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BIOPHYSICS LETTER

Ultrasound enhanced methanol penetration of zebrafish (*Danio rerio*) embryos measured by permittivity changes using impedance spectroscopy

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Abstract Studies on permittivity changes in fish embryos measured by impedance spectroscopy after ultrasound treatment during exposure to cryoprotectant is reported here for the first time. The permittivity changes of zebrafish embryos in cryoprotectant solutions before and after ultrasound treatment were measured using impedance spectroscopy. Zebrafish (*Danio rerio*) embryos at 50% epiboly stage were exposed to 2 M methanol for 25 min before ultrasound treatment for 5 min at 22°C. Embryos were treated with ultrasound in different frequencies (24 and 48 kHz) and voltages (50, 100, 150 and 175 V) combinations. The results showed a clear increasing trend of permittivity from voltage 50 to 175 V over lower impedance frequency range of 10–10^3 Hz indicating increased methanol penetration into the embryos after ultrasound treatment. The embryo survival was not compromised after ultrasound treatment under conditions used in the present study. The use of impedance spectroscopy technique provides a useful none-invasive tool for detecting changes of cryoprotectant penetration in fish embryos after ultrasound treatment. The technique is especially useful for the selection of the suitable cryoprotectants in embryo cryopreservation and may also allow quantitative measurements in embryo membrane permeability studies.

Keywords Ultrasound · Permittivity change · Impedance spectroscopy · Methanol · Zebrafish (*Danio rerio*) embryos

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Introduction

Cryopreservation and cryobanking of germplasm of aquatic species offers many benefits to the fields of aquaculture, conservation and biomedicine. Studies have shown that there are several obstacles to fish embryo cryopreseravtion: (a) the large size of fish embryos resulting in a low surface area to volume ratio, which reduces the rate of water and cryoprotectant movement during cryopreservation; (b) the complex membrane system and low permeability of fish embryos does not allow sufficient cell dehydration and cryoprotectant penetration resulting in lethal intracellular ice formation to the cells; and (c) fish embryos have a high yolk content that is linked to their high chilling sensitivity, making them more susceptible to injuries during exposure to sub-zero temperatures (Zhang and Rawson 1998; Liu et al. 1999). Successful cryopreservation of fish embryos requires sufficient embryo dehydration and cryoprotectant penetration. Several techniques have been applied for improving cryoprotectant delivery in fish embryos. However, approaches such as altering fish embryo membrane with aquaporin-3 protein and micro-injection are either inefficient or ineffective (Hagedorn et al. 2002; Robles et al. 2004; Kopeika et al. 2006).

Recent advances in ultrasound technology, especially its applications in transdermal drug delivery through mammal skins, are considered to have great potential for application in the delivery of cryoprotectants into fish embryos. Ultrasonic technology is currently used in medical practice for diagnostic and therapeutic purposes. Cavitation-level ultrasound has been used to enhance permeation of insulin-like protein across human skin (Mitragotri et al. 1995a). During the process of cavitation, oscillation and the collapse of air bubbles effectively disorganize the lipid bilayers allowing large molecules to diffuse though the skin (Mitragotri et al.

1995b). It was also shown that sonophoresis under low-frequency conditions (~20 kHz) enhances transdermal transport synergistically with sodium lauryl sulphate and allows the non-invasive transdermal extraction of glucose (Mitragotri et al. 1996). The use of ultrasound during cryoprotectant exposure to fish eggs and embryos is expected to enhance the transport of cryoprotectant (Alfaro et al. 2001). A recent experiment using ultrasound on rainbow trout has shown that cavitation-level ultrasound improves the access of membrane to substances such as calcein (Bart et al. 2001).

A rapid and non-invasive method for the measurement of cryoprotectant transportation during or after cryoprotectant treatment in fish embryos would help membrane permeability studies significantly. Conventional methods for assessing embryo membrane permeability involve the use of light microscopic measurement of volumetric change of embryos caused by exosmosis of cellular water and cryoprotectant penetration (Zhang and Rawson 1996, 1998). Other techniques have also been applied in fish embryo membrane permeability studies, including nuclear magnetic resonance (NMR) spectroscopy (Hagedorn et al. 1996, 1997). However, these methods are lengthy and reduce the capacity for multi-embryo measurement during an experimental run. Recent reports on the application of impedance spectroscopy for non-invasive measurement of cryoprotectant treated fish embryos have demonstrated the possibility of measuring levels of cryoprotants penetration in real-time (Wang et al. 2006; Zhang et al. 2006).

