectomy was performed with a Citelli punch to expose the dura-mater and allow the positioning of the ultrasound transducer. Two hours after the last sonication, animals were sacrificed by intravenous injection of pentobarbital salt (182 mg/100 mL). Laminectomies were then extended to extract the entire spinal cord for analysis of sonicated vs non-sonicated segments.

In silico simulations of the acoustic pressure distribution

From previously performed experiments in rabbit brain at our institution (Beccaria et al. 2013), we tested the nominal acoustic pressure to use for safe BBB opening. In this work, we hypothesized that similar acoustic pressure levels would be safe for BSCB opening. Nevertheless, due to the close proximity of vertebral body anterior to the spinal cord, ultrasound reflection could happen, amplifying the signal intensity. To quantify this phenomenon, *in silico* treatment simulations were performed *(Figure 1)*. The nominal treatment pressure amplitude delivered during treatments *(Table 1)* is defined as the pressure that would have been delivered in non-attenuative free-field conditions (water). However, the presence of distal bone in the acoustic field causes reflections and standing waves. To evaluate the actual spatial acoustic pressure distribution in the spine obtained for a given nominal pressure, simulations were conducted using a finite-difference time-domain wave propagation numerical model (Bouchoux et al. 2012). Three dimensional maps of the acoustic properties of heterogeneous media, including bone, were obtained using X-ray computed tomography scans of the C6, T4, T9, L2 vertebra of a New Zealand white rabbit. The scans were

segmented in three domains assigned with typical acoustic properties: soft tissues (speed of sound 1588 m/s, density 1000 kg/m^3, attenuation 7 Np/m), bone (3500 m/s, 2000 kg/m^3, 55 Np/m), and acoustically transparent gel (1524 m/s, 1000 kg/m^3, 0 Np/m).(Duck 2013) The laminectomy was modeled by removing an 8 mm-diameter cylinder of bone in front of the transducer. The simulated acoustic pressure distribution during the ultrasound exposure was evaluated in a 7-mm long region of interest (ROI) inside the targeted vertebra.

Ultrasound sonication setup

The ultrasound exposure parameters used in this study (25 ms pulse duration, 1 Hz pulse repetition frequency, 1.1 MHz ultrasound frequency, 150 s treatment duration) and the ultrasound transducer geometry (1-cm diameter piezocomposite, Vermon, France) were chosen to match the device used in a clinical trial for BBB disruption by our group (Carpentier et al. 2016). The ultrasound transducer was coupled to the exposed spinal cord through a 2-cm thick 1.6-cm diameter cylinder made from an acoustically transparent gel (Aquaflex, Parker Laboratories, NJ, USA). The gelpad was used to ease the positioning of the transducer, and to place targeted tissues in the acoustic far field of the 1 MHz unfocused transducer (>15 mm) to have a more uniform acoustic field at the intended sonication site. A polyisoprene envelope (Ultracover 88663, Microtek, MS, USA) covered the transducer and the gel pad to guarantee sterility. Acoustic gel was used to couple the transducer to the gel pad, the gel pad to the envelope, and the envelope to the targeted tissues.

The transducer was driven by a custom radiofrequency generator (CarThera, France) comprising a signal generator, a radio-frequency amplifier, an electrical power measurement system, and an electrical matching circuit. The complete setup was calibrated by placing the transducer/gel assembly in a degassed water tank at ambient

temperature and evaluating the relation between the electrical power and the acoustic pressure amplitude at the spatial peak measured (i.e. the nominal pressure, following our definition) with a hydrophone (HNC0200, Onda, CA, USA) mounted on a motorized positioning system (UMS, Precision Acoustics, UK). Simulated (1cm diameter piston) and measured acoustic field spatial distribution were almost identical: maximum pressure in free field, ie. nominal pressure, was measured at 15.4 mm from the transducer, vs. 15.5 mm obtained by simulation.

The nominal acoustic pressure varied from 0.3 MPa to 0.8 MPa according to the different experiments (*Table 1)*. Right at the beginning of each sonication, a bolus of 0.2 ml/kg of microbubbles (SonoVue, Bracco Imaging) was intravenously injected. As sonications were performed on each rabbit at different spinal positions, a delay of at least 15 minutes was used between each sonication to ensure washout of the contrast agent.

Evans Blue Dye (EBD)

BSCB disruption was measured by extravasation of EBD. After the last sonication, EBD was injected intravenously at a dose of 100 mg/kg in 60 mg/mL of saline (Beccaria et al. 2013). Animals were sacrificed 120 min after the dye injection. The spinal cord was immediately extracted and the meninges were discarded.

