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Brief Communication

Inhibition of Cellular Proliferation and Enhancement of Hydrogen Peroxide Production in Fibrosarcoma Cell Line by Weak Radio Frequency Magnetic Fields

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This study presents experimental data for the effects of weak radio frequency (RF) magnetic fields on hydrogen peroxide (H_2O_2) production and cellular growth rates of fibrosarcoma HT1080 cells in vitro. Cells were exposed either to 45 μT static magnetic fields (SMFs)-oriented vertical to the plane of growth or to SMFs combined with weak 5 and 10 MHz RF magnetic fields of 10 μT_{RMS} intensity perpendicular to the static field. Cell numbers were reduced up to 30% on Day 2 for the cells exposed to the combination of SMF and a 10 MHz RF magnetic field compared with the SMF control cells. In addition, cells exposed to 10 MHz RF magnetic fields for 8 h increased H_2O_2 production by 55%. The results demonstrate an overall magnetic field-induced biological effect that shows elevated H_2O_2 levels with accompanying decrease in cellular growth rates. Bioelectromagnetics 35:598–602, 2014. © 2014 Wiley Periodicals, Inc.

Key words: radio frequency; magnetic field effects; cellular growth curves; cancer cells; hydrogen peroxide; radical pair mechanism

Our previous work concentrated on low-level static field (LLF; 0.2–2 μT) effects on the response and function of endothelial cells and cancer cells [Martino et al., 2010a,b]. Static LLFs were shown to modulate cellular production of reactive oxygen species (ROS) in biological systems [Martino and Castello, 2011]. Modulation of ROS levels can potentially alter growth rates in cancer cells in vitro and in vivo [Fang et al., 2009]. ROS might accelerate DNA damage by elevating free-iron levels [Keyer and Imlay, 1996] and could play an important role for cancer cells to induce angiogenesis and tumor growth [Xia et al., 2007; Na et al., 2008]. Thus, the controlled modulation of ROS production with magnetic fields may open new venues of biomedical research and therapeutic strategies.

We investigated effects on hydrogen peroxide (H_2O_2) production with applied 5 and 10 MHz radio frequency (RF) weak magnetic fields and cellular proliferation of fibrosarcoma cells in vitro. The rationale for pursuing this work at these magnetic field intensities

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and frequencies is to validate our working hypothesis: ROS biochemical processes that involve radical pair intermediates may be influenced by magnetic fields via the free radical pair mechanism through hyperfine couplings. The radical pair singlet–triplet state product, either superoxide or H_2O_2, can have significant effects that determine the specific signaling pathway mediated by ROS [Fisher, 2009; Veal and Day, 2011]. External RF magnetic fields give rise to possible biological effects, such as changes in H_2O_2 homeostasis that may induce signaling events to mediate cellular growth rates [Usselman et al., 2014].

The magnetic field exposure conditions include control (Case I) exposed to 45 μT static magnetic fields (SMFs) along the *z*-axis (*z*-axis being normal to plane of growth of cells, *x*–*y* components were canceled), and one experimental condition: 45 μT SMF superimposed with 5 or 10 MHz, 10 μT_{RMS} horizontal RF magnetic field (Case II; Fig. 1). The rationale for these frequencies is that many biomolecules exhibit hyperfine splitting constants that range from 0.1 to 20 MHz, which most likely influence cellular chemical reactions [Cintolesi et al., 2003; Schleicher et al., 2010].

The first setup served to expose three 6-well cell culture plates to an SMF of 45 μT as a control (Case I) while the second one allowed for the simultaneous exposure to SMF and RF (Case II). A tri-axial Helmholtz coil was used to establish a uniform pre-set SMF inside the incubator. In order to superimpose electromagnetic fields in the RF band, we included a 5-turn square Helmholtz coil inside the main Helmholtz coils in one of the horizontal components of the SMF. A function generator (HP33120A; Hewlett-Packard, Palo Alto, CA) implemented the RF signal by connecting it directly to the 5-turn coil. A grounded Faraday cage surrounded the RF tri-axial square Helmholtz coil setup.

