Effect of high frequency and low intensity pulsed electric field on protecting SH-SY5Y cells against hydrogen peroxide and β-amyloid-induced cell injury via ERK pathway

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Abstract

Among various neurodegenerative diseases (NDDs), Alzheimer's disease (AD) is the most common form. Excessive aggregation of β-amyloid protein (Aβ) is thought to be a major cause in the pathogenesis of AD. A growing number of studies also have suggested that reactive oxygen species (ROS) play a critical role in the onset and progression of AD. The present study was aimed to investigate the neuroprotective effect of the high frequency and low intensity pulsed electric field (H-LIPEF) against hydrogen peroxide (H_2O_2) and Aβ-induced cytotoxicity in SH-SY5Y cells. The systematic study on frequency- and amplitude-dependent neuroprotective effect of pulsed electric field (PEF) was performed, and the result showed that H-LIPEF at 200 Hz conferred the best protective effect to the SH-SY5Y cells. The underlying mechanisms were confirmed to be due to the activation of extracellular signal-regulated kinase (ERK) pathway and the downstream prosurvival and antioxidant proteins. Given the fact that electric field can be modified to focus on specific area and applicable in a non-contact manner, we propose that H-LIPEF holds great potential for neuroprotection and curing the patients with NDDs. Furthermore, it is anticipated that the combination of such physical stimulation with drugs or natural compounds will be even more effective in treating NDDs.

Introduction

Neurodegenerative diseases (NDDs) are characterized by progressive deterioration of brain function, resulting from the gradual degeneration or death of neurons. Due to increased human lifespan, deaths due to NDDs have been increasing over the years, and caused significant impact on the quality of life of patients and their families. The World Health Organization (WHO) predicts that by 2040, NDDs will overtake cancer to become the second leading cause of death after cardiovascular diseases. Among various notorious NDDs, Alzheimer's disease (AD) is the most common neurodegenerative disorder. The typical symptoms of AD include the deposition of cortical plaques formed by aggregation of β-amyloid protein (Aβ) or hyperphosphorylation of Tau protein which finally formed the neurofibrillary tangles [1]. Scientists have long believed that these abnormal structures are prime suspects in damaging and killing nerve cells and cause progressive brain degeneration and deterioration of cognitive function among elderly people. However, there is currently no cure for AD or any other common type of NDDs. Existing medications can only help to control or delay the symptoms. One limitation for AD drug development is the presence of the blood-brain barrier (BBB), since more than 98% of small molecule drugs, and ~100% of large molecule drugs can not pass the BBB [2]. Therefore, effective treatment of AD remains one of the greatest challenges in modern medicine.

Our previous study used the non-invasive low intensity pulsed electric field (LIPEF) in combination with fucoidan to prevent oxidative stress-induced mouse motor neuron death [3]. It was found that LIPEF suppressed the hydrogen peroxide (H_2O_2)-enhanced expression of ROCK protein and increased the phosphorylation of Akt in the H_2O_2-treated NSC-34 cell line. In this work, the non-contact LIPEF stimulation was employed to study human neural cell line SH-SY5Y, which was widely used as the neurodegenerative damage model, and investigate the neuroprotective effect of LIPEF against oxidative damage induced by H_2O_2. Besides, we also extended the neuroprotective study to the AD disease model by Aβ protein administration. Interestingly, it was observed that the LIPEF parameters used in the mouse motor neuron are not effective in human neural cell line. The systematic study on frequency- and amplitude-dependent neuroprotective effect of LIPEF was performed, and the result found that the high frequency LIPEF (H-LIPEF) at 200 Hz conferred the best protective effect to the SH-SY5Y cells. Since the alternating current (AC) signal has only one frequency and intensity, it is time-consuming and painstaking to find the specific AC electric signal to achieve certain biological effect. Besides, the specific AC signal is definitely not enough to target complex signal transductions in a biological process or disease treatment. Therefore, such method will be confined to a small number of diseases which will discount its applicability. Unlike the AC signal, the

pulsed electric field (PEF) can be viewed as the composition of multiple sinusoidal subcomponents with different frequencies and intensities simultaneously [3]. We further propose that the multi-frequency component nature of PEF greatly facilitates the development of new disease therapeutics to fulfill different demands.

In this study, we applied the H-LIPEF stimulation to human neural cell line SH-SY5Y, and examined the prosurvival effect of such physical stimulation against oxidative damage induced by H_2O_2 and Aβ-induced cytotoxicity. The results showed that exposing the cells to H-LIPEF at 200 Hz significantly ameliorated the H_2O_2- and Aβ-induced cytotoxicity in SH-SY5Y cells. Examination of the underlying mechanism also showed that 200 Hz H-LIPEF could activate extracellular signal-regulated kinase (ERK) pathway and downstream neuroprotective proteins. These findings implicate that H-LIPEF is a promising electrotherapy, which sheds light on novel treatments for AD or other NDDs.

Materials and methods

Experimental setup for exposure of the cells to non-contact H-LIPEF

The LIPEF device described previously [3] was used for exposure of electric field with

various strengths and frequencies. The cells were placed between two copper flat and parallel electrodes. Consecutive pulses with pulse duration 2 ms, different electric field intensities and different frequencies were applied across the electrodes. Cells treated with continuous LIPEF were kept in a humidified incubator of 5% CO_2 and 95% air at 37℃. After optimization of the pulse parameters, we used the H-LIPEF stimulation to produce the electric field in a non-contact manner, and examined its neuroprotection effect against the oxidative insult caused by H_2O_2 to SH-SY5Y human neural cells.

Cell culture and treatment

The SH-SY5Y cell was purchased from Bioresource Collection and Research Center (Hsinchu, Taiwan) and maintained in MEM/F-12 mixture containing 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin, supplemented with 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. Cells were maintained in a humidified incubator composed of 5% CO_2 and 95% air at 37℃. Cells were harvested with 0.05% trypsin–0.5 mM EDTA solution (Gibco; Thermo Fisher Scientific, Inc.) and prepared for in vitro experiments. Cells were pretreated with H-LIPEF for 4 h and then challenged with 500 μM H_2O_2 in the continuous administration of H-LIPEF for another 24 h. For the AD disease model, the cells were pretreated with H-LIPEF for 4 h and then challenged with 25 or 50 μM $A\beta_{25\text{-}35}$ protein solution in the

continuous administration of H-LIPEF for another 4 days. The Aβ stock solution was prepared by solubilizing the $A\beta_{25-35}$ peptide (Sigma-Aldrich; Merck KGaA) in sterile deionized water to a concentration of 1 mM and then incubated at 37℃ for 3 days to allow self-aggregation before treatment. After the treatment, the cell viability was determined.

