Enhancement of astaxanthin incorporation by pulsed high-intensity ultrasound in LPS-stimulated macrophages

Ma, Xiaoqi ; Yamaguchi, Atomu ; Maeshige, Noriaki ; Uemura, Mikiko ; Noguchi, Hikari ; Kondo, Hiroyo ; Fujino, Hidemi

(Citation)
Journal of Medical Ultrasonics,49(2):125-132

(Issue Date)
2022-04

(Resource Type)
journal article

(Version)
Accepted Manuscript

Enhancement of astaxanthin incorporation by pulsed high-intensity ultrasound in LPS-stimulated macrophages

Xiaoqi Ma[1]; Atomu Yamaguchi[1]; Noriaki Maeshige[1]*, PhD; Mikiko Uemura[1], PhD; Hikari Noguchi[1]; Hiroyo Kondo[2], PhD; and Hidemi Fujino[1], PhD

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

[2] *Department of Food Science and Nutrition, Nagoya Women's University, Nagoya, Japan*

*** Corresponding author**

Noriaki Maeshige, PhD

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

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

Tel: +81 78 796 4582

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

Author contributions

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

Abstract

Purpose

Ultrasound (US) has been reported to improve the permeability of cell membranes to pharmaceuticals by causing cavitation. Astaxanthin (AX) potently terminates the induction of inflammation, but it has low oral bioavailability, which limits its incorporation in local cells and organs and its therapeutic potential. In this study, we aimed to investigate the contribution of US to AX incorporation to compensate for the limited incorporation of AX, and regulation of the pro-inflammatory factor interleukin-1β (IL-1β) by AX.

Methods

Murine bone marrow-derived macrophages were stimulated by lipopolysaccharide (LPS). After 2 h, cells were treated with 10 μM AX and/or pulsed high-intensity US irradiation. The cells were then incubated for another 3 h and harvested. AX incorporation in cells was measured by absorbance, and the expression of IL-1β was measured by qPCR. All values are expressed as means ± standard error of the mean.

Results

The combination of AX and US significantly increased AX incorporation in cells compared to AX alone ($p<.05$). In addition, this combination further suppressed the expression of IL-1β compared to AX alone ($p<.05$).

Conclusion

Pulsed high-intensity US irradiation combined with AX treatment promoted AX incorporation in cells and enhanced the anti-inflammatory effect on macrophages.

Key words

astaxanthin (AX), pulsed high-intensity ultrasound, inflammation

1. Introduction

Ultrasound (US) therapy has a long history, and its use is becoming increasingly widespread. It has also been reported that US can relieve pain, promote tissue healing, and bring fundamentally favorable safety characteristics to the clinic [1]. Based on previous studies, it has been reported that US can improve the permeability of cell membranes to pharmaceuticals, even allowing the delivery of drugs with low bioavailability in sufficient quantities because of the fast dynamics of cavitation bubbles produced by US on the membrane surface [2]. High-intensity US has been actively used to transfer the macromolecules, such as plasmid DNA, by enhancing cell membrane permeability [3]. The propagation of US waves with higher energy, intensity, and frequency through living tissues is useful for a variety of therapeutic applications. It is believed that high-intensity US can enhance the response of cells to therapeutic agents as a drug delivery tool by causing acoustic cavitation and microstreaming [4]. However, the effect of high-intensity US on the permeation of lipid-soluble nutrients has not been reported.

Astaxanthin (AX) has gained growing interest as a multi-target pharmacological agent against various diseases because of its strong antioxidant property, anti-inflammatory effect, anti-apoptotic effect, and immune modulation. AX is a natural red lipid-soluble pigment that belongs to the xanthophyll family of carotenoids. It is primarily biosynthesized by microalgae, phytoplankton, yeast, and bacteria, and it then accumulates in zooplankton, crustaceans, and subsequently fish [5]. It has been shown that AX is not genotoxic and can be safely consumed [6]. It is also safe for humans since it is well-tolerated [7], and no significant side effects have been reported in previous studies [8]. AX exhibits a variety of beneficial biological activities and effects. It is considered to have an extraordinary potential to protect the organism from a wide range of diseases such as cardiovascular problems, different types of cancer, and some diseases of the immune system. It is also a potential therapeutic agent against atherosclerotic cardiovascular disease, such as atherosclerosis, by inhibiting nuclear factor-kappa B (NF-κB) activation [9]. It has also been reported that AX can be a novel preventive and therapeutic strategy for chronic obstructive pulmonary disease by increasing the expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) [10]. However, AX has low oral bioavailability due to dissolution limitation in gastrointestinal fluids, dietary components, and so on, which limit its incorporation in local cells and organs and its therapeutic potential [11]. Thus, it is important and

meaningful to find a way to promote AX absorption to intensify the effects of AX in the target organs and cells to more successfully treat diseases. To develop an effective means, we hypothesized that US promoted AX incorporation, which may be a practical way to compensate for the limited incorporation of AX in the target cells and organs.

Macrophages are essential for the progression of inflammation, such as host defense against pathogenic infections [12]. Meanwhile, excess inflammation due to macrophages can be potentially harmful to the body, and it needs to be tightly regulated to avoid excessive tissue damage [13]. For example, macrophages play central roles in the initiation, progression, development, and rupture of atherosclerosis [14], which can be further exacerbated by interleukin-1β (IL-1β) [15]. IL-1β is a kind of pro-inflammatory cytokine, and the excessive production of pro-atherosclerotic cytokines can result in sustaining the inflammatory response [16]. Thus, it is important to inhibit the excessive production of pro-inflammatory cytokines to regulate the inflammatory responses and avoid various inflammatory diseases. Lipopolysaccharide (LPS) is widely used to induce inflammation in cells and is reported to stimulate macrophages and lead to the production of pro-inflammatory cytokines [13]. Moreover, macrophage inflammation can be suppressed by increasing Nrf2 expression and the inhibition of NF-κB activation by AX, so that the target inflammatory diseases can be improved [17][18]. However, the effect of high-intensity US on the permeation of AX in the macrophages and the accompanying anti-inflammatory effect are unknown. Therefore, in this study, we used LPS-stimulated macrophages as an experimental model to investigate the effect of high-intensity US on AX incorporation, and the following anti-inflammatory effects.

2. Materials and Methods

1. Cell culture

Bone marrow-derived macrophages were used in this study. 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 8 days to generate bone marrow-derived macrophages (BMDMs) [19]. Differentiated macrophages were harvested and plated 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. AX treatment and US irradiation

After 100 ng/mL of lipopolysaccharide (LPS) stimulation for 2 h, BMDMs were treated with AX and/or US irradiation in the presence of LPS. The cells were then incubated for another 3 h and harvested for the subsequent assays. AX oil (Fuji Chemical Industry Co., Ltd., Toyama, Japan) was dissolved in the culture medium using an ultrasonic cleaner (JP-009; Skymen Cleaning Equipment Shenzhen Co., Ltd., China) for 30 min and then added to the culture media in the dish to make the concentration of AX 10 μM. Cells were irradiated with US for 5 min just after administration of AX. US irradiation was performed by positioning the probe of a medical US device (SZ-100M; MINATO Medical Science Co., LTD, Japan) under the bottom of the culture dish. US energy transmitted through the dish bottom was around 82% of irradiated energy (Supplemental File 1-1). Coupling gel 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 (Fig. 1) [20]. US parameters were as follows: intensity of 3.0 W/cm^2, duty cycle of 20%, acoustic frequency of 1 MHz, repetition frequency of 100 Hz, and effective radiation area of 7.6 cm^2. The beam nonuniformity ratio, which is the ratio of the maximum intensity to the average intensity of the US, was 2.4, indicating the safety of the probe (Supplemental File 1-2). This US exposure could not improve the dissolution of AX in the medium (Supplemental File 2, Fig. 1). The temperature of the culture media was measured to monitor the drop below 37 degrees Celsius after US treatment (Supplemental File 2, Fig. 2). Handling of control and AX samples was identical to that of US-treated samples with or without AX to minimize discrepancies in temperature between groups with and without US treatment.

