The Effects of Bio-inspired Electromagnetic Fields on Normal and Cancer Cells

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Abstract

The electromagnetic field (EMF) is one of the many environmental factors, which earth creatures are exposed to. There are many reports on the effects of EMF on living organisms. However, since the mechanism has not yet been fully understood, the biological effects of EMF are still controversial. In order to explore the effects of bio-inspired EMF (BIEMF) on normal and cancer cells, various cultured cells have been exposed to BIEMF of different directions, *i.e.* vertical, parallel and inclined. Significantly reduced ATP production in Hela and A549 cancer cells is found for the parallel and vertical BIEMF. More careful examination on Hela cells has revealed a cell density dependent inhibition on colony formation. The morphological observation of BIEMF-exposed Hela cells has suggested that the retarded cell proliferation is probably caused by cell death *via* apoptosis. Together these results may afford new insights for cancer prevention and treatment.

Keywords: electromagnetic fields (EMF), bio-inspired electromagnetic fields (BIEMF), directionality, cancer, cell proliferation.

Introduction

Human and other creatures have adapted to the living environment on earth, which consists of air, water, temperature as well as the geomagnetic field. Yet the impact of the geomagnetic field is often neglected. Fortunately, more attention has been paid to the biological effects of the geomagnetic field and other forms of electromagnetic field (EMF) over the recent decades[1–6]. However, due to the unclear mechanism, there are many experimental results in bioelectromagnetics that are mutually conflicting without reasonable explanation[7–10]. Another reason is that there are limited scientific evidences to support the selection of the EMF signals used in the study.

Although the directionality is a crucial feature for a vector field like EMF, not until recent years have the bioelectromagnetic researchers begun to investigate the influence of the EMF direction on the bioelectromagnetic effects[11– 13]. Naarala *et al.* have reported that the relative orientation between static and extremely low frequency magnetic fields has an effect on cell proliferation and superoxide production[11]. Tian *et al.* have demonstrated that constant magnetic fields in different directions yield distinct effects on cell growth rates, depending on cell types[12]. Milovanovich *et al.* have shown that varying the direction of

homogeneous static magnetic fields causes physiological changes in various organs of mice[13]. However, the magnetic fields used in these works are of high intensity, far beyond the geomagnetic intensity in the range of 35 μT – 70 μT. The directionality effect of static or extremely low frequency magnetic fields at low intensity remains elusive. Hence, this work is proposed to examine the directionality effect of low-intensity EMF comparable to geomagnetic field (*e.g.* 35 μT – 70 μT).

The use of EMF and electric field in cancer treatment has also gained increasing interest recently, with a large body of literature showing that certain kinds of EMF or electric field hold the potential to be applied in cancer therapy[14–26]. Zimmerman *et al*. have identified tumor-specific amplitude-modulated EMF in the radiofrequency range and shown that such an EMF can inhibit cancer cell growth by modifying gene expression and interfering with mitosis[14–17]. After long-term studies, Kirson *et al*. have demonstrated that low-intensity electric fields of intermediate-frequency can depress cancer cell proliferation *in vitro* and *in vivo* by preventing cell mitosis[18–20]. This tumor-treating field is also able to prevent the metastasis of primary tumor and improve chemotherapy efficacy and sensitivity without increasing treatment-related toxicity[21,22]. Interestingly, both EMF and pulsed electric fields of 0.5 Hz can retard cancer in animals[27,28]. Although the reason for such a coincidence is still unclear, it is known that cancer cells have different subcellular structures than normal cells[26]. Hence, differences exist in the inherent vibrating or pulsation frequencies of cancer and normal cells[26]. It is postulated that 0.5 Hz is a frequency within the coherent zones of cancer cells but not for normal cells[25]. Small doses of EMF or electric field treatment operated at such a frequency may yield cancer-specific inhibition without side effects on normal tissue. However, studies on the role of such a bio-inspired EMF (BIEMF) in the development, progression, and treatment of cancer are still rare.