Cell viability assay

Viability of SH-SY5Y cells after treatments with or without H-LIPEF was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich; Merck KGaA) assay. Briefly, the medium was replaced with MTT solution (0.5 mg/mL in SH-SY5Y culture medium) and incubated at 37℃ for 4 h. During this period, the ability of mitochondrial dehydrogenases to reduce the MTT to formazan crystal is an indicator of cellular viability. Following MTT reaction, formazan dissolution was performed by using 10% sodium dodecyl sulfate (SDS) and 0.01 M HCl. The optical density of each well was then determined at 570 nm subtracting the background at 690 nm using an ELISA microplate reader. The cell viability was calculated based on the intensity of formazan, and expressed as percentage of the non-treated control.

Western blot analysis

After the treatments, cells were washed with phosphate buffered saline (PBS) and then harvested and lysed in RIPA lysis buffer (EMD Millipore) containing fresh protease and phosphatase inhibitor cocktail (EMD Millipore). The lysates were then centrifuged, and the supernatant was collected and used for the determination of protein concentration. Equal amounts of protein extracts were loaded in the 10% SDS-PAGE wells and transferred to polyvinylidene fluoride membranes. After drying for 1 h at room temperature (RT), the membranes were probed with ROCK (Abcam, plc.), p-ERK, Nrf2, p-CREB, Bcl-2, Bax (Cell Signaling Technology, Inc.), and GAPDH (GeneTex, Inc.) antibodies overnight at 4˚C. Subsequently, the washed membranes were incubated with horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) for 1 h at RT. All the antibodies were diluted at the optimal concentration according to the manufacturer's instructions. Finally, protein bands were visualized with an enhanced chemiluminescence substrate (Advansta, Inc.) and detected with the Amersham Imager 600 imaging system (GE Healthcare Life Sciences). The images were analyzed with Image Lab software (Bio-Rad Laboratories, Inc.). For normalization of proteins, GAPDH was used as an internal control.

Statistical analysis

Each data point represents the average from larger or equal to three independent

experiments and is expressed as the mean ± standard deviation. Statistical analyses were performed using OriginPro 2015 software (OriginLab). Differences of statistical significance were determined by a one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test. P-value < 0.05 was considered to indicate a statistically significant difference.

Results

In vitro-applied H-LIPEF

The H-LIPEF was applied in a non-contact manner with the apparatus consisting of two thin parallel conductive electrodes mounted on the insulating acrylic frame (Fig 1A). The culture dishes were placed between the parallel electrodes so the cells were not in contact with the electrodes directly. Therefore, the H-LIPEF stimulation device can provide the electric field in a non-invasive manner, avoiding the harmful effects caused by conduction current and Joule effect via contact electrodes. The pulsed electric signal was from a function generator and amplified by a voltage amplifier. In the study, the applied electric signal was characterized by a pulse train waveform (Fig 1B) with various field intensities (1-60 V/cm) and frequencies (2-499 Hz) for optimization of the treatment.

Fig 1. The experimental setup of H-LIPEF. (A) Image of the H-LIPEF stimulation device. The culture dishes were placed in the stimulation device constructed of two parallel electrodes. The blue arrows shown here indicate the schematic representation of non-invasive H-LIPEF exposure on cells. (B) Schematic representation of the pulse train waveform applied on the electrodes.

Effect of H-LIPEF exposure on H_2O_2− and Aβ−induced cytotoxicity in SH-SY5Y cells

The oxidative stress plays a critical role in the etiology and exacerbates the NDDs progression. We applied H_2O_2 to SH-SY5Y cells as an oxidative stress and looked into the neuroprotective effect of H-LIPEF technique. As shown in Fig 2A, administration of H_2O_2 on SH-SY5Y cells for 24 h decreased the cell viability in a dose-dependent manner. Treatment of SH-SY5Y cells with 500 μM H_2O_2 for 24 h reduced the cell viability to 52% compared to the control cells, which was used in the subsequent experiments. To examine whether H-LIPEF could produce protection and increase the cell viability against the oxidative insult, cells were pretreated with H-LIPEF for 4 h and then exposed to H_2O_2 in the continuous administration of H-LIPEF for another 24 h. In our previous study, the low frequency LIPEF with parameters of 2 Hz, 60V/cm,

and 2 ms pulse duration was used in the mouse motor neurons NSC-34 [3], but the result showed that this parameter combination is ineffective in the SH-SY5Y cells. In this study, we used H-LIPEF in our experiments, and found that the H-LIPEF stimulation using the parameters of 200 Hz, 10 V/cm, and 2 ms pulse duration produced significant protection against the oxidative insult caused by H_2O_2 to SH-SY5Y human neural cell line (Fig 2B). To examine whether the frequency and intensity are both critical parameters in the neuroprotective effect, the systematic study on frequency- and amplitude-dependent effects was performed. The intensity of H-LIPEF was firstly fixed at 10 V/cm and the frequency was varied from 2 Hz to 499 Hz. The upper limit was set to 499 Hz since for 2 ms pulse duration, two pulses will overlap for frequency greater or equal to 500 Hz. The results showed that for frequency less than or equal to 100 Hz, their survival rates were not significantly different from the H_2O_2-treated group. At high frequency region, the H-LIPEF conferred protective effect to the H_2O_2-treated SH-SY5Y cells, and the maximum protective effect was at near 200 Hz (Fig 2C). For the intensity-dependent effect, the frequency was fixed at 200 Hz, and the intensity varied from 1~60 V/cm was examined. As shown in Fig 2D, it was obvious that there was a maximum at 10 V/cm, indicating that the best protective effect was at 10 V/cm. Therefore, we conclude that both the frequency and intensity are both critical parameters in the neuroprotective effect, and the optimum protective parameters (200

Hz, 10 V/cm, and 2 ms pulse duration) were used in the following experiments. For AD disease model, it was found that Aβ treatment reduced the viability of SH-SY5Y cells due to the cytotoxicity of the aggregated Aβ (Fig 3A). As shown in Fig 3B and 3C, the H-LIPEF treatment greatly improved the viability and restored the cell morphology of Aβ-treated SH-SY5Y cells, indicating the curative effect of H-LIPEF in AD disease model in vitro.

