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A Study of Radiofrequency Effects on Gene Expression

Elizabeth A. Coln
Old Dominion University

A STUDY OF RADIOFREQUENCY EFFECTS ON GENE EXPRESSION

by

Elizabeth A. Coln
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Approved by:

_____
Karl H. Schoenbach (Director)

_____
Stephen J. Beebe (Member)

_____
Linda L. Vahala (Member)

ABSTRACT

A STUDY OF RADIOFREQUENCY EFFECTS ON GENE EXPRESSION

Elizabeth A. Coln
Old Dominion University, 2002
Director: Karl H. Schoenbach

Numerous studies have been completed over the past several years to look at the non-thermal effects of low-level radiofrequency exposure on biological systems. These studies were done to determine if exposure to radiofrequency fields are safe, or if they may lead to health problems or changes to any of the biological processes continually occurring within the human body. Responses measured include stress responses (1), gene expression (2), cell proliferation (3), cell morphology (4), and changes in DNA (5).

Dispersion is the most common model for explaining the electric behavior of biomaterials. It refers to the corresponding frequency domain concept of relaxation, which is the time required for dipolar molecules to orient themselves after an excitation signal. Small dispersions have been observed in protein solutions at 0.1 to 1 GHz (6). This observation led to the design of this study in which the objective was to determine if exposure to low-level radiofrequencies, in the range of 0.1 to 1 GHz, had any non-thermal effects on gene expression.

Effects on gene expression were determined by transient transfections of human cells with two different reporter plasmids. One type contained a firefly luciferase gene coupled to a CRE element and the other contained a renilla luciferase gene coupled to a Sp1RE element. The cells were then exposed to radiofrequencies of 800, 835, and 847.74 MHz at low power levels. After exposure, the cells were analyzed for gene

expression using a quantitative dual-luciferase assay. The dual-luciferase assay measures a photon of emitted light, and the quantity of light emitted is directly proportional to the level of luciferase gene expression. The results of this study showed that there were no effects in gene expression for the firefly luciferase gene after exposure to 800, 835, and 847.74 MHz at electric field strengths of 0.53, 0.83, and 1.67 V/cm.

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INTRODUCTION

It is well known that high levels of radiofrequency (RF) energy can produce biological damage through heating effects, also referred to as thermal effects. However, it is still unclear if lower levels of RF energy, referred to as non-thermal effects, may cause adverse health effects. The objective of this study was to determine if any non-thermal effects occurred in gene expression after exposure to low power RF energy.

There are many problems to face when conducting research in this area. Much of the existing evidence is conflicting or suffering from flaws in research methods. It is often difficult for experiments to be duplicated because they are not reported in enough detail. There are also many factors that are involved when working with biological systems and it is very important to have the least amount of variables present throughout the duration of the experiments. This thesis will give an overview of electromagnetic theory and discuss dispersion theory, followed by a review of some of the literature in this field of research and then a brief biological introduction. Methods and procedures will then be discussed followed by a results and discussion section, and finally, a conclusion.

The reference model used for this work is *IEEE Transactions on Plasma Science*.

ELECTROMAGNETIC WAVES AND ENERGY

Electromagnetic radiation consists of waves of electric and magnetic energy moving together through space at the speed of light. The electric and magnetic fields in an electromagnetic wave vary together. The fields are perpendicular to each other and to the direction of the wave.

Radiofrequency radiation, extending from 3 kHz to 300 GHz, is absorbed by biological systems, which are water-dominated dielectrics heavily packed with electrolytes and complex packaged polar and nonpolar molecules. Radiofrequency (RF) radiation, especially at microwave frequencies, can transfer energy to water molecules. At extremely high radiofrequency intensities, thermal energy is generated and the intense heating can lead to severe damage and even mortality of biological systems. Biological effects that result from heating of tissue by RF energy are referred to as thermal effects. Exposure to low levels of RF radiation is referred to as non-thermal effects. In recent years, research has focused on possible effects at non-thermal levels or under conditions in which physiologic temperature can be maintained by regulatory mechanisms of living systems. Evidence for production of harmful biological effects is still uncertain (7).

Radiofrequencies are a non-ionizing form of radiation. A single photon at radiofrequencies does not possess sufficient energy to break a chemical link. Ionization occurs when there is an interaction with high levels of electromagnetic energy, including X-rays and gamma rays, causing electrons to be stripped from atoms and molecules. This process can produce molecular changes that can lead to damage in biological tissues and molecules (8).

Exposure to electromagnetic fields in the microwave region is known to impose stresses on living cells when the specific absorption rate (SAR) is beyond the threshold level required to induce heating of cells and tissues. The amount of electromagnetic energy absorbed by a living organism is determined by the dielectric properties of the tissue, cell, and biological molecules. Tissue conducts electricity. Its resistance varies with frequency and the mechanism for conduction is the movement of ions. The cellular structure and cell membranes determine the electrical properties of the tissues (7,8,9,10).

Relaxation and Dispersion

The electrical properties of biological materials have been studied ever since suitable electrical techniques became available. After 1925 potential theory, originally developed for dielectrics, was applied to biological matter such as blood, tissue, and nerves, and led to a good understanding of the β-dispersion. Around 1935, investigations began of the concept of polar molecules originated by Debye. Several years later techniques became available for investigation of the electrical properties at ultrahigh and low frequencies.

Real and imaginary impedance components of tissues and other biological materials become comparable at frequencies above 100 kHz and so relatively simple bridge techniques have been sufficient, in the radiofrequency range, to provide accuracy for both components of the impedance. The impedance technique combines many basic and practical advantages such as the use of high analytical power and minimum interference with the biological medium, which can be kept under natural conditions. Measurement techniques at ultrahigh frequencies must use transmission line and

waveguide components and are based on controlled propagation of electromagnetic waves developed in the 1950s (11).

Molecules that carry a distribution of charges that can be simulated by a dipole will rotate with an alternating field. This rotation is associated with viscous losses and therefore establishes a mechanism for conductance. Orientation of polar molecules does not occur instantaneously. The force exerted by the field is in competition with forces due to Brownian movement and only partial orientation results. Therefore, the degree of orientation increases proportionately with the applied field strength. In other words, electrical properties due to polarity of molecules are linear unless saturation effects due to extreme field values are produced. The speed of the orientation depends on the size and shape of the polar molecule, viscosity, and temperature. The characteristic frequency of the dispersion decreases with increase in molecular size and weight (11).

Relaxation theory usually does not include resonance phenomena. Resonances are not usually found in macroscopic tissue samples in the frequency range of μHz to MHz. The concept of relaxation, according to Debye (12), refers to the time required for dipolar molecules to orient themselves and is linked with a step function excitation signal. After a system has experienced a disturbance, it will relax to a new equilibrium. Relaxation occurs in the time domain, after a step increase or decrease in the electric field strength. Relaxation time is dependent on the polarization mechanism. Proteins and other large organic molecules may have a particularly large permanent dipole moment because of the large distance between the charges. The rotation and twist in proteins can be slow since they are large molecules and have complex bonding structures.

The concept of dispersion refers to the corresponding frequency domain concept of relaxation. It is the permittivity as a function of frequency. In the Debye single dispersion model, the material in question has only one relaxation process with one characteristic time constant, and the polarization increases according to an exponential curve as a function of time (6,12,13).

When a step in electric field strength is introduced to a material, the response involves the physical displacement of charges. The response can be either relaxational or resonant in nature. In the Debye relaxation model, the simplest case is when the polarization of a sample will relax towards the steady state with a relaxation time τ:

$$D(t) = D_\infty + (D_o - D_\infty)(1 - e^{-t/\tau}) \tag{1}$$

where $D(t)$ is the charge density, D_∞ is the instantaneous response after the applied step (usually only effected by instantaneous polarization mechanisms), D_o is the response long after the step is applied, and τ is the time constant of the relaxation process that depends on the physical responses involved. However, some dielectric processes are not a single time constant in nature. In real physical and biological materials, several relaxation processes might occur in parallel and the total electrical response of the material may be characterized by several time constants.

A sample may depart from a single time constant behavior due to the presence of multiple relaxation processes, each with a different relaxation time. In concentrated suspensions, electrical interactions between the suspended particles will usually lead to a distribution of relaxation times in the bulk properties even though the individual particles

might show single time constant relaxation. In the simplest case, the dielectric response arises from the presence of independent first order processes:

$$D(t) = D_{\infty} + D_1(1 - e^{-t/\tau_1}) + D_2(1 - e^{-t/\tau_2}) + \ldots \quad (2)$$

There are three relaxation processes mainly responsible for the dielectric properties of tissues: interfacial polarization (Maxwell-Wagner effects), dipolar orientation, and counterion polarization. Maxwell-Wagner effects deal with the processes at the interface of different dielectrics. In this effect, if a material is electrically heterogeneous, a dispersion occurs in the bulk properties from the charging of the interfaces within the material. This effect is not from the dielectric relaxation but is instead a consequence of the boundary conditions on the fields at the interfaces between phases. These effects are typically observed in the dielectric properties of colloids and emulsions. The time constant for Maxwell-Wagner effects is in the form of τ = RC where R is the resistance and C is the capacitance. Maxwell-Wagner effects are typically dominant at radiofrequencies.

Dipolar relaxation is another polarization mechanism involving the partial orientation of permanent dipoles. The permanent dipoles in a polar dielectric experience a torque due to an applied electric field. The actual position of each dipole is determined by the externally created torque and the thermal motion of the dipoles. After an electric field is applied, the sample will approach thermal equilibrium leading to the Debye type dielectric relaxation behavior. Viscosity effects in the dielectric are considered to hinder

the rotational movement. The relaxation time τ, which characterizes rotational diffusion, can be estimated from:

$$\tau = \frac{4\pi\eta a^3}{kT} \tag{3}$$

where η is the viscosity of the medium, a is the radius of the dipole, k is Boltzmann's constant, and T is the absolute temperature. The time constant for dipolar relaxation ranges from picoseconds for small dipolar molecules, such as water, to microseconds for large globular proteins. Globular proteins typically have relaxation frequencies in the range from 1-10 MHz. Polar side-chains on protein molecules relax at higher frequencies due to their smaller size, which has been suggested as contributing to small dispersions observed in protein solutions at 0.1 to 1 GHz.

Counterion polarization is the ionic diffusion in the electrical double layers adjacent to charged surfaces. The surfaces of membranes and proteins are highly charged; therefore, counterion effects are important. These effects are typically observed in biological systems at kHz frequencies and below. The time constants for these effects are in the form of $\tau = L^2/D$ where L is the length over which diffusion occurs and D is the diffusion coefficient.

The electrical properties of tissue and suspensions of biological cells are determined at frequencies in excess of 100 MHz by their water, salt, and protein content. They are quite similar for all tissues of high water content and blood. Measurements of the dielectric properties of protein molecules at ultrahigh frequencies were conducted in the 1940s and 1950s and showed the existence of a spectrum of characteristic frequencies

from 40 to 3000 MHz. Data was obtained by extrapolation from measurements obtained at higher frequencies, using relaxation equations with one time constant. The data indicated that parts of the protein structure possess a sufficient degree of freedom to rotate partially on their own with the field and therefore contribute to the total polarization. These groups of molecules (side-chains) within the molecular complex (protein) rotate individually at much higher frequencies than the total molecule due to the fact that they are smaller in size than the total molecule (11).

Three effects add to the conductivity of tissues at frequencies greater than 100 MHz. The first is Maxwell-Wagner effects due to interfacial polarization of tissue solids through the tissue electrolyte. Another effect that adds to the conductivity is the dielectric loss of small polar molecules and polar side-chains on proteins. Dielectric relaxation of water will also add to the conductivity. The relaxation of water is centered around 20 GHz at 25°C.

