Indian Journal of Animal Sciences **86** (10): 1111–1114, October 2016/Article

Effect of low-intensity pulsed ultrasound on regenerative potential of transplanted ASCs –PCL construct in articular cartilage defects in sheep

PARVIZ VAHEDI[1], LEILA ROSHANGAR[2], SEYEDHOSEIN JAROLMASJED[3], HAJAR SHAFAEI[4], NASSER SAMADI[5] and JAFAR SOLEIMANIRAD[6]

Maragheh University of Medical Sciences, Maragheh, Iran

Received: 14 January 2016; Accepted: 18 March 2016

ABSTRACT

Articular cartilage is affected by weight loading and mechanical stimuli. Low intensity ultrasound promotes chondrogenesis in cartilage injury. This study was designed to show the effect of low-intensity pulsed ultrasound on chondrogenesis potential of transplanted adipose derived stem cells- polycaprolactone (ASCs –PCL) construct *in vivo*.The adipose tissue was obtained from infrapatellar fat pad of 5 male sheep. Adipose tissue derived stem cells (ASCs) at passage 2 were seeded in polycaprolactone (PCL). The cartilage defects were created on both sides of distal femoral articular cartilage. The right joint was chosen as control group. The left joint was chosen as experimental group and was exposed to low-intensity pulsed ultrasound with intensity 200 mW/cm², 10 min/day for 6 weeks. After 6 months, animals were euthanized to retrieve repaired articular cartilage tissue. Macroscopic appearance of defects was examined and samples of repaired cartilage tissue were analyzed by real time RT-PCR. The results showed that in the treated group with-LIPUS, cartilage defects were totally filled with relatively thin amorphous proliferative tissue and in the control group the defects were left as a dimple in the cartilage defects. Real time RT-PCR analysis showed that cartilage-specific genes expression levels are significantly increased by application of LIPUS after transplantation of ASCs-PCL construct *in vivo*. The results suggested that low-intensity pulsed ultrasound stimulates ASCs differentiation and induces chondrogenesis at the ASCs-PCL construct in *in vivo*.

Key words: Adipose tissue stem cells, Cartilage defects, Chondrogenesis, Scaffolds, Ultrasound therapy

Researches have shown that ultrasound signals consisting of 200 mW/cm^2 and 30 mW/cm^2 is capable to enhance the fracture healing process, including the endochondral phase of fracture healing (Fung *et al.* 2012). The studies supported this view that low-intensity ultrasound enhances transforming growth factor (TGF) mediated chondrocyte differentiation of mesenchymal stem cells *in vitro* (Cui *et al.* 2007). They strongly suggested that ultrasound have stimulatory effects on *in vivo* chondrogenesis and its clinical effects on cartilage are relevant because osteoarthritis makes joint stiff and painful in older adults. Low-intensity pulsed ultrasound was applied to repair soft tissues and its potential benefits in the repair of injured articular cartilage are reported frequently (Lee *et al.* 2006). In this study, we used the LIPUS with intensity 200mW/cm², 10 min/day on transplanted ASCs-PCL construct *in vivo*.

Present address: [1](pa.vahedi48@gmail.com), [2](lroshaengar@yahoo.com), Department of Anatomical Sciences, Maragheh Faculty of Medicine. [3]Umbilical Cord Stem Cell Research Center, [4](shafaei49@yahoo.com), [6](soleimanirj @yahoo.com), Department of Anatomical Sciences, [5](nsamudi @ualber.ac), Department of Biochemistry, Faculty of Medicine. [3](s.h.jarolmasjed@yahoo.com), [4]Department of Clinical Sciences, Faculty of Veterinary Medicine, Tabriz University of Medical sciences, Tabriz.

MATERIALS AND METHODS

Isolation of adipose tissue stem cells: We obtained adipose tissue from infrapatellar fat pad of 5 male sheep (Bergamasca–Massese). The pieces of adipose tissue were incubated by type 1 collagenase. After digestion, it was centrifuged and cell pellet was re-suspended in 100 µl medium. About 4×10^5cells were cultured and was incubated. The medium was replaced every 2 days. Then about 1×10^5 cells were seeded on polycaprolactone (PCL) at passage 2. After 12 h, they were evaluated by scanning electron microscopy.

