Ultrasound in Med. & Biol., Vol. ■, No. ■, pp. 1–10, 2015

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2015.10.021

● *Original Contribution*

EFFECTS OF FOCUSED EXTRACORPOREAL SHOCK WAVES ON BONE MARROW MESENCHYMAL STEM CELLS IN PATIENTS WITH AVASCULAR NECROSIS OF THE FEMORAL HEAD

Lei Zhai,*† Nan Sun,‡ Bo Zhang,§ Shui-Tao Liu,† Zhe Zhao,¶ Hai-Chao Jin,† Xin-Long Ma,‖ and Geng-Yan Xing¶

*Department of Orthopaedic Surgery, The Affiliated Hospital of Logistics College of Chinese People's Armed Police Force, Hedong District, Tianjin 300162, P. R. China; †Department of Orthopaedic Surgery, The General Hospital of Tianjin Medical University, Heping District, Tianjin 300052, P. R. China; ‡Department of Nephropathy, The Affiliated Hospital of Logistics College of Chinese People's Armed Police Force, Hedong District, Tianjin 300162, P. R. China; §Department of Immunology, Tianjin Key Laboratory of Cellular and Molecular Immunology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China; ¶Department of Orthopaedic Surgery, The General Hospital of Chinese People's Armed Police Force, Haidian District, Beijing 100039, P. R. China; and ‖Department of Orthopaedic Surgery, Tianjin Hospital, No 406 Jiefang Nan Road, Hexi District, Tianjin 300211, P. R. China

(*Received* 7 *May* 2015; *revised* 9 *October* 2015; *in final form* 26 *October* 2015)

Abstract—To observe the effect of extracorporeal shock waves (ESWs) on bone marrow mesenchymal stem cells (MSCs) in patients with avascular necrosis of the femoral head, we collected bone marrow donated by patients and then cultivated and passaged MSCs *in vitro* using density gradient centrifugation combined with adherence screening methods. The P3 generation MSCs were divided into the ESW group and the control group. The cell counting kit for MSCs detected some proliferation differences. Cytochemistry, alkaline phosphatase staining and Alizarin red staining were used to determine alkaline phosphatase content. Simultaneously, real-time polymerase factor α1, osteocalcin and peroxisome proliferator-activated receptor γ. Together, the results of our study first indicate that moderate ESW intensity, which is instrumental in enhancing MSC proliferation, inducing conversion of MSCs into osteoblasts, and inhibiting differentiation of MSCs into adipocytes from MSCs, is one of the effective mechanisms for treating avascular necrosis of the femoral head. (E-mail: zhangbomianyi@163.com) © **2015 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Extracorporeal shock wave, Femoral head, Bone marrow mesenchymal stem cells.**

INTRODUCTION

Avascular necrosis of the femoral head (ANFH) is a systemic defect associated with a decrease in bone marrow activity, rather than just the local change in the femoral head. The increasing conversion of mesenchymal stem cells (MSCs) to fat cells results in a corresponding decline in their ability to transform into osteoblasts.

Extracorporeal shock wave therapy (ESWT) has become a new non-invasive treatment because it does not require hospitalization, is associated with fewer complications and has a shorter treatment cycle with lower risk as well as higher cure rate. It is widely used and eutherapeutic in the treatment of tendinopathy, osteoarthritis, non-union and avascular necrosis, chronic low back pain and other diseases (Bannuru et al. 2014; Chen et al. 2009, 2014; Lee et al. 2014).

Extracorporeal shock wave therapy is novel in avascular necrosis research (Hsu et al. 2010; Vulpiani et al. 2012; Wang et al. 2013). The site of new bone formation is restricted not only to the region on which the waves are focused, but also in the surrounding area, when non-union and bone necrosis are being treated. Therefore, we believe that the osteogenesis is not only beneficial for creating local microfractures, but is also more likely to induce bone marrow stromal cells (BMSCs) into osteogenic differentiation in lesions. This indicates that ESWs exert biological effects on the human body through chemical signal transduction, to achieve tissue regeneration and restoration of function. However, the underlying molecular mechanism remains a matter of controversy.

Address correspondence to: Geng-Yan Xing, Department of Orthopaedic Surgery, The General Hospital of Chinese People's Armed Police Force, Haidian District, Beijing 100039, P. R. China. E-mail: zhangbomianyi@163.com

Bone marrow stem cells, as the seed cells in tissue engineering techniques, provide a brand new treatment for avascular necrosis of bone (Korda et al. 2008; Liu et al. 2013; Miao et al. 2014). Because of the low density of BMSCs in bone marrow, conventional chemical induction is not appropriate for clinical applications because it is toxic and complicates the operation process. For this reason, ESWT, as a physical therapy with no side effects, easy operation, and bone formation induction, has been widely studied in recent years.