Qualitative macroscopic evaluation (visual blue staining intensity) was performed on the whole spinal cord and on axial slices with respect to sonication location versus non sonicated areas. Transversal slices (3 mm long) were stored at -20°C until subsequent analysis.

Quantification of EBD was performed by UV-visible spectrophotometry using a Anthos *Zenyth 340 Microplate* Reader (Biochrom, Holliston, USA). The pharmacologist

performing the analysis (L.G) was blinded to the sonication levels. After thawing, spinal cord samples were weighed. Calibration standards and quality controls were spiked with appropriate dilutions of EBD (0 to 120 µg/mL). All samples were mixed with 100 µl of sodium chloride 0.9% (Fresenius Kabi France) and 750 µl of dimethylsulfoxide (Sigma-Aldrich, St. Louis, Missouri, United States). The mixture was then homogenized and centrifuged at 4,000 rpm for 10 min at 25°C. Absorbance in the supernatant due to EBD was measured using UV-visible spectrophotometry at a wavelength of 620 nm. Data *(table 1)* were expressed as EBD tissue concentration per wet tissue weight (µg/g). Sonicated spinal cord segments were compared to controls (non-sonicated segments).

Histological Study

Segments of the spinal cords were fixed in a 10% formalin solution for histological analysis. The neuropathologist (F.B.) was blinded to the US parameters. Axial sections of the spinal cord were embedded in paraffin. Three micron-thick sections were cut and stained by hematoxylin and eosin (H&E). Lesions were graded according to the classification proposed by Hynynen et al.(Hynynen et al. 2005) on a scale ranging from 0 to 3 : "0" : no detected damage, "1" : few erythrocytes extravasation, "2" : petechial hemorrhages; mild damage to the parenchyma, and "3" : hemorrhagic or non-hemorrhagic local lesions.

Repeated sonications and neurological evaluation

Repeated (x10) sonications were performed in one rabbit on the same spinal cord segment (D4). This rabbit had only one site of laminectomy, the skin was sutured and the rabbit was woken up. A 10-day interval was kept between surgery and sonications to evaluate the toxicity of the surgery independently of ultrasound. For sonications,

which were all performed the same day, the rabbit was anesthetized breathing ambient air, the scar was reopened and five sonications were performed at the D4 segment with a 20-minute interval between each sonication. The rabbit was woken up to allow clinical evaluation. A new anesthesia was performed, and five additional sonications were realized. Evan's blue dye was injected and the rabbit was sacrificed 120 minutes later.

For assessment of neurological function, a modified Tarlov scale was used (0 : no movement of the hind limbs, 1 : perceptible joint movement, 2 : good movement of the joints but inability to stand, 3 : ability to stand and walk, 4 : complete recovery = ability to hop (Tarlov 1972). Proprioception was evaluated by observing the placement of the limbs when brought to table edge. (Mancinelli 2015) Evaluation was performed before laminectomy, daily after laminectomy and 4 hours after the 5th sonication.

Statistical analysis

Statistical analysis of blue dye quantification results was performed using Graph Pad Prism 5.0 software. D'Agostino & Pearson omnibus normality test was used to determine normality of the data. To compare multiple groups, one way ANOVA with Tukey post-test was used for normal data, and Kruskal Wallis with Dunn's post test for non-parametric data. To compare two non parametric groups, Mann Whitney test was used. P-value <0.05 was considered significant. Results are presented as means ± standard error of the means (SEM).

RESULTS

Simulated acoustic pressure field in the targeted tissues

The simulated pressure field for an ultrasound exposure of a rabbit T4 vertebra after laminectomy through a 2-cm thick acoustically transparent gelatin pad is shown in *Figure 1*. As expected, an interference pattern typical for standing waves is clearly visible, with the

distance between stripes at about 0.8 mm (half of the wavelength). Reflection by the bone causes a heterogeneous pressure distribution in the targeted zone, including hotspots with acoustic pressure above the nominal pressure, which is defined as the maximum pressure amplitude measured in free-field conditions. Simulations for other vertebras (C6, T4, T9, L2) show similar pressure field patterns. Overall, 34±6% of the spinal canal ROI volume (mean±standard deviation for the 4 evaluated vertebras) received an acoustic pressure of 0-0.5x the nominal pressure, 47±5% between 0.5-1x, 17±3% between 1-1.5x, and 2%±1% above 1.5x. For comparison, if ultrasound reflection and attenuation are neglected (free-field condition), 48% of the spinal-canal ROI volume would have a pressure between 0-0.5x the nominal pressure and 52% of the ROI is exposed with a pressure between 0.5-1x the nominal pressure.