Dispersion data are based upon dielectric spectroscopy, the electrical examination of a biomaterial as a function of frequency. Three major dispersion regions can be found in typical tissues: alpha, beta, and gamma dispersion. Figure 1 shows the different dispersion regions for tissues (12). Tissues exhibit alpha dispersion at frequencies centered in the low kilohertz range. The effects of alpha dispersion can be attributed to several physical processes including polarization of counterions near charged surfaces in the tissue and possibly the polarization of large membrane-bound structures in the tissue. Other tissue-specific effects that might contribute to the alpha dispersion include active membrane conductance phenomena, the charging of intracellular membrane-bound organelles that connect with the outer cell membrane, and perhaps a frequency

dependence in the membrane impedance itself. The alpha dispersion is very apparent in the permittivity but barely noticeable in the conductivity of the tissue. Tissues are tremendously resistive even though they have such large permittivity values.

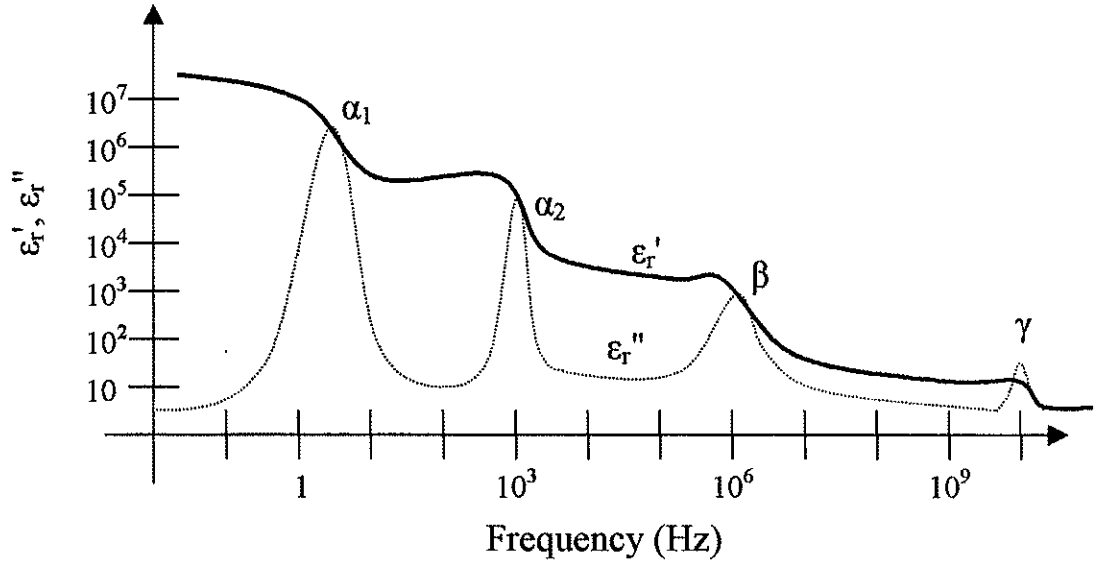


Figure 1. Dispersion regions for tissue. ε_r' represents tissue permittivity and ε_r'' represents the dielectric losses of the relaxation minus the dc conductance of the extracellular liquids found in living tissue (12).

The beta dispersion is exhibited by tissues at radiofrequencies, centered in the range of 0.1 to 10 MHz, and is apparent in both the permittivity and conductivity of the tissue. It is a Maxwell-Wagner relaxation effect. The beta dispersion is due to the polarization, or capacitive charging, of cellular membranes with the cytoplasmic and extracellular media serving as access paths for the charging current. Other contributions

include relaxation of protein-bound water, polarization of cellular organelles such as the nucleus and mitochondria, and a smaller part may come about at high radiofrequencies from the dipolar orientation of tissue protein. At low frequencies, the cell interior is effectively shielded by the cell membrane and the membrane becomes charged which results in a high induced dipole moment for the entire cell. This appears to rule out any direct interaction of external low frequency fields with the cell interior and make the membrane responsive to small field values of the order of 1 V/cm. At frequencies above the beta dispersion, the cell membranes have negligible impedance, and the current passes through both the intracellular and extracellular media.

At microwave frequencies, above 1 GHz, tissues exhibit gamma dispersion due to rotational relaxation of tissue water accounting for 80% of the volume of most soft tissues. Gamma dispersion is centered near 20 GHz at body temperature. There is another small dispersion, the delta dispersion, that overlaps both the beta and gamma dispersions. The delta dispersion has a characteristic frequency range from 0.1 to 3 GHz and is thought to be caused by a dipolar relaxation of water, rotational relaxation of polar side-chains, Maxwell-Wagner effects, and counterion diffusion along small regions of charged surfaces (6,12,13). Table 1 summarizes the different dispersion regions along with their characteristic frequencies and mechanisms.

Type of Dispersion	Characteristic Frequency	Mechanism
α	mHz - kHz	Counterion diffusion effects, dielectric losses, active membrane conductance phenomena, charging of intracellular membrane-bound organelles that connect with the outer cell membrane
β	0.1 – 100 MHz	Maxwell-Wagner effects, capacitance charging of cellular membranes in tissues, dipolar orientation of tissue protein
δ	0.1 – 3 GHz	Dipolar relaxation of water, rotational relaxation of polar side-chains, Maxwell-Wagner effects, counterion diffusion along small regions of charged surfaces
γ	0.1 – 100 GHz	Dipolar mechanisms in polar media (water, salts, proteins)

Table 1. Dielectric dispersion.

Specific Absorption Rate

Exposure to radiofrequency (RF) energy can be measured in several ways. Specific absorption rate (SAR) is the most common and represents a measure of the rate of absorption of RF energy by the body. In biological materials only the electric field contributes to energy absorption. Equation 1 represents the SAR value.

$$SAR = \frac{|E|^2 \sigma}{2\rho} = \frac{\omega \varepsilon_o \varepsilon'' |E|^2}{2\rho} \tag{4}$$

where σ is the electrical conductivity of the medium (Siemens/m), ε_o is the dielectric constant of free space (ε_o = 8.85 x 10^{-12} F/m), ε'' is the loss factor, $\omega=2\pi f$, f is the frequency in Hz, and E is the internal electric field in V/m. SAR is a simple and useful tool in quantifying the interactions of radiofrequency radiation and biological systems. The average SAR can be determined by measuring the total power absorbed in an object exposed in an electrically enclosed exposure chamber.

$$P_{abs} = P_{fwd} - P_{refl} - P_{trans} \tag{5}$$

where P_{abs} is the absorbed power, P_{fwd} is the forward power, P_{refl} is reflected power, and P_{trans} is transmitted power. SAR can also be determined through thermal measurements.

$$SAR = \frac{C\Delta T}{\Delta t} \tag{6}$$

where C is the specific heat, Δt is the exposure time, and ΔT is the increase in temperature. Equation 2 can be used for short durations of exposure (6,13).

REVIEW OF RESEARCH

Numerous studies have been conducted in which biological systems have been exposed to a wide variety of frequencies in the electromagnetic spectrum, ranging from extremely low frequencies to radiofrequencies and up to the microwave region. Biological effects have been discovered after exposure to frequencies in the radiofrequency range of 3 kHz to 1 GHz, also the region of interest for this study.

Daniells et al. (1) exposed worms, carrying a stress-inducible reporter gene, to 750 MHz and 0.5 W for 0, 2, 4, 8, or 16 hours. Worm cultures were exposed in 25-well plates in a transverse electromagnetic (TEM) cell. Stress responses were measured in terms of β-galactosidase reporter induction above control levels. β-galactosidase activity was detected by using a flourogenic MUG substrate (4-methylumbelliferyl-β-D-galacto-pyranoside) that is cleaved by the enzyme to a florescent product (4-methylumbel-liferone). Results showed a 2-fold increase in induction of reporter gene activity after a 2 hour exposure and a 2.3-fold increase after 16 hours of exposure. No significant increases were observed after 4 and 8 hour exposures. Data suggested that microwave radiation causes measurable stress to worms possibly reflecting increased levels of protein damage within cells, which is thought to be the common signal to trigger heat-shock protein (*hsp*) gene induction.

In another study conducted by Harvey et al. (2), a human mast cell line, HMC-1, was exposed to 864.3 MHz at a specific absorption rate (SAR) of 7 W/kg to determine if any non-thermal biological effects existed. Cells were exposed for 20 minutes three times daily, at four hour intervals, for seven days. Cells were exposed in a 75 cm^2 flask. The exposure chamber was a resonant chamber with a horizontal loop antenna mounted on a shelf at the mid-point of the chamber. Immunoflourescence and confocal microscopy were used to determine effects on F-actin distribution and cell morphology. Effects on gene expression were determined by a nucleic acid assay. Effects were seen on the localization of protein kinase C, as determined by western blot. There was an increase in the amount of immunoreactive protein kinase C in the membrane fraction of the exposed cells. Effects were also observed in expression of three genes, out of 588

screened, at temperatures well below those known to induce a heat shock response. A 36% increase was found in expression of the proto-oncogene *c-kit*, a 38% decrease in the expression of the transcription factor nucleoside diphosphate kinase B was found, and a 47% decrease was found in the expression of the apoptosis-associated gene, DAD-1 (defender against death). They concluded that exposure to low power microwaves may act on HMC-1 cells by altering gene expression via a mechanism that involves the activation of protein kinase C, and at temperatures well below those known to induce a heat shock response.

Kwee and Raskmark (3) exposed cell cultures of transformed human epithelial cells to 960 MHz at three different power levels resulting in SARs of 0.021, 0.21, and 2.1 mW/kg. Cell cultures were grown in 96-well microtiter plates and were exposed for 20, 30, or 40 minutes in a TEM cell. Effects on cell proliferation were determined by using a colorimetric assay based on the conversion of a tetrazolium salt by mitochondrial dehydrogenases in cells, to form a colored formazon product. They determined that a mimimum exposure time of 30 minutes was required in order to observe an effect. They found a decrease in cell growth of the exposed cells compared to that of the control and sham exposed cells for 30 and 40 minute exposures at all three SAR levels.

Donnellan et al. (4) exposed a mast cell line, RBL-2H3, to 835 MHz with a power density of 8.1 mW/cm^2 for 20 minutes three times per day, at four hour intervals, for seven days. They examined the effects of exposure on cell proliferation, F-actin distribution, cell morphology, and secretion of β-hexosaminidase. RBL-2H3 cells were maintained as an adherent monolayer. The exposure chamber consisted of an aluminum box with a circular loop antenna mounted horizontally at the center of the box. The

chamber was essentially a microwave cavity and so it had resonance modes. Immunoflourescence and confocal microscopy were used to determine effects of exposure on F-actin distribution and cell morphology. SDS-polyacrylamide gel electrophoresis was used to determine effects on protein expression. No differences were observed in protein expression between the control and the exposed samples. A 1.7-fold increase in β-hexosaminidase secretion was seen after 7 days of exposure. They also observed that exposure to these cells led to changes in cell proliferation, F-actin distribution, and cell morphology. Exposed cells showed increased cell spreading and prominent cell surface ruffles compared with the control. Changes in morphology, secretion, and proliferation occurred in the absence of obvious changes in protein expression and in the absence of heating of the cell culture medium and may have suggested a cumulative effect of microwaves at this power density. They proposed that exposure to 835 MHz may have affected molecules associated with the signal transduction pathways in those cells.

In studies conducted by Ivaschuk et al. (14), rat PC12 pheochromocytoma cells, treated with nerve growth factor, were exposed to a radiofrequency field of 836.55 MHz at power densities of 0.09, 0.9, and 9.0 mW/cm^2 with corresponding input powers of 17 mW, 0.17 and 1.7 W, respectively. Cells were exposed in 100 mm petri dishes in 20 mls of medium. Exposures were performed in a TEM cell with exposure times of 20, 40, and 60 minutes. The electric field was normal to the petri dishes, which were placed on the septum in order to receive a uniform electric field. After exposure, total cellular RNA was extracted and northern blot analysis was performed to assess the expression of the genes c-*fos* and c-*jun*. A significant effect was detected in the transcript level of c-*jun*.

They observed a 39% decrease after a 20 minute exposure to 9 mW/cm^2. They suggested that this change might be of interest because it may lead to a determination of the mechanism by which radiofrequency exposure can alter gene expression.

