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MONITORING THE EFFECTS OF CAVITATION ULTRASOUND ON *ARTEMIA SALINA* LARVAE

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A b s t r a c t

Ultrasound may produce a combination of chemical, thermal, and mechanical effects. The effects of two different ultrasound frequencies on larvae of the crustaceans *Artemia salina* were tested. The crustaceans *Artemia salina* belong to the *Anostraca* family group and live in inland salt lakes, salt pans, etc. They are used to test the effects of chemical substances on the environment. The effects of ultrasound were tested on freshly hatched larvae of 1 mm in length on average. The larvae hatched in 24 hrs at 25 °C in prepared "sea water". In addition, groups of 50 larvae were placed in cylindrical vessels with the bottom made of a plastic-sheet membrane and filled with 50 ml of "sea water". These samples were then insonated. A total of ten different insonation periods from 10 to 600 sec were used. To keep the larvae at a constant temperature during exposure, the vessels with larvae were placed in a cooling water and ice bath during experiments. The temperature of the samples was maintained between 20 and 21 °C. Subsequently, numbers of surviving *Artemia salina* larvae in individual samples were recorded at 24-hr intervals for five days. Insonation was performed at frequencies of 1 MHz and 3 MHz, in a continuous mode, using a 4 cm^2 insonation head. The instrument output was set at 0.5 W. cm^{-2}. During insonation, samples were intensively aerated (5 ml air per sec) to produce conditions enabling the creation of cavitation nuclei. Graphs of the relationship between the number of surviving larvae after 3 and 5 days into the experiment and the duration of insonation for the two frequencies used were plotted. The diagrams showed that the longer the insonation period, the fewer *Artemia salina* larvae survived; more pronounced effects were produced by ultrasound at 1 MHz frequency.

K e y w o r d s

Ultrasound, Cavitation, Brine shrimp, *Artemia salina*

INTRODUCTION

Ultrasound may produce a combination of chemical, thermal, and mechanical effects *(3).* Sonochemical reactions help to monitor the effects of ultrasound on biological systems, and are based on mechanisms similar to indirect effects of ionizing radiation. Ultrasound sonochemical effects include ultrasound cavitation, which is radial oscillations or collapses of microscopic gas bubbles produced by periodic changes in ambient pressure *(2).*

Artemia salina, the only representative of the family *Artemiidae*, belongs among the *Anostraca*, which is a characteristic group of crustaceans of the class Phyllopoda. They live in inland salt waters, e.g. the Great Salt Lake in the USA *(5)*. Their larvae known as "brine shrimps" are used to monitor toxicity because they are very sensitive to many chemical substances as well as to ionizing radiation *(1)*. The larvae hatch from permanent eggs called cysts, which are diapausing. Bought as feed for aquarium fish, cysts of high homogeneity can be obtained at a low price, compared with expensive commercially produced small-size "toxikits". An important factor is also the primary purity of cysts and their possible contamination with risk elements. Factors directly influencing hatching include temperature, agitation of the medium, and abundance of oxygen *(4)*.

METHODS

Larvae, hatched from commercially supplied cysts, were placed in a 200 ml beaker for 24 hrs at 25 °C in prepared "sea water" (see Table 1). The "sea water" was aerated throughout the hatching. Each sample for sonication consisted of 50 larvae placed into a plastic cylindrical vessel filled with 50 ml of "sea water". The samples were prepared at the University of Veterinary and Pharmaceutical Sciences in Brno. The samples were then shipped in a cool box to the Department of Biophysics of the Faculty of Medicine of Masaryk University in Brno for sonication. At the Institute, each sample was placed in a special 50-ml cylindrical vessel, the bottom of which was made of a plastic-sheet membrane to guarantee an optimum transfer of ultrasound energy; the samples were then insonated in a fixed position.

During sonication, samples were placed in an ice-and-water bath to guarantee a sample temperature between 20 and 21 °C. This eliminated the risk of the larvae dying because the *Artemia salina* can tolerate temperatures between 5 and 31 °C. A control group sample was shipped together with the samples for sonication.