Additional simulations were performed to evaluate a sonication through the bone, without laminectomy. The median pressure in the ROI was divided by a factor of 3.34±0.8 because of the bone in the acoustic path. The standing wave pattern in the spinal canal was similar to the one observed with a laminectomy.

BSCB Opening

Qualitative Macroscopic analysis showed blue colorations of the sonicated segments of the spinal cord by the EBD demonstrating the localized disruption of the BSCB *(Figure 2).* Non-sonicated spinal cord segments (controls) remained white. Similar to the effects previously observed in the brain, this coloration is more prominent in the grey matter (Beccaria et al. 2013)*(Figure 2, lower).* In sagittal sections, the blue coloration of the grey matter corresponded to the length of the acoustic field but was not always observed in white matter. In some samples, the coloration was unilateral, due to the orientation of the transducer. When

a surgical trauma occurred, (#4, 8, 12, 30) an intense coloration was observed in both white and grey matter associated with macroscopic hemorrhage. These four segments were excluded from all the statistical efficacy analysis. In rabbits ventilated with isoflurane and oxygen a blue coloration was seen in 3/8 segments. In this group, the blue was much less intense and more limited to the grey matter than in the ambient air group. The blue coloration was observed in 11/11 sonicated segments in rabbits ventilated with ambient air including segments with low acoustic pressure (0.3MPa).

Quantitative spectrophotometric measurements of EBD concentration revealed a statistically significant difference in EBD concentrations between the 19 sonicated samples (51.4 ± 6.1 µg/g) and the 7 controls (15.9 ± 1.8 µg/g), *p=0.0004*.(Mann Whitney test) The 11 segments of sonicated rabbits allowed to breath ambient air had a higher BSCB disruption (69.8 ± 5.6 µg/g) compared to the 8 segments of rabbits ventilated with isoflurane/oxygen (26.3 ± 2.5 µg/g), *p=0.006*. (Kruskall Wallis test – Dunn-s multiple comparaison post test) (*Figure 3).* When analyzing the segments with no or minimal histological lesions (*Grade 0-1),* the EBD concentration of the 13 sonicated segments was increased when compared to the five controls (44.8 ± 7.3 vs 15.8 ± 2.4 µg/g, *p=0.004, Mann Whitney test).* In segments with no or minimal histological lesions in rabbits breathing ambient air, EBD was even more increased (67.5 ± 8.6, vs 15.8 ± 2.4 µg/g) compared to reabbits breathing isofluran-oxygen. None of the control segments had a BSCB disruption.

Surgical exposition and ultrasound Toxicity

Histological toxicity was evaluated on 23 sonicated segments and seven non sonicated segments (controls) (*Table 1, figure 4*). In the control segments, 2/7 (29%) had moderate or severe lesions (Grade 2-3) due to surgical trauma. In the sonicated segments, 10/23 (43%) had moderate or severe lesions (grade 2-3). Four segments (#4,8,12,30) had severe lesions (Grade

3), macroscopically visible, due to the surgical procedure or ultrasound toxicity. Moderate damage (*Grade 2)* was observed in 6/23 (26%) segments in the sonicated group, and in 2/7(29%) segments of the control group.

Repeated sonication and clinical evaluation

The rabbit that received 10 sonications in the same spinal segment had a clinical evaluation before and after the first five sonications. No neurological worsening was observed (Tarlov modified scale 4/4) after five sonications when compared to the neurological status after laminectomy and before ultrasound. After sonications, the rabbit was able to hop (Tarlov modified scale 4/4) and had no proprioceptive defect four hours after five sonications. After a total of 10 sonications, EBD was injected and the animal was sacrificed. There was a clear opening of the BSCB (EBD : 95µg/g) with minimal histological lesions (extravasation of less than 20 erythrocytes - Grade 1).

DISCUSSION

This study is a proof of concept that the BSCB can be safely disrupted using a 1 MHz unfocused ultrasound device coupled with microbubbles. The optimal acoustic pressure (0.3-0.5MPa) is slightly lower to that used for BBB disruption in rabbits (0.5-0.6MPa).

Difficulties with the Rabbit Model

Simulations without laminectomy showed that propagation of the 1-MHz ultrasound wave through the bone causes a reduction of acoustic pressure by a factor 3.34±0.8. The very large standard deviation of this factor is due to the variability of acoustic transmission through the 4 vertebrae evaluated in silico, and suggests a large uncertainty of the in-situ acoustic pressure without laminectomy. Moreover, compensating for acoustic pressure loss due to transmission through the bone would likely cause mechanical and thermal side effects in intervening