Fig 2. Effect of H-LIPEF on H_2O_2-induced cytotoxicity in SH-SY5Y cells. (A) Dose-dependent curve of SH-SY5Y cells treated with different concentrations of H_2O_2 and the cell viability was measured by MTT assay at 24 h after the H_2O_2 treatment. (B) SH-SY5Y cells were pretreated using 200 Hz H-LIPEF with field intensity of 10 V/cm and challenged with or without 500 μM H_2O_2. The cell viability was also measured by MTT assay at 24 h after the H_2O_2 treatment. (C) SH-SY5Y cells were pretreated with 10 V/cm PEF at different pulse frequencies and challenged with 500 μM H_2O_2 for 24 h and then the cell viability was measured by MTT assay. (D) SH-SY5Y cells were pretreated using 200 Hz PEF with different field intensities and challenged with 500 μM H_2O_2 for 24 h and then the cell viability was measured by MTT assay. Data represent the mean ± standard deviation (n=3). $^{***}P < 0.001$.

Fig 3. Effect of H-LIPEF on Aβ-induced cytotoxicity in SH-SY5Y cells. (A) The cell viability of SH-SY5Y cells treated with 25 or 50 μM Aβ for 4 days. (B) The SH-SY5Y cells were pretreated with H-LIPEF for 4 h and then exposed to 50 μM Aβ in the continuous administration of H-LIPEF for another 4 days, and the cell viability was measured by MTT assay. (C) Representative light microscopy images of SH-SY5Y cells after Aβ and/or H-LIPEF treatment. The integrity of the cells was destructed by Aβ, and the H-LIPEF treatment caused the protective effect and retained the cell morphology. Scale bar = 100 μm. Data represent the mean ± standard deviation (n=3). $^{***}P < 0.001$.

Effect of H-LIPEF treatment on ROCK protein expression in SH-SY5Y cells

In our previous study, the neuroprotective effect of LIPEF was found to be associated with the inhibition of the H_2O_2-induced activation of ROCK pathway in the motor neuron cell line NSC-34 [3]. In this work, we examined the ROCK protein expressions in order to investigate whether the protective effect of high frequency LIPEF on human neuroblastoma SH-SY5Y cells was related to the ROCK pathway. As shown in Fig 4, the expression of ROCK protein is slightly higher after H_2O_2 treatment but almost

unaffected after the treatment of both H-LIPEF and H_2O_2. This phenomenon points out that although the H-LIPEF treatment can protect SH-SY5Y cells, the main mechanism is not associated with the ROCK pathway. Therefore, other prosurvival protein pathways should be considered in the following experiments.

Fig 4. Effect of H-LIPEF on expressions of ROCK protein in SH-SY5Y cells. (A) Western blot analysis of ROCK protein expressions. (B) Quantification of ROCK expression level after H-LIPEF, H_2O_2, or H-LIPEF+H_2O_2 treatment. The expression levels were normalized to GAPDH and each relative expression level was compared with control. Data represent the mean ± standard deviation (n=3).

Effect of H-LIPEF exposure on ERK-Nrf2/CREB signalling pathways in H_2O_2-treated SH-SY5Y cells

We then studied the signalling pathways that could participate in the neuroprotective mechanism of H-LIPEF stimulation. ERK is one of the major cascades of mitogen-activated protein kinases (MAPK) family and has been known to be widely associated with cell survival [4]. Upon phosphorylation, the active ERK proteins enter into the nucleus where they can phosphorylate certain transcription factors, thus regulating the expression of specific genes that contribute to cell survival [5]. Therefore, in our study,

we examined the phosphorylation of ERK and the downstream transcription factors including cAMP response element binding protein (CREB) and nuclear factor erythroid 2-related factor 2 (Nrf2) by western blot analysis.

As shown in Fig 5A, in comparison to the untreated control cells, the level of phosphorylated ERK (p-ERK) was increased in the cells exposed to H-LIPEF for 24 h. In contrast, the level of p-ERK in the cells was decreased when exposed to 500 μM H_2O_2 for 24 h without the treatment of H-LIPEF. In addition, the result showed that the H-LIPEF treatment induced a significant recovery of the p-ERK level in the cells exposed to 500 μM H_2O_2. This result indicates that the neuroprotection provided by H-LIPEF could be related to the recovery of the H_2O_2-induced decline of p-ERK. In the following, we further examined the expression levels of Nrf2 and p-CREB proteins using western blot analysis. Nrf2 is a key transcription factor that regulates the expression of antioxidant proteins participating in the protection of oxidative damage triggered by injury and inflammation. As shown in Fig 5B, the level of Nrf2 was also increased in H-LIPEF treated SH-SY5Y cells compared to the untreated cells. In contrast, the level of Nrf2 in the cells exposed to 500 μM H_2O_2 for 24 h but without the treatment of H-LIPEF was decreased. Meanwhile, the H-LIPEF treatment was found to recover the expression of Nrf2 in the cells exposed to 500 μM H_2O_2 obviously. This result showed that the neuroprotective effect was due in part to the H-LIPEF induced

Nrf2 expression and the antioxidant effect. Next, CREB is also an important transcription factor triggering the expression of prosurvival proteins. As shown in Fig 5C, in comparison to the untreated control cells, the level of p-CREB was dramatically increased when cells were under the H-LIPEF treatment alone. On the other hand, the level of p-CREB in cells was decreased when exposed to 500 μM H_2O_2 for 24 h without the treatment of H-LIPEF. Moreover, the result found that the H-LIPEF treatment obviously recovered the expression of p-CREB in the cells exposed to 500 μM H_2O_2. Furthermore, it is widely known that CREB phosphorylation regulates Bcl-2 family proteins including anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins, and the ratio of Bcl-2/Bax determines whether neurons undergo survival or death after an apoptotic stimulus. Therefore, the expressions of Bcl-2 and Bax protein levels were also examined in the study. As shown in Fig 5D, for SH-SY5Y cells under the H-LIPEF treatment alone, the expression levels of Bcl-2 and Bax were increased and decreased respectively, leading to a higher elevation of the Bcl-2/Bax ratio compared to the untreated control cells. Meanwhile, in comparison to the untreated control cells, the level of Bcl-2/Bax ratio of SH-SY5Y cells was found to decrease when exposed to 500 μM H_2O_2, indicating that the cells had undergone apoptosis. Moreover, it was observed that the H-LIPEF treatment notably restored the expression of Bcl-2/Bax ratio in the cells exposed to 500 μM H_2O_2. To sum up, the results showed that the H-LIPEF

treatment induced the anti-apoptotic effect by regulating the Bcl-2 family proteins via modulating the ERK/CREB pathways and thus promoted the cell survival against oxidative stress.

Fig 5. Effect of H-LIPEF on ERK/Nrf2 and ERK/CREB signalling pathways and related protein expressions. Western blot analysis of p-ERK (A), Nrf2 (B), p-CREB (C), Bcl-2 and Bax (D) protein expressions. The expression levels were normalized to GAPDH and each relative expression level was compared with control. Data represent the mean ± standard deviation (n=3). $^{***}P < 0.001$ and $^{**}P < 0.01$.

