Ultrasonic transparency of sonication tubes exposed to various frequencies: A metrological evaluation of modifications and uncertainty of acoustic pressures

Ultrasonic transparency of sonication tubes exposed to various frequencies: a metrological evaluation of modifications and uncertainty of acoustic pressures

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Abstract

Correlations between biological phenomena and ultrasonic exposure often involve mechanical and thermal factors. Cavitation proved capable of interacting with other factors, making awkward the evaluation of their individual effects. In microbiological research, the presence of a dual effect of ultrasound on microorganisms, namely bactericidal and stimulating, required development of methods enabling analysis of ultrasonic field effects, shielded from those of cavitation. This work shows how acoustic wave action may be analysed with a metrological approach, excluding cavitational effect and measuring acoustic pressure acting upon a sonication tube. Results show how such a goal was achieved in a repeatable and reproducible way, avoiding acoustic wave degeneration.

Keywords: ultrasounds; acoustic pressure; cavitation effect; hydrophone; uncertainty evaluation.

Introduction

Ultrasound (US) and ultrasonic techniques have a broad range of applications, covering e.g. mechanical, chemical, electronics, food industry typically for decontamination purposes, not to mention their usefulness in medical diagnostics and therapeutics. In medical applications, characterization of US activity on eukaryotic and prokaryotic still require substantial work. As far as biological effects are concerned, two independent parameters of the ultrasonic wave, intensity and frequency, determine treatment effectiveness; thus surgical effects on soft tissues require high frequency and high intensity levels, while stimulation of cellular metabolism calls for low frequency and low intensity [1]. Some regions of this two-parameters space were explored and related effects reported in literature, particularly in connection with prokaryotic cells metabolism. Thermosonic, manosonic and manothermosonic US treatments are mentioned by some authors for their anti-biofilm action, while diagnostic ultrasounds are sometimes described as enhancers of bacterial viability [1]. These ambiguous effects caused some misunderstandings about the influence of ultrasounds on prokaryotic cells. In the 2003 the works of Pitt and Piyasena showed clearly that, when bacteria are exposed to an ultrasonic field, both phenomena of destruction and stimulation may coexist and interfere [1,2,3]. This competition can have different outcomes owing to various influencing factors, such as bacterial species involved, nature of medium through which ultrasonic waves propagate, presence of cavitation phenomena and, last but not least, structure of bacterial community (planktonic or biofilm form) [1,4,5,6,7,8]. Cavitation ranks high among the most studied influencing factors [3,9,10,11]; it occurs when high intensity ultrasonic waves, with frequencies typically between 10 kHz and 1 MHz, originate negative pressure in a liquid medium. This leads to formation of gas and vapour bubbles which eventually collapse, generating shock waves and high temperature spots [12,13]. Cavitation bubbles typically target permeability of cellular membrane, with a strong bactericidal effect [10,11]. Action of acoustic pressure (p_{AC}) on living cells in absence of cavitation bubbles apparently attracted a minor amount of investigation [2,14]. Furthermore, p_{AC} seems to be associated to an increased cellular oxygenation and nutrient adsorption (favouring the growth rate) [2,15] but also to a greater antibiotic sensibility [16,17]. To our knowledge, precious few data are present in literature about antimicrobial effects of p_{AC} in non-cavitated media. The present work deals with a method aimed at assessing the influence of low intensity ultrasound on the metabolism of prokaryotic cells, *in vitro* planktonic and free floating forms being considered.

Experimental Apparatus and Methods

The effects of low-intensity US on micro-organisms was tested using a modified sonication bath (Branson 3200 Ultrasonic Cleaner, 30 x 15 x 15 cm^3). Built-in electronics of a commercial cleaning tank was replaced by an external broad-band amplifier driven by function generator. To avoid contaminations of culture media from the medium propagating ultrasonic waves, de-mineralised water in our case, prokaryotic cells were grown in test tubes, immersed in the sonication bath [15].

Exposition of culture medium to ultrasound is affected by position in the bath of test tubes, by their shape and their material. A standard measurement procedure was developed, aimed at measurement reproducibility with low uncertainty levels. As a first step, three different test tube shapes were tried out to assess their transparency to ultrasonic waves in a frequency range from 20 kHz to 40kHz.

Three test tubes were selected among those more frequently used in biological and chemical laboratories: a glass tube and polyurethane tube, both with hemispherical bottom and same size (17 mm dia. by 100 mm length), and a polyurethane tube with truncated cone bottom (17 mm dia. by 120 mm length).

A custom positioning system was added to the sonication bath accommodating an array of test tubes in well-defined locations in the host tank, enabling replacement of the tubes with minimal perturbation of the environment in terms of acoustic pressure level and field geometry (Fig. 1). Acoustic pressure measurements were performed using a piezoelectric needle hydrophone, 3 mm OD, equipped with a glass coating, previously calibrated in the working frequency range at the "O. M. Corbino" Institute in Rome for determining the mean sensitivity *S*, as the ratio of the output O_c to the calibration pressure p_c:

$$S = \frac{O_c}{p_c} \qquad (1)$$

Values of the mean sensitivity *S* for each frequency used are given in Table I, together with the related uncertainty intervals (95% confidence level). Table I shows also the hydrophone mean sensitivity S_{dB} expressed in decibel as ratio to a reference S_0 = 1 V/μPa, with the related uncertainty intervals. In formulas:

$$S_{dB} = 20\log_{10}\left(\frac{S}{S_0}\right) \qquad (2)$$

The sound pressure of an acoustic field can be obtained from a measurement of the electrical signal *O* produced by the hydrophone in the open-circuit condition, using the relevant sensitivity *S*:

$$p_{AC} = \frac{O}{S} = \frac{O}{S_0 \cdot 10^{\frac{S_{dB}}{20}}} \qquad (3)$$

Three preliminary measurement sessions, each session consisting of five replications, were performed with no test tubes in the bath, yielding a set of 15 measurements for each frequency. The electrical signals are expressed in microvolt, then, according to sensitivities shown in Table I, values of p_{AC} expressed in kilopascal may be derived. Table II shows mean values and uncertainty intervals (at 95% confidence level) of p_{AC} for each frequency. The expanded uncertainty of p_{AC} takes into account resolution and reproducibility of output, and hydrophone calibration uncertainty, see e.g. the uncertainty budget associated to 20 kHz frequency in Table III [18].

In particular, equation (3) represents the mathematical model considered, where O is the voltage amplitude from the hydrophone, S and S_{dB} are the frequency-dependent sensitivities.

