Low-intensity pulsed ultrasound promotes cell viability and inhibits apoptosis of H9C2 cardiomyocytes in 3D bioprinting scaffolds via PI3K-Akt and ERK1/2 pathways

Yugang Hu

Renmin Hospital of Wuhan University

Yan Jia

Renmin Hospital of Wuhan University

Hao Wang

Renmin Hospital of Wuhan University

Quan Cao

Renmin Hospital of Wuhan University

Yuanting Yang

Renmin Hospital of Wuhan University

Yanxiang Zhou

Renmin Hospital of Wuhan University

Tuantuan Tan

Renmin Hospital of Wuhan University

Xin Huang

Renmin Hospital of Wuhan University

Qing Zhou (✉ qingzhou@whu.edu.cn)

Renmin Hospital of Wuhan University <https://orcid.org/0000-0001-9796-468X>

Research Article

Keywords: low-intensity pulse ultrasound, 3D bio-printing, cell viability, gelatin methacryloyl, apoptosis

Posted Date: May 26th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1294111/v3>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Objective: The aim of this study was to investigate whether low-intensity pulsed ultrasound (LIPUS) promotes myocardial cell viability in three-dimensional (3D) cell-laden gelatin methacryloyl (GelMA) scaffolds.

Methods: Cardiomyoblasts (H9C2s) were mixed in 6% (w/v) GelMA bio-inks and printed using extrusion-based 3D bioprinter. These scaffolds were exposed to LIPUS with different parameters or sham-irradiated to optimize the LIPUS treatment. The viability of H9C2s was measured using Cell Counting Kit-8 (CCK8), cell cycle, and live and dead cell double staining assays. Western blot analysis was performed to determine the protein expression levels.

Results: We successfully fabricated 3D bio-printed cell-laden GelMA scaffolds. CCK8 and live and dead cell double-staining assays indicated that the optimal conditions for LIPUS were a frequency of 0.5 MHz and exposure time of 10 min. Cell cycle analysis showed that LIPUS promoted the entry of cells into the S and G2/M phases from the G0/G1 phase. Western blot analysis revealed that LIPUS promoted the phosphorylation and activation of ERK1/2 and PI3K-Akt. The ERK1/2 inhibitor (U0126) and PI3K inhibitor (LY294002) significantly reduced LIPUS-induced phosphorylation of ERK1/2 and PI3K-Akt, respectively, which in turn reduced the LIPUS-induced viability of H9C2s in 3D bio-printed cell-laden GelMA scaffolds.

Conclusion: A frequency of 0.5 MHz and exposure time of 10 min for LIPUS exposure can be adapted to achieve optimized culture effects on myocardial cells in 3D bio-printed cell-laden GelMA scaffolds via the ERK1/2 and PI3K-Akt signaling pathways.

Introduction

Ischemic heart disease (IHD) is one of the most common leading causes of morbidity and mortality worldwide^[1-3]. Because of the limited capability to regenerate cardiac tissues, the treatment options available for patients with end-stage heart failure caused by myocardial infarction typically involve grafting tissues from donors^[4-6]. However, the critical shortage of donor organs, immune rejection, limited durability, and high healthcare costs may prevent the wide use of grafting in clinical practice^[7-9], thus, researches on tissue engineering in order to grow tissue directly from cells have been widely motivated nowadays. Among them, as the key element for tissue engineering, a fully satisfactory cell-scaffold-microenvironment need simultaneously support the growth of different cell types, each with specific mechanical properties, cell populations, and geometric structures. Hence, there are two challenges for tissue engineering, fabrication methods and biomaterials. However, traditional fabrication methods for scaffolds, electrospinning, freeze-drying and so on, lack precise control of internal structural features and topology. three-dimensional (3D) bioprinting, an attached fabricating mechanics that applies a layer-by-layer method, has been widely proposed as an additive method for creating functional cardiac tissue^[10-12], due to its higher cell density, more accurate and controllable for the fabrication of multifunctional scaffolds. Biomaterials also play an important role on the cardiac tissue

engineering because they provide the necessary mechanical support and physical structure for cardiomyocytes to attach, grow and maintain their physiological functions. Among the synthesized biomaterials, gelatin methacryloyl (GelMA) has received great attention because of its good biocompatibility, low immunogenicity, and ability to promote cell growth. Nevertheless, various major challenges related to 3D bioprinting with GelMA for cardiac tissues on a macroscopic scale remain to be addressed, including ensuring cell viability and function and the multiple cell type usage, composition, and physical properties required to simulate the complex structure of the extracellular matrix ^[13, 14]. Maintaining sufficient cell viability and functionality for a long duration may be the most difficult task when the cells are transferred from 2D culture to a 3D environment. As a mechanical stimulus, low-intensity pulsed ultrasound (LIPUS) is considered as a secure and practical therapy for fracture healing and has been demonstrated to promote the proliferation of different cell types *in vitro* ^[15-18]. Guo *et al.* combined chondrocytes and alginate to construct a 3D scaffold with or without LIPUS exposure and found that LIPUS significantly enhanced the porosity and permeability of the 3D alginate scaffold, which is significant for cell growth ^[19]. Similar results were also demonstrated in 3D scaffolds containing human bone marrow mesenchymal stem cells ^[20, 21]. However, Wang *et al.* showed that a relatively low dose of LIPUS (70 mW/cm²) increased human omental adipose-derived mesenchymal stem cell viability, whereas a high dose of LIPUS (210 mW/cm²) promoted apoptosis ^[22]. LIPUS was also reported to inhibit the proliferation of osteosarcoma cells (30 mW/cm²) ^[23] and promote apoptosis of osteoclasts (30 mW/cm²) ^[24]. Thus, the optimal dose of LIPUS for various cell types and intensities must be determined. In addition, the effect of LIPUS on cardiac muscle cells in a 3D scaffold is unclear. Moreover, ERK1/2 and PI3K-Akt signaling pathways are important pathways that mediate cell cycle progression and promote cell survival and proliferation and LIPUS also had close relationship with these pathways in previous studies. Therefore, in the present study, a 3D bio-printed gelatin methacryloyl (GelMA) scaffold containing H9C2 cells was fabricated using a desktop 3D bioprinter. The effect of LIPUS on the apoptosis and viability of H9C2s was determined, and the roles of the ERK1/2 and PI3K-Akt signaling pathways were also examined. Our results support the applied of LIPUS to promote the viability of cardiac cells and may improve the application of LIPUS to generate functional cardiac tissues.