In this work, we have simulated a BIEMF at an extremely low frequency of 0.5 Hz. We name it as BIEMF for two reasons. First, the EMF intensity used in our experiments is in the range of 35 μT – 70 μT, consistent with the geomagnetic field[29]. Second, it is believed that the ultra-low frequency (as low as 0.5 Hz) is close to the inherent vibrating frequency of some cancer cells, whereas the normal cells' pulsation frequency is much higher than those of cancer cells[25]. Then, the intracellular ATP levels of various cells lines exposed to BIEMF of different directions have been measured to investigate the directionality effect of BIEMF. Subsequently, the influence of cell density on the inhibition of cancer cell proliferation by BIEMF has been examined. The possible pathway for the observed cell death has also been tentatively postulated. The results of these studies may afford new insights to the application of EMF in cancer prevention and treatment.

Materials and methods BIEMF setup and characterization

An EMF emitter, Magnafield (Model MF2200, MAGNACREUK LTD, Birmingham, United Kingdom), was chosen to provide an EMF that simulates the geomagnetic field for the cell culture experiments. The working mode of the emitter was designed as repeated cycles of 20-minute on and 20-minute off. The frequency of the BIEMF during the on-cycle is 0.5 Hz (Fig. S1). The Magnafield is mounted on the bottom steel plate in the incubator and the cells are placed on the shelves above. A three-axis magnetic field sensor (Mag690, Bartington Instrument Limited, Oxford, England) was used to detect the magnetic field intensity in the incubator and cell culture room. Five test points with intensity in the range of 35 μT – 70 μT were selected for BIEMF exposure. The setup of BIEMF exposure on cells is as shown in Fig. 1, and the details of the EMF intensity and direction at each test point are provided in Table 1. The cell culture room is surrounded by metal walls, so the external magnetic flux is shielded. The background EMF in the incubator without the emitter can be neglected, since it is less than 1 μT (Fig. S2).

Cell culture

Human cervical adenocarcinoma cell Hela (obtained from Guoqing Li, Jilin University), human bladder cancer cell EJ (obtained from Guoqing Li, Jilin University) and human intestine epithelial cell HIEC (purchased from Bei Na Culture Collection, China.) were cultured in RPMI 1640 medium containing 10% FBS and 1% Penicillin-Streptomycin. Human lung carcinoma cell A549 (obtained from Ranji Cui, The Second Hospital of Jilin University) was cultured in DMEM containing 10% FBS and 1% Penicillin-Streptomycin. Mouse fibroblast NIH/3T3 (purchased from Nanjing Beiruiji Biotechnology, China) was cultured in DMEM containing 10% NBS and 1% Penicillin-Streptomycin. Human lung fibroblast MRC-5 (purchased from Nanjing Beiruiji Biotechnology, China) was cultured in DMEM containing 10% FBS, 1% Penicillin-Streptomycin, 1% NEAA and 1 mM Nap. The culture medium was refreshed every 3 days. All cells were seeded in culture dishes of 3.5 cm in diameter for experiments. The cells for passage and control groups were placed in a humidified incubator (MCO-170AIVL-PC, Panasonic Healthcare, Japan) without BIEMF at 37 ˚C with 5% CO_2. The BIEMF-exposed cells were placed in another humidified incubator of same model but with BIEMF. Both BIEMF- exposed and unexposed cells were under the same humidified culture condition, *i.e*. 37 ˚C with 5% CO_2.

ATP measurement

Approximately 2×10^5 cells were seeded in each dish and placed in BIEMF 24 h later. After 48 h BIEMF exposure, the cellular ATP level was determined by the ATP Assay Kit (Beyotime, China) according to the manufacturer protocol. The assay is based on the luminescence produced when ATP is used in the catalysis of luciferin by luciferase. Briefly, cells were completely lysed by 200 μL ATP detection lysis buffer, and centrifuged at 12000 g at 4 ˚C for 5

min. The supernatant was collected for subsequent detection. Then, 100 µL ATP detection solution was added to each detection reaction, and was kept at room temperature for 5 min so that the background ATP was completely consumed. Finally, 20 µL sample or standard solution was added to the reaction and quickly mixed with a micropipette. A luminometer (BK-L96C-II, Beijing Zhongsheng Biake Scientific Instrument Technology, China) was used to detect the auto-luminescence of the samples.

