Ultrasound in Med. & Biol., Vol. 40, No. 9, pp. 2195–2206, 2014

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2014.05.001

● *Original Contribution*

DIFFERENTIATION OF NEURAL STEM/PROGENITOR CELLS USING LOW-INTENSITY ULTRASOUND

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(*Received* 29 *July* 2013; *revised* 30 *April* 2014; *in final form* 1 *May* 2014)

Abstract—Herein, we report the evaluation of apoptosis, cell differentiation, neurite outgrowth and differentiation of neural stem/progenitor cells (NSPCs) in response to low-intensity ultrasound (LIUS) exposure. NSPCs were cultured under different conditions, with and without LIUS exposure, to evaluate the single and complex effects of LIUS. A lactic dehydrogenase assay revealed that the cell viability of NSPCs was maintained with LIUS exposure at an intensity range from 100 to 500 mW/cm^2. Additionally, in comparison with no LIUS exposure, the cell survival rate was improved with the combination of medium supplemented with nerve growth factor and LIUS exposure. Our results indicate that LIUS exposure promoted NSPC attachment and differentiation on a glass substrate. Neurite outgrowth assays revealed the generation of longer, thicker neurites after LIUS exposure. Furthermore, LIUS stimulation substantially increased the percentage of differentiating neural cells in NSPCs treated with nerve growth factor in comparison with the unstimulated group. The high percentage of differentiated neural cells indicated that LIUS induced neuronal networks denser than those observed in the unstimulated groups. Furthermore, the release of nitric oxide, an important small-molecule neurotransmitter, was significantly upregulated after LIUS exposure. It is therefore reasonable to suggest that LIUS promotes the differentiation of NSPCs into neural cells, induces neurite outgrowth and regulates nitric oxide production; thus, LIUS may be a potential candidate for NSPC induction and neural cell therapy. (E-mail: iclee@mail.cgu.edu.tw or yingchih@gate.sinica.edu.tw)

Key Words: **Low-intensity ultrasound, Neural stem/progenitor cells, Neurite outgrowth, Neuron, Induction, Neuron network.**

INTRODUCTION

Neuron stem/progenitor cells (NSPCs) are self-renewing, multipotent cells that have the ability to differentiate into three major neural lineages: neurons, oligodendrocytes and astrocytes (Doetsch 2003; Jori et al. 2003; Li et al. 2003). The discovery of NSPCs in the central nervous system (CNS) and the potential of these cells to regenerate functional neural cells has raised hopes for the treatment of neurodegenerative diseases and injuries (Baetge 1993; Limke and Rao 2002). However, it is challenging to manipulate NSPC differentiation into the desired lineages. Niches regulate stem cell self-renewal and differentiation *in vivo* and are composed of supporting cells, extracellular matrix (ECM) components, surface topography and medium components. Cells express hundreds of different types of receptors to continuously monitor their chemical and physical microenvironments. Stem cells are particularly sensitive to their microenvironments, and their interactions have profound effects on stem cell potency (Li and Xie 2005).

Biochemical signals are widely used to regulate stem cell differentiation. Previous studies have suggested that the proliferation and differentiation of NSPCs isolated from the embryonic rat cerebral cortex are strongly influenced by intrinsic and extrinsic signals from medium components and cell–cell interactions (Heese et al. 2006; Nakayama and Inoue 2006). Defined medium containing growth factors, such as

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basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), can be used to enhance the differentiation efficiency of NSPCs (Egawa et al. 2011). Retinoic acid and nerve growth factor (NGF) were also found to be potent enhancers of neuronal differentiation, eliciting extensive outgrowth of processes and the expression of neuron-specific molecules (Schuldiner et al. 2001). However, the utilization rate of the growth factors was limited and resulted in heterogeneous populations of differentiated and undifferentiated cells.

