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1 # Protection of high-frequency low-intensity pulsed
2 electric fields and brain-derived neurotrophic factor for
3 SH-SY5Y cells against hydrogen peroxide-induced cell
4 damage

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7 Guan-Bo Lin [a,b], Wei-Ting Chen [a,b], Yu-Yi Kuo [a,b], You-Ming Chen [b,c],
8 Hsu-Hsiang Liu [b,c], and Chih-Yu Chao [a,b,c,*]

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10 [a] *Department of Physics, Lab for Medical Physics & Biomedical Engineering,*
11 *National Taiwan University, Taipei 10617, Taiwan*
12 [b] *Molecular Imaging Center, National Taiwan University College of Medicine, Taipei*
13 *10051, Taiwan*
14 [c] *Graduate Institute of Applied Physics, Biophysics Division, National Taiwan*
15 *University, Taipei 10617, Taiwan*

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17

18 *Abbreviations:* H-LIPEF, High-frequency low-intensity pulsed electric field; BDNF,
19 Brain-derived neurotrophic factor; NDDs, neurodegenerative diseases; ROS, Reactive
20 oxygen species; p-EGFR, phosphorylated epidermal growth factor receptor.

21 * Corresponding author.

22 *E-mail address*: cychao@phys.ntu.edu.tw (C.Y. Chao).

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24 # ABSTRACT

25 Neurodegenerative diseases have been a major threat to public health worldwide
26 nowadays. In particular, Alzheimer's disease is the most common type causing
27 dementia which has remained an incurable disease, despite improvement of the
28 symptoms via some therapies. The pathogenic mechanism of Alzheimer's disease is
29 deemed to be related to the aggregation of beta-amyloid peptide (Aβ), a major cause
30 for amyloid plaques, resulting in neuronal death via the generation of reactive oxygen
31 species (ROS). Scientists have regarded oxidative stress as a potential treatment target
32 in the development of novel remedies. In the study, human neural cell line SH-SY5Y
33 was used in probing the effect of combining non-invasive high-frequency
34 low-intensity pulsed electric field (H-LIPEF) and brain-derived neurotrophic factor
35 (BDNF) in protection against hydrogen peroxide (H_2O_2)-induced neuron damage. Our
36 result finds that the combination approach has intensified the neuroprotective effect
37 significantly, perhaps due to H-LIPEF and BDNF synergistically increasing the
38 expression level of the phosphorylated epidermal growth factor receptor (p-EGFR),
39 which induces the survival-related mitogen-activated protein kinases (MAPK)
40 proteins. The study confirmed the activation of extracellular signal-regulated kinase
41 (ERK), a member of MAPK family, and the downstream pro-survival and antioxidant
42 proteins as the mechanism underlying neuron protection. These findings have
43 highlighted the potential of H-LIPEF combining with BDNF in the treatment of
44 neurodegenerative diseases. Furthermore, the application of BDNF-mimetic drugs and
45 non-invasive H-LIPEF to patients is a promising approach worthy of further research,
46 and this points to strategies and mechanisms for selecting drugs or compounds to
47 cooperate with electric fields in the treatment of neurodegenerative disorders.

48

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49 # 1. Introduction

50 In line with the aging of global population, neurodegenerative diseases (NDDs)
51 have emerged as a mainstream medical issue, not only causing discomfort and even
52 death for patients but also disrupting the life of their families. Among these brain
53 diseases, Alzheimer's disease is the most common type and a major cause of dementia
54 in aged people. Alzheimer's pathogenic mechanism is believed to be related to the
55 aggregation of β-amyloid (Aβ), which leads to amyloid plaques, causing neuronal
56 death via the generation of reactive oxygen species (ROS) [1]. Another well-known
57 hypothesis is phosphorylation of tau protein, which forms neurofibrillary tangles in
58 nerve cell, damaging nerve cells and leading to progressive brain degeneration and
59 deterioration of cognitive function [2]. Although Alzheimer's disease has been
60 discovered for many years, there has yet to be an effective treatment, except a few
61 therapies to relieve its symptoms. Hence, there has been a global scramble for the
62 development of effective Alzheimer's disease therapy.

63 Neurotrophins are potential therapeutic agents for treating neurodegenerative
64 disorder of the central nervous system. Among these neurotrophins, brain-derived
65 neurotrophic factor (BDNF) is the most abundant neurotrophin in the human brain,
66 which has a remarkable capability to repair brain damage. With proven effect in
67 promoting neuronal survival, synaptic plasticity and neurogenesis, BDNF can resist
68 brain damage caused by oxidative inflammation-related factors [3]. Some studies
69 have found that BDNF produces neuroprotective effects against ROS-induced cell
70 death, even with the potential for treating NDDs [4-6]. However, it should be noted
71 that reduction in BDNF concentration can cause symptoms such as forgetfulness and
72 poor learning ability, and many studies have blamed insufficient BDNF contents for
73 depression and even Alzheimer's disease [7-9]. In addition, there has been evidence

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74 suggesting that Aβ can inhibit the proteolytic conversion of pro-BDNF to BDNF,
75 decreasing BDNF level [10]. Therefore, stimulation of BDNF secretion or use of
76 exogenous BDNF is a promising approach in the treatment of Alzheimer's disease.
77 Many studies have proposed treatments capable of increasing the production of
78 endogenous BDNF, thereby achieving nerves protection [11-13]. Meanwhile, the use
79 of exogenous BDNF is also a direct and effective approach in dealing with the
80 abnormal secretion of endogenous BDNF caused by the disease, as some studies have
81 confirmed the significant neuroprotection effect of exogenous BDNF [14-16].
82 However, a major problem is the short half-life of BDNF, which increases the
83 difficulty of many therapeutic applications [17]. Therefore, how to improve BDNF
84 efficiency will be an important issue.