Cell colony formation assay

Crystal violet staining solution (Beyotime, China) was used to stain Hela cell colonies according to the manufacturer protocol. Crystal violet can bind to DNA in the nucleus and dye the nucleus as dark purple. First, culture media were removed from the 3.5 cm culture dishes and cells were fixed with 4% paraformaldehyde for 15 min. Subsequently, the dishes were washed twice with distilled water and cells were stained with

Table 1 BIEMF characterization at five test points

Test point	$B\|_{MAX}$ (µT)	$B_{X)MAX}$ (µT)	$B_{Y)MAX}$ (µT)	$B_{Z)MAX}$ (µT)	Contribution by axis		
					X	*Y*	*Z*
V_L	39.5632	1	2	39.25	0.00064	0.00256	0.99681
V_H	64.2611	1.8	2.75	62.5	0.00079	0.00191	0.99730
I	65.8882	45	3.5	48	0.46646	0.00282	0.53072
P_L	35.0143	24	25	5	0.46982	0.50979	0.02039
P_H	59.3389	40	43	6	0.45911	0.53056	0.01033

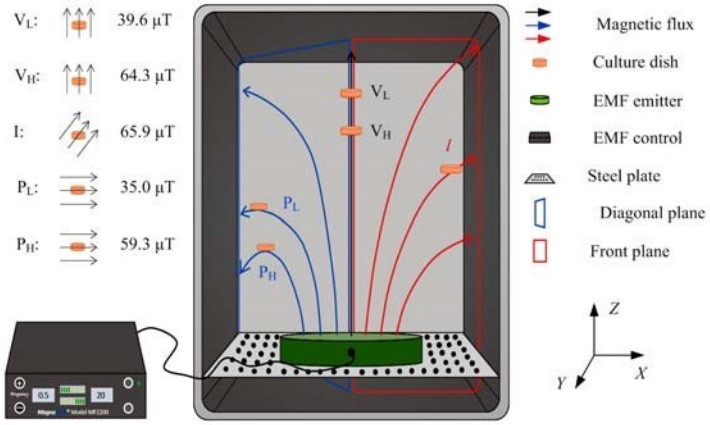


Fig. 1 Schematic illustration of BIEMF condition at each test point. The BIEMF directions and intensities are indicated on the left. The test point V_L and V_H are on the vertical central axis, where the magnetic field is perpendicular to the culture dish. The test point I is on the front plane and its EMF direction is inclined to the culture dish by roughly 45˚. The test point P_L and P_H are on the diagonal plane and their EMF directions are horizontal and parallel to the culture dish.

