



Facilitatory effect of low-pulse repetition frequency ultrasound on release of extracellular vesicles from cultured myotubes

馬, 晓琪

(Degree)

博士 (保健学)

(Date of Degree)

2025-03-25

(Date of Publication)

2026-03-01

(Resource Type)

doctoral thesis

(Report Number)

甲第9183号

(URL)

<https://hdl.handle.net/20.500.14094/0100496464>

※ 当コンテンツは神戸大学の学術成果です。無断複製・不正使用等を禁じます。著作権法で認められている範囲内で、適切にご利用ください。



博 士 論 文

Facilitatory effect of low-pulse repetition frequency ultrasound on release of extracellular vesicles from cultured myotubes

(培養筋管細胞外小胞の放出に対する低パルス繰り返し
周波数超音波の促進効果)

令和 7 年 1 月 16 日

神戸大学大学院保健学研究科保健学専攻

馬 晓琪 Xiaoqi Ma

Original article

Facilitatory effect of low-pulse repetition frequency ultrasound on release of extracellular vesicles from cultured myotubes

Xiaoqi Ma¹; Atomu Yamaguchi¹; Noriaki Maeshige^{1*}, PhD; Kento Tanida¹; Mikiko Uemura¹, PhD; Fuwen Lu²; Hiroyo Kondo³, PhD; and Hidemi Fujino¹, PhD

¹*Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences, Kobe, Japan*

²*Shanghai Yangzhi Rehabilitation Hospital (Shanghai Sunshine Rehabilitation Center), Tongji University School of Medicine, Shanghai, China*

³*Faculty of Health and Nutrition, Shubun University, Ichinomiya, Japan*

Full postal address of each affiliation:

1: 10-2 Tomogaoka 7-chome, Suma-ku, Kobe, Hyogo 654-0142, Japan

2: 2209 Guangxing Rd., Songjiang District, Shanghai, 201619, China

3: 72 Momo Higashiyashiki, Yamato-cho, Ichinomiya, Aichi, 491-0932, Japan

*** Corresponding author**

Noriaki Maeshige, PhD

Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences

7-10-2 Tomogaoka, Kobe, Hyogo, Japan 654-0142

Tel.: +81 78 796 4652

E-mail address: nmaeshige@pearl.kobe-u.ac.jp

Author contributions

This paper was coauthored by Xiaoqi Ma; Atomu Yamaguchi; Noriaki Maeshige, PhD; Kento Tanida; Mikiko Uemura, PhD; Fuwen Lu; Hiroyo Kondo, PhD; and Hidemi Fujino, PhD. About authors' contribution: Xiaoqi Ma (XM), Atomu Yamaguchi (AY), Noriaki Maeshige (NM), and Hidemi Fujino (HF) conceived and designed the experiments. XM, AY, NM, and Kento Tanida (KT) performed the experiments. XM, NM, AY, Mikiko Uemura (MU), and HF analyzed the data. NM, MU, Fuwen Lu (FL), Hiroyo Kondo (HK), and HF contributed by providing reagents, materials, and analysis tools. XM, AY, NM, KT, HK, and HF interpreted the data and wrote the paper. All authors approved the final version of the manuscript.

Abstract

Purpose

Extracellular vesicles (EVs) serve as carriers of intracellular factors with therapeutic effects, including tissue regeneration and attenuation of inflammatory responses. The majority of EVs *in vivo* are derived from skeletal muscle, which is reported to have anti-inflammatory effects. While high-intensity pulsed ultrasound (US) irradiation has been shown to promote EV secretion from myotubes, the impact of pulse repetition frequency, a US parameter affecting pulse length, on EV release remains unclear.

Hence, this study aimed to investigate the impact of pulse repetition frequency of US on the release of EVs from myotubes and the anti-inflammatory effect of the EVs on macrophages.

Methods

C2C12 myoblasts and bone marrow-derived macrophages (BMDM) were used in this study. After differentiation into C2C12 myotubes, US was performed for 5 min at an intensity of 3.0 W/cm², duty cycle of 20%, acoustic frequency of 1 MHz, and different pulse repetition frequencies (100 Hz, 10 Hz, or 1 Hz). After 12 h, EVs and cells were collected for subsequent analyses.

Results

US did not cause a reduction in cell viability across all US groups compared to the control. The concentration of EVs was significantly higher in all US groups compared to the control group. In particular, the highest increase was observed in the 1-Hz group on EV concentration as well as intracellular Ca²⁺ level. Moreover, 1Hz_EV suppressed expressions of pro-inflammatory factors in macrophages. RNA sequencing analysis revealed that protein processing in endoplasmic reticulum is significantly activated in both US groups, and Hsp90b1 was one of the upregulated genes of protein processing in endoplasmic reticulum under 1Hz while not under 100 Hz US irradiation.

Conclusion

This study concluded that a low-pulse repetition frequency of 1 Hz is the most effective for enhancing EV release from cultured myotubes with pulsed ultrasound. US irradiation with a pulse repetition frequency of 1 Hz significantly activated the pathway of protein processing in endoplasmic reticulum and intracellular Ca²⁺ concentrations, resulting in increased EV release and anti-inflammatory effects in macrophages.

Key words

pulsed ultrasound, pulse repetition frequency, extracellular vesicles (EVs), intracellular calcium, myotubes

1. Introduction

Pulsed ultrasound (US), a widely adopted therapeutic regimen of physical agents, offers noninvasive, painless, and favorable safety characteristics compared to other stimulation modalities, making it applicable to a broad range of patients [1]. With a rich history, it is increasingly used for angiogenesis, pain relief, tissue healing, and enhancing tissue properties [1-4]. Previous studies have demonstrated that cavitation is one of the underlying mechanisms of effects of US therapy [5,6]. Furthermore, cavitation has been utilized to facilitate the delivery of both small and large molecules, including proteins and DNAs into cells [7,8]. It has also been shown that US-induced transient membrane pores can enhance cellular calcium (Ca^{2+}) uptake [9,10], which is one of the initiators for the release of extracellular vesicles (EVs) by cells [11,12]. Indeed, it has been shown that cavitation triggers exocytosis and promotes EV release from cells [13], and US itself can promote the release of EVs [14].

EVs, which are composed of a lipid bilayer membrane, serve as carriers of donor cell proteins, lipids, nucleic acids, mRNA, and microRNA, facilitating intercellular communication [15,16]. They have been shown to regulate a diverse range of biological processes; promote tissue regeneration, cell proliferation, and differentiation; and attenuate inflammatory responses [17-20], emphasizing their critical role in cellular physiology and potential as targets for therapeutic intervention. Therefore, promoting release of EVs from skeletal muscle can exert various effects. Previous studies have highlighted that systemic exercise and hypoxic environments can promote EV release [21-23]. However, as systemic exercise training is not always feasible for elderly adults and patients, and oxidative stress due to hypoxic conditions can cause cellular damage, US therapy has been proposed as an alternative approach to enhance EV production.

In vivo, skeletal muscle is a significant source of EVs [18]. Skeletal muscle is the largest organ in the human body, accounting for approximately 40% of human body weight, and plays a vital role in metabolism and endocrine functions [24,25]. Furthermore, skeletal myotube-derived EVs can prevent macrophage inflammatory responses [20]. Macrophages are essential for the progression of inflammation, such as host defense against pathogenic infections [26]. Meanwhile, excess inflammation by macrophages can be potentially harmful to the body, it needs to be tightly regulated to avoid excessive tissue damage [27]. Thus, it is important to inhibit the excessive production of pro-inflammatory cytokines to regulate the inflammatory responses and avoid various inflammatory diseases and discover a method for promoting EV release from skeletal muscle cells. Notably, previous research has demonstrated that high-intensity US irradiation promotes EV secretion from cultured myotubes without cytotoxicity [14]. Meanwhile, the optimal pulse repetition frequency, a parameter that affects the form of US, has been poorly studied and is currently set empirically or fixed at 100 Hz [28]. By changing the pulse repetition frequency, the pulse duration and number of pulses can be adjusted without altering the duty cycle. Pulse duration, in particular, influences production of ultrasonic cavitation [29]. Since cavitation has been suggested to facilitate EV secretion [30], the pulse repetition frequency may influence EV release. Hence, this study aimed to investigate the effects of pulse repetition frequency of US on release of EVs from cultured myotubes and the anti-inflammatory effect of the EVs on macrophages.