Materials And Methods

GelMA bioink preparation

Cardiomyoblasts (rat heart myoblast H9C2 cells) obtained from Procell (Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, NY, USA) complemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 IU/mL penicillin (Servicebio, Wuhan, China), and 100 mg/mL streptomycin (Servicebio, Wuhan, China) at 37 °C in a 5% CO₂ atmosphere. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), GelMA powder, and the UV light source (405 nm) were purchased from the SuZhou Intelligent Manufacturing Research Institute (SuZhou, China). LAP was dissolved in phosphate-buffered saline (PBS, Servicebio, Wuhan, China) to a concentration of 0.25% (w/v) and heated

at 55 °C for 15 min to ensure complete dissolution. GelMA powder was dissolved in LAP solution at a concentration of 6% at 70 °C for 30 min and filtered through a filter membrane (0.22 µm, Millipore, Billerica, MA, USA) before use. All of these processes were conducted in the bechtop. Cardiomyoblasts were collected from the culture dish and homogeneously dispersed into the GelMA solution (37 °C) to form a single-cell suspension at a cell density of approximately 5×10^6 cells/mL. The cell-laden GelMA solution was then loaded into a 5-mL syringe equipped with a 25 G needle (SUNP BIOTECH, China), which was fixed at the extrusion printhead.

3D-bioprinting

Scaffolds were fabricated using a customized extrusion-based 3D printer BioMaker (SUNP BIOTECH, China). Computer-aided design software was used to produce a G-code file for printing. A 3D scaffold model with dimensions of 10 mm width, 10 mm length, and 1 mm height was designed with a 1-mm filament grid and 0.2-mm layer thickness. Before bioprinting, the cell-laden GelMA bio-ink was placed at 4 °C for approximately 30 s until it changed from liquid to semi-solid, and then placed in the 3D printer for bio-printing. The cell-laden GelMA bio-ink was extruded onto a moving platform based on the model described above, and Biomarker Software Suite 1.0.7 (SUNP BIOTECH, China) was applied to monitor and manage the printing process. The 3D GelMA scaffolds containing H9C2 cells were printed into a Petri dish and immersed in DMEM at 37 °C with 5% CO₂ after UV light exposure (wavelength: 405 nm) for 30 s to photo-crosslink the bio-inks to ensure their long-term stability. All printing processes were performed in the bechtop at room temperature with the nozzle set at a moving speed of 10 mm/s and the pressure was 0.5MPa. Moreover, everytime before bioprinting, we wiped the bioprinter with 75% alcohol totally and exposed it to the UV irradiation for 30 min for sterility.

Optimization of LIPUS treatment protocol and dosage

The LIPUS exposure device (Chongqing Haifu Medical Technology Co., Ltd., Chongqing, China) comprises an array of five transducers (34.8 mm in diameter), which is specifically drafted for a 6-well culture plate. A 6-well plate was deposited on the transducers with a thin layer of ultrasound coupling agent between them. The parameters of the LIPUS device were an intensity of 1.0 W/cm² (temporal average intensity), duty cycle of 20%, and pulse repetition frequency of 1 kHz; four frequencies (0.5, 1.0, 1.5, and 2.0 MHz) were used. All Scaffolds in the LIPUS group were exposed to LIPUS, whereas those in the control group received sham irradiation (LIPUS was turned off).

Cytocompatibility tests

A Cell Counting Kit-8 (CCK-8; Servicebio, Wuhan, China) assay was performed to measure the proliferation of H9C2 cells. After 1, 4, and 7 days of treatment with LIPUS or sham irradiation, scaffolds with H9C2 cells were incubated in CCK-8 solution for 4 h. Next, 10 µL of the supernatant was transferred into a 96-

well culture plate, and its absorbance at 450 nm was measured using a microplate reader (EnSight; Perkin Elmer, Waltham, MA, USA). To reduce the effect of non-clay adsorbing the staining solution, scaffolds without seeded cells were used as the blank group.

Cell viability was evaluated using the Live and Dead Cell Double Staining Kit (EFL-CLD-001, SuZhou) after treatment with LIPUS or sham irradiation for 1, 4, and 7 days. The scaffolds were washed twice with PBS, and then immersed in live/dead staining solution of 10 mL PBS containing 5 μ L calcein-AM and 5 μ L propidium iodide (PI) solution according to the manufacturer's instructions. After incubation at 37 °C for 45 min, the staining solution was removed, and the scaffolds were washed with PBS. Fluorescence staining was analyzed using confocal laser scanning microscopy (ECLIPSE TI; Nikon, Tokyo, Japan). Cell viability was calculated from the number of green (representing live cells) and red points (representing dead cells) by randomly five images of each groups.