Transplantation of ASCs-PCL construct: For transplanting ASCs-PCL construct, 10 male sheep (Bergamasca–Massese) were purchased. All animals received humane care in compliance with the "Principles of Laboratory Animals Care" formulated by the National Society for Medical Research. All aspects of the study were approved by the ethical committee of Tabriz University of Medical Sciences. Each sheep was sedated with Xylazine HCl (0.1 mg/kg) for surgical procedure. Both knee joints underwent surgery via a lateral parapatellar skin incision

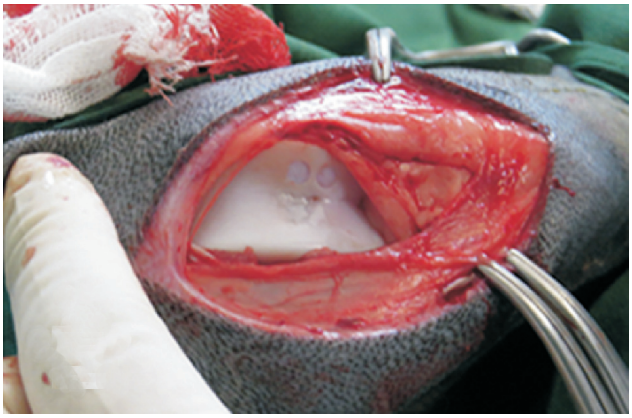


Fig.1. Creation of a lesion with 4 mm diameter in articular cartilage.

and the patellar was dislocated medially to expose knee joint. Two full-thickness cartilage defects (4 mm in diameter, 1 mm in depth) (Casper *et al.* 2010) were created (Fig. 1). ASCs- PCL construct were transplanted in both defects on each knee. Right knee was chosen as control group and left knee was chosen as treatment group.

Ultrasound therapy: The ASCs-PCL constructs were exposed to low-intensity pulsed ultrasound with intensity 200 mW/cm², 10 min/day for 6 weeks. The sheep were maintained for 6 months post-treatment.

Extract of RNA and design of primers: For extracting total RNA, RNX–PLUS solution was used and total RNA was converted into single strand cDNA by using reverse transcriptase and oliqo dT primer was used for synthesis of cDNA**.** All oligonucleotide primers were designed according to the mRNA sequence. So we used ensemble site to design the primers and performed primer blast in NCBI site. The primers sequence used are given in Table 1.

Real time RT-PCR: The real time PCR was performed by using SYBR-Green PCR Master Mix. The cDNA was amplified according to the following condition: 95°C for 15 sec, 56°C for 30 sec and 72°C for 20 sec for 40 to 45 amplification cycles. Fluorescence changes were shown with SYBR Green. A melting curve analysis was performed with 0.5°C/sec increase from 60 to 95°C with continuous fluorescence readings.

Toluidine blue staining: One drop of 1% toluidine blue was used and the sections were washed and fixed by alcohol and then mounted in Canada balsam.

Table 1. Primer sequences of the target genes

Gene	Primer nucleotide sequence	Product size (bp)
hAgg F	5' - GCCCAACTACCTCCGCCATCC-3'	145
hAgg R	5' - CACGATGCCTTTCACCACGACC-3'	
Coll 2 α1F	5' - CTCGTGGTGAACCTGGTACT-3'	81
Coll 2 α1R	5' - CTCCAGGGATTCCGTCAGTT-3'	
BGLAP F	5' - GGTGGTGAAGAGACTCAGGC-3'	136
BGLAP R	5'- GAAGCCGATGTGGTCAGCTA-3'	
SOX-9F	5' - GCACAACACACCCCCTAATC-3'	125
SOX-9 R	5' - CCGGAAGACCACATTGCTAC-3'	
18s F	5' - CCATCCAATCGGTAGTAGCG-3'	92
18s R	5' - GTAACCCGTTGAACCCCATT-3'	

Immunofluorescence staining of collagen Type 2: The sections were cut by cryostat in thick 10 µm and fixed with –20°C acetone and were washed by PBS (10 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4) three times. The slides were incubated with solution of 1% BSA plus 10% goat serum for 2 h. Then primary antibody was added and incubated for 18 h at 4°C. Secondary antibody was added to each section for 1 h and DAPI was added to each section. The slides were protected from light and incubated for 10 min at room temperature and slides were washed 2 times, 5 min each, in PBS.