85 In addition to traditional drug medication, physical therapy has gradually
86 emerged as a mainstream approach in many diseases, such as electric current
87 stimulation with proven value in treating muscle injury, wound, cancer, and neuronal
88 diseases [18-21]. Many studies have pointed out that external electric stimulation can
89 stimulate proteins to change their structure, affect their distribution, and even
90 influence relevant biochemical pathways [22-24]. On the other hand, it is worth
91 noting that epidermal growth factor receptor (EGFR) is a transmembrane protein
92 deemed to be relevant to cell survival pathways. Especially, EGFR signalling
93 abnormalities are believed to be associated with certain diseases, such as
94 atherosclerosis, Parkinson's disease, and Alzheimer's disease [25,26]. Furthermore, it
95 has been reported that the conventional electric current stimulation can affect cell
96 migration via EGFR and enhance the expressions of downstream survival-related
97 proteins, such as mitogen-activated protein kinases (MAPK) and phosphatidylinositol
98 3-kinase (PI3K) protein families in some medical fields [27].

99 To date, some issues remain unsolved for the application of electric current

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100 stimulation in many diseases. As the electric current stimulations are applied via
101 contact or even invasive method, electric current would pass through tissues directly,
102 causing the Joule effect [28]. It is known that invasive treatments are at higher risk
103 and difficult in execution for some tissues, especially brain. Therefore, the
104 development of non-invasive and non-contact electrotherapy is an inevitable trend.
105 Recently, our team has developed a unique non-invasive and non-contact
106 low-intensity pulsed electric field (LIPEF) stimulation for biomedical application. The
107 pulsed electric field (PEF) can be considered as a composition of multiple sinusoidal
108 subcomponents with different frequencies and intensities, which may interact with
109 molecules, proteins, or organelles to induce specific bioeffect at the same time.
110 Consequently, PEF stimulation can facilitate significantly the development of new
111 therapies to meet different needs of different diseases, with great potential for
112 applications in various medical fields. First, our team has demonstrated, for the first
113 time, the capability of LIPEF to combine with epigallocatechin-3-gallate (EGCG) in
114 inhibiting the pancreatic cancer cell line PANC-1 [29]. Second, low-dose curcumin,
115 along with LIPEF, also has good anticancer effect, underscoring LIPEF's capability to
116 work with various chemical molecules [30]. Third, ultrasound can be added to the
117 combination treatment, further boosting the anticancer effect [31]. Moreover, our
118 team has found another effective anticancer triple therapy, combining LIPEF,
119 chlorogenic acid (CGA), and thermal cycling-hyperthermia technique [32]. Besides,
120 LIPEF can join hands with fucoidan to modulate rho-associated protein kinase
121 (ROCK) and the downstream Akt protein, thereby protecting motor neurons NSC-34
122 against damage by oxidative stress [33]. On the other hand, in human neuronal
123 SH-SY5Y cells under hydrogen peroxide (H_2O_2) stress, we found that high-frequency
124 LIPEF (H-LIPEF) can modulate and activate the extracellular signal-regulated kinase
125 (ERK) pathway, thereby protecting neuronal cells against oxidative stress [34]. In

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126 sum, in addition to safety, LIPEF (or H-LIPEF) alone or in combination with
127 chemical molecules and/or other physical stimuli has significant potential for a wide
128 range of applications.
129 In this paper, our team first combined the features of BDNF and H-LIPEF
130 stimulation to study the effect of combination treatment on neuroprotection against
131 the oxidative insult caused by H_2O_2 to SH-SY5Y human neural cells. The result
132 showed that the combination of BDNF and H-LIPEF significantly inhibited the
133 H_2O_2-induced cytotoxicity in SH-SY5Y cells. Examination of the underlying
134 mechanism also indicated that the addition of BDNF is beneficial via regulation of
135 ERK, a member of MAPK family proteins, a possible reason for the enhanced
136 neuroprotective effect of combination BDNF and H-LIPEF treatment. Moreover, the
137 combination treatment was found to significantly upregulate the expression of
138 phosphorylated EGFR (p-EGFR) protein. These findings suggest that the combination
139 of neurotrophic-related chemical molecules and H-LIPEF promises to be an effective
140 therapy for neurodegenerative diseases.
141

142 2. Materials and methods

143 2.1 *Experimental setup for exposure of the cells to non-contact H-LIPEF*

144 The H-LIPEF stimulation device described in our previous study [34] was used
145 for exposure of the SY-SY5Y cells to electric field. In brief, the cells were placed
146 between two copper flat and parallel electrodes. The pulsed electric signal was
147 produced from a function generator and amplified by a voltage amplifier. In the study,
148 consecutive pulses with the electric field 10 V/cm, frequency 200 Hz, and pulse
149 duration 2 ms were applied across the electrodes for the treatment. Cells treated with
150 continuous exposure of non-contact H-LIPEF were kept in a humidified incubator of

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151 5% CO_2 and 95% air at 37℃.

152

153 2.2 *Cell culture*

154 The SH-SY5Y cells were purchased from American Type Culture Collection
155 (ATCC, Manassas, VA, USA) and cultured in MEM/F-12 mixture (HyClone; Cytiva,
156 Marlborough, MA, USA) supplemented with 10% fetal bovine serum (FBS)
157 (HyClone; Cytiva), 100 units/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.,
158 Waltham, MA, USA), 100 μg/ml streptomycin
159 (Gibco; Thermo Fisher Scientific, Inc.), 1 mM sodium pyruvate (Sigma-Aldrich;
160 Merck KGaA, Darmstadt, Germany), and 0.1 mM non-essential amino acids
161 (Gibco; Thermo Fisher Scientific, Inc.). Cells were maintained in a humidified
162 incubator composed of 5% CO_2 and 95% air at 37℃, and harvested with 0.05%
163 trypsin-0.5 mM EDTA solution (Gibco; Thermo Fisher Scientific, Inc.) for in vitro
164 experiments.

