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A Study of the Effects of Flux Density and Frequency of Pulsed Electromagnetic Field on Neurite Outgrowth in PC12 Cells

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Abstract. The aim of this study was to investigate the influence of pulsed electromagnetic fields with various flux densities and frequencies on neurite outgrowth in PC12 rat pheochromocytoma cells. We have studied the percentage of neurite-bearing cells, average length of neurites and directivity of neurite outgrowth in PC12 cells cultured for 96 hours in the presence of nerve growth factor (NGF). PC12 cells were exposed to 50 Hz pulsed electromagnetic fields with a flux density of 1.37 mT, 0.19 mT and 0.016 mT respectively. The field was generated through a Helmholtz coil pair housed in one incubator and the control samples were placed in another identical incubator. It was found that exposure to both a relatively high flux density (1.37 mT) and a medium flux density (0.19 mT) inhibited the percentage of neurite-bearing cells and promoted neurite length significantly. Exposure to high flux density (1.37 mT) also resulted in nearly 20% enhancement of neurite directivity along the field direction. However, exposure to low flux density field (0.016 mT) had no detectable effect on neurite outgrowth. We also studied the effect of frequency at the constant flux density of 1.37 mT. In the range from $1 \sim 100$ Hz, only 50 and 70 Hz pulse frequencies had significant effects on neurite outgrowth. Our study has shown that neurite outgrowth in PC12 cells is sensitive to flux density and frequency of pulsed electromagnetic field.

Key words: nerve growth factor, neurite outgrowth, PC12 cells, pulse frequency, pulsed electromagnetic field

Introduction

During the last two decades, there has been great interest in how biological systems can be influenced by the extrinsic low frequency electromagnetic fields. In particular, efforts have been directed to the study of whether and how low frequency fields alter living systems. The PC12 cell line, a basic model for studies on neuronal differentiation, can react reversibly to nerve growth factor (NGF) by differentiating and growing neurites. Some studies of Blackman *et al.* [1–3] found that 50 Hz AC fields (less than 0.04 mT) can inhibit neurite outgrowth induced by NGF; while promotion was also observed from other studies [4, 5]. Further they found that various frequencies from 15 to 70 Hz led to different flux-density-dependent effects.

Mcfarlane *et al.* [6] found that exposure to AC 50 Hz fields (4.35–8.25 μT) for one day during cell differentiation altered neurite outgrowth in PC12 cells while slightly higher fields (8.25-15.8 μT) did not. A study by Takastuki [7] demonstrated that AC 60 Hz fields (0.0333 mT) stimulated neurite outgrowth in PC12D cells. Others [8] have shown that NGF-induced neurite outgrowth in PC6 cells was significantly depressed by pulsed fields (2 Hz) with flux density of 0.3 mT. Systematic investigations on neurite outgrowth in PC12 cells have been performed to establish the critical exposure variables of AC fields. However, the critical exposure variables of pulsed fields are yet to be established. In this contribution, we studied whether pulsed fields with various flux densities (1.37, 0.19 and 0.016 mT peak values respectively) can affect neurite outgrowth. In addition, the PC12 cells were also exposed to pulsed fields at 1.37 mT at 1, 10, 30, 50, 70 and 100 Hz frequency to study whether various pulse frequencies can result in different neurite outgrowth.