2. Materials and Methods

2.1. Cell culture

C2C12 myoblasts (ATCC) were sustained in Dulbecco's modified Eagle medium (DMEM) combined with 10% fetal bovine serum. Upon attaining nearly 90% confluence, the differentiation of myoblasts into myotubes was initiated by altering the medium to DMEM supplemented with 2% horse serum, which was replaced every 2-3 days over a period of 7 days. Upon completion of differentiation, the C2C12 myotubes were subjected to pulsed US irradiation.

Bone marrow-derived macrophages (BMDM) were used in this study. The bone marrow cells collected from femurs and tibias of 7-week-old male C57BL/6 mice were harvested and cultured in Petri dishes with RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, 1% L-Glutamine, and 25% L929 cell supernatant for eight days to generate bone marrow-derived macrophages (BMDMs). Harvesting the differentiated macrophages and plating them in 35mm tissue culture dishes with macrophage culture media (RPMI 1640 supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% L-Glutamine, and 10% L929 cell supernatant) for subsequent experiments.

2.2. Ultrasound irradiation

After changing the culture medium to serum-free DMEM, US irradiation was conducted by positioning the probe of a customized medical US device (SZ-100M; MINATO Medical Science Co., LTD, Japan), which allows adjustment to selectable repetition frequencies (100 Hz, 10 Hz, 1 Hz), under the bottom of the culture dish for 5 min (Supplemental File 1- Fig. 1). The amount of US energy transmitted through the dish bottom was approximately 82% of the total irradiated energy [8]. Coupling gel (<1 mm) (Aquasonic 100 Ultrasound Transmission Gel; Parker Lab Inc., USA) was applied between the probe and the dish, and a piece of sterilized silicone was suspended in the culture media 2 mm above the cell monolayer [8,14]. The US parameters were as follows: intensity of 3.0 W/cm², duty cycle of 20%, acoustic frequency of 1 MHz, repetition frequencies of 1 Hz, 10 Hz, or 100 Hz (Fig. 1), and an effective radiation area of 7.6 cm². Probe safety was confirmed with a beam nonuniformity ratio of 2.4 [8], calculated as the ratio of maximum to average US output. Additionally, the temperature of the culture media was monitored with a thermometer (TM-947SDJ; SATOTECH Co., LTD, Japan) to be below 37°C during US irradiation (Supplemental File 1- Fig. 2). The temperatures between the US groups were also essentially the same.

2.3. EV extraction and analysis

To measure the particle concentration of EVs, twelve hours after US irradiation, the culture medium was collected and centrifuged at 1500 × g for 10 min, followed by centrifugating the supernatant at 10,000 × g for 30 min at 4°C. Subsequently, a 0.22-μm filter was used to remove organelles and protein aggregates [31]. The supernatant was then ultracentrifuged at 100,000 × g for 2 h at 4°C, and the obtained pellet was suspended in 200 μL of D-PBS.

The particle concentration and size distribution of EVs were measured using a resistive pulse

sensing device (qNano, Izon Science) with nanopores (NP200A, Izon Science). Calibration of size and concentration was accomplished using polystyrene beads (100 nm, Izon Science) with a concentration of 1.0×10^{10} beads/mL.

2.4. Cell viability

The assessment of myotube viability was performed 12 h after US irradiation using MTT assay and Zombie Red™ staining. In the MTT assay, the cells were incubated for 3 h with 1.3 mL of MTT solution (10×); 3-(4,5-Dimethyl-2-thizolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT; Wako Junyaku Co., Ltd., Japan) was dissolved in the culture medium at 5 mg/ml. After incubation for 3 h, dimethyl sulfoxide was added and the absorbance at 595 nm was measured (MTP-300; Kono Electric Co., Ltd., Japan). Cell viability was expressed as a percentage compared to the control.

In Zombie Red™ staining, the cells were washed with PBS twice and incubated for 15 min with Zombie Red™ Fiable Viability Kit (1:1000; BioLegend, CA, USA). Zombie Red™ is an amine-reactive fluorescent dye that is non-permeant to live cells but permeant to cells with a compromised plasma membrane. The cells were washed with PBS twice and incubated for 30 min at room temperature with 4% formaldehyde. Nuclei were counterstained with DAPI (1:1,000; Dojindo, Kumamoto, Japan) for 5 min. The stained cells were captured with a fluorescence microscope (BZ-X800; Keyence, Japan), and the mean fluorescence intensity was calculated.

2.5. Intercellular Ca^{2+} concentration

The concentration of intracellular calcium was assayed immediately following US irradiation. Briefly, cells were collected using a cell scraper and then centrifuged at 13,000 rpm for 20 s to remove the supernatant. One microliter of hydrochloric acid was added to 200 μl of RIPA buffer (RIPA Lysis Buffer; SCB Co., Ltd., Japan) to maintain a pH of 2-3. Following 30-min incubation, samples were centrifuged at 10,000 rpm for 10 min to analyze the supernatant for calcium concentration as the low pH dissociates calcium, which is then collected as the supernatant. Calcium concentrations were analyzed by measuring absorbance at a sample wavelength of 690 nm and a reference wavelength of 770 nm using the Metalloassay Calcium Measurement LS (CPZ III) (Metallogenics Corporation).

2.6. EV extraction for the macrophage treatment to test the anti-inflammatory effect of EVs

For the macrophage treatment, the culture medium was collected and centrifuged at $1500 \times g$ for 10 min, followed by centrifugating the supernatant at $10,000 \times g$ for 30 min at 4°C. Then the medium was concentrated approximately 100-fold by centrifugation with 100 kDa cut-off Amicon Ultra (Merck Millipore, USA) at $4000 \times g$ for 15 min. The concentrated liquid included EVs were further diluted 500-fold in the medium for macrophage treatment. After 1.5 h treatment by EVs, macrophages were incubated with macrophage culture media for 22.5 h. Subsequently, macrophages were treated with 100 ng/mL lipopolysaccharide (LPS) for 1.5 h for pro-inflammatory marker quantification. After that, mRNA was isolated to measure the expression of inflammatory factors.

2.7. Quantitative real-time PCR

To measure IL-1 β and IL6 expression in BMDMs, TRIzol RNA Isolation protocol was used to isolate mRNA. Then mRNA was used to make cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad). The StepOne™ Real-Time PCR System was used to analyze the samples under the following conditions: 95 °C (3 min), 40 cycles of 95 °C (10 sec), and 60 °C (30 sec). The relative expression value of IL-1 β and IL6 was calculated by normalizing to the expression of hypoxanthine phosphoribosyltransferase (HPRT). Data were analyzed using the delta/delta CT method. The obtained results are expressed as relative values to the LPS-unstimulated cells. qPCR primer sequences are shown in Table 1.

2.8. RNA sequencing of myotubes

Total RNA was extracted from the myotubes using TRIzol reagent (Takara Biotechnology, Japan). Raw RNA sequence data were obtained utilizing an Illumina NovaSeq 6000 machine. The fold change (mean expression of each RNA in the US group divided by the mean expression in the control group) and P value for each RNA were calculated after obtaining the raw data. These P values were then used to determine the false discovery rate (FDR) for each RNA, which was then applied as a filter to identify essential RNAs with an FDR < 0.05. RaNA-Seq and the R 3.5.3 program were utilized for pathway enrichment analysis. After performing pathway analysis using the Kyoto Encyclopedia of Genes and Genomes database, the 10 most enriched pathways associated with signal transduction were identified and used to explore related pathways.