Gene expression analysis: Experiments were performed in triplicate. Data were obtained, normalized to the house keeping gene and performed using Sigma Stat TM Software for Windows Version 11.5, SPSS Inc for statistical significance by independent T-Test and Leven2 s analysis. Data are expressed as mean ± SEM and $P<0.05$ is considered as significant.

RESULTS AND DISCUSSION

Scanning electron microscopy revealed that the seeded ASCs are well attached to scaffold construct (Figs. 1, 2). Evaluation of gross morphology of the defects revealed that the defect appeared smooth in group treated with LIPUS in comparison to control group (Fig. 3A, B). The defects in treated group were completely filled by a proliferative tissue while in control group the amount of repaired tissue was such that a shallow depression could still be observed (Fig. 3).

Real time RT-PCR analysis of samples from repaired tissue revealed the expression levels of cartilage specific genes. As it is shown in (Fig. 4A), the level of Aggrecan, relative to reference gene (18s) in the group treated with LIPUS during post-surgery period almost increased 4-folds in comparison to control group ($P<0.03$).

The level of Sox9, relative to reference gene (18s), also increased (Fig. 4B) tremendously in treated group in comparison to control group ($P<0.04$). The level of osteocalcin, a non-cartilaginous gene, relative to reference

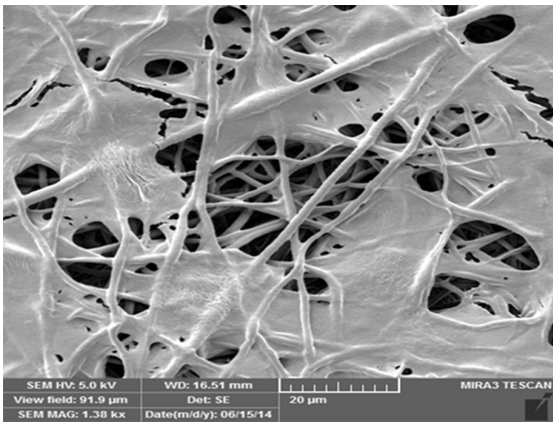


Fig. 2. Scanning electron micrograph of seeded ASCs on PCL nanofiber scaffold 1/38 Kx.

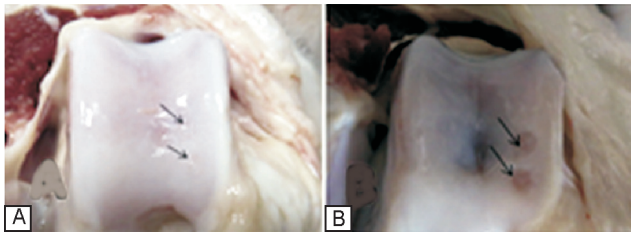
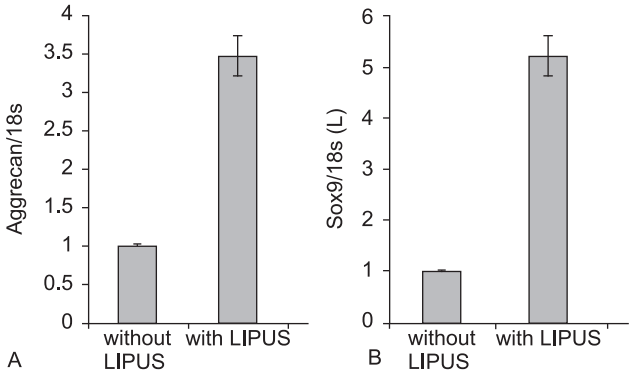


Fig. 3 (A-B). Post-surgical knee joint after 6 month in the LIPUS-treated group. (**A**) the defects were almost totally repaired with cartilaginous tissues, (**B**) in the control group, it shows that small amount of cartilage-like white tissues appeared at the defects.