The resolution of O measurements is 1 μV, their reproducibility, i.e. the standard deviation of 15 measurements, is 14 μV. The hydrophone calibration uncertainty is 2 dB (see Table I), which corresponds to a standard uncertainty equal to 0.63 dB (assuming 95% confidence level and 3 degrees of freedom). Further details on methods for uncertainty evaluation are given in [19].

Referring to Table II, frequencies which correspond to the lowest value of uncertainty in p_{AC} evaluation have been chosen, see Fig. 2 .

Three measurement sessions, with three different types of test tubes, with five replications each were performed for the chosen frequencies. Uncertainties on p_{AC} were calculated in the same way as the preliminary session without tubes; results are shown in Fig. 3(a-h).

Discussion and conclusions

A prerequisite to in vitro evaluation of low-frequency US effects on bacterial cell is identification of critical aspects liable to affect measurements.

The simplest system enabling work with US waves is a sonication bath, enabling to perform experiments with low frequency US in a controlled and calibrated system. Characterization of acoustic pressure within the bath is however made difficult by reflections and cavitation. Furthermore, bacterial metabolism may hardly be analysed by putting microorganisms directly inside the filling medium of the tank, owing to the sheer difficulty of controlling bacterial concentration within the bath, the inevitable contamination of the entire system, and the wide variability of effects due to broad variation of acoustic pressure within the bath, brought about also by cavitational bubbles.

Therefore placement of microorganisms within a small, well controlled volume in the sonication bath is required, in order to meet reasonable targets in terms of reproducibility and repeatability, and to reduce to an acceptable level the effects of cavitational shock waves.

In the current experiment cavitational effect is avoided mainly using low pressure (under 100 kPa), limiting measurement range well below the cavitation threshold. The main aim of the work was overall characterization of sonication bath in terms of repeatability of sound pressure levels at test tube locations, considering the position of hydrophone (always the same, in the reference system of the bath walls) as representative of the acoustic conditions in each tube. The final result is a consistent map relating voltage applied to the power amplifier and the acoustic pressure level in each test tube at a discrete set of frequency values. For each frequency, it is possible to identify the tube to be used to avoid an excessive alteration of the acoustic pressure (Fig. 3). In particular, all the tubes showed an adequate compatibility with the p_{AC} calculated for the *t0* condition (preliminary measurements) as confirmed by a normalized error (NE) evaluation [20]. Given the adequacy of the NE values, the tube with the lowest value of uncertainty was chosen for each frequency (e.g. the tube *t1* for the frequency 24 kHz). If all the three tested tubes show an unacceptable value of uncertainty, no tube is chosen (e.g. the case of frequency 36 kHz).

Such a calibration map may then be exploited to assess the effects of low-level ultrasound (below the cavitation threshold) on populations of living prokaryotic cells confined in the test tubes, maintained at the corresponding calibration positions. Further work shall be aimed at assessing the role of low-intensity ultrasound on bacterial growth, minimizing the effects of influence factors like environmental changes and cavitation activity.

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Captions

Figures

Fig. 1. Sonication bath with the custom positing system.

Fig. 2. Selected frequencies with the lowest value of uncertainty in p_{AC} evaluation.

Fig. 3 (a, b, c, d, e, f, g, h). Uncertainties on p_{AC} relevant to the preliminary measurement session (t_0) and to the three subsequent measurement sessions with different types of test tubes (t_1, t_2 and t_3).

Figure 1

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Figure 2

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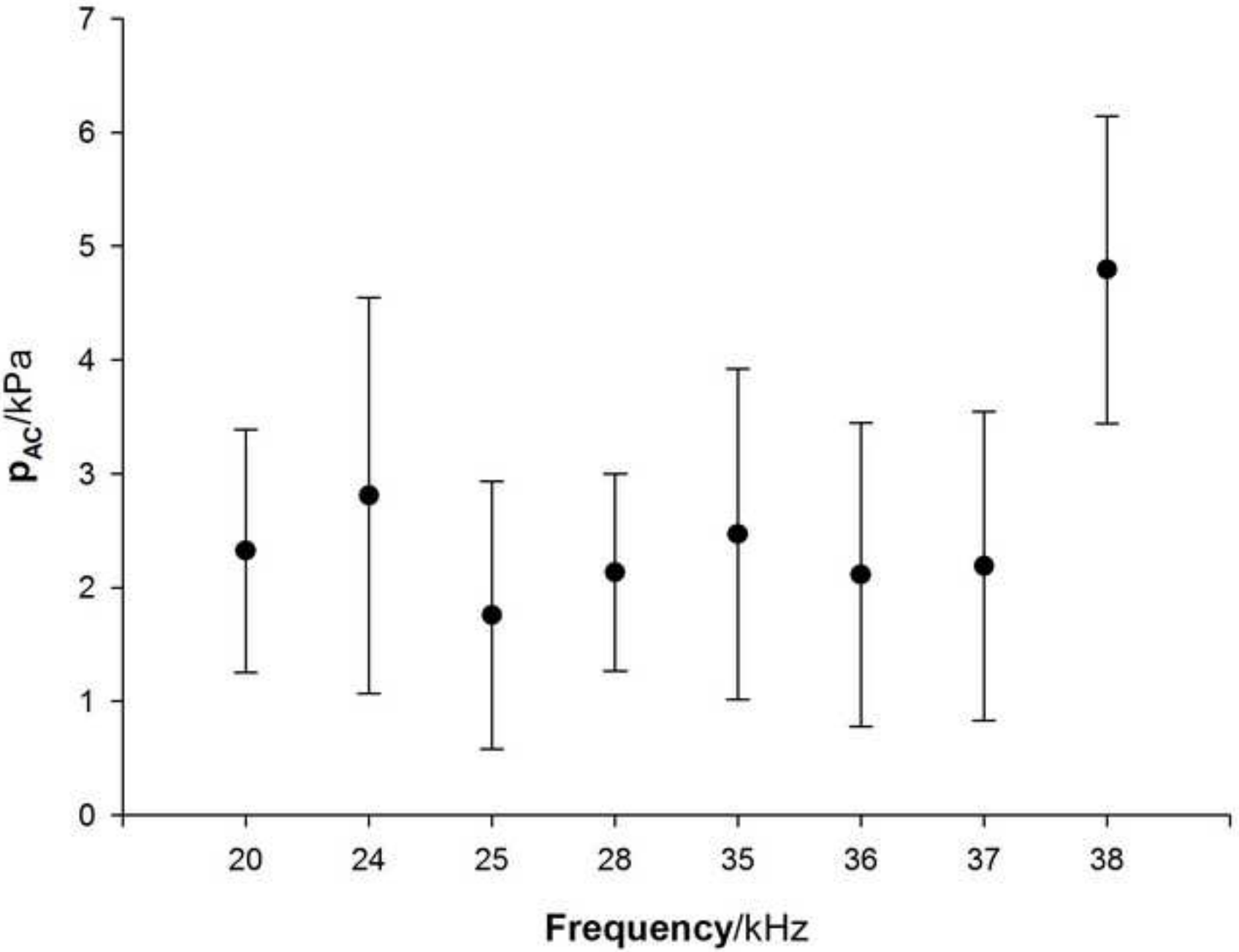