Flow cytometry

For the cell cycle assay, after LIPUS or sham irradiation treatment, cell-laden GelMA scaffolds were cut into small pieces and lysed using GelMA Lysis Buffer (EFL-GM-LS-001, SuZhou, China), harvested, and fixed with pre-cooled 75% ethanol at 4 °C overnight. After centrifugation, the cells were incubated with PI and RNase A at 37 °C for 30 min in the dark. The cell cycle stages were analyzed at an excitation wavelength of 488 nm using flow cytometry (BD Biosciences, San Jose, USA) and the data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Apoptosis of H9C2 cells was determined using an FITC Annexin V and PI double staining kit (Servicebio, Wuhan, China). After different treatments, the cells were collected, resuspended, and incubated with 5 μ L of Annexin V and 5 μ L of PI. After incubation for 20 min in the dark at room temperature, the cells were stained for 15 min, and a flow cytometer (BD Biosciences, San Jose, USA) was used to detect apoptosis. The data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

Western blot analysis

H9C2s were pre-treated with U0126 (20 μ mol/L) (specific inhibitors of the ERK1/2 pathway) or LY294002 (50 μ mol/L) (specific inhibitors of the PI3K-Akt pathway) for 1 h before 3D bioprinting. After bioprinting, the scaffolds were treated with LIPUS or sham irradiation and then lysed with GelMA Lysis Buffer for 2 h. The H9C2 cells were isolated by centrifugation and lysed in RIPA lysis buffer, after which the proteins were obtained using centrifugation. Protein concentrations were determined using the Bradford protein assay kit. The protein samples were separated using SDS-PAGE and then electro-transferred onto polyvinylidene fluoride membranes (Millipore). After washing, the membranes were blocked with 5% skim milk for 1 h, and then incubated overnight at 4 °C with specific primary antibodies for Bcl2, Bax, Caspase-3 (all from ASPEN, Wuhan, China), ERK1/2, phospho-ERK1/2, PI3K, phospho-PI3K, Akt, and phospho-Akt (all from ASPEN). After washing three times, the membranes were incubated for 1 h at room temperature

with secondary antibodies (ASPEN). The BeyoECL Plus kit was used for color development according to the manufacturer's instructions (Beyotime, Shanghai, China). The protein bands were quantified by densitometry scanning using the AlphaEaseFC 4.0 software (Alpha Innotech Corp, San Leandro, CA, USA). The quantitative densitometry values of all proteins were normalized to those of GAPDH.

Statistical analysis

At least three independent experiments were performed for all tests. All data were analyzed using SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) and expressed as the mean $\pm$ standard deviation. A two-sample t-test or one-way analysis of variance was used to analyze continuous variables across groups. All statistical tests were two-tailed, and a value of $P < 0.05$ was considered to indicate statistically significant results.

Results

Determination of the optimal frequency and exposure time of LIPUS

After successful bio-fabrication of the GelMA scaffolds (Figure 1), the scaffolds were transferred into 6-well plates for LIPUS exposure or sham irradiation. To determine the optimal frequency and exposure time of LIPUS for ensuring H9C2s viability, we first performed a CCK-8 assay. As shown in Figure 2A–C and Table 1, the mean optical density values of the LIPUS groups were much higher than those of the control group after 4 and 7 days of LIPUS exposure. The linear increase in absorbance indicated that cell viability was increased in both groups over 7 days. However, the trends in cell viability were not entirely consistent in the LIPUS and control groups; the absorbance of the former was much higher, indicating that performing LIPUS at different frequencies is beneficial for the proliferation of H9C2 cells when using the same cell number of cells in all groups. Moreover, GelMA scaffolds with H9C2 cells treated at a frequency of 0.5 MHz for 10 min showed significantly higher viability compared to that in the control group ($P < 0.05$) and other frequencies and exposure time groups; based on these results, the optimal conditions were determined.

We then performed flow cytometry to analyze the cell cycle distribution of the LIPUS-treated and control groups to confirm our results. As shown in Figure 3 and Table 2, the number of cells in G0/G1 phase after LIPUS treatment was significantly decreased ($P < 0.05$) compared to that in the control group, whereas cells in the S and G2/M phases were increased significantly ($P < 0.05$) after one day of LIPUS treatment or sham irradiation. The proportion of cells in the S and G2/M phases reached a maximum at a frequency of 0.5 MHz and exposure time of 10 min. These results demonstrate that LIPUS can induce H9C2 cells in GelMA scaffolds to enter the S and G2/M phases from the G0/G1 phase, indicating H9C2 cell proliferation. Thus, LIPUS at a frequency of 0.5 MHz and exposure time of 10 min was determined to be the optimal conditions and applied in the following experiments.

We confirmed our results by performing live/dead cell double staining, in which living cells and dead cells were labeled as green and red, respectively. At one day after LIPUS exposure or sham irradiation, the viabilities of cells in the LIPUS and control groups were $75.8 \pm 1.1\%$ and $66.8 \pm 1.8\%$, respectively ($P < 0.05$). After seven days of culture, the viability of cells in the LIPUS group increased by $90.8 \pm 2.1\%$, whereas that in the control group slightly increased to $69.8 \pm 1.2\%$ ($P < 0.05$, Figure 2D–E). 3D images of the cell-laden GelMA scaffolds were also obtained after culture for 7 days using Z-stack scanning to confirm these results.

Effects of LIPUS on apoptosis of H9C2s

To investigate the protective effects of LIPUS on cardiomyocyte apoptosis in GelMA scaffolds, Annexin V/PI analysis was performed after 24 h of LIPUS exposure or sham irradiation. As shown in Figure 4A and B, compared with the control group, cardiomyocytes in the LIPUS groups at all exposure times exhibited a significantly decreased apoptotic rate; the lowest rate was observed at 10 min of exposure time. Furthermore, the expression of apoptosis-related proteins was evaluated. LIPUS increased Bcl-2 levels and decreased the Bax and cleaved caspase-3 levels compared to that in the control group (Figure 4C–G). These results suggest that LIPUS inhibits the apoptotic signaling pathway.