In electrochemistry, impedance spectroscopy is a well-established method for characterising the electrical properties of materials and their interfaces exposed to electronically conducting electrodes (Angersbach et al. 1999, 2002; Wiegand 2000). Measurement of permittivity response of fish embryos during their exposure to cryoprotectants could provide important information on their membrane permeability. A direct correlation between permittivity and cell membrane potential at low frequencies in living cell suspensions has been reported by Prodan and Prodan (1999) and Prodan et al. (2004). The use of permittivity measurement in studies of cryoprotectant penetration into fish embryos has also been demonstrated (Wang et al. 2006). The changing trends of measured permittivity over time confirmed the penetration of cryoprotectants into the embryos after 3–10 min of exposure to methanol and dimethyl sulfoxide (DMSO).

In this study, the permittivity changes of zebrafish embryos in cryoprotectant solutions before and after ultrasound treatment were measured using impedance spectroscopy. The permittivity changes were used as indications for cryoprotectant penetration of the embryos. Methanol was used to study the suitability of selected frequency and output power on ultrasound device, as it was demonstrated as the most promising cryoprotectant for zebrafish embryos (Zhang et al. 1993).

Materials and methods

Zebrafish breeding and embryo collection

Adult zebrafish were held in a closed re-circulating system in 12 L tanks with 8–12 fish per tank. The temperature of the system water was maintained at 28°C and the photo period was fixed at 14 h light:10 h dark. Zebrafish were fed three times daily with TetraMin flake food (Tetra, Germany) and newly hatched brine shrimps. Embryos were collected from breeding trays each morning and maintained in embryo medium (60 μg/ml ocean salt in deionised water) at 28.5°C until 50% epiboly stage. Intact embryos at 50% epiboly stage were used in this study. The developmental stage of the embryos was examined using a laboratory microscope (Lecia, UK).

Ultrasound treatment during embryo exposure to methanol

A custom-built ultrasound system was used in this study. A signal generator (HP 8116A Pulse/Function Generator, USA) along with a wideband amplifier (Krohn-Hite 7500 Amplifier, Germany) was used to drive the transducer (piezoelectric disc, SJ Electronics, UK). The transducer (5 cm in diameter and 1.0 cm in thickness) was glued onto a PVC base and then sandwiched between two PVC cylinders (5 cm in diameter and 3 cm in height), which holds up to 50 ml water. A test tube holding rack with four 1 cm diameter holes were place on top of the transducer device (Fig. 1). Embryos were exposed to 2 M methanol for 25 min before loaded into the test tubes (in 1 ml 2 M methanol), which were then placed into the ultrasound unit for 5 min at 22°C. Embryos were treated with ultrasound in different frequencies (24 and 48 kHz) and voltages (50, 100, 150 and 175 V) combinations. Eighteen embryos were used in each treatment and each treatment was repeated six times. Embryos in 2 M methanol for 30 min without ultrasound treatment were used as controls.

After ultrasound treatment, 12 embryos were removed from the test tubes (six were used for permittivity measurements), they were washed three times in embryo medium and held in embryo medium in 100 ml beakers at 28.5°C for 4 days. Hatching rates of embryos with or without ultrasound treatment were assessed. Hatched larvae were examined under the microscope morphologically using the criteria described in the Zebrafish Book (Westfield 1995). Abnormal larvae were considered non-viable. Embryo survivals in embryo medium without ultrasound treatment were also assessed.

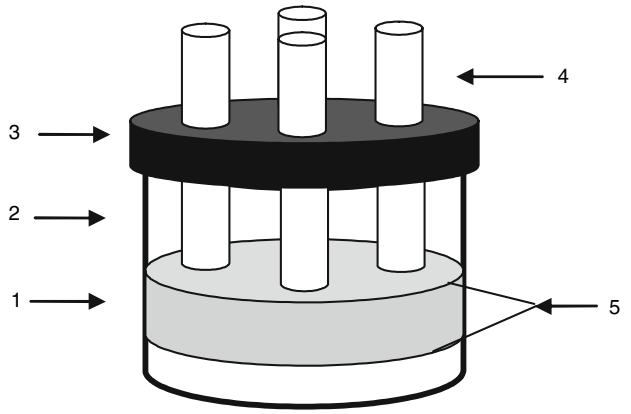

Fig. 1 Schematic diagram of the ultrasound device and embryo holding rack. *1* Transducer; *2* transducer holding case; *3* embryo holding tube rack; *4* embryo holding tube (1.0 cm diameter); *5* transducer connections