tissues. The simulations demonstrated that the bone thickness of the spine impedes ultrasound from reaching adequately the spinal cord, and prerequisite laminectomies were mandatory. Such laminectomies encountered several difficulties in this specific animal: highly hemorrhagic dissection of paravertebral muscles, narrowness of the spinal canal leading to possible severe spinal cord traumas during the surgical exposure (n = 4/30), and hemorrhagic lesions even on non-sonicated spinal cord segments. Larger series of animals were envisioned initially, but it appeared that the rabbit is a difficult model for spinal cord studies. In addition, due to ethical considerations for reducing the number of animals, each rabbit had multiple sites of sonication. Multiple sonication sites increased the total time of anesthesia and blood loss leading to acidosis (arterial pH often below 7 at the end of the procedure) and hyperkalemia (>7.5mmol/L). Three rabbits died of hemorrhagic shock or metabolic complication. Lastly, for the neurological evaluation, rabbits had to be kept alive after their laminectomy. Five rabbits initially received surgery for neurological evaluation but four developed a paraplegia seven to ten days after laminectomies, before any sonication due to spine instability. Only one had normal neurological status at 15 days after laminectomy and could be used for the ultrasound sonications.

Impact of anesthesia

Rabbits were first anesthetized with isoflurane and oxygen to limit hypoxemia. In such cases, the BSCB was poorly (26 ± 2.5µg/g) opened, and apart from surgical traumas, only one segment (#7) had a clear opening >30µg/g. Previous studies have shown that anesthesia agents influence the blood brain barrier disruption (McDannold et al. 2011). Isoflurane and oxygen are known to decrease the effect of the microbubbles on the blood brain barrier. This effect can be explained by vasoactive effects (including vasoconstriction, vasodilatation, blood flow) and decreases in microbubble circulation times.

In the second part of the experiment, we changed the ventilation method, letting rabbits spontaneously breath ambient air. With this procedure, results were much reliable. BSCB disruption was systematic (11 segments/11) and the EBD concentration was higher (70 ± 5.6 vs 26 ± 2.5 µg/g in the isoflurane/oxygen group). The difference of Evan's blue dye concentration was highly significant between these two methods of anesthesia *(p=0.0004)*

Evans blue dye

BSCB opening was confirmed by the uptake of EBD in the sonicated segments. A blue coloration of the spinal cord was observed on the sonicated segments. There was no visible leakage of coloration on non-sonicated segments confirming the BSCB's impermeability to this dye. This macroscopic evaluation was completed and confirmed by quantitative evaluation. The EBD concentration was significantly higher in the sonicated segments compared to controls (51.4 ± 6.1 vs 15.9 ± 1.8 µg/g *p=0.0004).* Evan's blue dye is a molecule with a very high affinity for albumin, thus once injected, it binds with albumin forming a 68 kDa complex which is a good model for protein permeability(Hoffmann et al. 2011; Wunder et al. 2012). In our previous results in the brain,(Beccaria et al. 2013) EBD was reliable in evaluating the BBB opening when compared to gadolinium enhancement. Indeed, just like the EBD, the gadolinium (1 kDa) uptake was limited to the sonicated location and predominate largely in the grey matter.

Comparison to the blood brain barrier disruption

An emitted acoustic pressure of between 0.3-0.4 MPa was found to be safe for disrupting the BSCB in rabbits. This pressure is slightly lower than the optimal acoustic pressure for the blood brain barrier (0.6 MPa) in a similar animal model (Beccaria et al. 2013; Beccaria et al. 2016). This difference could be due to the reflection of the ultrasound wave on the opposite

bone, which locally increases the acoustic pressure compared to the nominal treatment pressure as shown by the simulations presented in *Figure 1*.

Sonicated segments had an EBD concentration 4.4 higher than controls (69.8 vs 15.9 µg/g). This difference is similar to the one we previously found for the blood brain barrier: 4.1 higher (26.9 vs 6.5 µg/g).(Beccaria et al. 2013). Other parameters such as pulse duration, pulse repetition frequency or ultrasound frequency were not changed compared to our previous studies on the brain.

Tolerance of BSCB disruption by ultrasound

The acoustic intensity pattern due to standing waves predicted by simulation was not observed when examining the sonicated rabbits in EBD staining. The spatial distribution of petechia and hemorrhages, when observed, was also not in a striped pattern. In fact, despite the heterogeneity of the calculated acoustic field, the EBD distribution in tissues was fairly homogeneous. This could be due to diffusion of the dye in tissues, to the heterogeneity of tissues, or to the small motions of the hand-held transducer and of the animal during ultrasound exposures, which could spread the standing-wave pattern over the treatment duration. Due to the surgical traumas mentioned above, histological safety was delicate to evaluate. Histological lesions of grade 2-3 occurred in both sonicated segments and controls, and so could be attributed primarily to surgical damages, without exclusion of potential adverse events induced by ultrasound in the sonicated segments.