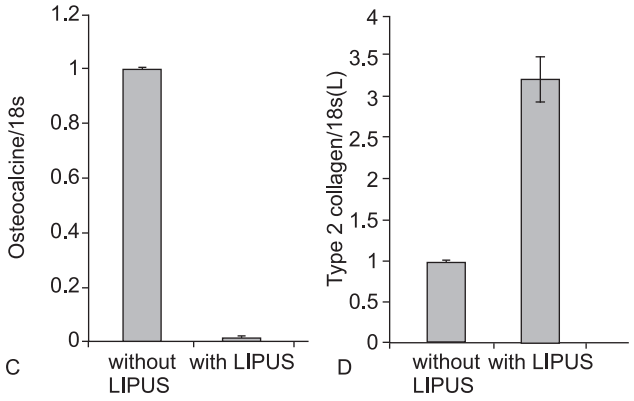


Fig. 4 (A-D). Expression of type 2 collagen (**D**), Aggrecan (**A**), Sox9 (**B**), Osteocalcine genes (**C**) relative to the reference gene18s.

gene (18s), in the treated group in comparison to non-treated group, decreased (Fig. 4C) significantly ($P<0.001$). The level of type 2 collagen, relative to reference gene (18s), had a ten-fold increase (Fig. 4D) in comparison to control group ($P<0.001$). The repaired tissue from articular defect (Fig. 5B) is hyaline cartilage-like when compared with normal hyaline cartilage (Fig. 5A).

Immunofluorescence staining of Collagen type 2 exhibited strong collagen type 2 positive staining and it showed that in the LIPUS-treated groups, Collagen type 2 highly increased in the pericellular regions (Fig. 6) and the negative control did not show Collagen type 2 expression (Fig. 7).

Low-intensity pulsed ultrasound activated chondrocyte phenotypes *in vitro* and it improved regeneration of cartilage

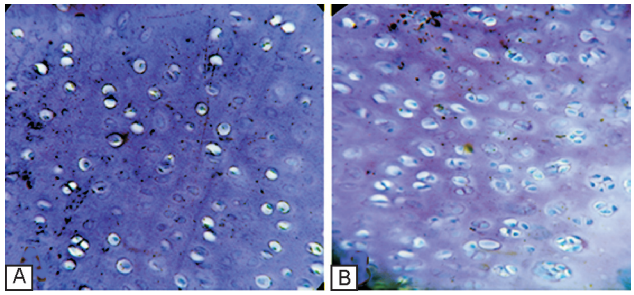


Fig. 5 (A-B). Photomicrograph from normal hyaline cartilage (**A**) and repaired tissue in implanted ASCs-PCL construct on cartilage defects after 6 month (**B**). Toluidine blue staining; original magnification, ×40.

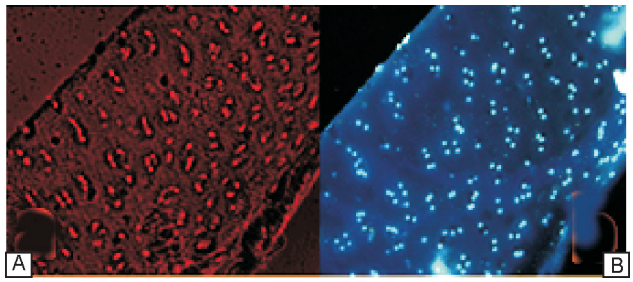


Fig. 6 (A-B). Immunofluorescence staining of collagen Type 2 in the LIPUS-treated groups (**A**), DAPI staining (**B**).

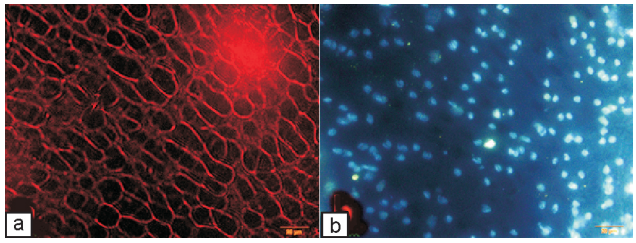


Fig. 7 (A-B). Immunofluorescence staining of collagen Type 2 in the negative control (**A**), DAPI staining (**B**).