Studies of permittivity spectra during embryos exposure to methanol

Permittivity spectra of embryos with or without ultrasound treatment were recorded using Solartron SI1260 Impedance/Gain-Phase Analyser (Novocontrol Technologies, Germany) with the impedance measurement range of 10–100 mΩ (accuracy 0.1%). AC output voltage of the system was set at 1.0 Vrms for current measurement across the frequency range 10–10^6 Hz with 12 logarithmic sweeping points per decade. Permittivity was measured automatically along with specific conductivity, serial/parallel impedance, capacitance and tan (d), temperature and actual time. A specially designed embryo holding cell and the associated electrodes connections were used (Wang et al. 2006).

Methanol (Sigma, UK) solution (2.0 M) was made up in embryo medium shortly before experiments. For each experiment, six embryos were loaded into the holding chamber with a pipette as it was confirmed as an optimal loading level in our previous study (Zhang et al. 2006). Both ultrasound treated and untreated embryos were measured in the same manner. The holding chamber was then quickly covered with a lid and secured between the two electrodes. A single sweep was set over the frequency range 10–10^6 Hz. All experiments were run under room temperature at 22 ± 1°C. The holding chamber was carefully cleaned and recalibrated with standard KCl solutions before each experiment.

Survival of embryos after ultrasound treatment and freeze-thawing

In these experiments, embryos were exposed to 2 M methanol for 25 min in test tubes before ultrasound treatment for 5 min at 22°C. Embryos were treated with ultrasound at different frequency (24 and 48 kHz) and voltage (150 and 175 V) combinations before loading into 0.5 ml plastic straws and frozen using the following protocol: cooling from 20 to −7.5°C at 2°C/min; manual seeding at −7.5°C and holding for 5 min; slow freezing to −15°C at 0.3°C/min. Thawing was carried out by direct immersion of straws in 28°C water bath for 10 s. After thawing embryos were washed three times in embryo medium and held 100-ml beakers at 28.5°C for 4 days. Control embryos were treated in 2 M methanol for 30 min without ultrasound before they were frozen using the same protocol. Hatching rates of embryos with and without ultrasound treatment were assessed using the method described above. Fifteen to twenty-two embryos were used in each treatment and each treatment was repeated nine times.

Statistical analyses

The overall effects of ultrasound at different frequency and voltage combinations and embryo survivals were statistically analysed. Student's *t* test (two tailed assuming unequal variances) was applied to determine differences between the treated and the controlled groups. Values of *P* less than 0.05 were considered to be statistically significant. Means, standard errors, threshold values for *t* test and variance were calculated using Excel.

Results and discussion

Effect of ultrasound treatment on permittivity changes in the embryos

The permittivity spectra of six embryos loaded in 2.0 M methanol at different ultrasound treatment combinations are presented in Figs. 2 and 3. An increasing trend of permittivity from voltage 50 to 175 V was seen over the lower impedance frequency range of 10–10^3 Hz. Under 24 KHz ultrasound treatment condition, the permittivity values at 10 Hz were increased from 3285.41 mF/m (Control) to 3667.94 mF/m (50 V), 4350.16 mF/m (100 V), 4896.79 mF/m (150 V) and 5928.58 mF/m (175 V) ($p < 0.05$). Relatively small increases or unchanged values were obtained between 10^3 and 10^6 Hz (Fig. 2). A similar trend was also obtained for 48 KHz ultrasound treatment experiment. The values were increased from 3285.4 mF/m (Control) to 4023.7, 4719.2, 5167.0 and 6490.75 mF/m for 50, 100, 150 and 175 V, respectively ($p < 0.05$).

The measured changes in permittivity reflected the changes in methanol penetration of the tested embryos after ultrasound treatment. The embryos were treated with 2 M methanol for 25 min prior to ultrasound treatment (5 min), which equilibrated the water and methanol movements between intra-embryonic and extra-embryonic fluid before