Still, seven segments had a significant BSCB disruption with no or minimal histological lesions.

Tolerance of repetitive sonications

The rabbit that received five sonications in the same spinal cord segment presented no neurological adverse events. Moreover, histological analysis after 10 sonications performed the same day did not show any secondary effects. Although these data were only in one animal, it represents the first report on clinical and histological tolerance after several spinal cord sonications in an animal model. Indeed, we performed surgery on four other animals but they became paraplegic before the planned sonication due to spinal instability after laminectomy. Ethically it was difficult to add more animals. Further studies will be performed in a different animal model. Nevertheless, this data on one animal is of importance and shows the promise of this technique for treatment of spinal cord diseases. Since repetitive, monthly BBB opening has been shown to be safe in humans (Carpentier et al. 2016) it is hypothesized that this technique may also be safe for repetitive drug delivery to the spine.

Existing literature

To our knowledge, there are three reports of ultrasound-mediated BSCB disruption in the literature. These three experiments used focused ultrasound devices. The first publication involved gene delivery to the spinal cord in a rat model (Weber-Adrian et al. 2015). Ultrasound was generated using a 1.114 MHz transducer for 5 minutes, with 10 ms bursts, at a repetition rate of 0.5 Hz and acoustic power of 0.73W. In this study, after one sonication, gene delivery of green fluorescent protein using a viral vector (20 nm) was increased in motor neurons (87% expression in sonicated regions vs 28% without ultrasound), and oligodendrocytes (36% vs 7%). Clinical tolerance was proven and histology at 13 days post sonication reported no toxicity.

The second study of BSCB disruption was also in a rat model.(Payne et al. 2017) BSCB disruption was assessed by measurement of gadolinium enhancement on magnetic resonance imaging (MRI). BSCB disruption was shown in 10/12 rats using contrast-enhanced MRI.

Three rats also had qualitative evaluation with Evan's Blue Dye. A correlation between Evan's Blue Dye and gadolinium was observed.

The third study evaluated the potential benefit of BSCB disruption on a rat model of leptomeningeal metastasis of breast cancer. (O'Reilly et al. 2018) In this study, BSCB disruption was obtain with acoustical pressure similar to those of our study (0.4MPa).

Future developments

Although surgical laminectomies are easy and routinely performed on humans without complications, further animal experiments will be performed on a different animal model such as mice, to avoid surgical laminectomy. In addition, several pathological models mimicking human diseases exist in mice for various neurological diseases such as amyotrophic lateral sclerosis.

In humans, the vertebral bone is thicker and more irregular than the skull which makes the trans bone passage of ultrasound very complex and hazardous. Unfocussed ultrasound could be delivered to the spinal cord with an implantable device placed directly in contact with the spinal cord.

CONCLUSION

The first safety data in a rabbit model of blood spinal cord barrier disruption using a 1 MHz unfocused ultrasound device was shown. Although the rabbit model, which required a narrow spinal canal surgical exposure induced spinal cord trauma in some animals, the BSCB opening procedure appeared to be safe in other animals, both clinically and histologically, even when repeated 10 times on the same location. Further research will be performed on mice to avoid surgical exposure and address the therapeutic potential in pathological models.

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DISCLOSURE

Sorbonne University and APHP (Alexandre Carpentier is an employee) submitted a patent application on this method in 2016.

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FIGURE CAPTIONS

Figure 1 : **Simulated acoustic field in the T4 vertebra of a 1 kg rabbit after laminectomy**

Legend: *A) Typical layout used for the simulations (T4 vertebra of a 1 kg rabbit): CT scan used to determine acoustic properties and geometry of bone (grayscale axial and transverse images), tissues are replaced by acoustic coupling gel in the hatched zone to model laminectomy and in-vivo experimental conditions. B) Simulated in-situ acoustic pressure (color overlay). C) Axial profile of acoustic pressure simulated for free-field and in-situ conditions; pressures are normalized by the maximum in the free-field. D) Geometry of the simulated free-field (water)*

Figure 2: Macroscopic analysis of EBD distribution

Legend: *Upper panel : external view of the spinal cord. Blue coloration is observed on the three sonicated segments of the spinal cord (arrows). Lower panel : on transverse section of the sonicated segment #22 (right), the coloration predominates on the grey matter. No macroscopic blue coloration is visible on the non sonicated segment. (left)*

Figure 3: EBD concentration and histological status according to the different procedures

Legend: Evans blue dye concentration (µg/g) in the control segments (no sonication), in the sonicated segments in rabbits anesthetized with isoflurane and oxygen and in the rabbits anesthetized with ambient air. Severe histological lesions (Grade 3) have been excluded from statistical analysis and this figure because they resulted in artificially high EBD concentrations. Moderate histological lesions (Grade 2) can be found in all groups due to the model fragility and surgical exposure. Clear BSCB opening was observed without

histological lesions. Performing the procedure with isoflurane and oxygen significantly decreased the BSCB opening. Data are presented as mean with SEM.