3. Cell viability analyses using Zombie Red™ immunofluorescence staining, MTT assay, and Trypan blue staining

BMDM viability was assessed 3 h after US irradiation with/without AX treatment or treatment with 1% povidone-iodine (positive control) using Zombie Red™. This reagent is an amine-reactive fluorescent dye that is non-permeant to live cells but permeant to cells with a compromised plasma membrane. BMDMs were washed twice with PBS, stained with Zombie Red™ (1:1000) (red) for 15 min, and fixed with 4% paraformaldehyde (PFA) for 30 min. Nuclei were counterstained with DAPI (blue) for 5 min. The image was captured with a 10× objective on a BX50 (OLYMPUS, Japan), and the numbers of total cells (blue) and dead cells (red) were counted. The percentage of live cells to total cells was calculated in all groups.

BMDM viability was evaluated 3 h after US irradiation with/without AX treatment by MTT assay. The cells were incubated for 3 h with 1.2 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 macrophage culture medium at 5 mg/ml. Cell viability was expressed as a percentage compared to the control.

Trypan blue staining was performed 3 h after US irradiation with/without AX treatment to distinguish live cells from dead cells and absolute cell counts for calculating cell viability.

4. Astaxanthin incorporation

AX incorporation in BMDMs was measured by absorbance index. According to the information from Astaxanthin Manufactures' Association, AX has a maximum absorption area at a wavelength of 460-480 nm [21]. To establish a suitable method for testing the absorption of AX, the absorbance of AX dissolved in dimethylsulfoxide (DMSO) was scanned with a photometer (NanoPhotometer NP80 Touch; IMPLEN GMBH Co., Ltd., Germany). The absorbance of AX detected the peak absorbance at the wavelength of 470-500 nm in the present experiment (Supplemental File 2, Fig. 3), and the absorbance at 470 nm showed linear change in an AX concentration-dependent manner (Supplemental File 2, Fig. 4a). The calibration line was also used to identify AX concentrations in the cell lysate (Supplemental File 2, Fig. 4b).

In this study, briefly, 3 h after US irradiation, BMDMs were washed three times with PBS, then 1 ml of DMSO was added to each dish to lyse BMDMs. After lysing for 5 min, the lysate was collected to measure the AX incorporation. Also, AX at different concentrations dissolved in culture medium was applied to cells, and the concentration-dependent AX incorporation in cells was measured using the same method as above to make another calibration line.

5. Quantitative real-time PCR

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

sequences are shown in Table 1.

6. Statistical analysis

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

3. Results

1. Cell viability

As shown in Fig. 2 and Table 2, US irradiation and AX treatment did not cause a reduction in cell viability in any of the groups.

2. Astaxanthin incorporation

The absorbance in the AX-treated group was significantly higher than that in the control and US groups. The absorbance in the AX+US group was significantly higher than that in the AX group (Fig. 3a). From the calibration line shown in Fig. 2b in Supplemental File 2, AX concentration in cells could be calculated using the formula, and the calculated results showed that the concentration of AX in DMSO cell lysate was 2.43-fold higher in the AX+US group compared to the AX group (Table 3). In addition, the absorbance in the AX+US group was equivalent to that at 49 μM AX treatment from the calibration line generated by the known AX concentrations in culture media (Fig. 3b).

3. Expression of inflammatory factor IL-1β

The expression of IL-1β significantly decreased in the AX group compared to the control group, and it was further suppressed in the AX+US group compared to the AX group (Fig. 4).

4. Discussion

In this study, the novel findings were as follows: (1) AX treatment with or without high-intensity US irradiation did not significantly reduce cell viability in any of the groups; (2) AX treatment combined with US irradiation increased the absorbance of AX in cells, which means the US irradiation promoted AX incorporation in BMDMs; and (3) AX treatment combined with US irradiation suppressed the expression of IL-1β, indicating enhancement of the anti-inflammatory effect of AX on macrophages. These results suggest that this combination treatment promotes AX incorporation in macrophages, leading to enhancement of anti-inflammatory effects.

Based on previous studies, the cellular uptake of drugs and genes is

increased when the region of interest is under US sonification [22], and US can enhance the efficacy of chemotherapeutic drugs because the ultrasonic cavitation effect of US directs the agent into the cells and permits the inflow of external agents [23]. In this study, we showed that when AX treatment was combined with US irradiation, the AX concentration in cells was 2.43-fold higher than that of AX treatment alone, which was almost equivalent to the effect of 5-fold AX concentration treatment. These findings demonstrated that US irradiation promoted AX incorporation in cells and compensated for the low absorption rate of AX. Also, it was reported that combination treatment with sodium butyrate and US showed a stronger anti-inflammatory effect on fibroblasts than sodium butyrate treatment alone [24]. In addition, AX has been reported to potently terminate the induction of inflammation. Yoshimi et al. also reported that higher concentrations of AX had a stronger inhibitory effect on IL-1β expression [25], which is consistent with the results in this study: combination treatment had a stronger inhibitory effect on IL-1β expression than AX treatment alone where US irradiation promoted AX incorporation, which means that this treatment could be used to intensify the anti-inflammatory effects of AX in target macrophages to more successfully treat diseases in the target organs. High-intensity US, in particular, has been actively used to enhance cell membrane permeability to allow for the transfer of macromolecules [22]; therefore, we surmised that the cavitation effect of high-intensity US would promote AX incorporation in BMDMs.

In the present study, US irradiation enhanced the anti-inflammatory effect of AX treatment. In a study by Riley et al., mitochondria were reported to orchestrate metabolism and inflammation as cellular organelles [26]. Kim et al. also reported that mitochondrial dysfunction underlies the endless cycle of inflammation [27]. These findings indicate that suppressing mitochondria dysfunction is essential to inhibit inflammation. AX is reported to maintain the metabolic efficiency of the mitochondria [27]. Hence, it is suggested that enhancement of AX incorporation by US prevents the mitochondrial dysfunction, leading to the anti-inflammatory effects. However, this hypothesis needs to be demonstrated in further experiments. In addition, this study was performed in vitro, and more research is needed to explore how this AX treatment combined with US irradiation can be applied in vivo and be therapeutically useful in the clinical setting. Since 1-MHz US can affect tissues up to 5 cm deep [28], when inflammation occurs, US could be used to irradiate the target organs after ingestion of AX, resulting in suppression of inflammatory responses. The dose and the time point for US irradiation need to be explored in further studies. In the present study, we used macrophages as the target cells of US to investigate the

immune response in inflammation. However, the tissue damage due to inflammation could be enhanced by immune cells and parenchymal cells synergistically [29][30], and the parenchymal cells could also have the therapeutic effects of AX facilitated by US along with immune cells upon irradiation. Therefore, the therapeutic effect of combination of AX and US on other types of cells should also be investigated in the future. In the present study, we confirmed the absence of cell damage due to the high-intensity US. However, this could change depending on the condition of the US target; therefore, investigations of the optimal and safe intensity are needed in further animal and human experiments.

5. Conclusion

This study revealed the facilitatory effect of pulsed high-intensity US on AX incorporation and the anti-inflammatory effect of AX. This treatment could be used to compensate for the low absorption rate of AX and further enhance the anti-inflammatory effect on macrophages.

6. Acknowledgments

We appreciate the members of our laboratory for their thoughtful technical advice. This study was supported by JSPS KAKENHI [grant numbers 21H03852, 20KK0227, and 19H03989].

7. Declarations

Ethical statements

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

The authors declare that they have no conflicts of interest.

References

1) Miller D, Smith N, Bailey M, et al. Overview of therapeutic ultrasound applications and safety considerations. J Ultrasound Med. 2012;31:623-34.

2) 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.