The time-varying RF magnetic field was measured by induction with a sensor comprised of two 1.5 cm turns in radius that was connected directly to an oscilloscope. The induced electric field, due to the time variation of the magnetic field, was also measured (in air): 0.1 mV/cm. The induced electric fields are two orders of magnitude below those used in bone growth applications [Pilla, 2006]. The background time-varying magnetic field at different locations inside the incubator was measured by induction in the location of the plates with a gauss meter (IDR-210; Integrity Design, Essex Junction, VT). The background noise present in the incubators where the plates rested registered between 1 to 2 μT at 60 Hz.

Fibrosarcoma HT1080 cells (#CCL-121; American Type Culture Collection [ATCC], Manassas, VA) were grown and maintained as previously described [Martino and Castello, 2011]. Cells were seeded and allowed to rest for 24 h under the same magnetic background conditions, after which timed magnetic exposures began. This time is denoted as t_0. The magnetic field exposure effects on cellular proliferation were determined by direct cell counts after each termination point. For the cell counting assay, six-well

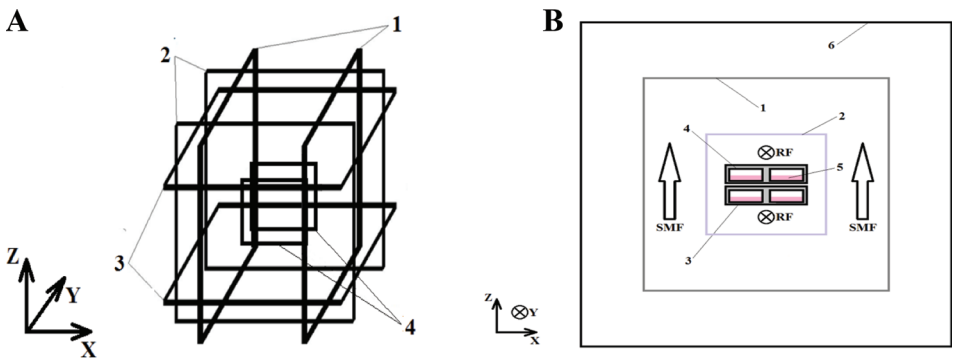
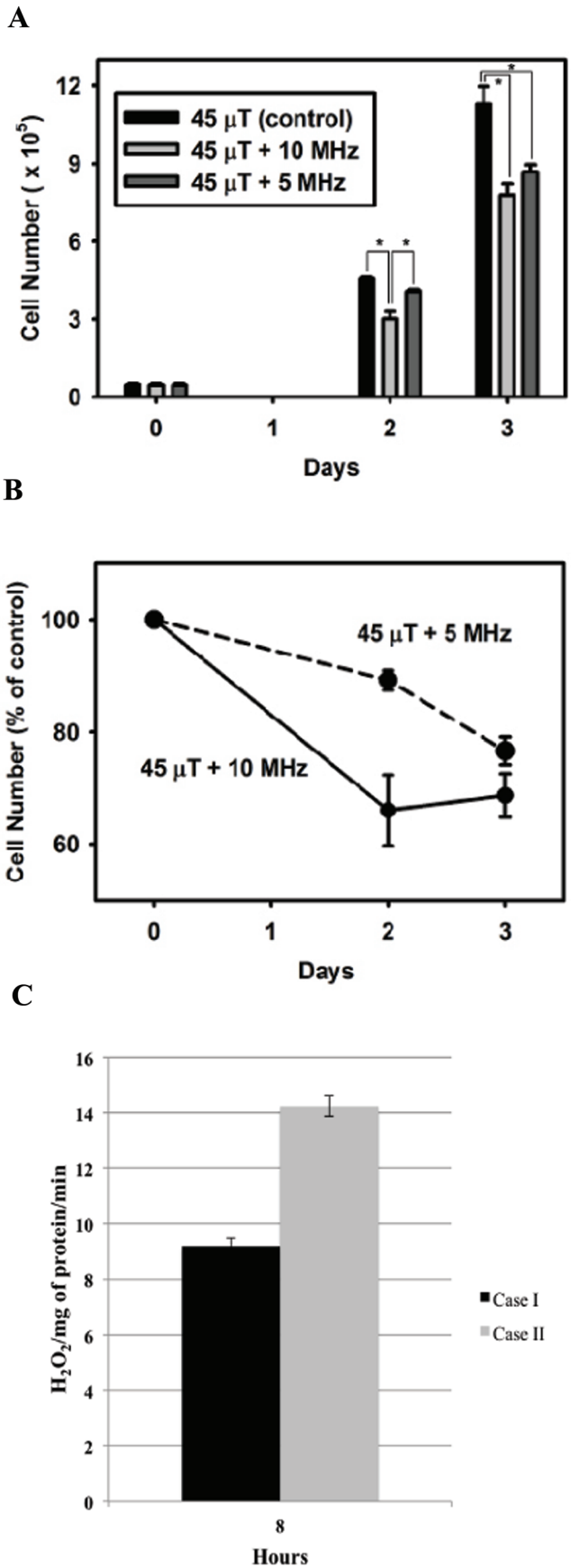


