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EFFECTS OF 100 mT STATIC MAGNETIC FIELD EXPOSURE ON CYTOSOLIC FREE Ca2+ LEVELS AND HSP70 REPORTER GENE EXPRESSION

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EFFECTS OF 100 mT STATIC MAGNETIC FIELD EXPOSURE ON CYTOSOLIC FREE Ca^{2+} LEVELS AND HSP70 REPORTER GENE EXPRESSION

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Abstract

Human exposure to electromagnetic fields has prompted investigations into possible effects of static magnetic fields (SMF)s on cellular processes. Discrepancies still remain between studies however, and are likely due to endpoint analysis on potentially small biological effects. The purpose of this research was to investigate the potential effect of SMF exposure on biological systems using real-time methods that are intrisicaly more sensitive than endpoint measurements. This research was divided into two main studies. The first study evaluated basal and activated levels of cytosolic free calcium in HL-60 cells before, during and after a 15 minute 100 mT SMF exposure. The second study used a heat shock protein (hsp70)/luciferase reporter system in NIH3T3 cells to detect changes in heat shock promoter activation before, during and after a 15 minute 100 mT SMF exposure. The first study found no effects of SMF exposure on resting or activated cytosolic free calcium concentration. However, a 100 mT SMF significantly decreased the activation of the heat shock protein promoter, but only when field exposure was combined with an additional heat insult.

Key words: Static magnetic field, cytosolic calcium, heat shock proteins (hsp70), bioluminescence, real-time analysis.

Co-Author Statement

Camilla Rozanski: Performed all manuscript writing, cellular exposure experiments, data collection, and analysis.

Michelle Belton: Assisted with experimental design and manuscript review

Frank Prato: Advisory committee member, assisted with experimental design and manuscript review.

Jeff Carson: Supervisor, assisted with experimental design and manuscript review.

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List of Abbreviations

$[Ca^{2+}]_c$	Cytosolic calcium concentration
DEM	Diethyl maleate
ELF-MF	Extremely low frequency magnetic field
EMF	Electromagnetic field
FWHM	Full width at half max
GSH	Glutathione (reduced form)
GSSH	Glutathione (oxidized form)
HL-60	Human leukemia – 60 cells
Hsp70/hsp70 (human/animal)	Heat shock protein mRNA
Hsp70/hsp70	Heat shock protein (human/animal)
HSE	Heat shock element
HSF	Heat shock factor
Luc	Luciferase
ROS	Reactive oxygen species
RPM	Radical pair mechanism
SMF	Static magnetic field

Chapter One

Introduction

1.1 – Survey of Literature Related to SMF Bioeffects

1.1.1 – Magnetic Fields: Introduction

Controversy over biological sensitivity to magnetic field exposure has prompted studies investigating possible interactions of magnetic fields (MF) with biological systems. Wertheimer and Leeper's work [Wertheimer and Leeper, 1979] exploring the association between neighborhood wiring and childhood leukemia, has been considered one of the original investigations in the area of magnetic field safety. They observed a higher incidence of childhood cancer in children who lived near high distribution electrical wiring, as compared to control children who did not live near such wiring. The reason for the correlation was uncertain, and thus prompted many successive studies regarding possible interactions of MF with biological systems. Since then, there have been numerous epidemiological and laboratory studies addressing possible biological effects of electromagnetic fields (EMF), pulsed or extremely low frequency magnetic fields (ELF-MF), and static magnetic fields (SMF). Furthermore, the development of Magnetic Resonance Imaging (MRI) which utilizes magnetic field gradients, pulsed radio-frequency fields, and a static magnetic field on the order of 0.2 T to 4 T, have raised public awareness and concern regarding safety issues and risks associated with human magnetic field exposure [De Wilde et. al., 2005; Guisasola et. al., 2002]. Reports by health agencies, such as the Environmental Health Criteria report from the World Health Organization Task Force on static EMFs, have served to raise public awareness and interest in the safety of human magnetic field exposure [De Wilde et. al., 2005]. Some laboratory studies have found evidence of magnetic fields affecting biological

systems. For example, ELF-MFs have been shown to act as co-carcinogenic factors when in the presence of known carcinogens [Loscher and Liburdy, 1998]. SMFs have been shown to affect the DNA repair mechanism in *Drosophila melanogaster* somatic cells resulting in increased mutant genotypes [Koana et. al., 1994], as well as inhibition of calcium channel function in cultured GH3 cells [Rosen, 1996]. However, many studies exist where no effects have been found [Belton et. al., 2008; Madec et. al., 2003; Schiffer et. al., 2003]. Both Madec et. al. and Schiffer et. al. used different cell lines then those used in this study, and evaluated different parameters, such as cell progression and calcium signalling. Differences in exposure systems and experimental conditions make it difficult to compare the results between studies. This is further compounded by the use of single end point analysis by most investigators, which is sensitive to biological variability. There are few studies that have used real-time analysis in the past [Belton et. al. 2007, Madec et. al. 2003, Carson et. al. 1990]. Real-time analysis provides immediate feedback on the dynamic changes occurring in the system, and is less likely to be subject to post-experimental variability. The objective of this thesis was to address the biological variability of end-point analysis by using real-time assays for evaluating the effects of SMF on biological systems.

1.1.2 –Previous Work on Biological Effects Induced by Static Magnetic Field Exposure

Although there have been many studies done on the effects of extremely low frequency magnetic fields at the cellular level, gaps still remain with regards to the interaction of static magnetic fields with biological systems. Some investigators have

found no effects. For example, two studies performed by Wiskirchen *et al.* [Wiskirchen et. al., 2000] assessed the effects of repetitive 0.2 T, 1 T or 1.5 T SMF exposure on human fetal lung (HFL) fibroblast proliferation. In one study, the HFL cells were exposed to 1.5 T SMF for 1 hour per day, three times a week, whereas in the second study, the HFL cells were exposed to 0.2 T, 1 T and 1.5 T SMF for 1 hour per day for 5 consecutive days. Population doublings were calculated in both studies. No effect of field was found on HFL cell proliferation, regardless of SMF strength or time of exposure. Studies on cellular proliferation were also conducted by Schiffer *et al.* [Schiffer et. al., 2003]. Fields relevant for magnetic resonance imaging (MRI) were examined for effects on cellular proliferation in two tumor cell lines: HL-60 and EA2. Cells were exposed to SMFs of 1.5 T and 7 T, ELF magnetic field gradients, and pulsed RF magnetic fields. Exposure times varied between 1 to 24 hours per experiment. Cell cycle distribution was analyzed using flow cytometry. Schiffer *et. al.* found no effects of SMF exposure on cell cycle distribution or cell proliferation. Therefore, regardless of discrepancies in protocol between Wiskirchen and Schiffer, there was a consistent lack of effect of magnetic fields on cell progression and proliferation in all three studies.

In other cases, an agreement regarding SMF bioeffects between studies is hard to reach. Studies by Fanelli *et al.* and Teodori *et al.* [Fanelli et. al., 1999; Teodori et. al., 2002] both investigated the potential influence of SMF exposure on cellular apoptosis. Fanelli exposed a number of human cell lines, including human-leukemia HL-60 cells (as used in this thesis), to 6 mT SMF. This study found that SMF exposure had the potential to increase cell survival and replication after the cells were exposed to apoptotic agents. Fanelli concluded that SMF exposure appeared to have a protective effect against

cellular apoptosis, mediated by an increased influx of Ca^{2+} ions from the extracellular medium. However, this explanation is limited to cell lines where calcium influx is said to have anti-apoptotic effects. Studies performed by Teodori *et. al.* are comparable to those performed by Fanelli's group. Teodori also exposed HL-60 cells to a 6 mT SMF in the presence of an apoptosis inducing agent, camptothecin (CPT). However, in this case SMF exposure did not demonstrate any protective effects against apoptosis. In fact, Teodori found the SMF exposure accelerated the rate of cell transition from apoptosis to secondary necrosis, resulting in a higher frequency of late apoptotic/necrotic cells following field exposure, compared to samples treated with CPT alone. The comparison between these two studies demonstrates the conflicting evidence of past studies regarding the effects of SMF exposure. The reason for this conflicting evidence is hard to determine, and may include differences in cell lines, time of field exposure, or mechanisms under investigation. Future studies are required to provide additional evidence in favor of a given hypothesis, or to provide an explanation for the variability between studies.

Other studies have investigated potential SMF effects on DNA damage and mutation [Zhang et. al., 2003; Zmyslony et. al., 2000], ion transport and plasma protein orientation [Aldinucci et. al., 2003; Rosen, 1996], metabolic activity [Onodera et. al., 2003; Sabo et. al., 2002] and cell morphology [Pacini et. al., 2003; Pacini et. al., 1999]. Investigations conducted by Zhang *et al.* [2003], found that strong SMFs had the potential to induce genetic mutations in E-coli cells through an elevated production of intra-cellular superoxide radicals. Additionally, DNA damage was investigated by Zmyslona *et. al.* [2000], where cells were exposed to SMF and $FeCl_2$, a known oxidant

which increased the number of reactive oxygen species in the cell. When cells were exposed to either the 7 mT SMF, or the $FeCl_2$, no DNA damage was observed. However, when cells were exposed to $FeCl_2$ and the SMF simultaneously, a significant increase in the number of damaged cells was observed. Both studies imply a relationship between SMF bioeffects and cellular free radical concentration.

Considering the effects of SMFs on DNA mutation and damage, it is reasonable to suggest a potential interaction of SMFs on gene expression. Studies by Salerno *et. al* (1999)., Hirai *et. al.* (2002), and Hiraoka *et. al.* (1992), investigated changes in gene expression by evaluating the changes in the levels of mRNA transcripts and proteins produced. Salerno *et. al.* investigated effects of 0.5 T SMF exposure on membrane antigen expression (CD69) and interleukin IL-4 release in human peripheral blood mononuclear cells. Both proteins act as signaling molecules expressed by white blood cells. This study found that after a 24 hour exposure period to 0.5 T SMF, there was an increased release of interleukin IL-4, and reduced expression of the membrane antigen CD69. Hiraoka *et.al.* (1992) found a correlation between the time of SMF exposure and the amount of the *c-fos* oncogene mRNA produced. Levels of the oncogene mRNA transcripts were induced in cells by magnetic field exposure for samples exposed to SMF for 2 hours to 24 hours at a time. The levels of mRNA transcripts produced were time dependent, with a peak production after 6 hours exposure time. SMF strengths varied between 180 mT and 200 mT, and so are comparable to the SMF flux density investigated in this thesis. Additionally, studies performed by Hirai *et. al.*(2002) used identical SMF conditions to those presented in this thesis. It was found that a 15 minute, 100 mT SMF exposure increased the DNA binding of the nuclear transcription factor

AP1 through elevated expression of Fra-2 and c-Jun, proteins necessary for the composition and assembly of AP1. Nuclear transcription factors are nuclear proteins which modulate the activity of RNA polymerase II, responsible for mRNA synthesis. Hirai's study provides a potential explanation for any change in mRNA production following SMF exposure, as seen in the study by Hiraoka *et. al.* (1992).

Another area of interest regarding SMF interaction involves ion transport across cellular plasma membranes. Some studies suggest that SMF have the potential to realign molecules with diamagnetic properties, found embedded within the cell membrane [Emura et. al., 2001]. Consequently, it was hypothesized that changes in plasma membrane properties can distort the function of ion channels, such as embedded calcium channels [Rosen, 1996]. It has also been hypothesized that the realignment of diamagnetically anisotropic molecules can affect the release of membrane-bound calcium ions [Azanza and del Moral, 1994]. The loss of membrane-bound calcium ions can in turn potentiate Ca^{2+}-activated potassium channels, resulting in membrane polarization. A study conducted by Rosen *et. al.*(1996), investigated whether exposing cultured GH3 cells to a 120 mT SMF could cause any changes in calcium ion channel function and transport. The most significant change observed was a slowing of the channel activation rate without any change in the inactivation rate. This resulted in a reduction in the peak calcium current amplitude, and a shift in the current-voltage relationship. Other evidence regarding the interaction of SMF's with ion channels and transport includes a study conducted by Aldinucci *et. al.* [Aldinucci et. al., 2003], where a 4.75 T SMF was found to influence changes in Ca^{2+} ion transport. A decrease in intracellular calcium concentration was observed following exposure in Jurkat cells. This decrease in $[Ca^{2+}]_c$

was shown to be associated with a corresponding decrease in cellular proliferation. It was hypothesized that decreased $[Ca^{2+}]_c$ was caused by field effects on the modification of the Ca^{2+} binding proteins and ion specific channels, or by changes in the function of Ca^{2+} ATPase. The importance of investigating the effects of SMF on ion transport, specifically, changes in Ca^{2+} homeostasis, can be attributed to the role of the calcium ion as an intracellular signaling ion, regulating many cellular activities such as cellular proliferation and differentiation [Chattopadhyay et. al., 1996].

1.1.3 – Potential Mechanisms for Magnetic Field Interaction

There are many suggested mechanisms for the method of magnetic field action. Much of the previous literature has focused on the mechanism of oscillating magnetic fields interaction with cellular membranes and proteins [Foster, 2003; Panagopoulos et. al., 2002]. These studies describe the forced-vibration of the free ions on the surface of a cell's plasma membrane, caused by an external oscillating magnetic field. Specifically, the oscillating force created by the external pulsed magnetic field will affect the free ions found in and around the plasma membrane. These ions are free to move across the membrane through transmembrane proteins, and therefore are susceptible to the oscillating magnetic force, which is likely to cause what is called "force-vibration" of the free ions. If the amplitude of the ion's forced-vibrations reaches a certain critical point, the oscillating ions can in turn trigger a false signal for gated ion channels in the plasma membrane, creating a disruption in the electrochemical balance of the plasma membrane. However, this theory is solely applicable to oscillating magnetic fields, generated by AC currents, and cannot be extended as a potential mechanism for the action of SMF.

There is no doubt however, that there is some degree of SMF interaction with biological systems. Many organisms are responsive even to the very weak magnetic field of the Earth, on the order of 0.05 mT, such as the role of this geomagnetic field in the orientation and navigation of migrating birds [Johnsen and Lohmann, 2008]. Some organisms are known to contain biogenic magnetite (Fe_3O_4) in their tissues, thought to be involved in magnetoreception, or the ability to sense the polarity of the Earth's magnetic field. Some leading suggestions for SMF interaction with biological systems include an interaction with the diamagnetic properties of organic molecules found in all biological systems [Barnes et. al., 2000; Dini and Abbro, 2005], and the Radical Pair Mechanism (RPM), applicable to most multi-cellular systems [Brocklehurst, 1997; Brocklehurst and McLauchlan, 1996; Eveson et. al., 2000b; Harkins and Grissom, 1994].

SMFs can affect biological processes by applying a force to charged particles or by orienting magnetic dipoles. One effect of a static magnetic field created by a DC (direct current) source is its ability to exert a torque on the magnetic dipoles of diamagnetic molecular structures such as those found in plasma membranes, thus influencing the molecular orientation [Barnes et. al., 2000; Rosen, 1996]. Diamagnetism is considered a very weak repulsion from an externally present magnetic field, and can be loosely applied to all materials, since it is evoked wherever paired electrons are present, such as those found in all atoms. These individual atomic diamagnetic properties can then contribute to a given material's magnetic response. The mechanism describing the diamagnetic anisitropic properties of membrane phospholipids most directly explains the observed SMF effects on plasma ion channels. Many of the reported SMF effects such as changes in cellular proliferation, differentiation, morphology and apoptosis can be

explained on the basis of alterations in membrane calcium ion flux across membrane bound channels. Alterations in membrane calcium ion flux can be explained by the reorientation of protein molecules, resulting in the deformation of the embedded ion channels and alteration in the channels' activation kinetics. It has been observed that calcium channels are more susceptible to SMFs then other ion channels [Rosen, 1996].