165

166 2.3 *H-LIPEF and BDNF treatment*

167 BDNF (Alomone Labs, Jerusalem, Israel) was dissolved in distilled water as a
168 stock solution and stored at -20℃. SH-SY5Y cells were pretreated with various
169 concentrations of BDNF and then subjected to H-LIPEF treatment for 4 h. Subsequently,
170 cells were challenged with H_2O_2 and put back again to the H-LIPEF stimulation device
171 for further treatment.

172

173 *2.4 Cell viability assay*

174 Viability of SH-SY5Y cells after treatments with or without H-LIPEF was

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175 assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
176 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) assay. Briefly, the medium was
177 replaced with MTT solution (0.5 mg/mL in SH-SY5Y culture medium) and incubated
178 at 37℃ for 4 h. During this period, the ability of mitochondrial dehydrogenases to
179 reduce the MTT to formazan crystal is an indicator of cellular viability. Following
180 MTT reaction, formazan dissolution was performed by using 10% sodium dodecyl
181 sulfate (SDS) in 0.01M HCl. The optical density of each well was then determined at
182 570 nm subtracting the background at 690 nm using Multiskan GO spectrophotometer
183 (Thermo Scientific, Hudson, NH, USA). The cell viability was calculated based on
184 the intensity of formazan, and expressed as percentage of the non-treated control.
185

186 2.5 *Western blot analysis*

187 After the treatment of H_2O_2, cells were washed with phosphate buffered saline
188 (PBS) and then lysed on ice for 30 m in lysis buffer (EMD Millipore, Billerica, MA,
189 USA) containing fresh protease and phosphatase inhibitor cocktail (EMD Millipore).
190 The lysates were centrifuged at 23,000 × g for 1 h at 4℃, and the supernatant was
191 collected and used for the determination of protein concentration. Equal amounts of
192 protein extracts were loaded in the 10% SDS-PAGE wells and transferred to
193 polyvinylidene difluoride (PVDF) membranes (EMD Millipore). The blots were
194 incubated overnight with primary antibodies at 4℃, followed by incubation with
195 horseradish peroxidase-coupled secondary antibodies (Jackson ImmunoResearch
196 Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 h. In this study,
197 the specific primary antibodies against p-EGFR, phosphorylated ERK (p-ERK),
198 nuclear factor erythroid 2-related factor 2 (Nrf2), phosphorylated cAMP response
199 element binding protein (p-CREB), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X
200 protein (Bax) (Cell Signaling Technology, Inc., Danvers, MA, USA), and GAPDH

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201 (GeneTex, Inc., Irvine, CA, USA) were used. All the antibodies were diluted at the
202 optimal concentration according to the manufacturer's instructions. Finally, protein
203 bands were visualized with an enhanced chemiluminescence substrate (Advansta, Inc.,
204 Menlo Park, CA, USA) and detected with the Amersham Imager 600 imaging system
205 (GE Healthcare Life Sciences, Chicago, IL, USA). The images were analyzed with
206 Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For
207 normalization of proteins, GAPDH was used as an internal control.
208
209 *2.6 ROS level detection*
210 After the treatments, the SH-SY5Y cells were collected and washed with PBS.
211 The fluorescent dye dihydroethidium (DHE) (Sigma-Aldrich; Merck KGaA) was used
212 to detect the intracellular ROS levels. Cells were resuspended in PBS and incubated
213 with 5 μM DHE dye for 20 min at 37°C in the dark. The fluorescence intensity was
214 measured by FACSCanto II system (BD Biosciences, San Jose, CA, USA) in the PE
215 channel. The ROS levels were expressed as mean fluorescence intensity for
216 comparison.
217
218 *2.7 Statistical analysis*
219 The results were presented as mean ± standard deviation and performed in
220 triplicate. Differences of statistical significance were determined by a one-way
221 analysis of variance (ANOVA), followed by Tukey's post-hoc test. P-value < 0.05
222 was considered to indicate a statistically significant difference. Analyses were carried
223 out using OriginPro 2015 software (OriginLab).
224

225 # 3. Results

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226 *3.1 In vitro-applied H-LIPEF*

227 To avoid the harmful effects caused by conduction current and Joule effect, the
228 culture dishes were placed in the non-invasive H-LIPEF device as described
229 previously [34]. The H-LIPEF stimulation was performed in a non-contact manner
230 with the apparatus consisting of two parallel conductive electrodes mounted on the
231 insulating acrylic frame (Fig. 1A). Therefore, the H-LIPEF device can provide the
232 electric field stimulation in a non-invasive manner, so that the cells were not in
233 contact with the electrodes directly. The pulsed electric signal was from a function
234 generator and amplified by a voltage amplifier that made pulsed electric signal
235 tunable. The applied electric signal was characterized by a pulse train waveform (Fig.
236 1B) with various field strengths and frequencies for different applications. In this
237 study, we used the electric field strength of 10 V/cm at 200 Hz, which was adopted in
238 our previous study to be the optimal H-LIPEF condition for neuroprotection on
239 SH-SY5Y cell line [34].

240

241 **Fig. 1. The experimental setup of H-LIPEF.** (A) Image of the H-LIPEF stimulation
242 device. The SH-SY5Y cells were placed in the H-LIPEF stimulation device
243 constructed of two parallel electrodes. The green arrows shown here indicate the
244 schematic representation of non-invasive and non-contact H-LIPEF exposure on cells.
245 (B) Schematic representation of the pulse train waveform applied across the
246 electrodes.