Discussion

The focus of this study was to investigate the neuroprotective effect of H-LIPEF on SH-SY5Y cells. The results demonstrated that treating the cells with high frequency (~200 Hz) and low intensity (~10 V/cm) pulsed electric field produced the most significant protection against H_2O_2- and Aβ-induced apoptosis in SH-SY5Y human neural cells. The western blot results showed that H-LIPEF triggered the ERK signalling pathway and mediated the activation of CREB and Nrf2 transcription factors.

The electric stimulation had long been used in medical applications, such as wound

healing [6], muscle relaxation [7], and increasing the blood circulation [8]. However, these treatments are often involving the electric current passing through the injury site caused by direct contact between body and implanted electrodes. The invasive or contact manner greatly prohibits the feasibility of the treatment or even causes harmful effects to the cells or body such as electrolysis, burns and muscle spasms [9]. Therefore, the electrotherapy with the non-invasive or non-contact manner will be a preferred modality. In our previous study, we had demonstrated a non-contact electric stimulation device [10]. The device operates as a parallel capacitor which generates electric field between two parallel electrodes (Fig 1A). Therefore, the electric field with such capacitive coupling mode can pass through biological samples to achieve specific biological effect without the actual current flowing through the cells or media via contact electrodes. It is worthy to note that the field strength passing through the culture dishes or media is the same as the provided voltage across the electrodes. Besides, the electric field waveform used here is a PEF, which is characterized by its multi-frequency component nature. A pulse train signal is composed of multiple sinusoidal subcomponents with different frequencies and intensities [3]. When the PEF enters the biological samples, it encounters different proteins, cells and tissues with different dielectric constants and is therefore dispersed to multiple frequencies electrically. The multi-frequency sinusoidal waves may interact with molecules, proteins, or organelles

simultaneously to induce specific bioeffect during the PEF exposure. In our previous studies, the non-contact LIPEF had shown promises in enhancing the activity of anticancer agent for pancreatic cancer treatment [11] and in the neuroprotection of mouse motor neurons [3]. In this work, we applied the LIPEF with different parameters to the human neural cells and examined its neuroprotective effect. Unexpectedly, the LIPEF parameter used in the mouse motor neurons NSC-34 is not effective in human SH-SY5Y cells. Actually, scientists had found that the mouse model is quite different from the human individuals [12]. The differences provide explanation for why some drugs or treatments that successfully treat mice do not work in people, which have been hampering the discovery and development of potential drugs or treatments of many diseases. Therefore, finding treatments for animal models or cell types closer to humans will be more important and meaningful. Here, our results showed that for human SH-SY5Y cells, the neuroprotective effect of LIPEF was more pronounced in the high frequency region especially near 200 Hz which was quite different from the parameter at 2 Hz for the NSC-34 mouse cell line. For higher frequency LIPEF, it consists more high frequency components in its composition. Therefore, the result points out that it could be due to such high frequency components participating in the neuroprotective effect of H-LIPEF in human SH-SY5Y neural cells. The results also showed that the therapeutic window of the H-LIPEF intensity for SH-SY5Y cells was near 10 V/cm

which was smaller than the most effective parameter (60 V/cm) for NSC-34 cells. Therefore, one can see that the pulse frequency and the field intensity are critical parameters for therapeutic applications. The suitable parameters (frequency, intensity, and pulse duration) of LIPEF could vary with different types of cells or diseases and need to be fine-tuned and optimized. Furthermore, we propose that one may also combine multiple pulses with different parameters to simultaneously treat the target region.

As for the neuroprotective mechanism, it was found previously that the LIPEF treatment obviously suppressed the H_2O_2-enhanced expression of ROCK protein in NSC-34 cells. However, for SH-SY5Y cells, the H-LIPEF simulation did not have significant impact on ROCK protein expression. It is thus known that the neuroprotective mechanism is not through the ROCK pathway. In the western blot results, the study proved that such neuroprotective effect induced by H-LIPEF was through activation of ERK pathway. The ERK pathway plays an important role in regulating of proliferation and differentiation as well as survival of various cell types [13]. It transmits external cues, including growth factors into signalling cascade via the phosphorylation of ERK. Several studies have suggested that the stimulation of ERK pathway may be a potential way for neuroprotection [4, 5]. Here, the study showed that the physical stimulation by H-LIPEF alone significantly enhanced the expression level

of p-ERK, indicating H-LIPEF could activate the ERK signalling pathway. Since the ERK pathway is initiated by the binding of epidermal growth factor (EGF) to the epidermal growth factor receptor (EGFR), we speculate that H-LIPEF could have an interaction with EGFR and cause the conformation change of EGFR, thus triggering the ERK pathway. Previous study had shown that magnetic field could alter the conformation of TRPV1 ion channel, making it more favorable for capsaicin binding [14]. The results implicate that the PEF or magnetic field with different parameters could activate different signalling pathways. In the study, H-LIPEF with 200 Hz frequency and 10 V/cm field intensity was found to significantly trigger the ERK pathway and produce the neuroprotective effect. Subsequently, the phosphorylation of ERK protein could result in the activation of its kinase activity and lead to phosphorylation of many downstream targets including certain transcription factors containing CREB and Nrf2 required for neuroprotection.

CREB is an important transcription factor activated by phosphorylation from ERK or other kinases. It triggers expression of neurotrophins and antiapoptotic proteins such as brain-derived neurotrophic factor (BDNF) and B-cell lymphoma 2 (Bcl-2) [15]. Besides, CREB downregulation is related to the pathology of AD [16], and thus increasing the CREB expression has been considered as a potential therapeutic strategy for AD. In the study, it was observed that H-LIPEF could increase the level of p-CREB

dramatically. Moreover, the result found that the H-LIPEF treatment notably recovered the expression of p-CREB in the cells exposed to 500 μM H_2O_2. On one hand, it is known that CREB phosphorylation regulates Bcl-2 family proteins including anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins. The antiapoptotic protein, Bcl-2, has been proposed to be a pivotal component for neuroprotection [17]. It suppresses apoptosis by heterodimerizing with Bax protein and neutralizes the effects of Bax. When Bcl-2 is present in excess, cells are protected against apoptosis. It should be also pointed out that when Bax is excessive and the homodimers of Bax dominate, cells are susceptible to programed cell death. Therefore, it is the ratio of Bcl-2 to Bax which determines the fate of a cell [18]. In the results, we found that the H-LIPEF treatment obviously restored the expression of Bcl-2/Bax ratio in the cells exposed to 500 μM H_2O_2, indicating the prosurvival effect of H-LIPEF in SH-SY5Y cells.