2.9. Statistical analysis

All values are expressed as means $\pm$ standard error of the mean (SEM). Statistical analysis was performed using Statistical 4 (OMS, Tokyo, Japan). For multiple comparisons, ANOVA (Tukey's multiple comparison test as post-hoc) was used. $p < .05$ was considered statistically significant.

3. Results

3.1. Waveforms of different pulse repetition frequencies of US

In this study, waveforms of US irradiation were measured and are shown in Fig. 2. The measured waveforms of US irradiated with the pulse repetition frequencies of 1Hz, 10Hz, and 100Hz over a period of 2 sec are presented in Fig. 2a, 2b, and 2c, respectively. They showed that the voltage of each pulse and the total irradiation time of the US was the same under different pulse repetition frequencies, but each pulse wave duration and the number of pulse waves were different under the different pulse repetition frequencies.

3.2. Cell viability

As shown in Fig. 3a, the percentage of cell viability was not decreased by US irradiation. Also, as shown in Fig. 3b and Table 2, the cell viability of myotubes was measured using Zombie Red™

immunofluorescence staining to examine cytotoxicity of US irradiation on myotubes. While the cells treated with 1% povidone-iodine showed a significant decline in viability, US irradiation did not elicit a reduction in cell viability across all groups.

3.3. EV concentration and size distribution

Isolated EVs were characterized using tunable resistive pulse sensing. In terms of EV concentration, all US groups showed a significant elevation relative to the control group. In particular, the highest increase was observed in the 1-Hz group, which was markedly greater than that in the 10-Hz group (Fig. 4a). Meanwhile, concerning the size of the extracted EVs, most of the particles were between 50-200 nm, the range of the EV size. There were no significant differences in EV size across all groups (Fig. 4b, Table 3).

3.4. Intercellular Ca^{2+} concentration

To clarify the mechanism of US irradiation on the promotion of EV release, the intracellular Ca^{2+} concentration in myotubes after US irradiation was measured. The intracellular Ca^{2+} concentration after US irradiation was significantly elevated in the 100-Hz and 1-Hz groups as compared with the control group. Moreover, the 1-Hz group showed the highest concentration, which was significantly greater than that observed in the 10-Hz group, among the US groups (Fig. 5).

3.5. The expression of the inflammatory factor IL-1 β and IL6

The expression of IL-1 β and IL6 tended to decrease in CON_EV and 100Hz_EV group, and significantly decreased in 1Hz_EV group compared to the LPS group (Fig. 6).

3.6. RNA-seq analysis of myotubes

RNA sequencing was performed to determine the mechanisms activated by US irradiation in myotubes after US irradiation. The differentially expressed RNAs between the 100 Hz group, the 1 Hz group and the control group were visualized using two volcano plots, respectively (Fig. 7a, b). The expression of 149 mRNAs was significantly changed after 100hz US irradiation (Fig. 7a), and 271 mRNAs was significantly changed after 1Hz US irradiation (Fig. 7b). Enrichment analysis revealed the ten most enriched KEGG pathways in the 100 Hz group and the 1 Hz group (Fig. 7c, d). The pathway of protein processing in endoplasmic reticulum is most activated in both US groups. In the factors of protein processing in endoplasmic reticulum, the expression of 16 genes was significantly upregulated by 1Hz, while only 13 was upregulated by 100Hz (Fig. 7e). Hsp90b1 was one of the the upregulated genes by 1Hz US irradiation only (Fig. 7e).

4. Discussion

In this study, we investigated the promotion of release of EVs from cultured myotubes using different pulse repetition frequencies of US. The results of the MTT assay indicated that US irradiation

at pulse repetition frequencies of 1 Hz, 10 Hz, and 100 Hz did not cause cell damage. This confirmed the non-injurious nature of US, which is consistent with a previous study in which cells were irradiated at a pulse repetition frequency of 100 Hz [14]. In the present investigation, the concentration of EVs was increased remarkably in all US groups, affirming the ability of US irradiation to stimulate the release of EVs. Additionally, the similarity in the size of the collected EVs among all groups suggested that the pulse repetition frequency of US did not influence the types of EVs. Of particular interest, the 1-Hz group exhibited a significantly higher concentration of EVs than the 10-Hz group and had a tendency to increase the concentration of EVs by approximately 1.4-fold compared to the 100-Hz group. This result indicates that US irradiation at a pulse repetition frequency of 1 Hz was the most effective in promoting release of EVs from cultured myotubes in this study.

In previous studies, US irradiation was reported to promote uptake of Ca^{2+} from the extracellular space [32,33]. Furthermore, an elevation in intracellular Ca^{2+} concentration was reported to encourage the release of exosomes, the most common type of EVs [34]. The present study confirmed that US irradiation elevated intracellular Ca^{2+} levels and the release of EVs, most notably at a pulse repetition frequency of 1 Hz. These findings suggest that the facilitation of Ca^{2+} uptake by US irradiation, in turn, increased the intracellular Ca^{2+} concentration and bolstered the excretion of EVs. Recently, the ESCRT complex, particularly ESCRT III, has been linked to exosome release via Ca^{2+} concentration increase [35,36]. A further exploration focusing on these factors is needed.

Within the confines of our study, the greatest impact on intracellular Ca^{2+} levels was observed at a pulse repetition frequency of 1 Hz. Previous research has suggested that membrane displacement activates mechanosensory channels and, consequently, intracellular Ca^{2+} influx [37]. Additionally, it has been suggested that lower pulse repetition frequencies provoke greater cell membrane displacement [38]. Therefore, we hypothesized that the increase in cell membrane displacement at a repetition frequency of 1 Hz resulted in a higher intracellular Ca^{2+} concentration. Furthermore, US cavitation, which generates bubbles and jets that impact the cell membrane, has been identified as another factor contributing to cell membrane displacement [39,40]. The jet stream generated by cavitation bubbles requires a certain degree of pulse duration, which is contingent on the acoustic frequency and intensity [41,42]. In our study, the concentration of intracellular Ca^{2+} was significantly elevated at the pulse repetition frequency of 1 Hz, which showed the longest pulse duration. Therefore, we propose that the pulse repetition frequency of 1 Hz provides enough pulse duration to generate a jet stream of cavitation under US irradiation conditions. To confirm our hypotheses, further research regarding cavitation dynamics is warranted.

In the present study, we could not detect any difference between 100 Hz and 10 Hz in terms of EV concentration and intracellular Ca^{2+} level. This means the length of the US pulse does not necessarily correlate to an increase in intracellular Ca^{2+} level. In addition, Ca^{2+} suppression experiments need to be conducted and factors related to mechanical stimulation analyzed in order to elucidate the proposed mechanism in the present study. Investigations on the effects of US on cavitation, membrane displacement, and mechanosensory channels will also be expected to clarify the biophysical action of low-pulse repetition frequency in future studies.

On the other hand, we found that 100Hz and 1Hz US irradiation activated protein processing in endoplasmic reticulum. Hsp90b1 was enriched in protein processing in endoplasmic reticulum in 1Hz group, which was not in 100Hz group. In previous studies, it is reported that Hsp90 is involved in membrane deformation processes that facilitate the release of EVs [43]. Also, the activation of the pathway of protein processing in endoplasmic reticulum can promote the secretion of EVs [44]. We suppose that the more enriched protein processing in endoplasmic reticulum is also the reason for 1Hz US irradiation can promote the release of more EVs.