Mouse C3H 10T½ fibroblasts and human glioblastoma U87MG cells were exposed to cellular phone communication frequencies to investigate any damage to DNA in *in vitro* cultures (5). Cultures were exposed to an 835.62 MHz frequency-modulated continuous wave (FMCW) for varying periods up to 24 hours. Exposures occurred in specially designed radial transmission lines (RTLs) that provided a relatively uniform exposure with a SAR of 0.6 W/kg. The RTL is a non-conventional electromagnetically shielded irradiator design consisting of a parallel-plate region driven by a centrally located conical antenna and terminated by microwave-absorbing material that is backed by aluminum. The electromagnetic waves propagate radially outward, applying an electric field normal to the cell layer inside each flask. The alkaline comet assay was used to measure DNA damage. Their results indicated that exposure of cultured mammalian cells to cellular phone communication frequencies under those conditions do not cause DNA damage as measured by the alkaline comet assay.

Goswami et al. (15) wanted to determine if radiofrequencies of 835.62 and 847.74 MHz, at a SAR of 0.6 W/kg, could elicit a general stress response in C3H 10T½ mouse embryo fibroblasts. Cells were exposed in specially designed radial transmission lines (RTLs). Changes in proto-oncogene expression were detected by measuring *fos*, *jun*, and *myc* messenger RNA (mRNA) levels, as well as by the DNA-binding activity of AP1, AP2, and NF-κB transcription factors. No effect was observed on the *jun* and *myc* mRNA levels or on the DNA-binding activity of the AP1, AP2, and NF-κB in

exponential cells in transit to plateau-phase growth. The exponential phase represented 30-40% confluent, transition to plateau phase represented 70-80% confluent, and the plateau phase represented 100% confluent at the beginning of microwave exposure. A 2-fold increase in *fos* mRNA for exponential and plateau phase cells exposed to a 835.62 MHz frequency-modulated continuous wave (FWCW) was observed, and a 1.4-fold increase was detected for cells in transit to the plateau phase exposed to 847.74 MHz was observed. Exposure of exponential and plateau phase cells to an 847.74 MHz code division multiple-access (CDMA) showed 1.4-fold and 1.9-fold increases in *fos*, respectively. The increase in *fos* expression could suggest that expression of specific genes could be affected by radiofrequency exposure.

The research that has been discussed in this section provides evidence that exposure to radiofrequencies do have effects on biological systems. Effects were observed on gene expression, cell proliferation, and cell morphology. The specific frequencies used in this study were based on this research and because the frequency range also falls into the range in which small dispersion of proteins have been observed (6). Based on this review of research, the experimental setup and design of this thesis evolved.

BIOLOGICAL REVIEW

Introduction to Biomolecules: Proteins and Nucleic Acids

Proteins can be classified as either fibrous or globular. Fibrous proteins are generally water insoluble, built of repetitive structures, and usually have static functions. They have elongated structures and are major structural components of cells and tissues.

Globular proteins are compact, roughly spherical macromolecules whose polypeptide chains are tightly folded. Their structure allows them to selectively bind other molecules. Globular proteins include most enzymes and most of the proteins involved in gene expression and regulation. They are generally dynamic and have the ability to catalyze reactions or change conformation. Nucleic acids are polymers, nucleotides, or phosphorylated nucleosides, that are covalently joined together to produce macromolecules. Two linear polymers of nucleotides combine to form deoxyribonucleic acid (DNA). DNA molecules serve as the repository of genetic information while ribonucleic acid (RNA) molecules participate in several processes by which genetic information is translated into functional proteins.

Nucleic acids are very large molecules, or macromolecules, and are responsible for passing on genetic information. The information that specifies the primary structure (i.e., functions) of proteins is encoded in the nucleotide sequence of DNA. Nucleic acids carry the information, while proteins provide the means for executing it. Each protein consists of a unique sequence of amino acids. These amino acids are joined together to form a chain by peptide bonds, which are created by the bonding of the carboxyl (COOH) group of one amino acid to the amino (NH_2) group of the next, and a water molecule (H_2O) is released (see figure 2). Since peptide bonds are covalent, they are relatively stable. Functions of many proteins can be explained based on their conformational structure, a spatial arrangement of atoms that depend on the rotation of a bond or bonds, and can change without the breaking of covalent bonds (16,17).

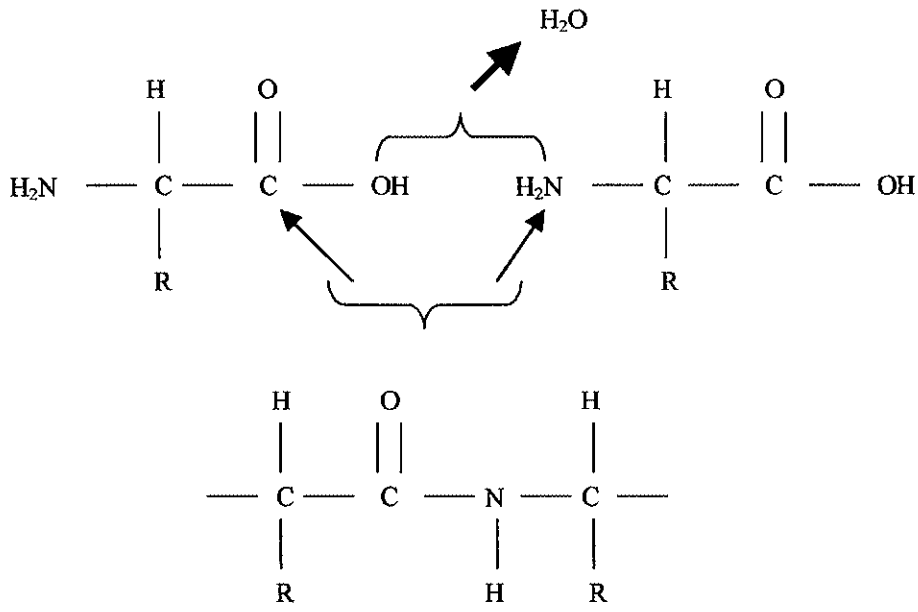


Figure 2. A peptide bond. Two amino acids are joined and water is released to form a peptide bond. R represents side chains.

Environmental changes or chemical treatments may cause a disruption in the native conformation of a protein with an associated loss of biological activity, often referred to as denaturation. The energy needed to cause denaturation is usually small, perhaps equivalent to the disruption of three or four hydrogen bonds. A protein can be denatured in several ways, most commonly by a change in the pH or by heating. Changing the pH can change the ionic state of ionizable side chains leading to the breaking of hydrogen bonds and in turn, creating regions of charge repulsion and disruption of ion pairs. Heating a protein solution causes an increase in vibrational and

rotational energy that can disturb the balance of weak interactions that stabilize the functional, folded conformation.

There are four groups of amino acids classified by their ionic charges: basic, acidic, neutral, and apolar neutral. One modification that can change the ionic charge of an amino acid is phosphorylation, which is the addition of a phosphate group, usually to the hydroxyl group of serine, tyrosine, or threonine. The phosphate group introduces negative charges, and therefore has a significant effect upon the electrostatic properties of the protein, often causing a conformational or functional change of the protein.

The biological properties of proteins are mainly determined by non-covalent interactions (hydrogen bonds, hydrophobic interactions, ionic bonds, and van der Walls interactions) that result in each molecule acquiring a unique three-dimensional structure. Each protein in a cell carries out a particular function which may be structural, enzymatic, or regulatory. Because of the folding of the polypeptide chain, the side chains produce a unique surface configuration of charged and hydrophobic groups. Usually, a localized portion of the surface, called a binding site, is responsible for an interaction between the protein and other molecules.

The forces responsible for maintaining the native conformations of complex cellular structures such as nucleic acids, proteins, and membranes are strong enough to maintain the structures, but weak enough to allow conformational flexibility. Covalent forces define the primary structures of these molecules while weak forces dominate the secondary structures or folded forms. The major forces that influence the stability of double-stranded DNA are hydrophobic interactions, base stacking, hydrogen bonding, and electrostatic repulsion. Most genomes are made up of double stranded DNA, which

is composed of two single-stranded polynucleotides. Each base of one strand is hydrogen bonded to a base pair of the opposite strand, forming a base pair. Guanine (G) pairs with cytosine (C), and adenine (A) with thymine (T). Hydrophobic interactions make an important contribution to the overall stability of double-stranded DNA. The stacked base pairs form van der Waals contacts in the interior of the molecule. In addition, an induced-dipole effect enhances stacking interactions. The strength of these interactions is base dependent, G/C base pairs are stronger than those involving A/T base pairs (16,17).

Gene Expression: Transcription and Transcription Regulation

Genetic information carried by DNA is expressed in two stages: transcription of one DNA strand into messenger RNA (mRNA), and translation of the mRNA into protein. Transcription is the process of generating a single-stranded RNA identical in sequence with one of the strands of the duplex DNA. mRNA is synthesized and matures in the nucleus and then moves to the cytoplasm where it functions. Translation is then accomplished by reading the genetic code. Each triplet of nucleotides of the mRNA is converted into one amino acid, and this process continues in order to make up a specific protein.

A eukaryotic transcription unit generally contains a single gene. A gene is the segment of DNA involved in producing a protein polypeptide chain. Genes are classified by their promoters, regions of DNA involved in the binding of RNA polymerase to initiate transcription by unique transcription factors. Each promoter contains characteristic sets of short conserved sequences (*cis*-acting elements) that are recognized by the appropriate class of factors (*trans*-acting elements) and/or RNA polymerase.

Promoters for RNA polymerase II, the major enzyme responsible for synthesizing the structural genes for mRNA, contain a variety of *cis*-acting elements, each of which is recognized by a *trans*-acting element. The *cis*-elements are located upstream of the TATA box, a septamer rich in adenine (A) and thymine (T) nucleotides, found approximately 25 base pairs before the startpoint of each eukaryotic transcription unit. The startpoint refers to the position on DNA corresponding to the first base incorporated into RNA.

Transcription factors have primary responsibility for recognizing the characteristic sequence elements of any particular promoter, and they serve in turn to bind the RNA polymerase and to position it correctly at the startpoint. At each type of promoter, the initiation complex is assembled by a series of reactions in which individual factors join (or leave) the complex. A competent transcription complex is formed when a promoter sequence in the DNA is recognized by one or more proteins that bind to the promoter and also to RNA polymerase. These DNA-binding proteins therefore assist RNA polymerase in binding tightly to the promoter. These proteins are known as transcription factors. Termination of transcription is signalled by information contained at sites in the DNA sequence being transcribed. RNA polymerase II transcribes all genes whose products are destined to be translated into proteins.

The phenotypic differences that distinguish the various kinds of cells in a higher eukaryote are largely due to differences in the expression of genes that code for proteins. Expression of these genes may be regulated at any one of the following stages: activation of gene structure, initiation of transcription, processing the transcript, transport to the cytoplasm, stability of RNA, or translation of mRNA. In characterizing groups of genes

under common control, the principle that emerges is that they share a promoter element (*cis*-element) that is recognized by a regulatory transcription factor (*trans*-activator). A DNA element that causes a gene to respond to such a factor is called a response, or *cis*-, element (RE). Response elements have the same general characteristics as upstream elements of promoters or enhancers, which are sequences involved in binding RNA polymerase to initiate transcription or sequences that increase the utilization of some promoters, respectively. They contain short DNA consensus sequences. Copies of the response elements found in different genes are closely related, but not necessarily identical. Binding of factors to specific sequences is followed by protein-DNA interactions and then by protein-protein interactions with other components of the general transcription complex.

A gene is regulated by a DNA sequence at the promoter or enhancer site (*cis*-element) that is recognized by a specific *trans*-activator protein (*trans*-element). The protein functions as a transcription factor needed for RNA polymerase to initiate. Active protein is available only under conditions when the gene is to be expressed; its absence means that the promoter is not activated by this particular system. *Trans*-activators determine when genes are expressed.

