Acoustic Properties of Small Animal Soft Tissue in the Frequency Range 12-32 MHz

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Abstract: Quality assurance (QA) phantoms are made of tissue-mimicking-materials (TMMs) whose acoustic properties mimic those of soft tissue. However, the acoustic properties of many soft tissue types have not been measured at ultrasonic frequencies above 9 MHz. With the increasing use of high frequency ultrasound for both clinical and preclinical applications, it is of increasing interest to ensure that tissue mimicking materials accurately reflect the acoustics properties of soft tissue at these higher frequencies. In this study, the acoustic properties of ex vivo brain, liver, and kidney samples from 50 mice were assessed in the frequency range of 12 - 32 MHz. Measurements were performed within 6 minutes of euthanasia in a phosphate buffer saline (PBS) solution maintained at 37.2 ± 0.2°C. The measured mean values for the speed of sound for all organs were found to be higher than the IEC guideline recommended value for TMMs. The attenuation coefficients measured from brain, liver and kidney samples were compared with the results of previous studies at lower frequencies. Only the measured kidney attenuation coefficient was found to be in good agreement with the IEC guideline. The information provided in this study can be used as a baseline upon which to manufacture a TMM suitable for high frequency applications.

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He has undertaken research in tissue characterisation

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13th of October, 2017

Dr. Holland

Editor-in-Chief

Ultrasound in Medicine & Biology

University of Cincinnati, Cardiovascular Research Center

Cincinnati, OH, USA

Dear Dr. Holland:

Please find attached my manuscript entitled the 'The acoustic properties of small animal soft tissue in the frequency range 12 – 32 MHz' for consideration for publication in Ultrasound in Medicine and Biology. This document include the reviewers comments.

I declared that the 'The acoustic properties of small animal soft tissue in the frequency range 12 – 32 MHz' have not been and will not be submitted elsewhere for publication.

I will advise the following for potential reviewers for the manuscript:

Dr. Jeff Bamber

Dr. Kumar Ramnarine

Dr. Bill O'Brien

Sincerely,

RABELL-MONTIEL Adela

***Detailed Response to Reviewers**

The authors are grateful to the Reviewers for their thorough review and constructive feedback on our manuscript.

The organisation of this response document will be as follows: answers to the questions raised by the Reviewers, will be indented and in italic font. Proposed modifications to the paper will be shown as underlined. These locations are based on the new manuscript.

Reviewers' comments:

Reviewer #1: This is a well-described report of a well-designed set of measurements of the ultrasonic attenuation coefficient and sound speed of several mouse soft tissues extending the frequency range above that currently published. The authors are to be applauded for the careful experimental design which gives confidence in the validity of the results, in an area of metrology in which attention to detail is not always present. Whilst sound speed is easy to measure, attenuation coefficient is not, because there are many traps for the unwary, and several sources of systematic error that need to eliminated to have confidence in the outcome. The following critique makes a few comments that the authors need to address before the paper can be finally recommended for publication.

Comments

On the use of the IEC guideline as the reference point. There are other guidelines and standards, of course. ICRU Report 61 suggests that the attenuation coefficient for 'average non-fatty soft tissue' should be taken to be 0.6 dB/MHz at 1 MHz, with a power law dependence on frequency of 1.2. The assumption of linear frequency dependence, however convenient, has never been supported by measured evidence from real tissues, even at the lower frequencies, let alone at the higher frequencies you have investigated. IEC (and FDA for that matter) have a regulatory need to oversimplify things and it is correct to critique such oversimplification. It is also important to emphasise consensus statements from other international bodies where they exist, especially when they are more firmly based on published measurements. (ICRU Report 61: Tissue Substitutes, Phantoms and Computational Modelling in Medical Ultrasound (1998).) On the question of the frequency dependence of attenuation coefficient. Your use of a power law fit of the form $Af+Bf^2$ is entirely appropriate. Unfortunately it gives difficulty in making comparison with previous data fitting, which sometimes used Af(exp b). In order to facilitate comparisons, it is appropriate to give results of both forms. Furthermore, comparison of the regression analyses will justify the choice of the better fit.

-We like to thank the reviewer for the helpful comments provided. For the frequency dependence of attenuation coefficient, a power law fit in the form $\underline{af^b}$, has been added into the analysis for comparison with published data.

-Information regarding the comparison with the ICRU 61 report has been added to the manuscript.

-Page 3 Line 34-36. *Also, the International Commission on Radiation Units and Measurements reports that for non-fatty tissues the attenuation at 1 MHz should be 0.6 dB* cm^{-1} *(ICRU, 1998)*

-Page 10 Line 182-183. The IEC recommended values are the most widely used, so, therefore in this study the acoustic properties of soft tissues were compared with these values.

-Page 12 Line 223-229. *Moreover, the attenuation versus frequency data measured in this study was re-expressed and extended to lower frequencies as a power law of the form af^b, where f is the frequency (MHz) and a and b are the coefficients of the fit. The power law fit calculated for the brain was 0.91 dBcm^{-1} MHz^{-1} (R^2=0.84). Kremkau et al., (1981) reported an attenuation of 1.08 dBcm^{-1} MHz^{-1}, Bamber et al., (1981) reported 1.1 dB cm^{-1} MHz^{-1} and Strowitzki et al., (2007) reported an attenuation of 0.94 ± 0.13 dBcm^{-1} MHz^{-1}. The maximum difference in the attenuation power law fit was with Bamber et al., (1979) by 4.2 dBcm^{-1} at 5 MHz (Figure 6).*

-Page 14 Line 267-271. *The attenuation versus frequency data for liver samples calculated in this study can also be expressed as a power-law of the form 1.08 dBcm^{-1} MHz^{-1} (R^2=0.66). This power-law was found to be in good agreement (± 0.42 dB cm^{-1}) with those power-laws reported from pig (1.2 dBcm^{-1} MHz^{-1}, López-Haro et al., (2009)), rat (1.3 ± 0.09 dBcm^{-1} MHz^{-1}, O'Brien et al., (1988)) and human (1.6 ± 0.21 dBcm^{-1} MHz^{-1}, Lu et al., (1999), 1.5 dBcm^{-1} MHz^{-1}, Gammell et al., (1977)) livers.*

-Page 15 Line 293-297. *The attenuation versus frequency data from kidney can be fitted to a power-law curve. The power law fit obtained was 0.73 dBcm^{-1} MHz^{-1} (R^2=0.81). This fit gave values of attenuation as 0.33 dBcm^{-1} MHz^{-1} higher than the attenuation measured from bovine and porcine kidney at 37°C and at 45°C (Goss et al., 1979; Worthington et al., 2001).*

On the use of the terms 'attenuation' and 'attenuation coefficient'. Check the script thoroughly to ensure you use these two terms correctly, especially noting that you have often quoted 'attenuation' in dB/cm, and not dB (though the context suggests that you meant attenuation coefficient).

-Agreed. The manuscript has been revised thoroughly to ensure the correct use of these two terms.
-Page 2 Line 23… *The attenuation coefficients measured from brain, liver and...*
-Page 3 Line 33… *and an attenuation coefficient of TMM…*
-Page 3 Line 50… *cellular matrix (ECM) attenuation coefficient of murine…*
-Page 4 Line 56… *and attenuation coefficient of soft tissue increases…*
-Page 7 Line145… *the attenuation coefficient [α in (dBcm^{-1})]…*
-Page 7 Line 149… *This enabled the attenuation coefficient of PBS…*
-Page 7 Line 150… *The SoS and the attenuation coefficient of degassed, deionised…*
-Page 7 Line 152… *The absolute attenuation coefficient of PBS…*
-Page 10 Line 181… *coefficient of the IEC agar-TMM…*
-Page 11 Line 193… *the attenuation coefficient of the soft tissue…*
-Page 12 Line 212… *At 32 MHz the attenuation coefficient difference…*
-Page 15 Line 301… *the attenuation coefficient with this study…*

On the matter of sample size. You do not state the lateral (radial) dimensions of the sample in comparison with the beam radius where the sample is placed. The aperture is 2.5 mm. It is left to be assumed that the beam is much smaller than this, and that the sample exceeds it. This needs to be stated. Atkins et al discuss the errors that may accrue from too small a sample lateral dimension. http://iopscience.iop.org/article/10.1088/1742-6596/279/1/012024/pdf

-Agreed. The approximate lateral (radial) dimensions of the samples were 0.5 cm for brain, 1 cm for liver and 0.5 cm for both dissection planes in the kidney. The beam was much smaller than the lateral dimensions of the sample. The spatial resolution of the beam profile was measured by Sun, (2012) using a hydrophone. At an acoustic spatial and temporal peak pressure of 1MPa, the measured 3dB beam radius was 0.14 mm.

-Page 5 Line 89-90. *The lateral (radial) dimensions of the samples were 0.5 cm for brain, 1 cm for liver and 0.5 cm for both dissection planes in the kidney.*

On the matter of non-linear effects. The reader needs to be reassured about the analysis of Sun et al. I note the use of the word 'significant'. This implies that you retained a source of systematic error which is un-accounted for. All broad-band attenuation experiments carry the potential that non-linear effects introduce systematic errors. Such effects increase with pulse amplitude, with distance and, most importantly, with frequency. If your experiment had been carried out at 3 MHz, you might have got away with a peak acoustic pressure of 1 MPa without introducing important errors. (By the way, I am impressed by the statement of acoustic pressure - usually this is a quantity of which those measuring attenuation coefficient are unaware!) Using the values of acoustic pressure, frequency and distance, and a guess of 5 as the focal gain of your system, the 'local distortion parameter' at the focus is just over 1.0 (see IEC 61949). This partially justifies your assertion that the acoustic design is appropriate from non-linear considerations. Incidentally, IEC 61949 suggests that sigma should be limited to 0.5 for measurement purposes, and that scaling from source pressures should be used if that cannot be achieved. This is because, at such levels of distortion, much acoustic energy has been removed from the fundamental band into higher harmonics. For sigma = 1.0, the second harmonic (60 MHz) component amplitude is -8dB (0.4) of the 30 MHz fundamental, and even the 3rd (90 MHz) component is only -12 dB. I suspect that your transducer and receiver electronics are not designed to handle such frequencies and that they disappear from the measurement chain.

If such losses are unaccounted for, they can result in an underestimate of the attenuation coefficient. Can you assure the reader that the assessment by Sun et al included explicitly an overall test of system linearity which resulted in the decision to operate at 10% of maximum power? And can you broadly quantify and justify the magnitude of errors that reside in your method from non-linear effects?

