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Ultrasound-Induced Blood–Spinal Cord Barrier Opening in Rabbits

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

17 **KEYWORDS:**

18 Blood spinal cord barrier, ultrasound, blood brain barrier, spinal cord, drug delivery.
19
20

1 INTRODUCTION

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

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

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

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

12 MATERIAL AND METHODS

13 Animal protocol and surgical spinal cord exposition

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

20 Since ultrasound is absorbed and distorted by the spinal sagittal median posterior
21 process, surgical removal of the bone was performed to insure reliable acoustic
22 exposures. Animals were anesthetized by intramuscular injection of Xylazine (5 mg/kg-
23 2% solution) and Ketamine (50 mg/kg-1000mg/10mL solution). If needed, additional
24 injections were performed during the experiments. Animals were either ventilated with
25 a mixture of isoflurane and oxygen using a laryngeal mask airway or allowed to
26 spontaneously breathe ambient air. A catheter was inserted into the marginal ear vein

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

10 **In silico simulations of the acoustic pressure distribution**

11 From previously performed experiments in rabbit brain at our institution (Beccaria et al.
12 2013), we tested the nominal acoustic pressure to use for safe BBB opening. In this
13 work, we hypothesized that similar acoustic pressure levels would be safe for BSCB
14 opening. Nevertheless, due to the close proximity of vertebral body anterior to the
15 spinal cord, ultrasound reflection could happen, amplifying the signal intensity. To
16 quantify this phenomenon, *in silico* treatment simulations were performed (*Figure 1*).
17 The nominal treatment pressure amplitude delivered during treatments (*Table 1*) is
18 defined as the pressure that would have been delivered in non-attenuative free-field
19 conditions (water). However, the presence of distal bone in the acoustic field causes
20 reflections and standing waves. To evaluate the actual spatial acoustic pressure
21 distribution in the spine obtained for a given nominal pressure, simulations were
22 conducted using a finite-difference time-domain wave propagation numerical model
23 (Bouchoux et al. 2012). Three dimensional maps of the acoustic properties of
24 heterogeneous media, including bone, were obtained using X-ray computed tomography
25 scans of the C6, T4, T9, L2 vertebra of a New Zealand white rabbit. The scans were

1 segmented in three domains assigned with typical acoustic properties: soft tissues
2 (speed of sound 1588 m/s, density 1000 kg/m³, attenuation 7 Np/m), bone (3500 m/s,
3 2000 kg/m³, 55 Np/m), and acoustically transparent gel (1524 m/s, 1000 kg/m³, 0
4 Np/m). (Duck 2013) The laminectomy was modeled by removing an 8 mm-diameter
5 cylinder of bone in front of the transducer. The simulated acoustic pressure distribution
6 during the ultrasound exposure was evaluated in a 7-mm long region of interest (ROI)
7 inside the targeted vertebra.

8 **Ultrasound sonication setup**

9 The ultrasound exposure parameters used in this study (25 ms pulse duration, 1 Hz
10 pulse repetition frequency, 1.1 MHz ultrasound frequency, 150 s treatment duration)
11 and the ultrasound transducer geometry (1-cm diameter piezocomposite, Vermon,
12 France) were chosen to match the device used in a clinical trial for BBB disruption by
13 our group (Carpentier et al. 2016). The ultrasound transducer was coupled to the
14 exposed spinal cord through a 2-cm thick 1.6-cm diameter cylinder made from an
15 acoustically transparent gel (Aquaflex, Parker Laboratories, NJ, USA). The gel pad was
16 used to ease the positioning of the transducer, and to place targeted tissues in the
17 acoustic far field of the 1 MHz unfocused transducer (>15 mm) to have a more uniform
18 acoustic field at the intended sonication site. A polyisoprene envelope (Ultracover
19 88663, Microtek, MS, USA) covered the transducer and the gel pad to guarantee
20 sterility. Acoustic gel was used to couple the transducer to the gel pad, the gel pad to the
21 envelope, and the envelope to the targeted tissues.

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

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

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

14 **Evans Blue Dye (EBD)**

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

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

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

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

11 **Histological Study**

12 Segments of the spinal cords were fixed in a 10% formalin solution for histological
13 analysis. The neuropathologist (F.B.) was blinded to the US parameters. Axial sections
14 of the spinal cord were embedded in paraffin. Three micron-thick sections were cut and
15 stained by hematoxylin and eosin (H&E). Lesions were graded according to the
16 classification proposed by Hynynen et al.(Hynynen et al. 2005) on a scale ranging from
17 0 to 3 : “0” : no detected damage, “1” : few erythrocytes extravasation, “2” : petechial
18 hemorrhages; mild damage to the parenchyma, and “3” : hemorrhagic or non-
19 hemorrhagic local lesions.