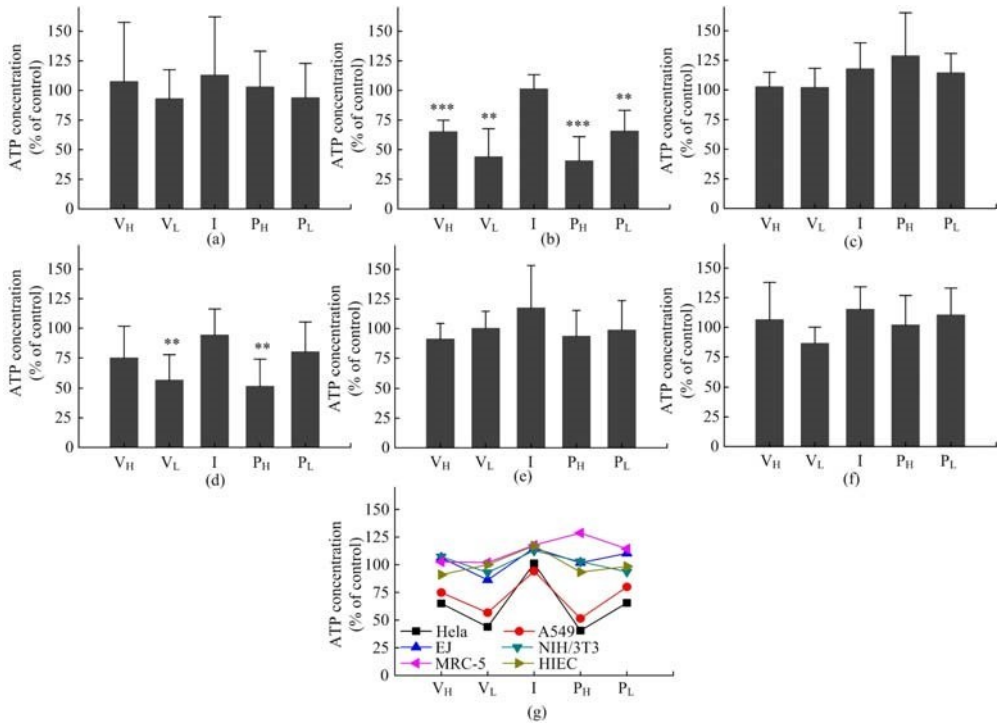


Fig. 2 Cellular ATP levels after 48 h exposure to BIEMF of different directions. Cell lines: (a) NIH/3T3, (b) Hela, (c) MRC-5, (d) A549, (e) HIEC, (f) EJ, and (g) all together. *n* = 6, which represents the total number of samples from three independent experiments. Data are shown as the average with standard deviation (SD) in (a) to (f). One factor two-side *t*-test was performed to determine the statistic significance of cellular ATP level changes as BIEMF-exposed groups *vs.* the control unexposed group. **: $P < 0.01$, ***: $P < 0.001$.

1 mL crystal violet staining solution for 10 min at room temperature. Then, the dishes were washed with distilled for three times and dried for observation and photographs.

Cell nuclear staining

DAPI staining solution (Beyotime, China) was used to stain Hela cell nucleus according to the manufacturer protocol.

When DAPI binds to double-stranded DNA, it produces strong fluorescence. First, culture media were removed from the 3.5 cm culture dishes and cells were fixed with 4% paraformaldehyde for 15 minutes. Subsequently, the dishes were washed twice with distilled water and cells were stained with 1 mL DAPI staining solution for 5 minutes at room temperature. Then, the DAPI staining solution was aspirated and cells were washed three times with PBS before imaging by a fluorescence microscope (IX83, Olympus, Japan).

Results and discussion Effect of BIEMF on cellular ATP levels

Cellular ATP provides direct energy source to almost all living organisms and thus its level is an important indicator of cell viability[30]. Previous works have demonstrated that electromagnetic fields can affects ATP production both *in vivo* and *in vitro*[3,31–33]. We first set out to examine whether BIEMF in different directions can yield differential ATP production in

six cultured cell lines, including NIH/3T3 (mouse fibroblasts), MRC-5 (human lung fibroblasts), HIEC (human intestine epithelial cells), Hela (human cervical adenocarcinoma cells), A549 (human lung carcinoma cells), and EJ (human bladder cancer cells). The first three are normal cells, whereas the others are cancerous. The cellular ATP levels after 48-hour BIEMF exposure are normalized to those of the control groups without exposure for comparison. No significant changes in ATP production are found for all normal cells examined in designated BIEMF orientations regardless of the intensity (Figs. 2a, 2c and 2e). It is not surprising since our BIEMF is similar to the geomagnetic field in nature, which these cells have adapted to. In contrast, cancer cells exhibit tumor-specific behaviors. The cellular ATP levels of Hela and A549 cells are significantly decreased by BIEMF in both vertical and parallel directions (Fig. 2b and 2d), whereas no ATP response is observed in EJ cells (Fig. 2f). It is interesting that the BIEMF of inclined orientation shows no significant effect on ATP production for three cancer cells investigated (Fig. 2g), probably because the attached cancer cells suffer more constraint from vertical and horizontal BIEMF than that of inclined direction. It should be noted that it is the first time that three representative EMF directions, which are perpendicular (vertical), parallel (horizontal) and diagonal (inclined by 45˚) to cell growth plane, are directly compared. The results indicate that the biological effects caused by EMF with low intensity and extremely low frequency are anisotropic, and such a direction-dependent feature should be carefully weighted in applying EMF in cancer treatment and reconciling the conflicting EMF research reports. The most evident inhibition on ATP production (by 30% – 60%) is observed for Hela cells at the vertical and parallel orientation of BIEMF (Fig. 2g).