Physical stimulation using mechanical stimulation (Knippenberg et al. 2005; Luu et al. 2009; Sim et al. 2007), electric fields (Egawa et al. 2011), magnetic fields (Boonen et al. 2010) and lasers (Li et al. 2008) have been popular approaches for inducing stem cell differentiation or stimulating cell functions. Mechanical stimulation is the most widely used method of biophysical stimulation (Desmaele et al. 2011; Huang et al. 2010). Among the methods of mechanical stimulation, low-intensity ultrasound (LIUS) is of particular interest because it is simple and cost-effective and has already been found to have therapeutic effects in promoting fracture repair and increasing mechanical strength. LIUS is also known to accelerate bone and tissue regeneration after injury (Malizos et al. 2006), to increase the matrix hardness of the healing tissue (Qin et al. 2006), to induce chondrocyte phenotypes *in vitro* (Lee et al. 2006) and to improve cartilage and bone repair in animal models (Shimazaki et al. 2000). Most studies of LIUS on cells have focused on connective tissue cells and mesenchymal stem cells (Choi et al. 2010; Lai et al. 2010; Yoon et al. 2009), and few studies have investigated neuron-related cells except for peripheral neurons and Schwann cells (Chang et al. 2005; Shimazaki et al. 2000; Zhang et al. 2009).

In the peripheral nervous system, previous studies have found that LIUS has positive effects on axonal regeneration in *in vivo* peripheral nerve injury trials (Chen et al. 2010). It was also demonstrated that ultrasound applied locally to the injured sciatic nerve could strongly increase the number of Schwann cells (Zhang et al. 2009). *In vitro* studies in Schwann cells have found that LIUS promotes a significantly greater number and area of regenerated axons (Chang et al. 2005; Tsuang et al. 2011; Zhang et al. 2009). Although it is known that LIUS accelerates peripheral nerve regeneration (Chang et al. 2005), the precise cellular mechanisms underlying this regeneration remain unclear. In previous literature, focused ultrasound has been reported to assist in the delivery of NSPCs from the blood to the brain by opening the blood–brain barrier (Burgess et al. 2011), indicating the potential for use in brain stem-cell therapy. However, the effects of LIUS on NSPCs have not been reported.

The detailed mechanism of ultrasound as a mechanical wave to assist chemical diffusion and membrane permeation is still not well understood, despite significant effort. Although eukaryotic cells are about one order of magnitude smaller than an ultrasound wavelength, ultrasound nonetheless exerts several effects on cells through a number of mechanisms. An ultrasound pulse directly causes entire cells to be compressed or rarefied due to this fact; however, the presence of a nearby bubble causes ultrasound to be rescattered, which proceeds to generate transient pores in the cell membrane (Donikov and Bouakaz 2010). Sonoporation (reviewed in Sheikh et al. 2011) has been used to introduce DNA and drugs into cells under study. In this process, the pores generated vary in size and duration. Typically, pores persist for seconds to minutes; however, their effects may last hours according to a recent study (Yudina et al. 2011). Those authors also note that sonoporation at sufficient intensities can induce pore formation in the absence of microbubbles. Pores exceeding certain sizes would be lethal to the cell (Donikov and Bouakaz 2010). Pores are generated both by shock waves and by microjets originating from collapsing inertial cavitational bubbles (Kudo et al. 2009; Zhou et al. 2012), as well as from shear stress from microstreaming as a result of stable cavitation (Newman and Bettinger 2007; van Wamel et al. 2008; Wolfrum et al. 2002). Recently, Zhou and colleagues (2012) reported that the ratio of the distance (D) from a bubble to a membrane to the bubble diameter (d) has a maximum value of 0.75 for membrane permeation to occur. The majority of cell permeation studies used bubbles introduced as ultrasound contrast agents. These contrast agents also nucleate additional cavitational sites. Ultrasound-induced endocytosis has also been cited as a possible mechanism in cellular uptake of materials (Lionetti et al. 2009; Meijering et al. 2009). The increased mixing from microstreaming and jets from inertial cavitation also lowers the diffusion-limited barrier to enzyme/substrate binding, increasing reaction rates around the cell (Easson et al. 2011). Improved mixing has been found to enhance the localized mass transport of nutrients (Schmidt et al. 2005).

Cell permeation triggers a repair process that requires the presence of external calcium ions (Zhou et al. 2008). External calcium ions enter the cell, inducing cellular responses that act to repair the plasma membrane over time (McNeil and Terasaki 2001; Meldolesi 2003; Togo et al. 1999), provided that the breaches are not fatal.

The synergistic effects of multiple physical or chemical cues on neurogenesis have not been determined, and the mechanisms by which this combination of cues affects cellular differentiation are unknown. Multistimulation by physical exposure and chemical treatment may provide an environment that selectively enhances neuronal differentiation and neurite outgrowth, even resulting in the formation of a neural network. Integrating