Another possible mechanism for neuroprotection of the H-LIPEF stimulation may be associated with the Nrf2 protein. Nrf2 is also an important transcription factor activated by ERK pathway. It regulates the expression of various antioxidant proteins and maintains their redox states in mammalian cells [19]. The results showed that the H-LIPEF treatment obviously recovered the expression of Nrf2 in the cells exposed to 500 μM H_2O_2, indicating that the neuroprotective effect was due in part to the H-LIPEF induced Nrf2 expression and the antioxidant effect.

In the study, the results confirmed the neuroprotective effect of H-LIPEF on SH-SY5Y cells. Given the fact that the electric field can be modified to focus on a targeted region, it is proposed that H-LIPEF treatment could be applied to different organs of the human body. In particular, the non-contact manner and the multi-frequency nature of the H-LIPEF technique will greatly reduce the risk and consuming time of the treatment, respectively. Besides, our results also demonstrated the ability of H-LIPEF to rescue the Aβ-induced cytotoxicity. Since the accumulation of misfolded proteins in brain is also the typical symptom of several NDDs, the study could become an inspiring paradigm for future exploration for optimal LIPEF conditions for the treatment of other NDDs.

In summary, the study demonstrates for the first time that the physical stimulation by H-LIPEF can protect SH-SY5Y human neural cells from H_2O_2- and Aβ-induced cytotoxicity. The underlying molecular mechanism for the neuroprotective effect is partially due to the activation of ERK pathway and the function of some downstream proteins, such as CREB, Nrf2, and Bcl-2. Further studies are necessary to confirm the optimal LIPEF conditions for other NDDs and the involvement of other proteins in the neuroprotective effect. Finally, it is also advisable for combining H-LIPEF with NDD drugs or natural compounds to attain better curative effect for NDD patients.

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Competing Interests

The authors have declared that no competing interests exist.

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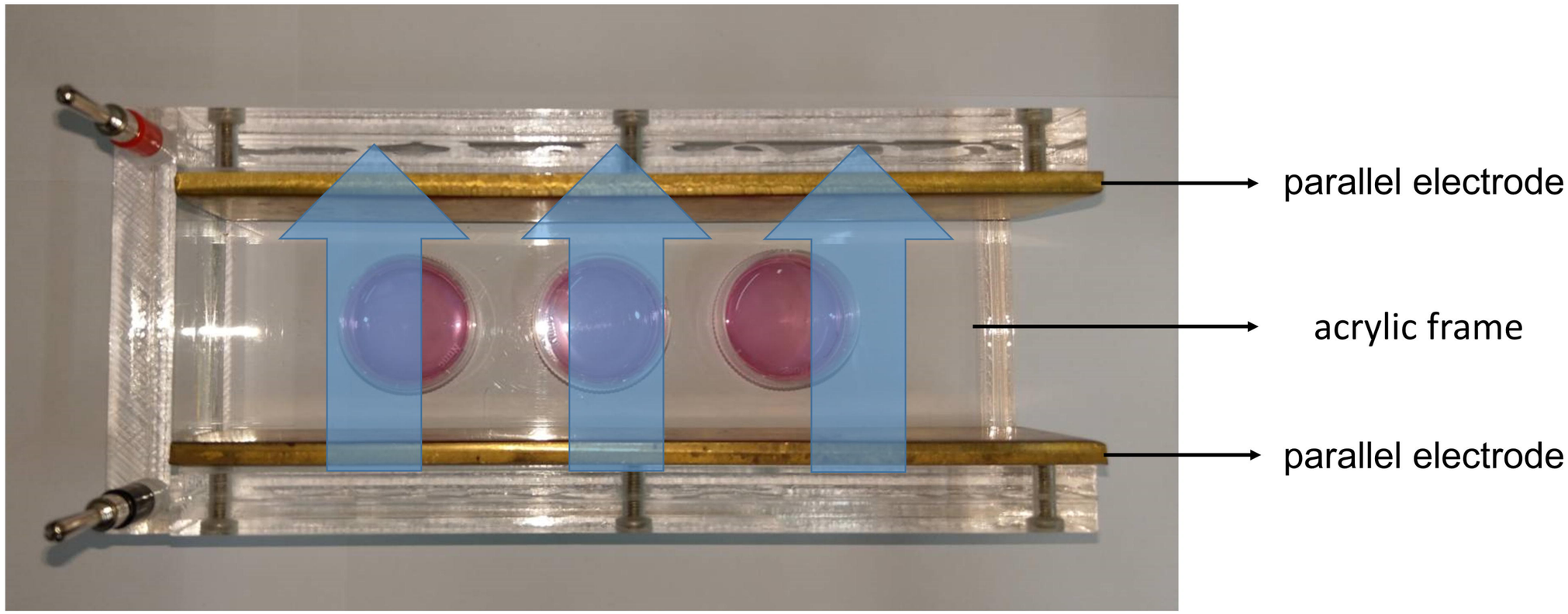
Figure 1