Moreover, although the directionality effect of EMF has not been clearly understood to date, three possible mechanisms have been proposed. One hypothesis is that radical pairs are magnetically sensitive and the EMF of distinct orientation may cause differential effect on radical pair reactions[34]. Another possible mechanism is based on biogenic magnetite, which is formed in organisms with anisotropic preference[35–38]. The third theory explains the directionality effect of EMF by the resonance between EMF and electric field ion cyclotron[39]. As for our work with extremely low EMF intensity and frequency, one mechanism of these three may work alone or in combination with each other. Nevertheless, we postulate that the radical pair mechanism is more likely in our case, since it is known that the EMF effect on some biochemical reactions, ATP biosynthesis in particular, is in line with the radical pair mechanism[34]. Indeed, the radical pair mechanism has been used in many reports to interpret the EMF effect on ATP production[10,40].

Effect of BIEMF on Hela cell proliferation

Given that Hela cells exhibit the most inhibition on cellular ATP level by BIEMF, we next decided to study the effect of BIEMF on cancer cell proliferation by placing Hela cells at the point V_L. Two parallel samples were set up for each test. Cell staining was performed after 6

days of BIEMF exposure. Interestingly, the inhibition of colony formation by BIEMF is found to be cell density dependent. In a pilot experiment, 100 cells were seeded per dish and no colonies could be seen on BIEMFexposed dishes, whereas small colonies were formed in the control group (Fig. 3a). Then, the cell seeding density was increased to see to what degree BIEMF was able to inhibit Hela cell proliferation (Fig. 3b). When the cells were seeded at 300 and 1000 cells per dish, no cell colonies could be observed after BIEMF exposure. In contrast, at the higher seeding densities of 3000 and 10000 cells per dish, abundant colonies were well formed even when they had undergone 6-day BIEMF exposure, similar to the control groups. These results suggest that BIEMF can inhibit the proliferation of Hela cells at low cell density and the inhibitory ability will be limited above a certain threshold of cell density, which is probably around 1000 cells per dish in our case. Moreover, together with the previous cellular ATP assay results of Hela at high cell densities, it can be inferred that the decrease in cellular ATP level is not the only cause for cell proliferation inhibition by BIEMF. Yet, the underlying mechanism of this density dependent effect is still unclear and needs further investigation. A previous report has shown that the effect of EMF exposure on cell proliferation is more evident at the log phase of cell growth but becomes less significant later, possibly because of the nutrient depletion[41]. However, the seeding densities of 3000 and 10000 cells per dish are still low for a 3.5 cm cell culture dish. So we postulate that it is probably due to the collective antagonism at these cell densities, which protects cancer cells from BIEMF inhibition.