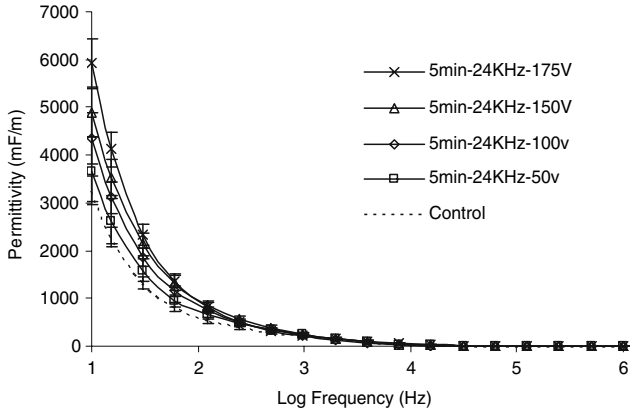

Fig. 2 Permittivity changes of six embryos after ultrasound treatment at 24 KHz and voltages of 50, 100, 150 and 175 V, respectively. Embryos were pre-treated with 2.0 M methanol for 25 min followed by 5 min ultrasound treatment in the same solution

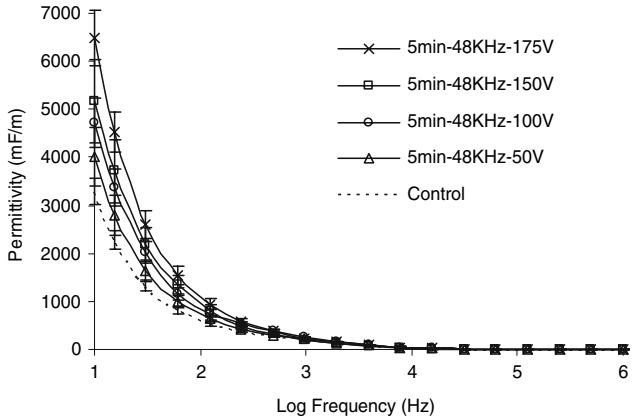

Fig. 3 Permittivity changes of six embryos after ultrasound treatment at 48 KHz and voltages of 50, 100, 150 and 175 V, respectively. Embryos were pre-treated with 2.0 M methanol for 25 min followed by 5 min ultrasound treatment in the same solution

ultrasound was introduced. The equilibrated conditions were used as control values in both 24 and 48 KHz experiments. The increased permittivity values after 5 min ultrasound treatment can be explained as the increased level of methanol in the embryos that changed the electrochemical property of these embryos. The changes were confirmed by the measured higher permittivity values than those obtained with the control groups. The increasing trend in permittivity with the applied voltages from 50, 100, 150 and 175 V demonstrated the positive impact of ultrasound force. It is also evident that the permittivity changes after ultrasound treatment are frequency-dependent, which is in agreement with our previous studies (Wang et al. 2006).

Ultrasound has been used in the medical field for several decades, recently there has been considerable interest in developing ultrasound as a technique to enhance transdermal drug delivery (Mitragotri et al. 1996) and the optimal conditions (frequency and power) of the ultrasound technique in drug delivery application have been thoroughly studied and defined. It is well known that low frequency high intensity ultrasound (conditions promoting collapse cavitation) is much more effective than higher frequencies for transportation of proteins and other large molecules. Electron microscopy on skin exposed to low frequency ultrasound revealed the removal of surface cells and the formation of large pores and pockets (~20 μm) (William et al. 2004). In the present study ultrasound frequencies of 24 and 48 KHz were used.

The differences in the permittivity spectra at different voltage levels applied in ultrasound treatment reflected different strength of the ultrasound force and its impact on membrane permeability. The transducer introduces cavitations on embryo membranes and creates micro air bubbles. The collapse of air bubbles effectively disorganises the membranes, which allowed methanol molecules to permeate. In reverse, the water molecules were transported out into the extra-embryo fluid driven by osmosis force. In the present study, the permittivity increased with increasing voltage (from 50 to 175 V) applied at both ultrasound frequencies (24 and 48 KHz), indicating 24 KHz/175 V and 48 KHz/175 V are the most effective conditions in the present study.

Effect of ultrasound treatment on embryo survival

Figure 4 shows 50% epiboly embryo survivals before and after ultrasound treatment at different frequency and voltage combinations. No significant differences in embryos survivals were found between ultrasound treated and the two control groups (both embryo medium and 2 M methanol, $p > 0.05$). Effective ultrasound treatment requires the increased cryoprotectant delivery into the embryos without compromising their viability. One possible negative impact of ultrasound treatment was heat generation during the treatment, which is normally caused by the energy release during the collapses of the micro bubbles (Machet et al. 1998; Meidan et al. 1999). Bart and Kywa (2003) also studied the effect of zebrafish embryo survival after ultrasound treatment and methanol exposure, however, as the methanol concentrations used in their study was exceptionally high (>10 M) and no methanol treated controls were given, it was impossible to identify the effect of ultrasound on embryo survival. In the present study, no negative impact of ultrasound on embryo survival was observed under the experimental conditions used.