The radical pair mechanism (RMP) describes the effect of a magnetic field on free radical orientation, propagation, and recombination. The importance of reactive free radicals in biology is well established. Free radicals like hydrogen peroxide (H_2O_2), the hydroxyl radical ($HO\cdot$), or superoxide anion (O_2^-) are generated by normal cellular processes, for example, in the electron transport system of the mitochondria [Chance et. al., 1979]. Free radicals are then implicated in the modification of cellular proteins, such as enzymes. Specifically, free radicals can be involved in converting enzyme substrates to products, as described in detail by Chance *et. al.*[1979]. Exercise has been shown to increase the rate of reactive oxygen species (ROS) production [Kourie, 1998]. Increased muscle exertion requires higher oxygen availability for energy or ATP production. This necessitates an increase in mitochondrial activity, generating higher levels of ROS as byproducts [Kourie, 1998]. Additionally, free radicals are known to be potent oxidizers and must be kept at a careful equilibrium in the context of biological systems, otherwise significant cellular oxidative stress can result [Nauser et. al., 2005a]. Such oxidative stress can result in a variety of detrimental biological effects including DNA damage [Kirkali et. al., 2007b], cell dysfunction [Valdez et. al., 2000b], apoptosis [Tirmenstein et. al., 2000b], and accelerated cell aging [Valdez et. al., 2000b].

Free radicals are a possible target for magnetic field interaction. The radical pair mechanism (RPM) has been formulated to describe the observed effects SMFs have on the reaction rates involving free radicals, which can in turn lead to an alteration in free radical concentration during SMF exposure [Eveson et. al., 2000b; Harkins and Grissom, 1994]. Generally speaking, the magnetic field can have an effect on the recombination and propagation rate of free radicals by affecting the spin properties of a correlated electron pair. During free radical production, a chemical bond involving two electrons is broken forming two radicals, each containing an unpaired electron. These electrons are said to have either parallel or anti-parallel "spin" properties. The recombination rate of the free radicals depends on the spin of the bonding electron, that is, a chemical bond between two radicals can only form if the spins on the bonding electrons are anti-parallel. It has been shown that an external magnetic field can modify the spin properties of the free electron, thus potentially increasing the number of electrons with parallel character, which can lead to a decrease in free radical recombination. Studies conducted by Harkins and Grissom [Harkins and Grissom, 1994] show decreased free radical recombination rates following SMF exposure in combination with ethanolamine ammonia lyase and an artificial substrate. Additionally, studies involving pulsed magnetic fields have shown magnetic field causing increased oxidative stress [Harkins and Grissom, 1994], increased DNA damage [Zmyslony et. al., 2000], and higher incidence of apoptosis [Jajte et. al., 2002] through the action of free radicals.

1.2 – Calcium Ion

1.2.1 – Significance of Calcium in Biological Systems

Calcium is responsible for controlling many cellular processes by acting as an intracellular signaling molecule. Its role among all cellular biological systems is universal by virtue of its versatility, and its ability to create a wide range of signals, both spatial and temporal [Berridge et. al., 2000]. It can trigger processes like fertilization, cell proliferation and differentiation. At basal levels, cytosolic calcium concentrations are kept very low, approximately 100 nM. The cell is very sensitive to fluctuations in basal cytosolic calcium levels, which are controlled by the activity of membrane channels and intracellular stores [Waring, 2005]. These specific fluctuations are part of a complex signal transduction pathway, which can lead to a variety of cellular alterations. If the cytosolic calcium concentration exceeds normal signaling fluctuations, cell death through apoptosis or necrosis can occur. Fertilization is an example of temporal calcium signaling. During fertilization, recurring and regular calcium spikes persist for about 2 hours after the sperm and egg interact, which initiates the completion of meiosis in a female egg, necessary for the male and female nuclei to fuse [Berridge et. al., 2000; Jones and Nixon, 2000]. In other cases, specific spatial increases in cytosolic calcium concentrations trigger cellular processes like differentiation. A study by Gardner *et. al.* [Gardner et. al., 1997a] investigated cellular differentiation in human leukemia HL-60 cells, a line of cells which continuously and slowly proliferate in a non-functional, non-specific manner. Gardner found that local induced changes in cytosolic calcium concentrations on the order of 100-300 nM, resulted in the differentiation of

promyelocytic HL-60 cells to monocyte-like cells. The sensitivity of cells to small changes in cytosolic calcium concentrations support the idea of monitoring changes in cellular calcium levels as a sensitive measure of cellular bioeffects induced by environmental stimuli, such as magnetic field exposure.

1.2.2 – Effects of SMF Exposure on Intracellular Calcium Ion Levels

Many studies have looked at the effects of SMF exposure on the calcium ion, whether through direct measurement of cytosolic calcium concentrations [Aldinucci et. al., 2003; Carson et. al., 1990a; Fanelli et. al., 1999], or by measuring alterations in calcium oscillations [Madec et. al., 2003]. There has been contradictory evidence of the significant effect of SMF exposure on cellular calcium levels. A study performed by Aldinucci *et. al.*[Aldinucci et. al., 2003], found that SMF can decrease cytosolic calcium concentrations in Jurkat cells, thus altering the calcium homeostasis of the cell, and decreasing cellular proliferation. Research performed by Fanelli *et al.* [Fanelli et. al., 1999] found that SMF exposure had the ability to enhance calcium influx into the cytosol of peripheral blood leukocytes. This increase in cytosolic calcium levels was shown to increase leukocyte survival by inhibiting apoptosis. Additionally, Fanelli found that cytosolic calcium levels increased in a dose dependent manner, where 3 mT and 6mT exposure increased cytosolic calcium concentrations by 50 nM and 90 nM respectively, from basal level [Fanelli et. al., 1999]. The study showed that SMF exposure had the ability to decrease cellular apoptosis by generating very small changes in cytosolic calcium concentrations. It is thus reasonable to monitor changes in cytosolic calcium

levels before, during and after SMF exposure, and use this information as a sensitive method of detecting magnetic field exposure bioeffects.

1.2.3 – Using Real-time cytosolic calcium measurements as a readout for SMF effects

There are significant advantages to measuring changes in a given parameter using real-time analysis. Endpoint analysis is more sensitive to experimental artifacts where effects may be misinterpreted as real when in fact they resulted from unwanted effects from the experimental protocol. A complicated experimental protocol can create many instances of cellular stress or sensitivity to environmental change, which can in turn cause additional $[Ca^{2+}]_c$ changes in addition to those induced by field exposure. For example, the study performed by Aldinucci *et. al.* [Aldinucci et. al., 2003] measured only endpoint cytosolic calcium concentrations. However, the experimental protocol involved many intermediate steps between cell culture preparation and calcium ion measurement, including washing with buffered saline solution twice, incubation at 25°C with the fluorescent dye fura-2, again washing three times with buffered saline solution and finally exposing cells to field. Following field exposure, the cells were placed in a fluorimetric cuvette containing saline solution, this time at 30°C, and immediately stimulated by the addition of 20 mM caffeine. Additionally, fura-2 leakage was estimated by quenching any extracellular dye with 0.2 mM $MnCl_2$. Only after this extensive protocol were calcium concentrations calculated by obtaining maximum and minimum fluorescence measurements using a calibration system. Final measurements of cytosolic calcium levels for sham and field exposed experiments were then compared to

determine SMF effects. In this protocol, a significant amount of time elapsed after field exposure and the actual cytosolic calcium measurement, with significant changes in the cells' environment, including temperature differences, air exposure, and the addition of solutions such as caffeine and $MnCl_2$. These changes could have increased environmental cellular stress apart from field exposure, thereby increasing the noise in the $[Ca^{2+}]_c$ calculated, thus reducing the precision and accuracy of the measurements. Addtionally, if the field did in fact induce increased cytosolic calcium concentrations, the cells could potentially have recovered from any field effects by the time actual calcium measurements were made.

Measuring changes in calcium levels in real-time eliminates the possibility of external factors other then field influencing the cells' response to SMF exposure. The environmental conditions of the cells can be kept constant before, during and after field application. The calibration of the system can also be performed and measured in real-time without removing the cells from their experimental environment. Also, if cellular activation is induced, the rate of change of cytosolic calcium levels can be calculated, allowing for a measure of the rate of cellular response and recovery. Additional parameters can also be measured, such as measuring calcium levels before, during and after field exposure within the same experiment, thus eliminating potential inter-experimental artifacts, i.e. using the sample as its own control.

1.3 – Heat Shock Proteins

1.3.1 – Role of Heat Shock Protein in Cells

Heat shock proteins (hsp) are involved in cellular protection from various environmental insults, such as heat, anoxia, or oxidative stress. Ritossa and Vonborstel [Ritossa and Vonborstel, 1964] first observed an increase in specific protein levels following heat exposure in *Drosophila melanogaster* salivary gland cells. These proteins had molecular weights of 70 and 26 kDa, and due to their sensitivity and over-expression following heat exposure, they were named "heat shock proteins". Further research in the area revealed additional proteins belonging to the heat shock family, with varying molecular weights. These proteins were also sensitive to cellular insults other than heat, such as glucose deprivation [Sciandra and Subjeck, 1983]

Heat shock proteins are found in all biological systems, including simple prokaryotic cells. It has been shown that these proteins are highly conserved among *all* eukaryotic cells, demonstrating a 60-78% amino acid identity [Bardwell and Craig, 1984; Kiang et. al., 1998]. Their prevalence amongst all cells, as well as their degree of conservation, validates the important role these proteins play in cell function and survival. Heat shock proteins act as molecular chaperones, aiding in protein folding of either newly synthesized proteins, or mature proteins that have undergone some degree of denaturation due to cellular stress, such as hyperthermia. These interact with partially denatured proteins, thus stabilizing the denatured protein by facilitating correct folding of protein subunits, and preventing inappropriate association of the denatured protein with surrounding molecules [Frydman, 2001]. Heat shock proteins are known to be produced

constitutively; however, their expression is increased by intracellular signals. As expected, these intracellular signals include increased levels of denatured proteins, a result of thermal stress to the cell. The 70 kDa heat shock protein (hsp70) has been shown to be present in all organisms, as opposed to hsp110 for example, which is present in mammalian cells only. Furthermore, the regulation and actions of hsp70 proteins have been extensively investigated and are very well understood, as opposed to some of their smaller counterparts, for example hsp 34, 47, or 56. An increase in hsp70 levels following heat exposure is well documented and supported by many groups [Lepock, 2005; O'Connell-Rodwell et. al., 2004; Ritossa and Vonborstel, 1964; Santoro, 2000].

The effect of magnetic field exposure on heat shock protein expression has become a popular area of research, largely due to the potential clinical benefits of controlled elevated levels of heat shock proteins [Robertson et. al., 2007]. The cytoprotective effects of heat shock proteins make them useful in precondition and protecting organs from certain forms of stress related to sugery, for example ischemic injurity or heat [Han et. al., 1998; Li et. al., 2006]. Furthermore, the potential of magnetic field exposure having an effect on heat shock protein synthesis is an attractive method of controlling the levels of hsps in cells, due to the relative safety and low damage of field exposure on human tissue [Han et. al., 1998]. Goodman *et. al.* [Goodman and Blank, 1998; Goodman and Henderson, 1988] and Alfieri *et. al.* [Alfieri et. al., 2006] were able to show that EMF were able to increase the levels of heat shock proteins in different cell types. The mechanism of magnetic field interaction with hsp production is not definite, and can occur at the DNA, mRNA or protein level. However, some studies suggest that there exists a specific DNA sequence on the hsp promoter

which is sensitive to magnetic field exposure, and thus may be responsible for alteration in heat shock protein expression [Lin et. al., 1999; Lin et. al., 2001].

1.3.2 –Bioluminescence as a Reporter for Heat Shock Protein Expression

The degree of genetic expression can be measured at two main stages – at the level of gene transcription yielding an mRNA product, and at the level of mRNA translation yielding a protein product. Levels of mRNA transcripts can be measured using well established methods, such as the Northern blot technique and reverse transcription polymerase chain reaction (PCR) [Alfieri et. al., 2006; Gottwald et. al., 2007]. The Western blot technique and 2D gel electrophoresis are examples of two common methods of measuring protein levels [Abdelmelek et. al., 2006; Alfieri et. al., 2006; Gottwald et. al., 2007; Han et. al., 1998]. However, there has also been much interest in using a bioluminescence construct as a reporter for heat shock gene expression, and more recently, it has been possible to detect luciferase in intact, living cells. The potential to use a luciferase/hsp reporter construct in live cells provides a novel way of detecting a potential interaction of MF with hsp expression.

Bioluminescence is a naturally occurring phenomenon whereby light is produced by a biological system via an ATP dependent reaction, during which chemical energy is converted into light energy. In the most commonly used system, bioluminescence is produced by the interaction of the luciferase enzyme with its substrate luciferin. When the luciferase gene is expressed, it produces luciferase protein. This enzyme then interacts with its substrate luciferin thereby catalyzing the oxidation of luciferin in the presence of ATP, oxygen, and Mg^{2+} [Beckham et. al., 2004]. This oxidation reaction

forms an electronically excited oxyluciferin. Light is emitted when oxyluciferin returns to its ground state, at a broadband emission spectrum of 500-700 nm [Hastings, 1996; Rice et. al., 2001]. Bioluminescent organisms such as the firefly (*photuris lucicrescens*), are a target for certain areas of research, including bioluminescent imaging, and, for the purposes of this research, genetically engineered reporter genes (described below). In fact, firefly luciferase is the most common form of the enzyme used in investigation of gene expression. There have been significant advances in the area of genetically engineered reporter genes [Brandes et. al., 1996; Contag et. al., 1997; Langridge et. al., 1994]. For example, O'Connel-Rodwell *et. al.* [O'Connell-Rodwell et. al., 2004] developed a reporter gene strategy in order to define cellular responses to varying hyperthermia conditions in NIH3T3 cells (Fig. 1.1). In this reporter construct, the heat shock 70 (*hsp70*) promoter sequence was fused to the coding sequence of the firefly luciferase gene. The heat shock promoter controls the degree of firefly luciferase (the biolouminescent reporter) transcription, by acting as an "on-off" switch [Beckham et. al., 2004; O'Connell-Rodwell et. al., 2004]. Specifically, when the *hsp70* promoter is activated due to cellular stress such as hyperthermia, it will induce the transcription and translation of the luciferase gene to produce the active enzyme, resulting in bioluminescence in the presence of luciferin. It has been shown that in the presence of excess luciferin, ATP, and Mg^{2+}, the light emission following enzyme substrate interaction is proportional to the concentration of luciferase protein produced [Beckham et. al., 2004; Brasier et. al., 1989].

There are recognized benefits of using a bioluminescent light emission system, rather than approaches such as fluorescence or phosphorescence. Quantitative data

analysis of fluorescence is complicated because of uncertainties in excitation light parameters and photobleaching. Alternatively, bioluminescence does not require external light excitation, and so provides a more direct measure of enzyme activity [Beckham et. al., 2004]. Additionally, because of the short half-life of the luciferase enzyme, it will not accumulate over time, a common artifact of the very stable green fluorescent protein (GFP). A shorter half-life provides a major benefit for tracking dynamic processes by enabling a more accurate light emission representation of changes in cellular gene expression. This feature is expected to be beneficial for the experiments performed for this thesis, since bioluminescence is measured in real-time, with a complete real-time analysis of the dynamic changes that occur in bioluminescence emission during manipulation of cells by field and heat over a 2 hour period. An additional benefit of using the described luciferase/hsp reporter system for detecting effects of MF exposure, is the potential interaction of the MF with the hsp promoter, as described previously [Lin et. al., 1999; Lin et. al., 2001]. Since the activation of the promoter is responsible for the changes in bioluminescence produced, any effective changes in promoter function as a result of MF exposure should be consequently detected.