Regulation of gene expression can take place at any point in the flow of biological information but occurs most often at the level of transcription. The initiation of transcription of regulated genes is controlled by the action of special regulatory proteins that are gene specific. Regulatory proteins are required in addition to the general transcription factors. These proteins regulate gene expression by binding to DNA, usually near the promoters or enhancers of the genes they control. Genes that are

regulated at the level of transcription initiation by regulatory proteins are of two basic types: negatively and positively regulated genes. Transcription of a negatively regulated gene is prevented by regulatory proteins called repressors and can be transcribed only in the absence of an active repressor. Transcription of a positively regulated gene can be activated by regulatory proteins called activators and is transcribed poorly or not at all in the absence of its activator. Transcription of some genes is regulated by multiple regulatory proteins that can associate with each other to form a complex at the promoter (16,17).

CREB and Sp1 Regulation

Cyclic AMP response element (CRE) binding protein (CREB) activates transcription of target genes in response to many different stimuli, including peptide hormones, growth factors, and neuronal activity. A variety of protein kinases can activate CREB regulated genes, including protein kinase A, mitogen-activated protein kinases, and Ca^{2+}/calmodulin-dependent protein kinases. All signaling pathways that activate CREB lead to phosphorylation of a particular residue, serine 133 (Ser133). Phosphorylation of Ser133 is required for CREB-induced gene transcription. CREB is critical for a variety of cellular processes, including proliferation, differentiation, and adaptive responses. CREB activity is important for hormonal control of metabolic processes, including regulation of glucogenesis by the hormones glucagon and insulin. It also functions in growth-factor-dependent cell survival and has been implicated in learning and memory, among many others (18,19,20).

Sp1 is also a transcription factor found in most cells. It prefers DNA sequences that are rich in the nucleotides guanine (G) and cytosine (C) and is involved in the regulation of the insulin receptor gene promoter, the human monoamine oxidase gene, and the glucose transporter-1 gene expression during rat trophoblast differentiation (21,22,23). Sp1 can also serve as both a positive and a negative regulator for the expression of hepatitis B virus (HBV) genes (24).

To investigate CREB regulation, reporter plasmids with a cyclic-AMP response element upstream of a firefly luciferase gene, pCRE-*fluc* (CRE), are transiently transfected into intact human cells. After transfection, CREB proteins, which are already present in the cells, will bind to the promoter regions of the CRE reporter plasmids (*cis*-elements). CRE is a response element; a specific nucleotide sequence that has the function of binding to a specific protein, CREB. This leads to the activation of transcription of a specific gene, firefly luciferase. The *trans*-element, CREB, acts as a dimer to bind to its response element, CRE (i.e., two CREB proteins will bind together). Once the CREB proteins are bound, they are phosphorylated at a single phospho-acceptor site, Ser133. This promotes transcription by recruiting the co-activator CREB-binding protein (CBP). CBP mediates transcription and allows for RNA Polymerase II to then bind and the process of making messenger RNA (mRNA) begins. Translation then begins in which a protein is synthesized on the mRNA template. The new protein, luciferase in this case, spontaneously folds to its natural conformation. The luciferase protein can then bind and cleave its specific substrate, luciferin, which releases light in a quantitative manner.

To investigate Sp1 regulation, reporter plasmids with a Sp1 response element upstream of a renilla luciferase gene, pSp1RE-*rluc* (Sp1RE), are transiently transfected into intact human cells. After transfection, Sp1 proteins, which are already present in the cells, will bind to the promoter regions of the Sp1RE reporter plasmids (*cis*-elements). The *trans*-element, Sp1, acts as a monomer to bind to its response element, Sp1RE. Once the Sp1 protein is bound, it must be phosphorylated and additional transcription factors will bind, not necessarily in that order. It then follows a similar transcription/translation pathway as CREB except that it will activate transcription of the renilla luciferase gene and the protein produced will bind and cleave the substrate coelenterazine, causing light to be released.

Theory of Electromagnetic Energy Effects on Biological Systems

In recent years, considerable effort has been devoted to defining a mechanism by which electromagnetic fields (EMFs) can couple to biological systems and generate so many effects. As a consequence, there has been a growing interest in EMF-induced alterations in gene expression.

Most biological molecules have some charge and are polarizable and can undergo an interaction with a local electric field. Biological structures in which the electric field will concentrate, like the cell membrane, are viewed as the most likely site of interaction. However, non-membrane macromolecules having a significant charge (e.g., DNA) can also be considered. One hypothesis is that combined chemical and electric field exposures might together alter biological systems. This means that the energy needed to cause biological changes does not necessarily need to come entirely from electric or

magnetic field energy. Instead, changes in chemical free energy can also contribute to these biological changes.

Menendez (25) hypothesized that the manner of folding of the protein chain can be altered if the protein synthesis is carried out under different environmental conditions. It is known that protein synthesis can be divided into three stages: (1) polypeptide chain intitiation, (2) chain elongation, and (3) chain termination. Menendez suggested that during the elongation stage of protein synthesis, when the chain is growing, torsions will be influenced by the interactions between the electrically charged groups from the amino acids (mainly from its side chains) and the electromagnetic and gravitational fields of the environment. Under different environmental conditions, some torsions could be more favored than others and therefore, non-covalent interactions may have to be established between parts of the polypeptide chain that may not be involved under usual conditions. Obviously, that would alter the manner of folding of the chain and thus, their three-dimensional structures and biological properties.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

In these studies, human cells were exposed to three different radiofrequencies at three different power levels to determine if there were any effects on CREB- and Sp1-regulated gene expression. The cells were exposed in a transverse electromagnetic (TEM) cell to frequencies of 800, 835, and 847.74 MHz. These specific frequencies were selected based on a review of previous research where effects on biological systems have been found.

Figure 3 shows a block diagram of the exposure system. The transverse electromagnetic (TEM) cell consists of a rectangular transmission line operating in the transverse electromagnetic mode. The cell is tapered at each end to a transition section that includes type N coaxial jacks for signal connection. The transmission line and tapered transmissions have a nominal characteristic impedance of 50 ohms, along their length, to ensure a minimum voltage standing wave ratio (VSWR), the ratio of maximum to minimum voltages along the transmission line. The electromagnetic field is developed inside the cell when radiofrequency energy is coupled to the transmission line from a transmitter connected at the cell input port. A matched 50-ohm load termination is connected to the output port. The size and precision design of the TEM cell allow for accurate maintenance and measurement of electromagnetic field levels (within +/- 1dB) without antennas. In addition, the typical VSWR is less than 1.25, where a VSWR of 1 represents a matched load. The electromagnetic field generated inside the cell can be calculated from E=V/D, where E is the electric field strength in volts/meter, V is the

input voltage to the cell in volts, and D is the distance between the septum and the top plate in meters (26). The upper limit of frequency for this TEM cell (2 GHz) is defined as the point of first resonance. Only the TEM mode can propagate over the entire rated frequency range of the cell. The TEM cell is designed so that it gives a homogenous exposure. It produces a spatially uniform exposure over the sample volume. To ensure that the test object sees a uniform field, its dimensions must remain within the maximum test object size of 2.36 in. x 2.36 in x 0.75 in. The test sample is literally placed within the space between the center conductor and outer conductor of the coaxial cable.

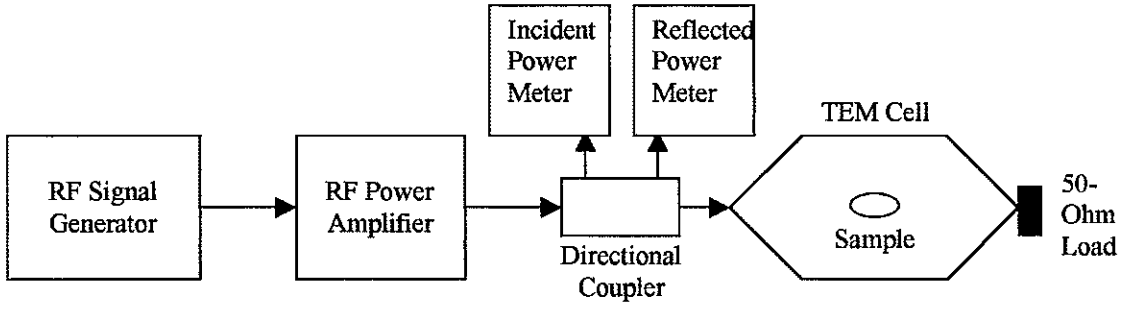


Figure 3. Block diagram of exposure system.

There are several advantages of using the TEM cell over other methods of radiofrequency exposure. The TEM cell is small and readily transportable, it is relatively inexpensive, it eliminates resonance problems, it allows the operator to remain outside of the test area, and it allows the measuring equipment and signal sources to be outside of

the test cell. The TEM cell manufactured by Instruments for Industry, model CC110EXX, works in the frequency range of DC – 2000 MHz. The TEM cell was terminated by a 50-ohm load, also provided by Instruments for Industry, which can handle up to 50 W and up to 4000 MHz.

The RF synthesized signal generator was manufactured by Leader Instruments, model LG3226. It has a frequency range of 100 kHz-2000 MHz and an output of –133.0 to +13 dBm. The RF power amplifier was manufactured by Kalmus, model 706FC-CE. It has an output of 6 W, a bandwidth of 0.5 – 1000 MHz and a gain of +36 dB. The high power directional coupler was manufactured by Microwave Communications Laboratories, Inc., model HDL-2-30/NMO. It covers a frequency range of 10 – 1000 MHz and has a coupling of 30 dB. The detector is a HP432A power meter, manufactured by Hewlett Packard and is used with a HP478A thermistor mount sensor. All connections were made with 18-inch RG 142 B/U coax double-shielded cables, purchased from Pasternack Enterprises.

In these experiments, the human cells were exposed in the TEM cell to frequencies of 800, 835, and 847.74 MHz. The input powers (into the TEM cell) used were 0.2, 0.5, and 2.0 W, equivalent to electric field strengths, at the sample, of 0.53, 0.83, and 1.67 V/cm, respectively. The electric field strength was calculated from $E = V/D$ where $V = (P*Z)^{1/2}$ where P represents the input power (W) into the TEM cell and Z represents the impedance of the TEM cell (ohms), as shown in equation 7. In the TEM cell model used, D is 0.06 meters and Z is 50 ohms. Each sample was exposed for 30 minutes.

$$E = \frac{V}{D} = \frac{\sqrt{P * Z}}{D} \tag{7}$$

Transfection Methodology

Human embryonic kidney cells (HEK-293) were used in these studies. They were transiently transfected with specific reporter plasmids, used as internal sensors, to detect gene expression. Transfection is the general process of bringing foreign DNA into cultured cells in order to monitor gene and protein expression. DNA transfection is essential for studying gene function and regulation. The transfection reagent is a multi-component lipid based transfection reagent that complexes with and transports DNA into the cell during transfection. HEK-293 cells were chosen primarily because they were readily available and they transfect well.

Reporter Plasmids

In these studies, the reporter plasmids, pCRE-*fluc* (CRE) and pSp1RE-*rluc* (Sp1RE), have been used as a sensor system and were transiently transfected into the HEK-293 cells. pCRE-*fluc* (CRE) contains 168 bases upstream of the α-glycoprotein hormone receptor promoter and was very generously given as a gift by S. McKnight. pSp1RE-*rluc* (Sp1RE) contains the herpes simplex virus thymidine kinase promoter and is 750 bases in length (Promega). Plasmids are self-replicating, circular DNA molecules. The plasmids for CRE and Sp1RE promoters, as shown in figure 4, are coupled to the firefly luciferase and renilla luciferase genes, respectively. Through gene expression, the luciferase gives off a quantum of light that can be quantitatively measured. The

expression plasmids, pCMV-PKACα (Cα) and pCMV-PKACγ (Cγ), are used as a positive control system to show that this model system is working. pCMV-PKACα (Cα) is the expression plasmid for the alpha isoform of cyclic AMP (cAMP) – dependent protein kinase catalytic subunit and pCMV-PKACγ (Cγ) is the expression plasmid for the gamma isoform of cAMP-dependent protein kinase catalytic subunit. Cα activates CRE-regulated transcription while Cγ activates Sp1RE-regulated transcription (19). The firefly and renilla luciferase genes are commonly used in expression assays because they can be detected with high sensitivity.