Materials and Methods

PC12 rat pheochromocytoma cells (CRL-1721, American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in 25-cm^2 flask (IWAKI, ASAHI Techno Glass, Japan) in 5 ml medium at 37°C in a incubator (SANYO-20AIC, Japan) containing 5% CO_2 and 95% air. The growth medium comprised of Dulbecco's modified Eagle medium (#12100046, Invitrogen, UK) supplemented with 10% horse serum (#16050122, Invitrogen, UK) and 5% fetal bovine serum (#10270106, Invitrogen, UK). The differentiation medium was comprised of DMEM medium, 1% horse serum and 0.5% fetal bovine serum supplemented with NGF (7S# N0513, Sigma-Aldrich, St. Louis, MO, USA). Before reaching confluence, cells were detached by washing with PBS containing 1mM EDTA. The suspension was centrifuged and the cells were plated at 2×10^4 cells/cm^2 with growth medium on a 24-well dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) coated with collagen (type I from rat tail cell, #C7661, Sigma-Aldrich, St. Louis, MO, USA). After 24 hours, the growth medium in the dish was removed and differentiation medium containing NGF of 30 ng/ml was added. We have confirmed that 30 ng/ml NGF concentration is the optimal concentration for studying the neurite outgrowth (unpublished work). Four wells in the dish were used to culture PC12 cells and each well (1.75 cm^{2}growth area) contained 1 ml medium. This dish was placed in the exposure system housed in the incubator. Another identical culture dish was placed in a second identical incubator without electromagnetic field as control sample. As soon as the differentiation medium was added to the culture dishes, the exposure started and continued for 96 hours. The differentiation medium with NGF was replaced once every 48 hours. The final cells were assayed after 96 hours NGF treatment. The experiment was repeated three times.

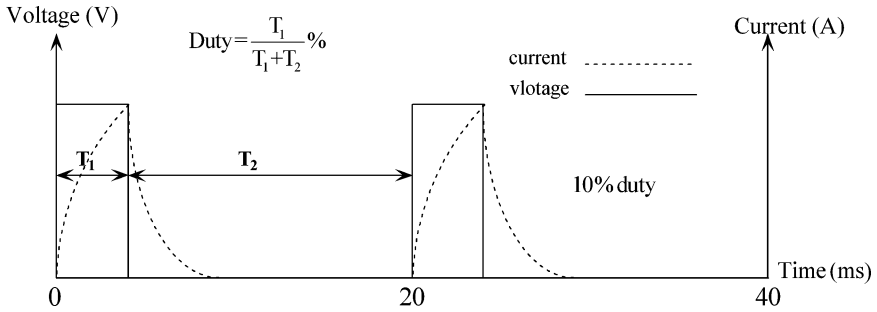

Figure 1. Waveform of 50 Hz pulse potential, electric current induced in coils and the definition of pulse duty. The saw tooth shape of electric current resulted from the inductance of coils. The time of one cycle is $T_1 + T_2$ (0.02 s for 50 Hz). For 10% duty, the rise time $T_1 = 2$ ms (10% of one cycle).

The electromagnetic exposure system was a pair of Helmholtz coils (13 cm in diameter): two identical coils (each consisted of 300 turns of enameled copper wire with a radius of 0.4 mm) were mounted coaxially at a distance of 13 cm from each other, in order to produce a highly uniform horizontal field in the area between the two coils. The coils were connected to a wave function generator (SONY Tektronix AFG310, Japan) with an amplifier (AR Worldwide 40AD1, Souderton, PA, USA). A positive pulse (repeating at different frequencies) with 10% duty was applied. The pulse duty was defined as the percentage of the time during which the voltage is switched on in one cycle. Figure 1 shows the waveform of 50 Hz output pulse potential and the periodic change of the electrical current (or EMF). Based on our calculation, when a pulse duty is below 10%, the current is not able to reach saturation and therefore the lowest pulse duty used in our work was 10%. Our previous work has established that the pulsed electromagnetic field with 10% pulse duty has significant effects on neurite outgrowth in PC12 cells (unpublished work). The dish was placed horizontally at the center between the two coils within the incubator to make sure that each well was exposed to a uniform field with field direction parallel to the surface of the medium. The flux density of 1.37 mT is the highest level of electromagnetic field for this system and was measured using a gaussmeter (Lakeshore 450, UK).

In our studies, two identical incubators were used for exposure and control respectively. In order to ensure the culture conditions of the two incubators were identical, we performed control experiments in the absence of the fields and found that the extent of neurite outgrowth in each incubator was the same.