Figure 4: Histological aspects

A-H Hemalun & eosin staining. A,C,E,G 20X magnification. Scale bar: 0,5mm. B,D,F,H 200X magnification. Scale bar 0,1mm. A,B. Absence of damage corresponded to grade 0. Erythrocytes are intravascular (arrowhead in B). C,D. Few extravasated erythrocytes are observed in grade 1 (arrowhead in C). E,F. Microscopic extravasation of erythrocytes is observed in grade 2 (arrowhead in E,F). G,H. Extensive hemorrhagic lesions are observed in grade 3 (arrowheads in G,H).

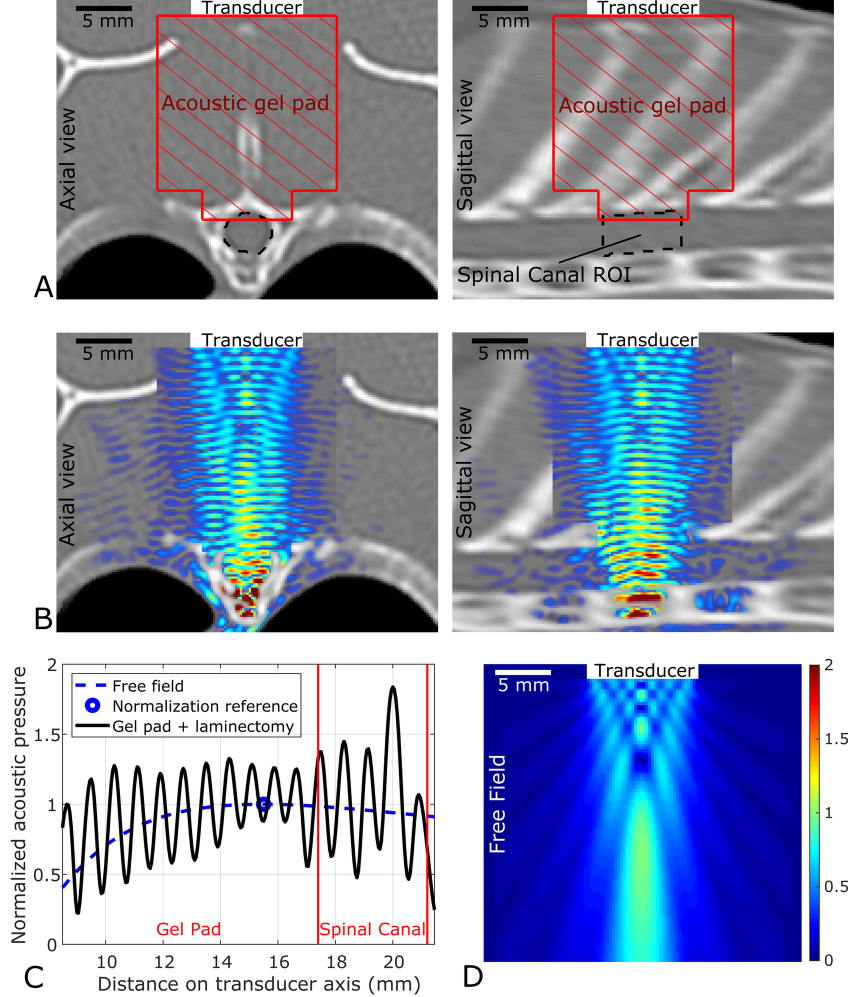
TABLE

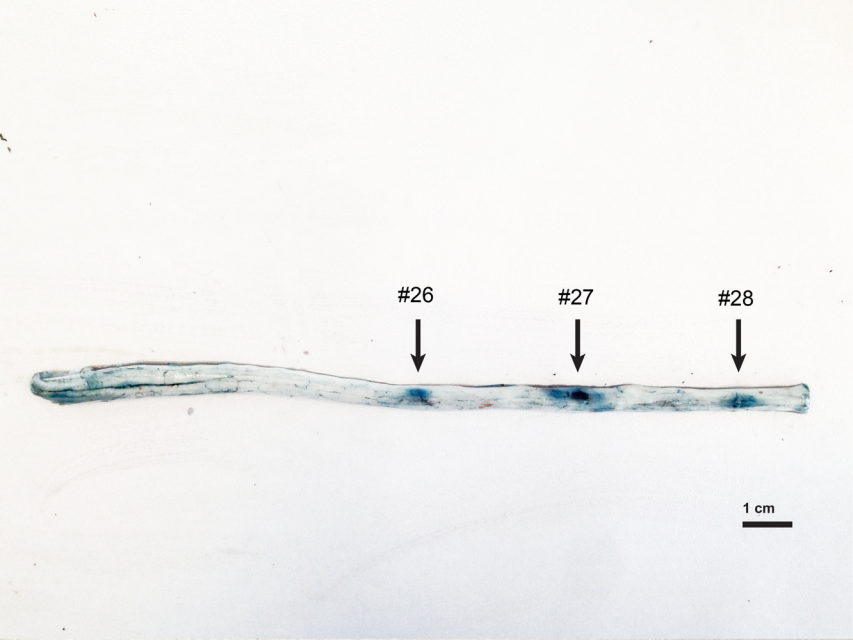
Table 1: experimental set up and results

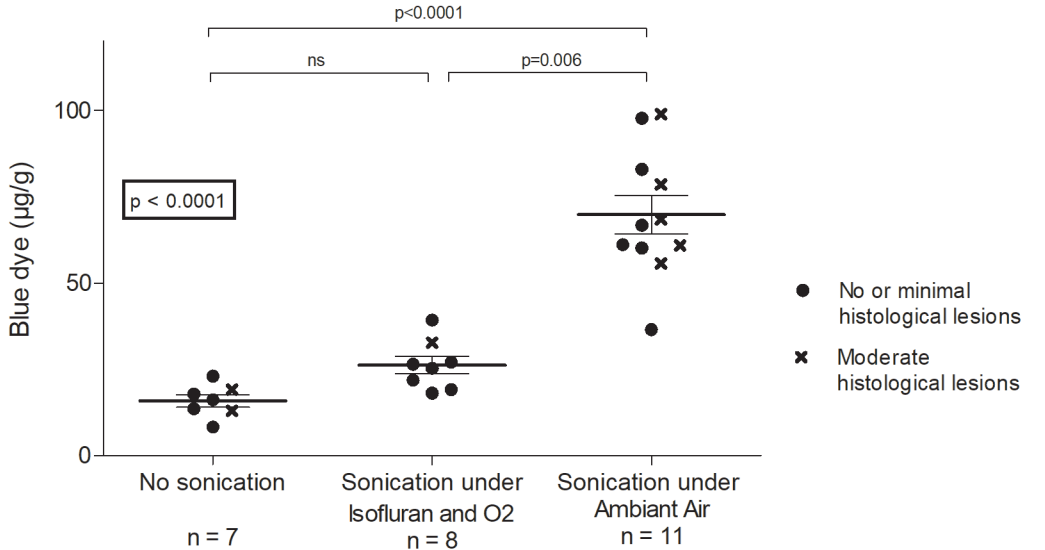
Ventilation	Sonicated Spinal cord segment Identification Nb.	Acoustic pressure (MPa)	Macroscopic Blue stain	Evan's Blue Dye (µg/g)	BSCB opening	Histology (Hynynen grade)