247

248 *3.2 Effect of the H-LIPEF and BDNF on H_2O_2-induced cytotoxicity in SH-SY5Y cells*

249 Because oxidative stress plays a key role in the etiology of neurodegenerative
250 diseases, reducing oxidative stress is often a top priority for neuroprotection. As

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251 shown in Fig. 2A, administration of H_2O_2 on SH-SY5Y cells for 24 h decreased the
252 cell viability in a concentration-dependent manner. Treatment of SH-SY5Y cells with
253 500 μM H_2O_2 for 24 h reduced cell viability to about 50% compared to the untreated
254 control group. In order to understand whether the H-LIPEF combined with BDNF
255 could attenuate the H_2O_2-induced neuron cell damage, we first applied various
256 concentrations of BDNF (0 to 100 ng/ml) in SH-SY5Y cells and see their
257 neuroprotection effect. As shown in Fig. 2B, we found a slight protective effect of
258 these concentrations of BDNF against H_2O_2-induced cell oxidation. On the other hand,
259 in the combination treatment, SH-SY5Y cells were pretreated with H-LIPEF and
260 BDNF for 4 h, and then exposed to 500 μM H_2O_2 in the continuous administration of
261 H-LIPEF for another 24 h. In present work, we applied the most effective H-LIPEF
262 parameters (200 Hz, 10 V/cm, and 2 ms pulse duration) previously used by our team
263 for neuroprotection study [34] to human neural cells SH-SY5Y. As shown in Fig. 2C,
264 the cell viability decreased to about half of the control group after treating SH-SY5Y
265 cells with 500 μM H_2O_2 for 24 h. When cells were treated by combination treatment
266 with H-LIPEF and BDNF, the cell viability was significantly rescued. Under
267 H-LIPEF stimulation, administration of 25 ng/ml and 50 ng/ml BDNF on SH-SY5Y
268 cells increased cell viability to approximately 80% and 90%, respectively. Our results
269 showed that H-LIPEF stimulation demonstrated a positive neuroprotective, while
270 administration of BDNF alone at low doses had a marginally protective effect on
271 SH-SY5Y cells. Noteworthily, the neuroprotective effect was further enhanced by the
272 H-LIPEF and BDNF combination treatment. Our goal is to achieve a better
273 therapeutic effect via H-LIPEF stimulation combined with a lower dosage of drug.
274 Therefore, based on these data, the H-LIPEF (200 Hz, 10 V/cm, and 2 ms pulse
275 duration) parameters in combination with the concentration of 25 ng/ml BDNF were
276 chosen to be used in all the following experiments.

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277

278 **Fig. 2. Effect of H-LIPEF and/or BDNF on H_2O_2-induced cytotoxicity in**
279 **SH-SY5Y cells.** (A) The concentration response of SH-SY5Y cells treated with
280 different concentrations of H_2O_2 and the cell viability was measured by MTT assay at
281 24 h after the H_2O_2 treatment. (B) SH-SY5Y cells were pretreated with different
282 concentrations of BDNF for 4 h and challenged with 500 μM H_2O_2 for 24 h and then
283 the cell viability was measured by MTT assay. (C) SH-SY5Y cells administered by 25
284 and 50 ng/ml BDNF were subsequently treated with or without H-LIPEF for 4 h and
285 then challenged with 500 μM H_2O_2, respectively. The cell viability was also measured
286 by MTT assay at 24 h after the H_2O_2 treatment. Data represent the mean ± standard
287 deviation (n=3). The concentration response curve of SH-SY5Y cells was statistically
288 analyzed using one-way ANOVA with Tukey's post hoc test. $^{**}P < 0.01$, $^{***}P <$
289 0.001.

290

291 *3.3 Combination of H-LIPEF and BDNF attenuates H_2O_2-induced ROS generation*

292 To the best of our knowledge, the damage of ROS accumulation to nerves is
293 known to be the main cause of many brain diseases, such as Alzheimer's disease and
294 Parkinson's disease. In view of this, we studied the effect of combination treatment
295 with H-LIPEF and BDNF on the regulation of ROS level by flow cytometry
296 measurements (Fig. 3A). As shown in Fig. 3B, the quantification results show that the
297 ROS level was increased to about 200% of the control level after exposure to 500 μM
298 H_2O_2 for 24 h. Compared to the H_2O_2 alone group, H-LIPEF treatment significantly
299 ameliorated the H_2O_2-induced increase in ROS level, while with BDNF involved, the
300 combination of H-LIPEF and BDNF further enhanced the effect. This result indicates
301 that the combination treatment (H-LIPEF+BDNF) is more effective in reducing the
302 oxidative stress of SH-SY5Y cells.

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303

304 **Fig. 3. Effect of H-LIPEF alone or in combination with BDNF on H_2O_2-induced**
305 **ROS generation in SH-SY5Y cells.** (A) ROS level was measured by flow cytometry
306 with DHE fluorescent dye. (B) Quantification of the ROS levels after H_2O_2,
307 H-LIPEF+H_2O_2, and H-LIPEF+BDNF+H_2O_2 treatment. Data represent the mean ±
308 standard deviation (n=4). Comparisons of the effect of H_2O_2, H-LIPEF+H_2O_2, and
309 H-LIPEF+BDNF+H_2O_2 on the ROS generation were analyzed using one-way
310 ANOVA with Tukey's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$.

311

312 *3.4 Role of the EGFR pathways in the neuroprotective effect of the H-LIPEF and*
313 *BDNF*

314 BDNF is an important neurotrophin that regulates the survival of existing
315 neurons and promotes the growth and differentiation of new neurons. It has been
316 previously reported that BDNF can activate EGFR proteins and cause intracellular
317 signalling pathways [35]. Consequently, to further identify whether the protective
318 effect of the H-LIPEF and BDNF on SH-SY5Y cells was related to the EGFR
319 pathway, we examined the protein expression of p-EGFR on human neuroblastoma
320 SH-SY5Y cells. As shown in Fig. 4, when SH-SY5Y cells were treated with H_2O_2
321 alone, the expression of p-EGFR was slightly decreased. In contrast, the H-LIPEF
322 treatment was found to induce a significant recovery of p-EGFR protein level in the
323 cells under H_2O_2 oxidative stress. Meanwhile, with the participation of BDNF, the
324 result finds that the combination treatment of BDNF and H-LIPEF can further
325 increase the expression level of p-EGFR protein significantly. The study suggests that
326 H-LIPEF could have an interaction with EGFR, stimulating the initiation of EGFR
327 function, or making EGFR itself more susceptible to phosphorylation. Besides, the
328 binding of BDNF to EGFR would further enhance the neuroprotective effect induced

