Vol. 189, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1992 Pages 437-444

^{3}H-Uridine Uptake in Human Leukemia HL-60 Cells Exposed to Extremely Low Frequency Electromagnetic Fields

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Received October 16, 1992

Human leukemia HL-60 cells *in vitro* were exposed or sham-exposed to a 60 Hz electric (3.40 mV/m) or magnetic 10 G (1 μT) field for 5 hr in an attempt to replicate the results of a previously published report (1) of increased uptake of [^{3}H] uridine in response to similar exposure conditions. The results of the present experiments indicated no treatment effect of the fields on uridine incorporation. The ability to detect differences in [^{3}H] uridine uptake were confirmed in a negative control experiment. A 'negative control' experiment demonstrated a statistically significant increase in ^{3}H-uridine uptake for cells at 37 °C relative to those at 4 °C.

Electrification of developed and developing countries has resulted in exposure of living organisms to power frequency (50-60 Hz) electric and magnetic fields which are considerably above the 10^{-4} V/m and 10^{-8} G ambient electric (EF) and magnetic fields (B), respectively (2). Possible adverse biological effects from exposure to such extremely low frequency electromagnetic fields (ELF-EMF) have been suggested by a few epidemiological studies but there are also a large number of reports which do not support this postulate (see Ref. 3 for discussion). However, the few positive results have generated considerable scientific interest and public concern regarding the potential impact of ELF-EMF on biological systems.

The present study was undertaken in an attempt to verify a recently published report (1) of striking enhancement of [^{3}H]-uridine uptake in HL-60 cells *in vitro* exposed to a 60 Hz magnetic field of 1 μT. The authors proposed that through Faraday induction in the outer annulus of the organ culture dish the 60 Hz 1

0006-291X/92 $4.00

μT magnetic field exposed cells *in vitro* were affected by a 60 Hz electric field of 3.40 mV/m. The maximum effect, of about a 70% increase in [^{3}H]-uridine uptake, occurred with a 5-hr exposure period. The percent enhancement of [^{3}H]-uridine uptake had standard errors of the means (S.E.M's) of 5-15%. Their exposure system consisted of a mu-metal shield encasing a solenoid; organ culture dishes were placed in a carrier inserted into the solenoid, which contained an incorporated water circulation system for temperature (37 °C) regulation. The control cultures were contained in a carrier placed in an incubator maintained at 37 °C.

The results of the Greene *et al.* (1) experiment have potentially important implications for health and safety considerations since the purported effective EF strength is well below the level induced in the body of people exposed to power frequency (50-60 Hz) transmission line fields of 1000 V/m (3). Because of this, the present experiments were conducted to attempt independent verification of the Greene *et al.* (1) results. The biological and field exposure aspects of the Greene *et al.* (1) experiments were duplicated as carefully as possible. The procedures and results of those experiments (1) became the hypothesis tested in the present study. The largest reported increase (70%) in [^{3}H]-uridine uptake was reported to occur with a 5-hr exposure (1); before or after the 5-hr exposure period there was less ^{3}H-uridine uptake. Thus, a 5-hr exposure period was used in the present study, and the subsequent results tested statistically for an increase in [^{3}H]-uridine uptake. Initially, we tested the postulate that a 5-hr exposure to a 60 Hz 3.40 mV/m EF would increase the amount of [^{3}H]-uridine uptake in HL-60 cells *in vitro*. Subsequently, when the EF-data failed to support the postulate, we then tested the postulate that a 5-hr exposure to a 60 Hz 1 μT MF would increase [^{3}H]-uridine uptake.