3) Taniyama Y, Tachibana K, Hiraoka K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: Enhancement of

transfection efficiency of naked plasmid DNA in skeletal muscle. Gene Ther. 2002;9:372-80.
4) Phenix CP, Togtema M, Pichardo S, et al. High intensity focused ultrasound technology, its scope and applications in therapy and drug delivery. J Pharm Pharm Sci. 2014;17:136-53.
5) Chang MX, Xiong F. Astaxanthin and its Effects in Inflammatory Responses and Inflammation-Associated Diseases: Recent Advances and Future Directions. Molecules. 2020;25:5342-14.
6) Edwards JA, Bellion P, Beilstein P, et al. Review of genotoxicity and rat carcinogenicity investigations with astaxanthin. Regul Toxicol Pharmacol. 2016;75:5-19.
7) Kishimoto Y, Yoshida H, Kondo K. Potential Anti-Atherosclerotic Properties of Astaxanthin. Mar Drugs. 2016;14(2):35.
8) Ambati RR, Phang SM, Ravi S, et al. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications--a review. Mar Drugs. 2014;12(1):128-52.
9) Ciapara IH, Valenzuela LF, Goycoolea FM. Astaxanthin: A review of its chemistry and applications. Crit Rev Food Sci Nutr. 2006;46:185-96.
10) Kubo H, Asai K, Kojima K, et al. Astaxanthin Suppresses Cigarette Smoke-Induced Emphysema through Nrf2 Activation in Mice. Mar Drugs. 2019;17(12):673.
11) Madhavi D, Kagan D, Seshadri S. A study on the bioavailability of a proprietary, sustained-release formulation of astaxanthin. Integr Med. 2018;17:38-42.
12) Funes SC, Rios M, Vera JE, et al. Implications of macrophage polarization in autoimmunity. Immunology. 2018;154:186-95.
13) Moghaddam AS, Mohammadian S, Vazini H, et al. Macrophage plasticity, polarization and function in health and disease. J Cell Physiol. 2018;233:6425-40.
14) Kishimoto Y, Tani M, Kondo HU, et al. Astaxanthin suppresses scavenger receptor expression and matrix metalloproteinase activity in macrophages. Eur J Nutr. 2010;49:119-26.
15) Grebe A, Hoss F, Latz E. NLRP3 inflammasome and the IL-1 pathway in atherosclerosis. Circ Res. 2018;122:1722-40.
16) Jinnouchi H, Guo L, Sakamoto A, et al. Diversity of macrophage phenotypes and responses in atherosclerosis. Cell Mol Life Sci. 2020;77:1919-32.
17) Pereira CPM, Souza ACR, Vasconcelos AR, et al. Antioxidant and anti-inflammatory mechanisms of action of astaxanthin in cardiovascular diseases (Review). Int J Mol Med. 2021;47(1):37-48.
18) Kobayashi EH, Suzuki T, Funayama R, et al. Nrf2 suppresses macrophage

inflammatory response by blocking proinflammatory cytokine transcription. Nat Commun. 2016;7:11624.
19) Langston PK, Nambu A, Jung J, et al. Glycerol phosphate shuttle enzyme GPD2 regulates macrophage inflammatory responses. Nat Immunol. 2019;20:1186-95.
20) 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.
21) Astaxanthin manufactures' Association [homepage on the Internet]. Japan: The Association; [cited 2012 Feb 25]. Astaxanthin Quality Standards;[about 3 screens]. Available from: http://www.asta-ma.jp/haematococcus/index.html.
22) Postema M, Gilja OH. Ultrasound-Directed Drug Delivery. Curr Pharm Biotechnol. 2007;8:355061.
23) Nan C, Zheng Y, Fan H, et al. Antitumorigenic effect of hsp90 inhibitor snx-2112 on tongue squamous cell carcinoma is enhanced by low-intensity ultrasound. Onco Targets Ther. 2020;13:7907-19.
24) Maeshige N, Koga Y, Tanaka M, et al. Low-Intensity Ultrasound Enhances Histone Acetylation and Inhibition of Interleukin 6 Messenger RNA Expression by the Histone Deacetylase Inhibitor Sodium Butyrate in Fibroblasts. J Ultrasound Med. 2017;36:879-85.
25) Kishimoto Y, Tani M, Kondo HU, et al. Astaxanthin suppresses scavenger receptor expression and matrix metalloproteinase activity in macrophages. Eur J Nutr. 2010;49:119-26.
26) Riley JS, Tait SW. Mitochondrial DNA in inflammation and immunity. EMBO Rep. 2020;21:e49799.
27) Kim SH, Kim H. Inhibitory effect of astaxanthin on oxidative stress-induced mitochondrial dysfunction-a mini-review. Nutrients. 2018;10:1137.
28) Hayes BT, Merrick MA, Sandrey MA, et al. Three-MHz Ultrasound Heats Deeper Into the Tissues Than Originally Theorized. J Athl Train. 2004;39(3):230-4.
29) Grumelli S, Corry DB, Song LZ, et al. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. PLoS Med. 2004;1(1):e8.
30) Vonlaufen A, Apte MV, Imhof BA, et al. The role of inflammatory and parenchymal cells in acute pancreatitis. J Pathol. 2007;213(3):239-48.

Appendices: Supplemental File 1 and Supplemental File 2.

Figure Legends:

Fig. 1 In vitro ultrasound irradiation system.

Fig. 2 Cell viability. (a) Zombie Red™ immunofluorescence staining in macrophages. BMDMs were exposed to US irradiation after LPS stimulation for 2 h. After US irradiation for 3 h, immunofluorescence staining of BMDMs was performed with Zombie Red™ dye. After fixation, the cells were counter-stained with DAPI. (b) MTT assay for measuring viability, which was expressed as a percentage compared to the control. (c) Trypan blue staining to distinguish live cells from dead cells. All values are presented as mean ± SEM.

Fig. 3 AX incorporation in macrophages and calibration lines for absorbance and AX concentration. (a) AX incorporation in BMDMs was measured by absorbance at the wavelength of 470 nm. Values are presented as mean ± SEM. **p<.01 vs. CON, ††p<.01 vs. US, ‡‡p<.01 vs. AX (n=4). (b) Different concentrations of AX were added to the cell culture medium, and the absorbance of concentration-dependent AX incorporation was measured to make a calibration line. The absorbance in the AX+US group (red line) was also measured.

Fig. 4 Anti-inflammatory effects of AX treatment with or without high-intensity US irradiation. LPS was used to induce inflammation, and then each treatment was performed. The expression of IL-1β was measured by qPCR. Values are presented as mean ± SEM. *p<.05 vs. CON, **p<.01 vs. CON, ††p<.01 vs. US, ‡p<.05 vs. AX. (n=4)

Table 1: Primer sequences for quantitative PCR.

Primer	Sequences
GAPDH-Forward	5'-CCAATGTGTCCGTCGTGGATCT
GAPDH-Reverse	5'-GTTGAAGTCGCAGGAGACAACC
IL-1β-Forward	5'-GCCTTGGGCCTCAAAGGAAAGAA
IL-1β-Reverse	5'-ATTGCTTGGGATCCACACTCTCC

Table 2: Cell viability analysis by Zombie Red™ immunofluorescence staining.