Activation of ERK1/2 and PI3K-Akt in LIPUS-induced GelMA scaffolds

Extracellular signal-regulated kinases (ERK1/2) and PI3K-Akt signaling pathways have been reported to play important roles in cell proliferation. Western blotting was conducted to determine whether the ERK1/2 and PI3K-Akt signaling pathways are involved in protective effects of LIPUS exposure. The phosphorylation of ERK1/2, PI3K, and Akt protein expression was markedly increased by LIPUS (Figure 5A, $P < 0.01$). The protein levels of phospho-ERK1/2, phospho-PI3K, and phospho-Akt significantly increased and peaked in 0.5MHz-10min LIPUS treatment group.; therefore, this treatment time was selected for subsequent western blot analysis. These results demonstrate that ERK1/2 and PI3K-Akt signaling is activated following LIPUS treatment.

Inhibition of ERK1/2 and PI3K-Akt signaling pathways reduces LIPUS-induced proliferation of H9C2s in GelMA scaffolds

To determine whether the ERK1/2 and PI3K-Akt signaling pathways contributed to the proliferative effect of LIPUS, H9C2s were pretreated with the inhibitors U0126 (10 $\mu\text{mol/L}$) or LY294002 (50 $\mu\text{mol/L}$) respectively, for 1 h before 3D-bioprinting. The protein levels of phospho-ERK1/2 and phospho-Akt in the LIPUS-treated group significantly decreased after pretreatment with U0126 and LY294002, respectively (Figure 5B–C, $P < 0.01$), indicating that these agents effectively blocked activation of ERK1/2 and Akt in LIPUS-treated H9C2s.

Cell proliferation was assessed using CCK-8 and live/dead cell double staining assays. Cell viability in the LIPUS-treated group was significantly higher than that in the LIPUS group pretreated with U0126 or LY294002 (LIPUS + inhibitor groups) and the control group after 1 day of LIPUS treatment ($P < 0.01$,

Figure 6A). The live/dead cell double staining assays also showed that the viable ratio of H9C2s in 3D-printed GelMA scaffolds increased significantly after LIPUS treatment compared to that in the control group, and that this increase was blocked by pre-treatment with U0126 or LY294002 ($P < 0.01$, Figure 6B–C). These results demonstrate that the ERK1/2 and PI3K-Akt signaling pathways are involved in promoting H9C2S cell proliferation following LIPUS of 3D-printed GelMA scaffolds.

Discussion

3D bioprinting is among the most advanced therapeutic strategies for fabricating complicated implants with biomimetic features, which exhibit both the native physiochemical and biomechanical characteristics of humans [25, 26]. Although the bioprinting technique is in its infancy and analogs with full biological functionality have not yet been generated, 3D bioprinting is widely used to create bone, cartilage, neural, and cardiovascular tissues and is employed in studies of the molecular basis of cardiac function, as well as to explore related signaling pathways [27–30]. Moreover, preparing myocardial cells with high viability and that function for a longer time in a 3D environment, which will enable 3D-bioprinting to be translated from the experimental stage to clinical practice, remains difficult. In this study, we first combined 3D bioprinting with LIPUS to investigate the impact of LIPUS on the proliferation of H9C2s in 3D printed scaffold-based cardiac patches and found that LIPUS promoted cell viability and inhibited apoptosis of cardiomyocytes via the PI3K-AKT and ERK1/2 pathways in 3D bioprinted scaffolds.

A simplified process of printing and tolerant conditions of shaping can reduce the time in which cells are not in the culture environment to prevent the loss of cell viability [31]. Thus, we performed this study using a desktop 3D-bioprinter with an acceptable speed range to shorten the printing time. Moreover, GelMA, which forms covalently crosslinked hydrogels under UV light exposure in the presence of a photoinitiator, has received attention in the field of biomedical applications for building constructs with various 3D architectures because of its good biocompatibility, low immunogenicity, and ability to promote cell growth [32–34]. Nevertheless, one of the main challenges to using GelMA in engineered scaffolds is to balance the physical printability and biological biocompatibility, as a high concentration of GelMA ($\geq 10\%$ w/v) has excellent printability but decreased cell viability. In contrast, low levels of GelMA ($\leq 5\%$ w/v) show high cell stability and viability and low viscosity of GelMA bio-inks, leading to a slow change from liquid to semi-solid. Because of this, low-concentration GelMA bio-inks exhibit poor processability during the 3D printing process [35, 36]. In this study, we selected 6% w/v as the final concentration of GelMA for 3D bioprinting. To improve printability, this material was placed at 4 °C for approximately 30 s to accelerate the change in state from liquid to semi-solid before 3D bioprinting and immediately after 3D bioprinting, and the GelMA scaffolds were exposed to UV light for 30 s, which is a relatively long time, to maintain the structural integrity and precision after printing. The cell-laden GelMA scaffolds maintained their structural integrity for more than 30 days, which may ensure the consistency of the cell growth space and provides a promising material for *in vivo* studies.