MATERIALS AND METHODS

Cell culture - An *in vitro* human promyelocytic leukemia cell line (HL-60) was obtained from the American Type Culture Collection (ATCC, 12301 Parklawn Dr. Rockville, MD 20852-1776). The cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum and antibiotics. All culture medium components were obtained from GIBCO Life Technology Laboratories, P.O. Box 68, Grand Island, NY 14072-0068. The cells were seeded into 25 cm^2 plastic

tissue culture flasks (Falcon, Oxnard, CA) containing 4 - 5 ml of complete medium and were maintained in a 5% CO_2, humidified, water-jacketed incubator at 37 ± 0.1°C. Cells from exponentially growing cultures were gently pipetted up and down against the bottom of the flask to obtain a monodispersed preparation; a 1 ml sample of the suspension was counted using a Coulter Counter (Model ZBI). The remaining suspension was centrifuged at 125 X g for 5 min, the pellet resuspended in complete medium to a final cell density of approximately 1 x 10^6 cells/ml, and recounted on the Coulter counter; 6 ml of this suspension were seeded into each of the exposure/sham-exposure dishes unless otherwise indicated. This study consisted of seven experimental series (see Table 1). #1] A negative control experiment. #2] An electrode-generated 60 Hz electric field of 3.4 mV/m. Subsequent experiments involved 60 Hz 10 G (1 μT) magnetic field exposures of HL-60 cells seeded in organ dishes and exposed immediately [#4], or seeded for 1 hour [#3,#5,#7] or overnight [#6] before exposure/sham-exposure. For the overnight experimental series [#6] approximately 5 x 10^5 cells/ml (6 ml total volume) were used in the dishes. For the 1 hour before exposure experiments, 3 ml [#3] or 6 ml [#5,#7] of a HL-60 cell suspension (1 x 10^6 cells/ml) were seeded in the organ dishes. Three replicate dishes per treatment (exposed, sham-exposed) were used in each of the 6 experimental series and each series was replicated 5 times (for a total of 6 trials per regimen), except in the seventh series which was replicated 11 times (for a total of 12 trials). The data were assessed for statistical significance using a one-sided Student's t-test for increased [^{3}H]-uridine uptake by the exposed culture.

Exposure/Sham-Exposure Dishes - Two types of vessels were used in this study. For the pure EF-field exposures the vessel consisted of a glass T-tube 14 cm long, 1.1 cm internal diameter and with an elevated 2 cm diameter T-port in the middle (Blaessing Glass Specialities, 645 Atlantic Ave., Rochester, NY 14609); planar type 304 stainless steel electrodes formed the two opposing ends of the tube. For the magnetic field study, organ culture dishes (Falcon No 3037, 60 mm in diameter) were used as the exposure/sham-exposure vessel; this is the type of dish used by Greene *et al.* (1). Cells contained in these dishes were exposed/sham-exposed to a 60 Hz, 10 G (1 μT) magnetic field. Cells were seeded in the outer radius of the organ dishes; through Faraday induction an approximate electric field of 3.40 mV/m would be generated (1, 3, 4).

Exposimetry - Electric Field - The stainless steel electrodes of the glass T-tube vessel were connected to a step-down 12.6 V A.C. power transformer (Model 273-1365, Radio Shack) and a 60 Hz Variac by a wire harness with alligator clips. A resistor (100 kΩ) was connected in series with the electrodes of the T-tube vessel. Voltage across the resistor (V_R) and electrodes (V_E) was measured with a digital multimeter (Model 330, Beckman). Ratios of these values were obtained and the results indicated a linear relationship for low voltages (*i.e.*, the ratio of V_R/V_E was constant). Measurement of a 60 Hz EF of 3.40 mV/m in the cell culture medium in the T-tube required a 100 kΩ resistor connected in series with the energized electrodes. A voltage of 0.476 mV across the electrode was used to achieve a 3.40 mV/m EF in the culture medium of the T-tube. **Magnetic Field** - This exposure system consisted of two cylindrical solenoids; each was 10 cm in diameter and about 27 cm in length, and doubly wound. The coils were connected to a 60 Hz Variac. One set of coils (sham exposure) had the twin coil winding connected so that the magnetic fields generated by the windings were 180° out of phase. In this coil, the magnetic fields were thus "canceled", but resistive heat generation would be the same as for the other energized cell. The other coil (exposure) was connected so that the magnetic fields generated by