1.4 – Objectives and Thesis Outline

To summarize, although there have been many papers published on the effects of magnetic field exposure on biological systems, no consensus has been reached with regards to biological magnetic field interaction, except perhaps on the possibility of RPM and magnetite. The purpose of this research is to investigate the potential interaction of static magnetic field with biological systems using a more powerful real-time method of

analysis. A few key ideas have been mentioned up to this point. First, there is a substantial amount of evidence to suggest an effect of magnetic field exposure on cellular calcium homeostasis. Additionally, a mechanism of static magnetic field interaction is described, specifically the Radical Pair Mechanism. We hypothesized that a cellular sensitization to magnetic field exposure follows an increase in free radical concentration, and may be measureable by observing a potential increase in cytosolic calcium levels. Second, we hypothesized that SMF may also increase the activity of heat shock proteins. The development of the heat shock protein/luciferase reporter system provides a novel method of measuring effects of SMF exposure. This thesis presents two main projects:

i) Measurement of changes in cytosolic calcium concentration between 0 mT and 100 mT magnetic field exposed cells after manipulation of the number of free radicals in the cell and after ATP activation

ii) Investigation of the effect of a 100 mT SMF exposure on the expression of the heat shock protein Hsp70, using a bioluminescent Hsp70/Luciferase reporter system,

All experiments were performed in a closed "black-box" environment, where cells were kept under highly controlled conditions of temperature, mixing, light exposure, and magnetic field intensity and uniformity. Both studies focus on the effect of SMF exposure in combination with a secondary stress, i.e. increased free radical concentration and heat respectively. A magnetic field strength of 100 mT was used in order to maintain consistency with previous experiments performed in our lab. Furthermore, 100 mT was

the maximum SMF that could be generated by the toroidal electromagnet without detectable heating effects on the water bath.

The body of this thesis is composed of two main papers, Chapter 2: **Real-time measurement of cytosolic free calcium concentration in DEM-treated HL-60 cells during static magnetic field exposure and activation by ATP**, and Chapter 3: **The effect of 100 mT SMF on activation of the Hsp70 promoter in the heat shock/luciferase reporter system.** Chapter 2 and Chapter 3 address the two main studies described above. Chapter 4 is a summary and discussion of the important finding of this thesis, the implications of the results, and potential future direction of this research.

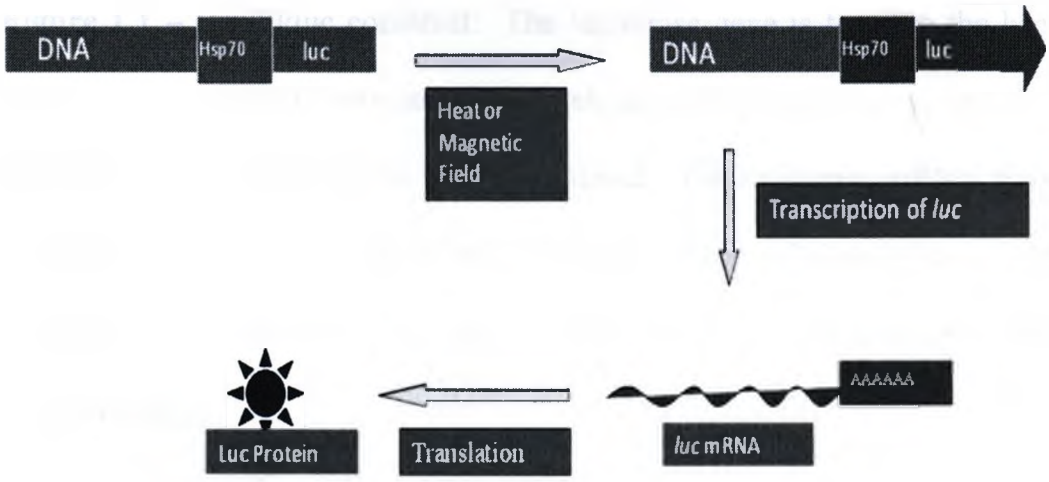
DNA
Hsp70
luc
Heat or Magnetic Field
DNA
Hsp70
luc
Transcription of luc
AAAAAA
luc mRNA
Translation
Luc Protein

Figure 1.1 – Hsp70/luc construct. The luciferase gene is fused to the hsp70 promoter. When the promoter is activated due to stress application (heat or magnetic field), the transcription of the luciferase gene is initiated. The luciferase mRNA transcript is then transported from the nucleus to the cytoplasm of the cell, where it is finally translated into the luciferase protein. Luciferase then interacts with its substrate luciferin to produce detectable light.

1.5 – References

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Chapter Two

Real-time measurement of $[Ca^{2+}]_c$ during SMF exposure

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Real-time measurement of cytosolic free calcium concentration in DEM-treated HL-60 cells during static magnetic field exposure and activation by ATP

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2.1 – Introduction

Human exposure to electromagnetic fields, increased through use of new technologies like MRI has prompted investigations into possible effects of static magnetic fields (SMF)s on cellular processes. This line of research has shown that SMF exposure can result in modification of cell proliferation [Aldinucci et. al., 2003; Chiu et. al., 2007], cell differentiation [Chiu et. al., 2007], and cell death by apoptosis or necrosis [Fanelli et. al., 1999; Jajte et. al., 2002; Tenuzzo et. al., 2006]. These results represent a collection of cellular responses that are known to be influenced by the intracellular concentration of ions and free radicals [Wong and Tepperman, 1994]. Several groups have chosen to look at cytosolic free calcium concentration as a readout for the effects of SMFs [Carson et. al., 1990b; Parkinson and Hanks, 1989; Walleczek and Budinger, 1992]. The calcium ion (Ca^{2+}) acts as a second messenger in signal transduction pathways related to neuronal excitability [Carson et. al., 1990b; Casteels and Raeymaekers, 1979; Gardner et. al., 1997b], muscle contraction [Casteels and

Raeymaekers, 1979], proliferation and differentiation [Gardner et. al., 1997b], and apoptosis [Lee et. al., 2006]. The low cytosolic free calcium concentration ($[Ca^{2+}]_c$) in resting cells compared to the much higher extracellular (and intracellular storage organelle) concentration of Ca^{2+} ions results in a large calcium gradient across the plasma membrane (and likewise across the calcium storage organelle membranes). Therefore, a change in membrane permeability due to the action of an external stimulus can result in an alteration in $[Ca^{2+}]_c$ [Carson et. al., 1990b].

One hypothesis for the method of action of SMF on biological systems is the radical pair mechanism (RPM). This hypothesis has been formulated by several groups based on evaluation of the chemistry literature where SMFs have been observed to affect reaction rates involving free radicals, which can lead to an increase in free radical concentration during SMF exposure [Eveson et. al., 2000a; Harkins and Grissom, 1994]. Free radicals are known to be potent oxidizers, which in the context of biological systems may result in oxidative stress on cells [Nauser et. al., 2005b]. Oxidative stress can result in a variety of biological effects including DNA damage [Kirkali et. al., 2007a], cell dysfunction [Valdez et. al., 2000a], apoptosis [Tirmenstein et. al., 2000a], and accelerated cell aging [Valdez et. al., 2000a]. To blunt the impact of free radicals, cells have evolved buffering mechanisms, the most important of which is the glutathione disulfide-glutathione (GSSH/2GSH) couple [Schafer and Buettner, 2001]. Perturbation of this buffering mechanism (e.g. by intracellular depletion of GSH) is known to lead to oxidative stress through the action of free radicals [Bizzozero et. al., 2006; Kirkali et. al.,

2007a], and lead to biological effects characteristic of free radical damage [Bizzozero et. al., 2006; Tirmenstein et. al., 2000a; Valdez et. al., 2000a].

Several groups have observed effects of SMFs that are consistent with the RPM. For example, exposure of ethanolamine ammonia lyase to a SMF resulted in changes in free radical activity, but only in the presence of an artificial substrate [Harkins and Grissom, 1994]. These observations are supported peripherally by studies on the effects of time-varying magnetic fields on cells, where there have been reports of field effects on oxidative stress [Harkins and Grissom, 1994], DNA damage [Zmyslony et. al., 2000], and apoptosis [Jajte et. al., 2002]. Although these reports suggested that magnetic fields influenced biological systems through the action of free radicals and hence the RPM, the direct measurement of magnetic field effects on free radicals in biological systems has yet to be demonstrated due to technical challenges. An easier approach would be to manipulate the free radical concentration in the biological system while observing the effect of the magnetic field. For example, this approach might be realized by experimental manipulation of the free radical buffering capacity of cells during SMF exposure. The work for this study was driven by the hypothesis that depletion of the cells buffering capacity toward free radicals should affect the cells' response to SMF exposure.

In this study, the free radical hypothesis was tested by evaluating if GSH depletion affected cells during exposure to a 100 mT SMF. The cells' response to SMF was monitored by measurement of $[Ca^{2+}]_c$ under conditions of rest and ATP activation. A transformed human myeloid leukemia cell line (HL-60) was used, due to the extensive calcium observations done with this cell line in the past, including in our lab [Belton et.

al. 2007, Gardner et. al. 1997, Sabo et. al. 2002, Carson et. al., 1990]. Specifically, $[Ca^{2+}]_c$ was measured in fura-2 loaded HL-60 cells by ratiometric, cuvette-based fluorescence spectroscopy. During experiments, cells were exposed to either a 100 mT SMF or a sham condition (i.e. 0 mT) in the presence of Diethyl Maleate (DEM), which has been shown to deplete intracellular GSH [Tirmenstein et. al., 2000a]. During exposure, ATP was added to the cells to facilitate changes in $[Ca^{2+}]_c$ through alterations in permeability of the intracellular calcium storage organelle membranes [Lee et. al., 2006].

2.2 – Methods

2.2.1 – Materials

The HL-60 human leukemia cell line and Iscove's Modified Dulbeccos Medium (IMDM) were purchased from American Type Tissue Collection (ATCC Manassas, VA). Fura-2 AM, Br-A23187, and Fetal Bovine Serum were purchased from Invitrogen (Burlington, ON). All other materials were purchased from Sigma Aldrich Canada (Oakville, ON). Buffered Saline Solution (BSS) consisted of D-[L]-Glucose (5 mM), HEPES (10 mM), NaCl (135 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1 mM), and sulfinpyrazone (0.225 mM). BSS was prepared in the laboratory and pH adjusted to 7.4 with NaOH pellets.

2.2.2 – Cell culture

Undifferentiated HL-60 cells were cultured in IMDM supplemented with 20% FBS in a humidified incubator at 37°C, 5% CO_2, and 95% air. Cell density was determined

daily. Typically, a 25 µL sample of cells in media was removed from the tissue culture flask and mixed with an equal volume of Trypan blue (0.4%) in a 1.5 mL Eppendorf tube. A volume of the mixture was transferred to a hemocytometer and viable cells were counted using the Trypan blue exclusion criteria. Cultures were diluted with fresh media as needed to keep cells within the density range of 2.5 x 10^5 to 1 x 10^6 cells/ml. Cells were centrifuged at 250 x g and re-suspended in new media approximately once per week.

2.2.3 – Sample preparation

Prior to each experiment, a volume of flask material containing 3.5x10^6 to 4.0x10^6 cells was taken from culture and centrifuged at 250 x g. The supernatant was removed. Cell pellets were combined and re-suspended in 1 ml BSS. The single tube of cells was centrifuged at 250 x g. The supernatant was removed and cells were re-suspended in 1 ml BSS. Fura-2AM (2 µg in 2 µl DMSO) was added to re-suspended cells, followed by gentle mixing and then incubation in a 37°C water bath for 30 min. Cells were then washed with 1 ml BSS twice to remove extracellular fura-2AM. The 1 mL of fura-2 loaded cell suspension was then transferred to a 1 cm x 1 cm disposable fluorescence cuvette and the volume was adjusted to 2.4 mL with BSS.

2.2.4 – Apparatus for measurement of cytosolic free calcium

Cytosolic free calcium in fura-2 loaded cell samples was measured with a modified ratiometric fluorescence spectroscopy system, comprised of a light source, chopper with filter assembly, bifurcated quartz fiber bundle, emission filter, photomultiplier tube detector, control electronics, and a desktop computer running *Felix32* data collection and

spectrometer control software (PTI, London, Ontario). The original commercial sample holder was replaced with a black acrylic enclosure. The enclosure contained an acrylic water bath, a cuvette holder, and a nonmagnetic optical assembly that delivered light and collected fluorescence from the sample. A toroidal electromagnet (15 mm pole gap, 16 AWG copper magnet wire, Arnold Engineering) was held within the water bath (kept at 36 ± 0.1°C by temperature controlled circulating water). The cuvette was held between the poles of the electromagnet. Water was pumped into the bath via an inflow port and flowed out through an overflow tube in order to ensure the electromagnet was completely submerged, but no water would overflow into the cuvette. Bath temperature was monitored with a thermistor and bridge circuit described elsewhere [Carson and Prato, 1996]. The electromagnet was driven by a power amplifier in current mode (7570 AE Techron, Elkhart, IN) and an arbitrary function generator (Model 75, Wavetek). A custom feedback circuit between the waveform generator and the amplifier was used to monitor and actively control the field (magnetic field error ± 0.05 mT). Cells were maintained in suspension with a computer-controlled mixer at 3 Hz, with a vertical displacement of 2 mm. The mixer was similar in design to a system described previously [Belton et. al., 2008]. The sample was excited alternately at 340 nm (calcium bound fura-2) and 380 nm (calcium free fura-2) using the spectrometer in combination with the custom optical assembly. Sample fluorescence was detected at 510 nm. Wavelength selection was performed with a 10 nm bandpass filter.

2.2.5 – Protocol for measurement of cytosolic free calcium

After fura-2 loading, cells were acclimatized to the 36°C bath environment at a SMF strength of 0 ± 0.05 mT for 1200 s prior to any experimental manipulation. At 1200 s, 24 μL of 8 mM DEM solution was added to the cell sample. At 2700 s, the field condition was applied, where the SMF either remained unchanged (sham or 0 mT), or was increased to 100 mT by manual adjustment of the DC signal from the waveform generator (adjustment took approximately 5-10 s and resulted in a dB/dt of 0.01-0.02 T/s). At 3000 s, 10 μL of 240 μM ATP was added to reach a final cuvette concentration of 1 μM. The magnetic field was returned to 0 mT at 3480 s for all experiments. The fura-2 signal was calibrated for each sample by the addition of Br-A23187 to a final concentration of 15 μM (3600 s) and EGTA (pH 8.4) to a final concentration of 0.6 M (4100 s). The cytosolic free calcium concentration was determined using the equation $[Ca^{2+}] = K_d \cdot Q \cdot (R-R_{min})/(R_{max}-R)$, where R was the ratio of fluorescence observed at excitation of 340 nm and 380 nm, K_d represented the dissociation constant for fura-2 (140 nM), and Q corresponded to the ratio of the fluorescence intensity at an excitation of 380 nm after adding EGTA and Br-A23187, respectively [Grynkiewicz et. al., 1985]. The calcium concentrations were plotted against time at a sample rate of 5 Hz. Cell viability at the end of each experiment was determined to be unchanged by trypan blue exclusion assay (data not shown). Ten paired experiments (i.e. two samples taken from the same culture flask, but in sequential order, approximately 2 h apart) were performed to assess systematic errors related to sample order and mock activation of the magnetic field. Mock activation referred to continued maintenance of the magnetic field at 0 mT from 2700 s to 3480 s. This was followed by 20-paired experiments where the magnetic field

was turned on for one of the samples from each pair. This resulted in 10 sham and 10 field-exposed samples that were drawn from the culture flask first and 10 sham and 10 field-exposed samples that were drawn from the culture flask second (approximately 2 h after the first sample was drawn from the flask).