Survival of embryos after ultrasound treatment and freeze-thawing

The survivals of embryos after ultrasound treatment and freeze-thawing are shown in Fig. 5. Although average

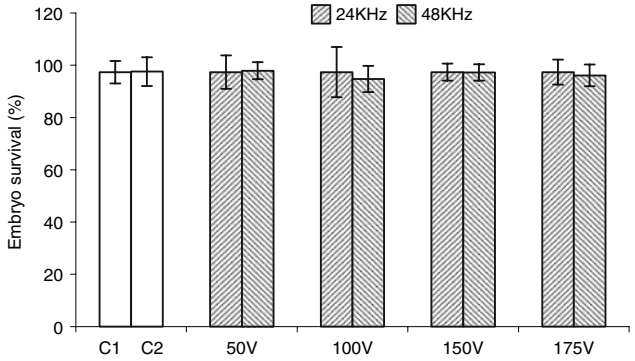

Fig. 4 Survival of 50% epiboly stage zebrafish embryos after ultrasound treatment at a range of frequency and voltage combinations. Fifty percent of epiboly embryos were exposed to 2.0 M methanol for 25 min followed by 5 min ultrasound treatment in the same solution at room temperature. Embryos were then washed and incubated for 4 days at 28.5°C before their hatching rates were assessed. *C1* embryo survival in embryo medium without ultrasound treatment (embryo medium control); *C2* embryo survival in 2.0 M methanol for 30 min without ultrasound treatment (2 M methanol control)

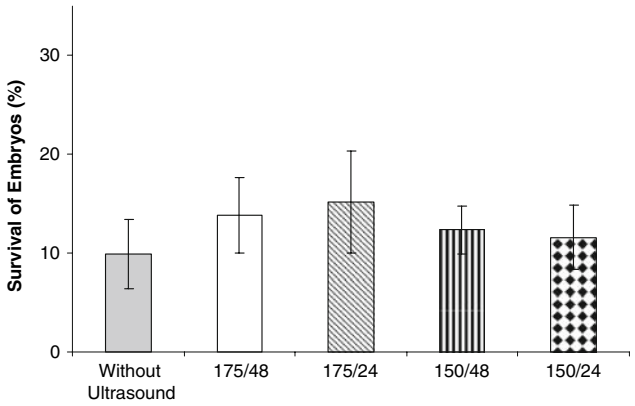

Fig. 5 Survival of embryos after ultrasound treatment and freeze-thawing. Embryos were treated in 2 M methanol for 30 min with or without ultrasound before they were frozen using controlled slow cooling. Embryo survival of room temperature control was 93.3 ± 2.2%

survivals for ultrasound treated embryos were higher for all frequency and voltage combinations when compared with embryos without ultrasound treatment, there were no significant differences between these results. Higher voltages may need to be applied to improve the results. Bart and Kywa (2003) suggested a 47 KHz and 420 V combination for ultrasound treatment of zebrafish embryos based on the survival rate of the embryos after ultrasound treatment (no cryoprotectant penetration was investigated in their report), however, as no statistical analysis results were provided in their study for all treatment conditions and especially those which provided ambiguous results, it is impossibly to be convinced that these conditions were suitable for zebrafish embryos. Further studies are needed to confirm the effect of higher voltage on embryo survival after ultrasound treatment and freezing.

Conclusions

The results presented in this study indicated that the ultrasound treatment has enhanced methanol penetration of the tested zebrafish embryos at 50% epiboly stage and especially when 24 HKz/175 V and 48 KHz/175 V combinations were used. The permittivity changes appeared to be frequency dependent. In the lower frequency range (10–10^3 Hz), permittivity values increased with the increasing level of ultrasound treatment power, which can be explained as increased methanol penetration. The present study provided foundations for identifying the optimum ultrasound treatment conditions for fish embryos to achieve maximum cryoprotectant penetration in their cryopreservation studies. The use of impedance spectroscopy provides a useful none-invasive tool for detecting changes of cryoprotectant penetration in fish embryos after ultrasound treatment. The technique is especially useful for the selection of the suitable cryoprotective chemicals and may also allow quantitative measurements in embryo membrane permeability studies. The combination of the use of ultrasound and impedance technologies could provide a unique tool to advance current cryopreservation studies on fish embryos.

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