Figure 3a

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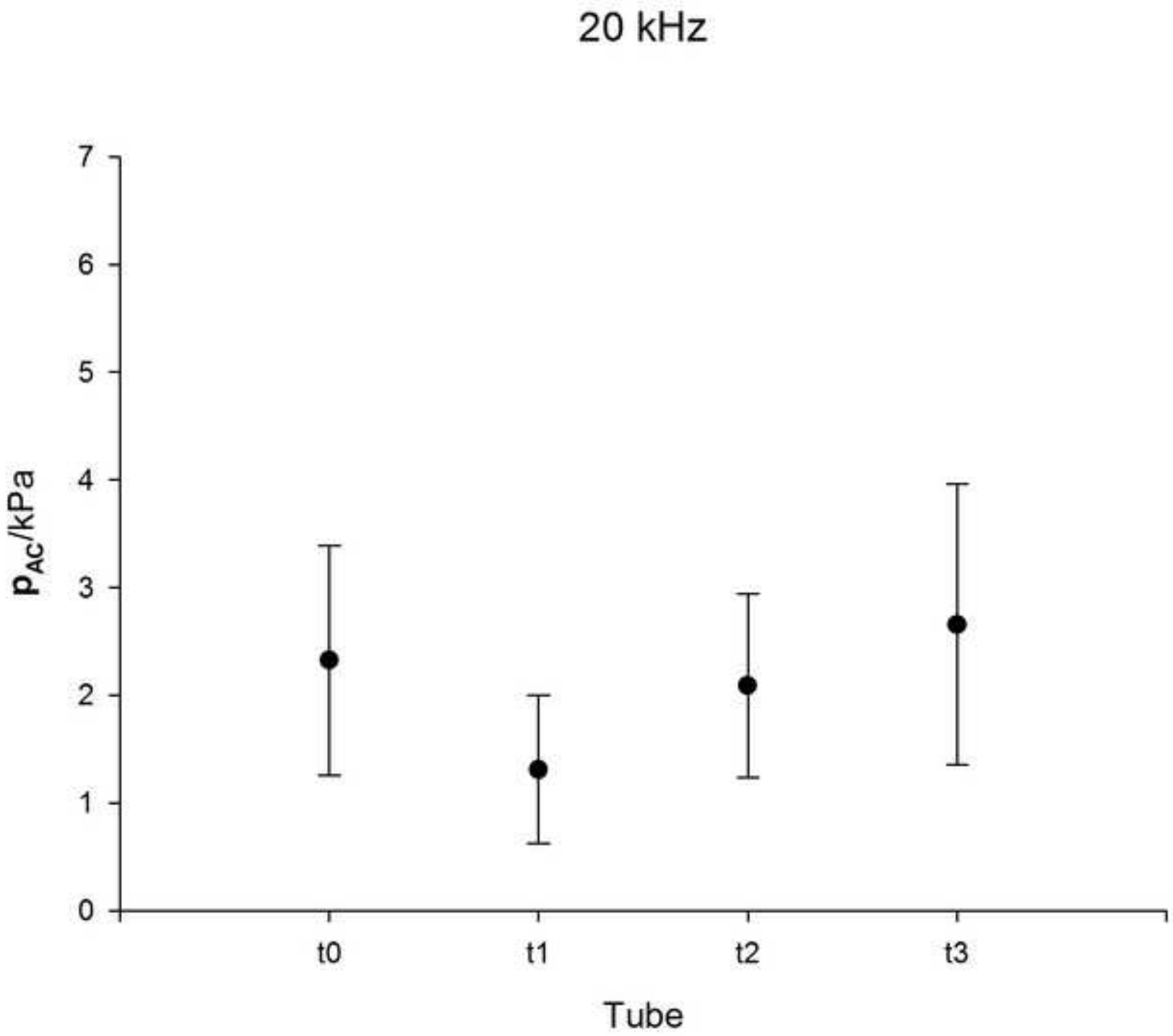