2.2.6 – Time series and statistical analysis

Five metrics were computed for each $[Ca^{2+}]_c$ time series: (1) the average before DEM (610 – 1190 s), (2) the average after DEM but before SMF condition (1210 – 2690 s), (3) the average during SMF condition but before ATP (2710 – 2990 s), (4) the peak value after SMF condition and after ATP (3000 – 3460 s), and (5) the full width half maximum (FWHM) of the peak shape after SMF condition and after ATP. All times series calculations were performed with custom software written in C++. Statistical analysis included two-factor ANOVA and paired t-tests (Excel, Microsoft Corp., Redmond, WA). Systematic differences due to sample preparation order (i.e. first vs. second sample taken from the same culture flask) and mock onset of the SMF (i.e. pre vs. post field condition for sham/sham experiments) were analyzed. Data for the two-factor ANOVA was grouped by SMF condition (sham vs. 100 mT) and sample preparation order (first vs. second sample taken from the same culture flask). Data for the paired t-test was grouped by SMF or sample preparation order. Reported errors represent standard errors of the mean.

2.3 – Results

2.3.1 – Temperature measurements

Consistency of the water bath temperature before, during and after field exposure was monitored in real-time using a thermistor and bridge circuit (Fig. 2.1A). The average water bath temperature was approximately 36.0°C before field exposure. During field exposure, the bath temperature increased by approximately 0.05°C. After the field was removed, bath temperature returned to 36.0°C almost immediately.

2.3.2 – Real-time data

Data collected by the ratiometric fluorescence spectroscopy system was first displayed in a real-time fluorescence trace, and then converted into a real-time cytosolic free calcium concentration trace, as represented by Fig. 2.1B/C. A linear increase in baseline $[Ca^{2+}]_c$ was observed during the experiment, which was likely due to leakage of fura-2 into the calcium-containing BSS bathing the cells. After addition of DEM (1200 s), a biphasic response was visible as a small increase in $[Ca^{2+}]_c$ followed by a decrease to a level above baseline. The biphasic response lasted approximately 400 s and resulted in a small, but long lasting, elevation in $[Ca^{2+}]_c$ above baseline values. After the addition of ATP (3000 s), there was a rapid and large increase in $[Ca^{2+}]_c$ that was followed by a fast decline, which slowed as $[Ca^{2+}]_c$ neared the baseline.

2.3.3 – Sham/sham experiments

The reliability of the protocol was tested by an investigation into potential systematic errors from mock onset of the SMF and sample ordering. Ten paired

sham/sham experiments were performed for a total of 20 runs. Pairing referred to the order in which the cell samples were removed from the culture flask before sample preparation. Fig. 2.2A represents the average $[Ca^{2+}]_c$ computed from time series data before (pre-DEM) and after (post-DEM) the addition of DEM for runs prepared from samples taken first or second from the flask. The average $[Ca^{2+}]_c$ for pre-DEM runs was 38 ± 5 nM for first samples and 38 ± 5 nM for second samples. The average $[Ca^{2+}]_c$ for post-DEM runs was 53 ± 5 nM for first samples and 53 ± 7 nM for second samples. The two-way ANOVA revealed a significant difference in $[Ca^{2+}]_c$ between pre vs. post DEM treatment ($P < 0.01$, $1-\beta = 0.78$), but no significant difference in $[Ca^{2+}]_c$ between the first and second samples ($P > 0.95$, $1-\beta = 0.05$). Furthermore, there was no significant interaction between the two factors ($P > 0.90$, $1-\beta = 0.05$). Fig. 2.2B represents the average $[Ca^{2+}]_c$ for the first and second samples before (Pre-Field condition) and during (Field condition) mock onset of the SMF, after the addition of DEM. The pre-field measurements were taken at the same time as the post-DEM measurements, and so result in identical values. The average $[Ca^{2+}]_c$ for Pre-Field condition runs was 53 ± 5 nM for first samples and 53 ± 7 nM for second samples. The average $[Ca^{2+}]_c$ for mock field condition runs was 58 ± 5 nM for first samples and 59 ± 7 nM for second samples. A two-way ANOVA revealed no significant effect on $[Ca^{2+}]_c$ measured before or after exposure to the mock SMF ($P > 0.80$, $1-\beta = 0.05$) and no significant effect when samples were taken first or second from the culture flask ($P > 0.30$, $1-\beta = 0.15$). No significant interaction between mock SMF and sample order was found ($P > 0.9$, $1-\beta = 0.05$). Following the addition of ATP to the cuvette (Fig. 2.2C), the peak $[Ca^{2+}]_c$ was 180 ± 13 nM for samples taken from the culture flask first, and 208 ± 18 nM for the samples taken

second. A paired t-test revealed a statistically significant difference in the mean peak $[Ca^{2+}]_c$ between these groups (P <0.05). The FWHM values derived from the shape of the peak $[Ca^{2+}]_c$ response were 49 ± 4 s and 53 ± 4 s for the first and second samples, respectively. A paired t-test revealed no significant difference between the mean FWHM values between the two groups ($P > 0.1$).

2.3.4 – SMF experiments

The next set of experiments was performed to compare the effect of a 100 mT SMF on the five $[Ca^{2+}]_c$-based metrics. Ten paired runs (first sample vs. second sample) were performed without SMF exposure, and 10 paired runs experienced a 100 mT SMF. In order to eliminate any potential ordering effects (for example, as found for the peak metric describing the $[Ca^{2+}]_c$ response to ATP), first sample experiments and second sample experiments were pooled, then divided into 0 mT and 100 mT experimental groups. From the pooled data (Fig. 2.3A), the average $[Ca^{2+}]_c$ measured prior to any SMF condition was 48 ± 2 nM for the sham group and 53 ± 2 nM for the 100 mT group. The average $[Ca^{2+}]_c$ measured during the SMF condition was 53 ± 2 nM for the sham group and 58 ± 2 nM for the 100 mT group. A two-way ANOVA revealed a significant effect on average $[Ca^{2+}]_c$ due to the onset (Pre-Field condition vs. Field condition) of SMF ($P <$ 0.01, $1-\beta = 0.81$), and a significant effect on average $[Ca^{2+}]_c$ due to the presence (0 mT vs. 100 mT groups) of the SMF ($P < 0.01$, $1-\beta = 0.72$). However, no significant interaction between the two factors was observed ($P > 0.90$, $1-\beta = 0.05$). Fig. 2.3B shows the peak and the FWHM of the $[Ca^{2+}]_c$ response after the addition of ATP to the sample cuvette. The peak $[Ca^{2+}]_c$ for the sham group was 189 ± 10 nM and 185 ± 9 nM for the

100 mT group. A paired t-test revealed no significant effect of SMF on peak $[Ca^{2+}]_c$ ($P > 0.75$). The FWHM for the sham group was 51 ± 3 s and 54 ± 3 s for the 100 mT group, but the means were not statistically different by a paired t-test ($P > 0.40$).

2.3.5 – Effect of SMF and sample order on peak $[Ca^{2+}]_c$ following ATP

Since a systematic error was observed on peak $[Ca^{2+}]_c$ during sham/sham experiments (Fig. 2.2C), the effects of SMF and sample order were analyzed for the experiments where conditions were sham and 100 mT exposure (Fig. 2.3C). Like the prior experiments (to allow for systematic error evaluation), pairing referred to the sample order, where the first sample in each pair represented cells taken from the culture flask first, and the second sample represented the cells taken from the same culture flask second (~2 hours later). The peak $[Ca^{2+}]_c$ following ATP for samples prepared from the first set of culture flask samples was 185 ± 11 nM for the sham group and 196 ± 17 nM for the 100 mT group. The peak $[Ca^{2+}]_c$ for the samples prepared from the second set of samples was 193 ± 17 nM for the sham group and 175 ± 7 nM for the 100 mT group. A two-way ANOVA resulted in no statistically significant effect of SMF ($P > 0.75$, $1-\beta = 0.06$), sample order ($P > 0.60$, $1-\beta = 0.08$), or interaction between SMF and sample order ($P > 0.30$, $1-\beta = 0.18$).

2.4 – Discussion

2.4.1 – Quality of $[Ca^{2+}]_c$ data and potential temperature artifacts

The data collected for this study was reliable and sensitive to changes in $[Ca^{2+}]_c$. Comparison of the real-time $[Ca^{2+}]_c$ trace to the real-time fluorescence data (Fig. 2.1

B/C)was consistent with the signal properties of fura-2 [Grynkiewicz et. al., 1985]. For example, after the addition of ATP, the fluorescent signals at 340 nm (i.e. calcium-bound fura-2) and 380 nm (i.e. unbound fura-2) increased and decreased, respectively. The computed increase in fluorescence ratio was consistent with the known behavior of fura-2 and to the known response of HL-60 cells to ATP [Belton et. al., In Press]. The data from this study was also not corrupted by potential artifacts from SMF-related changes in environmental temperature. No significant change in water bath temperature was observed before, during or after the application of current to the electromagnet either for the sham or 100 mT experimental runs (Fig. 1A).

2.4.2 – Basic findings –systematic sources of error intrinsic to the protocol

The results from sham/sham experiments confirmed that there was no systematic error for four of the five $[Ca^{2+}]_c$ metrics related to the order in which the samples were prepared (i.e. first vs. second preparation order). Specifically, differences in the measured values of $[Ca^{2+}]_c$ before DEM addition, immediately before field exposure, and after the onset of field did not depend on the sample preparation order. Likewise, the sample preparation order did not affect the FWHM of the $[Ca^{2+}]_c$ response to ATP. However, the peak $[Ca^{2+}]_c$ after ATP activation was dependant on the sample preparation order. The first sample on average had a peak $[Ca^{2+}]_c$ that was approximately 30 nM lower than the second sample. One explanation for the apparent increase in the peak $[Ca^{2+}]_c$ is the increased amount of handling the second sample of cells received compared to the first. A second explanation could be related to the opening of the flask during preparation of the first sample. This likely resulted in exchange of the CO_2 atmosphere in

the culture flask with atmospheric air inside the bio-safety hood, which may have affected the stress tolerance of cells. In either case, an increase in cellular stress or a decrease in the cells' resistance to stress could have resulted in an increase in $[Ca^{2+}]_c$. Therefore, with the existing protocol, the measurement of four of the five $[Ca^{2+}]_c$ metrics were likely to be reliable measures of SMF effects and free of measurable systematic sources of error. However, interpretation of potential positive SMF findings by estimates of the fifth metric on peak $[Ca^{2+}]_c$ needed to be interpreted with some caution and required inclusion of the sample preparation order in the statistical analysis.

2.4.3 – Basic findings – effect of SMF on HL-60 cells

Since potential systematic errors were not introduced by the protocol for most metrics, the data from the first and second sample preparation order groups was pooled. This procedure resulted in a doubling of the n for each group and increased the statistical power of the comparisons between the $[Ca^{2+}]_c$ metrics when the effect of field was examined. When the $[Ca^{2+}]_c$ levels were compared before and during field, there appeared to be a slight, but similar increase in $[Ca^{2+}]_c$ in both the sham and 100 mT groups. Since the sham group did not experience a change in field strength during the run, the similar increase observed in the 100 mT group was likely not related to the onset of the field exposure but due to another process that was present in both the sham and 100 mT groups. The most likely possibility was the slow upward drift in $[Ca^{2+}]_c$ related to the leakage of fura-2 from the cell into the calcium-containing BSS buffer. Another reason could include the length of time the cells were exposed to DEM. Field-Condition measurements were taken after significantly longer cellular DEM exposure, compared to

Pre-Field Condition measurements. Longer DEM exposure could result in higher average $[Ca^{2+}]_c$ in both sham and 100 mT exposed groups. When the effect of field was examined, there appeared to be a small increase in $[Ca^{2+}]_c$ in the 100 mT group over the sham group both prior to the onset of field and during field exposure. However, since no SMF was present in the 100 mT group prior to field onset, the increase over the sham group could not be due to the 100 mT field. The statistical analysis supported this interpretation since there was no interaction between the effect of field onset and the presence of the field. That is, one would have expected the difference in $[Ca^{2+}]_c$ between the sham and 100 mT groups to be larger after field onset than before the appearance of the field. The analysis of the metrics describing the $[Ca^{2+}]_c$ response to ATP also depended on the 100 mT field. Neither the peak $[Ca^{2+}]_c$ nor the FWHM were affected. Taken together the data suggested that there was no effect of a 100 mT SMF on resting or ATP-activated $[Ca^{2+}]_c$ in HL-60 cells pre–treated with DEM. This implied that depletion of GSH in HL-60 cells, which likely gave rise to an increase in free radical concentration, did not potentiate changes in $[Ca^{2+}]_c$ that may have been sensitive to a 100 mT SMF.

2.4.4 – Follow-up analysis on peak $[Ca^{2+}]_c$ after ATP

A follow-up analysis was then performed on the $[Ca^{2+}]_c$ peak following ATP activation, which was sensitive to the preparation order of the samples (Fig. 2.2C). The $[Ca^{2+}]_c$ peak data was grouped by sample preparation order (i.e. first and second) and the two SMF conditions (i.e. sham and 100 mT). Contrary to the findings in the sham/sham experiments, the statistical analysis for the sham/field experiments revealed no effect of sample preparation order, SMF, and a lack of interaction between sample order and SMF.

This confirmed our findings from the grouped data, but it also indicated that the systematic error observed in the sham/sham experiments (Fig. 2.2C) was not reproducible. This may have been related to the improved skill of the person handling the flasks between the first set of sham/sham experiments and the sham/field experiments, i.e. the flasks were opened for a shorter period of time, which left less time for gas exchange and resulted in a lower stress to the cells.

2.4.5 – Comparison to other studies

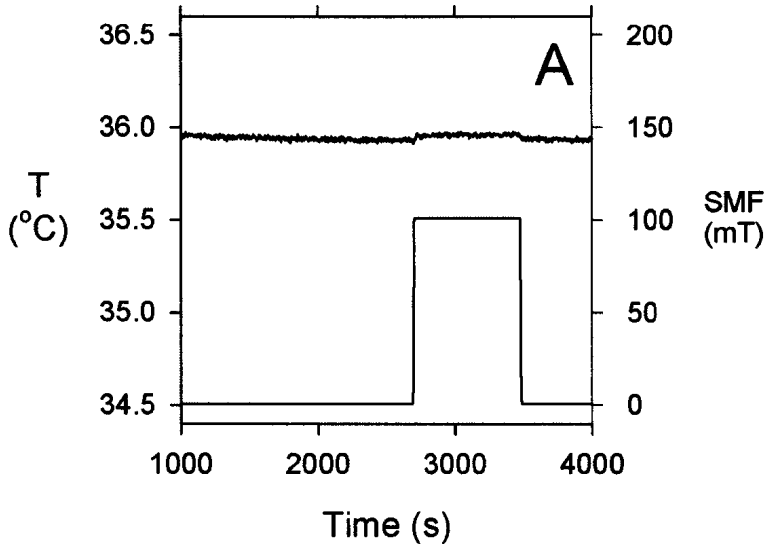
Previous studies have mainly investigated changes in basal $[Ca^{2+}]_c$. A study performed by Tenuzzo et. al. [2006], found that human lymphocytes were sensitive to SMF exposure, where a significant increase in basal $[Ca^{2+}]_c$ was observed, among other biological effects [Tenuzzo et. al., 2006]. However, the time of cell exposure used was on the order of 24 to 48h, significantly longer than the field exposures used in this study. These observations could imply that longer field exposure may be necessary in order to elicit a SMF effect in cells.