As a non-toxic and noninvasive form of mechanical stimulation, LIPUS has been widely used in fracture healing, wound healing, drug delivery, thrombolysis, and chemotherapy [37, 38]. Moreover, *in vivo* and *in vitro* studies demonstrated that LIPUS, depending on the irradiated parameters, promotes cell proliferation, osteogenic differentiation, and extracellular matrix formation [39]. Xie *et al.* found that LIPUS had a positive effect on the proliferation of human bone marrow mesenchymal stem cells at an optimal intensity of 50 or 60 mW/cm² (frequency = 1.5 MHz, exposure time = 5 min) [40]. Another study showed that the optimal working parameters for LIPUS were 150 mW/cm² when combined with 3D bioprinting tissue scaffolds [41]. Furthermore, Gao *et al.* verified the impact of LIPUS on different sources of mesenchymal stem cells and showed that both 250 and 750 mW/cm² had the same effect on cell proliferation; additionally, different types of mesenchymal stem cells responded differently to various LIPUS treatment regimens, with the ultrasound exposure duration less important than the intensity [42]. In addition to promoting cell proliferation, LIPUS was reported to inhibit cell growth. Katiyar *et al.* found that the optimal intensity of LIPUS for promoting proliferation was approximately 75 mW/cm²; when the intensity was increased to 465 mW/cm², LIPUS significantly reduced the proliferation of MC3T3-E1 cells [43]. Taken together, these studies suggest that different types of cells under varying conditions may respond differently to different LIPUS stimulation. In this study, we combined LIPUS and cell-laden 3D-bioprinted GelMA scaffolds and found that the optimal frequency was 0.5 MHz (intensity = 1.0 W/cm², exposure time = 10 min) in H9C2s.

Bcl-2 is a member of the Bcl-2 family of proteins that inhibits pro-apoptotic molecules and plays a vital role in cell survival [44]. Bax is also a member of the Bcl-2 family and induces apoptosis via cytochrome C-mediated cleavage of caspase-3 [45]. In this study, the effects of LIPUS exposure on the expression levels of Bcl-2, Bax, and cleaved caspase-3 were evaluated. Increased expression of Bcl-2 and decreased expression of Bax and cleaved caspase-3 were observed in H9C2s in response to LIPUS irradiation. We also found that LIPUS-activated ERK1/2 and PI3K-Akt signaling pathways induced H9C2s to enter the S and G2/M phases from the G0/G1 phase. ERK1/2 and PI3K-Akt signaling pathways are important pathways that mediate cell cycle progression and promote cell survival and proliferation. We investigated the roles of the ERK1/2 and PI3K-Akt signaling pathways in LIPUS-induced cell proliferation. The levels of p-ERK1/2 and p-Akt increased significantly after LIPUS irradiation compared to that in the control group. In addition, when LIPUS-treated H9C2s were pre-treated with the inhibitors U0126 and LY294002, the amounts of p-ERK1/2 and p-Akt were significantly decreased, thus inhibiting LIPUS-induced H9C2s proliferation in 3D-printed GelMA scaffolds. Therefore, LIPUS likely promotes H9C2s proliferation via phosphorylation and activation of ERK1/2 and PI3K-Akt. Although the precise mechanism by which LIPUS activates these signaling pathways remains unclear, it has been hypothesized that, initially, these acoustic radiation forces can sensitize the H9C2s in 3D-printed GelMA scaffolds through its mechanosensitive surface structure and, consequently, induce morphological deformation [46].

Conclusions

Our results suggest that LIPUS can promote the proliferation of H9C2s, and activation of the ERK1/2 and PI3K-Akt signaling pathways may be involved in this process. Our findings provide important experimental evidence for applying LIPUS to promote the proliferation of H9C2s.

References

- [1] Benjamin E J, Muntner P, Alonso A, et al. Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association[J]. *Circulation*, 2019,139(10):e56-e528.
- [2] Roerecke M, Rehm J. Ischemic heart disease mortality and morbidity rates in former drinkers: a meta-analysis[J]. *Am J Epidemiol*, 2011,173(3):245-258.
- [3] Roncella A. Psychosocial Risk Factors and Ischemic Heart Disease: A New Perspective[J]. *Rev Recent Clin Trials*, 2019,14(2):80-85.
- [4] Bergmann O, Bhardwaj R D, Bernard S, et al. Evidence for cardiomyocyte renewal in humans[J]. *Science*, 2009,324(5923):98-102.
- [5] Alraies M C, Eckman P. Adult heart transplant: indications and outcomes[J]. *J Thorac Dis*, 2014,6(8):1120-1128.
- [6] Elgendy I Y, Mahtta D, Pepine C J. Medical Therapy for Heart Failure Caused by Ischemic Heart Disease[J]. *Circ Res*, 2019,124(11):1520-1535.
- [7] Verheugt F W. Anticoagulation in patients with mechanical heart valves: follow the guidelines![J]. *Neth Heart J*, 2015,23(2):109-110.
- [8] Page A, Messer S, Large S R. Heart transplantation from donation after circulatory determined death[J]. *Ann Cardiothorac Surg*, 2018,7(1):75-81.
- [9] Pajaro O E, Jaroszewski D E, Scott R L, et al. Antibody-mediated rejection in heart transplantation: case presentation with a review of current international guidelines[J]. *J Transplant*, 2011,2011:351950.
- [10] Alonzo M, AnilKumar S, Roman B, et al. 3D Bioprinting of cardiac tissue and cardiac stem cell therapy[J]. *Transl Res*, 2019,211:64-83.
- [11] Liu N, Ye X, Yao B, et al. Advances in 3D bioprinting technology for cardiac tissue engineering and regeneration[J]. *Bioact Mater*, 2021,6(5):1388-1401.
- [12] Lee A, Hudson A R, Shiowski D J, et al. 3D bioprinting of collagen to rebuild components of the human heart[J]. *Science*, 2019,365(6452):482-487.
- [13] Derakhshanfar S, Mbeleck R, Xu K, et al. 3D bioprinting for biomedical devices and tissue engineering: A review of recent trends and advances[J]. *Bioact Mater*, 2018,3(2):144-156.