Oxygen - Isofluran	# 1	0 MPa (Control)	No	13,6	No	1
	# 2		No	17,9	No	0
	# 3		No	8,3	No	1
	# 4	0,4 MPa	Yes	76,2	Yes	3*
	# 5	0,5 MPa	No	26,5	No	0
	# 6		Yes	27,1	No	0
	# 7		Yes	39,3	Yes	0
	#8		Yes	83,3	Yes	3*
	#9		No	18,1	No	0
	#10		No	19,1	No	0
	#11		No	25,3	No	0
	#12		Yes	169,3*	Yes	3*
	#13	0.6 MPa	No	21.9	No	1
	#14	0,8 MPa	Yes	32,7	Yes	2
Ambient Air	# 15	0 MPa (Control)	No	19,1	No	2
	# 16		No	16,2	No	0
	# 17		No	13,0	No	2
	# 18		No	23,0	No	1
	# 19	0,3 MPa	Yes	82,9	Yes	0
	# 20		Yes	97,7	Yes	0
	# 21	0,35 MPa	Yes	98,9	Yes	2
	# 22		Yes	61,1	Yes	0
	# 23		Yes	55,7	Yes	2
	# 24	0,4 MPa	Yes	78,5	Yes	2
	# 25		Yes	36,5	Yes	0
	# 26		Yes	60,1	Yes	0
	# 27		Yes	66,7	Yes	1
	# 28		Yes	60,9	Yes	2
	# 29		Yes	68,4	Yes	2
	# 30	0,5 MPa	Yes	163,9*	Yes	3*
	# 31	0,4 MPa. 10 sonications	Yes	95,0	Yes	1