A


parallel electrode
acrylic frame
parallel electrode

B

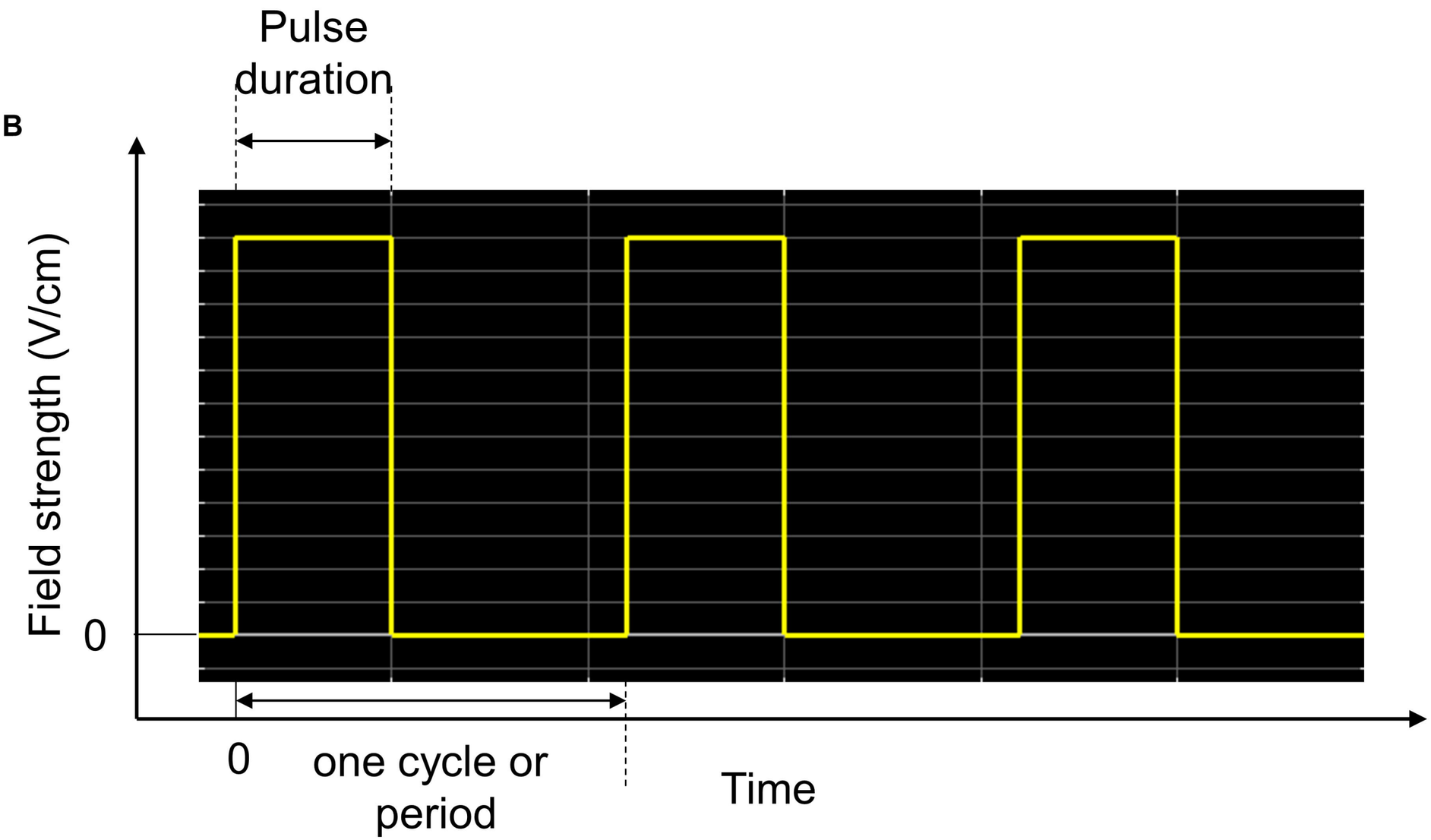

Pulse duration
Field strength (V/cm)
0
0
one cycle or period
Time

Figure 2

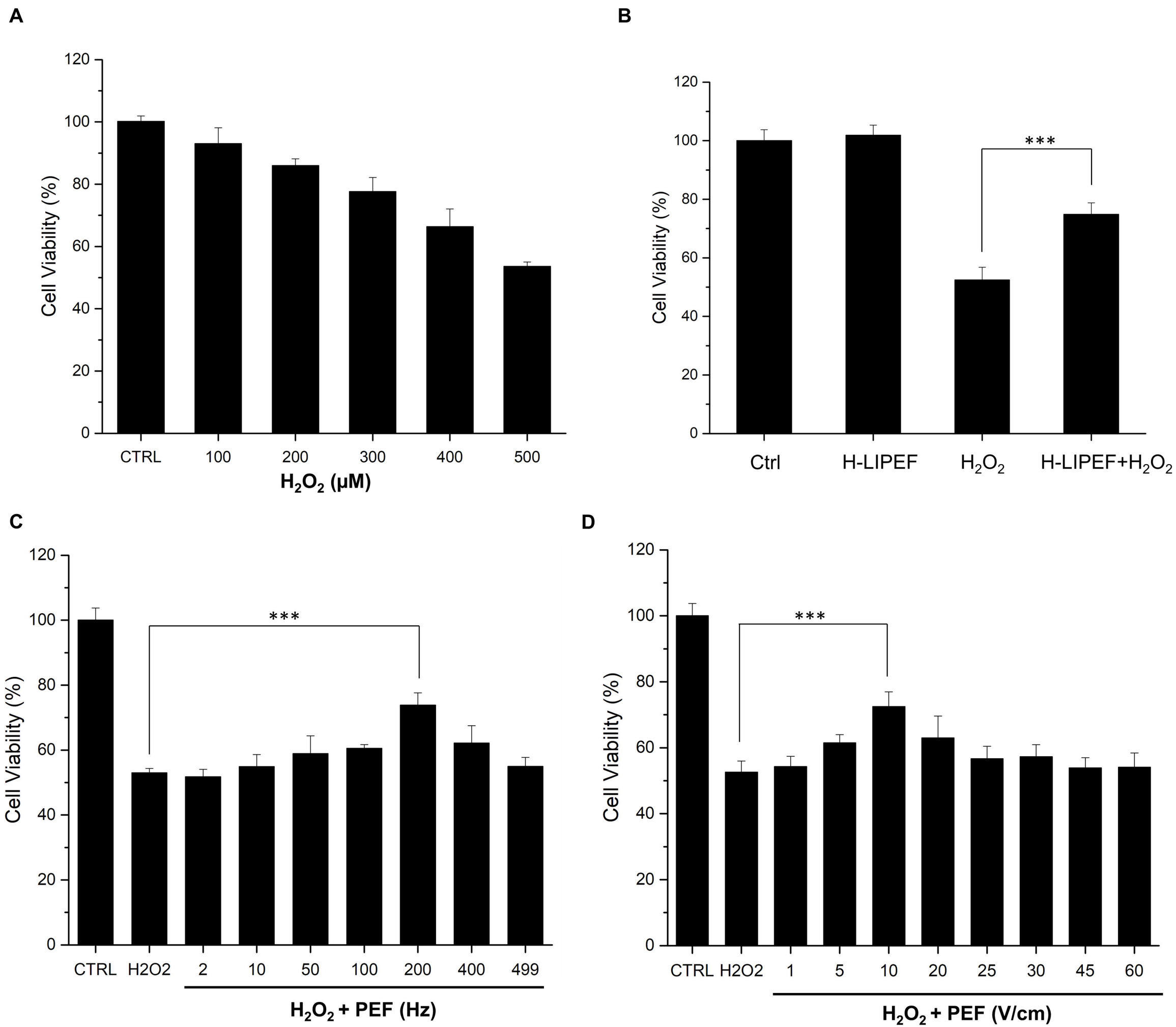

A
Cell Viability (%)
120
100
80
60
40
20
0
CTRL
100
200
300
400
500
H2O2 (μM)
B
Cell Viability (%)
***
Ctrl
H-LIPEF
H2O2
H-LIPEF+H2O2
C
Cell Viability (%)
***
CTRL
H2O2
2
10
50
100
200
400
499
H2O2 + PEF (Hz)
D
Cell Viability (%)
***
CTRL
H2O2
1
5
10
20
25
30
45
60
H2O2 + PEF (V/cm)