-The process of measuring the acoustic pressure can be found in Sun, (2012) and will be briefly explained here. The acoustic pressure was measured using a membrane hydrophone 0.2mm diameter active element (Precision Acoustics Ltd., Dorchester, UK). As a result there was no issues with the receiver electronics. The hydrophone was calibrated for frequencies between 2 – 60 MHz by the National Physical Laboratory (Teddington, UK). The measurements were performed by moving the hydrophone across the ultrasonic beam in a direction normal to the propagation direction. The maximum acoustic signal output was found and its position was determined to be the focus by adjusting the position of the hydrophone near the nominal focal position. The acoustic pulses were also recorded at different depths on the z-axis with a distance interval of 0.1 mm. The acoustic pulses were measured at different insonation powers from 3% - 100% at this nominal position. From Sun et al., (2012) the power output of 10% was considered a reasonable compromise between the generation of negligible nonlinear effects and adequate signal magnitude. Moreover when characterising this transducer in water it was found that the second harmonic component of the ultrasound beam was at least 30dB smaller in magnitude than the first harmonic (fundamental) (Sun, 2012; Rabell-Montiel et al., 2017).

Other specific remarks

L57 'Decay' in what way? 'Change' might be a better word

-Modified. Page 4 Line 60. Deteriorate.

L90 et seq. Viewing figure 1: The 1cm layer of Aptflex is not labelled. And the soft tissue seems to lay on top of the washer/tissue holder, not inside it. This relates in part to the comments above on the lateral dimensions of the tissue sample.

-Figure 1 has been modified and the Aptflex (absorber) was labelled.
-The washer/tissue holder was used to create a space between the sample tissue and the reflector as stated in Line 98-99. The sample tissue was not inside the washer/tissue holder, but lay on top of the washer.

L108. How confident are you in the measurement of acoustic pressure?

-From Sun,(2012), the hydrophone used to measure the acoustic pressure of the transducer attach to the Vevo 770® scanner was a membrane hydrophone with an active element of 0.2 mm diameter active element made of Polyvinylidene Fluoride (PVDF) (Precision Acoustics Ltd., Dorchester, UK). The hydrophone was calibrated in the frequency range 2 – 60 MHz by the National Physical Laboratory (Teddington, UK).

P255 Figures 7 and 8. The comparison with Wirtzfeld et al needs exploring further. Lin et al and Foster et al for example, seem to be in accordance with Wirtzfeld. I wanted to think that you are seeing the effects of scattering at higher frequencies, but the large difference, over all the whole frequency range, does not support this. It looks like some kind of systematic error. If Wirtzfeld was working with a highly non-linear beam, that might have caused his measurements to underestimate the true attenuation coefficient. On the other hand, are you sure that you accounted for all the possible causes for an increase in insertion loss in your experiment, including beam movement caused by refraction, or by interface losses etc.?

-Wirtzfeld et al., (2015) used a Vevo2100 and a MS550S linear array transducer (frequency bandwidth 15 – 35 MHz), but did not specify the acoustic pressure used when the measurements were undertaken. In addition, Wirtzfeld et al., (2015) measurements of SOS and attenuation were taken from the extra-cellular matrix (ECM) of the liver and the kidney so, the difference in attenuation values measured by Wirtzfeld et al., (2015) and this study may be due to the differences in cellular integrity of the tissues. Additionally, although the results from the study by Lin et al (1977) appear to agree with Wirtzfeld et al., (2015), Lin likewise did not quote the acoustic pressure at which the measurements were undertaken. The magnitude of the attenuation coefficient extrapolated to lower frequencies from our study is similar in to that of Foster et al., (1979). Moreover, in Figure 1 and Figure 2 below, we have included a highlighted ‘cone’ of attenuation versus frequency data from published studies and extended to higher frequencies. It can be seen that Wirtzfeld et al., (2015) falls at the lower limit whereas our results are largely in agreement with the extrapolated attenuation values of the extrapolated data from published studies at lower frequencies.

Additionally as far as possible, we have attempted to account for all the causes of error when using the broadband reflection substitution technique.

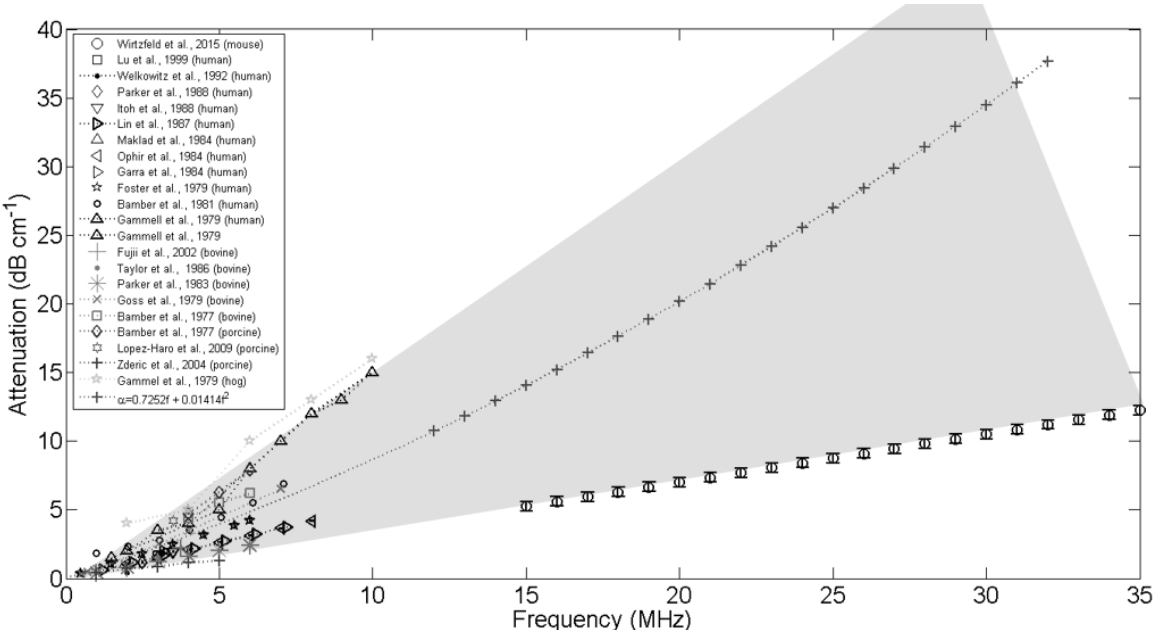

Figure 1. Figure 7 from the manuscript highlighting the area of the expected attenuation versus frequency based on those published studies.

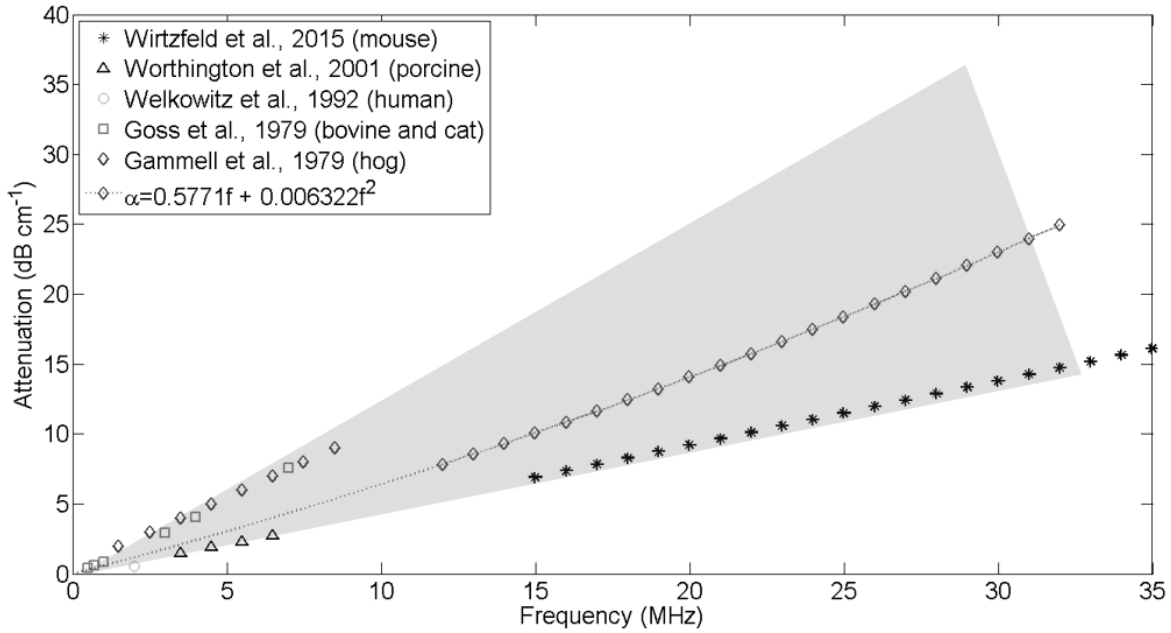

Figure 2. Figure 8 from the manuscript highlighting the area of the expected attenuation versus frequency based on those published studies.

P224. I am unclear about this statement. Do you mean that downward extrapolation from your attenuation coefficient results differs by no more than 1.8 dB/cm from all these other results throughout the range of frequencies from 1 to 7 MHz?

-When comparing the extended polynomial fit at lower frequencies, it does differ.

-Page 12 Line 222-224. *When compared to the extended polynomial fit at lower frequencies, the maximum difference was found to be 5 dB cm^{-1} at 5 MHz (Bamber et al., 1979).*

P249. Is there independent evidence that coagulation of blood increases its attenuation coefficient sufficiently to result in the high variability reported? This seems unlikely to me.

-The hardening of blood clots have been measured quantitatively by measuring their elasticity (Mfoumou et al., 2014). In that study, an increased in the Young's modulus was found over time (120 min). Also, when compared the elasticity curve measurements on thrombi (induction of venous thrombosis) with the surrounding muscle it was found that the Young's modulus varied from 1 kPa (at 10 min) to 25 kPa (at 14 days). The rigidity of the clots was reported to be statistically different from the baseline and after 50 min. Therefore, the variation in the attenuation versus frequency reported in our study are not due to the possible coagulation of blood, as the clots of blood do not change within the first 120 minutes according to Mfoumou et al., (2014). Furthermore, it is known that blood backscatter strongly depends on the shear rate (Foster et al., 1994). From Foster et al., (1994), at 50 MHz, the backscatter of blood is 0.4 with a shear rate of $0.16s_{-1}$ and 3.5 with a shear rate of $32s^{-1}$.

-Page 13 Line 251-253. The sentence has been deleted. *Therefore, it is not believed that the high variability (18 dBcm^{-1} at 32 MHz, see Figure 4) of the attenuation coefficient in this study derives from the production of gas due to autolytic decay.*

No information regarding the attenuation of blood with time has been found in the literature.