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329 by H-LIPEF on H_2O_2-treated SH-SY5Y cells. Subsequently, we studied the
330 downstream signalling pathways that could participate in the neuroprotective
331 mechanism of H-LIPEF and BDNF.
332
333 **Fig. 4. Effect of H-LIPEF alone or in combination with BDNF on expressions of**
334 **p-EGFR protein in SH-SY5Y cells.** (A) Western blot analysis of p-EGFR protein
335 expressions. (B) Quantification of p-EGFR protein expression level after H_2O_2,
336 H-LIPEF+H_2O_2, and H-LIPEF+BDNF+H_2O_2 treatment. H_2O_2 and BDNF
337 concentrations in treatments were 500 μM and 25 ng/ml, respectively. GAPDH was
338 used as the loading control, and the relative expression level of each group was
339 compared with the untreated control group. Data represent the mean ± standard
340 deviation (n=3). Comparisons of the effect of H_2O_2, H-LIPEF+H_2O_2, and
341 H-LIPEF+BDNF+H_2O_2 on p-EGFR protein levels were analyzed using one-way
342 ANOVA with Tukey's post hoc test. **P < 0.01, ***P < 0.001.
343
344 *3.5 Activation of the ERK pathway in the neuroprotective effect of the H-LIPEF and*
345 *BDNF*
346 It is well known that EGFR phosphorylation regulates the expression of its
347 downstream proteins such as MAPK family. ERK protein is one of the MAPK family
348 and has been known to be widely associated with cell survival. Upon phosphorylation,
349 activated ERK proteins enter the nucleus, where they can phosphorylate certain
350 transcription factors, thereby regulating the expression of specific genes that
351 contribute to cell survival [36]. As shown in Fig. 5, in comparison to the untreated
352 control cells, the expression level of p-ERK protein was decreased when cells were
353 exposed to 500 μM H_2O_2. Notably, the level of p-ERK was significantly restored in
354 the cells treated with H-LIPEF stimulation. Furthermore, the result found that p-ERK

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355 expression was further elevated significantly when SH-SY5Y cells were co-treated by
356 the BDNF and H-LIPEF combination treatment. Therefore, the study indicates that
357 the neuroprotection effect produced by the combination of BDNF and H-LIPEF could
358 be due to the enhanced activation of ERK pathway, thus inducing an even greater
359 recovery of p-ERK level in the cells under H_2O_2 oxidative stress.
360

361 **Fig. 5. Effect of H-LIPEF alone or in combination with BDNF on expressions of**
362 **p-ERK protein in SH-SY5Y cells.** (A) Western blot analysis of p-ERK protein
363 expressions. (B) Quantification of p-ERK protein expression level after H_2O_2,
364 H-LIPEF+H_2O_2, and H-LIPEF+BDNF+H_2O_2 treatment. H_2O_2 and BDNF
365 concentrations in treatments were 500 μM and 25 ng/ml, respectively. GAPDH was
366 used as the loading control, and relative expression level of each group was compared
367 with the untreated control group. Data represent the mean ± standard deviation (n=3).
368 Comparisons of the effect of H_2O_2, H-LIPEF+ H_2O_2, and H-LIPEF+BDNF+H_2O_2 on
369 p-ERK protein levels were analyzed using one-way ANOVA with Tukey's post hoc
370 test. ***$P < 0.001$.
371

372 *3.6 Effect of combination of H-LIPEF and BDNF on CREB, Nrf2, Bcl-2, and Bax in*
373 *H_2O_2-treated SH-SY5Y cells*

374 Our previous research pointed out that the protective effect of H-LIPEF on
375 SH-SY5Y cells was through activation of ERK pathway [34]. In present study, we
376 examined the effect of the H-LIPEF combined with BDNF on SH-SY5Y cells under
377 H_2O_2 oxidative stress. The ERK downstream proteins should be measured to
378 determine whether the combined treatment of H-LIPEF and BDNF can further
379 enhance the ERK signalling pathway. In the following, we further examined the
380 expression levels of p-CREB, Nrf2, Bcl-2, and Bax proteins using western blot

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381 analysis. As a transcription factor that regulates the expression of pro-survival
382 proteins, CREB plays an important role in neuronal survival [37]. As shown in Fig.
383 6A, the protein expression of p-CREB in SH-SY5Y cells was decreased when cells
384 were exposed to 500 μM H_2O_2. Meanwhile, we found that the expression of p-CREB
385 was restored significantly in SH-SY5Y cells treated with H-LIPEF stimulation. In
386 addition, the combination of BDNF and H-LIPEF was found to further enhance the
387 expression of p-CREB in H_2O_2-treated cells significantly. Next, Nrf2 is a key
388 transcription factor that regulates the expression of antioxidant proteins participating
389 in the protection of oxidative damage triggered by injury and inflammation [38]. As
390 shown in Fig. 6B, the level of Nrf2 was also decreased in H_2O_2-treated SH-SY5Y
391 cells compared to the untreated cells. In contrast with the cells exposed to 500 μM
392 H_2O_2, the expression level of Nrf2 in the cells treated with H-LIPEF was restored
393 significantly. Moreover, the result found that the combined treatment of BDNF and
394 H-LIPEF further increased the expression of Nrf2 in H_2O_2-treated cells significantly.
395 Furthermore, it is known that both phosphorylated CREB and Nrf2 proteins can
396 regulate the expression of Bcl-2 family proteins [39,40], and the Bcl-2/Bax ratio
397 determines whether the neurons undergo survival or death after being stimulated [33,
398 34,41]. Therefore, the expressions of Bcl-2 and Bax proteins were also measured in
399 this study. As shown in Fig. 6C, in comparison to the untreated control cells, the level
400 of Bcl-2/Bax ratio of SH-SY5Y cells was found to decrease when cells were exposed
401 to 500 μM H_2O_2. On the other hand, for SH-SY5Y cells treated with the H-LIPEF, the
402 expression levels of Bcl-2 and Bax were increased and decreased respectively, leading
403 to a higher elevation of the Bcl-2/Bax ratio compared to the H_2O_2-treated cells.
404 Moreover, it was observed that the combined treatment of BDNF and H-LIPEF
405 further enhanced the ratio of Bcl-2/Bax significantly in the cells under H_2O_2 oxidative
406 stress. The above data indicate that the combination treatment not only can greatly