the twin windings were in phase. The two coils were placed in a 5% CO_2, humidified, water jacketed incubator for field exposure study. The temperature of the growth medium was 37 ± 0.1 °C during the 5-hr exposure/sham-exposure to a magnetic field of 1 μT. An acrylic plastic ring was used to hold the 60 mm organ culture dishes stacked inside the solenoids. For 60 Hz magnetic field determination a small probe coil was inserted into each cylinder; the calibration of this probe was 1 mV/100 mGauss; 60 Hz fields in the sham exposure were <200 - 400 mG (2×10^{-5} - 4×10^{-5} T); the 'background' 60 Hz field in the incubator (apart from the coils) was 4 - 20 mG (4×10^{-7} - 2×10^{-6} T). All magnetic fields experiments were conducted double blindly. One person energized both coil systems, another person performed the biological assays. After the data were collected the code was broken. **Negative Control** - HL-60 cells were seeded in twelve 15 ml centrifuge tubes at a nominal concentration of 1 $x10^6$ cells/ml. Each tube had 3 ml total volume and was maintained in a 5% CO_2 incubator at 37 ± 0.1°C for 1 hr before experimental treatment. At the end of this period, half of the tubes were placed in an ice-water bath and the remainder were left in the 5% CO_2 incubator at 37 ± 0.1°C for an additional 45 min. HL-60 cells were plused labeled (15 min) before the end of the experiment by addition of 60 μl [^{3}H]-uridine at a final concentration of 20 μCi/ml.

Transcription Assay - T-tubes were seeded with HL-60 cells at a nominal concentration of 1 $x10^6$ cells/ml immediately before exposure/sham-exposure. Organ dishes were also seeded with HL-60 cells at approximately $1x10^6$ cells/ml immediately before, or 1 hr before exposure/sham-exposure of the cells. [^{3}H]-uridine (25.5 Ci/mmol) was purchased from NEN Research Products (DuPont/NEN, 331 Traceable Cove Rd., Billerica, MA 01862). T-tubes or organ dishes were removed from the incubator 15 minute before the end of the exposure/sham-exposure period and pulsed labeled by addition of 50 or 60 μl [^{3}H]-uridine at a final concentration of 4.2 or 20 μCi/ml, respectively. The [^{3}H]-uridine was mixed with the cell culture medium by gently swirling each dish or tube 20 times. This procedure took about 4 min. Dishes or T-tubes were replaced in their original locations in the incubator and were exposed/sham-exposed to the magnetic or electric field during the 15-min isotope application period. Cells from each triplicate T-tube or organ dish were collected by 5 min centrifugation at 125 g. Each cell pellet was resuspended and lysed in 3 ml of buffer of 1% sodium dodecyl sulfate, 10 mM Tris- HCl, 5 mM ethylenediamine tetraacetic acid at pH of 7.5. The lysate was vortexed and the material was precipitated by adding an equal volume of 20% trichloroacetic acid (TCA). The TCA precipitate from each dish was collected on a Whatman GF/B glass microfibre filter by vacuum filtration. Filters were washed three times with 3 ml of 5% TCA and once with 3 ml of 95% ethanol. Filters were dried under vacuum and the amount of radioactivity retained on the GF/B glass filter was determined by a scintillation counter, using Ecoscint H cocktail (National Diagnostics Inc., 305 Patton Dr., Atlanta, GA 30336). An experiment involved 6 trials, each involving 3 vessels per regimen (*e.g.*, 3 exposure, 3 sham exposure organ culture dishes). One experiment [#7] involved 12 trials.

RESULTS

Negative Control - This study was undertaken to determine if a difference in [^{3}H]-uridine incorporation could be detected under conditions known to effect transcription

(*i.e.*, low temperature). The expected decrease in uridine assimilation in the ice-treated HL-60 cells was statistically significant ($p < 0.00001$) relative to that of the 5% CO_2 incubator-treated cells at 37 °C (Table 1, No.1). It was concluded that the assay is capable of detecting changes in the rate of transcription in HL-60 cells.