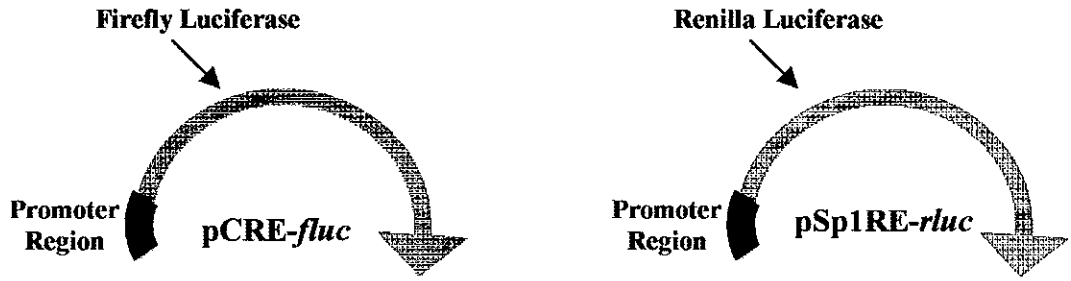


Figure 4. Reporter plasmids transfected into HEK-293 cells.

Human embryonic kidney cells (HEK-293) plate flat. They are an adherent cell line and are "fibroblast-like." HEK-293 cells contain CREB, but not Sp1. Therefore, pCRE-*fluc* serves as a test reporter and pSp1RE-*rluc* acts as an internal control (27). This

specific model system was chosen because CREB is a very common protein in the human body and plays an important role in many different genes. Sp1 was used because it could serve as an internal control.

Assay Design

The luciferase gene is widely used as a reporter gene to study the regulation of gene transcription. The assay for luciferase bioluminescence is very sensitive and background luminescence is very low in mammalian cells, allowing the detection of low levels of gene expression (28).

The MLX Microtiter Plate Luminometer (Dynex Technologies, USA) was used for the expression assay. It automates the initiation of a reaction, measures the light that is emitted, and calculates the assay results that are obtained when using luminescence technology and microtiter plates.

Light emitting chemical reactions and biological reactions have a wide range of analytical applications, ranging from detection of small molecules to the assay of large protein complexes. The type of assay used in these experiments was a dual flash assay - a compound flash assay that is triggered twice by using two different reagents. The intensity of emitted light is used to obtain quantitative measurements. A series of standards is run (blanks), and a calibration curve is prepared relating the resulting light intensity to the level in each standard. The intensity of light obtained for each sample is compared to the intensity of emitted light for the standards, and the level of each sample is then calculated.

The Dual-Luciferase Reporter Assay System (Promega) was used in these studies. It is a dual reporter gene assay that uses both the firefly and the renilla luciferase. The

firefly luciferase is used to measure the experimental sample while the renilla luciferase is used as a control against which the experimental sample can be normalized. By normalizing the activity of the experimental reporter, inherent variabilities of reporter assays, such as differences in cell number or viability, transfection and lysis efficiency, and volume transfer, are effectively eliminated (29).

The activities of firefly and renilla luciferases are measured sequentially from a single sample by adding specific substrates. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (luciferin) to generate a glow-type luminescent signal. After the firefly luminescence is quantified, this reaction is quenched, and then the renilla luciferase reaction is initiated by adding Stop & Glo Reagent (coelenterazine) to the same sample. This reagent also produces a glow-type signal from the renilla luciferase, which decays slowly over the course of the measurement. Renilla luciferase is generally used as a control. It can respond to some signals, but most likely not in these experiments because HEK-293 cells do not have Sp1.

Human embryonic kidney cells (HEK-293) do not have the luciferase protein or luciferase substrates. In these experiments, this protein is synthesized by transfecting the firefly luciferase gene or renilla luciferase gene activated by CREB or Sp1, respectively. In these experiments, the luciferase substrates, luciferin or coelenterazine, were added. Once the luciferase protein recognizes the substrate, it breaks a chemical bond. When this bond breaks, energy is released in the form of a photon. This photon is measured by the luminometer, giving quantitative results. Once the luciferin is cleaved, it is no longer recognized by the luciferase protein. The chemical reaction is shown in figure 5, where * represents the energized form of the protein.

a) $\text{Luciferin}^* + \text{ATP} + O_2 \longrightarrow \text{Luciferin} + \text{AMP} + PP_i + CO_2 +$ hv

b) $\text{Coelenterazine}^* + O_2 \longrightarrow \text{Coelenterazine} + CO_2 +$ hv

Figure 5. Chemical reactions for luciferin and coelenterazine. a) Chemical reactions for luciferin after recognition by the firefly luciferase protein. b) Chemical reaction for coelenterazine after recognition by the renilla luciferase protein.

METHODS

System Measurements

Prior to any equipment testing or exposure experiments, the equipment was setup as shown earlier in figure 3. The HP432A power meter was set to read zero by clockwise rotation of the meter adjustment screw, and the mount resistance switch was set to 200 ohms. The power meter was then turned on for at least one hour before any further use. This was to warm up the thermistor sensor so that it was stabilized and the power meter readings would be more accurate. Once the thermistor was warmed up, the power meter was then zeroed by setting the range selector to the course zero position and adjusting the screwdriver adjustment until the meter indicated zero. The range selector was then set to the desired range of use and the fine zero switch was depressed until the meter indicated zero. The accuracy of the power meter was +/- 1% of the full scale on all ranges.

The RF signal generator was turned on and set to the desired frequency of 800, 835, or 847.74 MHz. The amplifier was turned on prior to taking measurements. The RF

power amplifier had a minimum power gain of 36 dB. There were no adjustments on the amplifier so the power had to be controlled through the RF signal generator. The radiofrequency signal passed through the amplifier and then through the 30 dB directional coupler. To determine the input power, 30 dB was added to the dB reading on the power meter. For an input power (into the TEM cell) of 0.2 W (23 dB), the output of the signal generator was adjusted so that the reading on the power meter was –7 dB (-7 + 30 = 23 dB). For input powers of 0.5 W (27 dB), 2.0 W (33 dB), and 5.0 W (37 dB), the ouptuts of the signal generator were adjusted so that the readings on the power meter were –3 dB, +3 dB, and +7 dB, respectively. Reflected powers were also measured. These powers refer to the radiofrequency signals that went into the TEM cell and then were reflected back. For these measurements, the power meter was attached to the reflected power port of the directional coupler. These measurements were very small, in the order of μW, and were very difficult to read on the range of this power meter. Transmitted power measurements were also measured. The 50-ohm load was removed from the ouput port of the TEM cell and the power meter was connected. However, the maximum power that the power meter was capable of reading was 10 mW, and the transmitted powers exceeded that limit. An attenuator, or other device, was not available to reduce the amount of power going to the power sensor in order to get an accurate measurement for transmitted power. Due to the difficulty in determining both reflected and transmitted powers, only the electric field strength, at the location of the sample, was able to be calculated from these measurements for these specific experiements.

Cell Culture and Treatment Conditions

Human embryonic kidney cells (HEK-293) were maintained, in T-75 flasks, as an adherent monolayer in a humidified atmosphere at 37°C in 5% CO_2. The normal growth medium contained Dulbecco's Modification of Eagle's Medium (DMM) with 4.5 g/L glucose, L-glutamine, and without sodium pyruvate (Cellgro). This medium was also supplemented with 10% foetal bovine serum (FBS) and 1% L-glutamine. The cells were detached with trypsin (0.25%)/EDTA (0.1%) solution (Cellgro) and subcultured as needed. Cells were used only during early passages and were not passaged more than twelve times.

Experimental Protocols

NovaBlue competent cells (Novagen) were transformed with reporter genes pCRE-*fluc* (CRE) and pSp1RE-*rluc* (Sp1RE), and/or positive control plasmids pCMV-PKACα (Cα) and pCMV-PKACγ (Cγ). The NovaBlue is a strain of *E. Coli* that is ideally suited as an initial cloning host because of its high transformation efficiency which results in high yields of excellent quality plasmid DNA. The procedure was as follows: 50 µl of competent cells (Novagen) were thawed on ice. 1 µl of purified CREB DNA was added, gently mixed, and incubated on ice for 5 minutes. The tube was then heated for exactly 30 seconds in a 42°C water bath and then placed on ice for 2 minutes. 250 µl of SOC medium was added and then several different concentrations were plated on LB plates containing 1.5 g ampicillin. Plates were incubated overnight at 37°C.

Following transformation, a plasmid prep was done to isolate plasmid DNA. Plasmid purification was performed using the Qiagen Plasmid Maxi Kit protocol (30).

Essentially, this protocol is based on an alkaline lysis procedure of bacteria, followed by binding of plasmid DNA to Qiagen Anion-Exchange Resin under appropriate low-salt and pH conditions. Chromosomal DNA, RNA, proteins, dyes, and low-molecular weight impurities are removed by a medium-salt wash. Plasmid DNA is then eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation.

All of the cell culture procedures were done under the hood and under sterile conditions. To seed petri dishes for exposure experiments, T-75 flasks were removed from the incubator. The media was removed, and the cells were washed with 5 mls of Hank's solution (Cellgro), a salt buffer. The Hank's solution was then removed and the cells were trypsinized with 1 ml of trypsin (0.25%)/EDTA (0.1%) solution (Cellgro). 4 mls of growth media was added. The cells were removed from the flask and then counted using a hemacytometer and trypan blue dye. 6.0×10^5 cells (determined to be the most efficient cell density for these experiments) were seeded in 60 mm cell culture dishes (Corning). Growth media was then added up to a total volume of 4 mls. Culture dishes were then incubated at 37°C, 5% CO_2 for 20-24 hours, until plates reached 70-80% confluency.

For temperature experiments, the cell culture dishes were removed from the incubator after 20-24 hours, the media was removed, and 1.5 mls of fresh DMM was added. Experiments were done in sets of four, one control and three exposed samples. Samples were exposed for 30 minutes to 800, 835, and 847.74 MHz at input powers (into the TEM cell) of 0.5, 2.0, and 5.0 W equivalent to electric field strengths, at the sample, of 0.83, 1.67, and 2.64 V/cm, respectively. Temperatures were measured, using a thermometer, before and after exposures.

For cell viability experiments, the cell culture dishes were removed from the incubator after 20-24 hours, the media was removed, and 1.5 mls of fresh DMM was added. Experiments were done in sets of four, one control and three exposed samples. Samples were exposed for 30 minutes to 800, 835, and 847.74 MHz at input powers of 0.5, 2.0 and 5.0 W equivalent to electric field strengths, at the sample, of 0.83, 1.67, and 2.64 V/cm, respectively. All samples within one experiment were kept out of the incubator for a total of 1½ hours. Immediate viability experiments were done in which the cells were counted immediately after exposure. After all samples were exposed, the media was removed, 0.5 mls of trypsin/EDTA was added to get the cells into suspension and then the cells were counted using the hemacytometer. Overnight viability experiments were also done in which the cells were counted 22-24 hours after exposure. After all samples were exposed, an additional 2.1 mls of DMM and 0.4 mls of serum was added to each culture dish (for a total concentration of DMM with 10% FBS), and they were then incubated for 22-24 hours at 37°C, 5% CO_2. After the incubation period, the media was removed, 0.5 mls of trypsin was added to get the cells into suspension and then the cells were counted using the hemacytometer.