- [14] Puluca N, Lee S, Doppler S, et al. Bioprinting Approaches to Engineering Vascularized 3D Cardiac Tissues[J]. *Curr Cardiol Rep*, 2019,21(9):90.
- [15] Leighton R, Watson J T, Giannoudis P, et al. Healing of fracture nonunions treated with low-intensity pulsed ultrasound (LIPUS): A systematic review and meta-analysis[J]. *Injury*, 2017,48(7):1339-1347.
- [16] Wu Y, Gao Q, Zhu S, et al. Low-intensity pulsed ultrasound regulates proliferation and differentiation of neural stem cells through notch signaling pathway[J]. *Biochem Biophys Res Commun*, 2020,526(3):793-798.
- [17] Xie S, Jiang X, Wang R, et al. Low-intensity pulsed ultrasound promotes the proliferation of human bone mesenchymal stem cells by activating PI3K/Akt signaling pathways[J]. *J Cell Biochem*, 2019,120(9):15823-15833.
- [18] Sang F, Xu J, Chen Z, et al. Low-Intensity Pulsed Ultrasound Alleviates Osteoarthritis Condition Through Focal Adhesion Kinase-Mediated Chondrocyte Proliferation and Differentiation[J]. *Cartilage*, 2020:939066430.
- [19] Guo G, Lu L, Ji H, et al. Low intensity pulse ultrasound stimulate chondrocytes growth in a 3-D alginate scaffold through improved porosity and permeability[J]. *Ultrasonics*, 2015,58:43-52.
- [20] Zhou X, Castro N J, Zhu W, et al. Improved Human Bone Marrow Mesenchymal Stem Cell Osteogenesis in 3D Bioprinted Tissue Scaffolds with Low Intensity Pulsed Ultrasound Stimulation[J]. *Sci Rep*, 2016,6:32876.
- [21] Osborn J, Aliabouzar M, Zhou X, et al. Enhanced Osteogenic Differentiation of Human Mesenchymal Stem Cells Using Microbubbles and Low Intensity Pulsed Ultrasound on 3D Printed Scaffolds[J]. *Adv Biosyst*, 2019,3(2):e1800257.
- [22] Wang Y, Jiang L, Xu T, et al. p38 MAPK signaling is a key mediator for low-intensity pulsed ultrasound (LIPUS) in cultured human omental adipose-derived mesenchymal stem cells[J]. *Am J Transl Res*, 2019,11(1):418-429.
- [23] Matsuo T, Sato K, Matsui T, et al. Inhibitory effects of low-intensity pulsed ultrasound sonication on the proliferation of osteosarcoma cells[J]. *Oncol Lett*, 2017,14(3):3071-3076.
- [24] Suzuki N, Hanmoto T, Yano S, et al. Low-intensity pulsed ultrasound induces apoptosis in osteoclasts: Fish scales are a suitable model for the analysis of bone metabolism by ultrasound[J]. *Comp Biochem Physiol A Mol Integr Physiol*, 2016,195:26-31.
- [25] Ostrovidov S, Salehi S, Costantini M, et al. 3D Bioprinting in Skeletal Muscle Tissue Engineering[J]. *Small*, 2019,15(24):e1805530.

- [26] Matai I, Kaur G, Seyedsalehi A, et al. Progress in 3D bioprinting technology for tissue/organ regenerative engineering[J]. *Biomaterials*, 2020,226:119536.
- [27] Zhou X, Zhu W, Nowicki M, et al. 3D Bioprinting a Cell-Laden Bone Matrix for Breast Cancer Metastasis Study[J]. *ACS Appl Mater Interfaces*, 2016,8(44):30017-30026.
- [28] Zhou X, Cui H, Nowicki M, et al. Three-Dimensional-Bioprinted Dopamine-Based Matrix for Promoting Neural Regeneration[J]. *ACS Appl Mater Interfaces*, 2018,10(10):8993-9001.
- [29] Zhu W, Cui H, Boualam B, et al. 3D bioprinting mesenchymal stem cell-laden construct with core-shell nanospheres for cartilage tissue engineering[J]. *Nanotechnology*, 2018,29(18):185101.
- [30] Cui H, Miao S, Esworthy T, et al. 3D bioprinting for cardiovascular regeneration and pharmacology[J]. *Adv Drug Deliv Rev*, 2018,132:252-269.
- [31] Mann J L, Yu A C, Agmon G, et al. Supramolecular polymeric biomaterials[J]. *Biomater Sci*, 2017,6(1):10-37.
- [32] Yue K, Trujillo-de S G, Alvarez M M, et al. Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels[J]. *Biomaterials*, 2015,73:254-271.
- [33] Liu W, Zhong Z, Hu N, et al. Coaxial extrusion bioprinting of 3D microfibrous constructs with cell-favorable gelatin methacryloyl microenvironments[J]. *Biofabrication*, 2018,10(2):24102.
- [34] Liu W, Zhang Y S, Heinrich M A, et al. Rapid Continuous Multimaterial Extrusion Bioprinting[J]. *Adv Mater*, 2017,29(3).
- [35] Colosi C, Shin S R, Manoharan V, et al. Microfluidic Bioprinting of Heterogeneous 3D Tissue Constructs Using Low-Viscosity Bioink[J]. *Adv Mater*, 2016,28(4):677-684.
- [36] Liu W, Heinrich M A, Zhou Y, et al. Extrusion Bioprinting of Shear-Thinning Gelatin Methacryloyl Bioinks[J]. *Adv Healthc Mater*, 2017,6(12).
- [37] Leighton R, Watson J T, Giannoudis P, et al. Healing of fracture nonunions treated with low-intensity pulsed ultrasound (LIPUS): A systematic review and meta-analysis[J]. *Injury*, 2017,48(7):1339-1347.
- [38] Zhong Y, Zhang Y, Xu J, et al. Low-Intensity Focused Ultrasound-Responsive Phase-Transitional Nanoparticles for Thrombolysis without Vascular Damage: A Synergistic Nonpharmaceutical Strategy[J]. *ACS Nano*, 2019,13(3):3387-3403.
- [39] Man J, Shelton R M, Cooper P R, et al. Low intensity ultrasound stimulates osteoblast migration at different frequencies[J]. *J Bone Miner Metab*, 2012,30(5):602-607.