Previous studies reported that skeletal muscle derived EVs exert therapeutic effects in various dysfunctional organs [18]. Furthermore, since skeletal muscle EVs mediate crosstalk between skeletal muscle and other tissues [21], methods to enhance the release of skeletal muscle derived EVs are potentially beneficial in the treatment of various diseases and health management. In our study, we found that LPS-induced inflammatory responses was significantly suppressed by the treatment of 1Hz_EV, which contains the most amount of EVs among all the groups. It is reported that miRNA in EVs have various effects, including anti-inflammatory effect. For example, miR-206 is a member of the skeletal muscle-specific myo-miR family of miRNAs [45], it is reported that miR-206 can suppress macrophage inflammation [46]. Also, miR-378a is highly expressed in skeletal muscle and miR-378a-3p can contribute to the induction of anti-inflammatory macrophage reprogramming [47]. Furthermore, it is reported that treatment effects of miRNAs appear to be dose-dependent, and a certain concentration is necessary for their optimal anti-inflammatory action [48]. Thus, it is suggested that more anti-inflammatory miRNAs were delivered to macrophages via EV release facilitation, which is responsible for the anti-inflammatory effect in the 1Hz_EV group. Further analysis for the composition and changes of miRNAs profile in myotube EVs by 1Hz US irradiation is needed to confirm our proposal.

5. Conclusion

This study revealed the effect of different pulse repetition frequencies of US on the facilitation of EV release from cultured myotubes. US irradiation with a pulse repetition frequency of 1 Hz significantly activated the pathway of protein processing in endoplasmic reticulum and intracellular Ca^{2+} concentrations, resulting in increased EV release and anti-inflammatory effects in macrophages. Consequently, it is inferred that US irradiation with a pulse repetition frequency of 1 Hz is an effective way to facilitate EV release from skeletal muscles and use the EVs to reduce the inflammation response.

6. Acknowledgements

We appreciate the members of our laboratory for their thoughtful technical advice. This study was supported by JSPS KAKENHI [21H03852], Japanese Society for Electrophysical Agents in Physical Therapy [J202307], and JST SPRING [JPMJSP2148].

Declarations

Ethical Statement

This study was approved by the Institutional Animal Care and Use Committee and was performed according to the Kobe University Animal Experimentation Regulations.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Data Availability Statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

References

- 1) Miller DL, Smith NB, Bailey MR, et al. Overview of therapeutic ultrasound applications and safety considerations. *J Ultrasound Med.* 2012;31:623-34.
- 2) Izadifar Z, Babyn P, Chapman D, et al. Mechanical and biological effects of ultrasound: a review of present knowledge, *Ultrasound Med Biol.* 2017;43:1085-104.
- 3) Feril LB Jr, Yamaguchi K, Ikeda-Dantsuji Y, et al. Low-intensity ultrasound inhibits melanoma cell proliferation in vitro and tumor growth in vivo. *J Med Ultrason.* 2021;48:451-61.
- 4) Ouchi D, Mori S, Arakawa M, et al. Optimizing irradiation conditions for low-intensity pulsed ultrasound to upregulate endothelial nitric oxide synthase. *J Med Ultrason.* 2023;51:39-48.
- 5) Fu H, Comer J, Cai W, et al. Sonoporation at small and large length scales: Effect of cavitation bubble collapse on membranes. *J Phys Chem Lett.* 2015;6:413-8.
- 6) Tachibana K, Uchida T, Ogawa K, et al. Induction of cell-membrane porosity by ultrasound, *Lancet.* 1999;353:1409.
- 7) Paliwal S, Mitragotri S. Ultrasound-induced cavitation: applications in drug and gene delivery. *Expert Opin Drug Deliv.* 2006;3:713-26.
- 8) Ma X, Yamaguchi A, Maeshige N, et al. Enhancement of astaxanthin incorporation by pulsed high-intensity ultrasound in LPS-stimulated macrophages. *J Med Ultrason.* 2022;49:125-32.
- 9) Fan Z, Kumon R.E, Park J, et al. Intracellular delivery and calcium transients generated in sonoporation facilitated by microbubbles. *J Control.* 2010;142:31–9.
- 10) Zhou Y, Shi J, Cui J, et al. Effects of extracellular calcium on cell membrane resealing in sonoporation. *J Control.* 2008;126:34–43.
- 11) Atanassoff AP, Wolfmeier H, Schoenauer R, et al. Microvesicle shedding and lysosomal repair fulfill divergent cellular needs during the repair of streptolysin O-induced plasmalemmal damage. *PLoS ONE.* 2014;9:e89743.
- 12) Babiychuk EB, Monastyrskaya K, Potez S, et al. Intracellular Ca^{2+} operates a switch between repair and lysis of streptolysin O-perforated cells. *Cell Death Differ.* 2009;16:1126–34.
- 13) Yuana Y, Jiang L, Lammertink BHA, et al. Microbubbles-Assisted Ultrasound Triggers the Release of Extracellular Vesicles. *Int J Mol Sci.* 2017;18:1610.
- 14) Maeshige N, Langston PK, Yuan ZM, et al: High-intensity ultrasound irradiation promotes the

- release of extracellular vesicles from C2C12 myotubes: Ultrasonics. 2021;110:106243.
- 15) Ha D, Yang N, Nadithe V. Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges. *Acta Pharm Sin B*. 2016;6:287-96.
- 16) Estebanez B, Jimenez-Pavon D, Huang CJ, et al. Effects of exercise on exosome release and cargo in vivo and ex vivo models: A systematic review. *J Cell Physiol*. 2021;236: 3336-53.
- 17) Samir EA, Imre M, Xandra O, et al. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov*. 2013;12:347-57.
- 18) Rome S, Forterre A, Mizgier ML, et al. Skeletal Muscle-Released Extracellular Vesicles: State of the Art. *Front Physiol*. 2019;10:929.
- 19) Nakamura Y, Miyaki S, Ishitobi H, et al. Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. *FEBS Letters*. 2015;589:1257-65.
- 20) Yamaguchi A, Maeshige N, Yan J, et al. Skeletal myotube-derived extracellular vesicles enhance itaconate production and attenuate inflammatory responses of macrophages. *Front Immunol*. 2023;14:1099799.
- 21) Whitham M, Parker BL, Friedrichsen M, et al. Extracellular Vesicles Provide a Means for Tissue Crosstalk during Exercise. *Cell Metabolism*. 2018;27:237-51.
- 22) Bertoldi K, Cechinel LR, Schallenberger B, et al. Circulating extracellular vesicles in the aging process: impact of aerobic exercise. *Mol Cell Biochem*. 2018;440:115-25.
- 23) Lo Sicco C, Reverberi D, Balbi C, et al. Mesenchymal Stem Cell-Derived Extracellular Vesicles as Mediators of Anti-Inflammatory Effects: Endorsement of Macrophage Polarization. *STEM CELLS Translational Medicine*. 2017;6:1018-28.
- 24) Pedersen BK. Muscle as a secretory organ. *Compr Physiol*. 2013;3:1337-62.
- 25) Eleonora T, Valentina DF. Extracellular Vesicles: Delivery Vehicles of Myokines. *Front Physiol*. 2019;10:522.
- 26) Funes SC, Rios M, Vera JE, et al. Implications of macrophage polarization in autoimmunity. *Immunology*. 2018;154:186-95.
- 27) Moghaddam AS, Mohammadian S, Vazini H, et al. Macrophage plasticity, polarization and function in health and disease. *J Cell Physiol*. 2018;233:6425-40.
- 28) Manuel TJ, Kusunose J, Zhan X, et al. Ultrasound neuromodulation depends on pulse repetition frequency and can modulate inhibitory effects of TTX. *Sci Rep*. 2020;10:15347.
- 29) Lin Y, Lin L, Cheng M, et al. Effect of acoustic parameters on the cavitation behavior of Sono Vue microbubbles induced by pulsed ultrasound. *Ultrasonics*. 2017;35:176-84.
- 30) Yuana Y, Jiang L, Lammertink BHA, et al. Microbubbles-Assisted Ultrasound Triggers the Release of Extracellular Vesicles. *Int J Mol Sci*. 2017;18:1610.
- 31) Lesser C, Eldh M, Lotvall J. Isolation and Characterization of RNA-Containing Exosomes. *J Vis Exp*. 2012;59:e3037.
- 32) Yuana Y, Jiang L, Lammertink BHA, et al. Microbubbles-Assisted Ultrasound Triggers the Release of Extracellular Vesicles. *Int J Mol Sci*. 2017;18:1610.
- 33) Ambattu LA, Ramesan S, Dekiwadia C, et al. High frequency acoustic cell stimulation promotes