Reviewer #2: This paper is very well written and good to be published. It aimed to bridge the knowledge gap of various soft tissues' high frequency (above 10 MHz) ultrasound properties and largely achieved this goal in the experimental results and analysis. The results look consistent with previous published studies (mostly at lower frequency). This work will help establish the high frequency TMM requirement in IEC standard.

-The authors would like to thank the reviewer for the comment made on this research.

The acoustic properties of small animal soft tissue in the frequency range 12 – 32 MHz.

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ABSTRACT

Quality assurance (QA) phantoms are made of tissue-mimicking-materials (TMMs) whose acoustic properties mimic those of soft tissue. However, the acoustic properties of many soft tissue types have not been measured at ultrasonic frequencies above 9 MHz. With the increasing use of high frequency ultrasound for both clinical and preclinical applications, it is of increasing interest to ensure that tissue mimicking materials accurately reflect the acoustics properties of soft tissue at these higher frequencies. In this study, the acoustic properties of *ex vivo* brain, liver, and kidney samples from 50 mice were assessed in the frequency range of 12 – 32 MHz. Measurements were performed within 6 minutes of euthanasia in a phosphate buffer saline (PBS) solution maintained at 37.2 ± 0.2°C. The measured mean values for the speed of sound for all organs were found to be higher than the IEC guideline recommended value for TMMs. The attenuation *coefficients* measured from brain, liver and kidney samples were compared with the results of previous studies at lower frequencies. Only the measured kidney attenuation coefficient was found to be in good agreement with the IEC guideline. The information provided in this study can be used as a baseline upon which to manufacture a TMM suitable for high frequency applications.

Key words: ultrasound, high frequency, mice, brain, liver, kidney, speed of sound, attenuation.

INTRODUCTION

The purpose of tissue-mimicking-materials (TMMs) is to mimic the acoustic properties of soft tissue. Currently, the International Electrotechnical Commission (IEC, 2001) guideline recommends standard values for the speed of sound (SoS) (1540 ± 15 ms^{-1}) and an attenuation *coefficient* of TMM (0.5 ± 0.05 dB cm^{-1}) at frequencies up to 10 MHz. Also, the International Commission on Radiation Units and Measurements reports that for non-fatty tissues the attenuation at 1 MHz should be 0.6 dB cm^{-1} (ICRU, 1998). With the increasing use of high frequency ultrasound in both clinical (2 – 15 MHz) and preclinical (above 15 MHz) (Banchhor et al., 2016; Machet et al., 2009; Moran, 1995; Rhee, 2007; Schmitt et al., 2010; Sundholm et al., 2015; Xu et al., 2012) imaging applications there is a need to extend the frequency range of these recommended acoustic values. Furthermore, the development of phantoms which incorporate TMM that realistically mimics the acoustic properties of small animal soft tissue, will enable a reduction in the use of small animals to optimise ultrasound imaging techniques.

The acoustic properties of brain, liver, kidney amongst other organs, have previously been measured from small animals (Frizzel et al., 1981; Goss et al., 1979; Tervola et al., 1985; Foster et al., 2000; Gray et al., 2013; Szabo, 2014), humans (Bamber et al., 1979, 1980; Kremkau et al., 1981; Ludwig, 1950; Parker, 1983; Rajagopalan et al., 1979; Sehgal et al., 1986), chickens (Martínez-Valdez et al., 2015) and mammals (Bamber et al., 1977; Ghoshal et al., 2011; Goss et al., 1979; López-Haro et al., 2009; Martial et al., 2007). These studies measured the acoustic properties up to 9 MHz at either room temperature (22 – 26°C) or at human body temperature (37°C). Recently, Wirtzfeld, et al., (2015) measured the extra-cellular matrix (ECM) attenuation *coefficient* of murine liver and kidney across the frequency range 15 – 35 MHz, where a decellularised method was utilised, finding that the ECM of the organ contributes to the ultrasonic properties. Additionally, Frizzel et al., (1981), O'Brien, (1988) and Tervola et al., (1985) have performed very high frequency acoustical

measurements up to 100 MHz using a Scanning Laser Acoustic Microscope (SLAM). Measurements performed at 100 MHz were undertaken at room temperature (20 – 26°C).

It has been shown that the SoS and attenuation *coefficient* of soft tissue increases with increasing temperature (Bamber et al., 1979; Ghoshal et al., 2011; López-Haro et al., 2009; Rajagopalan et al., 1979; Suomi et al., 2016). However, there is no further increase in the SoS in soft tissue above 50°C (Duck, 2012). Furthermore, it is well-known that *ex vivo* soft tissue samples deteriorate with time after excision as gas bubbles form within the tissue, thus affecting its acoustic properties (Bamber, 1981; Duck, 2012). To prevent this, soft tissue should be excised and measured as soon as possible after euthanasia or stored at 4°C (Bamber et al., 1977, 1985; Foster et al., 1979).

The acoustic properties of soft tissue have also been measured *in vitro* or by embedding the organ sample in an ultrasound compatible acoustic material such as TMM (Bamber et al., 1977, 1979; Gross et al., 1980; Martínez-Valdez et al., 2015; Muleki-Seya et al., 2016; Sundholm et al., 2015; Suomi et al., 2016), but very few experiments have been undertaken using *ex vivo* tissue (Kumagai et al., 2014) or *in vivo* tissue (Kagadis et al., 2010; Zderic et al., 2004).

In order to address the current limited data on the acoustic properties of soft tissue, this study aims to measure the acoustic properties of *ex vivo* mouse brain, liver and kidney immersed in phosphate-buffer saline (PBS, Sigma-Aldrich, Saint Louis, MO, USA) at 37°C, over the frequency range of 12 – 32 MHz.

MATHERIALS AND METHODS

Soft tissue sample preparation

Twenty brains, 20 livers and 20 kidneys were analysed from 50 recently euthanized healthy male C57BL/6 mice, a common inbred laboratory mouse strain. The mice were euthanized by cervical dislocation under the auspices of the Animals (Scientific Procedures) Act 1986 (Schedule 1) approved by the University of Edinburgh Animal Welfare and Ethical Review Board (AWERB). Within 6 minutes of euthanasia, the organs were extracted, sliced in either coronal or transverse plane, and their acoustic properties measured. Excised mouse were sliced using a 1 mm adult rat brain acrylic slicer matrix (Zivic Instruments, Pittsburgh, PA).

Twenty brains were excised and sliced in the frontal plane at the *superior colliculus* which included the cerebral cortex (Figure 1a). For brain tissue, the sample thickness was 3 mm as thinner samples tended to disintegrate during handling. Acoustical measurements were made in the centre of each sample, within the grey matter. Twenty murine left lateral liver lobes were excised and sliced in the coronal plane, to a thickness of 2mm (Figure 1b). Twenty kidneys from 10 mice were excised and sliced (2mm) as follows: the right kidney was sliced in the coronal plane (Figure 1c) and the left kidney was sliced in the transverse plane (Figure 1d). Acoustical measurements were undertaken in the centre of each sliced kidney sample in an endeavour to ensure location within the *medulla* of the kidney. Only one tissue sample was collected from each organ. The lateral (radial) dimensions of the samples were 0.5 cm for brain, 1 cm for liver and 0.5 cm for both dissection planes in the kidney.

Experimental set-up using the high frequency Vevo 770® ultrasound scanner

A temperature controlled water-filled reservoir (Grant Instruments, Cambridge, UK) with dimensions of 15 x 33 x 19 cm was used to heat phosphate-buffered saline (PBS; Sigma-Aldrich, Saint Louis, MO, USA) to 37.2 ± 0.2°C. A smaller glass container (10 x 8 x 7.5 cm and 0.6 cm thick) was placed inside the water reservoir. A 1 cm layer of acoustic absorber (Aptflex F28, Precision Acoustics,

Dorset, UK) was fixed at the bottom of the glass container. A cylindrical acoustic reflector made from polymethylpentene (TPX; Boedeker Plastics, Texas, USA) with 2.5 cm diameter and 5 mm thickness was glued to the absorber. A circular washer made of the acoustic absorber, 1 mm thick, 2.5 mm inner diameter and 2.5 cm outer diameter, was attached to the top surface of the TPX reflector as shown in Figure 2. The circular washer acted as a tissue holder and ensured there was a space between the soft tissue sample and the TPX reflector. The aim of this separation was to allow the echoes from the tissue and from the TPX reflector to be differentiated during later analysis.

Acquisition and analysis of the acoustic data

The radio-frequency (RF) data from 60 soft tissue samples were acquired using a single-element high frequency probe RMV707B attached to the Vevo 770® ultrasound scanner (Visualsonics Inc., Toronto, Canada). The RMV707B probe has a centre frequency of 30 MHz, focal depth of 12.7 mm and a 3 dB bandwidth from 12 – 32 MHz (Rabell Montiel et al., 2017). The acoustic properties of the soft tissues were measured while immersed in PBS at 37.2 ± 0.2 °C. The TPX reflector was located at the focal point of the probe (Figure 2). Data was collected at 10% of maximum acoustic output power (peak negative pressure 1.05 MPa), which gave a satisfactory signal-to-noise ratio while avoiding significant non-linear propagation effects (Sun et al., 2012). Using a broadband pulse-echo substitution technique (AIUM, 2014) the data was analysed based on pre-selected regions of interest, (ROI). These ROIs were located at the front and rear of the sample and at the front surface of the TPX reflector with and without the sample placed in the acoustical path. Acoustic data was acquired from 10 ultrasonic data lines distributed equally across the ROIs and measurements were undertaken at 37.2 ± 0.2°C.

After slicing, the sample was immediately immersed and mounted in the tissue holder in the PBS tank, ready for acoustic measurements to be undertaken. Precise thickness measurements were obtained using the timing of the echoes from the front and rear surfaces of the sample. The tissue holder was necessary to enable these measurements to be made accurately and reproducibly.

Three measurements were undertaken for each sample immediately after immersion in PBS (t=0), after 5 minutes (t=5) and after 10 minutes (t=10). The PBS reference fluid was changed daily after each set of measurements. Up to 3 organ samples were assessed on any given day.

Acoustic properties of PBS

The PBS was prepared according to the manufacturer's recommendations (pH 7.4 at 25°C) (Sigma-Aldrich, Saint Louis, MO, USA). PBS was chosen as a physiological fluid in order to delay tissue deterioration, death and thus to minimise physiological and mechanical changes within the tissue during the measurement period (Bader et al., 2015; Edgeworth et al., 2009; Foster et al., 1979; Garcia-Duitama et al., 2016; Lay et al., 2003; Muleki-Seya et al., 2016; Wirtzfeld et al., 2015; Worthington et al., 2001).