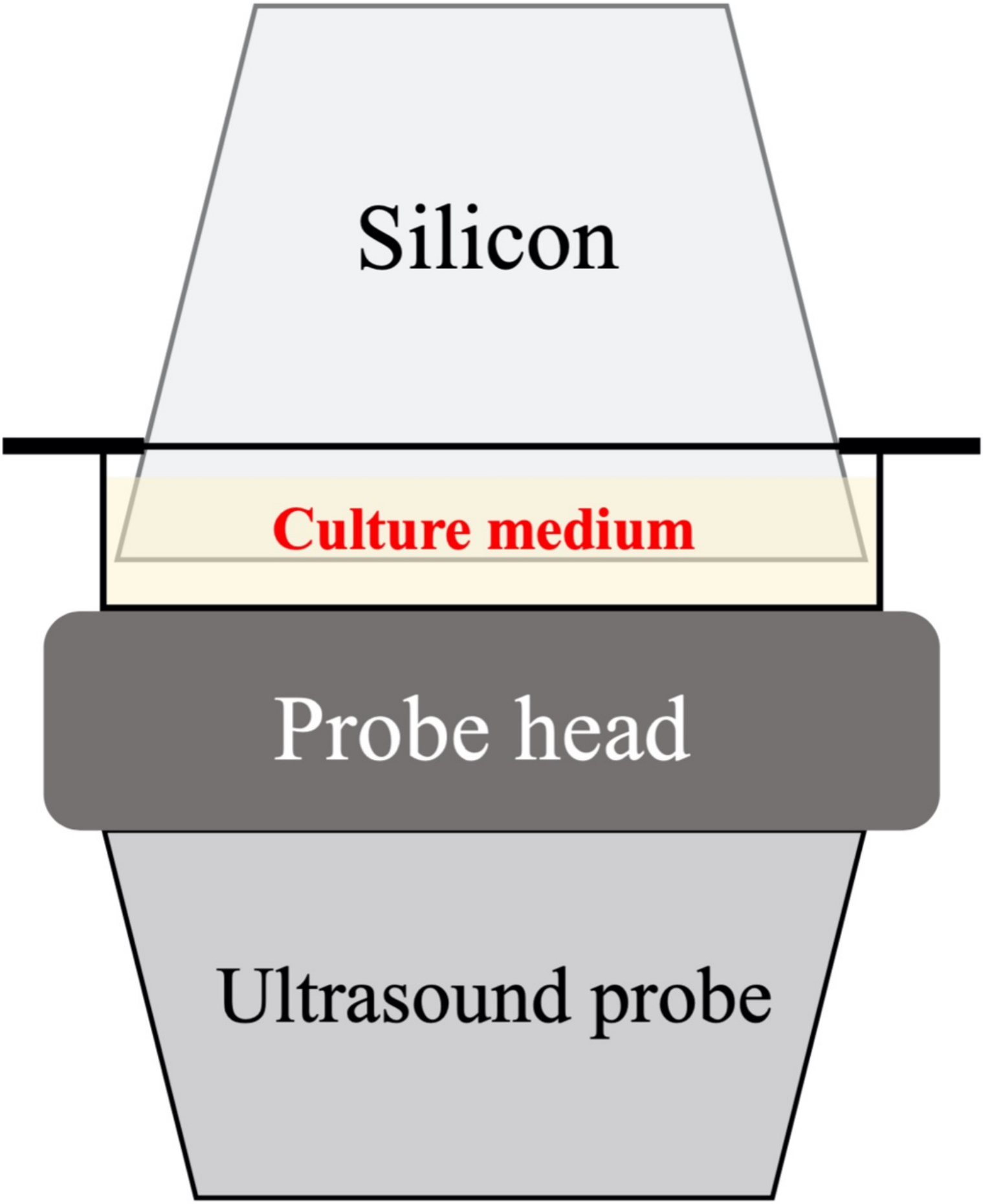
Group	Percentage of live cells to total cells (%)
CON	99.77 ± 0.15
US	99.75 ± 0.15
AX	99.81 ± 0.11
AX+US	98.21 ± 0.97

The percentage of live cells to total cells was calculated. All values are expressed as means ± SEM.

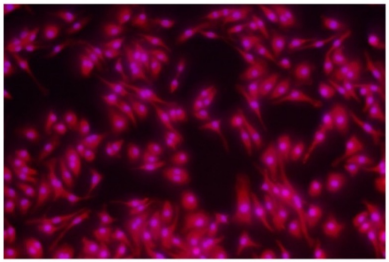
Table 3: AX incorporation in AX and AX+US groups.

	AX group	AX+US group
Absorbance	0.05	0.11
AX incorporation (μM)	0.19	0.47
Ratio	1.00	2.43

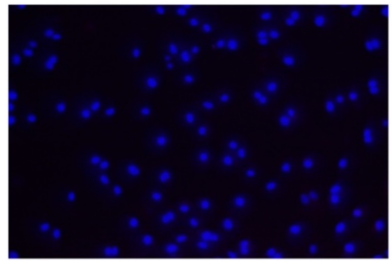
The absorbance was measured, and the AX incorporation was calculated using the formula shown in Fig. 2b in Supplemental File 2.