Figure 3

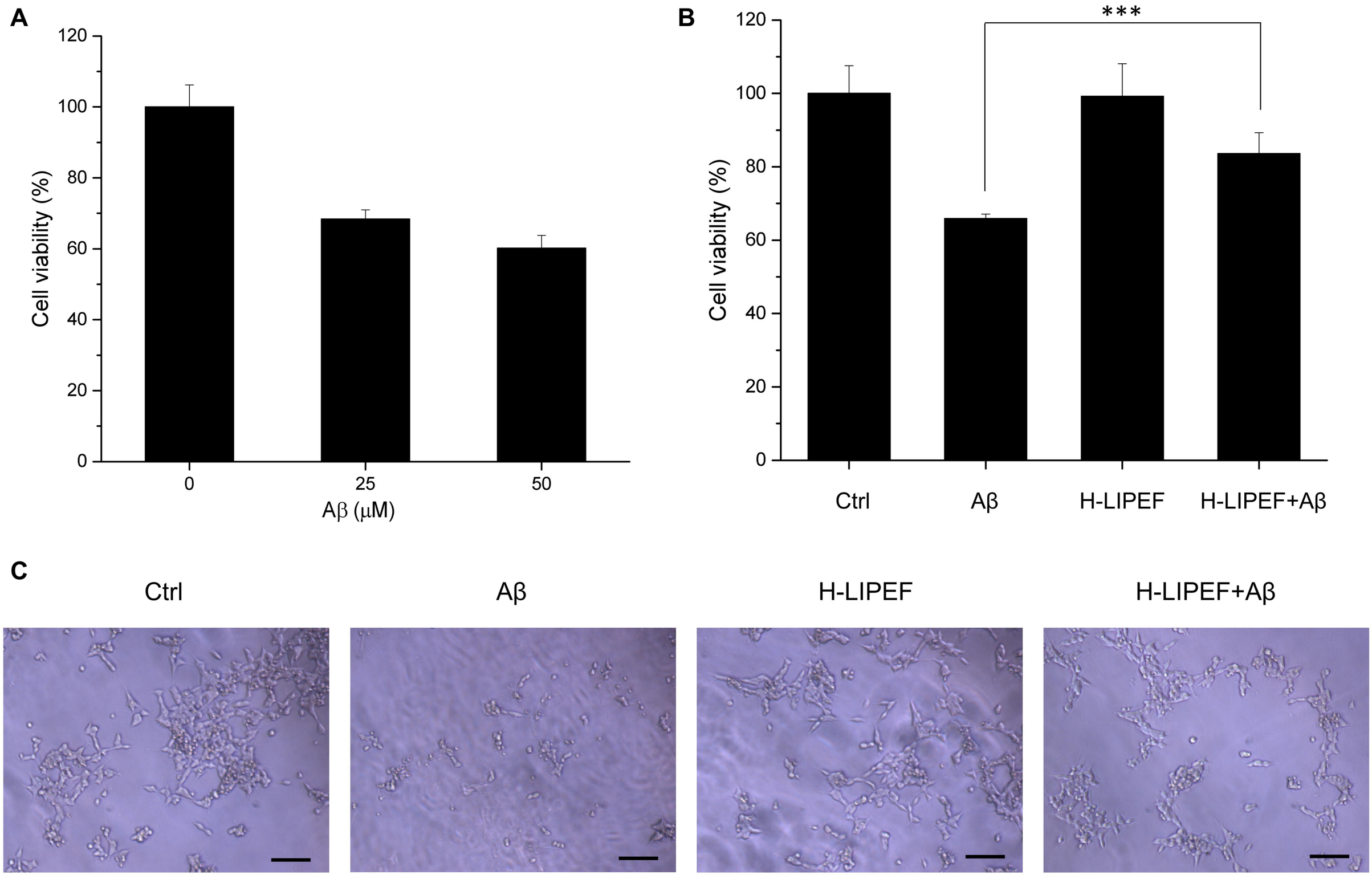

A
Cell viability (%)
120
100
80
60
40
20
0
0
25
50
Aβ (μM)
B
***
Cell viability (%)
120
100
80
60
40
20
0
Ctrl
Aβ
H-LIPEF
H-LIPEF+Aβ
C
Ctrl
Aβ
H-LIPEF
H-LIPEF+Aβ