20 **Repeated sonications and neurological evaluation**

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

1 which were all performed the same day, the rabbit was anesthetized breathing ambient
2 air, the scar was reopened and five sonications were performed at the D4 segment with a
3 20-minute interval between each sonication. The rabbit was woken up to allow clinical
4 evaluation. A new anesthesia was performed, and five additional sonications were
5 realized. Evan's blue dye was injected and the rabbit was sacrificed 120 minutes later.

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

12 **Statistical analysis**

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

20 **RESULTS**

21 **Simulated acoustic pressure field in the targeted tissues**

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

1 distance between stripes at about 0.8 mm (half of the wavelength). Reflection by the bone
2 causes a heterogeneous pressure distribution in the targeted zone, including hotspots with
3 acoustic pressure above the nominal pressure, which is defined as the maximum pressure
4 amplitude measured in free-field conditions. Simulations for other vertebrae (C6, T4, T9, L2)
5 show similar pressure field patterns. Overall, $34\pm 6\%$ of the spinal canal ROI volume
6 (mean \pm standard deviation for the 4 evaluated vertebrae) received an acoustic pressure of 0-
7 0.5x the nominal pressure, $47\pm 5\%$ between 0.5-1x, $17\pm 3\%$ between 1-1.5x, and $2\%\pm 1\%$
8 above 1.5x. For comparison, if ultrasound reflection and attenuation are neglected (free-field
9 condition), 48% of the spinal-canal ROI volume would have a pressure between 0-0.5x the
10 nominal pressure and 52% of the ROI is exposed with a pressure between 0.5-1x the nominal
11 pressure.

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

16
17

18 **BSCB Opening**

19 Qualitative Macroscopic analysis showed blue colorations of the sonicated segments of the
20 spinal cord by the EBD demonstrating the localized disruption of the BSCB (*Figure 2*). Non-
21 sonicated spinal cord segments (controls) remained white. Similar to the effects previously
22 observed in the brain, this coloration is more prominent in the grey matter (Beccaria et al.
23 2013)(*Figure 2, lower*). In sagittal sections, the blue coloration of the grey matter
24 corresponded to the length of the acoustic field but was not always observed in white matter.
25 In some samples, the coloration was unilateral, due to the orientation of the transducer. When

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

8 Quantitative spectrophotometric measurements of EBD concentration revealed a statistically
9 significant difference in EBD concentrations between the 19 sonicated samples (51.4 ± 6.1
10 $\mu\text{g/g}$) and the 7 controls ($15.9 \pm 1.8 \mu\text{g/g}$), $p=0.0004$. (Mann Whitney test) The 11 segments of
11 sonicated rabbits allowed to breath ambient air had a higher BSCB disruption (69.8 ± 5.6
12 $\mu\text{g/g}$) compared to the 8 segments of rabbits ventilated with isoflurane/oxygen (26.3 ± 2.5
13 $\mu\text{g/g}$), $p=0.006$. (Kruskall Wallis test – Dunn-s multiple comparaisn post test) (Figure 3).
14 When analyzing the segments with no or minimal histological lesions (*Grade 0-1*), the EBD
15 concentration of the 13 sonicated segments was increased when compared to the five controls
16 (44.8 ± 7.3 vs $15.8 \pm 2.4 \mu\text{g/g}$, $p=0.004$, Mann Whitney test). In segments with no or minimal
17 histological lesions in rabbits breathing ambient air, EBD was even more increased ($67.5 \pm$
18 8.6 , vs $15.8 \pm 2.4 \mu\text{g/g}$) compared to reabbits breathing isofluran-oxygen. None of the control
19 segments had a BSCB disruption.

20 Surgical exposition and ultrasound Toxicity

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

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

4 **Repeated sonication and clinical evaluation**

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

13 **DISCUSSION**

14 This study is a proof of concept that the BSCB can be safely disrupted using a 1 MHz
15 unfocused ultrasound device coupled with microbubbles. The optimal acoustic pressure (0.3-
16 0.5MPa) is slightly lower to that used for BBB disruption in rabbits (0.5-0.6MPa).