in animal models. Studies showed that the mechanical stimulus produced by low-intensity ultrasound improved the articular cartilage defects in human and animal models (Pu *et al*. 2014). The low-intensity ultrasound has more positive effects *in vitro* and *in vivo* on stem cells seeded into alginate or poly glycolic acid scaffolds. Other studies described that low-intensity ultrasound applied at a frequency of 0.8 –1MHz and 200 mW/cm^2 for 20 min or for 10 min a day increased the synthesis of type 2 collagen, PG and viability of chondrocytes and its effect decreased above 500 mW/cm^2 (Choi *et al.* 2006). In alginate culture of rabbit MSCs, low-intensity ultrasound stimulated synthesis of cartilage matrix and induced expression of markers such as aggrecan, Sox 9 and type2 collagen (Shafaei and Baghernezhad 2015). Low-intensity pulsed ultrasound increased the ability of cartilage cells to promote aggrecan gene expression and to synthesize proteoglycans. Further, when cartilage cells in 3 dimensional cultures are treated with LIPUS, it increased the ability of cartilage cells to synthesize type 2 collagen and the expression of type X

collagen is inhibited (Naito 2010). Studies showed that 200 mW/cm^2 of LIPUS (1 MHz, 10 min/day) has important effects for type 2 collagen synthesis and proteoglycans of chondrocytes more than 500 and 700 mW/cm^2 (Gessal *et al*. 2015). This study showed low-intensity pulsed ultrasound with intensity 200 mW/cm², 10 min a day, for 6 weeks accelerates differentiation of ASCs on PCL *in vivo* and produced hyaline cartilage-like tissue instead of fibrocartilage. According to the results, it is concluded that low-intensity pulsed ultrasound could promote differentiation of ASCs to chondrocytes and accelerate chondrogenic process *in vivo*.

ACKNOWLEDGEMENT

The authors are grateful to research department of Tabriz University of Medical Sciences for their financial support.

REFERENCES

Casper M E, Fitzsimmons J S, Stone J J and Meza A O. 2010. Tissue engineering of cartilage using poly-α- caprolactone nanofiber scaffolds seeded *in vivo* with periosteal cells. *Osteoarthritis Cartilage* **18** (7): 981–91.

Cui J H, Park S R, Park K, Choi B H and Min B H. 2007. Preconditioning of mesenchymal stem cells with low intensity ultrasound for cartilage formation *in vivo. Tissue Engineering* **13**: 351–56.

Choi B H. 2006. Low-intensity ultrasound stimulates the viability and matrix gene expression of human articular chondrocytes in alginate bead culture. *Journal of Biomedical Materials Research Part A* **79** (4): 858–64.

Fung C H, Cheung W H, Pounder N M, Ana F J and Harrison A. 2012. Effects of different therapeutic ultrasound intensities on fracture healing in rats. *Ultrasound in Medicine and Biology* **38**: 745–52.

Gessal, Irawan Y, Ilhamjaya P and Gatot L. 2015. Effect of low-intensity pulsed ultrasound on quality of life of knee osteoarthritis. *American Journal of Health Research* **3** (3): 166–69.

Hajar Shafaei and Hassan Baghernezhad. 2015. Ultrasound effect on gene expression of sex determining region Y-box 9 (SOX9) and transforming growth factor β isoforms in adipose stem cells. *Zahedan Journal of Research in Medical Sciences* **15**: 29–31.

Naito K. 2010. Low-intensity pulsed ultrasound (LIPUS) increases the articular cartilage type II collagen in a rat osteoarthritis model. *Journal of Orthopaedic Surgery and Research* **28** (3): 361–69.

Lee H J, Choi B H, Min B H, Son Y S and Park S R. 2006. Low intensity ultrasound stimulation enhances the chondrogenic differentiation in alginate culture of mesenchymal stem cells. *Journal of Artificial Organs* **30**: 707–16.

Pu C, Chang S, Sun J, Liu H and Zhu Y. 2014. Ultrasound-mediated destruction of LHRHa-targeted and paclitaxel-loaded lipid microbubbles for the treatment of intraperitoneal ovarian cancer xenografts. *Molecular Pharmaceutics* **11** (1): 49–58.