- exosome generation regulated by a calcium-dependent mechanism. *Commun Biol.* 2020;3:553.
- 34) Savina A, Furlan M, Vidal M, et al. Exosome Release Is Regulated by a Calcium-dependent Mechanism in K562 Cells. *J Biol Chem.* 2003;278:20083-90.
- 35) Larios J, Mercier V, Roux A, et al. ALIX- and ESCRT-III-dependent sorting of tetraspanins to exosomes. *J Cell Biol.* 2020;219:e201904113.
- 36) Scheffer LL, Sreetama SC, Sharma N, et al. Mechanism of Ca^{2+} -triggered ESCRT assembly and regulation of cell membrane repair. *Nat Commun.* 2014;5:5646.
- 37) Liao D, Li F, Lu D, et al. Activation of Piezo1 Mechanosensitive Ion Channel in HEK293T Cells by 30 MHz Vertically Deployed Surface Acoustic Waves. *Biochem Biophys Res Commun.* 2019;518:541-547.
- 38) Liao D, Hsiao MY, Xiang G, et al. Optimal pulse length of insonification for Piezo1 activation and intracellular calcium response. *Sci Rep.* 2021;11:709.
- 39) Yuan F, Yang C, Zhong P. Cell membrane deformation and bioeffects produced by tandem bubble-induced jetting flow. *Proc Natl Acad Sci U S A.* 2015;112:E7039-E7047.
- 40) Klaseboer E, Fong SW, Turangan CK, et al. Interaction of lithotripter shockwaves with single inertial cavitation bubbles. *J Fluid Mech.* 2007;593:33-56.
- 41) Atchley AA, Frizzell LA, Apfel RE, et al. Thresholds for cavitation produced in water by pulsed ultrasound. *Ultrasonics.* 1988;26:280-285.
- 42) Khokhlova T, Rosnitskiy P, Hunter C, et al. Dependence of inertial cavitation induced by high intensity focused ultrasound on transducer F-number and nonlinear waveform distortion. *J Acoust Soc Am.* 2018;144:1160-9.
- 43) Lauwers E, Wang YC, Gallardo R, et al. Hsp90 Mediates Membrane Deformation and Exosome Release. *Mol Cell.* 2018 Sep 6;71(5):689-702.e9.
- 44) Collett GP, Redman CW, Sargent IL, et al. Endoplasmic reticulum stress stimulates the release of extracellular vesicles carrying danger-associated molecular pattern (DAMP) molecules. *Oncotarget.* 2018 Jan 11;9(6):6707-6717.
- 45) Carpi S, Polini B, Nieri D, et al. Expression Analysis of Muscle-Specific miRNAs in Plasma-Derived Extracellular Vesicles from Patients with Chronic Obstructive Pulmonary Disease. *Diagnostics (Basel).* 2020 Jul 21;10(7):502.
- 46) Huang X, Gong F, Lu Z, et al. Downregulated MiR-206 expression promotes the proliferation and migration of macrophages by regulating IL-17A/REG3A pathway. *European Journal of Inflammation.* 2020;18.
- 47) Rückerl D, Jenkins SJ, Laqtom NN, et al. Induction of IL-4R α -dependent microRNAs identifies PI3K/Akt signaling as essential for IL-4-driven murine macrophage proliferation in vivo. *Blood.* 2012 Sep 13;120(11):2307-16.
- 48) Liao, Z., Zheng, R., Shao, G. Mechanisms and application strategies of miRNA-146a regulating inflammation and fibrosis at molecular and cellular levels (Review). *International Journal of Molecular Medicine* 51, no. 1 (2023): 7.

Figures:

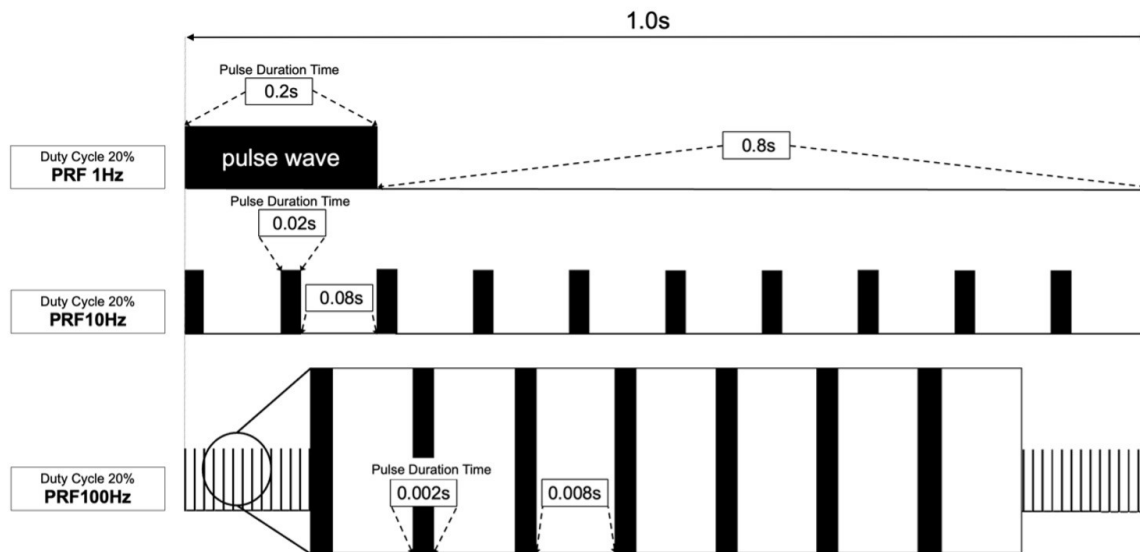
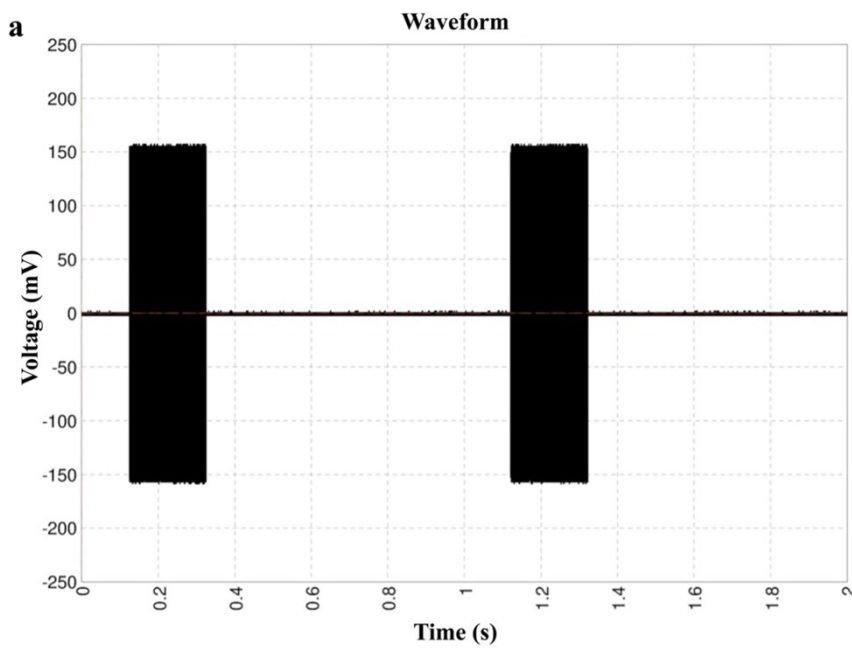


Fig. 1. Illustration of ultrasound irradiation with different pulse repetition frequencies.