The assay procedure for neurite outgrowth used in our work was as described by Greene and Tischler [9]. Neurite orientation with respect to the electromagnetic field was assayed with the aid of the labeling on the culture dish. Neurite outgrowth was evaluated from computer-captured images of each cell. Images were acquired by using a phase-contrast microscope (Zeiss Axiovert 25, Germany) equipped with

a digital camera (Nikon Coolpix 995, Japan). The neurite length and direction were analyzed with Leica image measurement software (Qwin2.0). The length measurement was calibrated using a hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) grid. At least 100 cells were assayed per well in a blinded fashion. We only counted the isolated cells, not the cell clumps, in order to obtain consistent results. A neurite-bearing cell was defined as a cell which generates at least one neurite with a length two times longer than the cell body. The neurite length was recorded in microns (μm). Statistical analyses were performed using the Student t-test, in which difference was considered as significant for $P < 0.05$. Data are expressed as mean $\pm$ S.E.

Results

Percentage of neurite-bearing cells

It is found that, exposure to pulsed field with high and medium flux densities (1.37 and 0.19 mT) led to significant inhibition of percentage of neurite-bearing cells. The inhibition was up to 14.29% (33.09 $\pm$ 1.21% compared to 38.03 $\pm$ 0.66%, $P = 0.023$ (see Table I)) and 20.96% (32.27 $\pm$ 0.72% compared to 40.83 $\pm$ 0.22%, $P = 0.000343$) for high and medium flux densities respectively, while exposure to low flux density (0.016 mT) led to a very low and insignificant inhibition of 2.1% (41.05 $\pm$ 0.79% compared to 41.94 $\pm$ 2.83%, $P = 0.78$), as shown in Figure 2. These results show that in samples exposed to high and medium flux density pulsed fields, the number of cells able to generate neurites is significantly inhibited. In contrast, there was no significant change in percentage of neurite-bearing cells when exposed under low flux density (0.016 mT). It should be noted that the inhibition of medium flux densities on percentage of neurite-bearing cells is significantly higher

Table I. Influence of pulsed electromagnetic field (1.37 mT) with different frequencies on percentage of neurite-bearing cells in PC12 cells treated with NGF at 30 ng/ml

	Percentage of neurite-bearing cells (%)			
Frequency (Hz)	Exposure[a]	Control	Change	P-value[b]
1	42.04 $\pm$ 1.10	41.58 $\pm$ 1.17	1.11 $\pm$ 0.56	0.79
10	52.52 $\pm$ 1.02	51.86 $\pm$ 1.30	1.27 $\pm$ 1.33	0.71
30	55.31 $\pm$ 0.84	56.18 $\pm$ 1.55	$-$1.55 $\pm$ 1.02	0.65
50	33.09 $\pm$ 1.21	38.03 $\pm$ 0.66	$-$14.29 $\pm$ 2.80	0.023
70	51.31 $\pm$ 0.94	58.05 $\pm$ 0.78	$-$11.61 $\pm$ 1.63	0.005
100	50.86 $\pm$ 0.75	51.54 $\pm$ 1.31	$-$1.32 $\pm$ 0.42	0.67

[a]Exposure, $n = 3$ (from 12 wells in 3 experiments); control, $n = 3$ (from 12 wells in 3 experiments).
[b]$P < 0.05$: significant difference.

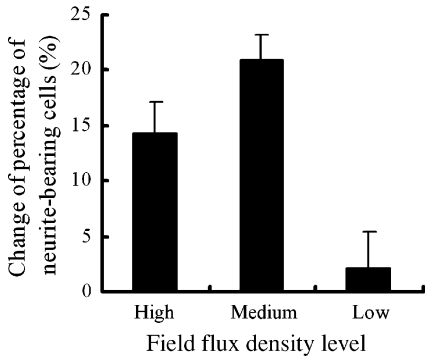

Figure 2. Decrease in the percentage of neurite-bearing cells at different flux density levels (high: 1.37 mT, medium: 0.19 mT and low: 0.016 mT).

(20.96% compared to 14.29%, $P = 0.046$) than that of high flux densities. Here we define the change (or inhibition) as the absolute value of the ratio of difference between the exposure data and the control data to the control data (($\text{data}_{\text{exposure}} - \text{data}_{\text{control}}$)/$\text{data}_{\text{control}}$).