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407 increase the expressions of the phosphorylated CREB and Nrf2 proteins, but also can
408 remarkably enhance the ratio of Bcl-2/Bax for better neuroprotection effect, compared
409 to the single H-LIPEF treatment. Consequently, the result suggests that the
410 combination of BDNF and H-LIPEF synergistically promotes the cell survival against
411 H_2O_2 oxidative stress via ERK signalling pathway.
412
413 **Fig. 6. Effect of H-LIPEF alone or in combination with BDNF on**
414 **survival-related proteins in SH-SY5Y cells.** Western blot analysis of p-CREB (A),
415 Nrf2 (B), Bcl-2 and Bax (C) protein expressions. H_2O_2 and BDNF concentrations in
416 treatments were 500 μM and 25 ng/ml, respectively. The expression levels of p-CREB
417 and Nrf2 were normalized to GAPDH and each relative expression level was
418 compared with control. Data represent the mean ± standard deviation (n=4).
419 Comparisons of the effect of H_2O_2, H-LIPEF+H_2O_2, and H-LIPEF+BDNF+H_2O_2 on
420 different protein levels were analyzed using one-way ANOVA with Tukey's post hoc
421 test. $^{**}P < 0.01$, $^{***}P < 0.001$.
422

423 # 4. Discussion

424 The study aimed to investigate the effect of combining BDNF and H-LIPEF in
425 protecting SH-SY5Y neural cells against oxidative stress. In the study, BDNF was
426 combined with the non-invasive and non-contact H-LIPEF with 200 Hz frequency
427 and 10 V/cm intensity, enhancing the survival rate of SH-SY5Y cells under oxidative
428 stress significantly. As one of the most abundant proteins in human brain, BDNF's
429 importance cannot be overstated. Many studies have pointed out that low-level of
430 BDNF can cause cognitive impairment in neurodegenerative diseases, such as
431 Alzheimer's disease, which has affected an increasing number of people worldwide

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432 [42-44]. Some studies on low endogenous BDNF expression caused by injury or
433 disease have found that employment of exogenous BDNF is effective in protecting
434 neuron cells or brain tissues [14,15,45]. In the study, with H_2O_2 as oxidative stress in
435 a cellular model, MTT data show that the combination treatment of H-LIPEF and
436 BDNF significantly increases the viability of SH-SY5Y cells under H_2O_2 stress, plus
437 significant improvement in stress-induced apoptosis and suppression of elevated ROS
438 level caused by H_2O_2.

439 As for the neuroprotective mechanism, our previous study showed that the
440 non-invasive H-LIPEF stimulation, within a specific range of electric field intensity
441 and frequency, can effectively increase the survival rate of SH-SY5Y cells under
442 H_2O_2 stress to achieve neuroprotection effect [34]. The main mechanism is attributed
443 to the ability of electric field to increase the expression of survival-related proteins
444 such as p-ERK and p-CREB, and thus improve the viability of SH-SY5Y cells. In this
445 work, we used H-LIPEF together with BDNF drug to protect SH-SY5Y cells against
446 H_2O_2-induced cell injury. This study employed the combination treatment using the
447 electric field with the optimal intensity and frequency identified in our previous study
448 [34] and tried to pinpoint more upstream sources of survival signals in SH-SY5Y cells
449 for neuroprotection.

450 In recent years, people have gradually come to know the importance of EGFR
451 protein in the nervous system, as some studies have found the correlation between
452 neurodegeneration and reduced EGFR expression [25]. Increased activation of EGFR
453 may be conducive to neuroprotection. It is known that EGFR has to be bound with a
454 specific ligand such as epidermal growth factor (EGF) for activation and enhancement
455 of the expression of its downstream MAPK family proteins [46]. Moreover, recent
456 literature has found that EGFR can also be triggered by BDNF to activate its
457 downstream signalling pathways [35]. In addition, it has been reported that electric

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458 current stimulation alone could trigger a ligand-independent activation of EGFR and
459 even cell migration [47]. Since activation of EGFR is thought to be associated with
460 cell survival, this study aims to investigate whether the combination of BDNF and
461 H-LIPEF treatment can further enhance the activation of EGFR signalling pathway
462 synergistically to intensify the ability of SH-SY5Y cells to cope with oxidative stress.

463 While most electrical stimulation applications called for direct contact of
464 electrode plate with tissues and cells, the study employed the non-invasive and
465 non-contact H-LIPEF to stimulate SH-SY5Y cells, demonstrating that H-LIPEF alone
466 can significantly enhance p-EGFR protein expression in SH-SY5Y cells under H_2O_2
467 oxidative stress (Fig. 4). Moreover, a further significant increase in the expression of
468 p-EGFR was observed in the presence of H-LIPEF in combination with BDNF. As
469 shown in Fig. 5, the data also demonstrated that H-LIPEF combined with BDNF
470 significantly boosting the expression of p-ERK, on top of the modulation of other
471 proteins, such as Nrf2 and p-CREB. It is known that Nrf2 is a cellular transcription
472 factor that regulates the expression of various antioxidant proteins. The combination
473 H-LIPEF and BDNF treatment can effectively activate Nrf2 protein to protect neurons
474 via the antioxidant pathways. The other protein, CREB, is also a transcription factor
475 activated by the phosphorylation from ERK, which can trigger an increase in Bcl-2
476 protein expression. Experimental results also show that the combination of H-LIPEF
477 and BDNF can have a greater effect in raising the Bcl-2/Bax ratio and exerting a
478 pro-survival effect on SH-SY5Y cells.