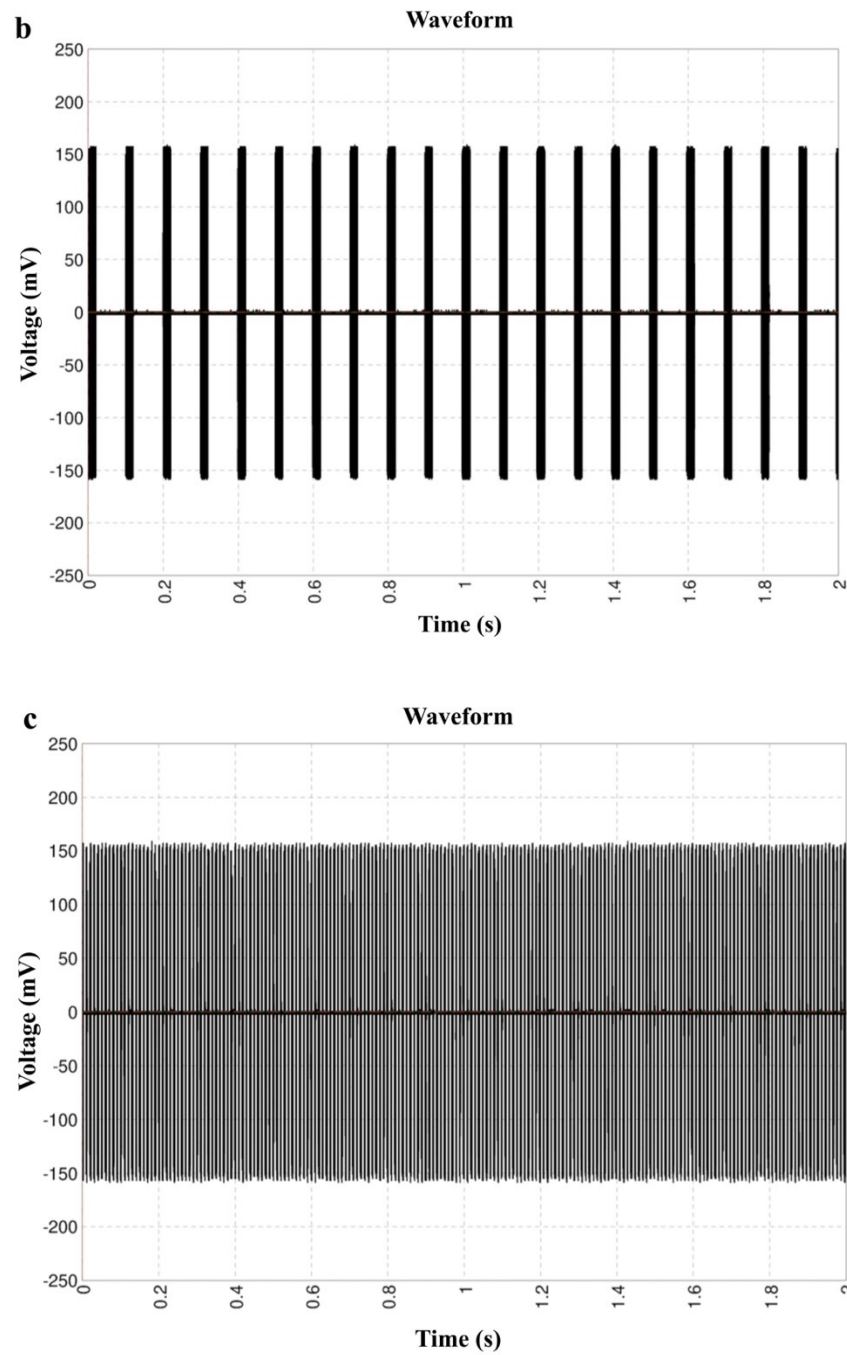


Fig. 2. Actual waveforms of the different repetition frequencies of US used in this study. a: The waveform of the repetition frequency of 1 Hz; b: The waveform of the repetition frequency of 10 Hz; c: The waveform of the repetition frequency of 100 Hz.

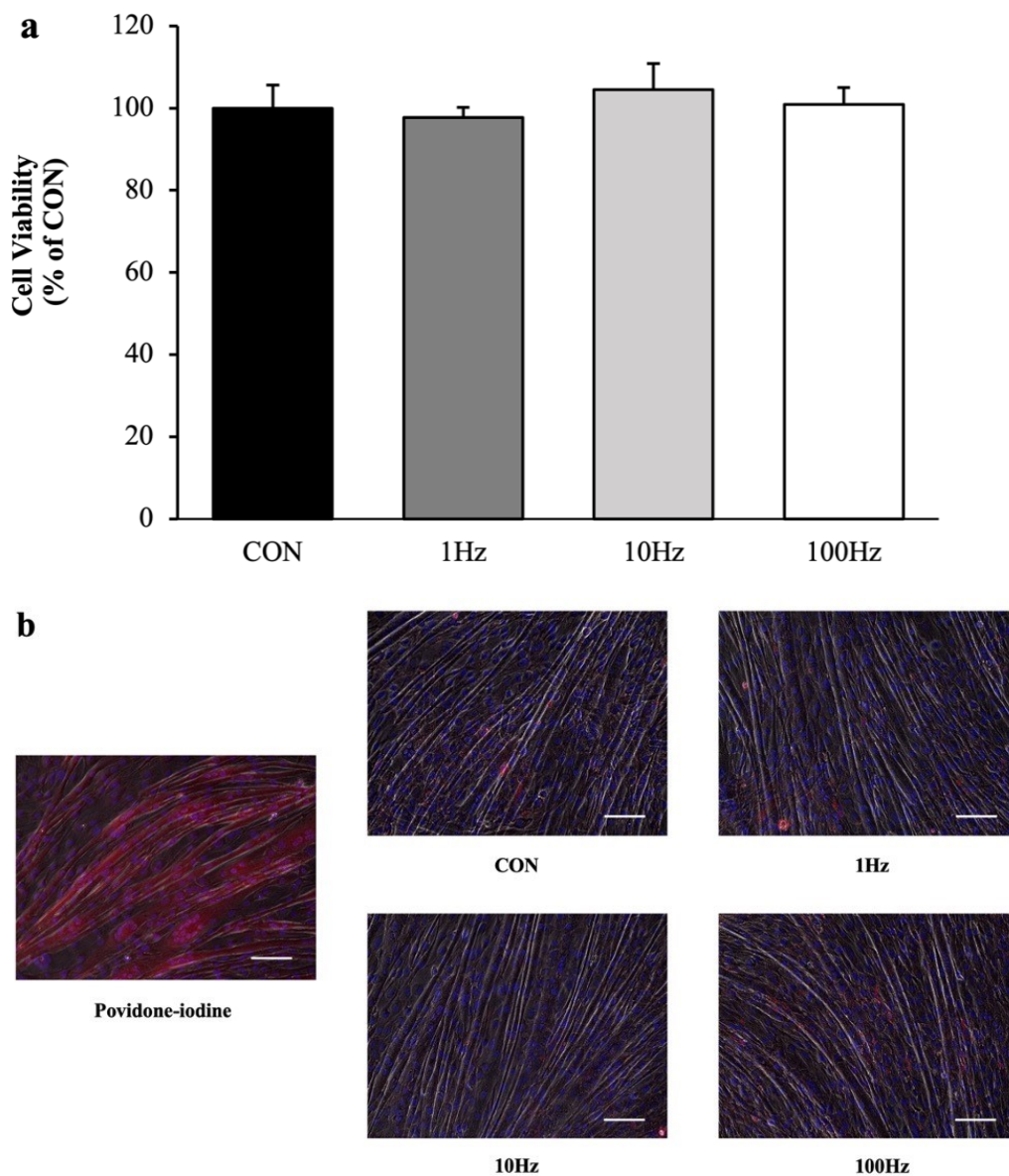


Fig. 3. Cell viability. a: MTT assay was performed at 12 hours after ultrasound (US) irradiation for measuring cell viability, which is expressed as a percentage compared to the control. All values are represented as mean $\pm$ SEM (n=4). b: Zombie RedTM immunofluorescence staining (red) in myotubes at 12 hours after US irradiation. After fixation, myotubes were counter-stained with DAPI (blue). Scale bar = 100nm.