To verify that BIEMF inhibits Hela cell proliferation in a density-dependent manner, we designed another cell colony formation assay. In this experiment, Hela cells were seeded at 500 cells per dish and divided into four groups from I to IV, which were subjected to four BIEMF exposure protocols as shown in Table 2. The symbol “+” means with BIEMF exposure, whereas the “–” sign represents no BIEMF exposure. Consistent with the previous results, cell colonies formed very well in group I without BIEMF exposure and no cell colonies were found in group IV after 6-day exposure (Fig. 4a). It is interesting that distinct results appeared in group II and III (Figs. 4a and 4b), where the total BIEMF exposure time was same as 3 days, but the order of “with exposure” and “without exposure” had been altered. Many small cell colonies can be seen in the group II dishes that were exposed to BIEMF for 3 days after 3-day ramping-up period, but no colonies can be identified from the group III dishes (Fig. 4a), which suffered a 3-day BIEMF exposure first and recovered without BIEMF for 3 days. Such a phenomenon is in line with our hypothesized density-dependent behavior and suggests that imposing a proper BIEMF exposure at an early stage of low cell density may inhibit cancer cell proliferation. The colony numbers of three independent verification experiments have been statistically compared, which shows a stronger difference between group I and III than that between group I and II (Fig. 4b). It is inferred from this observation that the inhibitory effect by BIEMF can be compromised by pre-amplified cancer cells, consistent with our hypothesis.

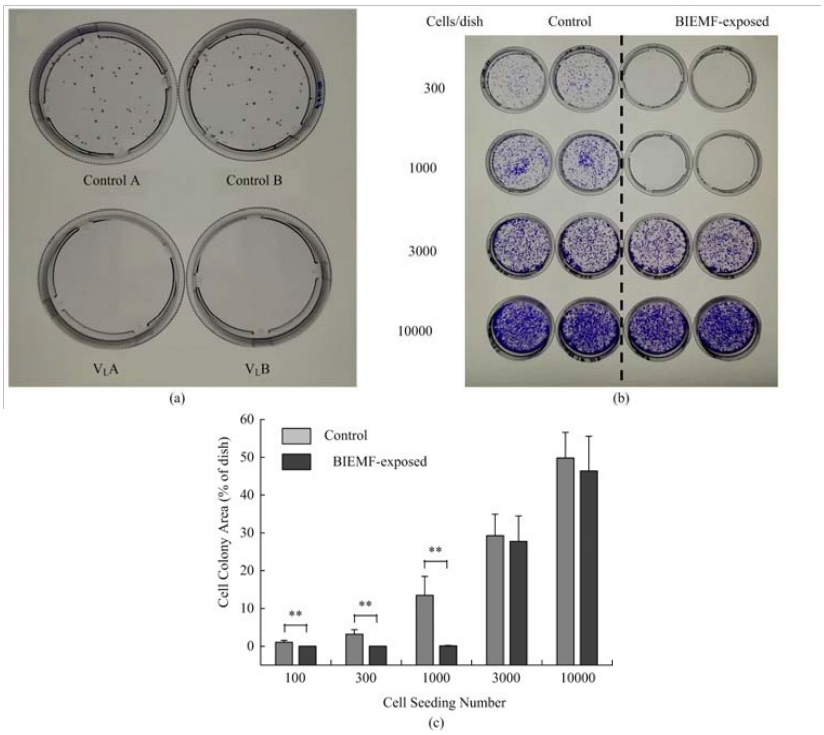


Fig. 3 Colony formation of Hela cells. (a) Colony formation at the seeding density of 100 cells per dish. (b) Colony formation at the seeding densities ranging from 300 to 10000 cells per dish. (c) Cell colony area at the seeding densities ranging from 300 to 10000 cells per dish. *n* = 6, which represents the total number of samples from three independent experiments. Data are shown as the average with Standard Deviation (SD). One factor two-side *t*-test was performed to determine the statistic significance between the BIEMF-exposed groups and the corresponding control unexposed group. **: $P < 0.01$.