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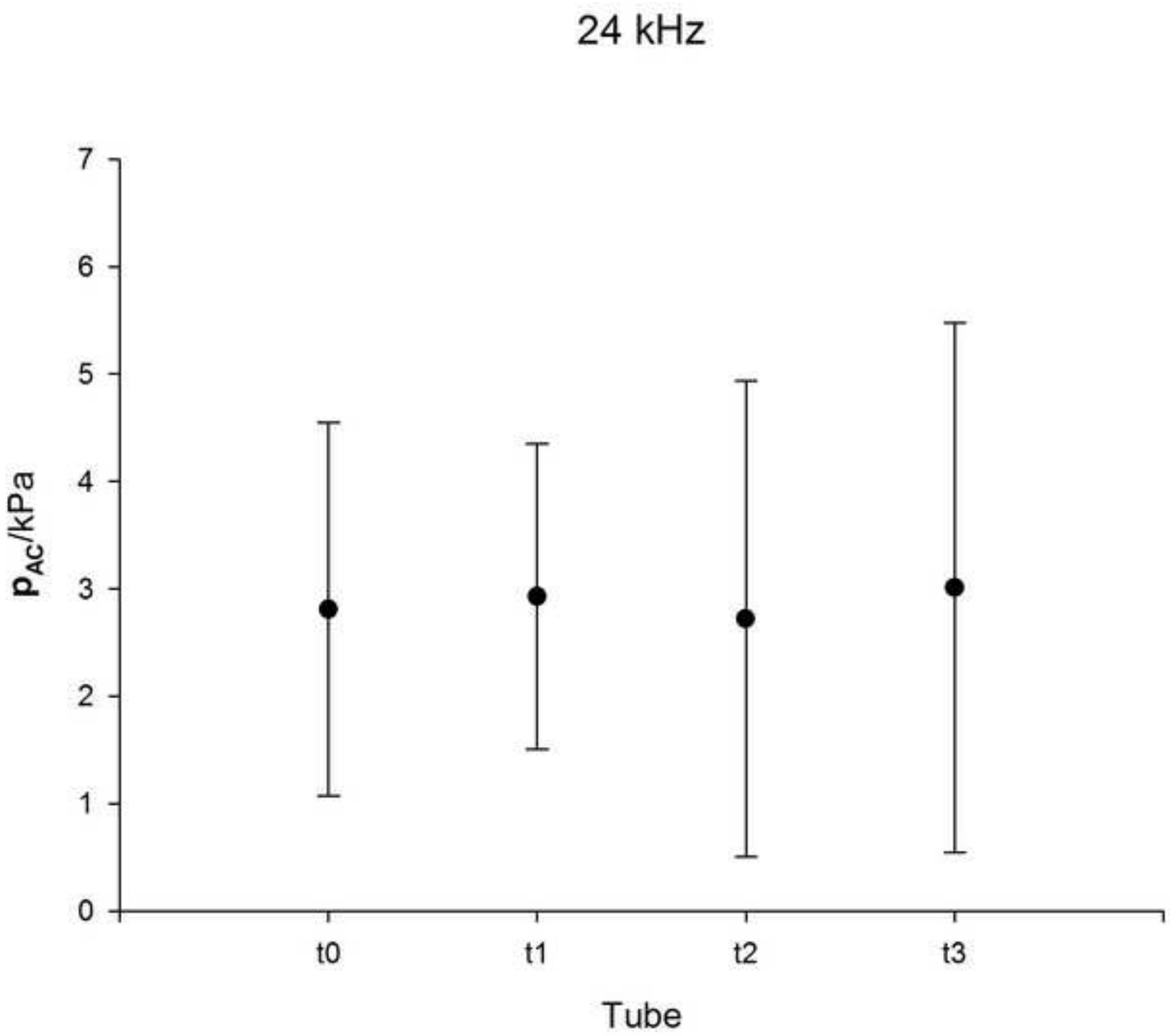

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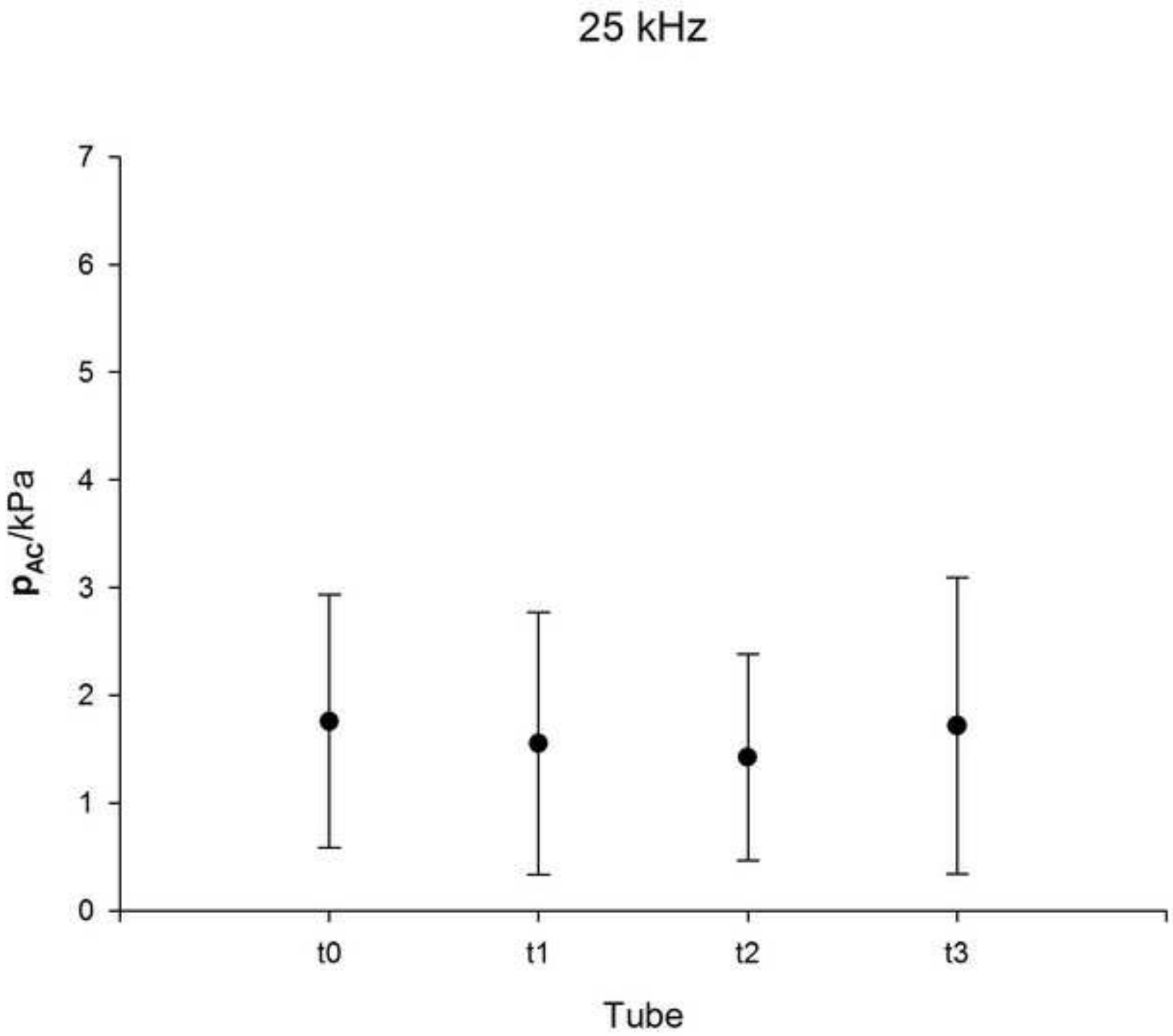