For exposure experiments, the cell culture dishes were removed from the incubator after 20-24 hours, the media was removed, and 4 mls of fresh DMM with 10% FBS was added for transfection experiments. For transfection experiments, the following was added, in order, to a 0.5 ml eppendorf tube: 88 µl DMM media, 6.0 µl Fugene reagent (Roche Molecular Biochemicals, USA), 1.0 µg CRE, and 0.1 µg Sp1RE. The tube was gently tapped and then sat at room temperature for 15 minutes. 100 µl of transfection mix was added to each dish and incubated for 4-6 hours. After the

incubation period, transfection was stopped by removing the media with transfection reagents. 1.5 ml of DMM was added and the sample was exposed. Experiments were done in sets of four, one control and three exposures. Some experiments were done at one frequency and exposing at three different power levels and some were done at one power level and exposing at three different frequencies. The control samples were treated the same way as the exposed except they were not irradiated. They were placed in the same room, out of the incubator, as the exposure equipment but kept at a distance. In order to minimize random variability in field strength, the quantity of culture medium in each plate and the position of the sample in the TEM cell were standardized between experiments as much as possible. After exposure, an additional 2.1 mls of DMM and 0.4 mls of serum was added to each culture dish (for a total concentration of DMM with 10% FBS), and they were then incubated for 18 hours at 37°C, 5% CO_2.

In the first group of experiments, the time that each sample, from one set of experiments, was out of the incubator and without serum was 1½ hours. In the second group of experiments, the samples were staggered so that each culture dish was removed from the incubator only for the exposure time of 30 minutes and then returned. Cells were exposed to 800, 835, and 847.74 MHz at input powers (into the TEM cell) of 0.2, 0.5, and 2.0 Watts that correspond to electric field strengths, at the sample, of 0.53, 0.83, and 1.67 V/cm, respectively. After 18 hours, the culture dishes were removed from the incubator, all media was removed, and the dishes were placed in the -80°C freezer until ready for the dual-luciferase assay.

For the dual-luciferase assay, the culture dishes were removed from the -80°C freezer and thawed. For the first group of experiments, the cells were then washed with

1.5 mls of Hank's solution. 1 ml of Hank's solution was then added to each dish and then sat at room temperature for 5-15 minutes to get the cells into suspension. The cells were then scraped and transferred into an eppendorf tube, one for each sample. An additional 0.5 mls of Hank's solution was added to each dish and any remaining cells were scraped and transferred to the same eppendorf tube. The tubes were then centrifuged at 14,000 rpm, 4°C for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl of 1X passive lysis buffer (Promega). The tube was vortexed to break up the cells and then sat at room temperature for 15 minutes. 40 µl of each sample was placed in a well of a tissue culture treated 96-well plate (Corning). 1X passive lysis buffer was used as a blank. Each sample was done in duplicates. The 96-well plate was inserted into the luminometer and the assay was programmed so that Luciferase Assay Substrate (Promega), luciferin, was added first and the intensity of CREB expression was detected. Then the Stop & Glo Reagent (Promega), coelenterazine, was added and the intensity of Sp1 expression was detected. The numbers were averaged for the duplicate samples.

For the second group of experiments, the culture dishes were removed from the -80°C freezer and thawed. The cells were washed with 1.5 mls of Hank's solution and were lysed directly in the plates. 300 µl of 1X passive lysis buffer was added to the cells and the dishes were placed on the shaker for 30 minutes. The cells were then scraped off and transferred to eppendorf tubes. The tubes were centrifuged at 14,000 rpm, 4°C for 1 minute to get the cell membranes down to the bottom. 40 µl of each sample was placed in a well of the 96-well plate, again in duplicates, and the procedure that followed was the same as in the first group.

Positive control experiments were done in the same way as the control samples of the second group of experiments with the exception of transfection experiments. For transfection, the following was added, in order, to a 0.5 ml eppendorf tube: 85 µl DMM media, 6.0 µl Fugene reagent, 1.0 µg CRE, 0.1 µg Sp1RE, 0.3 µg Cα (activates CRE), and 0.3 µg Cγ (activates Sp1RE). These experiments were not exposed to radiofrequencies but were done to show that the reporter plasmids were indeed working as expected.

Additional positive control experiments, with radiofrequency exposure, were also done in the same way as the second group of experiments with the exception of transfection experiments. For transfection, the following was added, in order, to a 0.5 ml eppendorf tube: 87.75 µl DMM media, 6.0 µl Fugene reagent, 1.0 µg CRE, 0.1 µg Sp1RE, and 0.05 µg Cα. One sample was a control and was not exposed to radiofrequencies. The remaining three samples, within an experiment, were exposed to an input power of 2.0 W at frequencies of 800, 835, and 847.74 MHz, respectively, for 30 minutes. The remaining procedures for analysis of these experiments followed those of the second group of experiments. These experiments were done not only to show that the reporter plasmids were working as expected, but also to show if exposure to radiofrequencies may have affected the CRE activators (Cα) or the process in which Cα activates CRE.

RESULTS AND DISCUSSION

TEMPERATURE EXPERIMENTS

The results of the temperature measurements are shown in table 2. Measurements were taken before and after 30 minute exposures at different frequencies and powers. All of the initial temperature readings were the same. The only increase in temperature observed was at an input power of 5.0 W for each frequency. However, the increase in temperature was very small, 1°C or less, and was considered to be an insignificant change. The results demonstrated that these experiments were looking at non-thermal effects of radiofrequency waves.

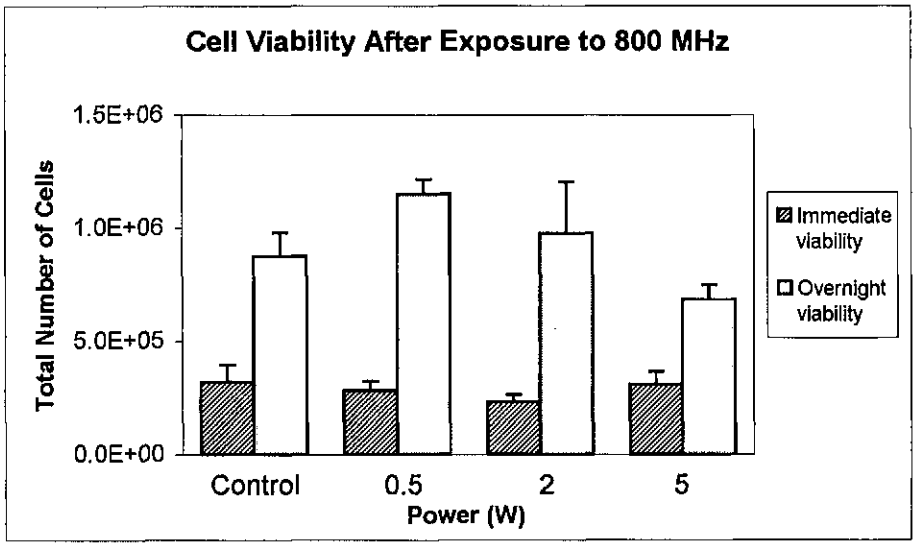
Frequency (MHz)	Input Power (W)	Initial Temp. (°C)	Final Temp. (°C)
800	0.5	24.5	24.5
	2.0	24.5	24.5
	5.0	24.5	25.0
835	0.5	24.5	24.5
	2.0	24.5	24.5
	5.0	24.5	25.0
847.74	0.5	24.5	24.5
	2.0	24.5	24.5
	5.0	24.5	25.5

Table 2. Temperature measurements. Cells with 1.5 mls fresh DMM were exposed to radiofrequencies for 30 minutes.

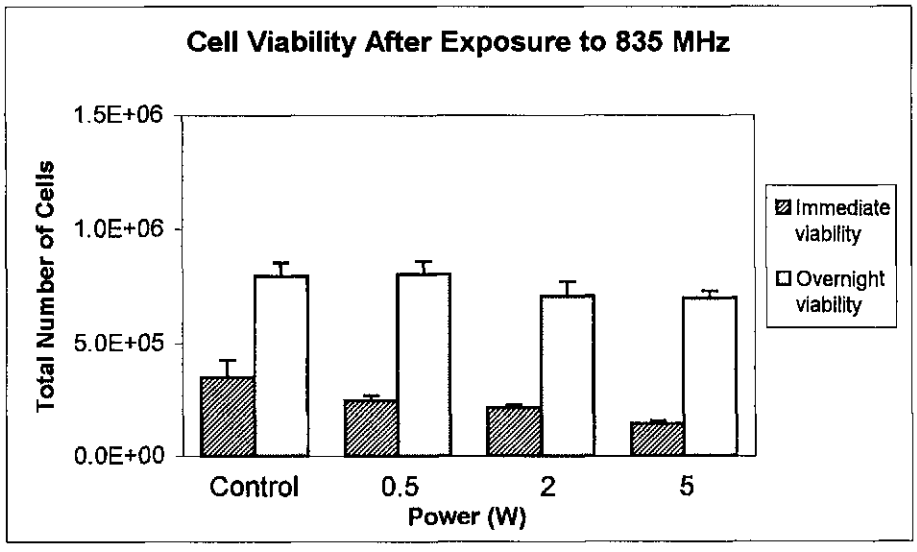
CELL VIABILITY EXPERIMENTS

Viability experiments were conducted at three different frequencies and at three different input power levels to determine if the radiofrequency exposure had any effects on cell viability immediately after and 22 hours (overnight) after exposure. Two experiments each were conducted, for the immediate and overnight viability data, and for exposure to 800 and 847.74 MHz. Four experiments were conducted for the immediate viability results of exposure to 835 MHz and three experiments for overnight viability. Initially, 6.0×10^5 cells were plated the day prior to exposure and incubated overnight. Immediately prior to exposure experiments, the media was removed and 1.5 mls fresh DMM was added for exposure.

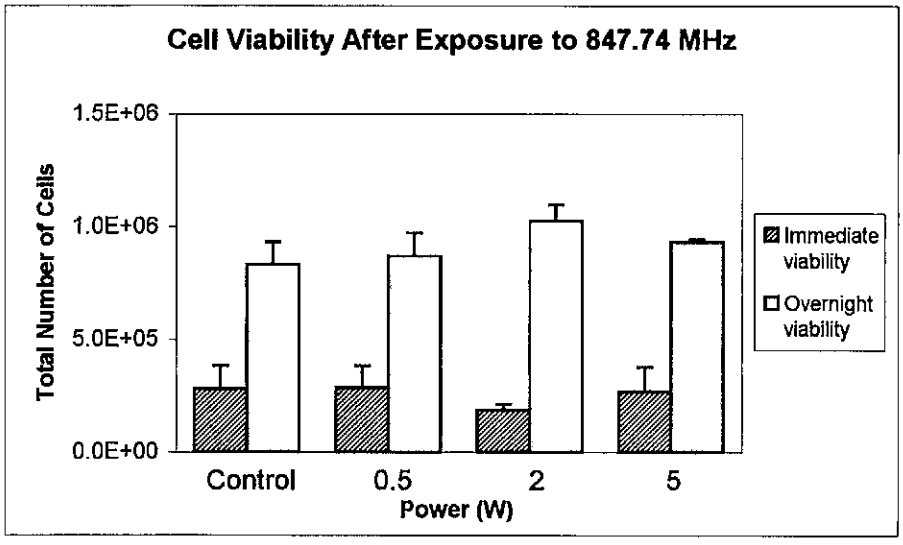
Graphs 1, 2, and 3 show the results of the cell count immediately after and 22 hours after exposure to 800, 835, and 847.74 MHz, respectively. The error bars represent standard error. In looking at the results of the cell count immediately after exposure, it was observed that the cell count was less than the initial number of cells plated (6.0×10^5). This was most likely due to the step prior to exposure in which the media was removed. Any cells that were not yet adhered to the bottom of the culture dish would have been removed. Since the dishes were only 70-80% confluent after seeding the plates and incubating them for 20-24 hours prior to exposure, this would explain the lower number of cells.



Graph 1. Cell viability results of experiments immediately after and 22 hours after exposure to a frequency of 800 MHz. Power represents the input power going into the TEM cell.



Graph 2. Cell viability results of experiments immediately after and 22 hours after exposure to a frequency of 835 MHz. Power represents the input power going into the TEM cell.



Graph 3. Cell viability results of experiments immediately after and 22 hours after exposure to a frequency of 847.74 MHz. Power represents the input power going into the TEM cell.