17

18 **Difficulties with the Rabbit Model**

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

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

17

18 **Impact of anesthesia**

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

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

6

7 **Evans blue dye**

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

20 **Comparison to the blood brain barrier disruption**

21 An emitted acoustic pressure of between 0.3-0.4 MPa was found to be safe for disrupting the
22 BSCB in rabbits. This pressure is slightly lower than the optimal acoustic pressure for the
23 blood brain barrier (0.6 MPa) in a similar animal model (Beccaria et al. 2013; Beccaria et al.
24 2016). This difference could be due to the reflection of the ultrasound wave on the opposite

1 bone, which locally increases the acoustic pressure compared to the nominal treatment
2 pressure as shown by the simulations presented in *Figure 1*.
3 Sonicated segments had an EBD concentration 4.4 higher than controls (69.8 vs 15.9 $\mu\text{g/g}$).
4 This difference is similar to the one we previously found for the blood brain barrier: 4.1
5 higher (26.9 vs 6.5 $\mu\text{g/g}$). (Beccaria et al. 2013). Other parameters such as pulse duration,
6 pulse repetition frequency or ultrasound frequency were not changed compared to our
7 previous studies on the brain.

8

9 **Tolerance of BSCB disruption by ultrasound**

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

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

23

24 **Tolerance of repetitive sonications**

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

12 **Existing literature**

13 To our knowledge, there are three reports of ultrasound-mediated BSCB disruption in the
14 literature. These three experiments used focused ultrasound devices. The first publication
15 involved gene delivery to the spinal cord in a rat model (Weber-Adrian et al. 2015).
16 Ultrasound was generated using a 1.114 MHz transducer for 5 minutes, with 10 ms bursts, at a
17 repetition rate of 0.5 Hz and acoustic power of 0.73W. In this study, after one sonication, gene
18 delivery of green fluorescent protein using a viral vector (20 nm) was increased in motor
19 neurons (87% expression in sonicated regions vs 28% without ultrasound), and
20 oligodendrocytes (36% vs 7%). Clinical tolerance was proven and histology at 13 days post
21 sonication reported no toxicity.
22 The second study of BSCB disruption was also in a rat model.(Payne et al. 2017) BSCB
23 disruption was assessed by measurement of gadolinium enhancement on magnetic resonance
24 imaging (MRI). BSCB disruption was shown in 10/12 rats using contrast-enhanced MRI.

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

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

6 **Future developments**

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

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

16 **CONCLUSION**

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

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7 Eiffelvet, Paris, France for providing rabbit CT-scans.

8

9 **DISCLOSURE**

10

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

13

14

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- 5

1 **FIGURE CAPTIONS**

2

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

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

10 **Figure 2: Macroscopic analysis of EBD distribution**

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

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

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

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

3 **Figure 4: Histological aspects**

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

9

1 **TABLE**

2 **Table 1:** experimental set up and results

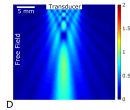
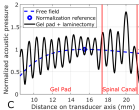
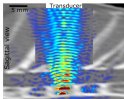
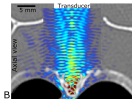
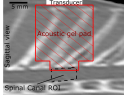
Ventilation	Sonicated Spinal cord segment Identification Nb.	Acoustic pressure (MPa)	Macroscopic Blue stain	Evan's Blue Dye (µg/g)	BSCB opening	Histology (Hynynen grade)
<i>Oxygen - Isofluran</i>	# 1	0 MPa (Control)	No	13,6	No	1
	# 2		No	17,9	No	0
	# 3		No	8,3	No	1
	# 4	0,4 MPa	Yes	76,2	Yes	3*
	# 5	0,5 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
<i>Ambient Air</i>	# 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		Yes	98,9	Yes	2
	# 22	0,35 MPa	Yes	61,1	Yes	0
	# 23	0,4 MPa	Yes	55,7	Yes	2
	# 24		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
	# 31	0,4 MPa. 10 sonications	Yes	95,0	Yes	1