AVERAGE LENGTH OF NEURITES

Figure 3 shows the effects of pulsed electromagnetic fields with various flux densities on average length of neurites in PC12 cells. It was found that exposure to both high and medium flux densities resulted in significant promotion of neurite length. For high flux density (1.37 mT), the promotion is up to 14.53% (60.01 ± 1.64 compared to 52.53 ± 1.97 μm, $P = 0.042$ (see Table II)) and for medium flux density (0.19 mT) the promotion is 12.32% (39.28 ± 0.66 compared to 34.97 ± 0.83 μm,

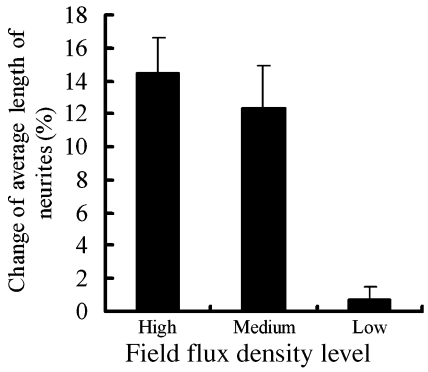

Figure 3. Change of average length of neurites at different flux density levels (high: 1.37 mT, medium: 0.19 mT and low: 0.016 mT).

Table II. Influence of pulsed electromagnetic field (1.37 mT) with different frequencies on average length of neurites in PC12 cells treated with NGF at 30 ng/ml

	Average length of neurites (μm)			
Frequency (Hz)	Exposure[a]	Control	Change	P-value[b]
1	48.49 ± 1.90	47.92 ± 0.55	1.19 ± 0.74	0.79
10	42.13 ± 0.69	42.33 ± 1.12	-0.47 ± 0.26	0.89
30	50.36 ± 0.87	52.20 ± 0.50	-3.50 ± 1.11	0.46
50	60.01 ± 1.64	52.53 ± 1.97	14.53 ± 2.11	0.042
70	55.70 ± 0.59	49.80 ± 0.78	11.85 ± 2.03	0.0038
100	46.70 ± 0.76	46.05 ± 0.12	1.40 ± 0.71	0.45

[a]Exposure, $n = 3$ (from 12 wells in 3 experiments); control, $n = 3$ (from 12 wells in 3 experiments).
[b]$P < 0.05$: significant difference.

$P = 0.016$). In this case, neurites were stimulated by a pulsed electromagnetic field and the cells in the exposure sample tended to extend longer neurites than those in the control sample, but unlike the percentage of neurite-bearing cells, the stimulation of neurite length caused by high flux density is significantly stronger (14.53% compared to 12.32%, $P = 0.028$) than that caused by medium flux density. Exposure to low flux density (0.016 mT) did not promote increased neurite length ($36.85 \pm 0.24\,\mu$m compared to $37.11 \pm 0.79\,\mu$m, $P = 0.77$), indicating there is no significant change (see Table II).

Directivity of neurite outgrowth

As reported by Macias *et al.* [10], neurite outgrowth may have preferred directivity when exposed to electromagnetic fields. In this work, we have analyzed the directivity of neurite outgrowth in the same method used by Macias. All the neurites were recorded in length and direction (relative to the direction of electromagnetic field). We divided 0 to 360° into four quadrants (Q_1 =315°–45°, Q_2 = 45°–135°, Q_3 = 135°–225° and Q_4 = 225°–315°). Since the field was applied in the direction of 0 and 180°, Q_1 and Q_3contained neurites which grew in the direction parallel to the electromagnetic field, while Q_2 and Q_4 contained neurites perpendicular to the electromagnetic field.

We found that exposure to high flux density (1.37 mT) resulted in an enhanced directivity of neurites along the field direction. For the control sample, the average length of neurites of Q_1 and Q_3 was almost equal to that of Q_2 and Q_4. But for the exposure sample, it was observed that the average length of neurites of Q_1 and Q_3 had a significant difference with that of Q_2 and Q_4, that is, PC12 cells produced longer neurites (65.79 ± 2.44 compared to $54.85 \pm 1.69\,\mu$m, $P = 0.02$ (see Table III)) along the direction of the pulsed electromagnetic field than those

Table III. Influence of pulsed electromagnetic field (1.37 mT) with different frequencies on directivity of neurites outgrowth in PC12 cells treated with NGF at 30 ng/ml