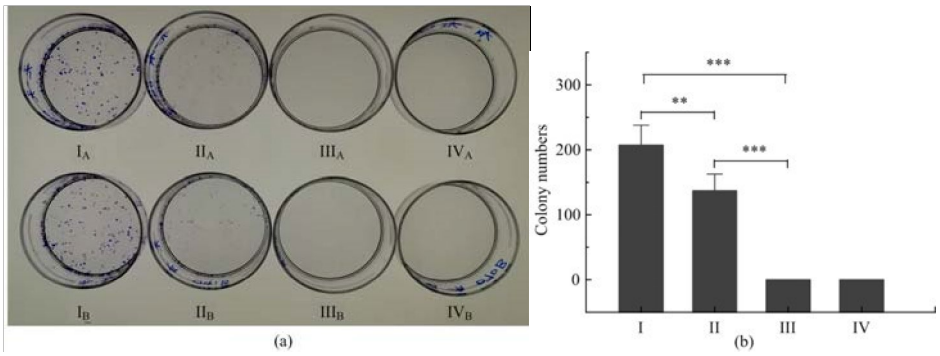


Fig. 4 Results of the verification test. (a) Colony formation of Hela cells with four BIEMF exposure protocols (seeding cell density is 500 cells per dish).

I_A and I_B, II_A and II_B, III_A and III_B, IV_A and IV_B are two parallel samples from one of the three independent experiments. (b) Colony numbers after different BIEMF exposure protocols. *n* = 6, which represents the total number of samples from three independent experiments. Data are shown as the average with Standard Deviation (SD). One factor two-side *t*-test was performed to determine the statistic significance among the groups with different BIEMF exposure protocols. **: $P < 0.01$, ***: $P < 0.001$.

Effect of BIEMF on Hela cell morphology

The observed inhibition of cell proliferation could result from either cell death or halted cell division[19,42]. Cell death is often accompanied by changes in cell morphology[43,44]. Thus,

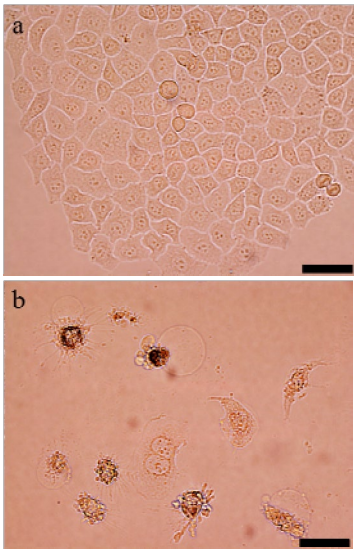
in order to determine how BIEMF inhibits Hela cell proliferation, we have compared the morphology of cells with or without 6-day BIEMF exposure. As shown in Fig. 5, the Hela cells with 6-day BIEMF exposure at the point of V_L show dramatic shrinkage with blebbing (Fig. 5b), which is a clear sign of cell death, whereas the unexposed control cells look healthy (Fig. 5a).

Like cell morphology, the morphological change of nucleus is also an important indicator for the type of cell death, *i.e*. apoptosis *vs*. necrosis[43,44]. Hence a time- course experiment was conducted to examine the cell morphology and nuclear morphology within 24 h to 72 h of BIEMF exposure at an interval of 12 h (Fig. 6). It is found that the nuclei started to condense after 60 h of BIEMF exposure (Fig. 6i). It can also be observed from the enlarged pictures of BIEMF-exposed cells (Figs. 6u and 6v) that the nuclei have condensed to smaller yet brighter dots, which can't be identified for unexposed control cells (Figs. 6w and 6x). Meanwhile, blebbing can be observed in BIEMF-exposed cells as shown in Figs. 6c and 6d as well as Fig. 5b. These observations suggest that the BIEMF-induced cell death probably undergoes apoptosis, which normally demonstrates the characteristics of nuclear condensation and blebbing without membrane disintegration at early stage[43,44]. The exact trigger of BIEMF-induced apoptosis in cancer cells is still under investigation.

Table 2 Setup of the verification test with four BIEMF exposure

Group	First 3 days	Last 3 days
I	–	–
II	–	+
III	+	–
IV	+	+

Fig. 5 Micrographs of Hela cells without (a) and with (b) 6 days of BIEMF protocols exposure at the point V_L. Seeding density is 1000 cells per dish. The scale bar is 50 μm.