Fig. 1. The diagram shown represents the experimental apparatus for magnetic field exposure. **A**: Tri-dimensional representation of the tri-axial set used for controlling static and alternating electromagnetic fields. Square coil pairs in a Helmholtz configuration are geometrically aligned to control the static magnetic field (SMF) and to compensate for fluctuations in the ambient magnetic fields in the (1) horizontal (X) direction, (2) horizontal (Y) direction, and (3) vertical (Z) direction. This diagram also depicts the placement of a square coil in Helmholtz configuration for the generation of RF magnetic fields (4). A Faraday cage was also used in the RF experiments to surround the setup to minimize RF reflections, but it is not shown in this diagram for clarity. **B**: This figure depicts the directions of the magnetic fields with respect to the biological samples. (1) A tri-axial set of square coils in Helmholtz configuration for SMF generation in all three dimensions; (2) square coils in Helmholtz configuration for RF generation in the horizontal (Y) direction; (3) an individual six-well plate; (4) individual wells; (5) culture medium; and (6) a Faraday cage.



plates were seeded at a concentration of $2.0–3.0 \times 10^3$ cells/cm^2 for HT1080 cells. The cells in three wells per termination point were counted twice with a hemocytometer (VWR, San Francisco, CA). The trypan blue exclusion method was used to assess the cell viability.

Cellular H_2O_2 production was measured with the horseradish peroxidase-linked Amplex Ultra Red (HRP-AUR; Invitrogen, Carlsbad, CA) fluorometric assay [Martino and Castello, 2011]. Cells were exposed to Case I and Case II magnetic fields for 8 h. Resorufin fluorescence was measured by a Gemini fluorescence microplate reader (Molecular Devices, Sunnyvale, CA). H_2O_2 calibration curves with HRP-AUR in RF magnetic fields do not show any difference compared to control, thus demonstrating that RF do not interact with the detection system. Cells that were assayed for H_2O_2 were also assayed for protein concentration [Castello et al., 2008].

Statistical analysis was performed with one-way ANOVA at a minimal confidence level of 0.05 statistical significance. Each experiment was performed at least three times with a minimum of three samples per termination point, per experiment. The data shown constitutes a representative sample of the experiments performed.

For a pilot study, we obtained a magnetic dose–response to investigate the ROS and growth specificity of the RF magnetic fields. The control Case I and the experimental Case II group (5 or 10 MHz RF at 10 μT intensity) were placed under the corresponding fields inside the incubator for the duration of the exposure. The combination of static and RF fields decreased the cell numbers significantly. Trypan blue exclusion method showed no significance in staining between groups. ANOVA tests revealed that cell growth after exposure to the 45 μT + 10 MHz signal is statistically different than the cell growth at both 45 μT (control) and 45 μT + 5 MHz groups on Day 2. However, on Day 3, the cell growth at both RF signals were