- [40] Xie S, Jiang X, Wang R, et al. Low-intensity pulsed ultrasound promotes the proliferation of human bone mesenchymal stem cells by activating PI3K/Akt signaling pathways[J]. J Cell Biochem, 2019,120(9):15823-15833.
- [41] Zhou X, Castro N J, Zhu W, et al. Improved Human Bone Marrow Mesenchymal Stem Cell Osteogenesis in 3D Bioprinted Tissue Scaffolds with Low Intensity Pulsed Ultrasound Stimulation[J]. Sci Rep, 2016,6:32876.
- [42] Gao Q, Walmsley A D, Cooper P R, et al. Ultrasound Stimulation of Different Dental Stem Cell Populations: Role of Mitogen-activated Protein Kinase Signaling[J]. J Endod, 2016,42(3):425-431.
- [43] Katiyar A, Duncan R L, Sarkar K. Ultrasound stimulation increases proliferation of MC3T3-E1 preosteoblast-like cells[J]. J Ther Ultrasound, 2014,2:1.
- [44] Bhutia S K, Dash R, Das S K, et al. Mechanism of autophagy to apoptosis switch triggered in prostate cancer cells by antitumor cytokine melanoma differentiation-associated gene 7/interleukin-24[J]. Cancer Res, 2010,70(9):3667-3676.
- [45] Wu H, Che X, Zheng Q, et al. Caspases: a molecular switch node in the crosstalk between autophagy and apoptosis[J]. Int J Biol Sci, 2014,10(9):1072-1083.
- [46] Zhang S, Cheng J, Qin Y X. Mechanobiological modulation of cytoskeleton and calcium influx in osteoblastic cells by short-term focused acoustic radiation force[J]. PLoS One, 2012,7(6):e38343.

Declarations

Funding: This work was supported by the National Natural Science Foundation of China (No. 81971624), Wuhan Municipal Science and Technology Bureau (No. 2019020701011477).

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Author Contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by HYG, JY, CQ, WH, YYT, ZYX, TTT, ZX and ZQ. The first draft of the manuscript was written by HYG and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Availability of Data and Material The data that support this study are available from the corresponding author upon reasonable request.

Ethics approval: Not applicable.

Tables

Table 1 Optical density (OD) values at 450 nm of each group at different days after LIPUS treatment (n=6)

Groups	Frequency	Exposure time		
		5min	10min	15min
Day 0				
Control group	0	0.296±0.025	0.290±0.021	0.306±0.032
LIPUS groups	0.5MHz	0.289±0.044	0.309±0.033	0.312±0.041
	1.0MHz	0.301±0.043	0.319±0.037	0.291±0.033
	1.5MHz	0.300±0.037	0.294±0.031	0.305±0.032
	2.0MHz	0.299±0.027	0.305±0.034	0.309±0.036
Day 1				
Control group	0	0.351±0.016	0.351±0.016	0.351±0.016
LIPUS groups	0.5MHz	0.389±0.036	0.416±0.028*	0.410±0.028
	1.0MHz	0.375±0.022	0.384±0.021	0.396±0.030
	1.5MHz	0.362±0.040	0.376±0.014	0.383±0.027
	2.0MHz	0.370±0.023	0.376±0.031	0.400±0.032
Day 4				
Control group	0	0.695±0.109	0.695±0.109	0.695±0.109
LIPUS groups	0.5MHz	1.201±0.029*	1.426±0.039*	1.367±0.062*
	1.0MHz	1.153±0.157*	1.126±0.068*#	1.145±0.076*#
	1.5MHz	1.129±0.146*	1.133±0.073*#	1.157±0.073*#
	2.0MHz	1.132±0.086*	1.166±0.071*#	1.228±0.079*
Day 7				
Control group	0	1.051±0.129	1.051±0.129	1.051±0.129
LIPUS groups	0.5MHz	1.483±0.065*	1.628±0.056*	1.480±0.100*
	1.0MHz	1.466±0.087*	1.413±0.066*#	1.427±0.073*
	1.5MHz	1.456±0.033*	1.473±0.081*	1.473±0.089*
	2.0MHz	1.469±0.079*	1.537±0.083*	1.431±0.788*

LIPUS: low-intensity pulsed ultrasound. Data were mean (SD) unless otherwise stated. For all analyses, Kruskal-Wallis test were used in this study and when the overall p values greater than 0.05, we did not perform group comparisons, p values for all analyses were adjusted for multiple comparisons to control the false-discovery rate using the adaptive Benjamini-Hochberg procedure. Compared with control group, * <0.05 and compared with 0.5MHz group, # <0.05 .