Frequency (Hz)	$Q_1 + Q_3$[a]	$Q_2 + Q_4$	Change (%)	P-value[b]
1	47.99 ± 1.62	48.76 ± 1.88	-1.58 ± 0.36	0.77
10	42.29 ± 1.33	41.85 ± 0.12	1.05 ± 0.96	0.76
30	50.38 ± 1.13	51.09 ± 0.57	-1.39 ± 1.08	0.61
50	65.79 ± 2.44	54.85 ± 1.69	19.97 ± 1.15	0.02
70	60.19 ± 0.49	51.29 ± 1.05	17.35 ± 2.18	0.0015
100	45.85 ± 0.66	46.87 ± 0.69	-2.18 ± 1.29	0.34

[a]Exposure, $n = 3$ (from 12 wells in 3 experiments).
[b]$P < 0.05$: significant difference.

oriented perpendicular to the electromagnetic field. For medium (0.19 mT) and low (0.016 mT) flux densities, no enhanced directivity of neurites along the field direction was observed.

Pulse frequency effects

In addition to 50 Hz pulse frequency, we also conducted experiments by using 1, 10, 30, 70 and 100 Hz frequency pulsed fields at flux density of 1.37 mT. We found that the pulsed electromagnetic field with 70 Hz frequency had the same effects on percentage of neurite-bearing cells, average length of neurites and neurite directivity as the 50 Hz frequency, while other frequencies had no noticeable effect on neurite outgrowth. The results of pulsed EMF with each frequency are listed in Tables I–III. Exposure to pulsed electromagnetic field with 70 Hz frequency resulted in a significant inhibition ($11.61 \pm 1.63\%$, $P = 0.005$) of percentage of neurite-bearing cells as well as a significant promotion ($11.85 \pm 2.03\%$, $P = 0.0038$) of average length of neurites. Furthermore, a significant enhancement (60.19 ± 0.49 μm compared to 51.29 ± 1.05 μm, $P = 0.0015$) of neurite outgrowth along the field direction was also observed for 70 Hz field exposure, as shown in Table III.

Discussion

Our data demonstrated that the pulsed electromagnetic fields with high (1.37 mT) and medium (0.19 mT) flux densities can lead to significant alterations of neurite outgrowth in PC12 cells, while low flux density field (0.016 mT) had no such effects. Both high and medium flux density fields resulted in the inhibition of percentage of neurite-bearing cells and the increase of the average length of neurites in PC12 cells. As mentioned in the introduction section, a study by McFarlane *et al.* [6] has demonstrated that 50 Hz AC field with 4.35–8.25 μT stimulated neurite outgrowth while slightly higher fields with 8.25–15.8 μT did not. Blackman *et al.*

has also reported the flux-density-dependence of biological effects of 50 Hz AC electromagnetic fields and they found that the fields between 5.0-10 μT inhibited neurite outgrowth [1] and fields between 2.2–4.0 μT stimulated neurite outgrowth, while lower fields had no effect [4]. Although they used the AC fields, not pulsed fields, these two studies, as well as our study, have confirmed that flux density plays an important role in biological effects on neurite outgrowth in PC12 cells. Only a field with high flux density (1.37 mT) led to significant enhancement of neurite directivity along the direction of electromagnetic field, while both the medium (0.19 mT) and low (0.016 mT) flux density fields had no effect on directivity of neurite outgrowth in PC12 cells. The directed neurite outgrowth induced by pulsed electromagnetic fields was also reported by other investigators [10, 11], but their research only focused on neurite outgrowth in DRG cells, not PC12 cells.

In the range from 1 to 100 Hz, a significant difference between the exposure and the control samples was observed at both 50 Hz and 70 Hz; while for 1, 10, 30 and 100 Hz frequencies, there was no significant difference between the exposure and the control samples. Although we used a different type of electromagnetic field, our studies are consistent with results of Blackman *et al.* [2] in which inhibition of neurite outgrowth was observed over 35–70 Hz frequencies (AC field), whereas between 15 and 30 Hz frequencies no significant effect was found.

Our studies have shown that neurite outgrowth in PC12 cells is sensitive to pulsed electromagnetic fields and this sensitivity is strongly dependent on the flux density and pulse frequency.

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