Results also showed that there were no significant differences in the number of cells between the control and exposed for samples exposed to 800 and 847.74 MHz (graphs 1 and 3). This was a good indication that the exposure was not immediately killing cells, or at least not in noticeable numbers. However, there was a slight downward trend in the number of cells as the power increased at the exposure to 835 MHz (graph 2). Table 3 shows the average number of cells, immediately after exposure, for each experiment. Statistics were done to determine if this trend was significant. The sign test was used and showed that this was not a significant difference in the number of cells ($p = 0.32$, $n = 4$). Further experiments would need to be done to show that this trend may be significant. It may be possible that exposure to 835 MHz had some effect on the ability of HEK-293 cells to adhere. Then, when the cells were washed after exposure,

any cells that were loosely adhered may have been washed off. Special care was given to attempt to treat each sample in the exact same way to help reduce any variabilities in counting the cells.

Input Power (W)	Cell Count 800 MHz	Cell Count 835 MHz	Cell Count 847.74 MHz
Control	3.19E+05	3.50E+05	2.81E+05
0.5	2.81E+05	2.46E+05	2.88E+05
2.0	2.31E+05	2.15E+05	1.88E+05
5.0	3.06E+05	1.44E+05	2.69E+05

Table 3. Cell viability data immediately after exposure to 800, 835, and 847.74 MHz.

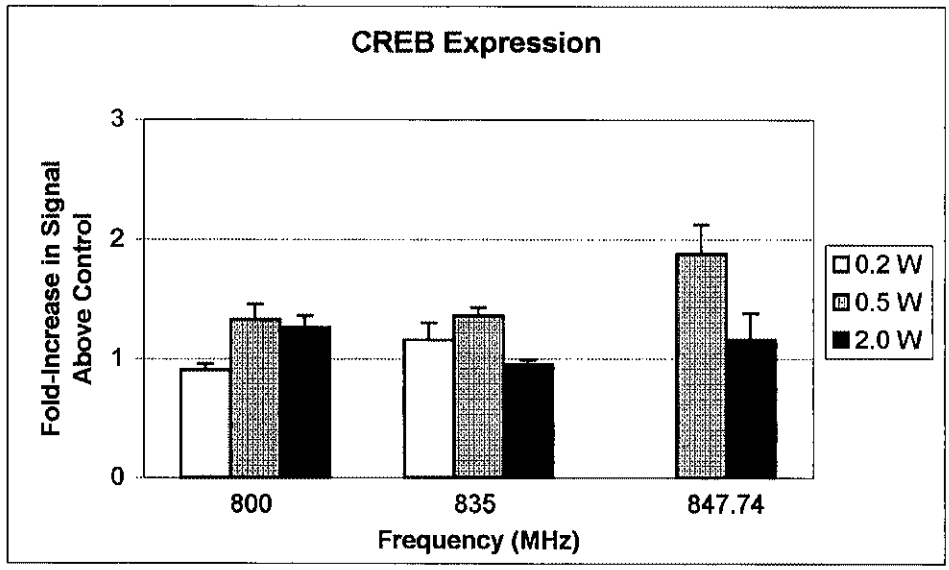
In looking at the viability effects 22 hours after exposure, there was not a significant difference between the control and exposed samples, or even between the different frequencies. However, the cell count 22 hours after exposure, for exposure to each different frequency and power, was at least 2-fold greater than the cell count immediately after exposure. This indicated that the cells were continuing to grow, even after 30 minutes of exposure to the various radiofrequencies and powers. Overall, these results suggested that there were no effects on cell viability immediately after or 22 hours after exposure to all three frequencies and at all three power levels. The downward trend that was observed for cell viability immediately after exposure to 835 MHz still needs to be investigated to see if these results can be reproduced. While this trend may not

necessarily represent viability of the cells, it may lead to other discoveries such as effects on the adherency of the cells.

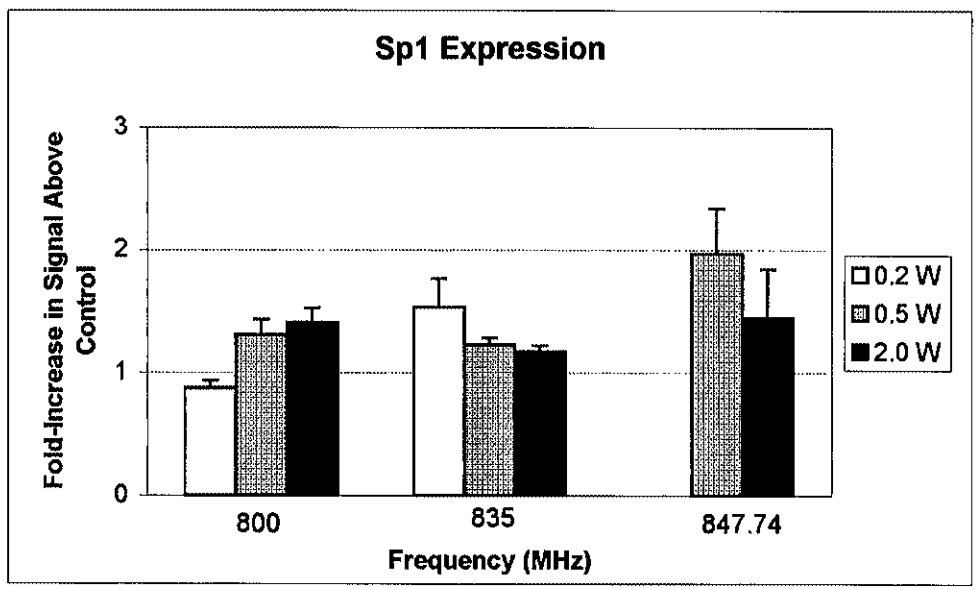
EXPRESSION EXPERIMENTS

The first group of experiments were done with all four samples of a single experiment out of the incubator for 1½ hours. Studies were done exposing transfected HEK-293 cells to radiofrequencies of 800 and 835 MHz, at three different power levels, 0.2, 0.5, and 2.0 W, for 30 minutes. Experiments at 800 MHz were repeated two, five, and four times each for exposures to 0.2, 0.5, and 2.0 W, respectively. Experiments at 835 MHz were repeated three, seven, and five times each for exposures to 0.2, 0.5, and 2.0 W, respectively. Studies were also done with exposures to 847.74 MHz at power levels of 0.5 and 2.0 W. All averages of similar experiments were calculated as well as the standard error.

Initially, results were examined after normalizing both CREB and Sp1 expression to the non-exposed control samples within the same experiment as shown in graphs 4 and 5. The y-axis represents the fold-increase in the signal above the control. Any bar above 1.0 had an increase in signal compared with the control. It was determined that these two graphs did not give a good indication of whether or not CREB or Sp1 expression was affected by radiofrequency exposure.



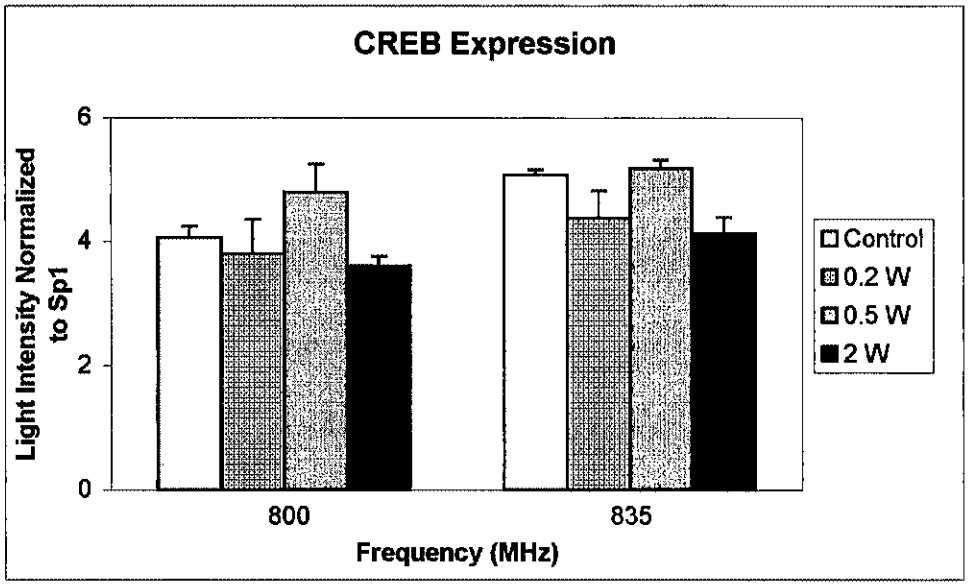
Graph 4. CREB expression after exposure to radiofrequency waves when normalized to the control.



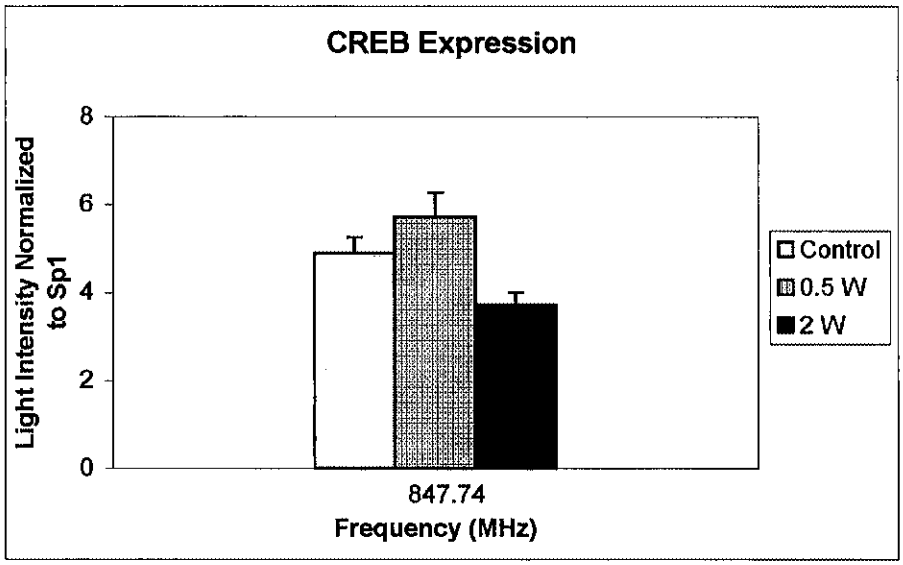
Graph 5. Sp1 expression after exposure to radiofrequency waves when normalized to the control.

There are several different parameters that may have had an impact on these results, other than radiofrequency exposure. Transfection is one variable that may have led to drastic variability within an experiment. Transfection is, to some extent, a random process. Not every plasmid will be transfected into the human cells, and so the transformation efficiency may vary between each sample culture dish within an experiment. Interpreting data in this way, normalized to control, may lead to difficulties in coming to conclusive results because of the high variability most likely in the transformation efficiency within the different samples of a single experiment. Other variables to consider include biological methods and the fact that each and every cell is not going to behave in the same way and at the same time. Attention was paid to the methods to make sure that each sample within an experiment, and overall, was treated the same.

After more research, it was discovered that Sp1 was not present in HEK-293 cells (27). Therefore, the Sp1RE could not be activated in these cells and was used as an internal control. This removed the variability of transfection within an experiment. Graphs 6 and 7 show CREB expression normalized to Sp1 expression within an experiment. The control sample was also normalized to Sp1 expression. These data are from the same experiments as those shown in graphs 4 and 5. Error bars represent the standard error.