Following sonication, each 50 ml sample was divided into five 10-ml samples each with 10 larvae in "sea water" and placed into five different Petri dishes. A microscopic assessment of the samples was made at 24-hr intervals for a total of 5 days and the numbers of surviving larvae in both the sonicated and the control samples were recorded. During the experiment, the *Artemia salina* larvae were not fed. On day 5 after sonication, 100 per cent of the larvae from the control group were still alive (*Fig. 1*). Later, however, some control group larvae died of starvation, and the experiment was therefore terminated after five days.

Fig. 1
Artemia salina larvae (age 5 days)

The source of ultrasound was the therapeutic instrument BTL-07p (Beautyline) (*Fig. 2*). Insonation was performed at 1 MHz and 3 MHz frequencies, in a continuous mode, a 4 cm2 insonation head was used. The instrument output was set at 0.5 W.cm-2, which is a level close to the cavitation threshold. During insonation, samples were intensively aerated (5 ml of air per sec) to produce conditions for the creation of cavitation nuclei. The sonication head was fixed in a position that allowed the vessel with the larvae to be placed on top of the head and sonicated through the vessel's plastic membrane bottom (*Fig. 3*).

Fig. 2
Therapeutic ultrasound instrument BTL-07p

Fig. 3
Fixation of sonication head, plastic cylindrical vesel

Table 1
Compound of the "sea water" used for experiments on Artemia salina larvae

Substance	$MgCl_2.6H_2O$	$CaCl_2.6H_2O$	KCl	$Na_2SO_4.10\ H_2O$	
Concentration [g/l]	10.830	2.250	0.68	9.060	
Substance	$NaHCO_3$	$SrCl_2.6H_2O$	KBr	H_3BO_3	NaCl
Concentration [g/l]	0.200	0.040	0.099	0.027	23.90

RESULTS

A total of ten different insonation periods from 10 to 600 sec for each of the two ultrasound frequencies were used. Plotting the data obtained on the graph produced a relationship between the number of surviving *Artemia salina* larvae and the length of the sonication period. For both ultrasound frequencies used, totals of four values obtained on days 3 and 5 post sonication were plotted. Taking the logarithm of the X axis produced a straight-line relationship between the number of surviving individuals of *Artemia salina* larvae and the time of sonication in all four cases (*Fig. 4*). Equations of all the four straight lines including the determination coefficient R^2 are given below:

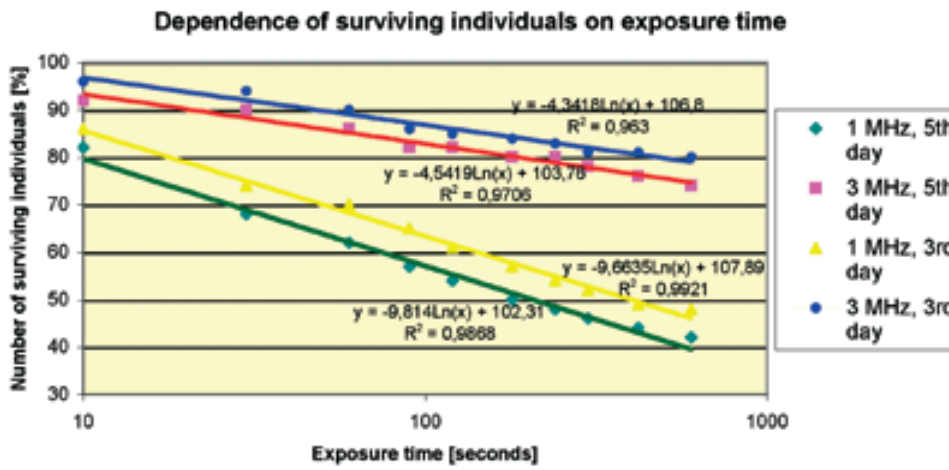

Fig. 4
Dependence of surviving individuals on exposure time

Equation for 3 MHz signal, Day 3 post sonication
$Y = -4.34 \ln(x) + 106.80$ $R^2 = 0.963$
Equation for 3 MHz signal, Day 5 post sonication
$Y = -4.54 \ln(x) + 103.76$ $R^2 = 0.971$

Equation for 1 MHz signal, Day 3 post sonication
$Y = -9.66 \ln(x) + 107.89$ $R^2 = 0.992$
Equation for 1 MHz signal, Day 5 post sonication
$Y = -9.81 \ln(x) + 102.31$ $R^2 = 0.987$

Calculations of the parameters of all the four straight lines were made using Microsoft Excel software. It follows from the parameters obtained for the determination coefficient R^2 that the values measured approximate an ideal straight line because they showed a minimum scatter only. A comparison of differences in death rates on days 3 and 5 post sonication for each frequency shows that the gradients of two mutually corresponding lines are almost identical; the minimum differences were probably due to measurement errors. The shift along the Y axis between days 3 and 5 post sonication is due to the mortality of larvae during the experiment. The diagrams showed conclusively that the longer the insonication period, the fewer *Artemia salina* larvae survived, thus the greater effects were produced by ultrasound at 1 MHz frequency.