The SoS of the PBS at 37°C was calculated using Equation (1) with a SD of 0.02 ms^{-1} (Coppens, 1981):

$$C = 1449.05 + (45.7t) - 5.21t^2 + 0.23t^3 + ((1.333 - 0.126t + 0.009t^2) * (10S - 35)) \quad \textit{Equation (1)}$$

where t is the temperature of the fluid (t = T/10, T in °C), and S is the salt concentration in g/100 cm^3. The salinity of the PBS was calculated as 0.41g/100 cm^3. At 37°C the SoS used in this study was calculated to be 1527.9 ms^{-1}.

The attenuation of PBS was measured using a pulse-echo substitution technique (AIUM, 2014) with a similar experimental set-up shown in Figure 2, but without the tissue holder in place. Fifty measurements were taken using the RMV707B and Vevo 770® scanner at 10% of maximum output power. The TPX reflector was placed at the focal depth of the transducer. Degassed, deionised water at 37°C was placed in the glass box, to act as a reference fluid. After acoustic measurement, the degassed deionised water was replaced with PBS, at the same temperature. Raw

RF data was collected from 10 lines within pre-selected ROIs located at the surface of the TPX reflector and the data was analysed offline using a MatLab script (MatLab R2013a MathWorks, Inc).

Using D_F as the distance between the transducer and the front surface of the TPX reflector, the attenuation *coefficient* [α_0 in (dBcm^{-1})] can be calculated (Equation 2):

$$\alpha_0(f) = -\frac{20}{2D_F} log_{10} \frac{A(f)}{A_0(f)} \qquad \textit{Equation (2)}$$

Where $A(f)$ and $A_0(f)$ are the magnitudes of the signal spectra from the TPX measured in degassed deionised water and in PBS fluid respectively and, D_F is the distance calculated using the return time intervals of the echoes from the TPX as described above.

This enabled the attenuation *coefficient* of PBS to be calculated relative to degassed, deionised water. The SoS and the attenuation *coefficient* of degassed, deionised water is well documented (Bilaniuk et al., 1992; Coppens, 1981; Del Grosso et al., 1972; Pinkerton, 1949; Rajagopal et al., 2014). The absolute attenuation *coefficient* of PBS at 37°C was calculated and fitted using a second degree polynomial as $\alpha_0 = 0.002127f^2 + 0.02076f$ (R^2=0.99).

RESULTS

The mean age of the animal organs used in this study was 8.5 ± 3.1 months for brains, 6.8 ± 4.9 months for the livers and 5.2 ± 3.6 months for the kidneys. The mean body weight across all the mice was 34.4 ± 6g (minimum 22.6 g, maximum 45 g).

Table 1 shows the mean SoS at t=0 and then at t=5 and t=10 minute intervals for brain, liver and kidney tissue samples. It can be seen that the variation in SoS as a function of time was less than 1.5 ms^{-1} across the soft tissue samples. Although the SD of the mean SoS values increased for the brain and the liver samples in the last measurement (approximately 16 minutes after euthanasia), a Student's *t*-test did not find that these values were statistically different ($p > 0.5$) at t=0, t=5 or t=10.

The mean and SD values of the SoS of the 20 soft tissue samples from brain, liver and kidney are shown in Table 2.

The difference in SoS between the centre of the left and right kidney samples (different dissection planes) was 0.97 ± 0.69 ms^{-1}.

Figure 3, Figure 4 and Figure 5 show the mean attenuation versus frequency at each time point for brain, liver, and kidney respectively. The displayed SD was calculated from the mean attenuation data averaged over all time points. A second degree polynomial fit was calculated to be the best fit over all the mean attenuation versus frequency data. The goodness of fit (R^2) for the mean attenuation versus frequency data over all time points varied between 0.70 – 0.85 for the small animal soft tissue. The best fits were found to be for the attenuation versus frequency data of brain tissue (R^2=0.85) and kidney tissue (R^2=0.83). Figure 3, Figure 4 and Figure 5 also show the polynomial fit calculated from the data of 20 brains, 20 liver and 20 kidneys, respectively. The polynomial fit was found to be $0.7533f + 0.006477f^2$ (R^2=0.85) for brain, $0.7252f + 0.01414f^2$ (R^2=0.70) for liver and $0.5771f + 0.006322f^2$ (R^2=0.83) for kidney in the frequency range 12 – 32 MHz.

178 Figure 6, Figure 7 and Figure 8 shows the polynomial fit previously calculated, from the
179 mean attenuation across all time points, with other published studies for each organ. The
180 polynomial fit found in this study has been extended to lower frequencies for comparison purposes.
181 Figure 9 shows the three polynomial fits calculated for each organ in this study in comparison with
182 the attenuation *coefficient* of the IEC agar-TMM (Rabell Montiel et al., 2017) in the frequency range
183 4.5 – 50 MHz and the IEC guideline (IEC, 2001). The IEC recommended values are the most widely
184 used, so, therefore in this study the acoustic properties of soft tissues were compared with these
185 values.

DISCUSSION

The aim of this study was to measure the acoustic properties of *ex vivo* small animal soft tissue. Twenty brains, 20 kidneys (10 left and 10 right kidneys) and 20 livers from 50 mice were extracted, sliced and their acoustic properties measured using a preclinical ultrasound scanner within 6 minutes post euthanasia. Table 3 shows the SoS of published studies of the acoustic properties of brain, liver and kidney from various sources at room and at body temperature.

An increase in either water or fat content results in a decreased velocity of ultrasound in soft tissue (Duck, 2012). For the brain and the liver samples, the SoS and the attenuation were analysed against the weight, the age of the animal and against the measured thickness of the sample (data not shown). Also the SoS and the attenuation *coefficient* of the soft tissue samples was analysed as a function of time after excision, up to 15 minutes. None of these variables demonstrated a relationship with the measured acoustic properties.

Acoustic properties of PBS

In other studies, the acoustic properties of PBS have been considered to be similar to those of degassed deionised water at the same temperature (Muleki-Seya et al., 2016). Also some studies have used saline (9% salinity) as their acoustic reference fluid (Kumagai et al., 2014) and have found a SoS of 1536 ms^{-1} at 36°C. However, saline has a higher salinity concentration, than PBS (4%).

The calculated SoS for PBS at 37°C used in this study was 1527.9 ms^{-1}. This SoS value was found to be 8.14 ms^{-1} less than the SoS for saline (Kumagai et al., 2014) and up to 4.5 ms^{-1} greater than the SoS for pure water (Bilaniuk et al., 1992; Del Grosso et al., 1972). Additionally, Worthington et al., (2001) measured a SoS for PBS at 37°C to be 1541 ms^{-1}, but using a salinity of 0.9% in Coppens, (1981) formula. This results in a SoS value of 13.1 ms^{-1} higher than the SoS value used in this study. The difference in the calculated SoS values between saline and PBS is likely due to the different salinity concentrations.

The attenuation data for PBS at 37°C calculated in this study was found to be similar to that of degassed deionised water, and was proportional to f^2 over the frequency range of 12 – 32 MHz. Previous published studies (Muleki-Seya et al., 2016; Worthington et al., 2001) which have used PBS as a reference fluid, assumed the attenuation coefficient to be the same as water (2.17×10^{-3} dB cm^{-1} MHz^{-2} at 20°C)(Duck, 2012). At 32 MHz the attenuation *coefficient* difference between pure water at 20°C and the attenuation calculated of the PBS at 37°C was found to be 0.67 $dBcm^{-1}$.

Brain

The SoS measured in the brain samples is in good agreement with Kremkau et al., (1981) where measurements were taken from human brain samples over the frequency range 1 – 5 MHz and measured at 37°C. However, the SoS measured in this study was 56 ms^{-1} higher than human brain tissue samples measured by Welkowitz et al., (1992).

For the brain attenuation, the largest inter-sample difference of 13.2 $dBcm^{-1}$ was found at 26 MHz. Extending the second degree polynomial fit calculated in this study to lower frequencies, it was found that the attenuation from this study agrees at 1 MHz with a 0.5 $dBcm^{-1}$ difference with Bamber, (1981), Goss et al., (1979), Kremkau et al., (1981) and Welkowitz et al., (1992). When compared to the extended polynomial fit at lower frequencies, the maximum difference was found to be 5 dB cm^{-1} at 5 MHz (Foster et al., 1979; Gammel et al., 1979). Moreover, the attenuation versus frequency data measured in this study was re-expressed and extended to lower frequencies as a power law of the form af^b, where f is the frequency (MHz) and a and b are the coefficients of the fit. The power law fit calculated for the brain was 0.91 $dBcm^{-1}$ MHz^{-1} (R^2=0.84). Kremkau et al., (1981) reported an attenuation of 1.08 $dBcm^{-1}$ MHz^{-1}, Bamber et al., (1981) reported 1.1 dB cm^{-1} MHz^{-1} and Strowitzki et al., (2007) reported an attenuation of 0.94 ± 0.13 $dBcm^{-1}$ MHz^{-1}. The maximum difference in the attenuation power law fit was with Bamber et al., (1979) by 4.2 $dBcm^{-1}$ at 5 MHz (Figure 6).

Liver

There have been extensive publications of the acoustical properties of liver at low frequencies, yielding a wide range of SoS and attenuation coefficient values. Based on those studies published for mammalian livers, at ultrasound frequencies ranging from 1 to 9 MHz with different temperatures (22°C and 37°C), the SoS varied between 1545 – 1639 ms^{-1} (Bamber & Hill, 1979, 1980; Chen et al., 1987; Frizzel & Gindorf, 1981; Kumagai et al., 2014; Martínez-Valdez et al., 2015; Welkowitz et al., 1992). The attenuation coefficient from those published studies (Figure 7) varied between 0.35 – 1.3 $dBcm^{-1}$ MHz^{-1} (Bamber et al., 1977; Fujii et al., 2002; Garra et al., 1984; Goss et al., 1979; Itoh et al., 1988; Lu et al., 1999; López-Haro et al., 2009; O'Brien, 1988; Ophir et al., 1984; Parker et al., 1988, 1983; Taylor et al., 1986; Welkowitz et al., 1992).

The SoS of the liver measured in this study was shown to be within 5 ms^{-1} with studies by Bamber et al., (1979) and Martínez-Valdez et al., (2015) and was up to 33 ms^{-1} higher than Bamber et al., (1980), Chen et al., (1987); Kumagai et al., (2014); Martínez-Valdez et al., (2015); López-Haro et al., 2009; O'Brien, (1988) and Sehgal et al., (1986). The largest difference was found to be with Welkowitz et al., (1992) who reported a SoS of 1510 ms^{-1} at 2 MHz.

It is known that gas is more likely to be introduced into the liver during excision than in any other organ due to its highly vascular structure and its tendency to produce gas during autolytic decay. The presence of gas in specimens is reported to be the greatest problem in the preparation of soft tissue samples for acoustical measurements (Bamber, 1981). Measurements in this study were initiated within 6 minutes post euthanasia and during measurement sequences, the samples were kept in PBS at 37°C. Therefore, it is not believed that the high variability (18 $dBcm^{-1}$ at 32 MHz, see Figure 4) of the attenuation *coefficient* in this study derives from the production of gas due to autolytic decay.