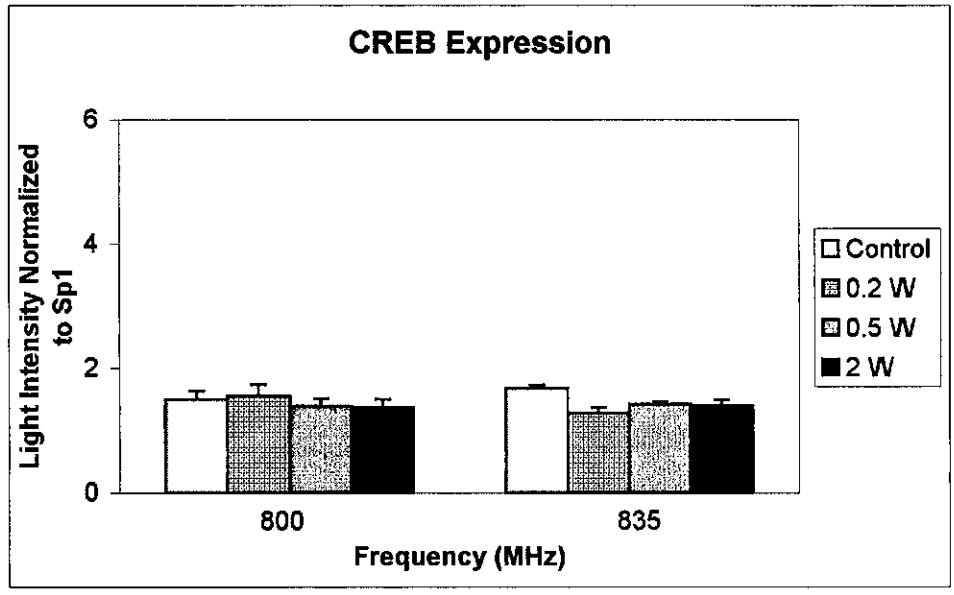
Graph 6. CREB expression normalized to Sp1 expression at exposure to 800 and 835 MHz. Samples were exposed to 0.2, 0.5, and 2.0 W at frequencies of 800 and 835 MHz for 30 minutes. These experiments represent data from the first group of experiments in which all four samples in a single experiment were kept out of the incubator for a total of 1½ hours.



Graph 7. CREB expression normalized to Sp1 expression at exposure to 847.74 MHz. Samples were exposed to 0.5 and 2.0 W at a frequency of 847.74 MHz for 30 minutes. These experiments represent data from the first group of experiments in which all four samples in a single experiment were kept out of the incubator for a total of 1½ hours.

After analysis of all the data from the first group of experiments, it was determined that the slight differences in the bar graphs were most likely attributed to natural cell activities, and did not indicate significant differences in the amount of CREB expression. It was concluded that there were no significant differences in CREB expression between the control and the exposed groups for all of the different frequencies and power levels tested.

In the second group of experiments, the experimental design was changed. First, each sample of a single experiment was out of the incubator for only 30 minutes, the exposure time. This was to minimize the effects of temperature and O_2/CO_2 changes on the cells. Second, the lysing process of the dual-luciferase assay was done directly in the cell culture plates. This was to eliminate several steps of handling the cells. Studies were done exposing transfected HEK-293 cells to radiofrequencies of 800 and 835 MHz, at three different power levels, 0.2, 0.5, and 2.0 W, for 30 minutes. Experiments at 800 MHz were repeated three times each for exposures to 0.2, 0.5, and 2.0 W. Experiments at 835 MHz were repeated twice for exposures to 0.2, 0.5, and 2.0 W. All averages of similar experiments were calculated as well as the standard error. Attention was also paid to the methods in these experiments to make sure that each sample within an experiment, and overall, was treated the same. Graph 8 shows the results. Error bars represent the standard error.



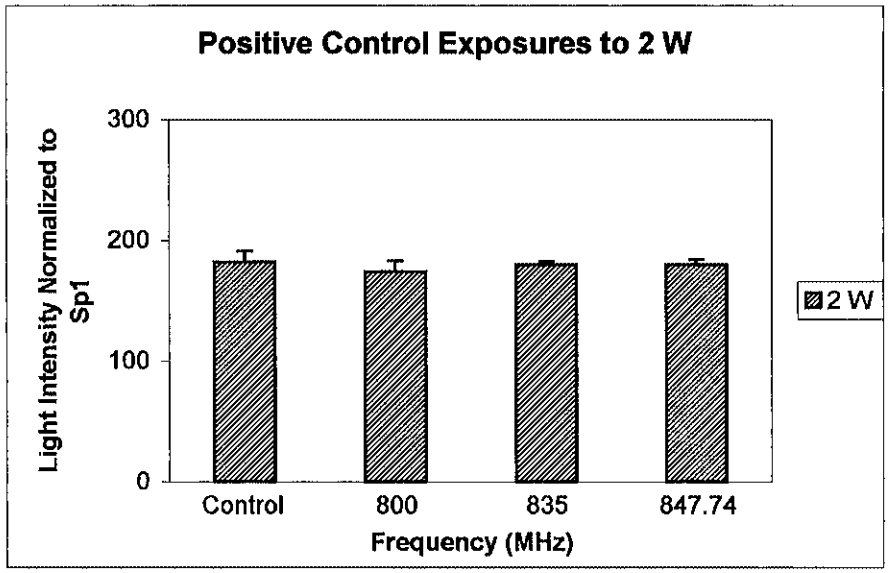
Graph 8. CREB expression normalized to Sp1 at exposure to 800 and 835 MHz for the second group of experiments. Samples were exposed to 0.2, 0.5, and 2.0 W at frequencies of 800 and 835 MHz for 30 minutes. These experiments represent data from the second group of experiments in which each sample in a single experiment was out of the incubator for the 30 minute exposure time and cell lysis (after exposure) was performed directly in the culture dish.

These experiments clearly indicated that there were no effects on CREB expression when exposed to radiofrequencies of 800 and 835 MHz at power levels of 0.2, 0.5, and 2.0 W. These results also showed that the methods of the second group of experiments gave results with much less variability than in the first group of experiments. This may indicate that most of the variability in the data is within the handling of the cells. Cells will be lost with each handling and be unaccounted for. It is interesting to note that the actual intensity values (normalized to Sp1) of CREB expression from the first group of experiments are 2- to 3-fold higher than those of the second group. The difference may have been due to the difference in time out of the incubator (30 minutes

versus 1½ hours) or due to the difference in the lysing process. It would seem unlikely that the time away from the incubator would have such an effect, or if so, that the effect might be the opposite- meaning that more time away from the incubator may have led to more cell death and there would be less expression. However, that was explained in the cell viability experiments in which no effects were seen on cell viability after exposure. On the other hand, the difference in the lysing process meant a more diluted sample (3 times more diluted) for the dual-luciferase assay that may explain the lower value of expression in the second group of experiments. In conclusion, there was strong evidence that there was no effect on CREB expression in HEK-293 cells when exposed to radiofrequencies of 800, 835, and 847.74 MHz at power levels of 0.2, 0.5, and 2.0 W. No conclusion could be made about radiofrequency effects on Sp1 expression since there was no Sp1RE activity in HEK-293 cells and the Sp1 was used as an internal control.

POSITIVE CONTROL EXPOSURE EXPERIMENTS

Positive control samples were co-transfected with CRE, Sp1RE, and Cα, the expression plasmid known to activate CRE- regulated transcription by phosphorylating CREB. Positive control experiments were done for several reasons: to determine if exposure to radiofrequencies had an effect on Cα which may have led to an effect on the phosphorylation of CREB; to determine if radiofrequency exposure caused cells to send or initiate CRE signals; and to determine if radiofrequency exposure inhibited strong CRE signals which may have altered a CRE response. The results of positive control exposure experiments are shown in graph 9. Error bars represent the standard error.



Graph 9. Positive control exposure experiments. Samples were co-transfected with CRE, Sp1RE, and Cα and exposed to radiofrequencies of 800, 835, and 847.74 MHz at a power level of 2.0 W for 30 minutes.

Positive control samples were exposed to radiofrequencies of 800, 835, and 847.74 MHz at a power level of 2.0 W for 30 minutes. Experiments were repeated three times. These experiments were exposed to the highest power level (2 W) due to the thought that a higher power may have more of an effect than a lower power. More studies need to be done in order to support that statement. The results showed that there was no significant difference between the control and the exposed samples. This suggested that not only was there no observable effect on exposure to CREB expression, but even with the Cα subunit present during exposure, the gene was still expressed and did not appear to be any different than the non-exposed control sample.

CONCLUSION

The main objective for this research was to determine if exposure to low-level radiofrequency waves had any non-thermal effects on gene expression. These results showed that there were no effects on gene expression for the firefly luciferase gene when exposed to 30 minute radiofrequencies of 800, 835, and 847.74 MHz at electric field strengths of 0.53, 0.83, and 1.67 V/cm with corresponding input power levels (into the TEM cell) of 0.2, 0.5, and 2.0 W. No conclusion can be made about gene expression for the renilla luciferase gene since there was no Sp1RE activity in HEK-293 cells and therefore, Sp1 was used as an internal control.

The results of this study are not in agreement with several of the studies from previous literature discussed earlier in this thesis, in which biological effects were observed after radiofrequency exposure (4,5,14,15). The difference in results can be due to several reasons. One obvious reason is the different biological system being analyzed. For example, in the experiments done by Ivaschuk et al. (14), a signficant effect was found in the expression of the c-*jun* gene in rat cells when exposed in a TEM cell to 836.55 MHz with an input power of 1.7 W, which is very similar to the setup in this study. Another obvious reason is in the different frequencies and power levels used. It is very possible that a single radiofrequency may target specific cell lines, certain proteins or genes, or even specific signalling pathways while having no effect on others. An additional reason that may have led to different results is the experimental setup and design. Other studies have used radial transmission lines (5,15) or aluminum chambers with antennas mounted at the center of the box (2,4) in comparison with the TEM cell

that was used in this study. The TEM cell gives a uniform electric field at the location of the sample, however, that may not be the case in the other exposure designs.

As far as dispersion theory, molecules with permanent dipole moments, such as water or proteins, are going to undergo a torque after application of an electric field, and they will tend to align with the electric field. The relaxation time will be in the range of nanoseconds. Essentially, the electromagnetic energy is being converted into vibrational energy of the dipoles. This vibrational energy (of the dipoles, not the vibrational oscillations of the molecules) must be converted into thermal energy, creating a localized thermal effect, while the overall effect of exposure to electromagnetic fields is still considered a non-thermal effect. To support this idea, new experiments would need to be designed to measure any localized heating. Because the relaxation time is so short, equipment would be needed that can measure almost instantaneous heating effects.

While no radiofrequency effects on gene expression were found in these experiments, this research can be used as an inspiration for future studies. Research should continue to look at a wide array of different genes, as well as other biological systems, for possible effects from radiofrequency exposure. The radiofrequencies used in this experiment represent only a small portion of the electromagnetic spectrum. Applications of electromagnetic fields have been used in a positive sense to help heal and repair damaged biological systems. Conversely, exposure to electromagnetic fields may also lead to harmful biological effects such as cancer. Further research must be done in support of the benefits or harmful effects of exposure of biological systems to electromagnetic fields.

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CURRICULUM VITA
for
ELIZABETH A. COLN

DEGREES:

Master of Science (Electrical Engineering), Old Dominion University, Norfolk, Virginia, May 2002

Bachelor of Science (Bioengineering), University of Illinois at Chicago (UIC), Chicago, Illinois, May 1997

PROFESSIONAL CHRONOLOGY:

United States Navy, Portsmouth, Virginia
Surface Nuclear Warfare Officer, April 1997 – July 2000

Panasonic Factory Automation, Franklin Park, Illinois
Co-Op Student, January 1997 – May 1997

Mechanical Engineering Department, University of Illinois, Chicago, Illinois
Research Assistant, May 1996 – December 1996

SCIENTIFIC AND PROFESSIONAL SOCIETIES MEMBERSHIP:

Bioelectromagnetics Society, Member, 2000 – Present
Golden Key National Honor Society, Member, 1997 to Present
Tau Beta Pi (Engineering Honors Society), Member, UIC, 1996 to Present
Biomedical Engineering Society, Member, 1995 to Present
Society of Women Engineers, Vice President, UIC, 1996 to 1997
Biomedical Engineering Society, Vice President, UIC, 1995 to 1997

HONORS AND AWARDS:

Graduate Assistance in Areas of National Need (GAANN) Fellowship, 2000 – 2001
United States Navy Achievement Medal, 2000

SCHOLARLY ACTIVITIES COMPLETED:

Electromed 2001 Conference, poster session, Portsmouth, Virginia May 2001