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● *Original Contribution*

INFLUENCE OF DONOR AGE AND STIMULATION INTENSITY ON OSTEOGENIC DIFFERENTIATION OF RAT MESENCHYMAL STROMAL CELLS IN RESPONSE TO FOCUSED LOW-INTENSITY PULSED ULTRASOUND

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Abstract—A focused low-intensity pulsed ultrasound (FLIPUS) was used to investigate the effects of stimulation period, acoustic intensity and donor age on the osteogenic differentiation potential of rat mesenchymal stromal cells (rMSCs). rMSCs from 3- and 12-mo-old female Sprague Drawly rats were isolated from bone marrow and stimulated 20 min/d with either 11.7 or 44.5 mW/cm^2 (spatial average temporal average intensity) for 7 or 14 d. Osteogenic differentiation markers, *i.e.,* Runt-related transcription factor 2 (RUNX2), osteocalcin (OCN) and degree of matrix calcification were analyzed. On day 7 of stimulation, OCN gene expression was enhanced 1.9-fold in cells from young rats when stimulated with low intensity. The low intensity also led to a 40% decrease in RUNX2 expression on day 7 in aged cells, whereas high intensity enhanced expression of RUNX2 on day 14. FLIPUS treatment with low intensity resulted in a 15% increase in extracellular matrix mineralization in young but not old rMSCs. These differences suggest the necessity of a donor-age related optimization of stimulation parameters. (E-mail: kay.raum@charite.de) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Aging, Low-intensity pulsed ultrasound, Acoustic intensity, Mesenchymal stromal cells, Regeneration.**

INTRODUCTION

The skeleton has a considerable repair competence, and a complex interplay of various cellular, humoral and mechanical factors enable the scarless repair of bone injuries to restore pre-fracture properties under optimal conditions (Giannoudis et al. 2007). However, bone is not resistant to the aging process, and its regeneration potential progressively declines with increasing age (Gruber et al. 2006; Strube et al. 2008). Although age-related skeletal impairments are rarely fatal, they compromise quality of life and diminish the ability to participate socially. Thus, there is a clear medical demand for optimizing existing therapeutic options or developing new approaches for bone regeneration for elderly patients in particular (Satija et al. 2009).

At the cellular level, age-related changes in skeletal health can be attributed largely to declines in both number and function of mesenchymal stromal cells (MSCs) in the bone marrow (D'Ippolito et al. 1999; Kasper et al. 2009; Shen et al. 2011). MSCs are highly proliferative multipotent progenitor cells and have the ability to differentiate into various mesoderm-type cells such as osteoblasts, chondrocytes, adipocytes (Pittenger et al. 1999) and hematopoiesis-supportive stromal cells (Dexter et al. 1977). Hence, they are thought to be the major progenitor cells for intramembranous and endochondral bone formation (He et al. 2013). Besides their capacity for differentiation, MSCs support tissue regeneration by the modulation of immune and injury responses *via* the secretion of various proteinases and growth factors (Caplan and Dennis 2006; Geissler et al. 2012; Kasper et al. 2007; Li et al. 2012; Philippou et al. 2012). The functional behavior of MSCs not only depends on biochemical factors but also is strongly influenced by mechanical forces in their microenvironment. Mechanical stress in form of compression, stretching, vibration, shear force or traction on the extracellular matrix (ECM) regulates signaling mechanotransduction pathways, affecting proliferation, differentiation and migration of MSCs and other bone-forming cells (Discher et al. 2005; Engler et al. 2006; Hadjipanayi et al. 2009; Winer et al. 2009). However, MSCs from aged donors often have a

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distorted cellular homeostasis (Geissler et al. 2013), leading to dire consequences: the resulting increased intracellular stress not only compromises the cells' general proliferation (Fehrer and Lepperdinger 2005) and differentiation potential (Bonab et al. 2006; D'Ippolito et al. 1999; Sethe et al. 2006; Stolzing et al. 2008; Zhang et al. 2008) but also impairs their ability to sense and adapt to mechanical stimuli.