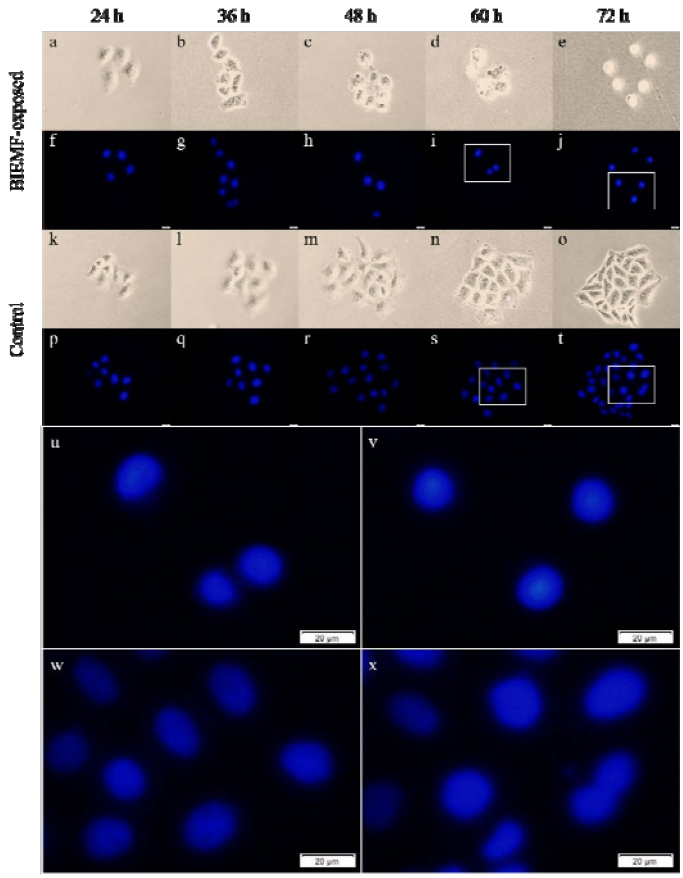


Fig. 6 Bright-field images ((a) – (e) and (k) – (o)) and DAPI-staining fluorescence images ((f)–(j) and (p)–(t)) of Hela cells with ((a)–(j)) and without ((k)–(t)) BIEMF exposure at the designated time points. Images (u), (v), (w) and (x) are the enlarged pictures of the boxed areas in image i, j, s and t respectively. Seeding density is 1000 cells per dish. BIEMF-exposed cells were placed at the point V_L. The scale bar is 20 μm for all images.

In this work, we started from the perspective of bionics and found that BIEMF can inhibit the proliferation of Hela cells. On one hand, this finding can help to understand the role of the geomagnetic field on human health and thus, provides another line of evidence for applying EMF, particularly BIEMF, in the prevention and treatment of certain cancers. On the other hand, we have confirmed that the directionality of low-intensity extremely low-frequency electromagnetic fields can also affect certain bioelectromagnetic effects, implying that the anisotropic nature of EMF should be considered in future research. The underlying mechanism for BIEMF-induced apoptosis in cancer cells also requires further investigation.

Conclusion

In summary, inspired by the natural geomagnetic field and the inherent vibrating of cancer cells, we generated a BIEMF of low-intensity (35 μT – 70 μT) and extremely low-frequency (0.5 Hz) and then conducted a series of experiments to determine the effects of BIEMF on normal and cancer cells. For the first time, differential effects on ATP production have been found for BIEMF of distinct orientations, *i.e*. vertical, parallel and inclined directions. Yet the inhibition on ATP synthesis by BIEMF demonstrates cell type preference, showing stronger impact in Hela cells. Interestingly, the colony formation assay with Hela cells has revealed that BIEMF is able to inhibit cancer cell proliferation in a cell density dependent manner. The morphological changes of cells and nuclei during cell death suggest that BIEMF-induced cell death probably undergoes apoptosis. Owing to its GMF-like intensity and extremely low frequency, the BIEMF we proposed here is safer than other forms of EMF in the field of cancer treatment. Hence, these results can not only help to re-understand the influence of the geomagnetic field on the health of humans and other creatures, but also afford new approaches for cancer prevention and treatment.

Acknowledgement

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