Additionally, a previous study in our lab also looked at changes in $[Ca^{2+}]_c$ response to ATP in HL-60 cells, during varying strengths of SMF exposure [Belton et. al., In Press]. The applied SMF strengths varied between 1 mT to 100 mT, and so are directly comparable to the magnitude of the SMF used in this investigation. It was found that the application of SMF did not significantly change the cytosolic free calcium levels following ATP activation. Likewise, results presented in this paper provide evidence in support of these previous findings. Moreover, an additional variable in our experiments was included: DEM. It was hypothesized that since DEM depletes free radical

scavengers (e.g. GSH), it should indirectly increase the number of free radicals in the cell. This hypothesis was supported by the observable increase in cytosolic free calcium concentration immediately following the addition of DEM. It is well established that DEM depletes GSH [Termenstein et. al. 2000]. Additionally, it has been shown that GSH depletion caused an increase in cytosolic free calcium concentration [Wong et. al. 1994], and so we concluded that the observed increase in $[Ca^{2+}]_c$ was an indication of depleted GSH levels and the free radical equilibrium was likely affected. Although an increase in $[Ca^{2+}]_c$ after DEM addition was observed, this stress did not have an effect on overall cellular response to field exposure. Since these results are comparable to the results of previous studies done in our lab in the absence of DEM, it can be hypothesized that decreasing the number of free radical scavengers in the cell does not exclusively induce enough intracellular free radicals for the SMF to exert significant effects through the radical pair mechanism as observed by measurement of $[Ca^{2+}]_c$. Furthermore, other studies conducted by Chater et. al. [2006], imply that environmental factors other than DEM can affect intracellular GSH concentrations, such as SMF exposure. After exposing rat hepatocytes to a 128 mT SMF, this group observed a significant increase in intracellular GSH concentration [Chater et. al., 2006]. Thus, the opposing effects of DEM and SMF on GSH concentrations may result in no significant changes in cellular GSH levels, and so unaltered free radical numbers. This could compromise any cell sensitization to SMF exposure, providing a potential explanation for the lack of a statistically significant difference in $[Ca^{2+}]_c$ between sham and field exposed experiments.

2.4.6 – Conclusions and Future Work

Based on the results from this study, it was observed that $[Ca^{2+}]_c$ did not change during SMF exposure in DEM-treated HL-60 cells either at rest or after activation with ATP. The findings supported the hypothesis that a 100 mT SMF had no effect on resting or activated $[Ca^{2+}]_c$ in HL-60 cells even when the intracellular free radical concentration was manipulated. There are other possibilities however. For example, (1) an effect of SMF might have been present but not measurable with the $[Ca^{2+}]_c$-dependent metrics measured in the study, and (2) potential effects of SMF on HL-60 cells may not be influenced by the presence or absence of DEM. Two streams for future work can be suggested from these possibilities. The experiments could be repeated over a greater range of magnetic field strengths to include values above and below 100 mT. Also, repetition of the experiments at a variety of doses of DEM and with GSH potentiators such as L-NAC and glutathione diesters would allow for a greater range of GSH levels to be tested. This last possibility is driven by the hypothesis that a threshold free radical concentration exists where the action of SMF becomes apparent.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36.5
36.0
35.5
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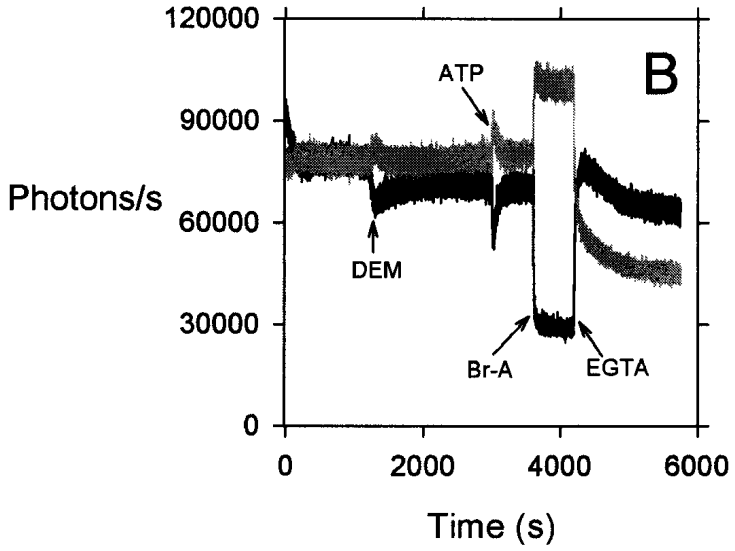
Time (s)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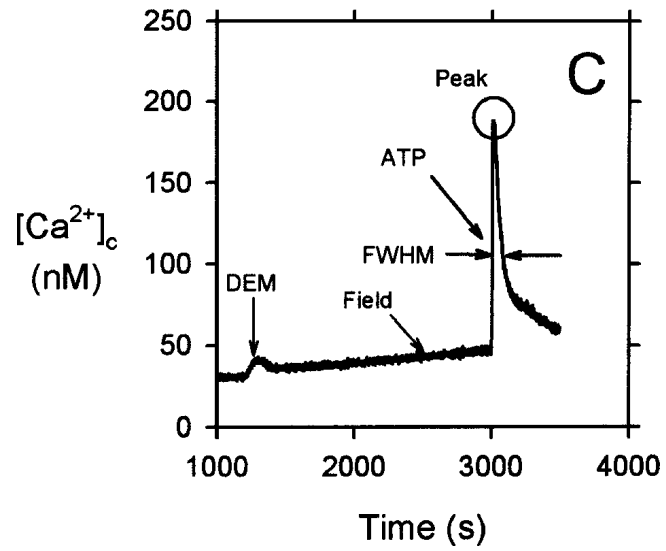
C
250
200
150
100
50
0
[Ca2+]c
(nM)
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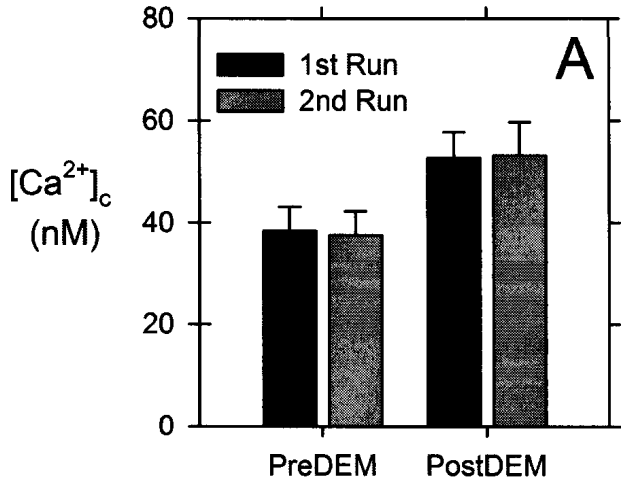
3000
4000
Time (s)

Figure 2.1 - Water bath temperature during SMF exposure and an example of real-time data. **A**. Representative real-time temperature and static magnetic field trace. Upper trace demonstrates consistency in water bath temperature before, during, and after SMF exposure in a sample experiment. The SMF intensity is represented by the lower trace. SMF was turned on at 2700 s, and turned off at 3480 s. Water bath temperature increased ~0.05°C while SMF was turned on. **B**. Real-time ratio-metric fluorescence trace, demonstrating response to DEM and ATP (1 μM final concentration) added to the cuvette at 1200 s and 3000 s, respectively. Upper grey trace represents calcium-bound fura-2, lower black trace represents calcium unbound fura-2. Addition of DEM, 1 μM ATP, Br-A and EGTA are marked with arrows. **C**. Corresponding real-time calcium trace, demonstrating $[Ca^{2+}]_c$ response to DEM and 1 μM ATP added to the cuvette at 1200 s and 3000 s, respectively. Addition of DEM, field, and 1 μM ATP, as well as the FWHM of the peak ATP response are marked with arrows. Measurements of $[Ca^{2+}]_c$ were averaged over four different times intervals: Pre-DEM (610 – 1190 s), Post-DEM/Pre-Field condition (1210 – 2690 s), Post-DEM/Field condition (2710 – 2990 s), and ATP peak (3000 – 3460 s).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0
[Ca2+]c
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1st Run
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A
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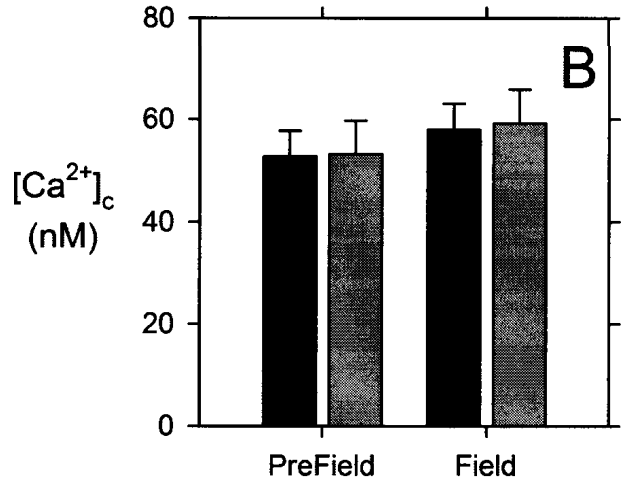
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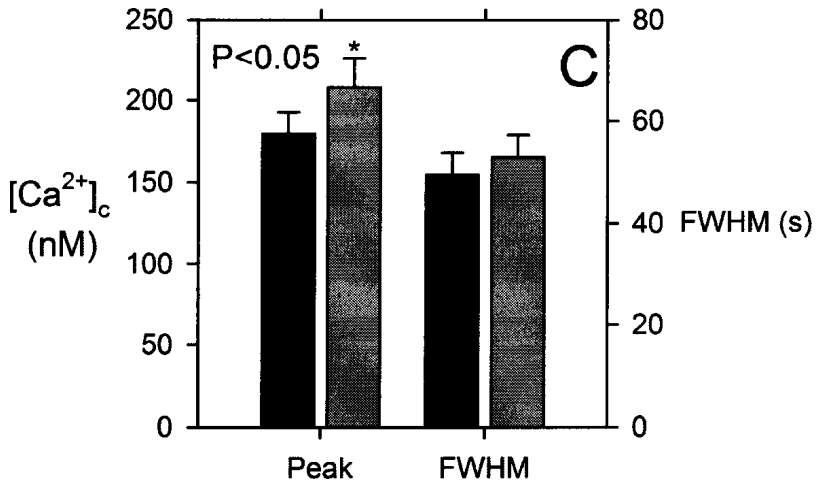
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0
[Ca2+]c
(nM)
P<0.05
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C
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40
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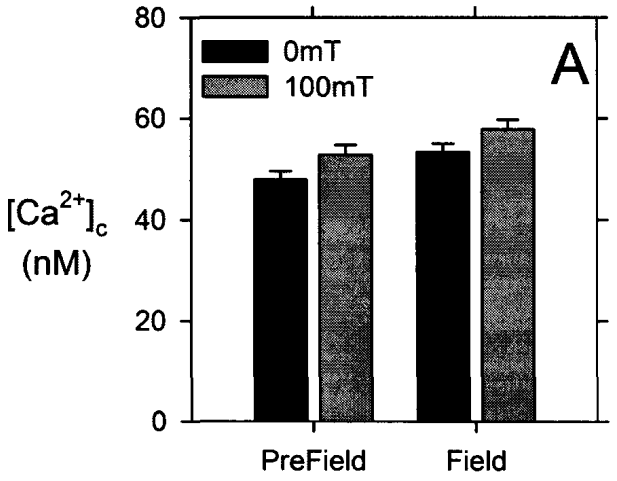
0
FWHM (s)
Peak
FWHM

Figure 2.2 - Dependence of $[Ca^{2+}]_c$ on sample ordering. In all panels, black and grey bars represent first and second sample of cells removed from culture flask, respectively. Each bar with error represents the mean ± standard error of the mean for n = 10 experiments. For all field conditions, mock SMF exposure was used (i.e identical to sham [0 mT] experiments). **A**. Average $[Ca^{2+}]_c$ measured before (Pre-DEM) and after addition of DEM (Post-DEM). **B**. Average [Ca2+]c measured after the addition of DEM but before (Pre-Field condition) and after mock SMF exposure (Field condition). **C**. Average peak $[Ca^{2+}]_c$ (Peak) and average full width at half maximum (FWHM) of the $[Ca^{2+}]_c$ response following ATP activation. Each bar is representative of n=10 experiments.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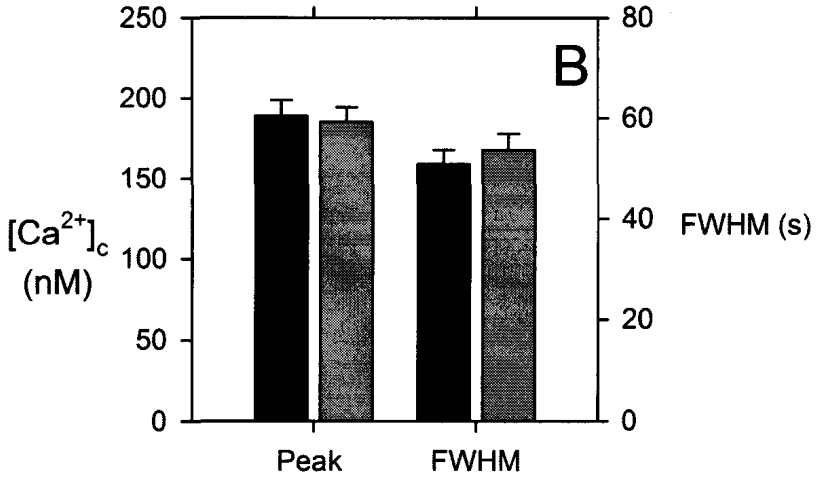
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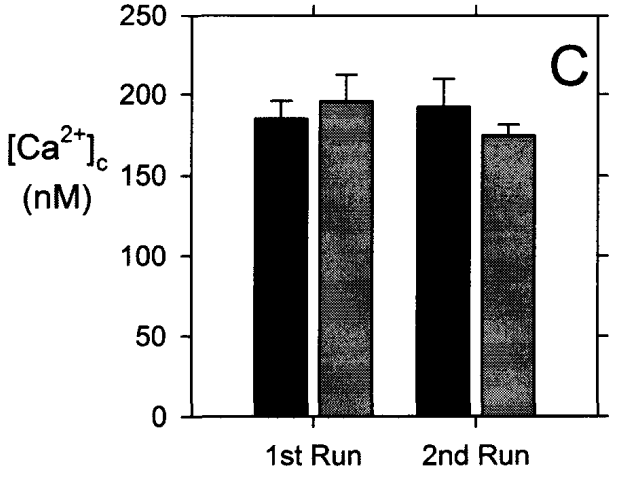
(nM)
FWHM (s)
Peak
FWHM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C
[Ca2+]c
(nM)
1st Run
2nd Run

Figure 2.3 - Comparison of $[Ca^{2+}]_c$ between sham (0mT) and 100 mT field condition experimental groups. **A.** Average $[Ca^{2+}]_c$ measured after DEM addition but before (Pre-Field) and during SMF condition (Field). **B.** Average peak $[Ca^{2+}]_c$ (Peak) and average full width at half maximum (FWHM) of the $[Ca^{2+}]_c$ response following ATP activation. **C.** Average peak $[Ca^{2+}]_c$ following ATP activation for sham and 100 mT experiments, grouped by preparation order (i.e. first vs. second). For all panels, the black and grey bars represent mean values for sham and 100 mT experimental groups, respectively. Error bars represent SEM for each bar. Each bar is representative of n = 20 experiments.