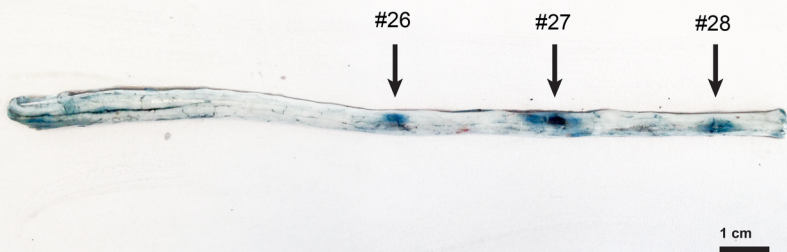
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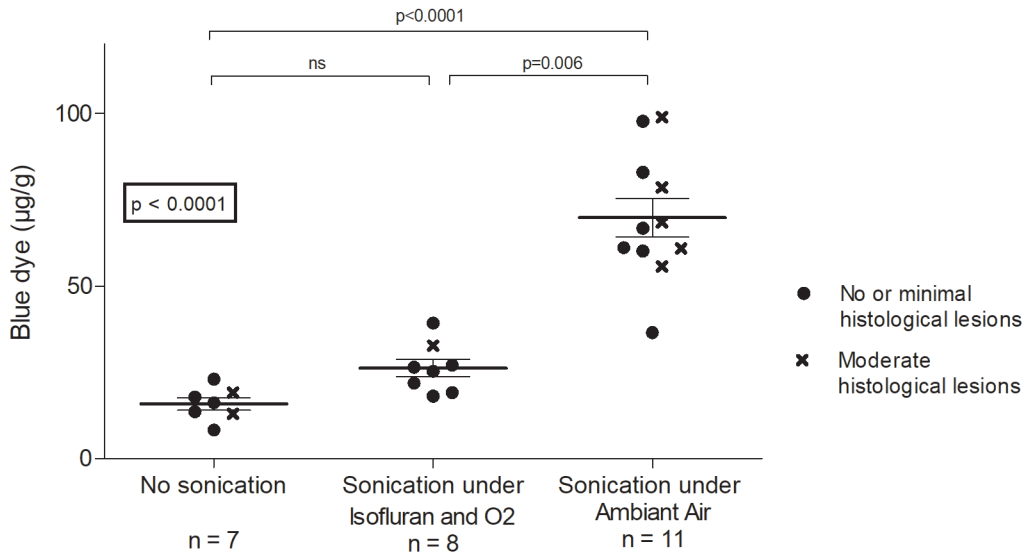
4 *In total, 23 segments had sonications and were compared to 7 controls. Sonications #4 to 14*
5 *were performed on rabbits breathing a mixture of isofluran and oxygen while sonications*
6 *#19 so #30 were with ambient air. 4 segments(#4#8#12#30) had severe histological lesions,*
7 *probably due to surgical trauma. These lesions resulted in high level of EBD. These data(*)*

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

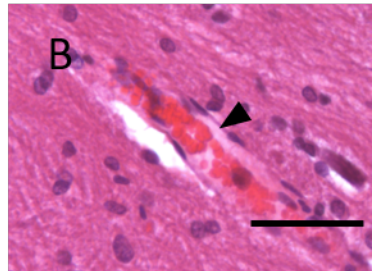
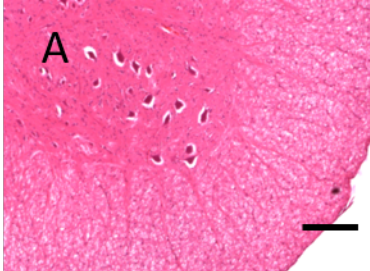
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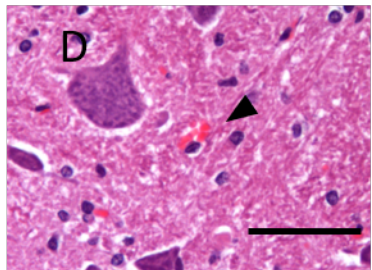
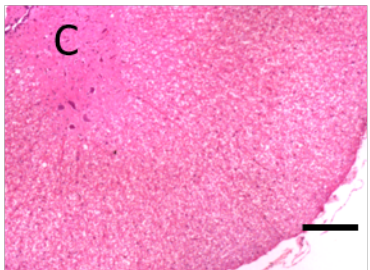




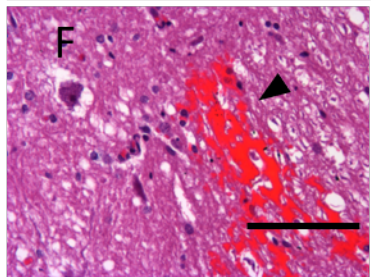
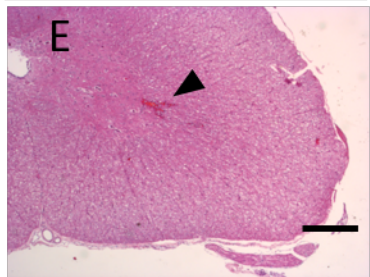
Grade 0



Grade 1



Grade 2



Grade 3