Silicon
Culture medium
Probe head
Ultrasound probe

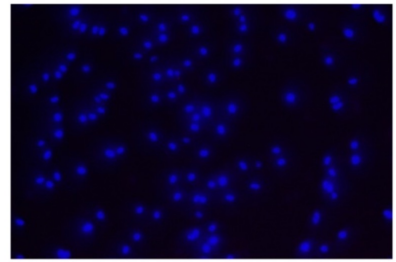
a



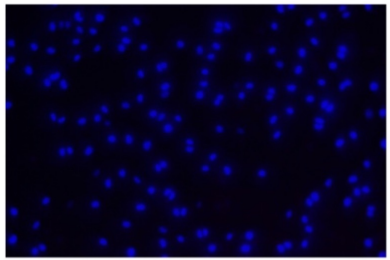
Povidone-iodine



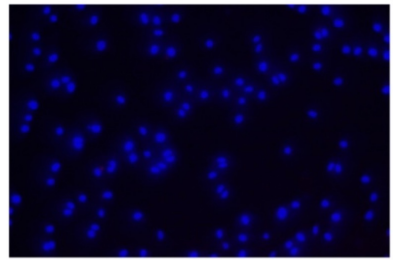
CON



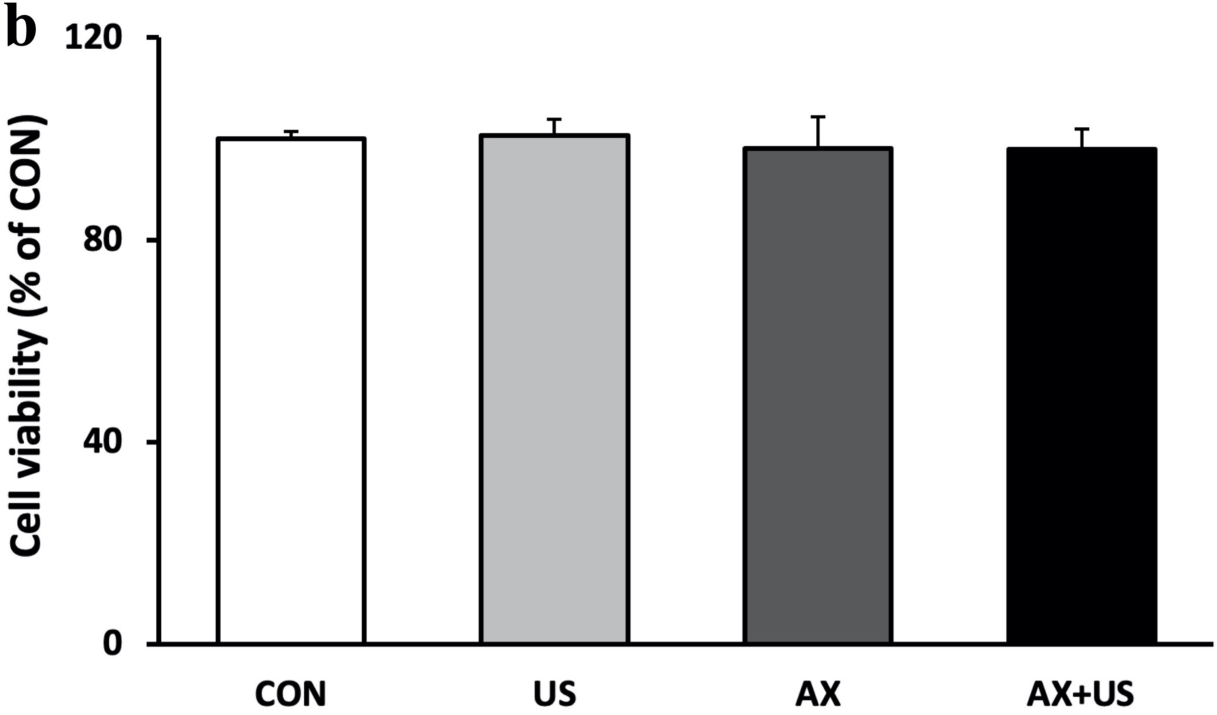
US

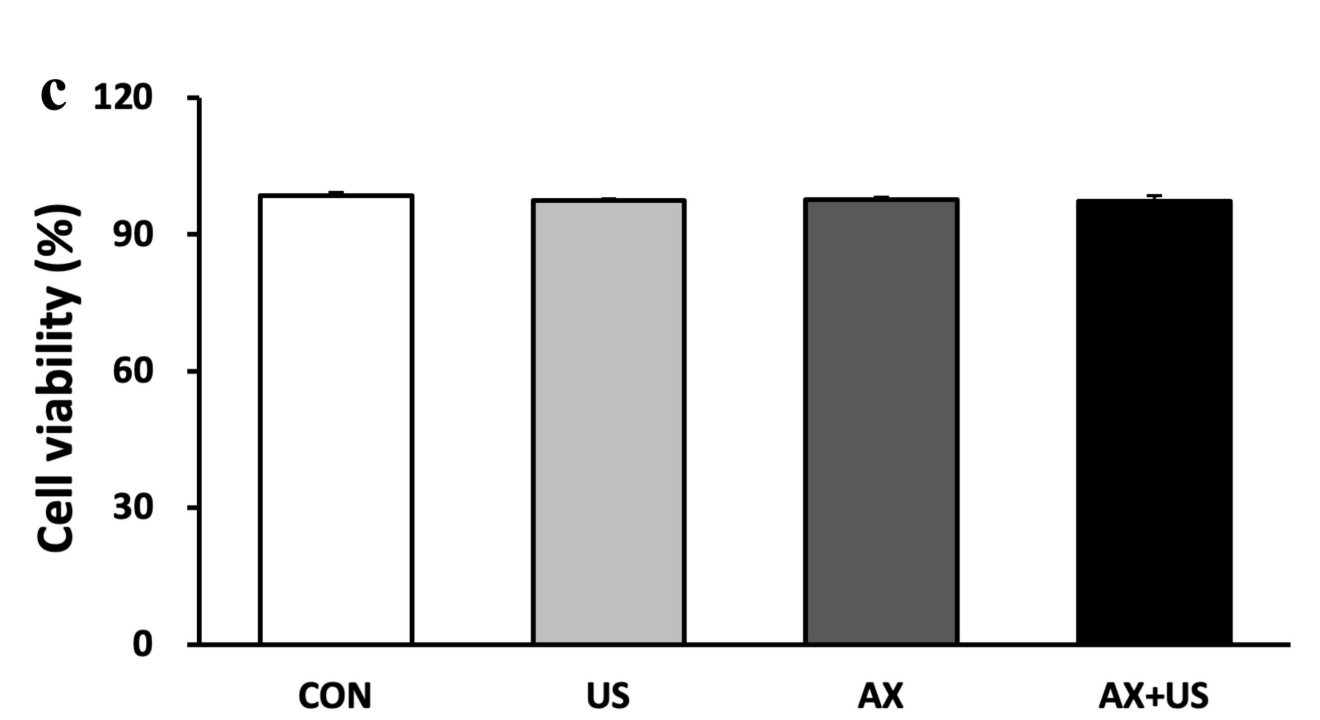


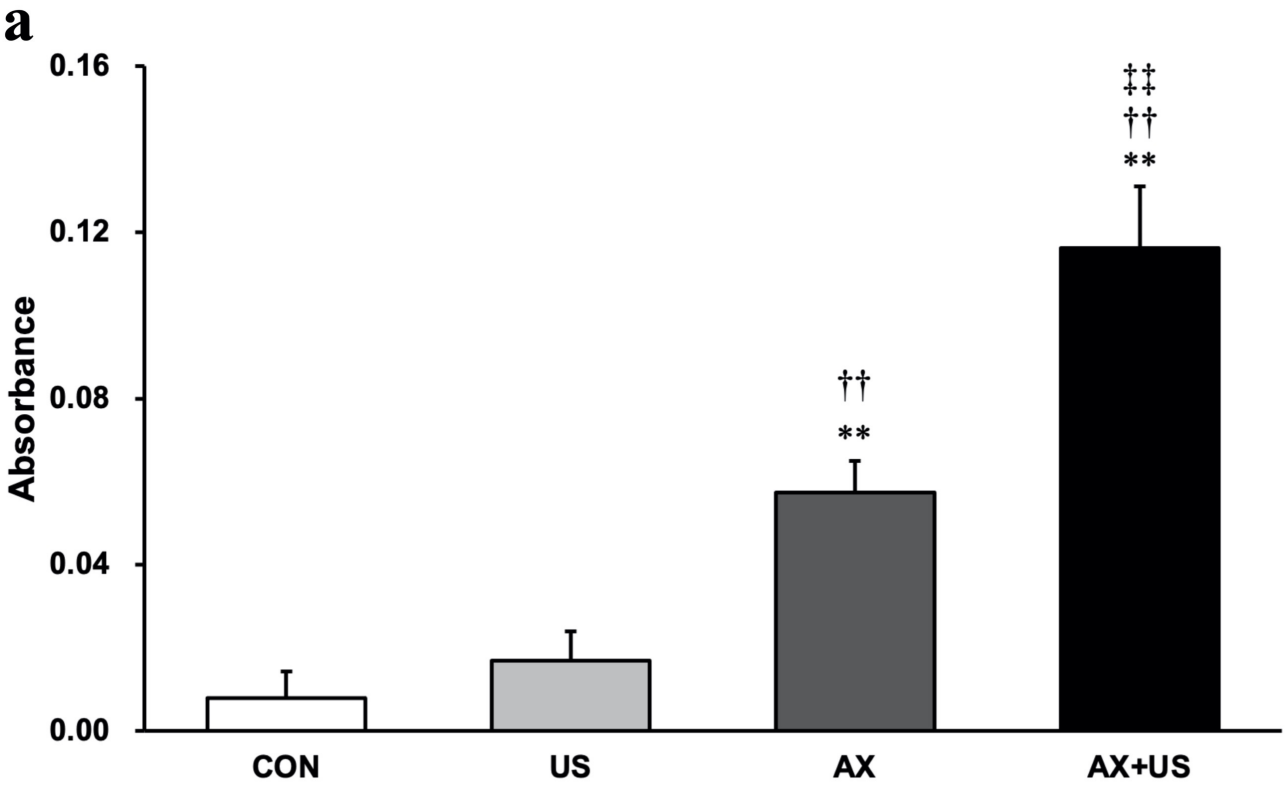
AX

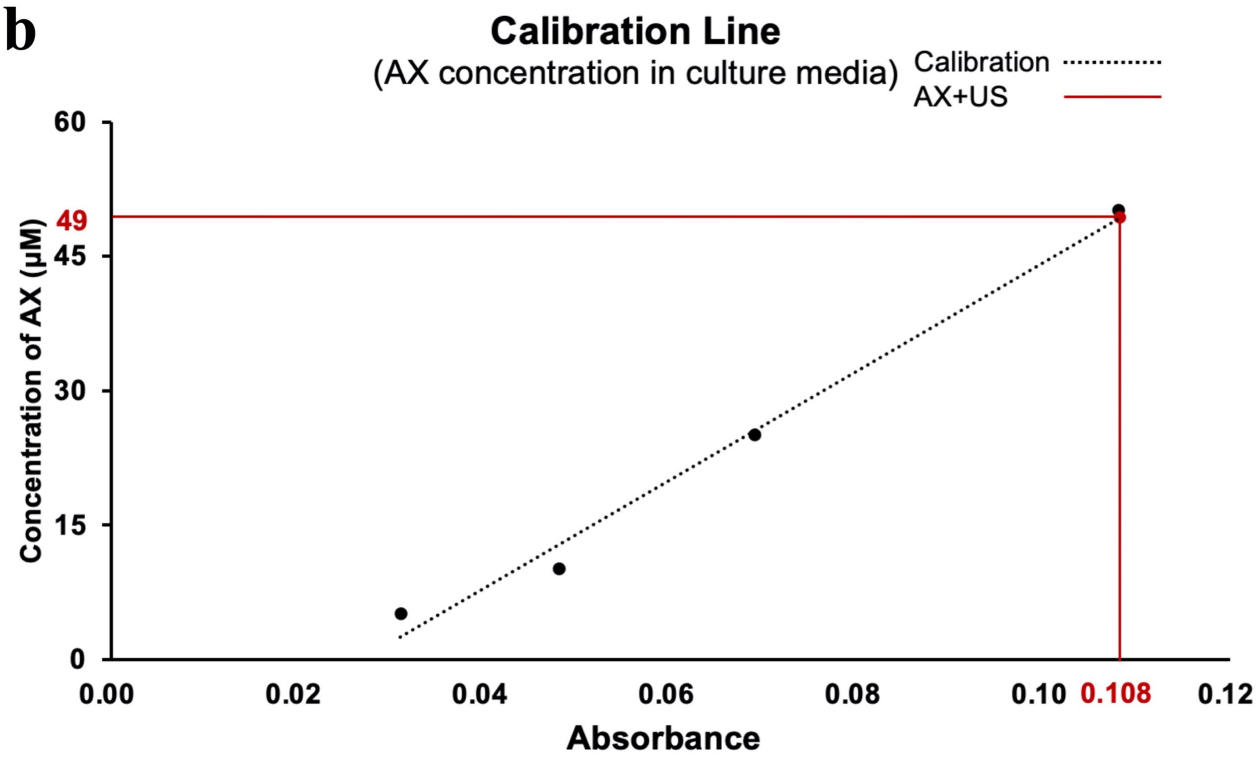


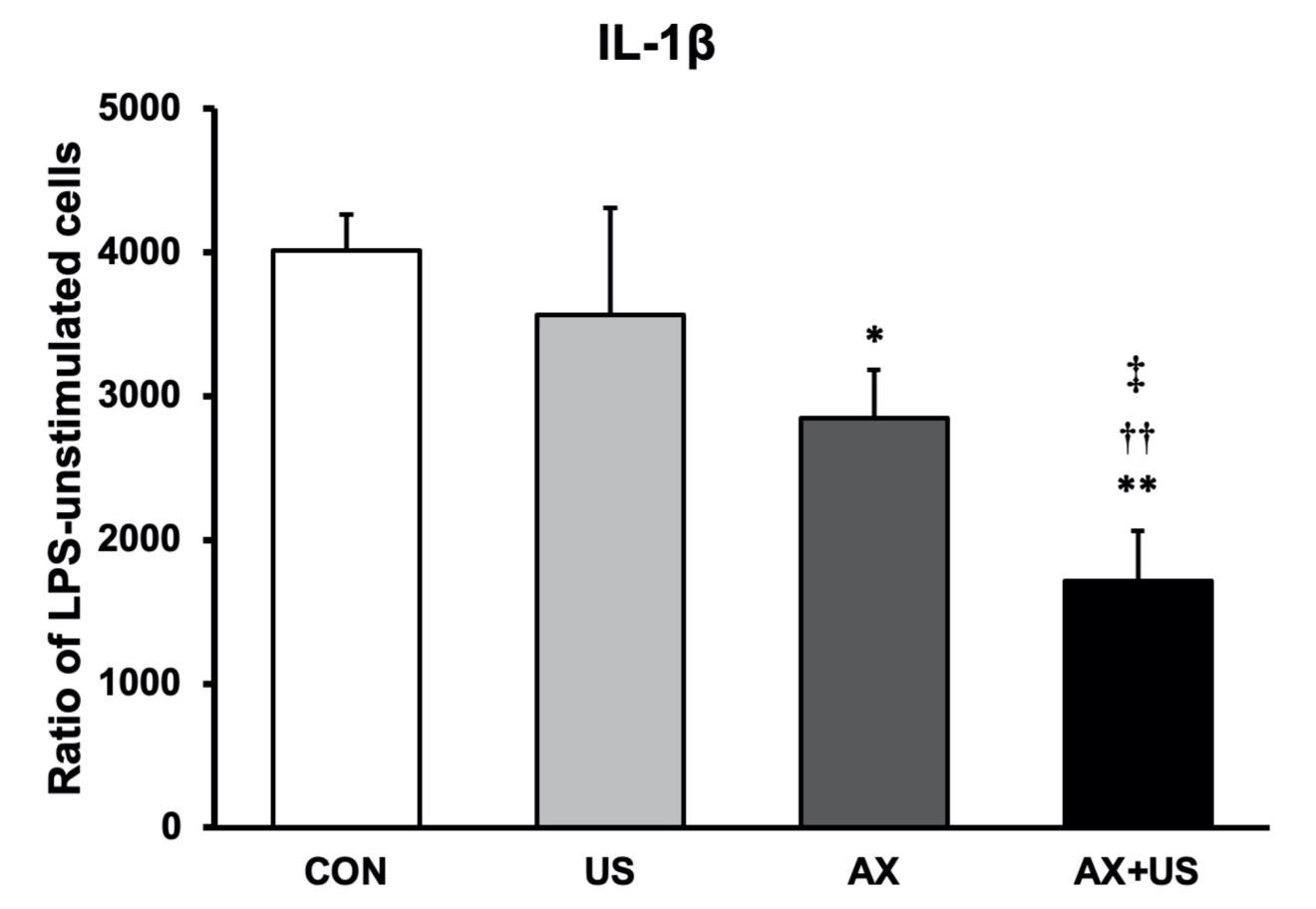
AX+US


b
120
80
40
0
Cell viability (% of CON)
CON
US
AX
AX+US


c
120
90
60
30
0
Cell viability (%)
CON
US
AX
AX+US


a
0.16
0.12
0.08
0.04
0.00
Absorbance
CON
US
AX
AX+US
††
**
‡‡
††
**


b
Calibration Line
(AX concentration in culture media)
Calibration
AX+US
60
49
45
30
15
0
Concentration of AX (μM)
0.00
0.02
0.04
0.06
0.08
0.10
0.108
0.12
Absorbance


IL-1β
Ratio of LPS-unstimulated cells
5000
4000
3000
2000
1000
0
*
‡
††
**
CON
US
AX
AX+US

Supplemental File 1

1. Actual output of the ultrasound

We measured the actual output of ultrasound using an Ultrasound Power Meter (Ohmic Instruments, USA) in degassed water. The actual output of ultrasound was 3.25 ± 0.06 W/cm^2. After passing through the dish, the intensity of the ultrasound was 2.66 ± 0.03 W/cm^2 due to the attenuation of intensity caused by the cell culture dish. Ultrasound energy transmitted through the dish bottom was around 82% of irradiated energy. Values are expressed as means ± standard error of the mean.

2. BNR and ERA measurements

1. Definition of beam non-uniformity ratio (BNR):

In JIS T 0601-2-5 based on IEC 60601-2-5, BNR is defined as follows:
The ratio of the square of the maximum sound pressure RMS value to the spatial mean of the square of the sound pressure RMS value. The spatial average is obtained from the effective emission area.