DISCUSSION

Logarithms of sonication periods were used in final diagrams. In this way an exponential dependence of the number of surviving larvae on the duration of sonication periods was linearly transformed by plotting in a semilogarithmic scale, to allow for a more accurate assessment of the results obtained. It follows from the graph that the number of surviving larvae of Artemia salina decreases with the increasing duration of sonication periods. The decrease in the number of surviving larvae is caused by a combination of various effects of ultrasound, but it is not yet quite clear which is the dominant one. The influence of cavitation is, however, clear from the greater effect at 1 MHz frequency: cavitation occurs more readily at lower frequencies, and cavitation thresholds increase with an increase in ultrasound frequency (practically no cavitation can occur at frequencies above 10 MHz because the resonance diameter of bubbles is too small – such bubbles cannot exist in an aqueous liquid medium).

CONCLUSIONS

Biological effects of exposure to ultrasound were greater at the application of 1 MHz than 3 MHz frequency. It follows from the evaluation of parameters of the final graph that the determination coefficients of all four measured dependences

are high (over 96 %) and the resulting scatter of the values measured is minimal. We believe that experiments should continue to allow for the differentiation and quantification of individual effects of ultrasound frequency and exposure, and thus to help identify factors that are the most important in the insonation and have the greatest influence on survival rates.

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SLEDOVÁNÍ ÚČINKU KAVITAČNÍHO ULTRAZVUKU NA LARVY ARTEMIA SALINA

Souhrn

Cílem této práce bylo zjištění účinku ultrazvuku na larvy korýše *Artemia salina* při dvou různých frekvencích ultrazvuku. Ultrazvuk se projevuje kombinovanými chemickými, tepelnými a mechanickými účinky. Korýš *Artemia salina* patří mezi žábronožky (*Anostraca*) a vyskytuje se ve slaných kontinentálních vodách. Je využíván pro testování vlivu chemických látek na životní prostředí. Účinek ultrazvuku byl sledován na čerstvě vylíhnutých larvách o průměrné velikosti 1 mm. Líhnutí larev probíhalo po dobu 24 hodin při teplotě 25,0 °C v připravené „mořské vodě". Poté bylo vždy 50 larev umístěno do 50 ml „mořské vody" ve válcové nádobě, jejíž dno bylo tvořeno mikrotenovou membránou, a takto připravený vzorek byl ozvučován. Zvolili jsme celkem deset různých ozvučovacích časů od 10 do 600 sekund. Teplotu vzorků jsme se snažili při expozici zachovat pokud možno stejnou, proto jsme nádobku se vzorky ponořovali během expozice do chladicí vodní a ledové lázně. Teplota vzorků se proto pohybovala v intervalu 20,0 až 21,0 °C. Poté byl v každém vzorku po dobu pěti dnů v intervalu 24 hodin zaznamenán počet přežívajících jedinců *Artemia salina*. Jako zdroj ultrazvuku sloužil terapeutický přístroj firmy Beautyline BTL-07p. Ozvučování probíhalo při frekvencích 1 MHz a 3 MHz, v kontinuálním režimu s ozvučovací hlavicí o ploše 4 cm^2. Výkon přístroje byl nastaven na 0,5 $W.cm^{-2}$. Během ozvučování byly vzorky intenzivně provzdušňovány (5 ml vzduchu za sekundu), z důvodu zajištění podmínek pro tvorbu kavitačních jader. Výsledkem této práce jsou pro obě použité frekvence grafy počtu přežívajících organizmů po 3 a 5 dnech experimentu v závislosti na délce ozvučování. Z grafů vyplývá, že se vzrůstající dobou ozvučování přežívá méně jedinců *Artemia salina*, přičemž výraznější účinky měl ultrazvuk o frekvenci 1 MHz.

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