Table 2 Effects of LIPUS on the cell cycle phase distribution of H9C2 cells in 3D-printed GelMA scaffolds

Group	Frequency/ET	Cycle phase distribution (% , n = 3)			
		G0/G1	S	G2/M	S+G2/M
Control	Sham irradiation	75.49±2.06	18.73±2.51	5.79±0.58	24.51±2.92
LIPUS groups	0.5MHz/5min	62.37±1.53*	26.81±1.15*	10.81±1.36	37.62±1.89*
	0.5MHz/10min	56.14±2.21*	31.57±2.98*	12.29±1.74*	43.86±1.52*
	0.5MHz/15min	60.94±1.57*	26.56±1.34*	12.50±1.78*	39.06±1.66*
	1.0MHz/5min	63.66±2.50*	24.35±2.68*	11.99±1.18*	36.34±2.08*
	1.0MHz/10min	65.28±2.24	24.32±2.91*	10.39±1.65	34.72±1.53#
	1.0MHz/15min	64.70±2.49	24.30±2.49	10.99±1.17*	35.30±2.16*
	1.5MHz/5min	66.38±1.96	23.02±1.15	10.59±1.89	33.62±1.49#
	1.5MHz/10min	66.11±2.11	24.44±1.58*	9.44±1.09	33.88±1.96#
	1.5MHz/15min	68.04±2.08#	23.45±1.76	8.51±2.19#	31.96±2.89#
	2.0MHz/5min	72.13±2.78#	20.08±1.49#	8.12±1.73#	28.40±2.63#
	2.0MHz/10min	70.33±1.51#	20.18±2.60#	9.43±3.21#	29.61±1.94#
	2.0MHz/15min	71.61±6.78#	19.75±2.08#	8.71±3.21#	28.57±2.11#

LIPUS: low-intensity pulsed ultrasound. Data were mean (SD) unless otherwise stated. For all analysis, Kruskal-Wallis test were used in this study and p values were adjusted for multiple comparisons to control the false-discovery rate using the adaptive Benjamini-Hochberg procedure. Compared with control group, * <0.05 and compared with 0.5MHz/10min group, # <0.05 .

Figures

Figure 1

Schematic illustration of bio-ink formulation, scaffold printing and LIPUS treatment. From left to right: images of GelMA and H9C2s and their formulation process; simple illustration of extrusion-based 3D printing working principle and UV light for photo-crosslink; the photo and optical microscopic image of 3D-bioprinted GelMA scaffolds; scale bar=100µm; Apparatus and diagram of LIPUS-exposure system.

Figure 2

Effects of LIPUS on the proliferation of H9C2s in 3D-bioprinted cell-laden GelMA scaffolds. The LIPUS group and the control group were treated with different frequencies of LIPUS (frequency=0.5, 1.0, 1.5 and 2.0MHz) and sham irradiation (control) for 5 minutes (A), 10 minutes (B) or 15 minutes (C) and the proliferation of H9C2s in the LIPUS group and the control group were measured using a Cell Counting Kit-

8 assay before and 1, 4 and 7days after LIPUS treatment (n = 6). * $P < 0.05$ and ** $P < 0.01$ vs Control; mean $\pm$ SEMSD. (D) Cell

viability of H9C2s in LIPUS group and control group after 1, 4 and 7 days

for LIPUS treatment (frequency=0.5MHz, time=10min) or sham

irradiation (n = 3). (E) 2D and 3D confocal images of live (green,

calcein-AM)/dead (red, PI) stained cells in 3D-bioprinted cell-laden GelMA

scaffolds after 1, 4 and 7days for LIPUS treatment (frequency=0.5MHz,

time=10min) or sham irradiation, scale bar=100 μ m.

Figure 3

Effects of LIPUS on cell cycle phase distribution measured by flow cytometry in the control and LIPUS groups with different exposure parameters. Cells harvested were fixed and stained with propidium iodide, and their DNA contents were analyzed by flow cytometry. The result of one representative assay from three similar independent experiments is shown. x- and y-axes denote DNA content and cell number, respectively. Each phase was calculated by using the cell ModFit software.

Figure 4

Effect of LIPUS on cell apoptosis in 3D-bioprinted cell-laden GelMA scaffolds. (A) Representative graphs of the flow cytometry analysis of cell apoptosis after the treatment of LIPUS for different exposure time (n=3). (B) The statistical results of the flow cytometry analysis of cell apoptosis. (C) and (D) Western blotting of apoptosis-related factors (n=3). (E-G) Quantitative analysis of Bcl-2, Bax and cleaved Caspase

3 in different groups. Error bars represent the SD of the mean. Compared with control group, * $P<0.05$, ** $P<0.01$.

Figure 5

Phosphorylation of extracellular signal-regulated kinases (ERK1/2) and PI3K-Akt in H9C2s in 3D-bioprinted cell-laden GelMA scaffolds. (A) H9C2s were stimulated by LIPUS (frequency=0.51MHz, ET=10 min). Cells were collected for Western blot analysis at the indicated time point after LIPUS treatment. Phosphorylation of ERK1/2 and PI3K-Akt induced by LIPUS were analyzed by Western blot (n=3). (B, C) H9C2s were stimulated by LIPUS (frequency=1MHz, ET=10 min) in the presence of the specific inhibitors of ERK1/2 (U0126, 10 μ mol/L) and PI3K-Akt (LY294002, 50 μ mol/L). ERK1/2, phospho-ERK1/ 2, PI3K, phospho-PI3K, Akt and phospho-Akt were analyzed by Western blot at 10 min after LIPUS treatment and sham irradiation (n=3). * $P<0.05$ and ** $P<0.01$.

Figure 6

Effects of the two inhibitors (U0126 and LY294002) on LIPUS-induced proliferation of H9C2s in 3D-bioprinted cell-laden GelMA scaffolds. (A) Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay before LIPUS irradiation and at 7 days after irradiation in the presence or absence of U0126 and LY294002 (n=6). Cell proliferation was further detected by live and dead cell double staining assays in the presence or absence of (B and D) LY294002 and (C and E) U0126 (n=3). * $P < 0.05$ and ** $P < 0.01$. Scale bar=100 μ m.