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Chapter Three

Hsp70/luciferase construct as reporter for SMF exposure

3.1 – Introduction

3.1.1 – Heat Shock Protein Background

The induction of heat shock proteins (hsp) in cellular systems following hyperthermal conditions is a well understood phenomenon [Kiang and Tsokos, 1998; Lepock, 2005]. It was first observed in 1962 by Ritossa *et. al.* in Drosophila salivary gland cells [Ritossa and Vonborstel, 1964]. When these cells were exposed to heat conditions of 42°C or more, a transient and consistent increase of gene expression and protein synthesis was observed. The proteins synthesized had molecular weights of 26 and 70 kDa. Upon further investigation, it was determined that these proteins were always over-expressed following an application of heat stress to the cells. These proteins were thus aptly named "heat shock proteins". Since then, there have been numerous additional discoveries of new proteins belonging to the heat shock family. Members of the heat shock family are referred to by their molecular weight (i.e. hsp70) with molecular masses ranging from 20 to 110 kDa.

Heat shock proteins are found endogenously in all cells, both prokaryotic and eukaryotic. They are highly conserved between all cell types, with a 68-70% amino-acid identity among eukaryotic cells [Bardwell and Craig, 1984; Kiang and Tsokos, 1998]. Their highly conserved structure suggests that these proteins play a very important role in cell function and survival. HSPs with molecular weights of 60, 70, 90 and 110 kDa are better understood than others, and are considered the major and most commonly expressed heat shock proteins. Indeed, the transcription and translation of these major heat shock proteins is constantly under activation throughout the cells' life cycle under

normal physiological conditions, such as cell differentiation or proliferation [Kiang and Tsokos, 1998; Santoro, 2000].

3.1.2 – Activation and Regulation of Heat Shock Proteins

The 70 kDa heat shock protein is one of the most well understood and conserved proteins in the heat shock protein family. Levels of hsp70 mRNA and protein have been shown to be affected following a variety of external stress stimuli, including food-deprivation [Cara et. al., 2005], insulin exposure [Li et. al., 2006], high hydrostatic pressure [Kaarniranta et. al., 1998; Kaarniranta et. al., 2000], and electromagnetic field exposure [Alfieri et. al., 2006; Goodman and Blank, 1998; Gottwald et. al., 2007; Han et. al., 1998; Junkersdorf et. al., 2000; Pipkin et. al., 1999; Tokalov and Gutzeit, 2004] or pulsed magnetic field stimulation [Tsurita et. al., 1999]. Initiation of hsp70 gene expression is dependent on the activation of the hsp70 promoter. The activation of the promoter involves two main elements: the heat shock factor (HSF), and the heat shock element (HSE). Heat shock proteins are typically bound to HSFs when they are inactive. This heat shock protein/HSF complex is located in the cytosol of the cell, under unstressed conditions. During heat exposure (typically 42°C or higher) or other stress stimuli, the heat shock protein dissociates from the HSF. The released heat shock protein is then free to assist in protein folding and stabilization, whereas the HSF is phosphorylated and then enters the nucleus of the cell. The nucleus is where the first stage of gene expression occurs: transcription. During unstressed condition, the heat shock element, HSE, which is a region of the hsp70 promoter, remains unbound thus inhibiting transcription. When there is an increase in phosphorylated HSFs entering the

nucleus, the HSF will interact with the HSE. The interaction of the HSF with HSE prevents the inhibiting action of the HSE, allowing transcription of hsp70 mRNA to begin in the nucleus. The transcribed hsp70 mRNA then leaves the nucleus and enters the cytosol of the cell, where translation, the final step of protein synthesis takes place. Consequently, when the levels of hsp70 increase in the cell, the heat shock proteins will bind to the HSFs, resulting in less HSF/HSE interaction and thus inhibiting further protein production via a negative feedback mechanism [Kiang and Tsokos, 1998].

3.1.3 – Heat Shock Proteins and Magnetic Field Exposure

Recently, there has been an interest in the area of magnetic field induced heat shock proteins [Alfieri et. al., 2006; Coulton et. al., 2004; Goodman and Blank, 1998; Gottwald et. al., 2007; Han et. al., 1998; Junkersdorf et. al., 2000; Pipkin et. al., 1999; Robertson et. al., 2007; Tokalov and Gutzeit, 2004]. Investigation into safe and regulated method of elevating Hsp levels is largely inspired by the clinical benefits of controlled elevated levels of heat shock proteins. Many studies describe the clinical relevance of preconditioning organs and tissues at risk with increased heat shock protein levels [Gottwald et. al., 2007; Han et. al., 1998; Li et. al., 2006; Robertson et. al., 2007]. Due to the cytoprotective effects of these proteins, it has been shown that increased levels of heat shock proteins are useful in protecting organs and tissues from ischemic injury, metabolic stress or heat [Han et. al., 1998; Li et. al., 2006]. The induction of heat shock proteins by magnetic field exposure is considered a safe and controlled way of preconditioning organs to environmental stress often associated with surgery or treatment [Han et. al., 1998], with comparatively little or no damage to the tissue. However, there has been

contrasting evidence on the consistency of heat shock protein induction by magnetic field exposure. Work done by Goodman *et. al.* [Goodman and Blank, 1998; Goodman and Henderson, 1988] and Alfieri *et. al.* [Alfieri et. al., 2006] have consistently shown that low-frequency electromagnetic field exposure can affect the degree of hsp70 expression. Since genetic expression is a multi-step process, it has been suggested that magnetic field interaction can occur either at the level of mRNA, or protein synthesis. Studies by Blank *et. al.* describe electromagnetic fields interacting at the DNA level directly [Blank and Goodman, 2002]. In fact, it has been suggested that there exists a specific DNA sequence on the promoter region of certain proteins (such as heat shock proteins), which is sensitive to electromagnetic field exposure [Lin et. al., 1999; Lin et. al., 2001]. However, it has been shown that the level of mRNA transcribed is not always representative of the level of heat shock protein produced [Alfieri et. al., 2006; Gottwald et. al., 2007; Han et. al., 1998]. Alfieri et. al. [2006] suggests that magnetic field interaction occurs post-transcription, where electromagnetic field exposure decreases proteosome activity, thus decreasing hsp70 mRNA degradation, leading to elevated hsp70 protein levels. However, not much research has been done with regards to SMF on hsp expression, as addressed in this thesis.

Other studies have found that magnetic fields alone had no effect on the expression of heat shock proteins; however, when combined with additional environmental stress such as heat, an observable change in hsp70 expression was seen [Junkersdorf et. al., 2000; Tokalov and Gutzeit, 2004; Tsurita et. al., 1999]. Here, we report on the effect of a combination of heat shock plus magnetic field exposure, as well

as magnetic field exposure alone on heat shock gene expression, specifically, hsp70 promoter activation.

3.1.4 – Heat Shock Protein/Bioluminescence Reporter System

Protein levels have been traditionally measured using methods such as the Western blot technique, or 2D gel electrophoresis [Abdelmelek et. al., 2006; Alfieri et. al., 2006; Gottwald et. al., 2007; Han et. al., 1998]. However, recently there has been an interest in using bioluminescence as a reporter for heat shock protein expression. Bioluminescence is a naturally occurring phenomenon whereby light is produced by a biological system as the result of a typically ATP dependent chemical reaction, during which chemical energy is converted into light energy. The best known example of bioluminescence occurs when of the luciferase enzyme interacts with its substrate luciferin. Bioluminescent organisms such as the firefly (*Photuris lucicrescens*), have been exploited for their luceriferase/luciferin genetic coding, and some groups have been successful in developing genetically engineered luciferase reporter genes [Brandes et. al., 1996; Contag et. al., 1997; Langridge et. al., 1994]. Recently, O'Connel-Rodwell et. al. [2004] were able to measure cellular responses to varying heat exposures in NIH3T3 cells, using an hsp70/luc reporter gene construct (Fig. 1.1). In this reporter system, the hsp70 promoter region is fused to the firefly luciferase gene. Activation of the promoter leads to the expression of the luciferase gene (the bioluminescent reporter), and the resulting enzyme then interacts with its substrate, luciferin, to produce detectable light [Contag and Ross, 2002; Contag et. al., 1997; O'Connell-Rodwell et. al., 2004]. Specifically, when the hsp70 promoter is activated due to cellular stress such as

hyperthermia, it will induce the transcription and translation of the luciferase gene. It has been shown that in the presence of excess luciferin, ATP, and Mg^{2+}, the light emission following enzyme substrate interaction is proportional to the concentration of luciferase and hsp70 protein produced [Beckham et. al., 2004; Brasier et. al., 1989].

For this investigation, NIH3T3 cells, stably tranfected with the hsp70/luc construct (as described above) were used for all bioluminescence experiments. It was hypothesized that a 100 mT SMF exposure will have a significant effect on the rate of the activation of the hsp70 promoter, measurable throught the degree of bioluminescence detected, using a sensitive, real-time, luminescence spectroscopy system. Effects of a 100 mT SMF alone and in combination with a 42°C heat insult, both before and during SMF exposure, were evaluated.

3.2 – Methods

3.2.1 – Materials

The NIH3T3 stable cell line was a kind gift from Dr. Christopher Contag, Stanford University, California. Dulbecco's modified Eagle medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Invitrogen (Burlington, ON). The luciferin substrate (D-luciferin) was purchased from Promega. All other materials were purchased from Sigma Aldrich Canada (Oakville, ON).

3.2.2 – Cell culture

The stably transfected NIH3T3 cells were cultured in DMEM supplemented with 10% FBS and antibiotics in a humidified incubator at 37°C, 5% CO_2, and 95% air. This

monolayer adherent cell line was passaged every 2 days in order to maintain a 60-70% confluency. Typically, the media was removed from the culture plate, and the cell monolayer was washed twice with 2 mL of phosphate-buffered saline (PBS). A 2 mL solution of 0.25% Trypsin/EDTA in PBS was then added to the monolayer for approximately 1 minute in order to detatch the cells from the plate. The trypsin/PBS mixture was then removed, and 5 mL of DMEM was added to the plate. The cells were dispersed into the media using a 1 mL pipette until they were suspended in solution. Depending on cell confluency, appropriate aliquots of the suspended cell solution were removed and mixed with fresh media on a new culture plate, for a total volume of 5 mL.

3.2.3 – Sample preparation

Cells were grown to 60-70% confluency for each experiment. The media was aspirated, and the cell monolayer was washed twice with PBS. A 10% solution of Trypsin in PBS (2 mL) was added to the monolayer for 1 minute. This solution was then removed, and 3 mL of DMEM was added to the culture plate. Cells were mixed with the media using a 1 mL pipette. A volume of 2.4 mL cell suspension was removed from the plate and added to a disposable, plastic cuvette (1 cm x 1 cm). To initiate bioluminescence, 0.075 mL D-Luciferin was added to the cuvette. Additionally, cell density was determined before each experiment. A 25 μL aliquot was removed from the remaining 0.6 mL cell suspension in the plate and mixed with an equal volume of Trypan blue (0.4%) in a 1.5 mL Eppendorf tube. A volume of the mixture was transferred to a hemocytometer and viable cells were counted using the Trypan blue exclusion criteria. Typically, 1.5×10^6 to 2.5×10^6 cells were used per experiment. Cell viability at the end

of each experiment was assumed to be unchanged, since the levels of bioluminescence production remained constant until the end of each experiment, implying no significant decrease in cellular viability or function.

3.2.4 – Apparatus for measurement of bioluminescence

Bioluminescence in luciferin loaded cell samples was measured using a modified luminescence spectroscopy system, comprised of a photomultiplier tube detector, control electronics, and a desktop computer running *Felix32* data collection and spectrometer control software (PTI, London, Ontario). The original commercial sample holder was replaced with a black acrylic enclosure. The enclosure contained an acrylic water bath and a cuvette holder. A toroidal electromagnet (15 mm pole gap, 16 AWG copper magnet wire, Arnold Engineering) was held within the water bath (kept at 37 ± 0.1°C or 42 ± 0.1°C) by temperature controlled circulating water. The cuvette was held between the poles of the electromagnet. Water was pumped into the bath via an inflow port and flowed out through an overflow tube in order to ensure the electromagnet was completely submerged, but no water would overflow into the cuvette (Fig. 3.1). Bath temperature was monitored with a thermistor and bridge circuit described elsewhere [Carson and Prato, 1996]. The electromagnet was driven by a power amplifier in current mode (7570 AE Techron, Elkhart, IN) and an arbitrary function generator (Model 75, Wavetek). A custom feedback circuit between the waveform generator and the amplifier was used to monitor and actively control the field (magnetic field error ± 0.05 mT). Cells were maintained in suspension with a computer-controlled mixer at 3 Hz, with a vertical

displacement of 2 mm. The mixer was similar in design to a system described previously [Grundler et. al., 1977].

3.2.5 – Protocol for measurement of bioluminescence

Cells were acclimatized to the 37°C bath environment at a SMF strength of 0 ± 0.05 mT for 1800 s prior to any experimental manipulation. At 1800 s, water temperature was either maintained at 37°C or increased to 42°C at a rate of 1°C/min. At 3600 s, water temperature was either returned to 37°C, or maintained at 42°C to 7200 s. At 5400 s, the field condition was applied, where the SMF either remained unchanged (sham or 0 mT), or was increased to 100 mT at a rate of 0.01-0.02 T/s by manual adjustment of the DC signal from the waveform generator. The magnetic field was returned to 0 mT at 6300 s for all experiments. The luminescence levels were detected using the photomultiplier tube (PMT) and plotted against time at a sample rate of 0.5 Hz. Experimental groups were as follows: 16 experiments were performed with no heat application (water bath maintained at 37°C), of which 8 included exposure to 100 mT SMF (5400 s – 6300 s), and eight were maintained at 0 mT (sham). A second group of 16 experiments were performed with a 0.5 h heat application of 42°C as described above, of which 8 included exposure to 100 mT SMF (5400 s – 6300 s), and 8 were sham exposed. A third greoup of 16 experiments were performed with a 1.5 h heat application as described above, of which 8 were exposed to 100 mT SMF (5400 s – 6300 s), and 8 were sham exposed.

3.2.6 – Time series and statistical analysis

Each bioluminescence real-time raw data acquisition represented the number of photons measured per second by the PMT, as a function of time. Moreover, the derivative

of the raw data trace was calculated, providing measurements of the average instantaneous rate of change of photon acquisition rate (# photons/s^2) for all metrics. The rate of change was computed at four time periods: (1) before field exposure (4900 s – 5200 s), (2) the beginning of a given field condition - 0 or 100 mT (5400 s – 5700 s), (3) the end of a given field condition (5800 s – 6100 s) (4) after the given SMF condition (6600 s – 6900 s). The rate of change of the photon acquisition rate is representative of the rate of change of hsp70 promoter activation. The average measurements obtained during early field, late field, and after the given field condition were then normalized to the average measurements before the given field condition. All times series calculations were performed with custom software written in C++. Statistical analysis included two-factor ANOVA (Excel, Microsoft Corp., Redmond, WA). Changes in the rate of bioluminescence detected over time were analyzed and compared between sham and 100 mT exposed experiments. Data for the two-factor ANOVA was grouped by SMF condition (sham vs. 100 mT) and time of data analysis (early-field/late-field/post-field). Reported errors represent standard errors of the mean.