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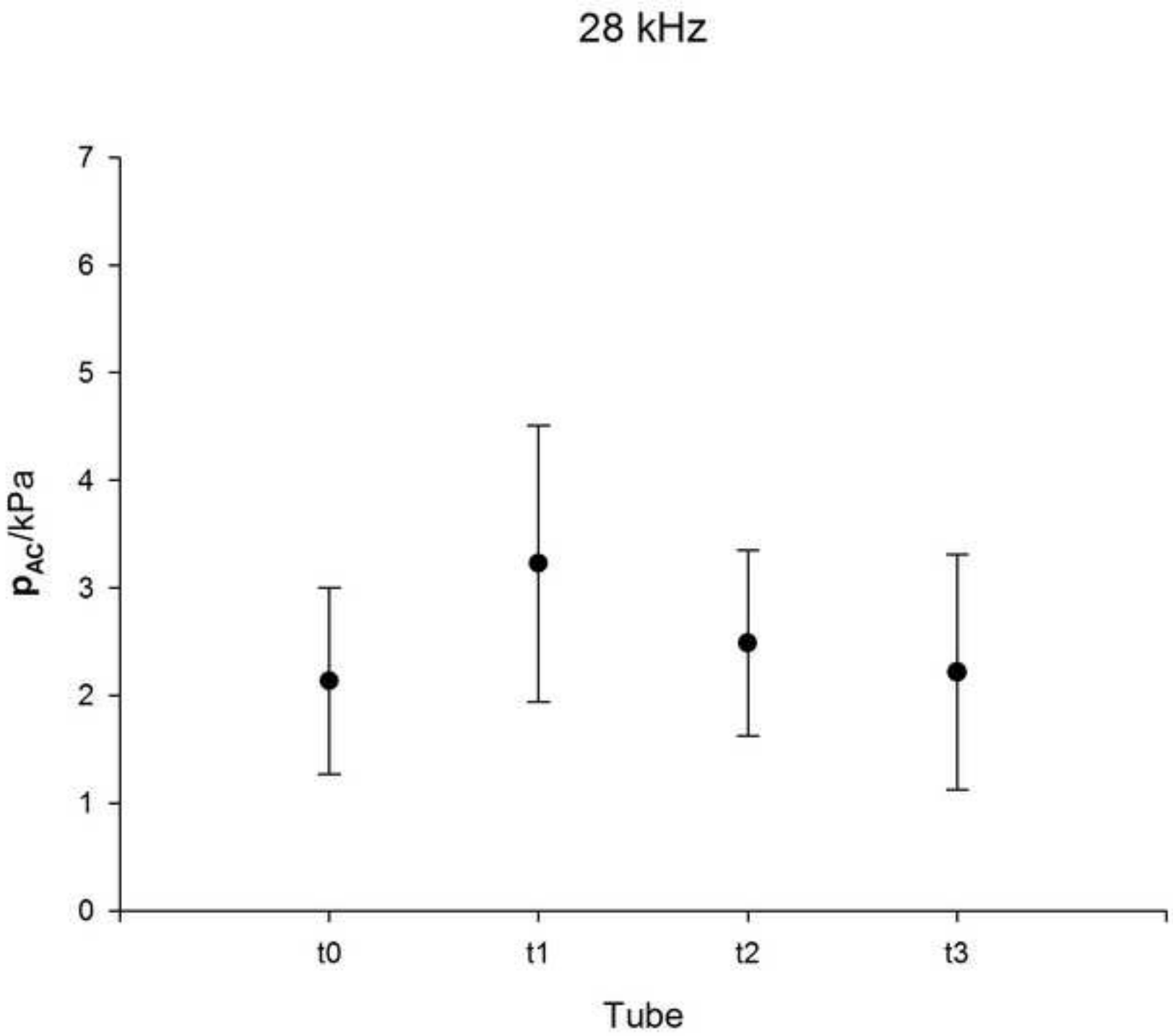

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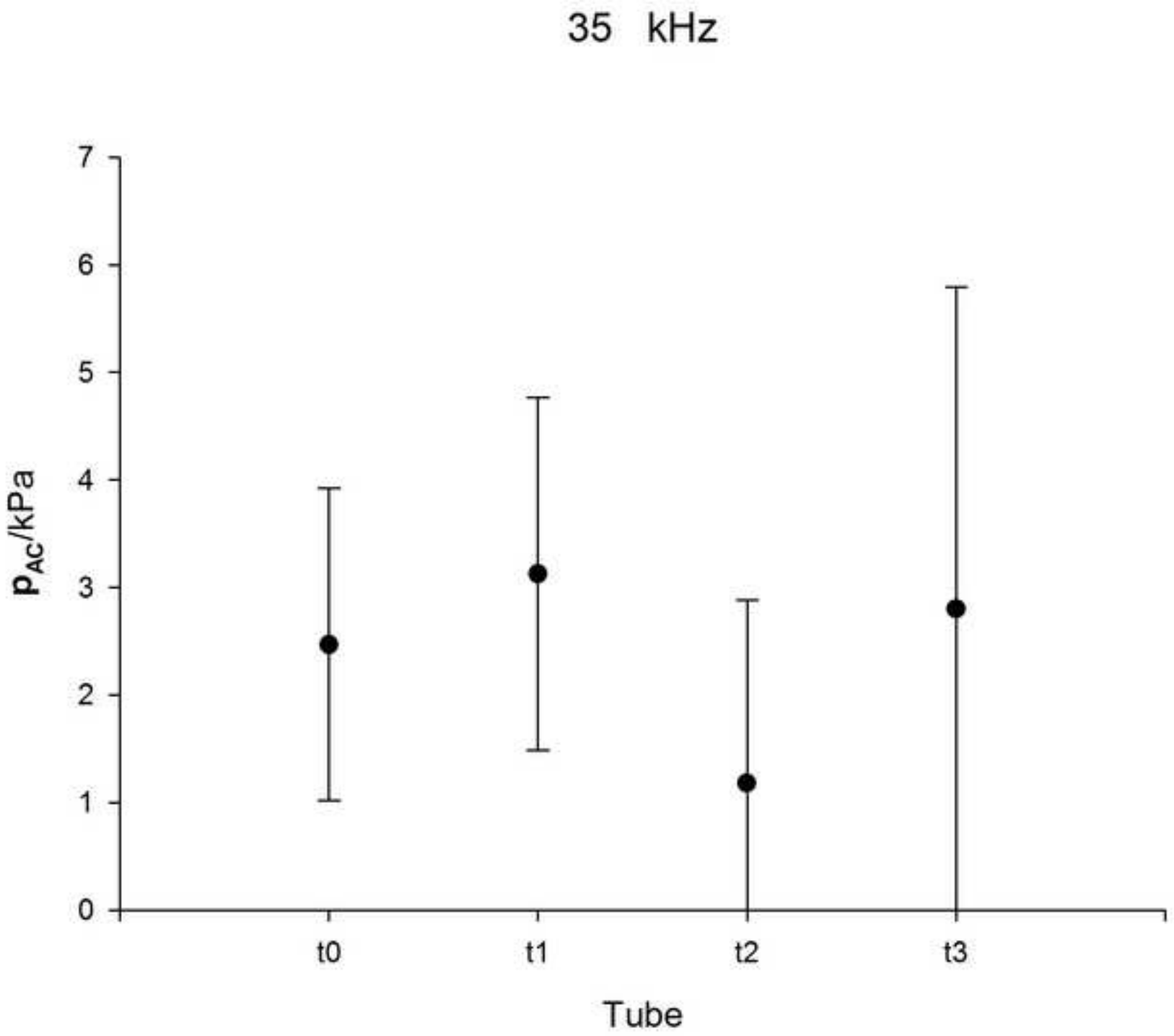