479 Recently, the importance of EGFR in neuroprotection has been on the rise, which
480 may be a noteworthy trend for the development of neurodegenerative drugs [25].
481 Besides, the study demonstrates the potential of combining H-LIPEF and BDNF in
482 activating EGFR and related downstream survival proteins, leading to higher survival,
483 which is an approach that may augment drug efficacy and reduce drug dosage. This

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484 suggests that the non-invasive H-LIPEF treatment can synergize with BDNF drug to
485 achieve desirable effect at low drug dosage, minimizing side effects. On the other
486 hand, it should be mentioned that conventional electric stimulation was conducted
487 mainly via contact or invasive manner using direct currents, limiting its applications
488 significantly, especially in some organs, such as brain. Therefore, it is believed that
489 the development of non-contact, non-invasive electric field therapy will be inevitable,
490 which can also be further applied in conjunction with drugs for various therapeutic
491 purposes. Previously, our team has achieved synergistic inhibitory effects on cancer
492 cells by combining such electric fields with herbal extracts such as EGCG, CGA, and
493 curcumin, and has also accomplished satisfactory results in repairing mouse motor
494 neuron cell NSC-34, demonstrating the versatility of non-contact, non-invasive
495 electric field stimulations [29-33]. In application, the electric field's strength and
496 frequency would vary among different cellular tissues, depending on accompanying
497 drugs, tissue structure, and protein response, which needs further research.
498

499 ## 5. Conclusions

500 The study demonstrates that H-LIPEF can work together with BDNF, creating a
501 synergy effect in protecting human neuronal cells SH-SY5Y against antioxidant stress.
502 The underlying mechanism is believed to involve activation of EGFR and ERK
503 pathway to regulate ROS level, thereby enhancing cell survival. The study also
504 suggests further research on the combination of H-LIPEF and BDNF-mimetic drugs
505 for treating neurodegenerative brain diseases effectively.
506

507 ## Declaration of Competing Interest

508 The authors declare no conflict of interest present in this study.

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515

516 ## CRediT authorship contribution statement

517 **Chih-Yu Chao:** Conceptualization, Data Curation, Formal analysis, Funding
518 acquisition, Investigation, Supervision, Writing – original draft, Writing – review &
519 editing. **Guan-Bo Lin:** Data Curation, Formal analysis, Investigation, Validation,
520 Writing – original draft. **Wei-Ting Chen:** Formal analysis, Investigation. **Yu-Yi Kuo:**
521 Formal analysis. **You-Ming Chen:** Formal analysis. **Hsu-Hsiang Liu:** Formal
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523

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528

529 ## References

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669

670

Figure 1

A

parallel electrode

acrylic frame

parallel electrode

B

Pulse duration

Field strength (V/cm)

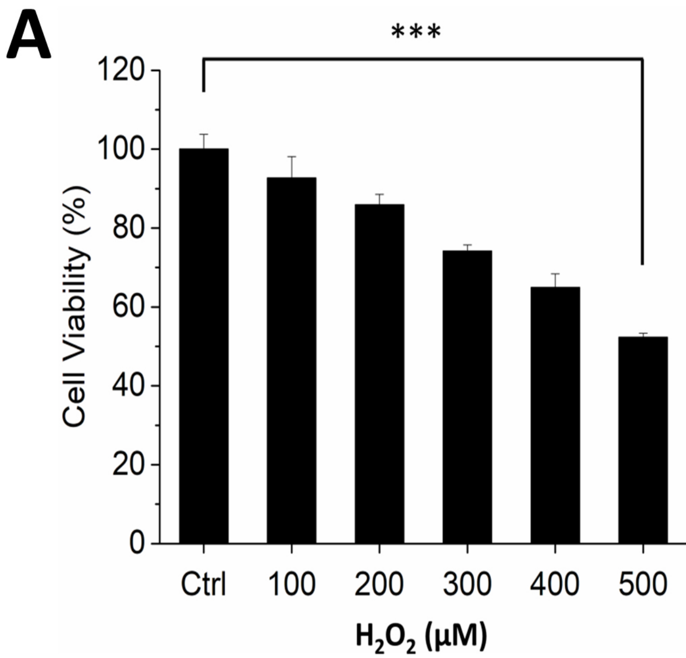
0

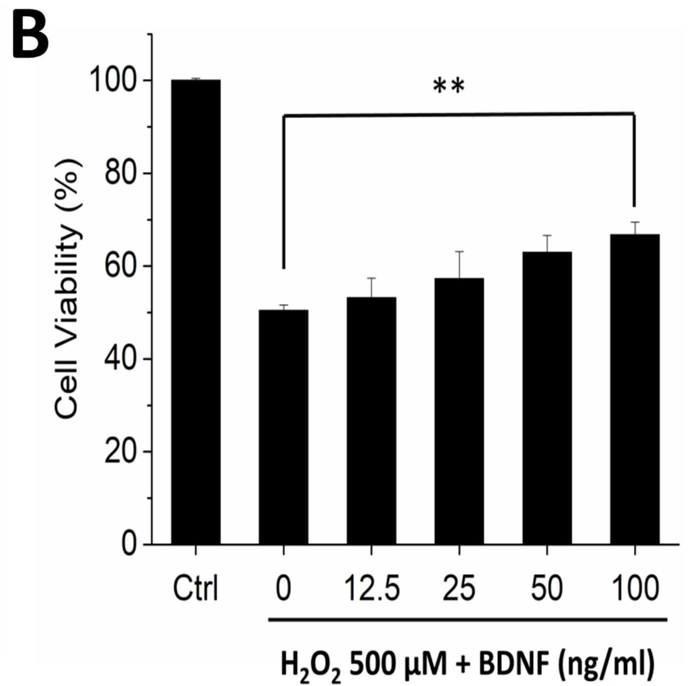
One period

0

Time

Figure 2





C

Ctrl

H-LIPEF

***

**

**

Cell Viability (%)

100

50

0

Ctrl

0

25

50

H_2O_2 500 μM + BDNF (ng/ml)