Previous studies found an attenuation coefficient ranging between 0.44 – 0.65 $dBcm^{-1}$ MHz^{-1} (Itoh et al. 1988; Lu et al. 1999; Parker et al. 1988; Fujii et al. 2002). Even though the attenuation of liver has been studied extensively in various publications, there is an 8.8 $dBcm^{-1}$ variability in the attenuation coefficients at 9 MHz (Garra et al. 1984; Itoh et al. 1988; Lu et al. 1999; Maklad et al. 1984; Parker et al. 1988; Taylor et al. 1986). The attenuation of the liver has also been studied at similar frequencies to those used in this study. Wirtzfeld et al., (2015) found a difference of 26.5 $dBcm^{-1}$ at 32 MHz when compared with the results of this study. This difference could be due to the decellularised method used by Wirtzfeld et al., (2015) versus the fresh tissue *ex vivo* method used in this study. Furthermore, extending the second degree polynomial fit found in this study to lower frequencies (Figure 7), the data from this study was found to be in good agreement with the data published of bovine and human liver at 37°C up to 9 MHz (Foster et al., 1979; Fujii et al., 2002; Gammell et al., 1979; Goss et al., 1979; Lu et al., 1999). Also, the second degree polynomial fit calculated in this study was found to be in agreement within ±6 $dBcm^{-1}$ with pig, rat and human livers measured up to 9 MHz by López-Haro et al., (2009), O'Brien et al., (1988), Lu et al., (1999) and Gammell et al., (1977).

The attenuation versus frequency data for liver samples calculated in this study can also be expressed as a power-law of the form 1.08 $dBcm^{-1}$ MHz^{-1} (R^2=0.66). This power-law was found to be in good agreement (± 0.42 dB cm^{-1}) with those power-laws reported from pig (1.2 $dBcm^{-1}$ MHz^{-1}, López-Haro et al., (2009)), rat (1.3 ± 0.09 $dBcm^{-1}$ MHz^{-1}, O'Brien et al., (1988)) and human (1.6 ± 0.21 $dBcm^{-1}$ MHz^{-1}, Lu et al., (1999), 1.5 $dBcm^{-1}$ MHz^{-1}, Gammell et al., (1977)) livers.

Kidney

The difference in SoS values between the left and right kidney, using different dissection planes, was 0.97 ms^{-1}. Based on the second polynomial fit, the difference in attenuation coefficient was found to be a maximum of 1.31 dB cm^{-1} between the planes across the frequency range 12 – 32 MHz. Despite measuring the acoustic properties from different dissection planes the mean

attenuation values did not show a consistent variation. Previous work has shown the variation in the acoustic properties of the kidney are associated with five sections across the longitudinal axis in canine renal anatomy (Sarvazyan et al., 1983). In that study, the SoS showed a difference of 5 ms^{-1} and a difference of 0.5 $dBcm^{-1}$ at 8.8 MHz in dog's kidney (from the *cortex* through to the *renal veins*).

In this study, an endeavour was made to ensure measurements were undertaken within the medulla in both dissection planes. The limited variation in our measurements would suggest that this has been achieved. The acoustic properties found for both the left and the right kidney were combined by taking the mean value and compared with those shown in the literature. The mean magnitude of the SoS values from the kidney was found to lie within the range of values obtained from studies published on human and mouse kidneys at different temperatures (Table 3). The inter-sample attenuation as a function of frequency was found to vary up to 5 $dBcm^{-1}$ at 30 – 32 MHz and the smallest difference, 1 $dBcm^{-1}$, was seen at 3 MHz. In Figure 8, the polynomial fit calculated in this study is compared with published studies. The magnitude of the attenuation data calculated using the second degree polynomial fit calculated in this study fall within the magnitude of attenuation found in the published studies. This polynomial fit was found to be smaller by 2.7 $dBcm^{-1}$ with data from Gammell et al., (1979), Goss et al., (1979) and Welkowitz et al., (1979) and higher by up to 1.6 $dBcm^{-1}$ with data reported by Worthington et al., (2001) in the frequency range from 1 – 9 MHz. *The attenuation versus frequency data from kidney can be fitted to a power-law curve. The power law fit obtained was 0.73 $dBcm^{-1}$ MHz^{-1} (R^2=0.81). This fit gave values of attenuation as 0.33 $dBcm^{-1}$ MHz^{-1} higher than the attenuation measured from bovine and porcine kidney at 37°C and at 45°C (Goss et al., 1979; Worthington et al., 2001).* These differences could be attributable to differences in animal kidneys or to the difference due to the temperature at which the studies were undertaken up to 65°C (Worthington et al., 2001). The kidney has been studied up to 35 MHz by Wirtzfeld et al., (2015), the difference in the attenuation *coefficient* with this study was found to be up to 10.2 $dBcm^{-1}$ at 32 MHz.

Comparison with TMM

The frequency range used in this study (12 – 32 MHz) falls out-with the range over which the IEC guidelines give recommended values (2 – 10 MHz). However, assuming that dispersion is insignificant, the biggest difference in the SoS from recommended TMM SoS values was found in liver tissue (64 ms^{-1}).

For the attenuation coefficient, the polynomial-fits calculated from the brain, liver and kidney tissue data were compared with previously published acoustical measurements from the IEC agar-TMM (Figure 9) up to 50 MHz. The attenuation of kidney matched with the IEC agar-TMM with a consistent difference of 0.5 $dBcm^{-1}$ in the frequency range 12 to 32 MHz. This difference falls within the 2 dB cm^{-1} SD specified for the IEC agar-TMM attenuation (Rabell Montiel et al., 2017). The biggest difference in the attenuation coefficient was found to be with liver tissue of 14 $dBcm^{-1}$ at 32 MHz when compared with the IEC agar-TMM (Rabell Montiel et al., 2017).

CONCLUSIONS

The acoustical properties of mice soft tissue samples (brain, liver and kidney) were measured over the frequency 12 – 32 MHz while immersed in PBS at 37°C. The samples were obtained from recently euthanized C57BL/6 healthy male mice with a mean age of 6.9 ± 3.9 months. Measurements were undertaken within 6 minutes after euthanasia and then at 5 and 10 minute time-points after the first measurement.

The measured SoS of the brain, liver and kidney was found to be 1566.3 ± 9.9 ms^{-1}, 1604.7 ± 16.8 ms^{-1} and 1574.9 ± 10.8 ms^{-1} respectively. For all the small animal soft tissues, the SoS results were comparable with those published at lower ultrasound frequencies (1 – 9 MHz).

The attenuation of the small animal soft tissue samples was shown to increase with increasing frequency. The attenuation coefficient was found to be nonlinear as a function of frequency and was modelled as second degree polynomials: $0.7533f + 0.006477f^2$ (R^2=0.85) for brain, $0.7252f + 0.01414f^2$ (R^2=0.70) for liver, and $0.5771f + 0.006322f^2$ (R^2=0.83) for kidney.

Research into the acoustical properties of soft tissue based on the structure of the organ during normal and abnormal function is vitally important (Sarvazyan et al., 1983) as this information is useful for diagnosis (Kumagai et al., 2014).

Finally, quality assurance (QA) phantoms are made of TMM which mimics the acoustic properties of soft tissue. The use of high frequency ultrasound for both clinical and preclinical applications has increased in recent years resulting in a need to develop a relevant TMM suitable for use at these high frequencies. The acoustic properties of soft tissue have been previously assessed up to 9 MHz and at 15 – 35 MHz. Establishing the acoustic properties of soft tissue at high frequency is a required first step in the development of a suitable TMM QA phantom. Currently, the IEC guideline does not provide the necessary guidance data to develop a TMM suitable for frequencies above 10 MHz. Furthermore, to reproduce the acoustic properties of small animal soft tissue using

344 the IEC agar-TMM as a base, a modification in the IEC agar-TMM recipe must be generated to match
345 the SoS of the brain, liver and kidney at these higher frequencies. Therefore, the data provided in
346 this study can be used as a basis upon from which a recipe for TMM, which is representative of
347 tissue properties at high frequencies, can be based.

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LIST OF FIGURES

the acoustical data collected from 20 mouse livers and was extended to low frequencies (dotted line) for comparison purposes.

Figure 8. Attenuation versus frequency of the kidney soft tissue data published in the literature and the second degree polynomial fit calculated in this study. The polynomial fit was calculated from the acoustical data collected from 20 mouse kidneys (10 left and 10 right) and was extended to low frequencies (dotted line) for comparison purposes.

Figure 9. Attenuation versus frequency of polynomial fit found in this study, comparison with the attenuation data for IEC agar-TMM (IEC, 2001; Rabell-Montiel et al., 2017).

Table 1

Table 1. The SoS and SD (ms^{-1}) measured within 6 minutes post euthanasia (t=0) and then at t=5 and t=10 minutes. Measurements were performed using a Vevo 770® preclinical ultrasound scanner over the frequency range of 12 – 32MHz.

Organs	Mean SoS ± SD (ms^{-1})		
	first measurement (t=0)	**t=5 minutes**	**t=10 minutes**
Brain	1565.9 ± 9.6	1566.1 ± 9.5	1566.9 ± 11.2
Liver	1604.4 ± 16.5	1603.8 ± 15.9	1604.7 ± 18.2
Kidney	1575.3 ± 10.8	1574.8 ± 11.9	1574.1 ± 9.5

Table 2

Table 2. Mean SoS and SD (ms^{-1}) of the small animal soft tissue samples, brain, kidney and liver measured using the Vevo 770® preclinical ultrasound scanner over the frequency range of 12 – 32MHz.

Organs	**Brain**	**Liver**	**Kidney**
SoS ± SD (ms^{-1})	1566.33 ± 9.9	1604.7 ± 16.8	1574.9 ± 10.8

Table 3

Table 3. Values of SoS (ms^{-1} ± SD) of the small animal soft tissue samples, brain, liver and kidney from published studies. The values measured in this study has been added for comparison purposes only. Blank spaces indicate that no information is available.