Low-intensity pulsed ultrasound (LIPUS) is a widely used technique for regeneration of fresh fractures, delayed-union bone, non-union bone and other osseous defects (Heckman et al. 1994; Kristiansen et al. 1997). Recent *in vivo* and *in vitro* studies confirmed that LIPUS could trigger signaling events involved in bone healing (Angle et al. 2011; Chow et al. 1998; El-Mowafi and Mohsen 2005; Pounder and Harrison 2008) and stimulate migration (Wei et al. 2014), proliferation and differentiation (Bandow et al. 2007; Padilla et al. 2014; Roussignol et al. 2012; Suzuki et al. 2009; Unsworth et al. 2007) of cells with osteogenic properties. However, both experimental and clinical results remain contentious, limiting the understanding of LIPUS effects on tissue regeneration (Padilla et al. 2016). Most of the *in vitro* experiments established for the investigation of LIPUS stimulated bone-healing mechanisms use planar transducers, exposing cells to risks of uncontrolled heat transfer, standing waves generation, and near-field signal variations (Padilla et al. 2014). These unwanted effects may lead to inadequate translations of *in vitro* findings to *in vivo* applications. In our previous work, we have developed a focused low-intensity pulsed ultrasound (FLIPUS) *in vitro* set-up, which prevents or minimizes the LIPUS-associated artifacts described above and allows for standardized investigation of the physiobiological regenerative mechanisms induced by LIPUS in adherent cells (Puts et al. 2016b). Recently, we have shown that proliferation of bone-forming cells was enhanced when they were cultured in media containing reduced serum supply and stimulated by FLIPUS (Puts et al. 2014). Moreover, increased expression of osteogenic markers in response to a spatial average temporal average intensity (I_{SATA}) of 44.5 mW/cm^2 was observed in rat MSCs (Puts et al. 2016b).

Although LIPUS has been successfully used in clinics, there are still a number of studies questioning its healing potential (Emami et al. 1999; Lubbert et al. 2008; Rue et al. 2004) and compromising the clinical acceptance of LIPUS. The differences noted in these studies could be attributed to generalization of the treatment protocol, which consisted of I_{SATA} = 30 mW/cm^2 delivered at 1 kHz pulse repetition frequency (PRF) with 20% duty cycle (DC) for 20 min, which could be in need of further optimization, depending on fracture type and its fixation or patient properties such as sex and age (Watanabe et al. 2010).

To address the last parameter, we used our recently developed focused LIPUS (FLIPUS) *in vitro* set-up to study age-related differences in the osteogenic differentiation potential of female rMSCs upon stimulation at two different acoustic intensities for 20 min/d for 7 or 14 consecutive d.

MATERIALS AND METHODS

FLIPUS cell stimulation conditions

The stimulation of cells was carried out with a custom-built FLIPUS system (Fig. 1), which has been described in detail in Puts et al. (2016b). This system permits stimulation of adherent cell cultures with FLIPUS under sterile conditions (at 37°C, 95% air and 5% CO_2 supply). Briefly, the system consists of an array of four focused transducers (center frequency: 5-MHz; diameter: 19 mm; epoxy lens with geometric focus R at 22.8 mm; epoxy backing; STT Richter, Mühlanger, Germany) that are placed below the well-plate chamber. The system is controlled by means of a high-precision scanning stage and delivers acoustic sound waves in a temperature-controlled water tank simultaneously into four wells. The well plate was placed at a distance of 13.3 mm above the focus point of the transducer, *i.e.,* the cells were stimulated in the diverging far field of the transducers. With this configuration, the −6-dB cross-sectional stimulation area of approximately 0.81 cm^2 of each transducer ensured a smooth and homogenous intensity distribution within the stimulated wells. By variation of well-transducer array distance and the signal amplitude of the signal generator, the cross-sectional stimulation area can be adjusted, either to the diameter of the well or to local stimulations. The diverging wave front prevents the development of standing waves between well bottom and the upper liquid–air interface inside the well. For the described configuration, the transmission characteristics through the well-plate have been analyzed and optimized previously by means of lipstick hydrophone measurements directly above the well-plate bottom and by numerical sound propagation simulations (Puts et al. 2016b). No measurable intensity levels have been detected in wells adjacent to the stimulated ones. Significant decreases of the transmitted intensity levels were observed at 3.0, 4.5 and 5 MHz due to reverberations inside the well plate bottom. In contrast, 3.6 MHz provided the most optimal transmission, *i.e.,* the most homogenous intensity distribution within the well. The simulations revealed that standing waves and reverberations were much smaller for the focused beam compared to configurations using a planar transducer. The system has been calibrated for I_{SATA} up to 60 mW/cm^2, which is the range typically applied in LIPUS studies (Puts et al. 2016b). FLIPUS was applied daily for 20 min at 3.6 MHz frequency, 100 Hz PRF, 27.8% DC,