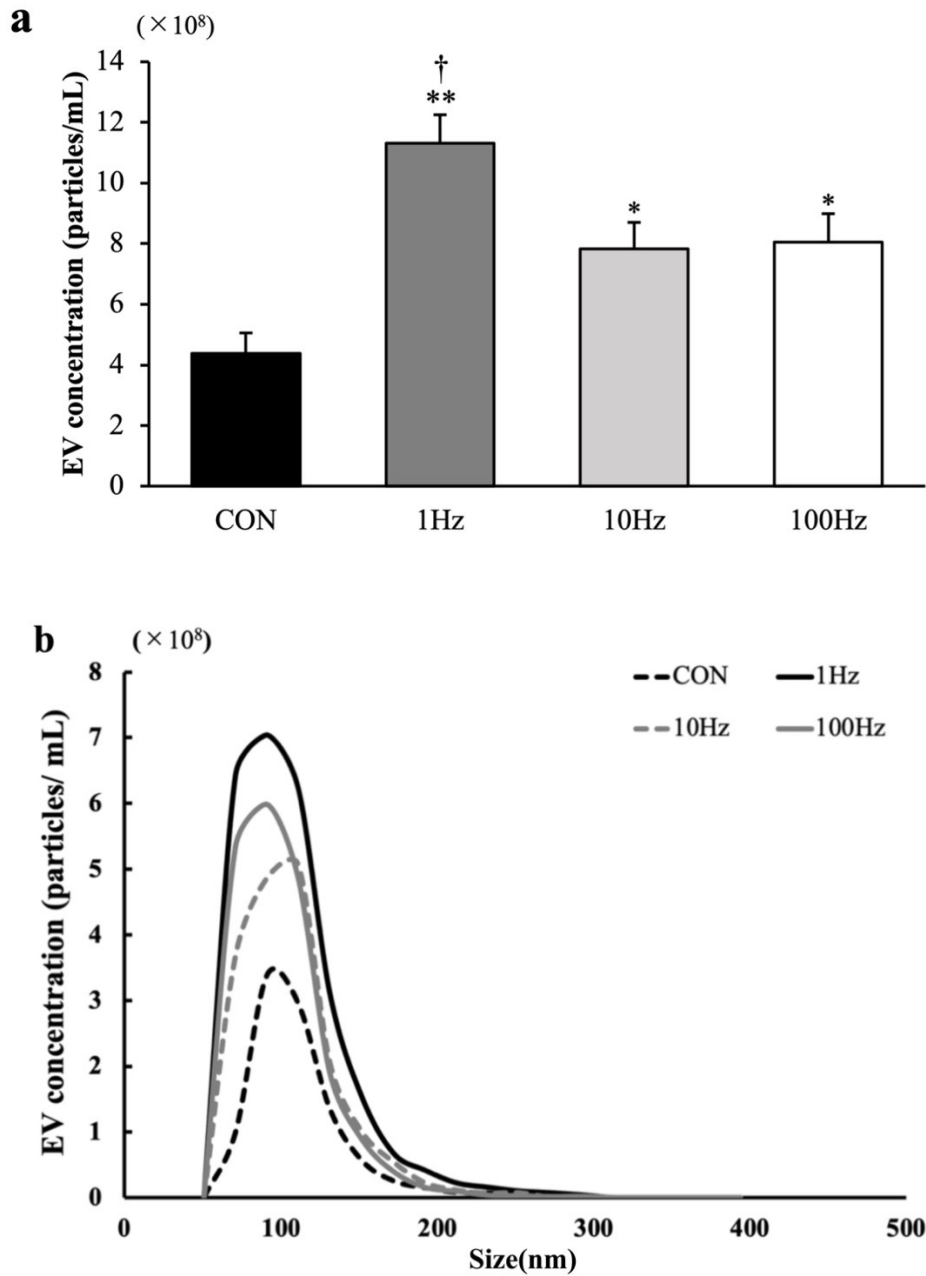


Fig. 4. The concentration of extracellular vesicles (EVs). a: The concentration of EVs from myotubes at 12 hours after ultrasound (US) irradiation was measured using qNano. All values are represented as mean $\pm$ SEM. * $p < .05$ vs. control, ** $p < .01$ vs. control, † $p < .05$ vs. 10 Hz (n=9). b: EV concentration and size distribution. The concentration and size distribution of EVs from myotubes at 12 hours after US irradiation was measured (n=9).

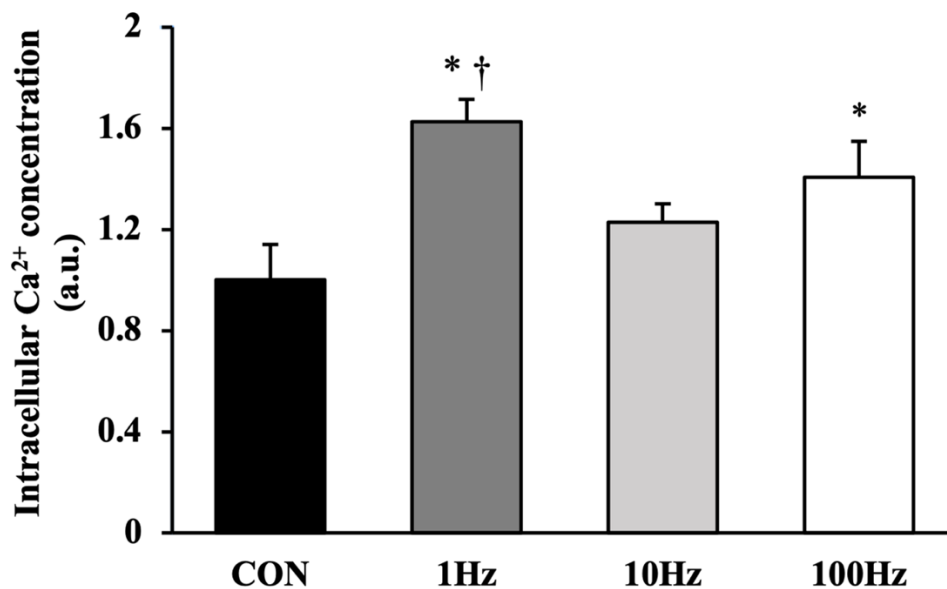


Fig. 5. The concentration of intracellular Ca²⁺ was measured after ultrasound (US) irradiation. All values are represented as mean $\pm$ SEM. * $p < .05$ vs. control, † $p < .05$ vs. 10 Hz (n=4).