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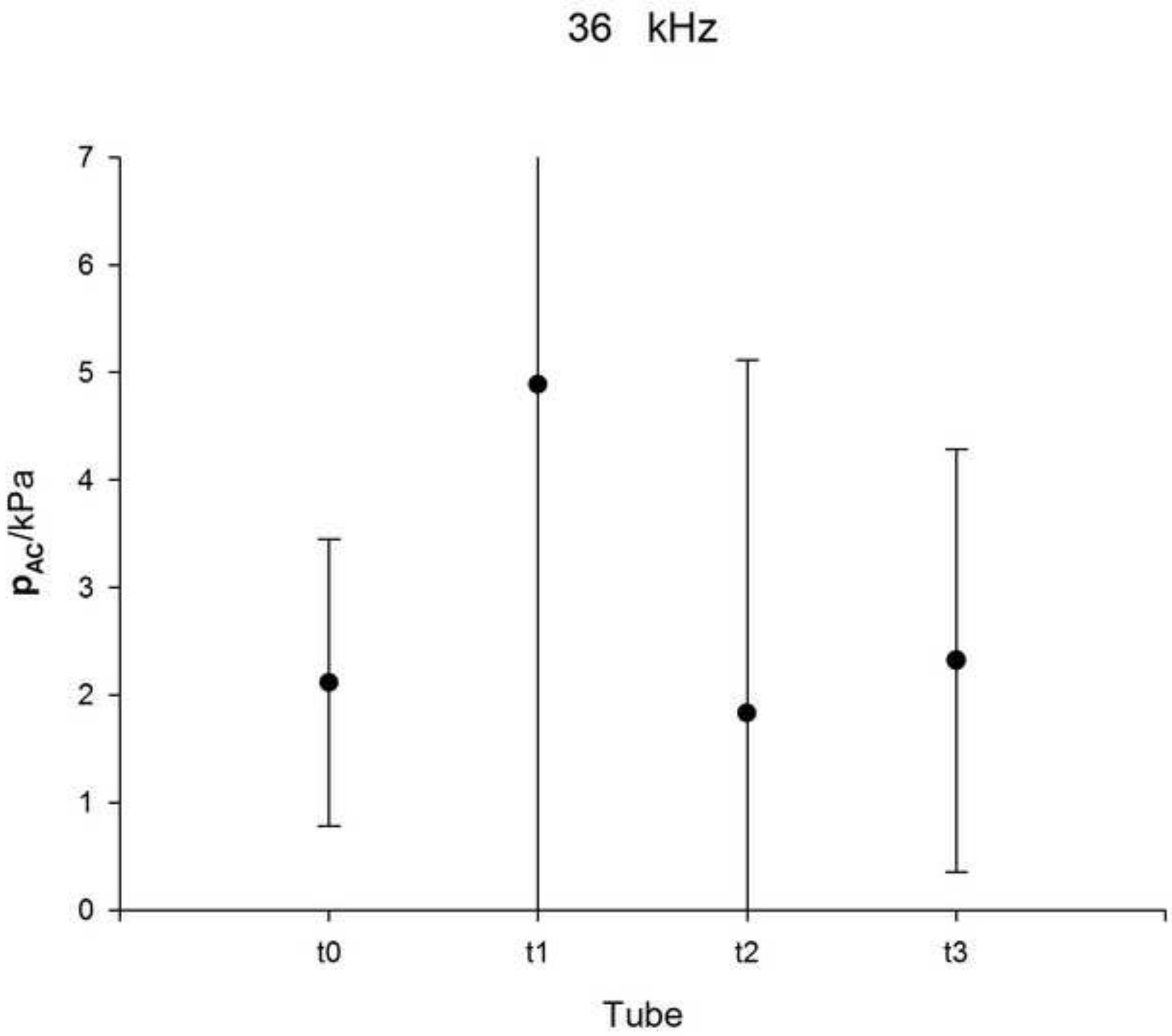