Core-binding factor α1 (Cbfα1) is a critical gene in osteoblast differentiation, which has a close relationship to osteoblasts, chondrocytes and osteoclasts. This gene also plays a crucial role in the maturation of bone cells and angiogenesis (Muruganandan et al. 2009; Pan et al. 2009; Zhou and Lin 2014). To date, osteocalcin (OCN) is the only known bone extracellular matrix protein produced by osteoblasts. MSCs are the common precursor cells for osteoblasts and adipocytes. Between the bone marrow and adipocytes, there is a signal transduction pathway, which regulates the differentiation of MSCs into osteoblasts and adipocytes. The transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is thought to play an important part in regulating the differentiation of MSCs into fat adipocytes, as well as in stimulating differentiation of MSCs into osteoblasts and inhibiting their differentiation into adipocytes (Yu et al. 2012).

In the method of Donald and Arnold (2006), MSCs of patients with ANFH are cultured *in vitro*, and the impact of ESWs on their activity, proliferation and differentiation is observed, as are the changes in levels of expression of Cbfα1, OCN and PPARγ. Using this method, we explored the molecular mechanism underlying ESWT of ANFH, which provided a theoretical basis for the future clinical application.

METHODS

Bone marrow samples

The study was approved by the Affiliated Hospital of Logistics College of Chinese People's Armed Police Force ethics committee, as well as an institutional review board for the collection of human samples. Twenty bone marrow samples were collected from patients with ANFH aged from 25 to 50 initially. After exclusion of trauma, blood system diseases, tuberculosis and tumor-invasion, six bone marrow samples were finally used for this study. The Association Research Circulation Osseuse (ARCO) classifies ANFH into phases 0–IV. The samples used here, from 4 men and two women, were phases I and II. The aseptic proximal parts of femur bone marrow materials were drilled during the core decompression of femoral head surgery. Afterward, heparin (200 U/mL) was pumped into the material, and then 10 mL equivalent bone marrow material was injected into a heparinized sterile sample bottle, which was placed in oscillation at low temperature under the super-clean bench as soon as possible.

Culture of cells by separation and purification and determination of cell surface molecules

The anticoagulant bone marrow material was removed from bone marrow debris after being washed and filtered. The filtered solution was mixed with Percoll liquid (density = 1.073 g/mL) in the ratio 1:2 after centrifugation at 1,800 rpm for 18 min to draw the intermediate buffy coat. The cells in the sediment were resuspended in DMEM/LG culture, which included 10% fetal bovine serum (FBS) and Green streptomycin (PAA—The Cell Culture Company, Brisbane, Australia). The cells were inoculated at a density of 1–2 $\times$ 10^5/mL density. The primary culture was denoted P0, and subcultures were timely recorded as P1–P7.

After isolation and purification, MSCs from the P3 generation were digested with 0.25% trypsin (HyClone, Logan, UT, USA) and centrifuged (1,000 rpm, 5 min), and the cells were resuspended. After the concentration was adjusted to 1 $\times$ 10^6/L, the cells were reacted with monoclonal antibody tagged with fluorescein isothiocyanate (FITC), anti-CD14, CD34, CD44, CD45, CD90, CD105 (Sigma-Aldrich, St. Louis, MO, USA), at room temperature for 30 min. Flow cytometry was used to detect cell surface molecules.

Grouping of cells and intervention

The primary cells were added to DMEM/LG medium (HyClone) and whipped into a single-cell suspension count after trypsinization and centrifugation at 1,000 rpm for 5 min. Afterward, the supernatant was discarded. The cells were subcultured at a density of 1–1.5 $\times$ 10^4/mL, and labeled P1–P7. The P3 cell suspension was divided into two groups: the intervention group and the control group, eacg containing 5 mL liquid. The intervention group was treated with ESWs for 10 min (0.16 mJ/mm^2, 500 impulses, 1 Hz, electrohydraulic MFL 5000 Lithotriptor, Dornier Medizintechnik, Wessling, Germany) (Fig. 1). We chose these parameters because the flux density was in line with the best data from the previous study, and the intervention group exhibited significant differences compared with the control group (Hofman et al. 2008). There was no intervention in the control group.

Cell proliferation was detected using a CCK-8 kit (Molecular Probes, ThermoFisher Scientific, Grand Island, NY, USA). Suspensions of the two cell groups were injected into 96-hole culture plates, each at a density of 2 $\times$ 10^4/mL. Ten holes containing cells were selected