Organ	Temperature (°C)	Frequency (MHz)	SoS ± SD (ms^{-1})	Source of tissue	Reference
Brain	37	1 – 5	1562 ± 1.2	human	Kremkau et al., 1981
		1	1510		Welkowitz et al., 1992
		12 – 32	***1566.33 ± 9.9***	***mouse***	***THIS STUDY***
Liver	22	100	1570 ± 10	rat	O'Brien et al., 1988
	room	100	1550		Tervola et al 1985
	36.3		1596 ± 4.8	human	Kumagai et al., 2014
		2	1510		Welkowitz et al., 1992
	37	3	1578.3 ± 5.4		Chen et al., 1987
	37.2		1578.1 ± 2.9		Sehgal et al., 1986
	20	1 – 6	1577 ± 11		Bamber & Hill, 1980
	37	1 – 7	1607		Bamber & Hill, 1979
		1 – 7	1597-1639	bovine	Bamber & Hill 1979
		3.5	1579	pig	Lopez-Haro et al., 2009
	23-26	100	1565 ± 7.8 and 1567 ± 13.2	sheep/cat	Frizzel & Gindorf 1981
	21.8	5	1588.2	chicken	Martinez-Valdez et al., 2011
	46	5	1609.8		
	37.2	***12 – 32***	***1604.7 ± 16.8***	***mouse***	***THIS STUDY***
Kidney		2	1560	human	Welkowitz et al., 1992
	37.2	100	1560.2 ± 1.8		Rajagopalan et al., 1980
	37	3.5 – 7	1571	pig	Worthington et al., 2001
	23 – 26	100	1586 ±10.7	***mouse***	Frizzel & Gindorf 1981
	37.2	***12 – 32***	***1574.9 ± 10.8***		***THIS STUDY***

Figure 1

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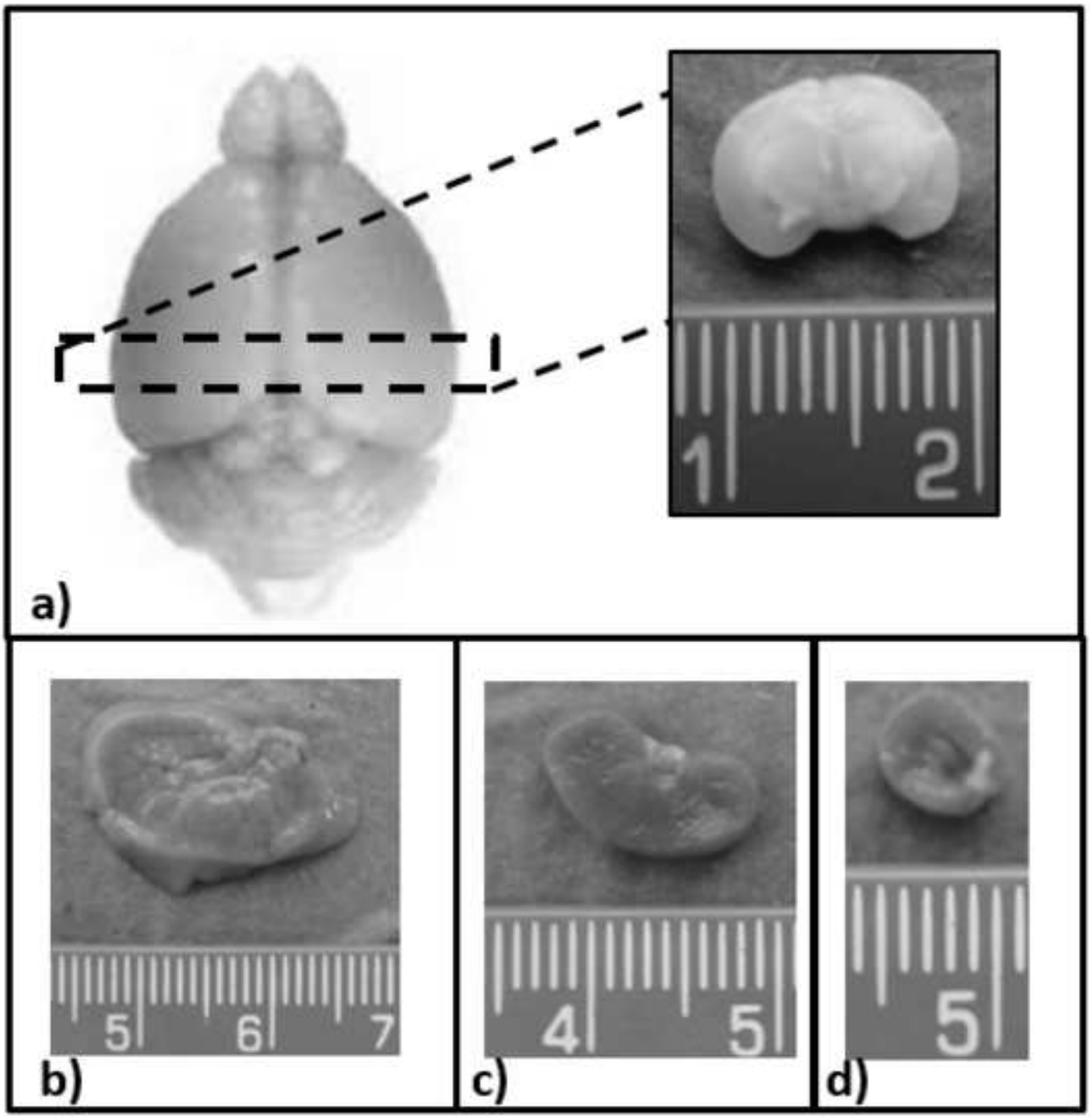

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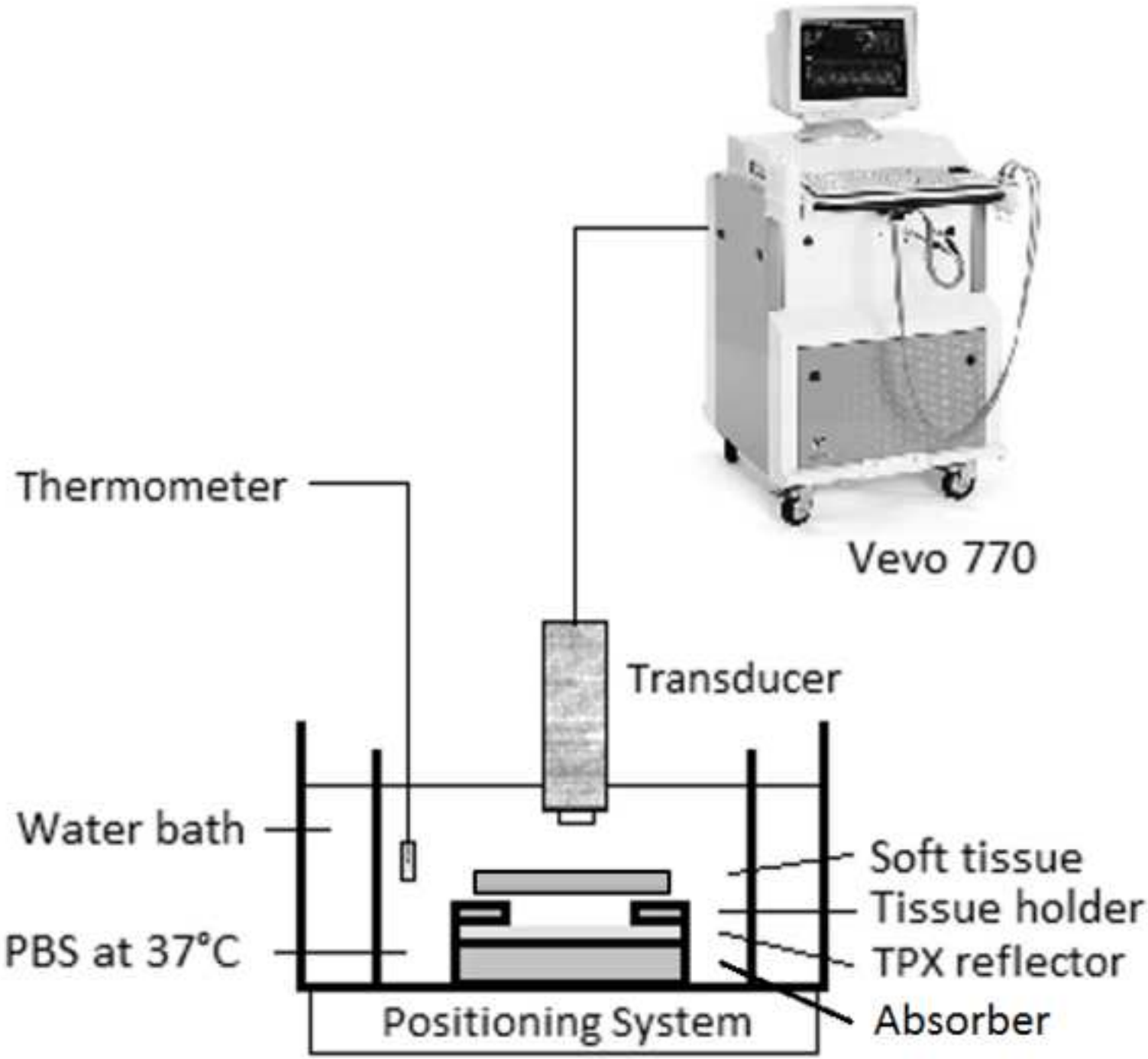