Fig. 2. **A**: The effect of the 5-10 MHz RF signals was investigated. HT1080 cells were exposed continuously in the incubator under the control condition (45 μT) and exposure groups (45 μT + 5 MHz; 45 μT + 10 MHz). RF magnetic field exposure inhibits growth rates of HT1080 cells. ANOVA test showed no specific cell growth inhibition by the RF signals by Day 3 while statistically significant to control ($n = 3$, $P < 0.05$ for Day 3 for both signals on Day 3). *Statistical significance among groups considered. **B**: Inhibition of cell growth by RF signals. Number of cells were taken from panel A and plotted as % of control. **C**: RF magnetic field-induced hydrogen peroxide production in HT1080 cells. RF magnetic fields induced production of H_2O_2 by over 50% on Day 3 of exposure as determined by AUR assay. Data are representative of a sample of three independent experiments ($n = 6$).

indifferent while still statistically significant when compared to the control 45 μT (Fig. 2A and B). For the remainder of the study, the 10 MHz signal was used due to its effectiveness on Day 2.

The fibrosarcoma cells, when exposed to Case II 10 MHz magnetic fields at an intensity of 10 μT for 8 h, produced 14.24 ± 0.32 pmol of H_2O_2/mg of protein/min, while the control Case I (45 μT) produced 9.17 ± 0.37 pmol of H_2O_2/mg of protein/min (Fig. 2C). Thus, levels of H_2O_2 production increased 57% after an 8 h exposure to 10 MHz RF magnetic fields compared to the proper controls at 45 μT ($P < 0.05$). Catalase was added as a negative control at a concentration of 40 U/ml. Addition of external catalase suppressed the RF magnetic field effects on H_2O_2 production; catalase brought RF magnetic fields levels of H_2O_2 production to control levels (data not shown). Protein concentration and resorufin fluorescence were measured concomitantly, with the production of H_2O_2 was normalized to protein content.

Our present work demonstrates differential effects on cellular proliferation and H_2O_2 production of HT1080 fibrosarcoma cells exposed to RF magnetic fields. In our previous work, static LLFs inhibited growth rates of cancer cells in vitro [Martino et al., 2010b]. Moreover, reduction of the Earth's magnetic field suppressed H_2O_2 production in cancer cells and PAEC [Martino and Castello, 2011], where LLF effects were compared previously to proper controls at 45 μT. In our present work, RF magnetic fields (Case II) demonstrated a suppressive effect on HT1080 fibrosarcoma cell growth while enhancing the production of H_2O_2. Cellular viability measured by trypan blue exclusion suggested that apoptosis did not lead to reduction in cell numbers.

Extracellular quantification of H_2O_2 may be an indirect measure of the production and decomposition of superoxide [Castello et al., 2008]. Therefore, we suggest in this study that superoxide radicals may be involved in the magnetic sensitivity of HT1080 fibrosarcoma cells. The data presented here measure a possible secondary byproduct of magnetically modulated superoxide production, namely extracellular H_2O_2. H_2O_2 can freely pass through cellular membranes, where the concentration levels can be detected outside of the cell. The varying levels of H_2O_2 are an indication of oxidative stress to the cells and can be used as a metric for magnetic fields effects in ROS production. Inhibition of fibrosarcoma cell growth rates together with enhancement of H_2O_2 production provides supporting evidence for magnetic effects in spin radical pair product distribution. Elevated H_2O_2 levels might influence a biochemical pathway that involves cell growth rates, and preliminary evidence suggests a connection between H_2O_2 and superoxide RF-mediated production by the spin radical pair mechanism [Usselman et al., 2014].

We have demonstrated that relatively weak magnetic fields can alter cellular growth rates and H_2O_2 production. We showed that RF magnetic fields inhibited the proliferation of HT1080 fibrosarcoma cells in vitro. The exposure levels were below standard threshold levels of electromagnetic thermal effects. The molecular mechanism of interaction remains unclear, but we present supporting evidence that the underlying mechanism of ROS-mediated spin pairs could be altered, given the evidence that RF magnetic fields affect H_2O_2 steady-state production.

This work revealed possible oxidative stress-induced magnetic field effects. We believe that this is an important result in light of current medical interest in the plausible harmful or beneficial effects of magnetic fields. These results could open up the possibility for new therapies that take advantage of elevated levels of H_2O_2 production in response to relatively weak, magnetic field stimulations; for example, remote-controlled ROS production by magnetic field stimulations or magnetically modulated energy delivery.

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