Figure 4

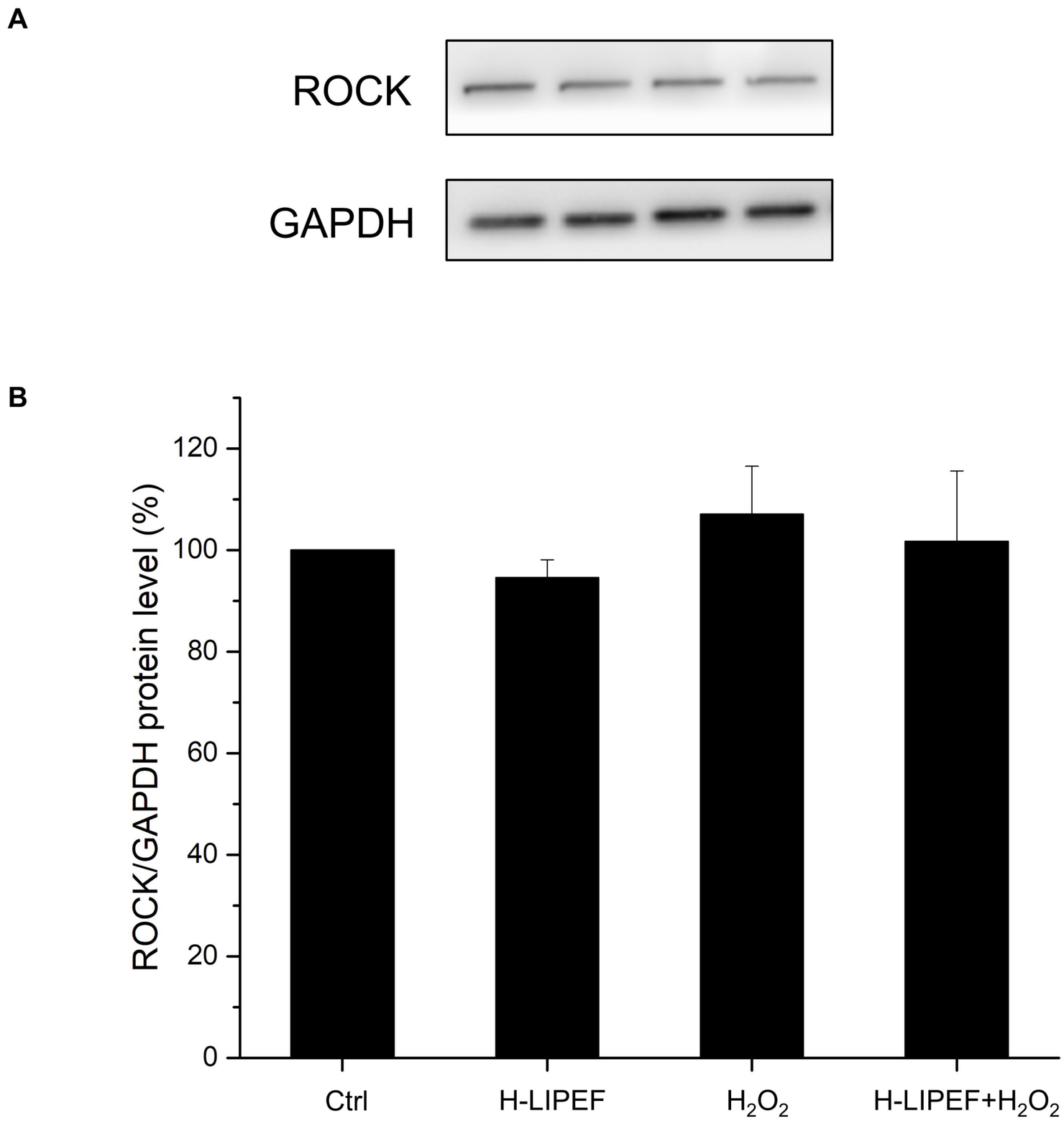
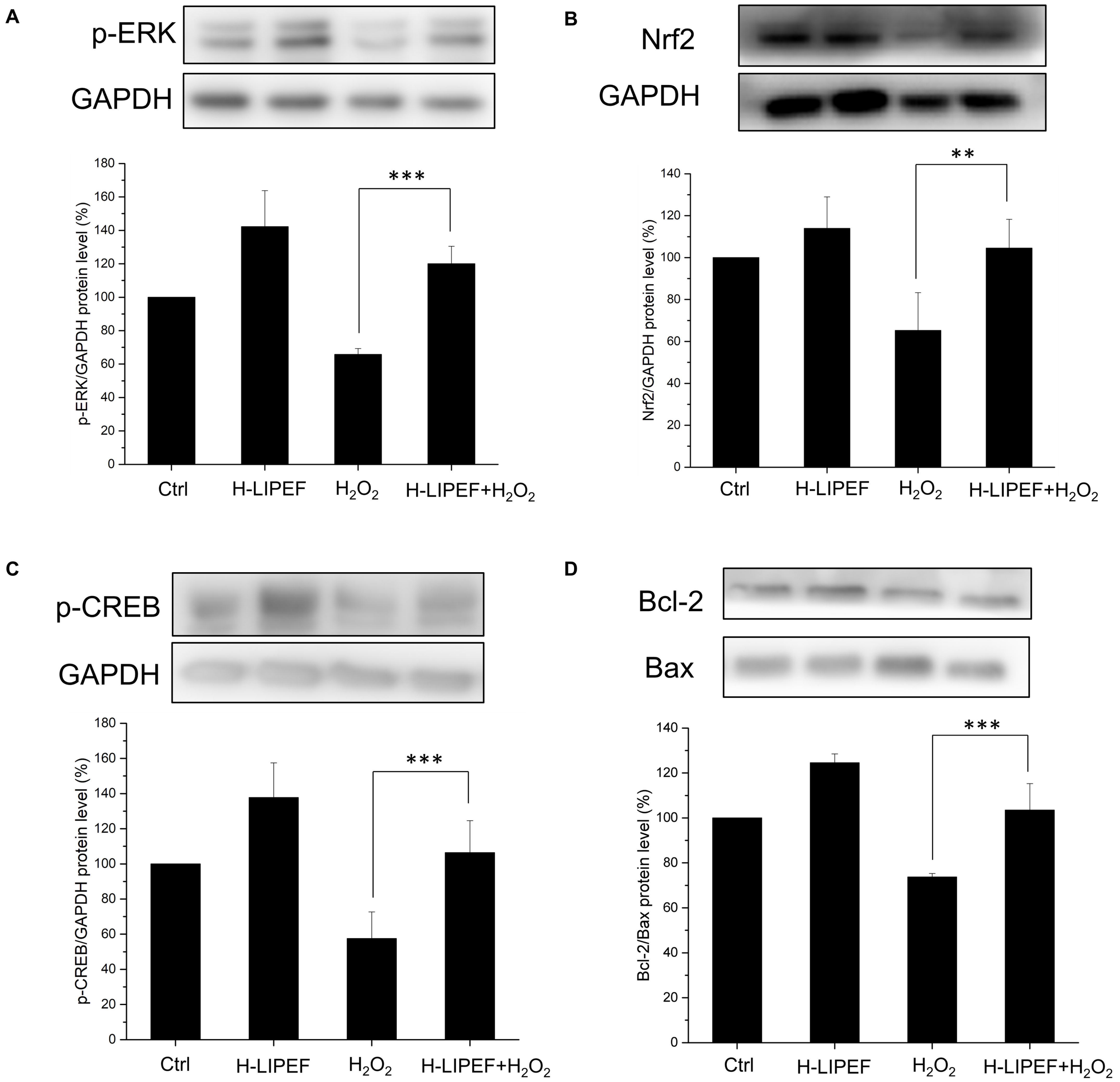

A
ROCK
GAPDH
B
ROCK/GAPDH protein level (%)
0
20
40
60
80
100
120
Ctrl
H-LIPEF
H_2O_2
H-LIPEF+H_2O_2

Figure 5


A
p-ERK
GAPDH
p-ERK/GAPDH protein level (%)
0
20
40
60
80
100
120
140
160
180
***
Ctrl
H-LIPEF
H2O2
H-LIPEF+H2O2
B
Nrf2
GAPDH
Nrf2/GAPDH protein level (%)
0
20
40
60
80
100
120
140
**
Ctrl
H-LIPEF
H2O2
H-LIPEF+H2O2
C
p-CREB
GAPDH
p-CREB/GAPDH protein level (%)
0
20
40
60
80
100
120
140
160
180
***
Ctrl
H-LIPEF
H2O2
H-LIPEF+H2O2
D
Bcl-2
Bax
Bcl-2/Bax protein level (%)
0
20
40
60
80
100
120
140
***
Ctrl
H-LIPEF
H2O2
H-LIPEF+H2O2