Figure 3g

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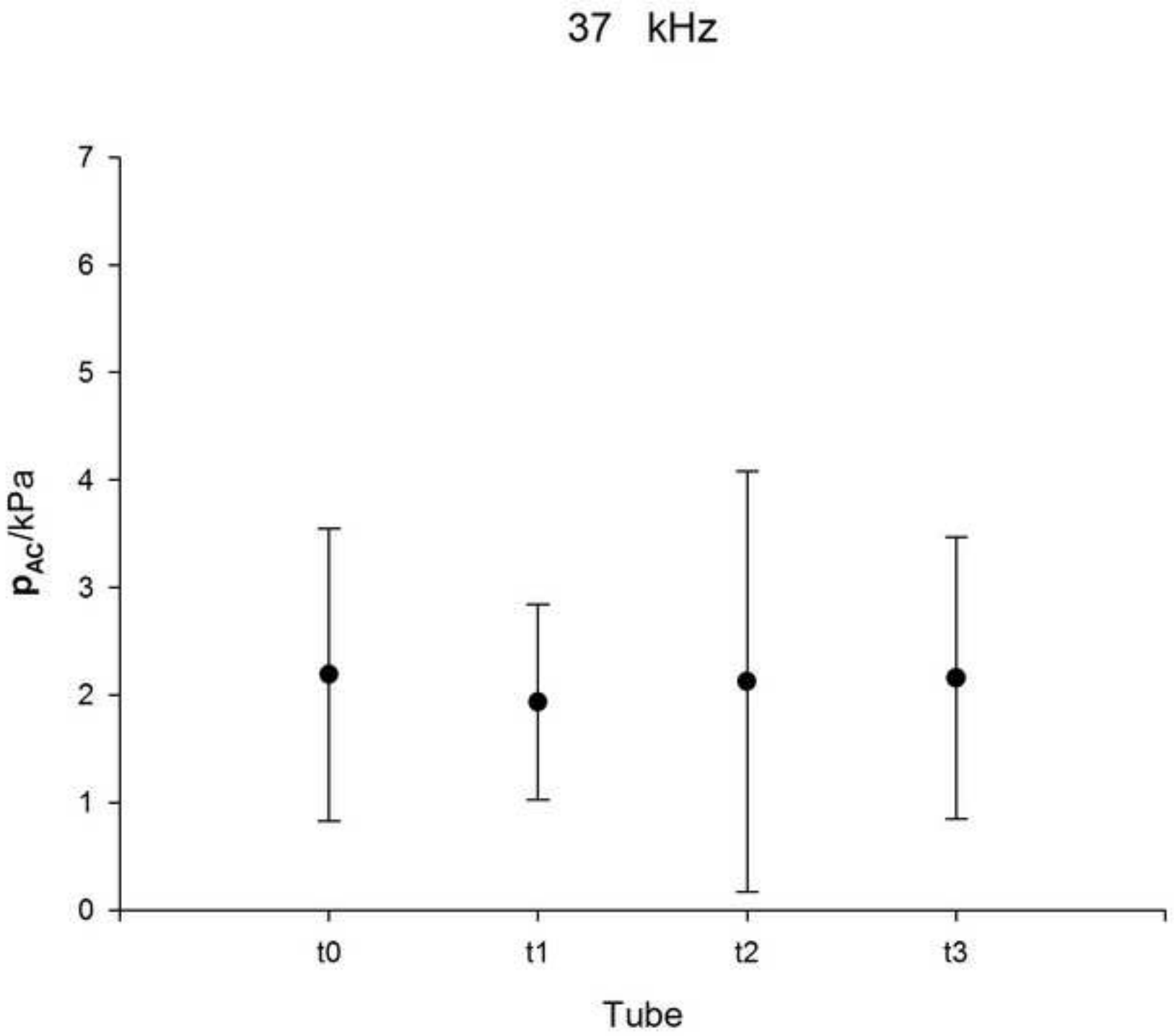

Figure 3h

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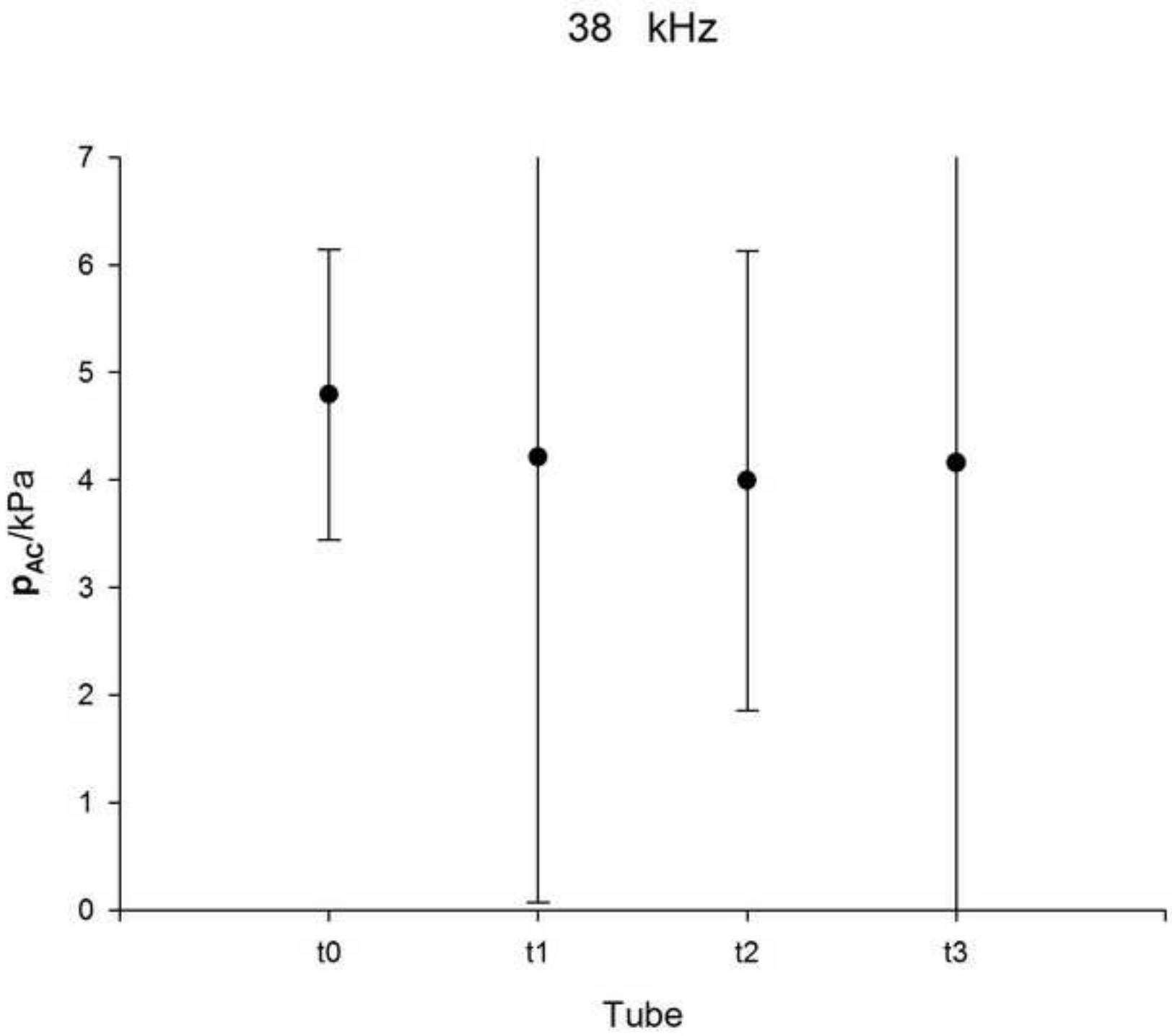

Table(s)

Table I. Hydrophone calibration. Mean values m and lower limit L_L and upper limit U_L of 95% confidence intervals (uncertainty intervals of sensitivity S), expressed as dB re 1V/µPa and as µV/Pa, at explored frequencies.