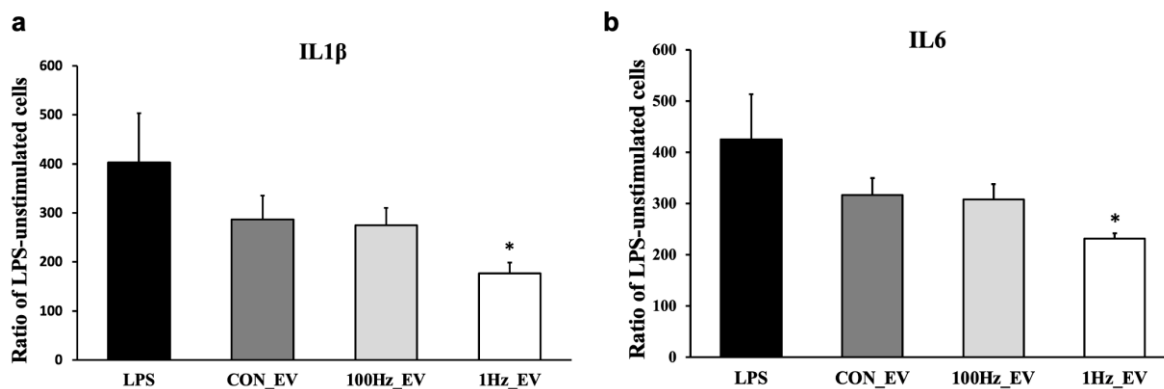


Fig. 6. The expression of inflammatory factors. a: The expression of IL-1 β . b: The expression of IL-6. All values are represented as mean $\pm$ SEM. * $p < .05$ vs. LPS (n=4).

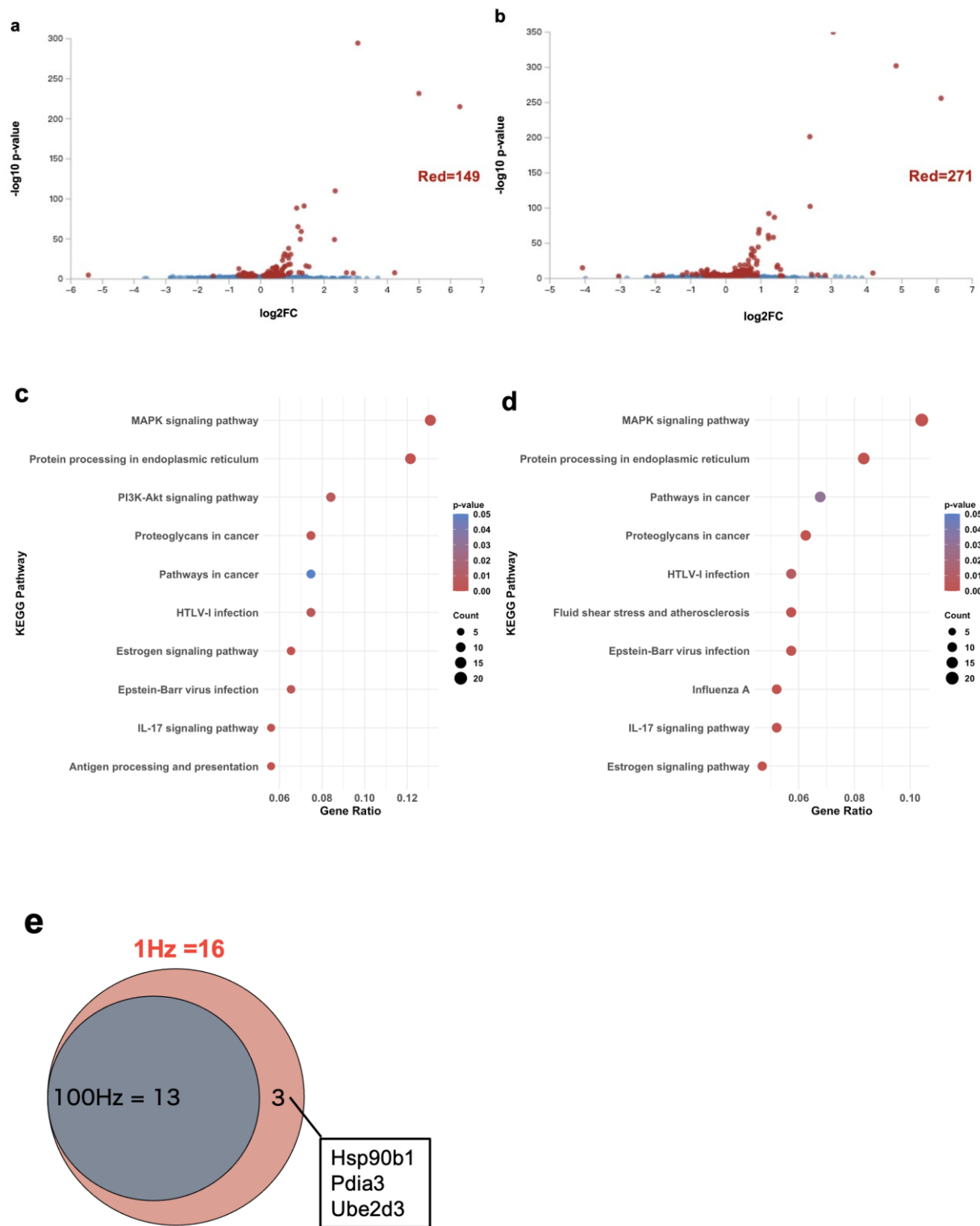


Fig. 7. RNA sequencing analysis of myotubes after ultrasound (US) irradiation. a: Volcano plot of

differentially expressed RNAs in control group vs. 100Hz group. Red dots represent RNAs with statistically significant difference, and blue dots indicate RNAs with no statistically significant difference between the CON and 100Hz groups. b: Volcano plot of differentially expressed RNAs in CON group vs. 1Hz group. c: Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed on differentially expressed RNAs between the CON and 100Hz groups, with the 10 most enriched pathways presented. d: KEGG analysis was performed on differentially expressed RNAs between the CON and 1Hz groups, with the 10 most enriched pathways presented. e: A Venn diagram showing unique and overlapping differentially expressed genes between 1Hz and 100Hz groups in the pathway of protein processing in endoplasmic reticulum.

Table 1: Primer sequences for quantitative PCR.

Primer	Sequences
HPRT-Forward	GCTTGCTGGTGAAAAGGACCTCTCGAAG
HPRT-Reverse	CCCTGAAGTACTCATTATAGTCAAGGGCAT
IL-1 β -Forward	GCCTTGGGCCTCAAAGGAAAGAA
IL-1 β -Reverse	ATTGCTTGGGATCCACACTCTCC
IL-6-Forward	ACAAAGCCAGAGTCCTTCAGAGA
IL-6- Reverse	TTGGATGGTCTTGGTCCTTAGCC

Table 2: Cell viability analysis using Zombie RedTM immunofluorescence staining. Mean fluorescence intensity of Zombie RedTM in myotubes is shown.

Group	Mean fluorescence intensity (% of povidone-iodine)
Control	0.39 $\pm$ 0.12
100 Hz	0.38 $\pm$ 0.17
10 Hz	0.40 $\pm$ 0.08
1 Hz	0.41 $\pm$ 0.17

Values are presented as percentage of povidone-iodine and expressed as means $\pm$ SEM.

Table 3: Extracellular vesicle (EV) distribution and size.

Group	Mean (nm)	Mode (nm)
Control	113.1 $\pm$ 4.9	98.2 $\pm$ 4.6
100 Hz	109.0 $\pm$ 3.2	92.7 $\pm$ 4.0
10 Hz	112.3 $\pm$ 4.1	92.9 $\pm$ 5.4
1 Hz	114.8 $\pm$ 4.5	94.1 $\pm$ 4.1

The size distribution of EVs was measured. The mean and mode size of EVs in each group are shown in the table. All values are expressed as means $\pm$ SEM.