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● *Original Contribution*

PRE-TREATMENT WITH EITHER L-CARNITINE OR PIRACETAM INCREASES ULTRASOUND-MEDIATED GENE TRANSFECTION BY REDUCING SONOPORATION-ASSOCIATED APOPTOSIS

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Abstract—Sonoporation, the use of ultrasound to alter the permeability of cell membranes, is a non-viral technique used to facilitate gene delivery, possibly by opening transient pores in the cell membrane. However, sonoporation may have negative bio-effects on cells, such as causing apoptosis, which limits its efficacy in gene delivery. In this study, we investigated whether pre-treatment with either L-carnitine or piracetam could protect cells from undergoing apoptosis after sonoporation and the possible mechanisms. We found that either L-carnitine or piracetam can promote gene transfection without reducing cell viability, possibly by reducing cavitation-induced reactive oxygen species generation, reversing alterations of mitochondrial membrane potential, preventing caspase-3/7 activity and facilitating mitochondrial ATP production. In conclusion, pre-treatment with either L-carnitine or piracetam could protect cells from sonoporation-associated apoptosis by preserving mitochondrial function. (E-mail: b88401062@ntu.edu.tw)

Key Words: **Carnitine, Piracetam, Ultrasound, Gene, Apoptosis.**

INTRODUCTION

Gene therapy involves the delivery of genetic material into cells to provide an effective treatment for various diseases such as cancer and congenital diseases. For successful gene therapy, it is necessary to develop techniques that can deliver therapeutic genes to tissues or cells efficiently, selectively and safely. In recent years, ultrasound (US) combined with microbubbles has been reported to be a non-viral method for delivering genes *in vitro* and *in vivo* (Lentacker et al. 2014; Li et al. 2009; Rychak and Klibanov 2014; Tomizawa et al. 2013). US-mediated delivery (also called *sonoporation*) has low immunogenicity and invasiveness, site specificity, localization and high penetration and safety for repeated treatments compared with other delivery techniques (Delalande et al. 2013; Rychak and Klibanov 2014). Sonoporation, which is induced by the disruption of microbubbles exposed to ultrasound, promotes the transfer of extracellular materials into cells by opening transient pores in the cell membrane (Lentacker et al. 2014). However, it may have negative bio-effects on cells, such as cell destruction (Hassan et al. 2010), free radical formation (Honda et al. 2004), cell cycle arrest (Chen et al. 2013; Zhong et al. 2011), necrosis (Hassan et al. 2010) and apoptosis (Honda et al. 2004; Hutcheson et al. 2010; Miller and Dou 2009; Zhong et al. 2011, 2013). Previous studies have indicated that sonoporation can perturb endoplasmic reticulum (ER) homeostasis, cause mitochondria to lose their membrane potential ($\Delta\psi_m$), increase mitochondria-activated apoptosis initiators (caspase family members) and finally result in the initiation of apoptosis (Honda et al. 2004; Zhong et al. 2013). Therefore, protection of cells from the negative effects of sonoporation is an important issue when using sonoporation for therapeutic purposes.

To protect mitochondria in particular from the negative bio-effects of sonoporation, L-carnitine and piracetam may be feasible candidates as they have been reported to protect cells from undergoing apoptosis through the

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preservation of mitochondrial function (Chang et al. 2005; Costa et al. 2013; Keil et al. 2006; Kurz et al. 2010; Leuner et al. 2010; Moosavi et al. 2016; Stockburger et al. 2013; Ye et al. 2010). L-Carnitine could protect cells from various stresses through the reduction of oxidative injury, mitochondrial dysfunction, caspase activation and, finally, inhibition of apoptosis (Chang et al. 2005; Costa et al. 2013; Moosavi et al. 2016; Ye et al. 2010). Furthermore, L-carnitine significantly increased the activity of endogenous antioxidant enzymes (Ye et al. 2010) and ATP production (Bremer 1983) and attenuated aging (Arockia Rani and Panneerselvam 2001). With respect to piracetam, it is a nootropic drug commonly used in a clinical context, and is used to improve cognitive impairment in aging, brain injuries, Alzheimer's disease and ischemia (Keil et al. 2006; Leuner et al. 2010; Stockburger et al. 2013). Piracetam has been found to improve mitochondrial membrane potential and the impairment of mitochondrial function and apoptosis after oxidative stress (Costa et al. 2013; Keil et al. 2006; Kurz et al. 2010; Leuner et al. 2010; Stockburger et al. 2013). Furthermore, the combination of L-carnitine and piracetam has additive effects on the protection against PC3 cell necrosis induced by simvastatin (Costa et al. 2013). Although L-carnitine and piracetam have been reported to protect mitochondrial function in different circumstances, their roles in mitochondrial protection from sonoporation have not been investigated. Therefore, we aimed to test whether L-carnitine or piracetam could protect cells against the negative bio-effects induced by sonoporation.