Figure 3

A

Ctrl

H_2O_2

H-LIPEF+H_2O_2

H-LIPEF+BDNF+H_2O_2

Cell numbers

Fluorescence intensity of DHE

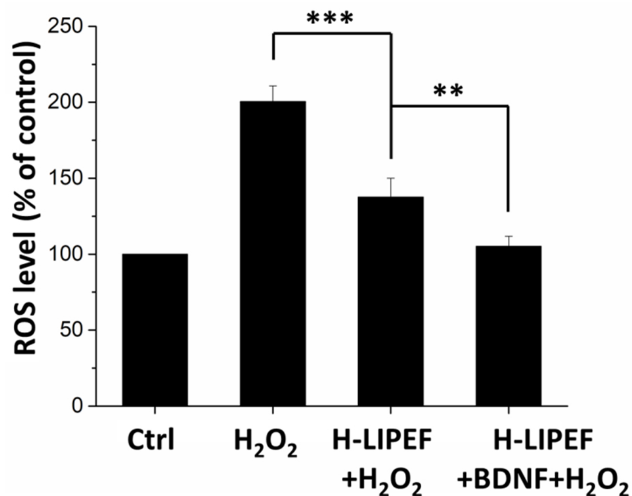
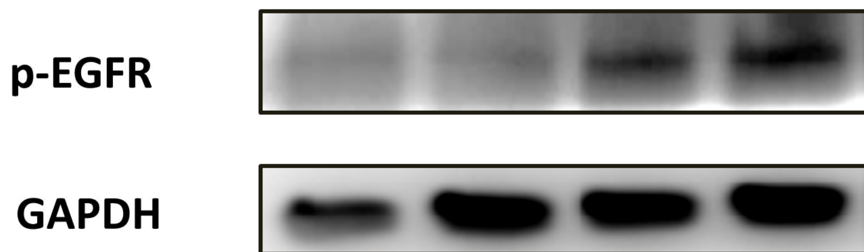


Figure 4

A

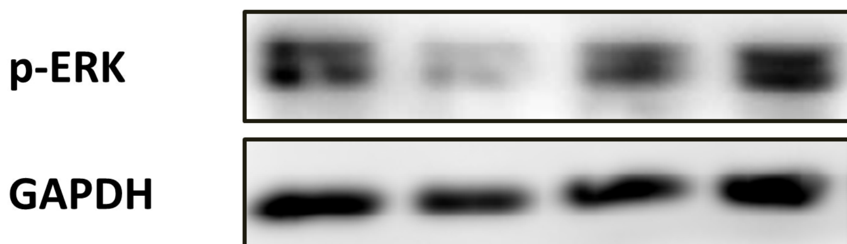


B

**
***
180
160
140
120
100
80
60
40
20
0
p-EGFR/GAPDH protein level (%)
Ctrl
H_2O_2
H-LIPEF+H_2O_2
H-LIPEF+BDNF +H_2O_2

Figure 5

A

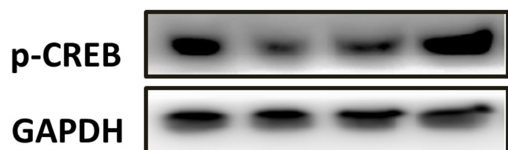


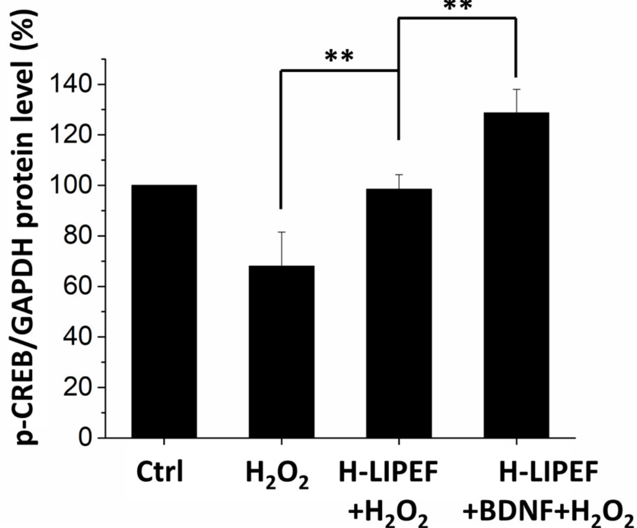
B

***
***
p-ERK/GAPDH protein level (%)
0
20
40
60
80
100
120
140
Ctrl
H_2O_2
H-LIPEF+H_2O_2
H-LIPEF+BDNF +H_2O_2

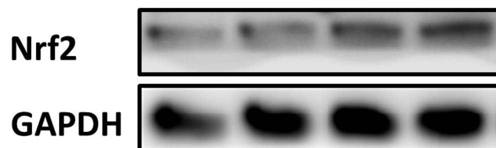
Figure 6

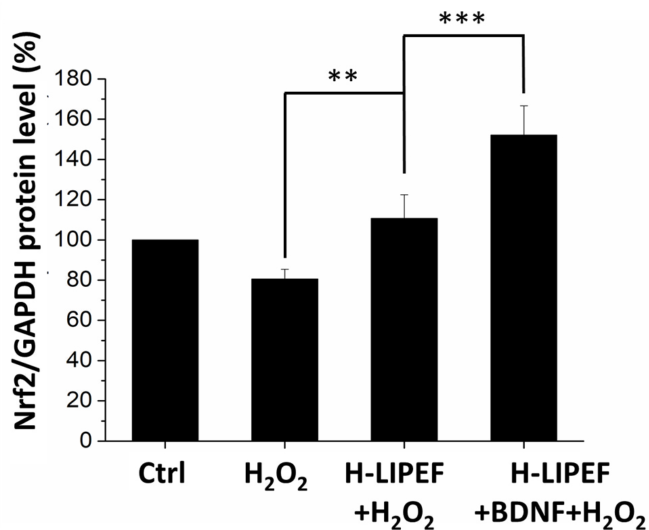
A





B





C