Electric Field Exposure - HL-60 cells were seeded in a T-tube immediately before exposure/sham-exposure to 3.40 mV/m EF for 5 hr. The result of the one sided t-test (Table 1, No. 2) indicated no statistically significant increase in transcription rate in the EF-exposed cells relative to the sham-exposed cells.

TABLE 1. Individual Student t-tests for pooled raw counts of ^{3}H-uridine from HL-60 cells:1) Negative control, and 2-7) Exposed/Sham-exposed to 60 Hz electric (EF) or magnetic (B) fields

No.	Experimental Series	Treatment	Mean (cpm)	*S.E.M. ±	**t	P value
1.	Negative Control: 20 µCi/ml [^{3}H]-uridine					
		Ice-treated Cells	6452	1137	-20.65	<0.00001
		Incubator-treated cells	417294	19858		
2.	EF (3.40 mV/m); 4 µCi/ml [^{3}H]-uridine					
		Exposed	174335	19451	-0.02	0.51
		Sham-exposed	175008	22621		
3.	B (10 G; 1µT); 20 µCi/ml [^{3}H]-uridine; 1-hr pre-exposure incubation					
		Exposed	91615	10404	-0.42	0.66
		Sham-exposed	99174	14625		
4.	B (10 G; 1µT) ; 4.2 µCi/ml [^{3}H]-uridine; immediate exposure					
		Exposed	75175	8798	0.15	0.44
		Sham-exposed	73329	8984		
5.	B (10 G; 1µT); 4.2 µCi/ml [^{3}H]-uridine;1-hr pre-exposure incubation					
		Exposed	82737	4148	0.39	0.35
		Sham-exposed	79298	7784		
6.	B (10 G; 1µT); 4.2 µCi/ml [^{3}H]-uridine;overnight pre-exposure incubation					
		Exposed	130364	13314	-0.52	0.70
		Sham-exposed	144329	23202		
7.	B (10 G; 1µT); 4.2 µCi/ml [^{3}H]-uridine; 1-hr pre-exposure incubation					
		Exposed	250649	19033	-1.25	0.89
		Sham-exposed	284280	19065		

*S.E.M. = ± standard error of mean; **Student's t-test (one - sided) for an increase in counts per minute (cpm) for the incubator-treated or exposed cells.

Magnetic Field Exposures - Several slightly different regimens were implemented. 1) Log phase cells were seeded in the organ dishes 1 hr before exposure/sham-exposure to 10 G magnetic field, and pulsed labeled with a final concentration of 20 μCi/ml of [^{3}H]-uridine. This regimen (Table 1, No. 3) had produced the maximum effect in the Greene *et al.* (1) study. There was no statistically significant increase in the rate of transcription of exposed relative to that of the sham-exposed cells. 2) Log phase cells were seeded in the organ dishes and exposed/sham-exposed immediately to the 60 Hz 10 G (1 μT) field for 5 hr. There was no statistically significant increase in the rate of transcription in the exposed relative to that of sham-exposed cells (Table 1, No 4). 3) Cells were seeded in the organ dishes and incubated 1 hr or overnight prior to exposure/sham-exposure; [^{3}H]-uridine at a final concentration of 4.2 μCi/ml was used. The results showed no statistically significant enhancement in the rate of transcription in the exposed as compared to the sham-exposed cells (Table 1, Nos. 5 and 6). 4) As a further check 12 trials were conducted with a 1-hr delay between seeding and a 5-hr magnetic field exposure; one coil was energized for 6 trials and then the condition was reversed for an additional six trials according to a randomized scheme. There was no statistically significant difference for [^{3}H]-uridine uptake between exposed or sham-exposed regimens (Table 1, No. 7).