3.3 – Results

3.3.1 – Temperature measurements

The temperature of the water bath was monitored in real-time throughout each 2 hour experiment, using a thermistor and bridge circuit. The average water bath temperature was approximately 37°C at basal levels, and was increased to 42°C at t = 1800 s, in two groups of experiments. This water bath temperature remained elevated

until t = 3600, or t = 7200 s, depending on time of heat exposure (0.5 hrs or 1.5 hrs). During field exposure, the bath temperature increased by approximately 0.1°C, as described previously [Rozanski et. al., In Press]. After field was removed, bath temperature returned to 37/42°C almost immediately.

3.3.2 – Real-time data and measurements

Data collected by the luminescence spectroscopy system was first displayed in a real-time bioluminescence trace, where the number of photons per second (# photons/s) detected by the photomultiplier tube were plotted against time, at a rate of 1 measurement every 2 seconds (Fig. 3.2A/B). Next, the instantaneous rate of change of the raw-data (the derivative) was calculated and plotted against time (Fig. 3.2C/D). In the raw data trace, the degree of bioluminescence detected over time was dependent on the heat exposure (Fig. 3.2A/B). For the 0 h heat experiments, no observable increase in bioluminescence was detected. The most significant increase in bioluminescence was observed for the 0.5 h heat experiments, for both sham and 100 mT groups. An intermediate increase in bioluminescence was observed for the 1.5 h heat experiments, for both sham and 100 mT groups. Notably, the change in the rate of bioluminescence detected occurred after approximately t = 3600 s, for both the 0.5 h and 1.5 h heat exposed experiments.

In Fig. 3.2C and Fig. 3.2D, the instantaneous rate of change, or slope, of photons collected/second is presented as a function of time. There was a constant slope, or constant rate of bioluminescence produced, between t = 0 and t = 3600 s for all three heat conditions. For the 0 h heat exposed experiments, the slope of the bioluminescent raw

data remained constant throughout the whole experiment, which is consistent with the measurements observed for the raw-data trace. For the 0.5 h heat exposed experiments, the slope of the bioluminescent raw data increased significantly between t = 3600 s and t = 6900 s . After this time, the slope leveled off until the end of the experiment, t = 7200 s. This observation was true for both sham and 100 mT exposed experiments. Again, the rate of change of the slope (Fig. 3.2C/D) was consistent with the corresponding raw-data trace. Similarly, for the 1.5 h heat exposed experiments, the slope of the bioluminescent raw data increased significantly after t = 3600 s, and did not level off until the very end of the experiment. Again, this observation was true for both sham and 100 mT exposed experiments, and the rate of change of the slope (Fig. 3.2C/D) was consistent with the corresponding raw-data trace.

3.3.3 – SMF data and analysis

All experiments conducted in the study were grouped according to two variables: duration of heat exposure and SMF exposure. For each of the heat conditions of 0 h, 0.5 h, and 1.5 h exposed experiments, half of the experiments were sham exposed, and the other half were exposed to 100 mT SMF. Specifically, 16 experiments were performed for each of the heat conditions, of which 8 experiments were exposed to 100 mT SMF, and 8 were sham exposed. A total of 48 experiments were performed in this study. To analyze the data, a real-time trace of the instantaneous rate of change of bioluminescence detected (Fig. 3.2C/D) was derived. From this trace, the slope before, during (early-field/late-field), and after each respective field condition (sham or 100 mT) was calculated. The slopes calculated during the field condition and post-field condition,

were normalized to the slope measured during the pre-field condition. Time ranges of slope measurements have been noted in the methods section. In Fig. 3.3, there are three panels representing three different heat exposure conditions. Average normalized slope measurements were calculated over early-field, late-field, and post-field time intervals for both sham and 100 mT grouped experiments. For the 0 h heat exposed experiments (Fig.3.3A), the average normalized slope measured during the early-field condition was 1.36 ± 0.6 for the sham group and 1.77 ± 0.3 for the 100 mT group. The average normalized slope measured during the late-field condition was 0.97 ± 0.3 for the sham group and 1.26 ± 0.2 for the 100 mT group. The average normalized slope measured during the post-field condition was 1.78 ± 0.4 for the sham group and 1.83 ± 0.4 for the 100 mT group. The large errors observed for this group of experiments are likely a result of the small bioluminescence measurements acquired, relative to the 0.5 h and 1.5 h heat conditions. A two-way ANOVA revealed no significant change in average normalized slope values between time of data analysis (early-field/late-field/post-field) ($P > 0.9$, $1-\beta = 0.05$), no significant change in average normalized slope values between sham vs. 100 mT exposed experiments ($P > 0.3$, $1-\beta = 0.3$), and no significant interaction between time of data analysis (early-field/late-field/post-field) and field condition (sham vs. 100 mT) were found ($P > 0.8$, $1-\beta = 0.05$).

For the 0.5 h heat exposed experiments (Fig. 3.3B), the average normalized slope measured during the early-field condition was 1.23 ± 0.02 for the sham group and 1.16 ± 0.03 for the 100 mT group. The average normalized slope measured during the late-field condition was 1.40 ± 0.02 for the sham group and 1.27 ± 0.03 for the 100 mT group. The average normalized slope measured during the post-field condition was 1.63 ± 0.03 for

the sham group and 1.43 ± 0.04 for the 100 mT group. A two-way ANOVA revealed a significant change in average normalized slope values between time of data analysis (early-field/late-field/post-field) ($P < 0.001$, 1-β = 0.99), a significant change in average normalized slope values between sham vs. 100 mT exposed experiments ($P < 0.001$, 1-β = 1.00), and no significant interaction between time of data analysis (early-field/late-field/post-field) and field condition (sham/100 mT) were found ($P > 0.200$, 1-β = 0.20).

For the 1.5 h heat exposed experiments (Fig. 3.3C), the average normalized slope measured during the early-field condition was 1.13 ± 0.03 for the sham group and 1.04 ± 0.05 for the 100 mT group. The average normalized slope measured during the late-field condition was 1.17 ± 0.06 for the sham group and 1.08 ± 0.07 for the 100 mT group. The average normalized slope measured during the post-field condition was 1.23 ± 0.12 for the sham group and 1.24 ± 0.12 for the 100 mT group. A two-way ANOVA revealed no significant change in average normalized slope values between time of data analysis (early-field/late-field/post-field) ($P > 0.6$, 1-β = 0.07), no significant change in average normalized slope values between sham vs. 100 mT exposed experiments ($P > 0.2$, 1-β = 0.2), and no significant interaction between time of data analysis (early-field/late-field/post-field) and field conditions (sham vs. 100 mT) were found ($P > 0.6$, 1-β = 0.07).

3.4 – Discussion

3.4.1 – Quality of bioluminescence data and potential temperature artifact

The data collected in this study showed that the hsp70/luc reporter was reliable and sensitive to changes in heat exposure. The observed increase in bioluminescence

following a heat insult was significant and substantial. An increase in hsp70 promoter activation was to be expected following hyperthermia, as determined by other studies [Lepock, 2005; O'Connell-Rodwell et. al., 2004; Santoro, 2000]. Due to the nature of the hsp70/luciferase construct, the increase in promoter activation should correspond to a representative increase in bioluminescence production, which is what was observed for all of the heat exposed experiments. The data from this study was also not corrupted by potential artifacts from SMF-related changes in water bath temperature. No significant change in water bath temperature was observed before, during or after the application of current to the electromagnet either for the sham or 100 mT experimental runs, as described previously [Rozanski et. al., In Press].

3.4.2 – Basic findings – effects of SMF on Hsp70/bioluminescence construct

The data collected for the three heat conditions varied significantly between each heat exposure. In Fig. 3.3A, significantly larger error bars are reported than for the remaining heat conditions (Fig. 3.3B/C), with large variability in the average normalized photons/s^2 for both field conditions. This was likely due to the measurements being taken at baseline values, since no induction of a heat response was observed through increased bioluminescence measurements. Therefore, the reported number of photons detected per second is many times lower for the 0 h heat experiments, and thus more susceptible to greater error. Also, there is no significant effect of field on average normalized photons/s^2 values. This observation supports data found in previous studies, where an effect of field was observed only when cells are pre-stressed with an additional insult, such as heat or exposure to free radicals [Han et. al., 1998; Junkersdorf et. al.,

2000]. In this set of experiments, cells had not been pre-stressed, and so it is expected that the effect of SMF exposure would be negligible.

The statistical analysis of the data represented in Fig. 3.3B has very high significance, with very small error bars compared to the other heat condition measurements (Fig. 3.3A/C). In this set of experiments, there was a very significant effect of time of data analysis (early-field/late-field/post-field) and a very significant effect of field exposure (sham vs. 100 mT) on average normalized photons/s^2 values. The significant difference in average normalized photons/s^2 values between the early-field, late-field, and post-field conditions, can be attributed to the steeply increasing slope, as seen in Fig. 3.2C/D, until the termination of the experiments. During the time gap between the early-field, late-field, and post-field measurements , the rate of photons/s detections increased substantially, as demonstrated by the steep slope, resulting in significant measured changes in average photons/s^2 over time. The significant effect of field in this case is also supportive of data found in other studies [Han et. al., 1998; Junkersdorf et. al., 2000], and coincides with the explanation provided for the 0 h heat results. In this case, the cells had been pre-stressed with 0.5 h heat exposure. They were then allowed to recover for another 0.5 hrs, before being stressed again with 100 mT SMF exposure. These results show that the experiments with an additional field stress had significantly lower average photon/s^2 measurements then the control (sham) experiments. This implies that the rate of photon/s production did not increase as much for 100 mT experiments, as compared to sham experiments. Taking this observation together with the nature of the hsp70/luc construct, this would imply that the rate of hsp70 promoter activation was higher for sham then 100 mT exposed experiments, thus

suggesting that exposure to the field caused a decrease in hsp70 promoter activity. This could be explained by the results found in a study by Alfieri et. al. [Alfieri RR, 206]. This study found that magnetic field exposure increased the stability of the hsp70 proteins, and decreased proteosome activity leading to less hsp70 protein degradation. Therefore, if the field is in fact reducing hsp70 protein degradation and increasing its stability, it is eliminating the need for more hsp70 protein production, thus, less activation of the hsp70 promoter is necessary, leading to lower average photon/s^2 measurements.

The data presented in Fig. 3.3C shows measurements for the 1.5 h heat exposed experiments. Here there are slightly lower values for average normalized photons/s^2values, when compared to the data represented for the 0.5 h heat exposure in Fig. 3.3B. Additionally, there is no significant difference in average normalized photons/s^2 values between the times of data analysis (early-field/late-field/post-field). This can be explained by the fact that in the 1.5 h heat exposed experiments, the rate of increase of detected photons/s is not as robust as the real-time traces for the 0.5 h heat exposed experiments (Fig. 3.2A/B). If the slope does not increases as steeply, there will be a smaller change in measured average slope values between the time ranges over which the average slopes are calculated (early-field/late-field/post-field). Contrary to the corresponding results observed for the 0.5 h heat exposed experiments, the time gap between the three time ranges (early-field/late-field/post-field) did not potentiate the rate of photons/s^2 measurements. In addition, there was no significant effect of field observed for these experiments. Although the cells were stressed before field exposure, the stress was maintained throughout the additional field insult. In this case, although the field may have been stabilizing some of the hsp70 proteins, the simultaneous addition of heat was

equally causing some additional degradation. Additionally, the level of heat stress is significantly more powerful then the field stress by comparison, likely overriding any field effects.

3.4.3 – Comparison to other studies

The results in this study suggest that there is a significant effect of a 100 mT SMF exposure on activation of the heat shock protein promoter, as measured by the degree of bioluminescence produced. However, this observation holds true only if the cells had been preconditioned with a 0.5 h heat insult and allowed to recover before the applied 100 mT SMF exposure. These results are consistent with other studies, where cells had to be pre-stressed before significant effects of field exposure could be observed [Han L 1998, Junkersdorf B 2000]. Conversely, other studies show that a secondary stress in combination with magnetic field exposure does not potentiate magnetic field effects on hsp70 expression [Coulton LA 2004]. However, this study used human leukocytes in whole blood for the analysis. In this case, whole blood contains erythrocytes and platelets, which undergo lysis and secrete products into the blood. These are additional factors that could affect the production of hsp70 in the leukocytes, and override any observable field effects.

Another factor that needs to be considered when comparing studies, is the type of magnetic field used for exposure. Most of the studies described in this paper use either low frequency ELF exposure, or repetitive pulsed magnetic field stimulation. Only one study has observed effects of SMF exposure on hsp70 expression [Abdelmelek H 2005]. Their study used a 67 mT and 128 mT exposure systems on rat skeletal muscle. There

was no observable effect of the SMF exposure on the expression of heat shock proteins. However, the method used to determine hsp70 protein levels required freezing muscle samples, homogenizing them in a ground-glass- homogenizer, and determining protein content according to a method developed by Lowry et al. [1972]. The additional stress and time it took to complete the sample analysis could affect the results of the SMF exposure. The hsp70/luciferase construct used in this research allows for a real-time monitoring of the dynamic changes occurring during the exposure to both heat and SMF, for each 2 h long experiment. This real-time method guarantees a very controlled environment for the cells, without additional environmental stressors during or post experimental exposure.

3.4.4 – Conclusions and future work

Based on the results in this study, it was observed that SMF exposure significantly affected the rate of hsp70 promoter activation after the cells had been pre-stressed with a 0.5 h, 42°C heat insult, and allowed to recover for an additional 0.5 hrs before field application. SMF alone had no affect on hsp70 promoter activation. When cells were exposed to 1.5 h of heat insult, during which an additional SMF was applied simultaneously, no observable changes in hsp70 promoter activation were noted. Additional work can be done using different preliminary heat-stress conditions in order to see if a potential threshold exists where the action of SMF becomes apparent. Furthermore, in order to validate the hsp70/luciferase reporter system, it would be useful to evaluate the levels of endogenous hsp70 produced, and compare it to the level of luciferase produced via the hsp70 promoter reporter system. Such studies should be done

under varying heat and SMF exposures in order to ensure the system works under a wide range of experimental conditions. However, the small change observed in our real-time system may not be measurable by traditional means. Results should demonstrate that the level of luciferase produced is directly correlated to the level of endogenous hsp70 expression. This work is currently being undertaken in the lab, and will hopefully provide additional validation to the elegance and precision of the exposure and analysis system used in this study.