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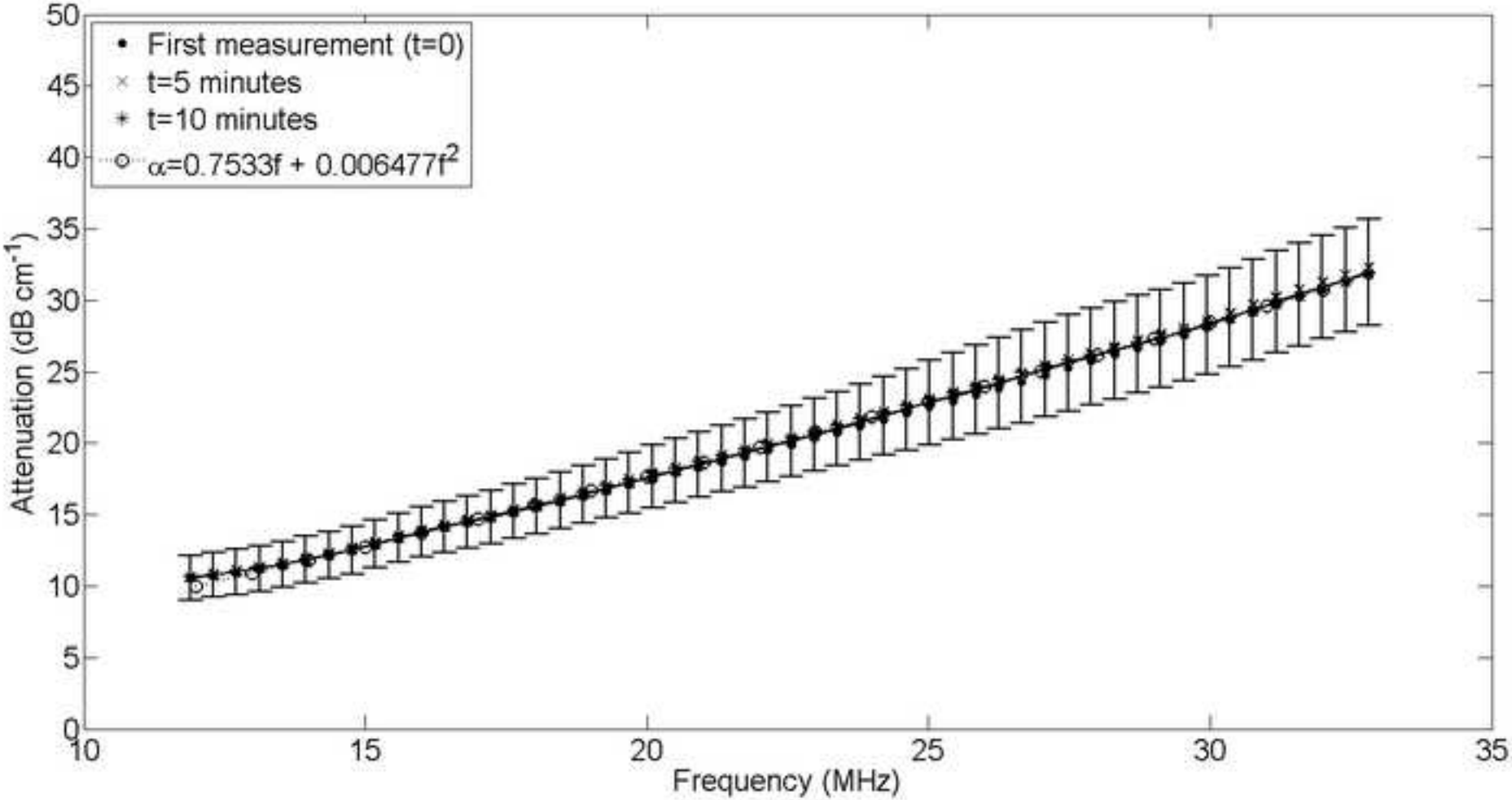

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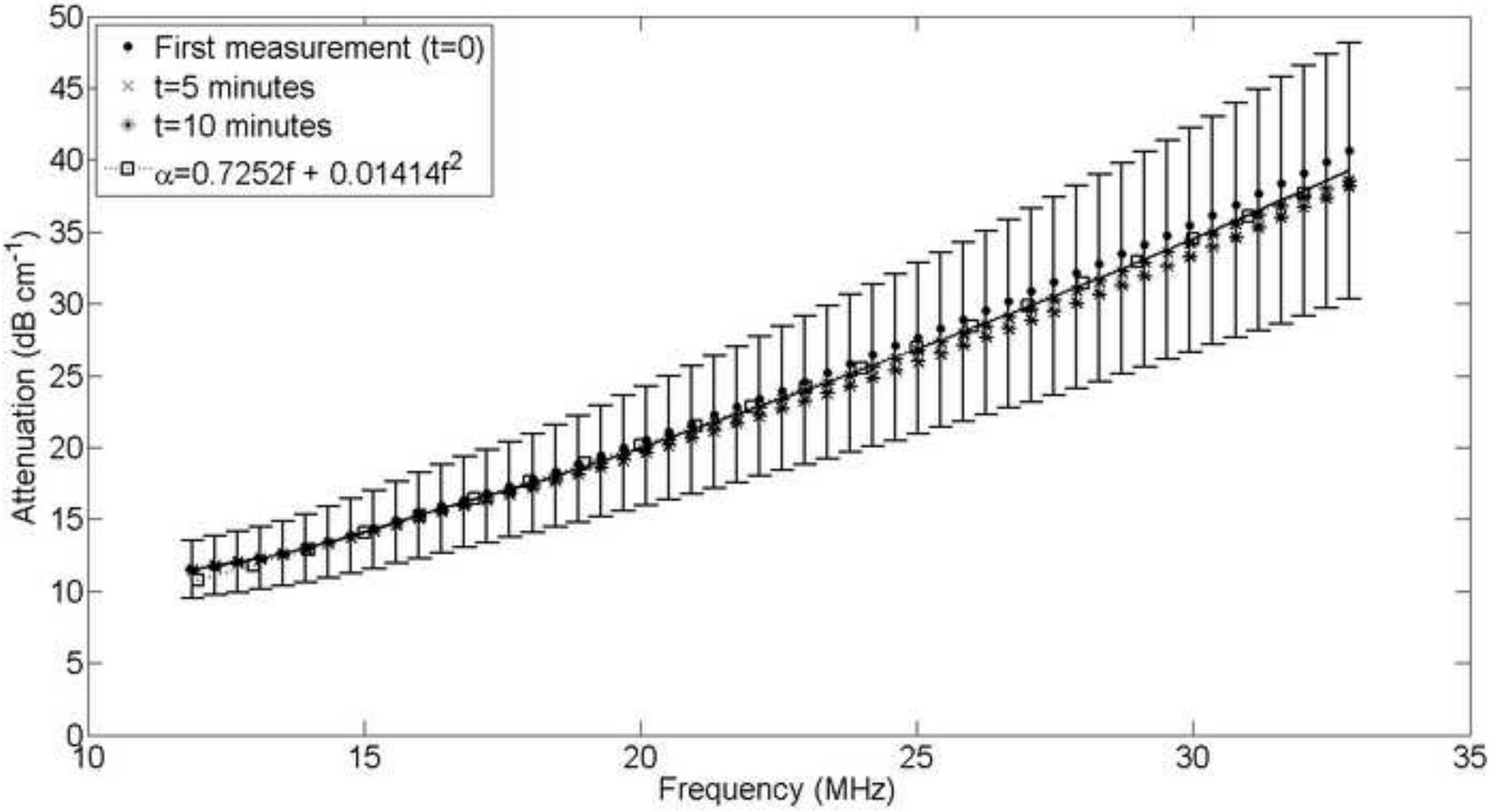

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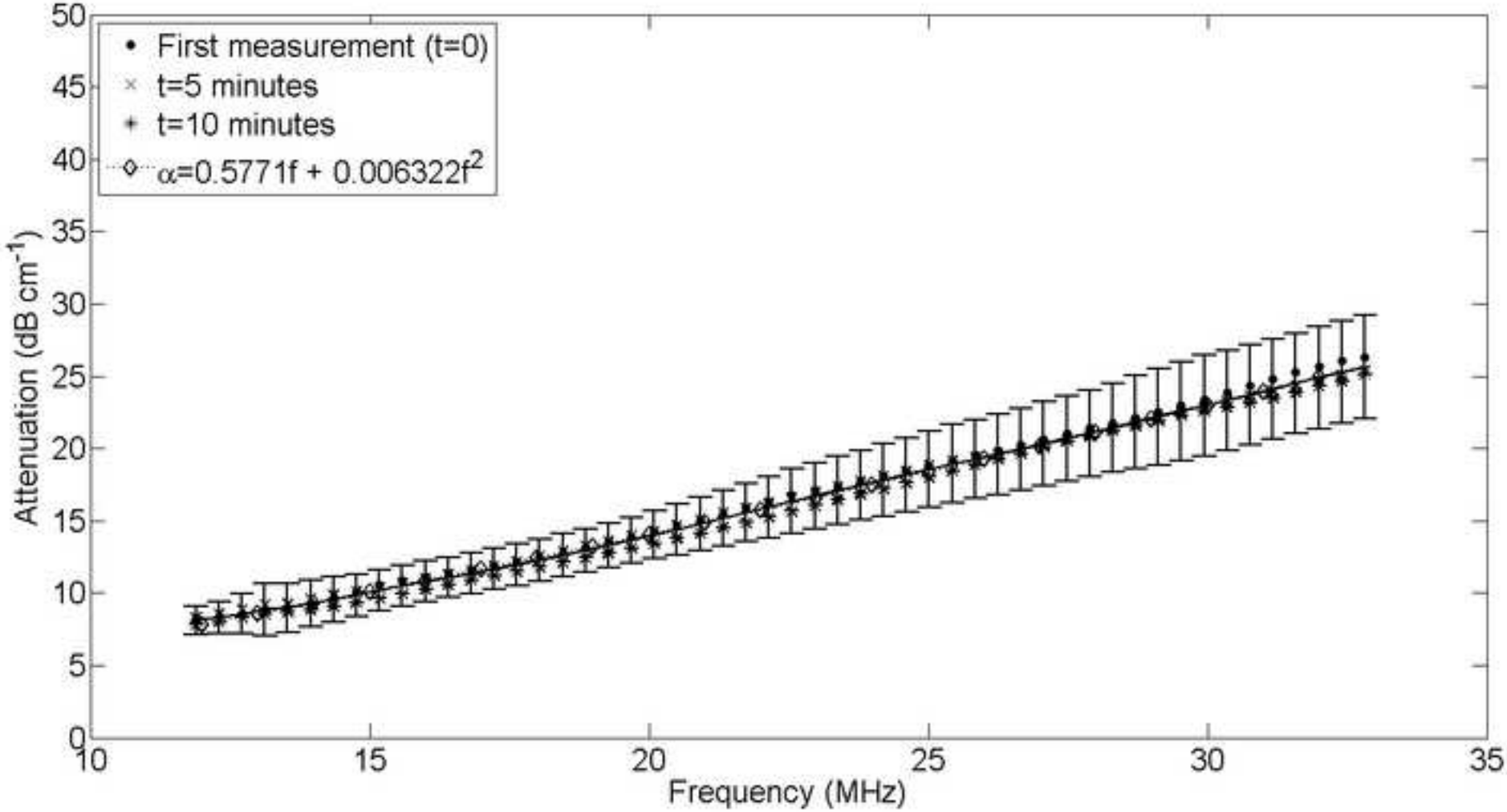