Frequency/kHz	S_{dB} / (dB re 1V/µPa)			S/ (µV/Pa)		
	m	L_L	U_L	m	L_L	U_L
20	-270.6	-272.6	-268.6	$2.96 \cdot 10^{-2}$	$2.35 \cdot 10^{-2}$	$3.73 \cdot 10^{-2}$
21	-272.6	-277.9	-267.4	$2.33 \cdot 10^{-2}$	$1.28 \cdot 10^{-2}$	$4.26 \cdot 10^{-2}$
22	-274.4	-278.2	-270.6	$1.90 \cdot 10^{-2}$	$1.23 \cdot 10^{-2}$	$2.95 \cdot 10^{-2}$
23	-272.6	-274.9	-270.4	$2.34 \cdot 10^{-2}$	$1.80 \cdot 10^{-2}$	$3.03 \cdot 10^{-2}$
24	-268.6	-270.3	-266.8	$3.73 \cdot 10^{-2}$	$3.06 \cdot 10^{-2}$	$4.55 \cdot 10^{-2}$
25	-269.5	-275.8	-263.3	$3.34 \cdot 10^{-2}$	$1.63 \cdot 10^{-2}$	$6.83 \cdot 10^{-2}$
26	-269.6	-271.8	-267.3	$3.32 \cdot 10^{-2}$	$2.56 \cdot 10^{-2}$	$4.31 \cdot 10^{-2}$
27	-266.8	-269.3	-264.3	$4.55 \cdot 10^{-2}$	$3.41 \cdot 10^{-2}$	$6.07 \cdot 10^{-2}$
28	-265.2	-266.0	-264.4	$5.51 \cdot 10^{-2}$	$5.03 \cdot 10^{-2}$	$6.04 \cdot 10^{-2}$
29	-267.3	-273.1	-261.4	$4.33 \cdot 10^{-2}$	$2.20 \cdot 10^{-2}$	$8.50 \cdot 10^{-2}$
30	-267.5	-271.3	-263.7	$4.22 \cdot 10^{-2}$	$2.71 \cdot 10^{-2}$	$6.57 \cdot 10^{-2}$
31	-267.2	-267.8	-266.5	$4.38 \cdot 10^{-2}$	$4.06 \cdot 10^{-2}$	$4.72 \cdot 10^{-2}$
32	-265.5	-266.4	-264.6	$5.32 \cdot 10^{-2}$	$4.79 \cdot 10^{-2}$	$5.89 \cdot 10^{-2}$
33	-263.9	-264.5	-263.4	$6.36 \cdot 10^{-2}$	$5.96 \cdot 10^{-2}$	$6.79 \cdot 10^{-2}$
34	-264.0	-264.8	-263.3	$6.29 \cdot 10^{-2}$	$5.76 \cdot 10^{-2}$	$6.88 \cdot 10^{-2}$
35	-265.0	-266.6	-263.3	$5.63 \cdot 10^{-2}$	$4.65 \cdot 10^{-2}$	$6.82 \cdot 10^{-2}$
36	-264.6	-266.3	-262.8	$5.91 \cdot 10^{-2}$	$4.85 \cdot 10^{-2}$	$7.21 \cdot 10^{-2}$
37	-263.2	-265.5	-260.9	$6.94 \cdot 10^{-2}$	$5.34 \cdot 10^{-2}$	$9.01 \cdot 10^{-2}$
38	-263.3	-264.6	-262.0	$6.86 \cdot 10^{-2}$	$5.90 \cdot 10^{-2}$	$7.98 \cdot 10^{-2}$
39	-264.3	-265.8	-262.8	$6.10 \cdot 10^{-2}$	$5.15 \cdot 10^{-2}$	$7.22 \cdot 10^{-2}$
40	-262.3	-263.0	-261.6	$7.67 \cdot 10^{-2}$	$7.09 \cdot 10^{-2}$	$8.30 \cdot 10^{-2}$

Table II. Mean values m and lower limit L_L and upper limit U_L of 95% confidence intervals (uncertainty intervals) of p_{AC}. Computed negative values, devoid of physical meaning, are replaced with 0*.

Frequency/kHz	p_{AC} /kPa		
	m	L_L	U_L
20	2.3	1.3	3.4
21	5.1	1.9	8.3
22	6.4	3.2	9.7
23	6.7	0*	14.3
24	2.8	1.1	4.5
25	1.8	0.6	2.9
26	3.8	0*	8.7
27	2.0	0*	4.7
28	2.1	1.3	3.0
29	1.5	0*	3.3
30	3.0	0.4	5.6
31	5.4	0.4	10.5
32	2.9	0.1	5.7
33	3.2	0.1	6.3
34	3.6	0.8	6.5
35	2.5	1.0	3.9
36	2.1	0.8	3.4
37	2.2	0.8	3.5
38	4.8	3.4	6.1
39	5.2	2.4	8.0
40	3.7	0*	7.9

Table III. Uncertainty table for p_{AC} relevant to the frequency 20 kHz, showing main contributions and resulting expanded uncertainty. For the resolution the value k_{aj} was set to 3 as a uniform distribution was assumed.

	x_j		s_j	a_j	k_{aj}	$u^2(x_j)$	c_j	$u_j^2(p_{AC})$	ν_j	$u_j^4(p_{AC})/\ \nu_j$
Symbol	**Value**	**Note**								
O	69	Res		0.5	3	$8.3\cdot10^{-2}$	$3.4\cdot10^{-2}$	$9.5\cdot10^{-5}$	100	$9.1\cdot10^{-9}$
		Repr.	14.0			$2.0\cdot10^{2}$	$3.4\cdot10^{-2}$	$2.2\cdot10^{-1}$	14	$3.6\cdot10^{-3}$
S_{dB}	-270.6	Acc.	0.63			$4.0\cdot10^{-1}$	$-2.7\cdot10^{-1}$	$2.8\cdot10^{-2}$	3	$2.6\cdot10^{-4}$
p_{AC}	2.3		Variance of p_{AC}, $u^2(p_{AC})$					$2.5\cdot10^{-1}$	Σ	$3.9\cdot10^{-3}$
			Standard deviation of p_{AC}, $u(p_{AC})$					$5.0\cdot10^{-1}$	ν_{pAC}	16
			Confidence level					95%		
			Coverage factor (Student's t)					2.1		
			Expanded uncertainty, $U(p_{AC})$					1.1		