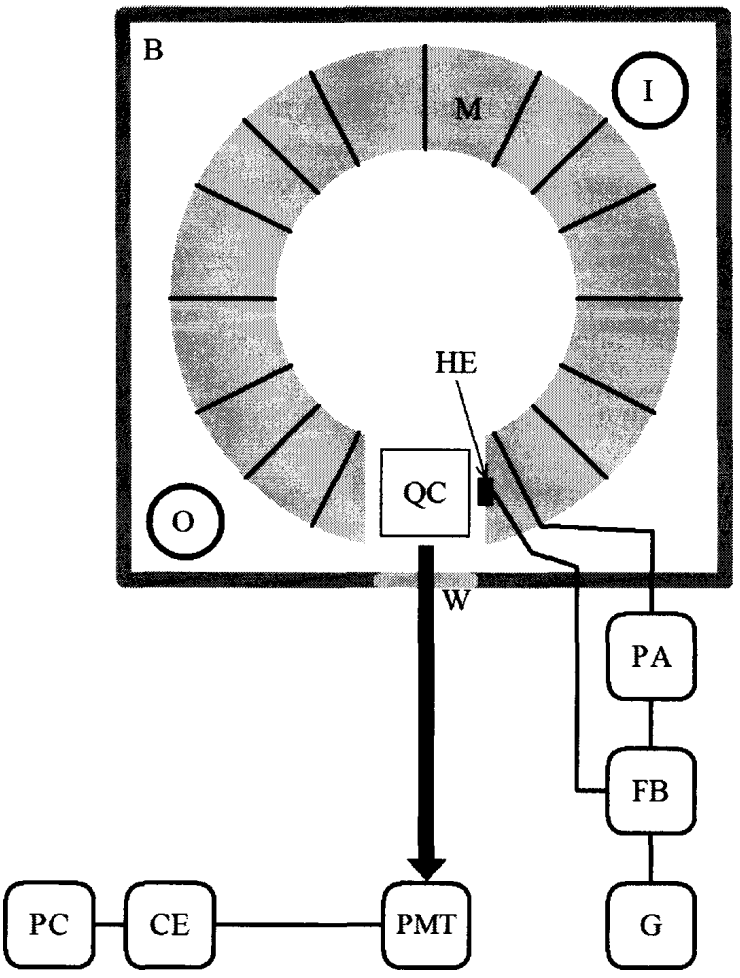
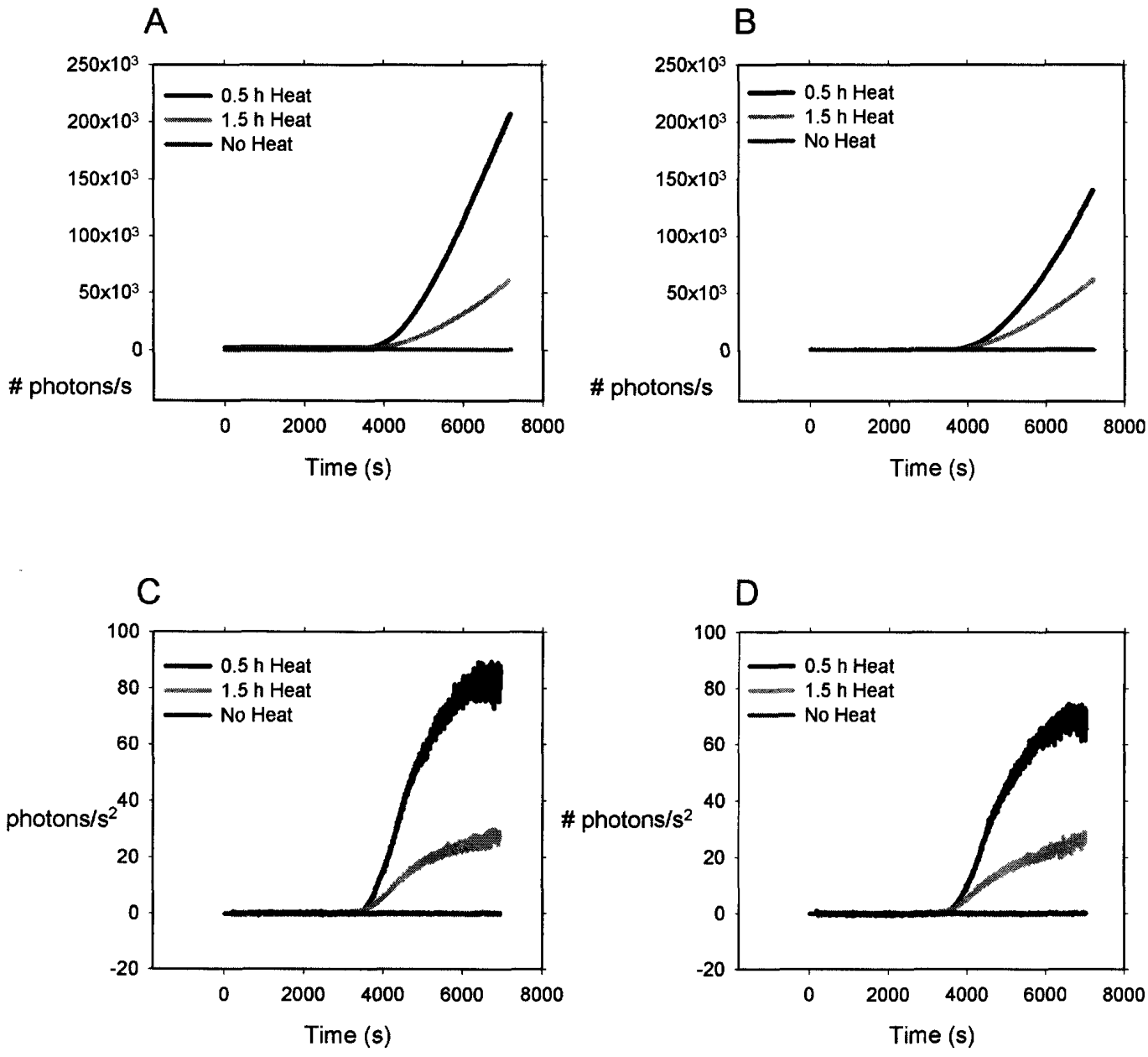

B
M
I
HE
QC
O
W
PA
FB
PC
CE
PMT
G

Figure 3.1 - Exposure apparatus used for hsp70/luciferase experiments. Toroidal electromagnet (M) was placed in a circulating water bath (B) connected to an inflow (I) and an outflow (O) port. Controlled temperatures were maintained at either 37°C or 42°C. A disposable cuvette (QC) containing transfected NIH3T3 cells was placed between the poles of the electromagnet. The magnetic field was controlled using the power amplifier (PA), the feedback circuit (FB), and the signal generator (G). The applied SMF was monitored using a Hall Effect sensor (HE). Magnet and cuvette were enclosed by a black box, excluding all external light. Photons emitted by cells passed through a small window (W), were collected by the photomultiplier tube (PMT), transformed into an electronic signal via control electronics (CE), and displayed on the PC screen in real-time.


A
250x10³
200x10³
150x10³
100x10³
50x10³
0
photons/s
0.5 h Heat
1.5 h Heat
No Heat
0
2000
4000
6000
8000
Time (s)
B
250x10³
200x10³
150x10³
100x10³
50x10³
0
photons/s
0.5 h Heat
1.5 h Heat
No Heat
0
2000
4000
6000
8000
Time (s)
C
100
80
60
40
20
0
-20
photons/s²
0.5 h Heat
1.5 h Heat
No Heat
0
2000
4000
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8000
Time (s)
D
100
80
60
40
20
0
-20
photons/s²
0.5 h Heat
1.5 h Heat
No Heat
0
2000
4000
6000
8000
Time (s)

Figure 3.2 - Representative real-time bioluminescence traces and corresponding real-time instantaneous rate of change trace. **A/B**. Real-time raw data trace representing the number of photons detected per second. **A** represents three sham exposed experiments, with varying duration of heat exposure. **B** represents three 100 mT exposed experiments, with varying duration of heat exposure. **C/D**. Corresponding instantaneous rate of change of the slope, derived from the raw data represented in **A/B**. These two panels represent the change in the photon acquisition rate. **C** represents three sham exposed experiments, with varying degrees of heat exposure. **D** represents three 100 mT exposed experiments, with varying degrees of heat exposure.

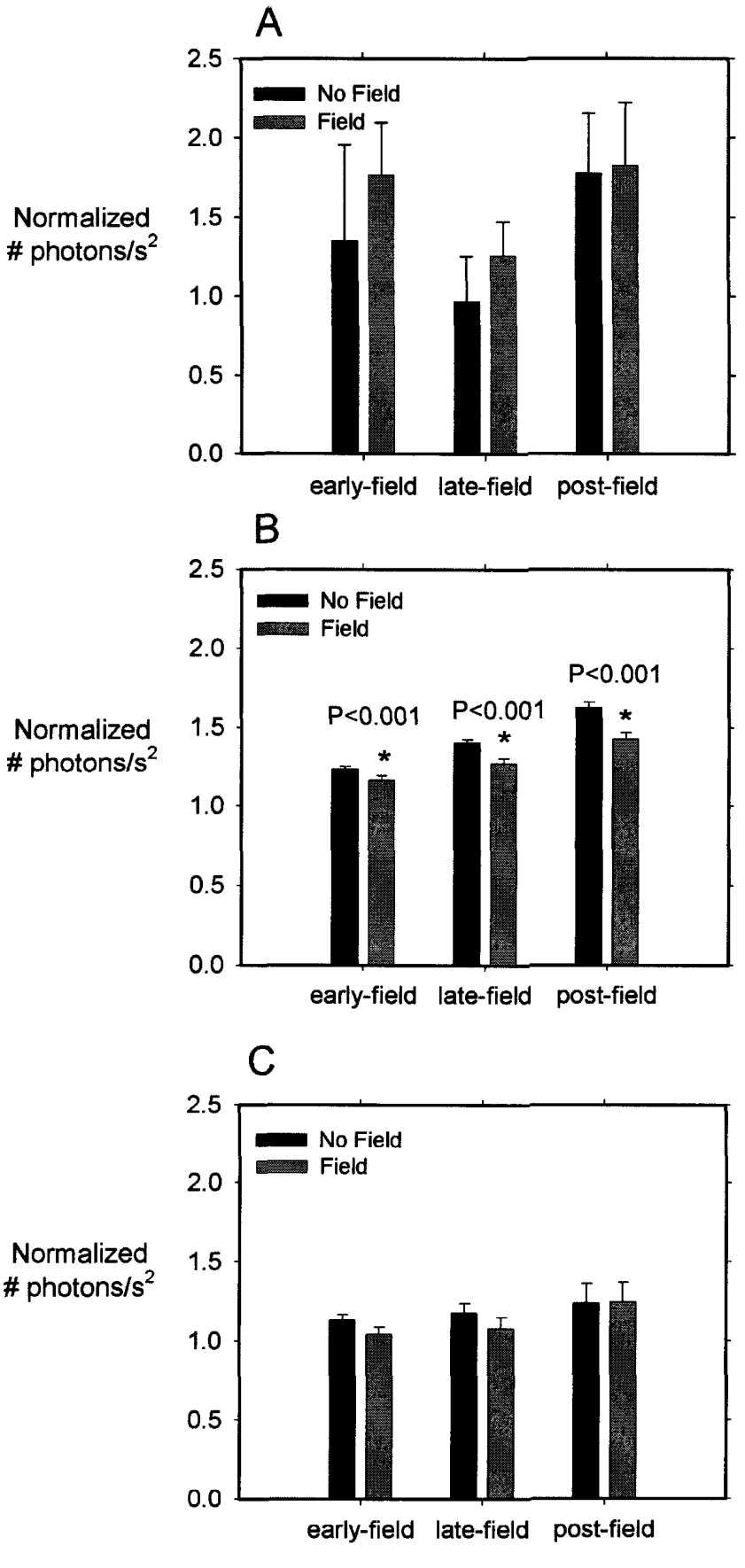
A
2.5
2.0
1.5
1.0
0.5
0.0
No Field
Field
Normalized
photons/s²
early-field
late-field
post-field
B
2.5
2.0
1.5
1.0
0.5
0.0
No Field
Field
P<0.001
P<0.001
P<0.001
*
*
*
Normalized
photons/s²
early-field
late-field
post-field
C
2.5
2.0
1.5
1.0
0.5
0.0
No Field
Field
Normalized
photons/s²
early-field
late-field
post-field

Figure 3.3 - Comparison of average normalized photon/s^2 acquisition rates between sham (0 mT) and 100 mT field condition experimental groups. **A.** Average normalized photon/s^2 measured during early-field, late-field, and post-field time intervals. Experiments were not exposed to any additional heat insult. **B.** Average normalized photons/s^2 measured during early-field, late-field, and post-field time intervals. Experiments were exposed to heat for 0.5 h, and allowed to recover before the additional SMF parameter. **C.** Average normalized photons/s^2 measured during early-field, late-field, and post-field time intervals. Experiments were exposed to heat for 1.5 hrs, and the field parameter was added simultaneously 3600 s into heat application. Significant difference between bars is represented by '*'.

3.5 – References

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Chapter Four

Conclusions and Future Work

Summary, Conclusions and Future Work

This thesis investigated the effects of a 100 mT SMF on two different biological systems, using accurate and precise real-time spectroscopy methods. Much of the controversy regarding potential biological effects of magnetic field exposure can be attributed to a lack of uniformity across experimental protocols, including experimental variables such as the length of magnetic field exposure, the strength and type of magnetic field exposure (for example, extremely low frequency-EMF vs. SMF), the type of parameter or organism under analysis. Additionally, most investigations use end-point analysis which gives limited insight into the active changes that may be occurring throughout experimental exposure. This thesis evaluated a powerful real-time analyses of the potential dynamic changes induced by SMF, using two optical methods: (1) Real-time ratiometric fluorescence spectroscopy, and (2) Real-time bioluminescence spectroscopy.

In Chapter 2, it was demonstrated that a 100 mT SMF exposure had no effect on resting or activated cytosolic calcium concentrations, even after the cells had been subjected to free-radical manipulation by Diethyl Maleate (DEM). It was concluded that DEM did not induce enough free-radicals in the cells in order to exploit the radical pair mechanism as a means for SMF interaction, measureable through changes in cytosolic calcium concentration. This set of experiments generated new questions regarding the degree of free radical manipulation as a result of GSH (free-radical scavenger) depletion. For example, further depletion of GSH may be necessary to observe significant cell sensitaization to SMF, by the addition of higher DEM concentrations. Additionally validation studies regarding cellular free radical levels would be beneficial to provide a

more absolute and direct analysis of free-radical concentrations in the cell, leading to more precise conclusions regarding the interaction of SMF via the radical pair mechanism in biological systems. Additionally, the strength of the applied SMF is an important variable to investigate. Typically, human exposure to SMF's in clinical systems such as Magnetic Resonance Imaging, include SMF strengths of 0.5 T or more. It would be beneficial to investigate the biological effects of a broad spectrum of varying SMF flux densities to determine if a threshold for optimal SMF interaction exists.

In Chapter 3, effects of SMF exposure on heat shock protein (hsp70) expression were evaluated. It was concluded that a 100 mT SMF decreased the activation rate of the hsp70 promoter, as measured by the rate of bioluminescence detected via the hsp70/luc construct. However, this SMF effect was only observed for experimental conditions where the cells had been pre-stressed with a 0.5 hour heat insult, allowed to recover (0.5 hours), and then exposed to SMF. When the SMF was applied without heat, no observable change in heat shock promoter activation was observed. Additionally, when SMF was applied at the same time as the heat insult (lasting 1.5 hours), no effect of field was observed. These results support the hypothesis that cells need to be pre-conditioned by an additional stress and allowed to recover, before SMF effects become apparent. It would be beneficial to attempt replicating these findings using different cell lines with the same hsp70/luc construct, in order to determine if the effects of SMF in combination with heat have the same effect on a wider biological scale. Furthermore, the testing of SMF effects on the hsp70/luc construct in an animal model, would benefit the development of exploiting the heat shock proteins' cytoprotective effects for future clinical use. The use of hsp70/luc construct in a small mouse model has already been performed in some

studies [Contag et. al. 2002, Contag et. al. 1997]. Replicating similar *in vivo* conditions and exposing the animal to heat and SMF stress, would provide valuable insight on SMF effects on living mammals. In order to advance to an animal model, additional validation studies would need to be conducted demonstrating the direct correlation between increased detected bioluminescence, and increased hsp70 protein synthesis. Both luciferase and hsp70 protein levels could be measured using the Western blot technique following the SMF/heat exposure experiments. Proof of a direct correlation between the degree of luciferase and hsp70 protein expression would provide appropriate justification for developing this reporter system in a more sophisticated animal model.

In conclusion, the goal of this research was to build upon past findings and apply a new and more powerful experimental technique in order to investigate conditions under which SMF effects can be detected. However, the findings demonstrated in this thesis are not enough to conclusively describe effects of SMF exposure. Nonetheless, this research provides the foundation and motivation to further investigate effects of SMFs under varying exposure conditions and experimental designs. Ideally, the results described in this thesis will serve as intermediate evidence for future magnetic field investigation.