2. Definition of effective radiating area (ERA):

In JIS T 0601-2-5 based on IEC 60601-2-5, ERA is defined as follows (five formulas):

"A value obtained by multiplying the beam cross section of $A_{BCS}(0.3)$ measured at a distance of 0.3 cm from the front of the probe head by the dimensionless coefficient of 1.354"

1. Square the measured data at each point and obtain the sum of them. Define this total(P^2) as P^2.

$$total(P^2) = \sum_{n=1}^{number\ of\ measurements} (\ measured\ data\ at\ n\ th\ -\ noise\ correction\ value\ )^2 \cdots ①$$

2. Multiply P^2 by 0.75 and call it $P^2 75$.

$$P^2 75 = P^2 \times 0.75 \cdots ②$$

3. Arrange the squares of the measured data in descending order (larger order) and add until it becomes larger than $P^2 75$.
   Define this added value as P_n, count the number of n when $P_n \geq P^2 75$.

$$P_n = \sum_{n=1}^{n\ value} (\ nth\ data\ in\ descending\ )^2 \quad (\ P_n \geqq P^2 75\ ) \cdots ③$$

4. A_{BCS} (0.3) is the value obtained by multiplying the n value counted above by the unit area of scanning [cm^2].
   The unit area of the scan can be calculated by the square of the scan pitch.

$$A_{BCS}(0.3) = n \times (scan\ pitch)^2 \cdots ④$$

5. By multiplying this by the standard dimensionless coefficient of 1.354, the A^{ER} (ERA in the catalog notation) is obtained.

$$A^{ER} = 1.354 \times A_{BCS}(0.3) \cdots ⑤$$

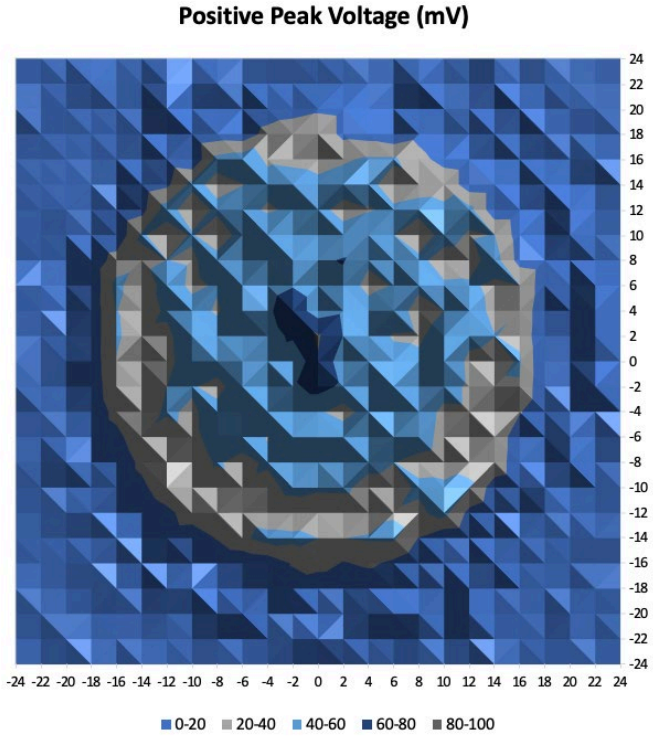
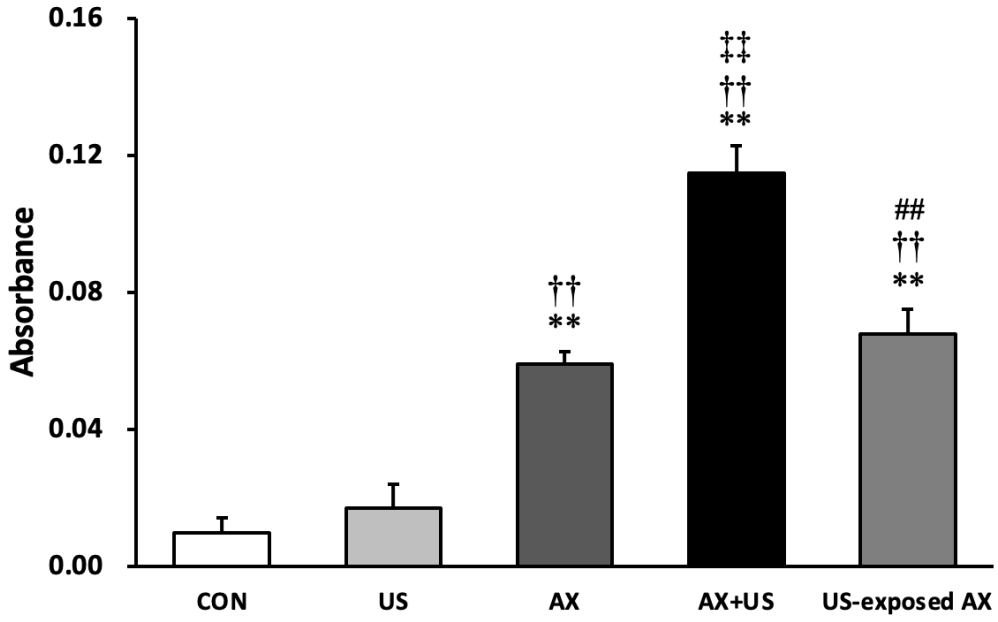


Figure: Actual energy mapping of ultrasound at 3.0 W/cm^2. The measurement pitch shown with graduation lines is 2 mm.

BNR and ERA were measured in degassed water using an acoustic intensity measurement system (AIMS III Hydrophone Scanning System; ONDA, USA) and hydrophone (PVDF hydrophone; EASTEK, Japan).

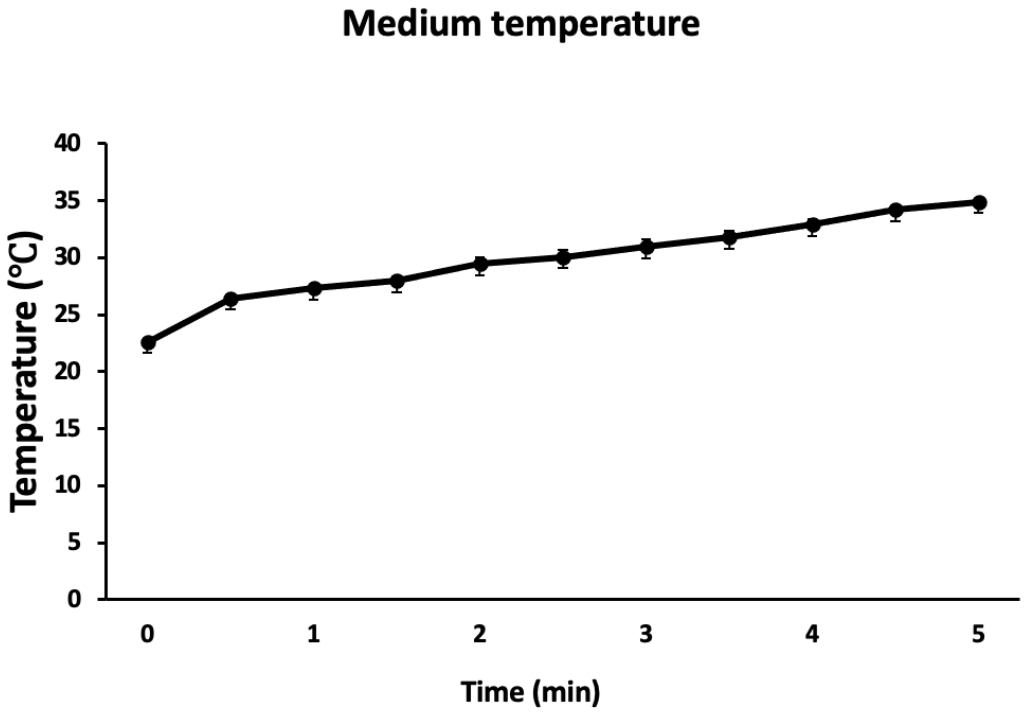
Supplemental File 2

Supplemental File 2-1: US exposure (SZ-100M; MINATO Medical Science Co., Ltd., Japan) for 5 min did not improve the dissolution of AX in the medium to enhance incorporation of AX in the cells. The absorbance in the US-exposed AX group showed no difference from that in the AX group and was significantly lower than that in the AX+US group.