DISCUSSION

The hypothesis that a 60 Hz EF of 3.40 mV/m would significantly enhance the transcription rate in exposed HL-60 cells *in vitro* was formulated in a recently published report (1). The present study was undertaken to test this hypothesis by measuring the assimilation of [^{3}H]-uridine during the last 15 min of exposure/sham-exposure. Treatments initially included exposure to a 60 Hz electric field of 3.40 mV/m as a direct test of Greene *et al.*'s (1) hypothesis. Because the results of this initial test failed to support the hypothesis, subsequent experiments sought to replicate the results of the initial report by exposing the cells in organ culture dishes to a 60 Hz magnetic field of 10 G (1 μT) for 5 hr. In each experiment, there was an exposure

accompanied by a sham-exposure regimen. A one-sided t-test was used to assess the data for increase in the rate of [^{3}H]-uridine uptake in the exposed cells. From the results of six experiments, it was concluded that exposure of human promyelocytic leukemia (HL-60) cells to a specific 60 Hz electric or magnetic field did not result in an increased cellular transcription rate relative to that of sham-exposed cells. Thus, the present data do not support the hypothesis.

Whenever an attempt is unsuccessful in verifying a reported positive result from another laboratory, procedural differences could be a possible reason for the disparate results. Exact duplication of experimental conditions between laboratories is difficult to obtain. However, the hypothesis can easily be tested and a consensus of support generally is requisite for acceptance of novel findings. In fact, some effects are so readily obtainable as to be used as positive controls -- *e.g.*, X-rays and chromosomal aberrations, UV-light and DNA dimer production.

There are many similarities between the reportedly effective (1) and present protocols for ascertaining whether a specific ELF-EMF exposure of HL-60 cells *in vitro* results in increased cellular transcription. These similarities include the same: 1) cell line , 2) exposure duration, 3) concentration of radioactive tracer, 4) duration of isotope application, 5) size and type of organ culture dish for exposing cells to the magnetic fields, 6) frequency and flux density of the magnetic field, 7) type of analysis for assessment of ^{3}H-uridine uptake (scintillation counting), 8) variability in data (S.E.M = 5 -16% of mean), 9) volume of exposed medium, 10) cell concentration, and 11) cell culture medium and temperature for maintaining and exposing the cells. There were, of course, differences between the two studies: Greene *et al.* (1) used 1) ten dishes per exposure regimen, we used three, 2) up to five trials per point, we used six except for twelve in one instance, 3) no statistical evaluation, we used a one-sided Student's t test, 4) one volume of exposed medium, we used the same and an additional volume, 5) one concentration of [^{3}H]-uridine, we used the same and an additional concentration and, 6) only relative rates of [^{3}H]-uridine uptake whereas we report counts per minute (cpm). Additionally, we undertook a negative control experiment to demonstrate the assay's effectiveness in detecting a statistically significant difference in [^{3}H]-uridine uptake.

Our approach was to test the Greene *et al.* (1) regimen most likely to yield a positive result; this regimen appeared to be their 5-hr exposure of cells to a 60 Hz magnetic field of 10 G (1 μT). The reported increase was 70% above control level (their Figure 3A) and the postulated effective 60 Hz electric field was 3.40 mV/m. Thus, there were two ways to test the Greene *et al.* (1) hypothesis: expose the cells to a comparable electric or magnetic field. Neither test resulted in the expected increase in ^{3}H-uridine uptake. Other magnetic field exposures of cells in slightly different nutritional status (fresh medium, 17-hr old medium) failed to elicit an effect. A negative control analysis indicated our ability to detect differences in [^{3}H]-uridine uptake but the absence of any indication of positive effects with the purported maximally effective field intensities, exposure duration and pre-exposure delays precluded further investigation.

ACKNOWLEDGMENTS

Support for this research was provided by Rochester Gas and Electric Corporation, 89 East Ave. Rochester, N. Y. 14649. We acknowledge the cooperation of Dr. J. J. Greene in helpful telephone discussions.

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