METHODS

Cell culture and plasmid preparation

NIH3T3 cells (from Bioresource Collection and Research Center, Taiwan), an immortalized mouse fibroblast cell line, were maintained in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DMEM, high glucose, Invitrogen, USA), supplemented with 10% bovine calf serum (Invitrogen, USA), and a 1 × antibiotic–antimycotic (Invitrogen, USA) at 37 °C in 5% CO_2.

BNL CL.2 (from Bioresource Collection and Research Center, Taiwan) is a normal embryonic liver cell line derived from a BALB/c mouse. BNL cells were maintained in high-glucose DMEM supplemented with 10% fetal bovine serum (Invitrogen, USA) and a 1 × antibiotic–antimycotic at 37 °C in 5% CO_2.

Firefly luciferase gene (*luc*, Promega, Madison, WI, USA) was subcloned into the pCI-neo mammalian expression vector (Promega) and called pCI-neo-*luc*. Plasmid DNA was prepared using the EndoFree Plasmid Mega kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

US exposure

The US field was generated by a 1-MHz plane piezoelectric transducer with an active diameter of 3.8 cm (A392 S, Panametrics, Waltham, MA, USA). A 24-well plate was placed 5.4 cm above the transducer, and only one well at a time was exposed to US. We chose the 5.4-cm distance to ensure that the culture well was within the −3-dB pressure distribution in the near field of the transducer. The volume inside the exposure well was 2 mL. The duty cycle was set to 20%. The pulse length was fixed at 27.5 ms for exposure conditions. A needle hydrophone (SPEH-S-0500, ONDA, Sunnyvale, CA, USA) was located near the sonicated well and used to detect the cavitation noise in the medium. The received signal was amplified, digitized, transferred to a computer and analyzed with the Labview program. The cavitation activity was quantified using an inertial cavitation index (CI). The designed cavitation regulation system was based on the instantaneous calculation of the inertial cavitation level. This feedback loop process imposes a precise cavitation level for providing better control of cavitation. Further details of the acquisition device, calculation of CI and feedback control system can be found in previous reports (Lo et al. 2014; Sabraoui et al. 2011).

Cultured NTH3 T3 cells or BNL cells were initially plated in a 24-well plate at a density of 10^5 cells/well for 18 h. The medium was replaced with 2 mL of fresh medium, and cells were treated with the indicated doses of L-carnitine (Sigma, St. Louis, MO, USA) or piracetam (Sigma) for 4 h before US exposure. The medium was then immediately supplemented with 10 μg of pCI-neo-*luc* plasmid and 5 μl of SonoVue contrast agent (Bracco, Milan, Italy). The cells were exposed to US bursts with various CIs (CI = 10, 15) for 110 s.

At various post-exposure points (2 and 18 h), the cells were harvested for further analysis, as described in the following sections. The 18-h point was chosen as it takes time for gene expression to produce sufficient proteins for analysis. It also takes time for cell culture to test viability and caspase activity. On the other hand, the membrane potential alteration, ROS generation and ATP generation induced by US may return to baseline in a short time and cannot be detected after 18 h. Cell proliferation after18 h of incubation may also interfere with ATP detection. Therefore, we analyzed membrane potential alteration, ROS generation and ATP generation in 2 h.

Cell viability assay

Cell viability on US exposure was measured using the alamarBlue assay (Thermo Fisher Scientific, USA). After 18 h of incubation, NIH3T3 cells were washed twice with phosphate-buffered saline solution and incubated with 10% v/v alamarBlue medium. The plate was incubated under standard conditions of 37 °C and 5% CO_2 for 30 min,