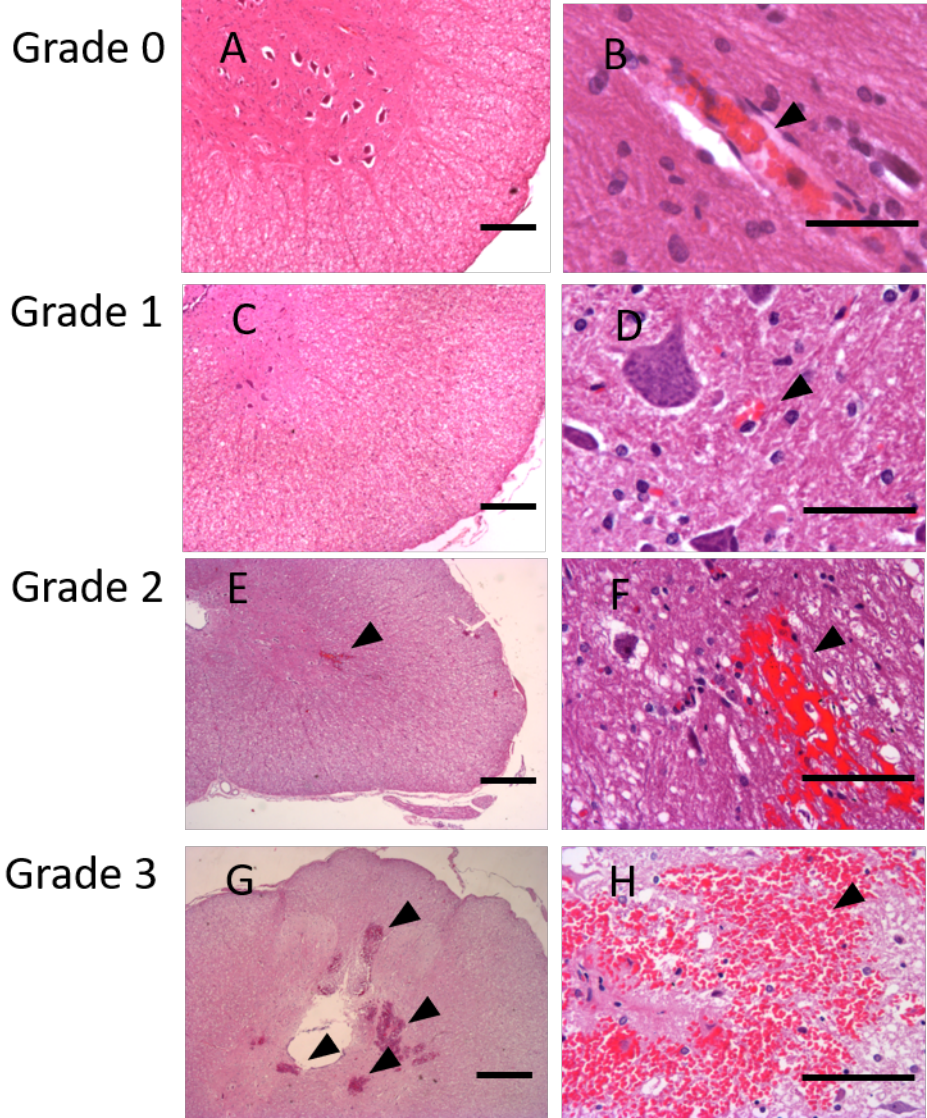
In total, 23 segments had sonications and were compared to 7 controls. Sonications #4 to 14 were performed *on rabbits breathing a mixture of isofluran and oxygen while sonications #19 so #30 were with ambient air. 4 segments(*#4#8#12#30) *had severe histological lesions, probably due to surgical trauma. These lesions resulted in high level of EBD. These data(*)*

were excluded for statistical analysis. BSCB was determined to be opened when EBD concentrations exceeded 30μg/g.


5 mm
Transducer
Acoustic gel pad
Axial view
Sagittal view
Spinal Canal ROI
A
B
Free field
Normalization reference
Gel pad + laminectomy
Normalized acoustic pressure
Gel Pad
Spinal Canal
Distance on transducer axis (mm)
C
Free Field
D


#26
#27
#28
1 cm


p<0.0001
ns
p=0.006
100
50
0
Blue dye (µg/g)
p < 0.0001
No sonication
n = 7
Sonication under Isofluran and O2
n = 8
Sonication under Ambiant Air
n = 11
No or minimal histological lesions
Moderate histological lesions


Grade 0
A
B
Grade 1
C
D
Grade 2
E
F
Grade 3
G
H