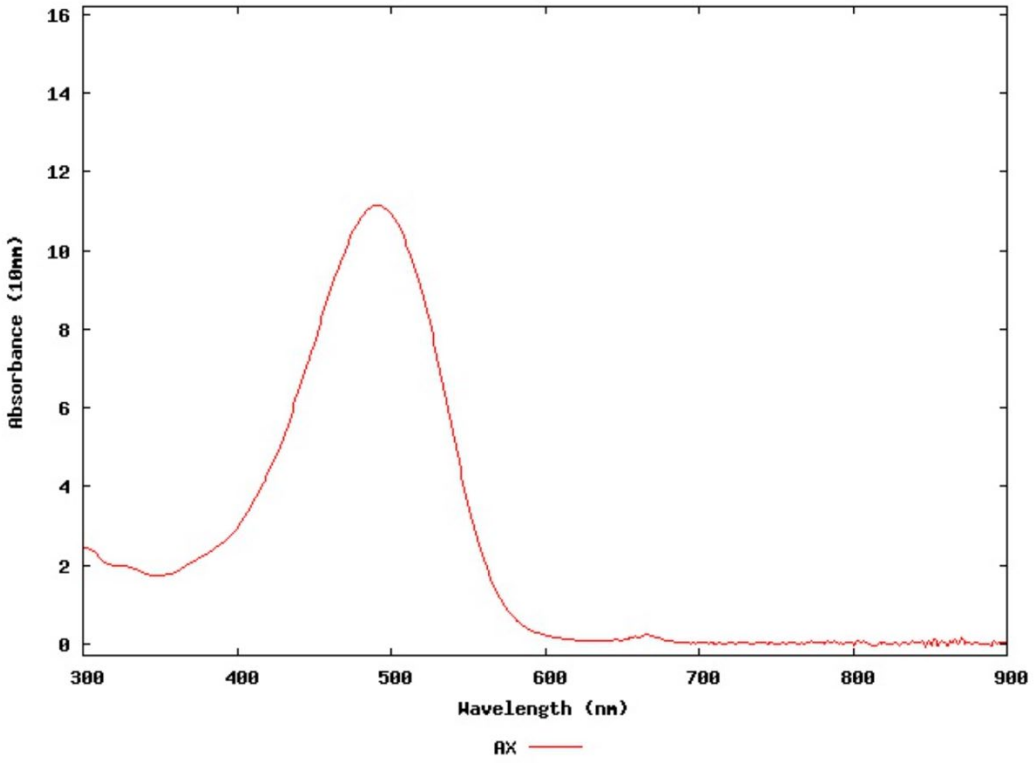
Supplemental Fig. 1 AX incorporation in BMDMs in different groups. US-exposed AX group: AX oil was dissolved in the medium using ultrasound (JP-009; Skymen Cleaning Equipment Shenzhen Co., Ltd., China) for 30 min and added to the culture medium to make the concentration of AX 10 μM. Then, the culture medium was added to the dish and irradiated by US exposure (SZ-100M; MINATO Medical Science Co., Ltd., Japan) for 5 min. Lastly, the medium was transferred to the dish with BMDMs. AX incorporation was measured by absorbance at the wavelength of 470 nm. Values are presented as mean ± SEM. **p<.01 vs. CON, ††p<.01 vs. US, ‡‡p<.01 vs. AX, ##p<.01 vs. AX+US (n=4).

Supplemental File 2-2: The medium temperature was below 37 degrees Celsius after US irradiation.



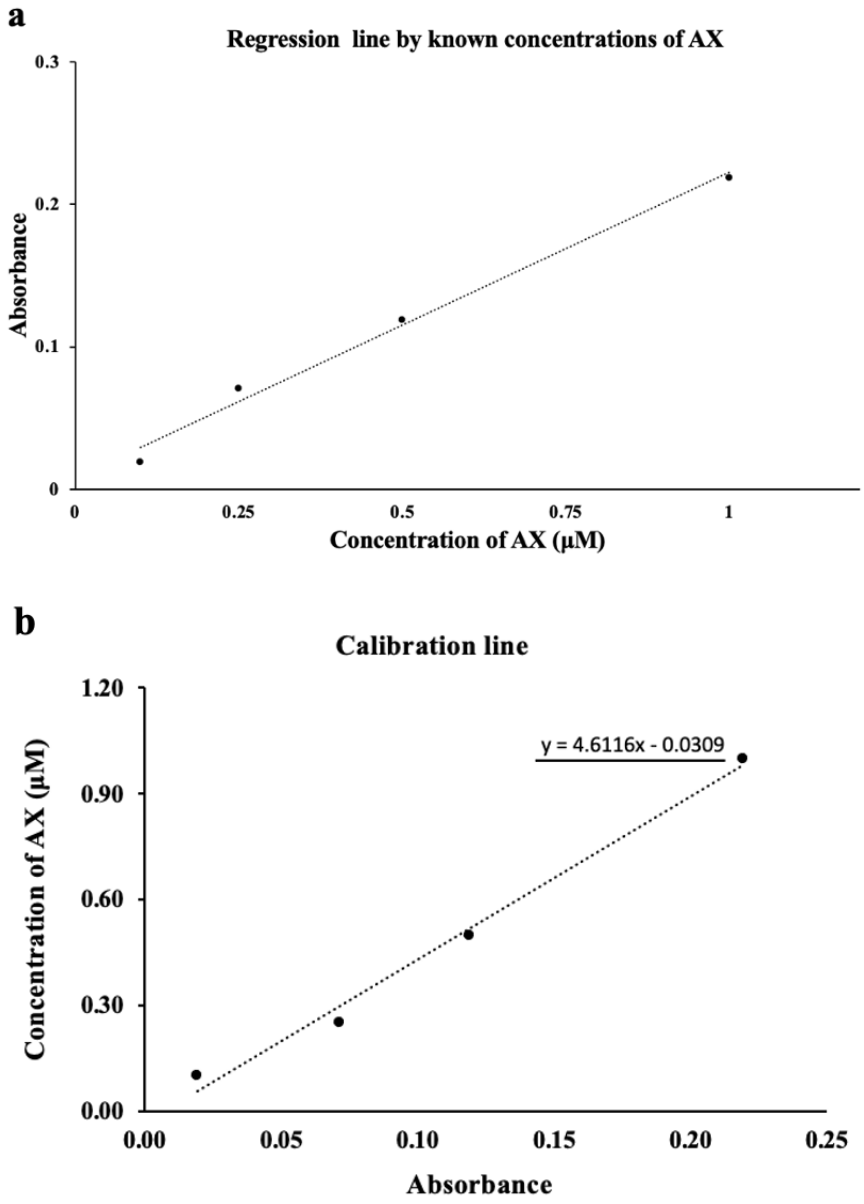
Supplemental Fig. 2 The temperature of the culture medium. During the US irradiation for 5 min, the temperature was measured at 0 min, 0.5 min, 1 min, 1.5 min, 2 min, 2.5 min, 3 min, 3.5 min, 4 min, 4.5 min, and 5 min (n=3).

Supplemental File 2-3: The absorbance of AX peaked roughly at the wavelength of 470-500 nm.



Supplemental Fig. 3 The absorbance of AX under the wavelength at 300 nm - 900 nm. AX dissolved in DMSO was scanned with a photometer (NanoPhotometer NP80 Touch; IMPLEN GMBH Co., Ltd., Germany).

Supplemental File 2-4: The magnitude of the absorbance of AX dissolved in DMSO can indicate the concentration of AX, and the AX concentration can be calculated by absorbance.



Supplemental Fig. 4 The AX concentration-dependent regression line and the calibration line. (a) AX was dissolved in DMSO at different concentrations and was measured at the wavelength of 470 nm, which showed a concentration-dependent regression line. (b) The formula for absorbance and AX concentrations, which shows the method of calculating AX concentration by absorbance.