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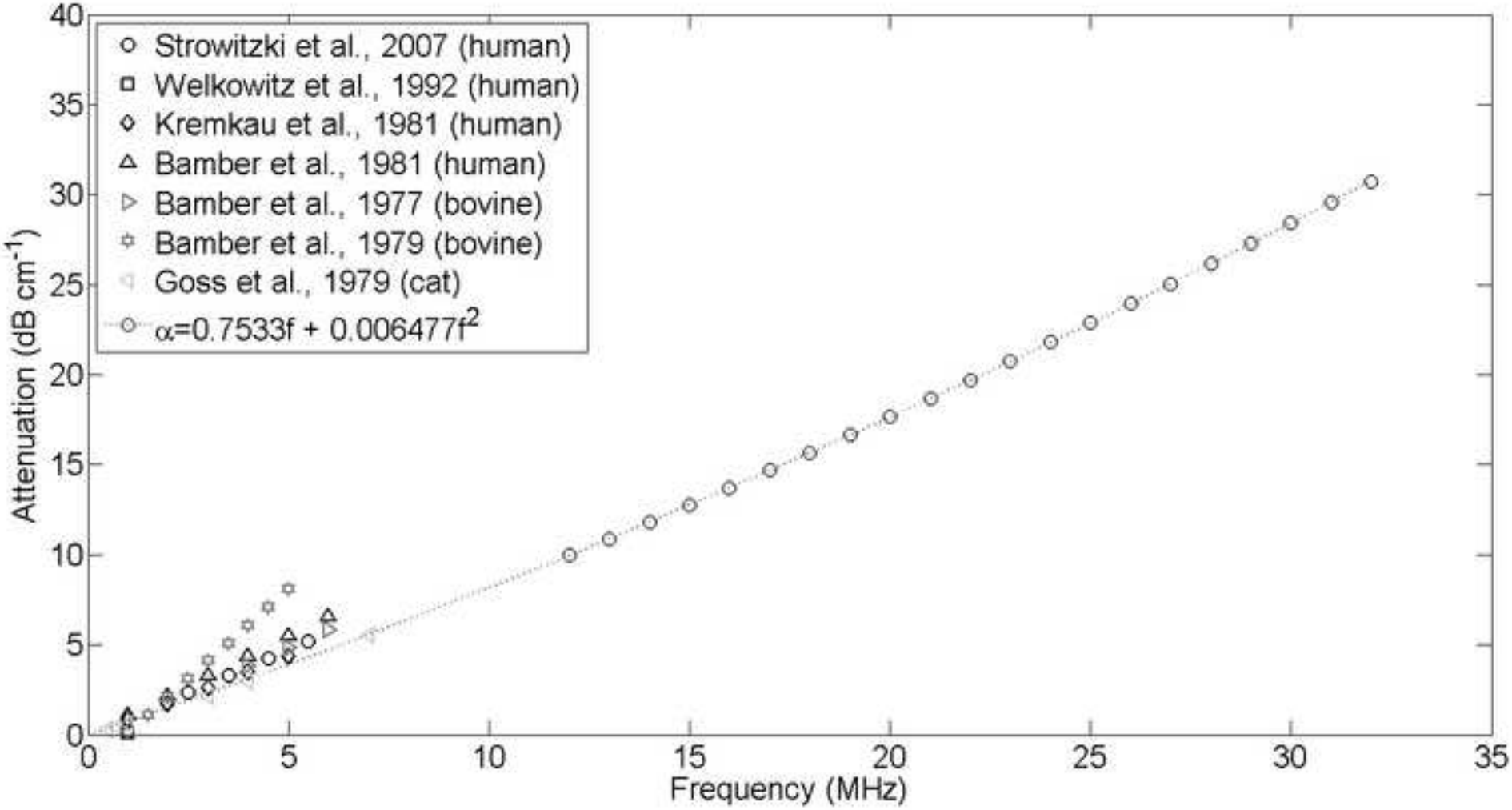

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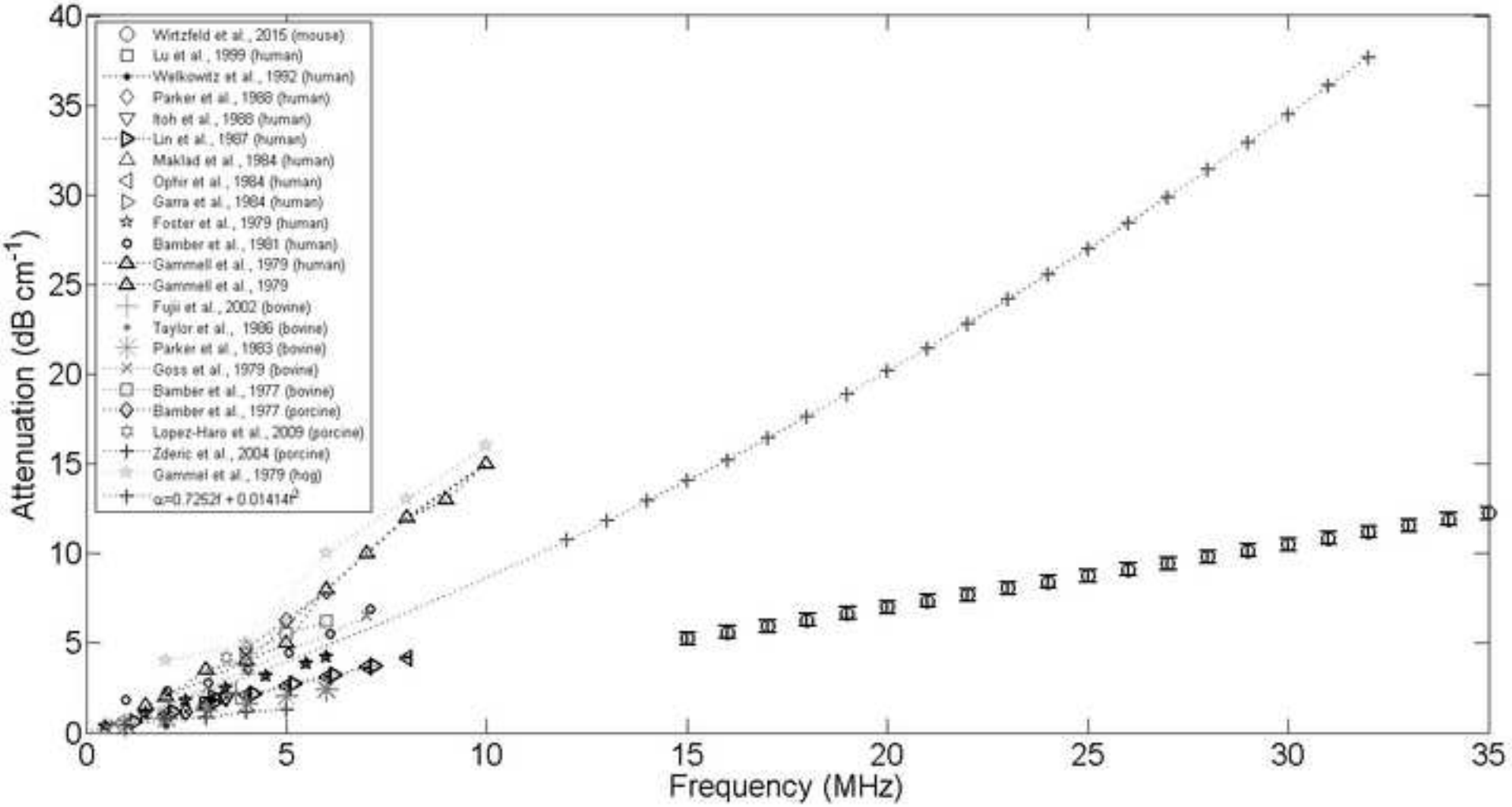

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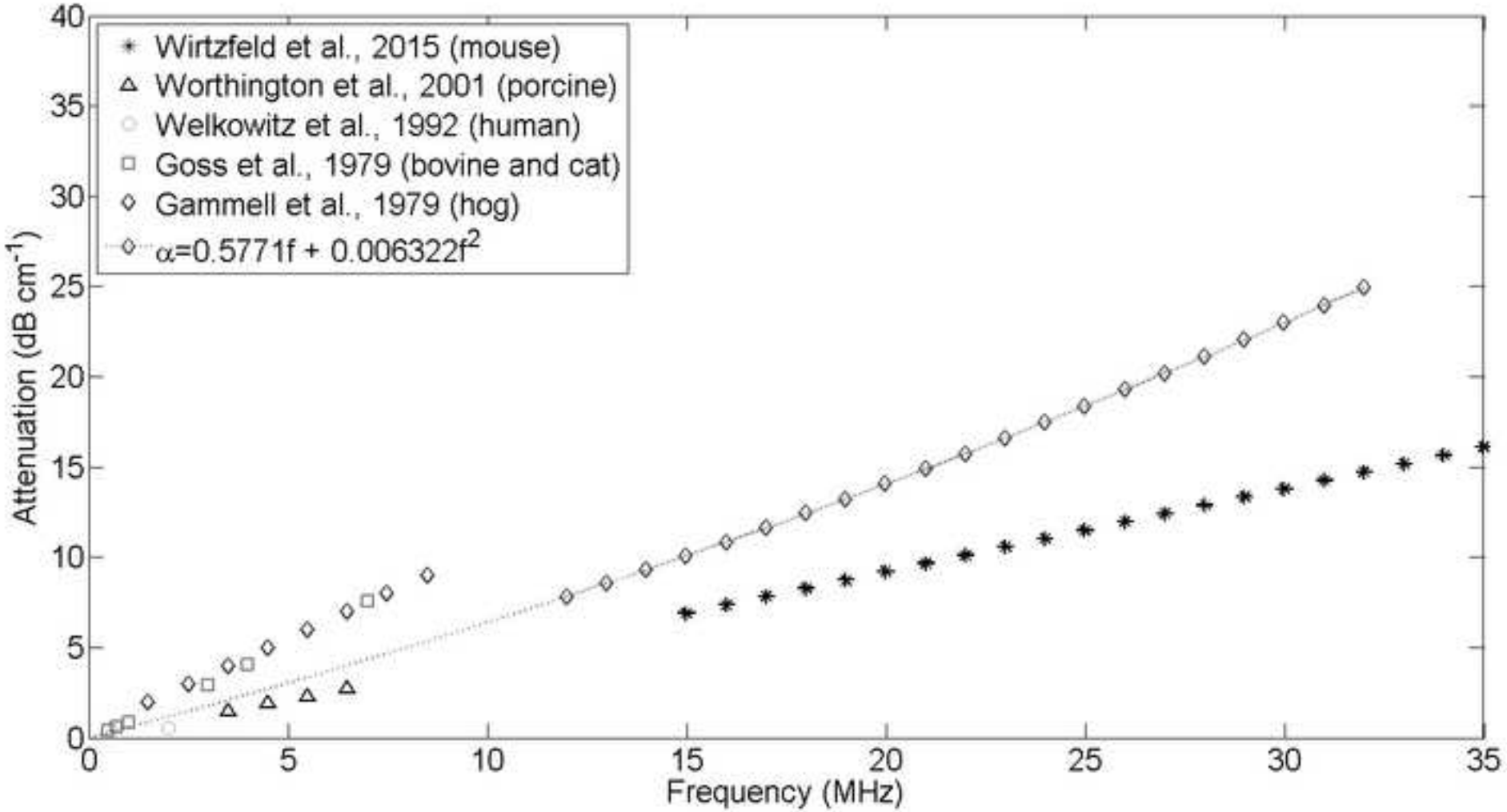

Figure 9

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